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(Follitropin)



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- ✓ 국내 최초 고함량 면역글로불린
- ✓ 대용량 제형으로 편의성 향상
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Product Information

【성상】 무색 또는 담황색이고 투명 또는 약간 혼탁한 액이 무색 투명한 바이알에 든 주사제입니다. 【원료약품의 분량】 1mL 중 주성분 : 사람면역글로불린-(β) 100mg, 안정제 : 글리신 (Kp) 18.8mg, 용제 : 주사용수 (EP) 적 량 【효능·효과】 1. 저 및 무감마글로불린증 2. 중증감염증에 항생물질 병용 3. 특발성신장질환(신장기능이 무효로서 현저한 출혈경향이 있고, 외과적 처치 또는 출산 등 일시적 지혈관리를 필요로 하는 경우) 4. 길항바체 증후군(급성특발성발신경염) 5. 가외사기병(관상동맥질환 병용 예정목적) 【용법·용량】 1. 저 및 무감마글로불린증 : 1회 사람면역글로불린-β 1000mg을 3~4주 간격으로 정적 정맥주사 또는 직접 정맥주사합니다. 2. 중증감염증에 항생물질 병용 시 : 1회 사람면역글로불린-β 1000mg, 소아 50~150mg/kg을 정적 정맥주사 또는 직접 정맥주사합니다. 3. 특발성신장질환(신장기능) : 이 약으로서 1일 1000mg/kg, 2일간 사용하더라도 증상의 개선이 보이지 않으면 중단합니다. 4. 길항바체 증후군 : 400mg/kg 1일 1회 5일간 투여합니다. 5. 가외사기병 : 1일 400mg/kg, 5일간(중간), 혹은 2000mg/kg을 1회 정적 정맥주사합니다. (전역간격), 가외사기병에 사용하는 경우에는 병용 후 7일 이내에 투여를 개시하는 것이 바람직합니다. 2000mg/kg을 1회 투여할 경우 기본적으로 아래 투여속도를 준수하는 것으로 하지만 기온으로서 12시간 이상에 걸쳐 정적 정맥주사합니다. 투여속도 초기 30 분 동안은 0.01~0.02mL/kg/min으로 투여하고 (예, 체중 60kg 인 경우 0.6~1.2mL/min 투여) 환자의 상태에 이상이 없으면 0.06mL/kg/min까지 점차적으로 투여속도를 증가할 수 있습니다. 투여 후 환자의 상태를 충분히 관찰하고, 만약 투여속도 증가로 인해 환자의 상태에 이상이 발생할 경우 즉시 투여속도를 줄이거나 증상이 호전될 때까지 투여를 중단합니다. 【사용상의 주의사항】 1. 경고 1) 이 약은 사람 혈장으로부터 제조되어 현재의 과학기술 수준에서 혈액 매개 바이러스 또는 다른 종류의 감염원(이론적으로 CJD)의 감염 위험을 완전히 배제할 수 없습니다. 따라서 혈우병 환자 또는 면역기능이 현저히 저하된 환자는 A형 간염 백신 등 적절한 백신 접종이 권장되며, 동 제제 투여 시 의사는 정기적으로 감염 여부를 모니터 해야 합니다. 또한 사람 혈액을 원재료로 하고 있는 것에 의한 감염증 전파의 위험을 완전히 배제할 수 없으므로 투여시 환자에게 충분한 설명을 하고 질병 치료상의 필요성을 충분히 검토한 후에 필요한 최소한의 사용에 그쳐도 합니다. 2) 이 약 투여를 통한 혈전증 발생 위험은 완전히 배제할 수 없으며, 위험요인 및 투여 경로에 무관하게 발생할 수 있습니다. 고령자 등 혈전증 발생 위험요인(고령, 장기간 부동상태, 과용과 상태, 정맥 또는 동맥 혈전증 병력, 에스트로겐 사용, 중심정맥카테터 삽입, 고고도 및 심혈관계에 위험요인이 있는 환자)의 경우, 가능한 최소농도를 최저 주입속도로 신중 투여하여야 합니다. 또한 투여 전 환자가 적절한 수분을 섭취할 수 있도록 하여야 하며, 투여 후 혈전증 증상 및 징후를 관찰하고 고정성 위험이 있는 환자의 혈액검사를 평가하는 등 환자의 상태를 관찰하여야 합니다. - 첨부문서 참조. 【포장단위】 10, 25, 50, 100, 200 mL/Vial x 1 【저장방법 및 유효기간】 밀봉용기에 넣어 1~25℃에서 차광하여 보관 제조일로부터 18개월까지입니다. ※ 유효기간 또는 사용기한이 경과되었거나 변질, 변태 또는 오손된 제품은 약국, 병·의원, 도매상에 한하여 각 영업소에서 교환하여 드립니다. ※ 이 첨부문서의 작성일자(2017년 07월 03일) 이후 변경된 내용은 홈페이지(www.greencross.com)나 소비자 상담실 (☎ 080-260-8232)로 문의하시기 바랍니다.

PERGOVERIS® PRE-FILLED PEN

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DETAIL MAKES UP A FORMULA YOU CAN RELY ON

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제품 주요 정보

■ **제품명:** 페고베리스® 펜주 (300/150 IU, 450/225 IU, 900/450 IU) ■ **원료약품 및 그 분량:** 1) 페고베리스® 펜주 300/150 IU 1 프리필드펜(0.48mL) 중 유효성분: 폴리티로핀알파(재조합-인간난포자극호르몬) (별규) 21.8μg(300IU) 및 루트로핀알파(인황체형성호르몬)(유전자재조합) (별규) 6.6μg(150IU), 10% Overage 첨가제: 벡당 18.5mg(안정화제), 페놀 2.4mg(보존제), L-아르기닌염산염 5.69mg(안정화제), 인산일수소나트륨이수화물, 인산이수소나트륨일수화물, 수산화나트륨, 인산, 플록시마188, L-메티오닌, 주사용수(용제) 첨부용: 1회용주사침(별규) 5개 2) 페고베리스® 펜주 450/225 IU 1 프리필드펜(0.72mL) 중 유효성분: 폴리티로핀알파(재조합-인간난포자극호르몬) (별규) 32.8μg(450IU) 및 루트로핀알파(인황체형성호르몬)(유전자재조합) (별규) 9.9μg(225IU), 10% Overage 첨가제: 벡당 27.7mg(안정화제), 페놀 3.6mg(보존제), L-아르기닌염산염 8.53mg(안정화제), 인산일수소나트륨이수화물, 인산이수소나트륨, 수산화나트륨, 인산, 플록시마188, L-메티오닌, 주사용수(용제) 첨부용: 1회용주사침(별규) 7개 3) 페고베리스® 펜주 900/450 IU 1 프리필드펜(1.44mL) 중 유효성분: 폴리티로핀알파(재조합-인간난포자극호르몬) (별규) 65.5μg(900IU) 및 루트로핀알파(인황체형성호르몬)(유전자재조합) (별규) 19.8μg(450IU), 10% Overage 첨가제: 벡당 55.4mg(안정화제), 페놀 7.2mg(보존제), L-아르기닌염산염 17.06mg(안정화제), 인산일수소나트륨이수화물, 인산이수소나트륨일수화물, 수산화나트륨, 인산, 플록시마188, L-메티오닌, 주사용수(용제) 첨부용: 1회용주사침(별규) 14개 ■ **효능·효과:** 내인성 혈장 황체형성 호르몬(LH) 농도 1.2IU/L 미만인 중증의 황체형성호르몬(LH)과 난포자극호르몬(FSH) 결핍 여성에서 난포 발달의 자극 ■ **용법·용량:** 이 약의 치료는 반드시 불임증의 치료에 병행하여 실시해야 합니다. 이 약은 난포자극호르몬(FSH)과 황체형성호르몬(LH)이 결합된 여정(hypogonadotrophic hypogonadism)에서 태반형성신자극호르몬(hCG) 투여 후에 난포세포를 배출하는 한 개의 상측 그라프(Graafian) 난포를 발달시키는 것을 목적으로 한다. 이 약은 여말 투여해야 한다. 대상 환자들은 무월경이고 내인성 에스트로겐 분비가 적으므로, 치료는 어느 때나 시작할 수 있다. 치료는 환자 개개인의 반응에 따라 조절되어야 하며, 각 환자의 반응은 (i) 초월화로 특징된 난포 크기(II) 에스트로겐 반응으로 평가된다. 치료 시작 용량은 이 약의 추천 용량에 해당 투여하는 것이다. 추천 용량보다 적은 용량을 투여할 시에는 LH의 함량 부족으로 인해 난포 발달이 충분하지 않을 수 있다. FSH 용량 증가 요구된다면, 폴리티로핀 알파 제제로 7-14일 간격으로 37.5-75IU씩 증량한다. 투여기간은 한 주 당 5주까지 연장 가능하다. 최적 반응이 얻어지면, 마지막으로 이 약을 주사한 후 24-48시간에 hCG 5,000-10,000IU를 1회 주사한다. 환자에게 hCG 투여 당일부터 그 다음날에 상교가 추진되며, 자궁내 인공수정(Intrauterine insemination, IUI)도 고려할 수 있다. 배란 후에 황체자극 물질(LH/hCG)이 결핍되면 황체기 미성숙으로 인해 임신 실패를 유발할 수 있으므로 황체가 지지 요법도 고려할 수 있다. 반응이 과도하다고 판단되면 치료는 중단되어야 하고 hCG는 투여하지 않는다. 다음번 주기 치료는 이전 주기보다 더 낮은 FSH 용량으로 시작해야 한다. 임신시점에서 중증의 난포자극호르몬과 황체형성호르몬 결핍 증상은 내인성 황체 LH 농도 1.2IU/L 미만으로 정의되었다. 단, 연구자들의 LH 측정방간의 차이가 존재한다는 것을 고려해야 한다. 이 임신시점에서의 주기당 배란율은 70~75%였다. ■ **사용상의 주의사항:** 다음 환자에는 투여하지 말 것 1) 활리트로핀알파, 루트로핀알파 또는 이 약의 다른 성분과 과민증이 있는 환자 2) 시상하부 또는 뇌하수체에 종양이 있는 환자 3) 다낭성난소질환(PCOD)과 무관한 난소의 비대 또는 난소낭종이 있는 환자 4) 원발성 또는 2차성 난소기능 부진 환자 5) 난소암, 자궁암 또는 유방암 환자 6) 원발성 난소기능성 환자 7) 임신할 수 없는 생식기관의 병행이 있는 환자 8) 임신할 수 없는 자궁의 선유종(fibroid tumors)이 있는 환자 9) 알부, 수술부 ■ **개정년월일:** 2019년 6월 14일 후 차폐한 허가사항은 색인제 문의문의국(www.medic.go.kr) 또는 제품설명서를 참조하시기 바랍니다. 미국 주지사 서클시 강남구 테헤란로 508 애성빌딩 5층 TEL:02-2185-3800 FAX:02-2185-3830 www.merck.co.kr Merck Ltd. Korea 5th Floor Haesung-2-Building, Teheran-Ro 508, Gangnam-gu, Seoul, 135-725, Korea



CERM

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Aims and Scope

Clinical and Experimental Reproductive Medicine (CERM) is an international peer-reviewed journal for the gynecologists, reproductive endocrinologists, urologists and basic scientists providing a recent advancement in our understanding of human and animal reproduction. CERM is an official journal of Pacific Society for Reproductive Medicine, the Korean Society for Reproductive Medicine and Korean Society for Assisted Reproduction. Abbreviated title is *Clin Exp Reprod Med*. The aims of CERM are to publish the high quality articles that facilitate the improvement of the current diagnosis and treatment in couples with reproductive abnormalities through human or relevant animal model research. Its scope is the infertility, reproductive endocrinology, urology, andrology, developmental biology of gametes and early embryos, basic reproductive science, reproductive physiology, reproductive immunology, genetics and biology of stem cell.

Background

CERM continues the *Korean Journal of Reproductive Medicine* (pISSN: 2093-8896, 2007-2010) that continues the *Korean Journal of Fertility and Sterility* (pISSN: 1226-2951) launched in 1974. It was initially published annually, biannually from 1983, tri-annually from 1994, and quarterly from 2000. It is published quarterly on the 1st day of March, June, September and December. This journal is supported by the Korean Academy of Medical Sciences and the Korean Federation of Science and Technology Societies (KOFST) Grant funded by the Korean Government. All or part of CERM is indexed/tacked/covered by PubMed, Emerging Sources Citation Index (ESCI), SCOPUS, KoreaMed, KoMCI, CrossRef and Google Scholar.

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Fertility-sparing treatment in women with endometrial cancer

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Endometrial cancer (EC) in young women tends to be early-stage and low-grade; therefore, such cases have good prognoses. Fertility-sparing treatment with progestin is a potential alternative to definitive treatment (i.e., total hysterectomy, bilateral salpingo-oophorectomy, pelvic washing, and/or lymphadenectomy) for selected patients. However, no evidence-based consensus or guidelines yet exist, and this topic is subject to much debate. Generally, the ideal candidates for fertility-sparing treatment have been suggested to be young women with grade 1 endometrioid adenocarcinoma confined to the endometrium. Magnetic resonance imaging should be performed to rule out myometrial invasion and extrauterine disease before initiating fertility-sparing treatment. Although various fertility-sparing treatment methods exist, including the levonorgestrel-intrauterine system, metformin, gonadotropin-releasing hormone agonists, photodynamic therapy, and hysteroscopic resection, the most common method is high-dose oral progestin (medroxyprogesterone acetate at 500–600 mg daily or megestrol acetate at 160 mg daily). During treatment, re-evaluation of the endometrium with dilation and curettage at 3 months is recommended. Although no consensus exists regarding the ideal duration of maintenance treatment after achieving regression, it is reasonable to consider maintaining the progestin therapy until pregnancy with individualization. According to the literature, the ovarian stimulation drugs used for fertility treatments appear safe. Hysterectomy should be performed after childbearing, and hysterectomy without oophorectomy can also be considered for young women. The available evidence suggests that fertility-sparing treatment is effective and does not appear to worsen the prognosis. If an eligible patient strongly desires fertility despite the risk of recurrence, the clinician should consider fertility-sparing treatment with close follow-up.

Keywords: Endometrial neoplasms; Fertility preservation; Uterine neoplasms

Introduction

The most common gynecologic malignancy in developed countries is endometrial cancer (EC). Although typically diagnosed in postmenopausal individuals, 3%–14% of EC cases occur in those younger than 40 years. The overall incidence of EC has increased, most rapidly in the under 40 age group, the members of whom frequently are nulliparous and strongly desire to keep their fertility [1-5].

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Because EC in young women tends to be early-stage and low-grade, a good prognosis is anticipated for such cases [6,7]. The standard treatment for EC is total hysterectomy, bilateral salpingo-oophorectomy (BSO), pelvic washing, and/or lymphadenectomy [8]. Although this treatment is highly effective, it results in the permanent loss of reproductive potential, which is problematic in young patients wishing to preserve their fertility. Given both this fact and the increasing incidence of EC in younger patients, conservative management has drawn attention and has been increasingly investigated.

The core of fertility-sparing treatment is progestin therapy, as unopposed estrogen is the main cause of EC. In fact, numerous studies on various dosages of progestin and other medicines have been published [9-16]. Although it is agreed that fertility-sparing treatment can be considered for select young women with early-stage disease, the matter is complicated by the lack of evidence-based

consensus or guidelines regarding target patients, treatment methods, and surveillance [17]. In this review, we will summarize data drawn from the recent literature and derive, both therefrom and from our own experience, answers to the aforementioned unresolved issues regarding fertility-sparing treatment.

Ideal target patients

Selecting ideal candidates for fertility-sparing treatment is crucial. Candidates should have a minimal risk of metastatic disease or local invasion and therefore a higher chance of regression; thus, the ideal candidates for fertility-sparing treatment have been suggested to be young women with grade 1 endometrioid adenocarcinoma confined to the endometrium. Few studies [4,18-20] have reported the outcomes of fertility-sparing treatment for patients with more advanced disease. Park et al. [18] reported the outcomes of fertility-sparing treatment for grade 2–3 EC with or without superficial myometrial invasion. The rates of complete response (CR) to fertility-sparing treatment were 76.5%, 73.9%, and 87.5% for patients with stage IA (without myometrial invasion) grade 2–3 disease, patients with stage IA (with superficial myometrial invasion) grade 1 disease, and patients with stage IA (with superficial myometrial invasion) grade 2–3 disease, respectively [18]. Chae et al. [4] reported pregnancy outcomes of fertility-sparing treatments and demonstrated that a higher grade was also closely associated with pregnancy failure. Although a few reports have indicated that fertility-sparing treatment can be safe and effective for EC patients with grade 2–3 disease or superficial myometrial invasion [4,18-20], expansion of the criteria for target patients is not yet recommended due to the paucity of high-quality evidence.

Several groups have provided target-patient selection criteria that differ only marginally. The Japan Society of Gynecologic Oncology, the European Society of Gynecological Oncology, and the Society of Gynecologic Oncology have stated that fertility-sparing treatment can be considered for women with grade 1 endometrioid adenocarcinoma suspected of being confined to the endometrium [21-24]. The British Gynecological Cancer Society has suggested that fertility-sparing treatment may be safe in the short term for women exhibiting grade 1 endometrioid adenocarcinoma with superficial myometrial invasion [25]. The Korean Society of Gynecologic Oncol-

ogy has recommended fertility-sparing treatment for grade 1 endometrioid adenocarcinoma limited to the endometrium if the patient strongly desires it [26]. All of these criteria account for only grade 1 endometrioid adenocarcinoma, while the criteria of these organizations regarding the degree of invasion, as alluded to above, differ only slightly. The recommendations are summarized in Table 1.

Drawing together the above criteria, we believe that the proper target patients for fertility-sparing treatment are young women exhibiting grade 1 endometrioid adenocarcinoma without myometrial invasion who strongly desire preservation of their fertility.

Appropriate pre-management evaluation

After confirmation of the histologic type of a tumor, imaging testing should be performed to rule out myometrial invasion and extrauterine disease before starting fertility-sparing treatment. Although ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI) can be used, contrast-enhanced MRI is known to be the superior method and offers the highest efficacy [27,28], especially in determining the presence of myometrial invasion. Lin et al. [29] reported that fused T2-weighted and diffusion-weighted MRI had an 88% accuracy in the assessment of myometrial invasion. CT can be a good tool for the assessment of the extrauterine encroachment of EC; however, Zerbe et al. [30] reported that the sensitivity of CT for the detection of adnexal involvement of EC was only 60%. Therefore, some authors have argued that diagnostic laparoscopy should be performed to rule out the presence of extrauterine disease before initiating fertility-sparing treatments [15]. They advocated that the occurrence of synchronous or metachronous endometrioid ovarian cancer in stage I EC limited to the endometrium in up to 25% of cases is not negligible [15,31,32]. In contrast, the Korean Gynecologic-Oncology Group (KGOG) conducted a multicenter, retrospective study that showed the incidence of synchronous ovarian cancer in women under 40 years old to be 4.5% (21/471), which is much lower than reported elsewhere [32]. Additionally, that study showed that in patients with low-risk early EC on pretreatment (no myometrial invasion, normal or benign-looking ovaries, normal CA-125, and grade 1 endometrioid histology), which generally can be considered suitable for fertility-sparing treatments, no synchronous ovarian cancer was identified at all (0/21) [33]. On that basis, the KGOG concluded

Table 1. Target-patient selection criteria for fertility-sparing treatment for endometrial cancer published by different groups

Group	Target-patient selection criteria
KSGO, JSGO, ESGO, and SGO	Grade 1 endometrioid adenocarcinoma confined to the endometrium
BGCS	Grade 1 endometrioid adenocarcinoma confined to the endometrium (or with only superficial myometrial invasion)

KSGO, Korean Society of Gynecologic Oncology; JSGO, Japan Society of Gynecologic Oncology; ESGO, European Society of Gynecological Oncology; SGO, Society of Gynecologic Oncology; BGCS, British Gynecological Cancer Society.

that diagnostic laparoscopy is not mandatory in low-risk patients for fertility-sparing treatments [33]. To summarize, MRI is the best method available for the identification of myometrial invasion, and diagnostic laparoscopy prior to fertility-sparing treatments seems not to be mandatory.

Treatment efficacy and primary modality

To ascertain the efficacy of fertility-sparing treatments, a meta-analysis of 32 studies was performed in 2012 and found that fertility-sparing treatment for EC was associated with a regression rate of 76.2% and a relapse rate of 40.6% [6]. However, no consensus exists regarding which agent, dose, or duration of treatment is most effective. Generally, the most commonly employed agent is medroxyprogesterone acetate (MPA) at 400–600 mg daily or megestrol acetate (MA) at 160–320 mg daily, as shown in Table 2. Park et al. [11] conducted a retrospective study showing that 115 of 148 patients (77.7%) achieved CR with oral MPA or MA and that MPA was associated with a lower risk of recurrence than MA (odds ratio, 0.44; 95% confidence interval, 0.22–0.88; $p=0.021$). Interestingly, because no patients showed clinical progression at the time of recurrence, the authors concluded that fertility-sparing treatment is safe. The response rates to MPA have varied widely by study group. According to a prospective study conducted by Ushijima et al. [34], the first of its kind, CR was achieved in 55% of women with EC who took 600 mg of MPA and low-dose aspirin orally. The outcomes of studies on fertility-sparing treatment with oral progestin [11,34–39] are summarized in Table 2. As these results were unsatisfactory, other options have been investigated. For example, the levonorgestrel-intrauterine system (LNG-IUS) has been suggested, either as an alternative to or in combination with an oral agent. This system can reduce systemic adverse effects and increase local effectiveness. In a prospective trial involving the daily administration of 500 mg of oral MPA with LNG-IUS, Kim et al. [12] reported a CR rate of 87.5% (14/16 patients) and an average time to CR of 9.8 ± 8.9 months. Subsequent to that study,

the KGOG conducted a multicenter prospective investigation [9] to evaluate the efficacy of combined oral MPA/LNG-IUD treatments. However, the CR rate at 6 months was only 37.1% (13/35 patients). This lower CR rate may have been due to the short treatment and follow-up periods [9].

A few other studies have considered agents other than progestin. Metformin, as an example, can also be used for the treatment of type 2 diabetes mellitus. A meta-analysis revealed that metformin was associated with improved overall survival in EC patients [14]. Mitsuhashi et al. [40] reported that a regimen of MPA with metformin elicited a better prognosis than treatment with MPA alone with respect to relapse-free survival. A gonadotropin-releasing hormone (GnRH) agonist combined with another agent also can be used. Several studies have reported the successful treatment of EC with a GnRH agonist along with an aromatase inhibitor or LNG-IUS [16,41,42]. Additionally, others have reported positive outcomes of treatment incorporating photodynamic therapy [13] and of hysteroscopic resection of the lesion combined with progestin [43] or a GnRH agonist [44]. Table 3 shows a summary of the studies on fertility-sparing treatment using various methods. However, the data on drugs other than progestin are not yet sufficient to assess their efficacy and safety for fertility-sparing treatment. In summary, given that a number of studies have established the effectiveness of systemic progestin, we recommend high-dose oral progestin (MPA 500–600 mg/day or MA 160 mg/day) as the primary choice of fertility-sparing treatment.

Method of evaluation of post-treatment response

Evaluation of the response is crucial, though no universally accepted standard protocol currently exists. Various follow-up intervals have been reported [45,46], the most frequent being 3 months [47]. Endometrial re-evaluation at 3 months can be performed with dilation and curettage (D&C), endometrial aspiration biopsy (EAB), or hysteroscopic biopsy. According to the literature [48], no significant

Table 2. Studies on fertility-sparing treatment of endometrial cancer with oral progestin

Study	Progestin dose (mg/day)	Treatment duration (mo)	Number of patients	CR	RE	Median follow-up duration (mo)
Ushijima et al. (2007) [34]	MPA 600	< 6–7	22	12 (54.5)	2 (16.7)	47.9
Eftekhar et al. (2009) [35]	MA 160–320	6–12	21	18 (85.7)	3 (16.7)	42
Mao et al. (2010) [37]	MPA 250–500 or MA 160	3–9	6	4 (66.7)	0	50
Shirali et al. (2012) [36]	MA 160–320	1–43	16	10 (62.5)	0	Unknown
Park et al. (2013) [11]	MPA 500 or MA 160	2–31	148	115 (77.7)	35 (30.4)	41
Wang et al. (2014) [38]	MA 160	8–20	37	30 (81.1)	15 (50.0)	78.6
Ohyagi-Hara et al. (2015) [39]	MPA 400–500	3–6	16	11 (68.8)	9 (81.8)	45.5

Values are presented as number (%).

CR, complete response; RE, relapse; MPA, medroxyprogesterone acetate; MA, megestrol acetate.

Table 3. Studies on fertility-sparing treatment of endometrial cancer with various attempted methods

Study	Method	Number of patients	CR (%)	RE (%)	Median follow-up duration (mo)
Kim et al. (2013) [12]	MPA 500 mg/day+LNG-IUS	16	14 (87.5)	2 (14.3)	Mean \pm SD, 31.1 \pm 11.8
Kim et al. (2019) [9]	MPA 500 mg/day+LNG-IUS	35	13 (37.1)	0	6
Mitsuhashi et al. (2019) [40]	MPA 400 mg/day+metformin (750–2,250 mg/day)	42	40 (95.2)	7 (17.5)	57
Zhou et al. (2017) [16]	IM injections of GnRH agonist every 4 wk+LNG-IUS	17	15 (88.2)	1 (6.7)	18.7
Zhang et al. (2019) [42]	IM injections of GnRH agonist every 4 wk+oral aromatase inhibitor 2.5 mg/day	6	6 (100)	0	48
Choi et al. (2013) [13]	Photodynamic therapy	16	12 (75.0)	4 (33.3)	Mean, 78
Falcone et al. (2017) [43]	Hysteroscopic resection followed by MA 40–160 mg/day or LNG-IUS	28	25 (89.3)	2 (8.0)	92
Tock et al. (2018) [44]	Hysteroscopic resection followed by IM injections of GnRH agonist every 4 wk	9	5 (55.6)	1 (20.0)	40.7

Values are presented as number (%) unless otherwise indicated.

CR, complete response; RE, relapse; MPA, medroxyprogesterone acetate; LNG-IUS, levonorgestrel-intrauterine system; SD, standard deviation; IM, intramuscular; GnRH, gonadotropin-releasing hormone; MA, megestrol acetate.

difference in accuracy exists between D&C and EAB at the time of the initial diagnosis of EC.

However, concerns have been raised that not enough endometrial tissue is collected with EAB, due to progestin-induced endometrial atrophy at the time of re-evaluation. In fact, Kim et al. [49], based on their prospective study comparing the diagnostic accuracy of D&C with EAB in patients treated with high-dose oral progestin along with LNG-IUS, reported a diagnostic concordance of only 33% ($\kappa=0.27$). Thus, EAB might not be reliable as a follow-up evaluation method; instead, re-evaluation with D&C at 3 months is recommended over EAB.

Necessity of maintenance treatment

Regression has been reported to take 3 to 6 months to achieve with initial fertility-sparing treatments [50]. This notwithstanding, no consensus yet exists on the necessity of maintenance treatment. A meta-analysis of 29 studies reported a relapse rate for fertility-sparing treatment of 40.6% regardless of maintenance treatment [6]. According to a study by Park et al. [51], relapse rates were 31%, but patients undergoing maintenance treatment (either a combined oral contraceptive [OC] or LNG-IUS) did not experience recurrence. On that basis, they concluded that maintenance treatment with cyclic OC or LNG-IUS can be administered to prevent recurrence. Several other studies [11,34,37] have also supported maintenance treatment. For patients who do not desire to conceive, maintenance treatment with OC or LNG-IUS should be recommended to lower the risk of recurrence. Furthermore, no consensus exists regarding the duration of maintenance treatment; as shown in Table 2, several studies with various treatment durations have been conducted. Therefore, patients achieving CR should attempt to conceive immediately if possible.

If patient with CR does not want to conceive soon, the clinician should decide when to stop the maintenance treatment. Recently, Chae et al. [4] reported that 49 patients who had experienced CR after treatment with 500 mg of MPA once daily tried to conceive, and 22 of those patients (44.9%) became pregnant. In that study, the maintenance treatment was stopped when two serial iterations of D&C at 3 months showed no carcinoma. The maximum duration of maintenance treatment was 49 months, and the duration of maintenance treatment was shown to have no effect on pregnancy. Although it is reasonable to consider maintaining progestin therapy until pregnancy, individualization may be required due to the lack of consensus.

Safety of fertility treatment

The safety of ovarian stimulation for EC patients remains uncertain. Clomiphene citrate is the most frequently used drug in the initial protocol for ovulation induction and may be used with gonadotropins [52]. As these drugs lead to increased estrogen production during the follicular phase, concern exists that they will increase the risk of EC [53]. Silva Idos et al. [54], reporting a large cohort study of 7,355 women among whom 43% were treated with ovulation-stimulation drugs, stated that increased EC risk may be associated with increasing cumulative dose of clomiphene citrate and, possibly, the number of cycles. Park et al. [55] examined 141 EC patients after fertility-sparing treatment. Among them, 44 patients tried to conceive with the aid of ovarian stimulation drugs, none of whom experienced recurrence and 38 of whom became pregnant. Additionally, no significant differences in 5-year disease-free survival were present between the ovarian stimulation group and the non-medication group ($p=0.335$). Recently, Kim et al. [56] reported that 26.3% of pa-

Table 4. Summary of unresolved issues of fertility-sparing treatment for endometrial cancer

Issue	Description
Ideal target patients	Patients with endometrioid-type cancer that is grade 1 and confined to the endometrium
Appropriate pre-management evaluation	Magnetic resonance imaging
Treatment efficacy and primary modality	High-dose oral progestin (MPA 400–600 mg/day or MA 160–320 mg/day)
Method of evaluation of post-treatment response	D&C every 3 months
Necessity of maintenance treatment	Yes; however, no consensus exists regarding duration.
Safety of fertility treatment	May be safe.
Post-childbearing necessity of hysterectomy	Yes
Safety of ovarian preservation in young women	May be safe.

MPA, medroxyprogesterone acetate; MA, megestrol acetate; D&C, dilation and curettage.

tients (6/22) who underwent *in vitro* fertilization after fertility-sparing treatments experienced recurrence over the course of a median 41-month follow-up period, and all six were then treated with hysterectomy. Particularly noteworthy was the lack of significant differences in the total duration of gonadotropin use or total gonadotropin use between patients with and without recurrence. Although that recurrence rate (26.3%) is not surprisingly high when compared with the rates of a cohort that achieved pregnancy after fertility-sparing treatment without fertility treatment, Kim et al. [56] stated that longer durations with more cautious follow-up are needed in order to avoid missing cases of recurrence. To summarize, although various opinions on the safety of ovarian stimulation drugs exist, they do appear to be safe when combined with close follow-up.

Post-childbearing necessity of hysterectomy

No controversy exists regarding the necessity of hysterectomy after childbearing is completed, because recurrence rates after CR remain high [57]. One meta-analysis reported a relapse rate of 40.6% despite maintenance treatment [6]. BSO, however, is indeed controversial. Conventionally, BSO was performed to lower the risk of recurrence for EC patients. However, many studies have reported that pre-menopausal oophorectomies were correlated with increased risk of premature death, coronary heart disease, stroke, and cognitive impairment [58–61]. Lee et al. [62] studied the impact of ovarian preservation on the recurrence and survival rates of premenopausal women with EC. Among 495 patients, 176 were in the ovarian-preservation group, and no differences in recurrence-free survival ($p=0.742$) or overall survival ($p=0.462$) were found between the ovarian-preservation group and the BSO group [62]. Similarly, another study reported that 402 women with early EC who underwent ovary-preserving treatment showed no differences in survival compared to 2,867 women with early EC who underwent BSO [63]. Although the data supporting ovarian preservation in cases of EC are limited, ovarian preservation does seem to be both safe and advan-

tageous in cases of oocyte retrieval and surrogacy [17].

Conclusion

The unresolved issues regarding the fertility-sparing treatment of EC are summarized in Table 4. As emphasized above, a lack of high-quality evidence exists regarding the efficacy and safety of fertility-sparing treatments; therefore, no evidence-based consensus or guidelines have been published. The available evidence suggests that fertility-sparing treatment is effective and does not appear to worsen prognosis. If an eligible patient strongly desires fertility despite the potential risk of recurrence, the clinician should consider fertility-sparing treatment with close follow-up.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Nanotechnology in reproductive medicine: Opportunities for clinical translation

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In recent years, nanotechnology has revolutionized global healthcare and has been predicted to exert a remarkable effect on clinical medicine. In this context, the clinical use of nanomaterials for cancer diagnosis, fertility preservation, and the management of infertility and other pathologies linked to pubertal development, menopause, sexually transmitted infections, and HIV (human immunodeficiency virus) has substantial promise to fill the existing lacunae in reproductive healthcare. Of late, a number of clinical trials involving the use of nanoparticles for the early detection of reproductive tract infections and cancers, targeted drug delivery, and cellular therapeutics have been conducted. However, most of these trials of nanoengineering are still at a nascent stage, and better synergy between pharmaceuticals, chemistry, and cutting-edge molecular sciences is needed for effective translation of these interventions from bench to bedside. To bridge the gap between translational outcome and product development, strategic partnerships with the insight and ability to anticipate challenges, as well as an in-depth understanding of the molecular pathways involved, are highly essential. Such amalgamations would overcome the regulatory gauntlet and technical hurdles, thereby facilitating the effective clinical translation of these nano-based tools and technologies. The present review comprehensively focuses on emerging applications of nanotechnology, which holds enormous promise for improved therapeutics and early diagnosis of various human reproductive tract diseases and conditions.

Keywords: Nanomedicine; Nanosensors; Nanotherapy; Reproductive health; Translational research

Introduction

Reproductive medicine is an emerging field that focuses on male and female reproductive tract function and associated clinical issues

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such as fertility preservation, infertility, pubertal development, menopause, and sexually transmitted infections (STIs), including AIDS (acquired immunodeficiency syndrome) and cancers [1]. Over the years, reproductive medicine has come a long way, as remarkable technological advancements have given the gift of parenthood to infertile couples by empowering them to manage their fertility [2]. However, the objectives of reproductive medicine are not confined to only conception and delivery-related issues, but also include fertility-sparing treatments, selection and micromanipulation of gametes and embryos, *in vitro* culture, preimplantation genetic testing, and reproductive cryopreservation and associated diseases and conditions [3,4]. Despite recent advances in this particular field of clinical medicine, the existing challenges are still enormous [5]. To confront such challenges, nanoengineering-based tools and technologies that offer minimally invasive detection and treatment of reproductive tract-associated pathologies are indispensable and warrant an

innovative plan of action for the development of therapeutic interventions depending upon the stage and type of diseases and conditions [6]. In order to put forward any such strategy, it is essential to design and develop novel applications using functionalized nanomaterial-based methodologies that are rapid, selective, and sensitive in nature. Of course, the effective implementation of such interventions will require careful validation in control, experimental, and field settings.

The concept of nanotechnology dates back to 1959, when an American theoretical physicist, Professor Richard P. Feynman, at the Annual Physical Society Meeting at Caltech emphasized a conceptual framework that involves direct manipulation of individual atoms using synthetic chemistry to develop denser computer circuitry-based imaging platforms that could visualize tinier objects with higher resolution [7]. Although he never used the term “nanoscience,” his pioneering views laid the foundation of the newly emerging field. In later years, Professor Norio Taniguchi, a MIT-based American engineer, drew significant attention from a wider audience to the nanotechnological field. During the 1990s, many nanotechnological applications were derived from a specialized form of materials science [8]. However, these materials are currently used for a number of applications in various fields such as energy, electronics, food and agriculture, cosmetics, and healthcare. With the availability of enormous opportunities and resources at public-funded institutions and in the industrial research and development sector, the field of nanotechnology is growing at an exponential scale. The trends in the development of improved nanotechnological techniques over conventional research and therapeutic approaches will inexorably encourage the rapid broadcasting of the nanobiotechnological “vision” to a growing number of research and clinical disciplines [9,10].

Over the last decade, the advent of new applications and technologies linked to biomedical nanotechnology have revolutionized existing preventive, diagnostic and treatment approaches. This innovative approach has paved the way for development of improved and very sensitive tools for the investigation of the mechanisms underlying the biology of various diseases. This has been the major driving force leading to the expansion of global health sector [11-13]. The global market of healthcare nanotechnology (including nanomedicine) accounted for about 78.54 billion USD in 2012, which was expected to rise to 177.60 billion USD in 2020 with a compound annual growth rate of 12.3% from 2012 to 2020 [14]. A plethora of work is currently under progress in the healthcare sector. This includes a spectrum of systems that target specific cells or proteins from the systemic circulation to frameworks that incorporate multiple drugs and diagnostic agents, all the way to developing novel formulations that can deliver a diverse set of agents through the oral route. Some of these nanoproducts developed by the biotechnological and phar-

maceutical industry have demonstrated remarkable improvements in drug delivery systems, medical imaging, theranostics, biodegradable implantable materials, tissue regeneration strategies, and diagnostic platforms [15,16].

Among the potential clinical applications of nanomaterial-based approaches, the use of investigational nanobiotechnological tools in reproductive medicine has already resulted in encouraging outcomes in the treatment of several high-impact conditions, opening significant opportunities for alternative non-invasive or minimally-invasive treatments for several traditionally “surgical” pathologies. The use of such tools has immensely contributed towards improved technologies for fertility preservation and the diagnosis and treatment of infertility and other clinical problems associated with puberty, menopause, STIs (including HIV [human immunodeficiency virus]), and cancers of the reproductive tract [17]. In reproductive biology, the availability of versatile delivery vehicles with a large loading capacity and spontaneous internalization into target cells has created unprecedented possibilities to explore and manipulate the fine mechanisms underlying reproduction and early embryo development for research purposes. Of late, a few experimental investigations using nanodrug vectors for endometriosis, uterine fibroids, ectopic pregnancy, and trophoblastic diseases have also been reported. Most of the trials involving nanoengineering methods for fertility regulation and treatment of reproductive cancers are still at the initial stage [18].

The present review focuses on all emerging aspects of nanomaterial-based approaches for the precise identification, therapy, and monitoring of reproductive tract-associated disorders, which are at the center of attention in reproductive medicine, along with their bench-to-bedside translational potential to the point-of-care setting in a comprehensive fashion.

Assisted reproductive technologies in clinical practice

Reproductive medicine is an emerging discipline that not only deals with the application of novel approaches to address issues related to successful pregnancy and preservation of fertility, but also helps in the diagnosis and treatment of disorders or ailments that disrupt the normal functioning of the reproductive tract. In light of advances in the field of reproductive medicine, assisted reproductive technology (ART) has emerged as a successful and widely performed treatment paradigm throughout the world that involves a set of procedures that assist with infertility-related issues in males and females [6,19,20]. ART includes the use of *in vitro* fertilization (IVF), zygote intrafallopian transfer (ZIFT), gamete intrafallopian transfer (GIFT) and intracytoplasmic sperm injection (ICSI). IVF is generally applicable for

women with low ova production or men with a low sperm count. In IVF, women are treated with multiple drugs that lead to the production of numerous eggs, which are removed and fertilized *in vitro* and the healthy embryos are finally implanted in the uterus. ZIFT involves a similar procedure to IVF with the striking difference that the embryos are transferred in the fallopian tube. GIFT involves the transfer of eggs and sperm in the fallopian tube where the fertilization takes place. ICSI is often appropriate for couples affected by severe inadequacy of the sperm count, wherein a single sperm is injected into a mature egg followed by implantation to the uterus or transfer to the fallopian tube [1,21].

The past few decades have witnessed a substantial augmentation in the frequency of ART procedures, and ART has made it possible for countless infertile couples to experience parenthood. However, excessive usage is generally accompanied by elevated perinatal risk and problems related to multifetal gestations. Cases of multiple gestations are often found to be associated with risks of maternal and fetal morbidity and mortality due to factors such as pre-term birth, pre-eclampsia, and pregnancy-related complications. However, evidence supporting the generation of risk associated with multi-fetal gestations arising due to the application of excessive ART is limited. Apart from the aforementioned problems, the increased use of ART may also lead to the potential danger of monozygotic twinning, which may contribute to growth abnormalities and twin-to-twin transfusion [22,23]. Such complications could be overcome by appropriate consideration of the maternal condition as well as a thorough assessment of the patient's obstetric history prior to ART procedures. In addition to these, determination of pre-existing disorders of the cardiopulmonary system and common ailments such as diabetes, hypertension, epilepsy, obesity, maternal medical status, treatment regimen, and other aspects of care may have productive effects on pregnancy outcomes and significantly reduce risk to the woman's life and health. In men, detection of oligospermia and azoospermia, which are indicative of male infertility, by testing for cystic fibrosis, Y micro-deletion and karyotyping are essential to prevent any further complications in ART procedures such as IVF and ICSI. In addition to tests to identify any conditions that may cause complications prior to commencement of the ART procedures, couples must be counselled about the risks associated with the procedures [24,25].

Biomarkers indicative of reproductive impairment

Biomarkers indicative of reproductive disorders might aid in the real-time assessment of exposure, early detection, identification of patients who require immediate treatment, prediction of outcomes, monitoring of disease progress, and stratification of the population

on the basis of the etiology and severity of diseases. Despite the aforementioned benefits of biomarkers, the field of reproductive medicine has received remarkably little attention in the development of novel molecular signatures. This may be due to the limited number of novel molecular entities entering clinical investigations. With the increase in the number of molecular markers that have been identified in reproductive science, it is highly imperative to understand the pathway from discovery to translational impact of a disease-associated signature, as most markers may not be clinically relevant due to a variety of complexities [26,27]. Therefore, extensive evaluation, standardization, and validation are required before establishing a molecular signature as clinical biomarker for disease assessment and monitoring. In the most recent decade, biomarker research has witnessed a significant resurgence in interest due to advancements in "omics" methodologies, which involve generating voluminous amounts of data by high-throughput systems. These advances have made it possible to establish fascinating association of novel biomarkers and disease mechanisms in a relatively easy way for early screening [24,28]. The challenge is to validate such associations and to move them into the clinical setting. Reproductive medicine is still in its infancy in terms of integrating novel biomarkers into precision medicine. However, concerted efforts and speedy advancements in the research in reproductive medicine might narrow the gap of the translational process.

Pursuit of nanotechnology in reproductive medicine: accomplishments and challenges

Increasingly many studies have affirmed that impotence and reproductive disorders, such as endometriosis, adenomyosis, polycystic ovary syndrome, and uterine fibroids, negatively affect pregnancy. Repeated surgical interventions may reduce the likelihood of natural and assisted conception, which may result in an increasing dependence on third-party reproductive methodologies. The combination of nanotechnology-based approaches with reproductive medicine has led to the development of safer strategies for improving diagnosis and increasing precision and responsiveness [29]. Several studies have demonstrated the potential of nanomaterial-based methodologies that offer efficient noninvasive detection, treatment and monitoring of common disorders that may affect women of reproductive age (Figure 1).

1. Endometriosis

Endometriosis and fibroids represent the most common female reproductive disorders; endometriosis is present in approximately 10%–15% of reproductive-age women and in 70% of women with chronic pelvic pain. Endometriosis represents the commonest form

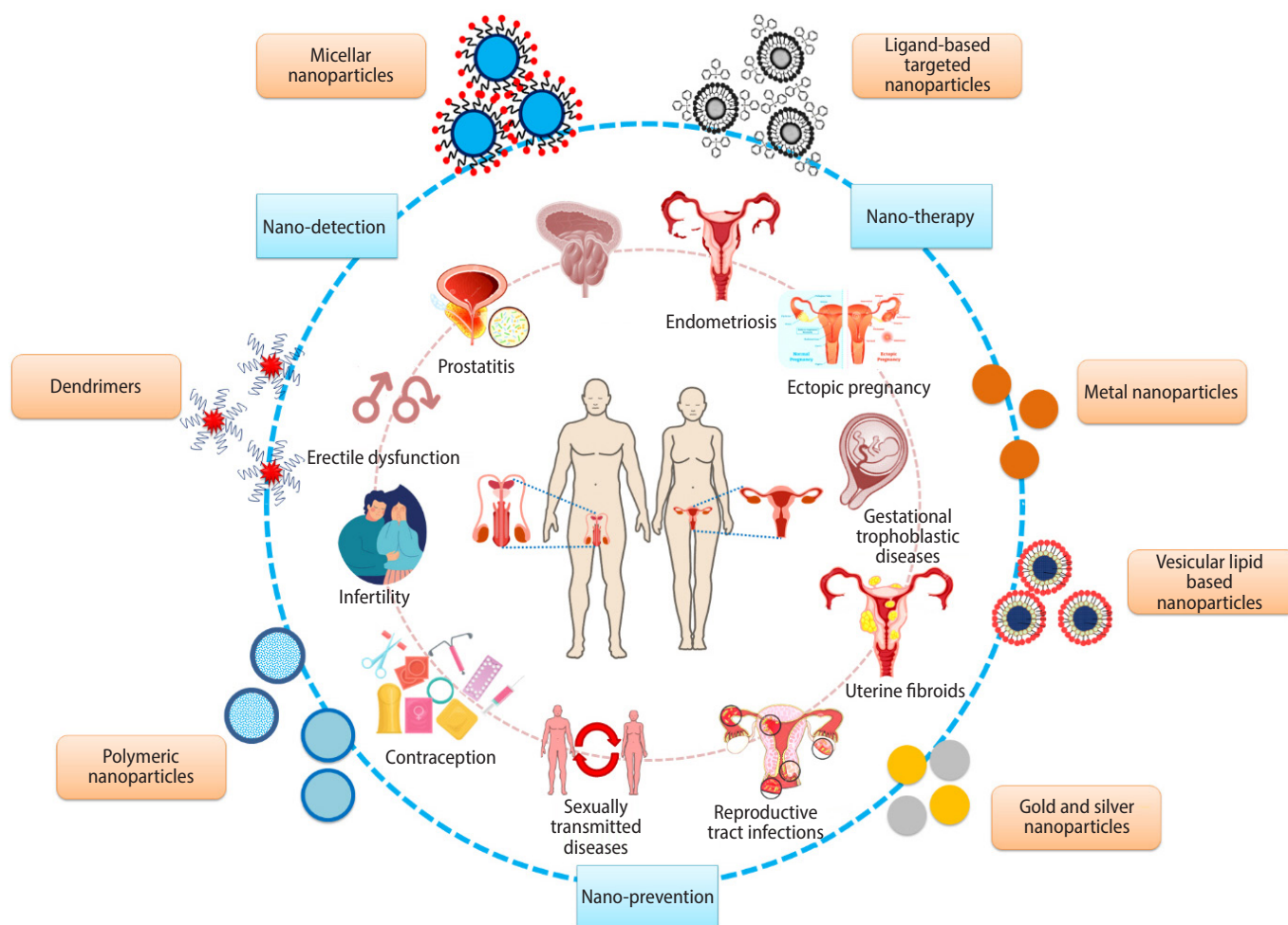


Figure 1. Nanotechnological strategies for addressing emerging issues in male and female reproductive health.

of pregnancy-related complication and affects both the endometrial glands and the stroma outside of the endometrial cavity. The increase in the percentage of women affected by endometriosis is due to the late diagnosis of the disease, which results in significant reduction in the quality of life due to symptoms including non-menstrual pelvic pain, dyspareunia, and infertility [30,31]. Since the majority of women report the symptoms of endometriosis in adolescence, early detection and timely treatment may significantly alleviate pain and avert progression of the disease, thereby preserving fertility. The treatment approach has recently been gradually shifting from surgical ablation to medication control, and it is therefore highly desirable to develop a non-surgical mode of diagnosis. Several investigative studies have reported the application of nanomaterial-based approaches for the treatment of endometriosis and uterine fibroids. For instance, a novel mitigation approach using nanoceria (cerium oxide nanoparticles) was demonstrated in an endometriosis-induced mouse model by Chaudhury et al. [32], who reported a considerable reduction of endometrial glands, microvessel density in the perito-

neum, and endometrial lesions upon treatment with nanoceria, highlighting the antioxidant and anti-angiogenic potential of the developed system, both *in vitro* and *in vivo*. The system also exhibited a promising reduction in the adverse effects related to endometriosis on the quality of oocytes, which is a critical factor for successful pregnancy. A novel herbal nanoformulation comprising a *Copaifera langsdorffii* oil-resin nanocomposite showed evidence of reduction of cell viability, alterations of cellular morphology, and induction of necrosis and apoptosis in proliferative primary endometrial and endometriotic stromal cells [33,34]. Singh et al. [35] reported a combinatorial approach for the treatment of endometriosis that consisted of dual drug-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles combining the anti-angiogenic and antioxidant properties of epigallocatechin gallate and the targeted matrix metalloproteinase inhibitory activity of doxycycline. A novel laser-mediated photothermal ablation therapy of endometriosis was reported to show enhanced permeability and retention and targeted delivery of gold nanoparticles to endometriotic sites using the TNYL peptide, which

has a strong affinity for overexpressed EphB4 receptors in endometriosis lesions [36].

The increase in the number of women experiencing discomfort due to endometriosis is attributable to late diagnosis; this problem is exacerbated if the ovaries are involved, resulting in the formation of cysts, often termed as endometriomas. The discomfort and complications associated with endometriosis may be avoided if it is diagnosed at an early stage, providing better possibilities for pain and infertility management. Conventional diagnostic procedures such as operative laparoscopy and biopsy are often invasive in nature, which is a major cause of distress among patients that hinders early screening [37]. Such situations could be avoided by employing nanomaterial-based sensing approaches, which offer real-time assessment of the disease. Several nano-sensing strategies have been proposed by investigators for effective detection of endometriosis, enabling timely commencement of therapy. The presence of endometriosis, owing to its inflammatory characteristics, is often characterized by disturbances in the immunological environment, which is strongly reflected by alterations in the expression levels of various cytokines, angiogenic factors, matrix metalloproteases, tumor suppressor genes, and circulating nucleic acids [38,39]. Cathepsins (in particular cathepsins B, D, and G), which belong to the class of proteases, have been quantitatively evaluated in patients with proliferative eutopic endometrium using a surface plasmon resonance (SPR) imaging technique, since cathepsins have been found to have a positive impact on the establishment of endometriotic lesions. The results of the study demonstrated a significantly higher expression of cathepsin G (CatG) in eutopic endometriotic patients than in the control group [40,41]. In view of this, an SPR-chip-based biosensor was developed by Grzywa et al. [42] for the selective determination of CatG in endometrium samples of patients and healthy controls. The gold-chip was surface-tethered with a phosphonic-type inhibitor that offered selective determination of active CatG in the sample at a picomolar concentration. For the *in vivo* detection of endometriotic lesions, hyaluronic acid (HA)-attached magnetic iron oxide nanoparticles (HA-Fe₃O₄ NPs) were developed as novel contrast agents for magnetic resonance imaging (MRI) in rodents. The results of the study highlighted the potential of the developed systems for hyperthermal treatment of endometriosis in the future [43].

2. Uterine fibroids

Uterine fibroids, often known as uterine leiomyomas, are non-cancerous benign tumors that often appear during pregnancy. After endometriosis, uterine fibroids are among the most commonly occurring solid tumors in women of reproductive age [31]. These neoplasms occur in about 77% of women, including 25% of those above the age of 45 years. The presence of fibroids in the uterus is often ac-

companied by symptoms with a major impact on quality of life, such as irregular and prolonged menstrual bleeding, anemia, pelvic discomfort, pelvic masses, bowel and bladder dysfunction, and obstetric complications. The presence of uterine fibroids has been strongly correlated with infertility and abortion, which significantly affect women's health and quality of life and may necessitate the use of ART [44,45]. Early treatment of fibroids, which hamper fertility, might increase the likelihood of future pregnancies. Shalaby et al. [46] demonstrated an effective noninvasive adenovirus-based alternative for the treatment of uterine fibroids using a combination of viral-based gene delivery with nanotechnology. A targeted magnetic nanoparticle-based approach was adopted for the efficient transduction of adenovirus under an external magnetic field. The novel combinatorial method offered a paradigm shift in therapeutic interventions for uterine fibroids, as it may significantly eliminate tumor-forming fibroid stem cells, which currently pose a major challenge to treatment.

3. Pregnancy-related complications: ectopic pregnancy and gestational trophoblastic diseases

Ectopic pregnancy is the most common cause of death among women during the early stages of pregnancy. An ectopic pregnancy occurs when the fertilized egg/embryo is implanted outside of the uterine cavity. The overwhelming majority of ectopic pregnancies (97%) involve implantation in the fallopian tube, while in the remaining 3% of cases, implantation occurs in the cervix, ovary, peritoneal cavity, or uterine scars. This pregnancy-related complication can result in rupture of the tube, leading to life-threatening internal bleeding. Ectopic pregnancies account for about 4% to 10% of pregnancy-related deaths and are characterized by a high incidence of ectopic site gestations in subsequent pregnancies [47]. Although several advances have been made in the detection and treatment of ectopic pregnancy, not all can be considered highly effective. Therefore, there is an urgent need for the development of diagnostic and therapeutic interventions that focus on precisely identifying and addressing the causes of the disease without compromising the other reproductive organs. Nanomedicine comprises an amalgamation of medicine and nanotechnology for the development of methodologies or strategies to improve the safety and efficacy of the conventional drugs by achieving targeted delivery to the site of action. Such precise targetability is urgently required for the development of novel methods of drug transport in women with pregnancy-related complications or diseases [48,49]. Fertility preservation-based surgical interventions are a major challenge in initial-stage ectopic pregnancy cases, especially among women with rare locations of extra-uterine pregnancies. This challenge has led medical practitioners to seek alternative methods for treatment. For instance, doxorubi-

cin-loaded EnGeneIC delivery vehicles were prepared for targeted delivery to the epidermal growth factor receptor expressed on placental cells. *In vitro*, *ex vivo*, and *in vivo* studies demonstrated significant inhibition of the trophoblastic tumor cell proliferation mediated by an increase in apoptosis, which offers a novel alternative to ectopic pregnancy treatments [50]. Complications could be avoided if ectopic pregnancy is diagnosed at the earliest possible stage. In recent years, significant advances in the field of nanotechnology have sparked research interest in the development of early detection approaches using nano-biosensing. Studies have reported the detection of human chorionic gonadotropin as an important protein marker indicative of pregnancy-related complications such as ectopic pregnancy, miscarriage, fetal abnormalities, and testicular tumors using ultrasensitive and highly selective carboxyl-graphene oxide-based SPR aptasensor and electrical double layer-gated field effect transistor-based chip sensor [51].

Another major pregnancy-related complication is gestational trophoblastic diseases (GTDs) which comprise a spectrum of tumors with a wide range of biological behavior and potential for distant metastases that arise from placental trophoblastic tissue after normal or abnormal fertilization [52]. Gestational choriocarcinoma is the most prevalent GTD. Despite progress in the field of modern reproductive medicine, gestational trophoblastic tumors continue to pose a significant challenges both for diagnosis and treatment owing to their irregularity, broad range of differences in diagnoses, and the uncertainty of detection of the precursor lesions [53]. These factors have fueled the need for extensive research on the development of precise and selective strategies for identifying and treating these tumors through nanotechnology. A glycosaminoglycan, placental chondroitin sulphate A (pLCSA), is highly expressed in a wide range of cancer cells and placental trophoblasts, highlighting its potential as a target in the treatment of GTDs [54]. Recently, a study investigated the efficient delivery of methotrexate to placental cells using synthetic pLCSA binding peptide (pLCSA-BP)-tethered lipid-based nanoparticles as a novel targeted approach for the treatment of pregnancy-related complications such as ectopic pregnancy and choriocarcinoma. The results of the research illustrated efficient binding of the pLCSA-BP attached to the methotrexate-loaded nanoparticles to human placental syncytiotrophoblasts and mouse trophoblasts throughout gestation after targeted delivery of the drug to the placenta of the mouse model without any fatal effects on the fetus [55]. Likewise, a similar study illustrated the development of pLCSA-BP-decorated polymeric core-lipid shell nanoparticles for the targeted delivery of doxorubicin to the placental choriocarcinoma (JEG3) cells [54]. Therapeutic interventions in patients with gestational trophoblastic choriocarcinoma often involve the application of systemic anti-neoplastic agents at high doses, which results in arbi-

trary distribution of the drugs to other organs and consequent systemic toxicity. To avoid such adverse effects, in light of the targeting potential of pLCSA-BP, Zhang and co-investigators demonstrated the efficient delivery of doxorubicin by nanoparticles to choriocarcinoma cells as a novel targeted cancer therapeutic approach. The results of the investigation reported competent internalization in the lysosomes resulting in increased *in vitro* anti-cancer action, rapid localization in the tumors, inhibition of primary tumor growth, and suppression of metastasis when observed *in vivo* using a rodent model [56].

4. Sexually transmitted infections and HIV

Reproductive tract infections (RTIs) are a concealed epidemic affecting the quality of life of both women and men, as well as compromising health and economic conditions throughout the world by means of their severe consequences, which include pelvic inflammatory disease, infertility (in both women and men), ectopic pregnancy, and adverse pregnancy outcomes such as miscarriage, stillbirth, preterm birth, and congenital infections. According to World Health Organization estimates, about 200 million cases of RTIs among women due to sexual transmission are reported every year in developing countries [5,57]. The reproductive-age population is highly susceptible to developing RTIs, especially women. RTIs include endogenously occurring infections such as bacterial vaginosis; infections transmitted due to sexual contact such as gonorrhoea, chlamydia, syphilis, chancroid, trichomoniasis, genital herpes, genital warts, and HIV; and iatrogenic infections such as pelvic inflammatory disease. Prompt diagnosis and treatment would reduce the transmission of these diseases [5,58]. Several studies have reported the use of nano-material-based treatment and monitoring approaches for safeguarding reproductive health and improving their quality of life. There is ample evidence of the usage of nanoparticles for the prevention, treatment, and early detection of RTIs, including STIs. For instance, oxygen vacancies comprising zinc oxide tetrapod nanoparticles have been fabricated for the nano-immunotherapy of genital herpes in females. This nano-system offered a great potential as an intravaginal microbicide/vaccine, leading to a significant reduction in vaginal infections and animal deaths, as well as an increase in the T cell-mediated and antibody-mediated responses, which subsequently suppressed re-infections [59]. Another group reported the treatment of genital herpes using acyclovir as a model drug encapsulated in polyvinyl pyrrolidone-Eudragit RSPO hybrid polymeric nanoparticles in the form of an *in situ* gel system. The developed formulation demonstrated a controlled release of the drug with improved permeability and viability for the vaginal epithelial cell lines, as well as a two-fold increase in the bioavailability of the drug in rat models in comparison to the pure drug, highlighting its potential for clinical therapy

[60]. Several polyphenolic compounds exhibit anti-viral properties, among which tannic acid was used by Orłowski et al. [61], for the development of a synergistically acting formulation against herpes simplex virus 2 (HSV-2). For this, silver nanoparticles, with inherent anti-microbial activity, were synthesized using green chemistry followed by modification with tannic acid, which in mice models demonstrated an increment in levels of interferon-gamma-positive CD8+ T cells, activation of B cells and plasma cells, and reduction of viral titers in vaginal tissues shortly after the treatment. Another therapeutic approach for HSV-2-induced genital infections using exclusively customized zinc oxide tetrapod nanoparticles was described by Agelidis et al. [62], who reported a significant inhibitory effect on viral vaginal infections in female Balb/c mice. The proposed microbio-vac system is a promising platform for the development of live virus vaccines. A novel polymeric nano-carrier system developed by Ganda et al. exhibited excellent potential for the delivery of peptide vaccines. The system comprised fourth-generation hydroxyl-terminated polyamidoamine (PAMAM) dendrimers (G4OH) conjugated with an ester bond to a chlamydial glycolipid antigen mimicking peptide (peptide 4; Pep4). The G4OH and Pep4 bond, upon dissociation in the intracellular environment, led to antigen presentation, which induced chlamydia-specific serum antibody recruitment after subcutaneous immunization. The action of the formulation demonstrated an increased anti-chlamydia antibody response in mice owing to enhanced and sustained Pep4 immunogenicity as a consequence of ester bond dissociation by phagolysosomes [63]. Several reports have described examples of nanoparticle-based delivery to the reproductive organs, which offer bioavailability and biodistribution to a higher degree, as well as prolonged release/action and retention of the drug in comparison to the drug alone. In light of this, Park et al. [64], demonstrated the potential of an anti-retroviral drug, elvitegravir, loaded surface-tailored bio-adhesive poly(lactic acid)-hyperbranched polyglycerols (PLA-HPG) nanoparticle formulation for prolonged intravaginal delivery. The developed formulation exhibited a remarkable improvement (roughly five-fold) in the sustained delivery of the drug in comparison to the non-adhesive alternative formulation. The results of the research highlighted the potential of the adhesive PLA-HPG nano-formulation for intravaginal therapeutics to treat and prevent STIs [65]. Aside from STIs associated with HSV, Wagner et al. [66] developed a nanoparticle-based targeted approach that comprised microbicidal drugs encapsulated in mucous-penetrating PLGA-PEG nanoparticles for intravaginal inoculation against STIs. Soler et al. [67] illustrated the development of a rapid nano-plasmonic biosensor that offered simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. The nano-biosensor comprised an array of gold nano-hole sensors that allowed precise detection and quantification of the levels of the two afore-

mentioned bacterial strains in an amplification-free fashion.

STIs are among the most prevalent diseases around the world. Despite extensive research, STIs such as HIV and HSV still haunt a large population due to their incurability. Considering the present situation, the global health initiative programs have primarily focused on reducing the incidence of STIs, especially in highly susceptible female populations [68]. Several nanotechnology-based therapeutic, preventive, and early diagnostic approaches have emerged as potential alternatives to conventional orally administered medications. For instance, Mandal et al. [69] reported that a conventional anti-retroviral drug, emtricitabine, was encapsulated in PLGA polymeric nanoparticles to circumvent the limitations associated with the large volume of distribution, short plasma life, low bioavailability, and cytotoxic nature of the drug to improve its efficacy for the therapeutic treatment of HIV infection. Similar studies have demonstrated the encapsulation of conventional anti-retroviral drugs such as zidovudine, a combination of three agents (zidovudine, efavirenz, and lamivudine); and stavudine in PF-68-coated alginate nanoparticles, and lactoferrin nanoparticles and gelatine liposomal nano-formulations have been reported as strategies for enhanced anti-HIV therapy [70-72]. Efficient microbicide action was displayed by a combination of a HIV reverse transcriptase-inhibiting drug (efavirenz) and an integrase inhibitor (elvitegravir) encapsulated in a graft copolymer of methoxy-polyethylene glycol-polylysine with a fatty hydrophobic core. The developed nano-formulation exhibited a significant reduction of cytotoxicity and *in vivo* bio-distribution upon topical intravaginal administration [73]. A similar effect was demonstrated by Mirani et al. [74], wherein a nano-formulation comprising a lipidic nano-microbicide gel was loaded with tetrahydrocurcumin. The formulation exhibited significant stability in the release profile and quick time-independent intracellular uptake, highlighting its potential for anti-retroviral therapy. A recent study illustrated a potential multiplexed *in vivo* treatment approach for HIV and HSV infections using a composite system consisting of methoxypoly(ethylene glycol)-b-poly(lactide-co-glycolide) Griffithsin nanoparticles incorporated in polycaprolactone fibers adjoined with polyethylene oxide fibers [75].

With advancements in the field of nanomaterials for diagnostic purposes, a surge of studies have been reported that offer rapid, and minimally-invasive methods for the early detection of STIs such as HIV and HSV. For the detection of human immunodeficiency virus type 1 (HIV-1) DNA, as a potential molecular signature of retroviral disease, a novel, user-friendly lateral flow assay was developed by Fu et al. [76] that utilizes surface-enhanced Raman scattering for precise identification and quantification. Another group reported the application of fluorescence resonance energy transfer for the detection of viral DNA using a combination of single-strand DNA (ssDNA)-tethered silver nanocrystals as donor and carbon nanoparticles as accep-

tor species. The combination exhibited desorption of the fluorescence in the presence of the target analyte, while restoration of the fluorescence in the absence of the same led to a highly sensitive diagnosis [77]. Fluorescence quenching as a mode of detection of HIV-1 DNA was employed by Deng et al. [78] and Fang et al. [79], who used fluorescent quantum dot-based lateral flow strips and surface DNA-decorated fluorescent silver nanocrystals, respectively. The HIV-related p24 antigen has emerged as another highly specific marker for the prompt detection of HIV in the recent years. Nano-detection approaches, such as a sandwich immunoassay amalgamating optoplasmonics and a micro-cantilever-based nanoplatform, have been developed for the precise detection of the HIV-1 capsid antigen p24 directly from human serum samples [80]. A similar sandwiched immunoassay developed by Chunduri et al. [81], involving the application of surface streptavidin-decorated europium-doped fluorescent silica nanoparticles, demonstrated a 100-fold improvement in detection of the HIV-1 p24 antigen in clinical samples compared to conventional enzyme-linked immunosorbent assay.

5. Gene therapy for reproductive disorders

Efficient gene therapy for the treatment of endometriosis was demonstrated by Zhao et al. [82], through the application of pigment epithelium-derived factor plasmid encapsulated in lipid-grafted chitosan micelles. An *in vivo* study in a rat model showed a significant diminishment of endometriotic lesions, as well as atrophy and degeneration of ectopic endometrium with no cytotoxic effect on the reproductive organs. A similar gene therapy for endometriosis was reported by Wang et al. [83], using endostatin-loaded PAMAM (PAMAM-Es) dendrimers in a non-invasive animal model. The results demonstrated a significant reduction in endometriotic lesions owing to the anti-angiogenic mechanisms of the PAMAM-Es in comparison to the traditional gene carrier, Lipofectamine, both in *in vitro* and *in vivo* settings. A novel polymeric gene delivery system, comprising HA-tethered polyethylenimine-grafted chitosan oligosaccharide nanoparticles encapsulating small interfering RNA (siRNA) was reported to considerably reduce the size of endometriotic lesions, with atrophy and degeneration of the ectopic endometrium. Endometriotic rat models exhibited a noteworthy decline in the expression of CD44 expression in the treated group with respect to the control endometriotic population [84]. Gene therapy using cyclic arginine-glycine-aspartic acid (cRGD)-attached fifth-generation PAMAM dendrimers to efficiently deliver siRNAs to spermatogonial stem cells (SSCs) demonstrated great potential for promoting auto-transplantation of SSCs with genetically modified cells as a curative approach for male infertility caused by genetic disorders [85].

6. Treatment of male associated disorders: erectile dysfunction

Reproductive disorders in men include benign prostatic hyperplasia (BPH) or prostate enlargement in general, prostatitis, and male infertility issues such as erectile dysfunction, testosterone deficiency, undescended testicle, varicocele or dilated veins around the testicle, and hydrocele or fluid around the testicle. BPH is often characterized by the increased growth of connective tissue, smooth muscle, and glandular epithelium upon histological visualization. As it progresses, BPH results in compression of the urethral pathways, leading to bladder outlet obstruction, which may subsequently result in lower urinary tract infections, retention, and other undesirable effects. Evidence supports the potential role of diet, lifestyle, and genetic factors, as well as metabolic syndrome and erectile dysfunction, on the incidence of hyperplasia of the prostate [86,87]. Although many choices of treatment are available, ranging from laser ablation methods medications (e.g., α -blockers, 5- α reductase inhibitors), and combination therapy to surgical procedures and medical devices (e.g., Prolieve and Urolift), the treatment options are often accompanied by unwanted effects like retrograde ejaculation, complications during urination, urinary tract infections, erectile dysfunction, and even incontinence (very rarely). In order to circumvent these undesirable events, nanomaterial-based therapies have been developed in recent years. For instance, de Sousa et al. [88] reported that PLGA nanoparticles and clay nano-systems encapsulating babassu oil were prepared for targeted delivery to hyperplastic tissue. Both the developed systems demonstrated an efficient encapsulation of about 90% with excellent bioavailability, highlighting the potential of the system for BPH therapeutics. Another study corroborated the ability of gold nanoparticles to target inflammation and angiogenesis for the development of novel BPH therapies. Adult male rats with experimentally induced BPH that received treatment with gold nanoparticles demonstrated a significant reduction of BPH in a size-dependent fashion with inhibition of inflammation, angiogenesis, and prostatic cell proliferation [89]. This could be a remarkable milestone for the progression of nanomaterial-based therapies towards clinical investigation for bench-to-bedside translation.

Prostatitis is a common urinary tract condition following prostate cancer and BPH that involves therapeutic challenges. Prostatitis accounts for about 8.2% of men, with an estimated expenditure of \$84 million on its diagnosis and treatment excluding pharmaceutical aids. Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) occurs in 90% to 95% of men with prostatitis, of whom 10% to 14% are severely affected. The symptoms of CP/CPPS range from a combination of acute and chronic bacterial prostatitis, CPPS, and none whatsoever (in asymptomatic patients). This medical condition is often diagnosed based on the patient's history, a physical assessment, urine specimen culture and testing, and pre- and post-prostatic massage.

After diagnosis, CP/CPPS is treated with medical agents such as antimicrobials, alpha blockers, and anti-inflammatory drugs. However, the conventional diagnostic and therapeutic approaches are often questioned due to limited availability of evidence supporting their effectiveness [90,91]. The effectiveness of these treatments could be improved by exploiting the targeting potential of the nanoparticles to prostatic tissue. With the targeting potential of nano-systems in mind, disease-relevant antigenic T2 peptide-conjugated biodegradable PLGA nanoparticles modified with poly ethylene-alt-maleic anhydride were prepared by Cao et al. [92] and were demonstrated to be an effective strategy for the treatment of prostatitis in a CP/CPPS-induced mouse model. A similar study by Cheng et al. [93] illustrated the preparation of PLGA nanoparticles coupled with auto-antigen peptide T2 for targeted action, initiation of immune tolerance, and amelioration of disease symptoms in a mouse model with induced CP and CPPS. Both studies presented a potential mitigation approach and an economically feasible tool for treatment of CP/CPPS.

Erectile dysfunction is a common problem impacting millions of men and is defined as the incapability of a man to achieve or maintain sufficient erection for satisfactory sexual performance. Despite the availability of various treatment options, ranging from first-line treatments with oral agents such as sildenafil, tadalafil, and vardenafil to second-line approaches such as intracavernosal injections and intra-urethral therapy, a significant proportion of men discontinue treatment. The discontinuation is directly attributed to the expensive nature of these therapies, as well as the fact that they involve discomfort, lack of spontaneity, prolonged erections, and priapism. However, with the advancements in the pharmaceutical industry, the search for alternatives for the delivery of vasoactive and vasodilatory medications is continuing [94,95]. Topical delivery has proven to be highly effective for the treatment of erectile dysfunction because local action avoids systemic effects and is easy to use. However, the applicability of topical methods is often hindered by the capability of the treatments to cross the barrier due to the penile skin and tunica albuginea, which inhibits the therapeutic efficacy of the drug. This limitation could be surmounted if efficient delivery of the drug is achieved, which is the reason for the success of nanoparticles in this field. A noteworthy example involved treatment and monitoring of erectile dysfunction by human mesenchymal stem cell- labelled super-paramagnetic iron oxide nanoparticles (SPION-MSCs) using MRI. The SPION-MSCs transplanted in the cavernosa of rats exhibited retention for about 4 weeks after cavernous nerve injury and enabled an efficient recovery of erectile dysfunction that could be monitored by MRI in an *in vivo* setting [96]. A formulation (NanoShuttle) comprising a complex of adipose-derived stem cells and magnetic nanoparticles demonstrated effective *in vivo* cell tracking in the cor-

pus cavernosum under the effects of a magnetic field for up to 3 days and was shown to be an efficient stem cell therapy for erectile dysfunction in an animal model [97]. The dendrimer-based delivery of vardenafil hydrochloride was demonstrated by Tawfik et al. [98], and sustained release of the drug was found, with a 3.7-fold improvement in bioavailability. Among the pharmaceutical nano-formulations, lipid-based systems have attracted significant attention in the field of reproductive medicine owing to their capability to improve the solubility, bioavailability, and biocompatibility of poorly water-soluble medications. Fahmy's work illustrated the development of a biocompatible nanoethosome formulation of vardenafil for enhanced permeation via the transdermal route and improved bioavailability. The developed system, which exhibited a 3.05-fold improvement in permeation, consisted of a vardenafil powder-containing film. A two-fold increment in the transdermal bioavailability was found when the topical nanoethosome film was used in comparison to an aqueous suspension of vardenafil. This transdermal delivery system provides a potential method of curing erectile dysfunction and managing impotence [99]. A similar study using nano-transferosomal transdermal films as a mode of delivery of sildenafil citrate reported a significant enhancement in the *ex vivo* permeation characteristics, controlled release, bioavailability, and absorption of the drug [100]. Another study demonstrated the transdermal delivery of papaverine to the penis using an ultra-flexible liposomal formulation (nano-transferosomes) for the effective diagnosis and treatment of erectile dysfunction. The prepared formulation exhibited remarkable potential as a therapy for male impotency [101]. A considerable improvement in the bioavailability and transdermal delivery of avanafil, a first-line drug for the treatment of erectile dysfunction, was observed when it was delivered in the form of solid lipid nanoparticle-based hydrogel film [102].

7. Nano-based approaches for treatment and diagnosis of male infertility

Infertility is a condition wherein a couple is unable to become pregnant despite being sexually active and avoiding contraception. Male factor infertility can result from a low sperm count, poor sperm quality, or both. According to global statistics, about 15% of couples suffer from infertility-related issues, and in 50% of these couples, the male partner is affected by aberrations in sperm properties, count, vitality, and morphology [103,104]. This fact underscores the need to develop novel methodologies for the early identification of the causes of infertility and approaches for the personalized treatment of infertility. In this aspect, nanotechnology has come to the forefront in recent years, offering better solutions for infertility related issues. One such solution was described in the work of Moridi et al. [105], who found that the deleterious effect of malathion, a common or-

ganophosphorus pesticide, on the male reproductive system was remarkably reduced by the application of cerium dioxide nanoparticles (CeNPs). Restoration of testicular changes was observed upon treatment with CeNPs in malathion-exposed male rats, and the nanoparticles exerted a protective effect on sperm count, motility, and viability. Another study utilized the magnetic properties of surface charged Fe_3O_4 nanoparticles for the development of a method of controlling sperm motility as a novel and simplified approach for the improvement of fertility in infertile males [106]. Rapid and precise detection of semen abnormalities is crucial for diagnosing male infertility and arranging customized care. The size and abundance of samples, however, impose a number of detection constraints [104]. One such solution was proposed by Vidya and Saji [107], wherein a quick screening approach utilizing an environment-friendly, minimally-invasive, and label-free heparin gold nanoparticle-based colorimetric biosensor was developed for the detection of semen protamines as effective biomarkers of male infertility. The nano-biosensor demonstrated noteworthy changes in plasmon absorption spectra upon specific detection of protamines in real semen and serum samples. A similar colorimetric nano-biosensing approach for human semen analysis was proposed by Sun et al. [108]. The nano-biosensor comprised zirconium metal-organic frameworks (Zr-MOFs) coupled with single-stranded DNA-decorated gold nanoparticles (ssDNA-AuNPs), and precise detection was enabled by the possibility that the target proteins in the test sample would hamper the co-precipitate formation of Zr-MOFs and ssDNA-AuNPs. This change manifests as an alteration in the color of the supernatant, offering the potential to accurately identify possible cases of male infertility with utmost simplicity and high sensitivity.

8. Nanomaterials in contraception

Contraception has been a major topic of debate for decades, as promoting birth control and family planning not only benefits individuals' health and well-being, but also helps facilitate economic growth. The current methods of birth control usually involve administration of hormonal contraceptive medications through the oral, transdermal, intravaginal, and intrauterine routes. Several other approaches such as female sterilization, male condoms, and intrauterine devices (IUDs) have attracted significant attention as potential alternatives to conventional drugs. However, these approaches are faced by challenges such as an increased risk of blood clots or breast cancer associated with the long-term usage of contraceptive pills, the risk of failure of condoms, and the irreversible nature of vasectomies [109,110]. This situation clearly indicates the increasing need for new developments in both male and female contraceptive methods [111]. The past decades have witnessed remarkable improvements in contraceptive methods owing to nanotechnological advance-

ments. Chitosan nanoparticle-based immuno-contraceptive vaccines have exhibited efficient peptide and protein delivery with increased uptake by dendritic cells and retention in the lymph nodes. The vaccines target luteinizing hormone-releasing hormone, thereby increasing the generation of antibodies that hamper reproductive capabilities; thus, this framework offers a potential system of contraception development [112]. Another study used biodegradable polymer-based polyethylene sebacate particulates as effective carriers for the delivery of an HSA peptide-1 vaccine [113]. The improved immunogenic activity of an mCRISP1 DNA contraceptive vaccine was demonstrated by chitosan-DNA nanoparticles that exhibited high effectiveness and safety [114]. Apart from vaccine delivery, photo-thermal therapy using *in situ* testicular injection of methoxypoly (ethylene glycol)-modified gold nano-rods under near-infrared light in male mice led to male infertility. The high temperature induced by plasmonic nanomaterials resulted in complete destruction of the morphological characteristics of the testes or seminiferous tubules, leading to loss of fertility [115]. Remarkable achievements have been made in combining IUDs with nanomaterials to develop effective and long-term contraceptives. For instance, nano-copper and polymeric substances such as polydimethylsiloxane, silicone rubber, and low-density polyethylene-based nanocomposite have been developed for potential use in IUDs as novel, safe and pragmatic alternatives for contraceptive applications [116-118]. A cocktail-inspired medium-term, reversible male contraceptive strategy was proposed by Bao et al. [119], wherein four layers of nanomaterials were injected sequentially into the vas deferens. The first layer comprised a hydrogel for the formation of a physical barrier to sperm, the second layer contained gold nanoparticles for heating on irradiation, the third layer was made up of ethylenediaminetetraacetic acid (EDTA), which functioned as a system for breaking the hydrogel and exterminating the sperm, and the fourth layer comprised gold nanoparticles. An *in vivo* study of this approach in a male rat model exhibited inhibition of impregnation of females for more than 2 months. The reversible nature of the approach was confirmed, as near-infrared irradiation led to dissolution of all the layers, allowing the rats to conceive [119]. A biodegradable polymer-based point-of-care micro-needle patch encapsulating contraceptive hormones was developed by Li et al. [120] and demonstrated sustained release for more than a month.

Conclusion

In the modern world, maintaining sustainable health of both men and women is a major challenge due to rapidly evolving lifestyle changes and environmental impact. Several maternal and fetal factors exhibit a strong correlation with adverse outcomes of children

Table 1. Summary of conventional and nano-based approaches used for diagnostic and therapeutic applications in reproductive medicine

Domain	Detection	Treatment	Reference
Conventional approaches used in reproductive medicine			
1. Reproductive tract associated ailments			
a. Endometriosis	<ul style="list-style-type: none"> • Pelvic examination • Transvaginal ultrasound imaging • MRI • Laparoscopy • Ultrasound imaging and MRI • Hysterosonography • Hysterosalpingography • Hysteroscopy 	<ul style="list-style-type: none"> • Administration of NSAIDs such as ibuprofen or naproxen sodium • Hormonal therapy • Conservative surgery • Hysterectomy with removal of the ovaries • Administration of medications such as gonadotropin-releasing hormone agonists; progesterin-releasing intrauterine devices; tranexamic acid and NSAIDs • MRI-guided focused ultrasound surgery • Minimal invasive methods such as uterine artery embolization; radiofrequency ablation; myomectomy and endometrial ablation 	[121]
b. Uterine fibroids			
c. Ectopic pregnancy	<ul style="list-style-type: none"> • hCG blood test • Transvaginal and abdominal ultrasound imaging 	<ul style="list-style-type: none"> • Administration of medications • Laparoscopic surgery (e.g., salpingostomy and salpingectomy) 	[124,125]
d. Gestational trophoblastic diseases	<ul style="list-style-type: none"> • Blood and urine tests of hCG • Placental examination • Lumbar puncture or spinal tap test • Imaging procedures such as ultrasound, MRI, chest X-ray, computed tomography, and positron emission tomography 	<ul style="list-style-type: none"> • Dilatation and curettage with suction evacuation • Hysterectomy • Chemotherapy • Radiation therapy 	[52]
2. Sexual health			
a. Reproductive tract infections and sexually transmitted diseases	<ul style="list-style-type: none"> • Microscopy • Immuno-chromatography • Tissue culture • Nucleic acid hybridization • Nucleic acid amplification • Physical examination • Blood and urine analysis • Ultrasonography • Nocturnal penile tumescence test 	Treatment with antibacterial, antifungal, and antiviral agents	[17,126]
b. Erectile dysfunction		<ul style="list-style-type: none"> • Oral administration of drugs such as sildenafil, tadalafil, vardenafil, etc. • Testosterone therapy • Penile injections • Intra-urethral medications • Vacuum erection devices • Penile implants • Surgery 	[127,128]

(Continued to the next page)

Table 1. Continued

Domain	Detection	Treatment	Reference
c. Male infertility	<ul style="list-style-type: none"> • Physical examination • Analysis of semen • Scrotal and transrectal ultrasound • Hormone (testosterone level) testing • Post-ejaculation urinalysis • Genetic analysis • Testicular biopsy 	<ul style="list-style-type: none"> • Antibiotic treatment of infections • Fertility improving medications • Hormonal therapy • Surgery • Assisted reproductive technology 	[129,130]
3. Contraception	<ul style="list-style-type: none"> • Oral, transdermal, intravaginal, and intrauterine administration of hormonal contraceptive medications • Use of injectable contraceptives, implants, intrauterine devices, contraceptive vaginal rings, and barrier methods • Surgical methods 		[110,131]
Applications of nanotechnology in reproductive medicine			
1. Reproductive tract-associated ailments			
a. Endometriosis	<p>Lipid nanoparticles encapsulating chemotherapeutic agents for targeted action on endometriotic foci [132]</p> <p>Chitosan oligosaccharide-g-stearic acid polymer micelles encapsulated in nano-structured lipid carriers for the delivery of P2X3 receptor antagonist, A-317491 for reduction of endometriotic pain. [133]</p> <p>A combinatorial near-infrared fluorescence and photothermal therapy-based (SiNC nano-platform was developed, composed of SiNC and a polymeric nanoparticle for the systemic administration in the endometriosis tissue. [134]</p> <p>A nonsurgical adenovirus-magnetic nanoparticles-based complex system was developed for the treatment of uterine fibroids. [46]</p> <p>EnGeneC delivery vehicles are nanocells containing doxorubicin for targeted delivery to epidermal growth factor receptor and treatment of ectopic pregnancy. [50]</p>		[55,56]
d. Gestational trophoblastic diseases	<p>Lipid based and polymeric core-lipid shell nanoparticles loaded with anti-neoplastic agents have been developed for the targeted delivery to placental trophoblasts.</p>		
2. Sexual health			
a. Reproductive tract infections and sexually transmitted diseases	<p>Several point-of-care setups are available for the detection of sexually transmitted diseases. Alere Determine Syphilis TP is a rapid lateral flow test from Abbott Laboratories Inc. (USA) for the detection of syphilis.</p> <p>DPP Syphilis Screen & Confirm Assay is a TP and non-TP-based point-of-care test for syphilis. The test involves a unique combination of protein A and anti-human immunoglobulin M antibody conjugated to colloidal gold particles.</p> <p>A microfluidic sperm sorting system was developed for the ultrahigh-throughput selection and separation of motile, DNA-intact, and functionally competent sperm. The low-dose insemination of microfluidic sorted spermatozoa improved fertility, leading to successful production of live births.</p>		[135]
b. Erectile dysfunction and male infertility	<p>An <i>in situ</i> depot system was developed, composed of biodegradable poly(lactide-co-glycolide) and poly (lactic acid) polymers encapsulating a contraceptive agent (levonorgestrel) for sustained delivery via intravenous administration.</p>		[136]
3. Contraception			[137]

MRI, magnetic resonance imaging; NSAIDs, nonsteroidal anti-inflammatory drugs; hCG, human chorionic gonadotropin; SiNC, silicon naphthalocyanine; TP, treponema pallidum.

and mothers. Increasingly many studies have reported that infertility and reproductive disorders such as endometriosis, polycystic ovary syndrome, and uterine fibroids have a harmful effect on conception, starting from the first events of implantation and lasting until term delivery. These risks have led to the increasing use of ART; however, the use of ART is often accompanied by other deleterious effects that directly or indirectly hamper fertility or pregnancy-related outcomes. In addition to this, the tendency for women to become pregnant at older ages has contributed to an increased risk of gestational complications. Therefore, it has become more important to develop novel strategies for the management of reproductive health. Therapeutic, preventive, and diagnostic approaches in combination with nanotechnology have provided remarkable opportunities for the development of superior strategies in reproductive medicine, with the projected aim of improving the quality of life of both men and women (Table 1). Despite enormous advances, nanotechnological approaches in reproductive health face several shortcomings, such as the limited availability of validation studies of both diagnostic and therapeutic interventions and toxicity issues related to the use of nanomaterials. Most trials involving nanoengineering methods for reproductive health management are still at the nascent stage. There is a sharp dearth of evidence supporting the effective clinical translation of these approaches; therefore, it is highly essential to conduct meticulous research in the future to establish the potential applicability of nanotechnological methodologies for the real-time prevention, treatment, and monitoring of disease. In order to bridge the gap between translational research and product development, a careful choice of qualified partners will be highly essential. Strategic partnerships with the insight and ability to anticipate challenges early on, as well as in-depth understanding of the impact of early processes on later-stage development, will have considerable advantages in overcome the regulatory gauntlet and technical hurdles, thereby facilitating effective clinical translation of these engineered nano-particles for use in reproductive medicine. Nonetheless, several serious concerns linked to the potential risks of the systemic and local toxicity of engineered nanomaterials, the disruption of metabolic function, and potential immune pathophysiological consequences of long-term usage must be addressed. Therefore, the utilization of nanomaterials to treat any form of reproductive ailment must be done after careful compliance with regulatory pharmacological and toxicological guidelines. There is not an iota of doubt that nanotechnology will help to bring about specific, sensible, and cost-effective clinical interventions that are suitable, acceptable, and more personalized in the future.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Autophagy in the uterine vessel microenvironment: Balancing vasoactive factors

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Autophagy, which has the literal meaning of self-eating, is a cellular catabolic process executed by arrays of conserved proteins in eukaryotes. Autophagy is dynamically ongoing at a basal level, presumably in all cells, and often carries out distinct functions depending on the cell type. Therefore, although a set of common genes and proteins is involved in this process, the outcome of autophagic activation or deficit requires scrutiny regarding how it affects cells in a specific pathophysiological context. The uterus is a complex organ that carries out multiple tasks under the influence of cyclic changes of ovarian steroid hormones. Several major populations of cells are present in the uterus, and the interactions among them drive complex physiological tasks. Mouse models with autophagic deficits in the uterus are very limited, but provide an initial glimpse at how autophagy plays a distinct role in different uterine tissues. Herein, we review recent research findings on the role of autophagy in the uterine mesenchyme in mouse models.

Keywords: Autophagy; Mesenchyme; Mice; Uterus; Vessel

Introduction

The uterus is composed of multiple cell types that respond distinctively to hormones and growth factors [1]. The epithelial lining, the innermost layer facing the lumen, is where the embryo attaches for implantation. The underlying stroma contains a sparse population of fibroblasts that secrete extracellular matrix proteins to fill the space among blood vessels and lymphatic vessels. The stroma contains numerous capillaries and spiral arteries, as well as diverse types of leukocytes, in the deep regions. The outermost layer, the myometrium, consists of smooth muscle cells; the uterine length and diameter can

be regulated by coordinated actions of longitudinal and circular layers of smooth muscle cells. The uterine capillaries are surrounded by a single endothelial layer, similar to other capillaries found throughout the body, but larger vessels found in deep stromal regions and the myometrium are also surrounded by vascular mural cells (i.e., smooth muscle cells and pericytes). Blood flow to the uterus and vascular permeability are thought to be regulated by ovarian steroid hormones and vasoactive factors [2].

As a dynamic organ that responds to cyclic changes of the reproductive cycle and pregnancy, the uterus experiences tissue remodeling occurring at regular intervals. The uterus also ages with waning levels of steroid hormones depending on the species. Multiple female-specific pathologic conditions involving the uterus exist that are related or unrelated to pregnancy [3]. Its collective complexity makes the uterus a difficult-to-study organ system in gene-deletion studies. In this review, I attempt to present the possible outcomes of autophagic deficit in uterine tissue-specific mouse models based on previous studies and unpublished observations.

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Autophagic process in brief

Three types of autophagic processes exist; microautophagy, macroautophagy, and chaperone-mediated autophagy [4]. Macroautophagy, the main bulk degradation pathway involved in turning over macromolecules and organelles, will hereafter be referred to as autophagy. The autophagic process is governed by proteins encoded by autophagy-related (*Atg*) genes, along with other associated factors [5]. The process begins with the formation of a horseshoe-shaped stretch of membrane near a target within the cytoplasm. This membrane grows with the addition of lipids from various subcellular sources until the two ends meet to form a closed vesicle called an autophagosome [6]. Initially, the contents within the autophagosome and double membrane structure are visible at the ultrastructure level [7]. Next, the autophagosome fuses with other vesicular structures such as endosomes or lysosomes. As degradation of the intravesicular contents begins, the double membrane structure and targets are no longer clearly visible. Therefore, it is customary to examine the ultrastructure of cells when attempting to demonstrate autophagic activation. This is considered the most direct evidence of autophagy [8].

Atg proteins and others involved in the pathway are subjected to post-translational modifications such as phosphorylation, acetylation, lipidation, and ubiquitination [5]. The most widely used method of visualizing heightened autophagy is to show the ratio of LC3B-I and LC3B-II changes in cells or tissues of interest on western blots. Pro-LC3B is first processed by *Atg4* to LC3B-I, which, in turn, is lipidated by the *Atg7-Atg3* complex. An increased ratio of LC3B-II/LC3B-I generally indicates an increased autophagic rate. Autophagy has a clear endpoint: the degradation of intravesicular contents and recycling. Trapped protein targets are tagged with a marker of destruction called sequestosome-1 (SQSTM1/p62) protein. SQSTM1 is a polyubiquitin-binding protein that links protein targets to autophagic degradation [9]. When autophagic flux is high and works well, the amount of SQSTM1 is maintained at a low level. In contrast, any problem that occurs in the autophagic process, such as the deletion of an important upstream *Atg* (*Atg5* or *Atg7*) or defective fusion between an autophagosome and endosome/lysosome, results in the accumulation of SQSTM1 in cells. In mammalian cells, an increased ratio of LC3B-II/ LC3B-I, along with a decrease in SQSTM1 levels under certain conditions, is considered to indicate increased autophagic flux [8].

Immunofluorescence staining of LC3B or SQSTM1 is also widely used, but interpreting the data requires caution. As autophagic vacuoles at different maturation stages form small vesicular structures within cells, the presence of LC3B-II on the membranes of autophagic vacuoles or SQSTM1 within them should exhibit puncta-like pat-

terns in the cytoplasm, rather than a diffuse pattern. Extensive guidelines for the use and interpretation of autophagy assays are available [8].

Searching for a suitable *in vivo* model system

Various autophagy-deficient mouse models are available for *in vivo* studies [10,11]. Systemic deletion of *Atg* genes in core conjugation machinery generally leads to neonatal lethality, precluding the possibility of studying gene function in adult tissues and organs [11]. Tissue-specific knockout of floxed *Atg7* using various *Cre* models has been widely used to study the physiological roles of autophagy [10,12].

Atg7, an E1 ubiquitin-activating enzyme, activates LC3B and *Atg12* during autophagosomal membrane elongation. As one of the major components of the *Atg* conjugation system, the deletion of *Atg7* shuts down autophagy during the initial stages of autophagosome formation, leading to neonatal lethality in knockout mice [12]. *Atg5* is also a crucial component of the conjugation system, and its systemic deletion leads to neonatal lethality [13]. The *Atg5* knockout mouse model was later further exploited in a study where the rescue of neonatal lethality was attempted by re-expressing transgenic *Atg5* in neurons [14]. The neuron-specific re-expression of *Atg5* in the *Atg5* knockout background averted the neonatal death of the knockout mice, while still retaining an autophagic deficit in the rest of the body. In this model, a diverse array of organ abnormalities was observed. One remarkable phenotype was hypogonadism in both male and female transgenic mice [14].

The pituitary gland and gonads all showed high levels of SQSTM1 accumulation in these transgenic mice, suggesting that these organs heavily depend on autophagy for normal functions. The uteri of these *Atg5* knockout mice were severely hypoplastic, which may be due to hypogonadism [14]. This study presented clear evidence that the reproductive hormonal axis requires normal autophagic flux to support fertility. Therefore, to establish how autophagy is involved in normal uterine functions, a uterine-specific knockout model was in demand.

Several cell type-specific expression models of *Cre* recombinase gene are available to study gene function in the uterus. Anti-Mullerian hormone type 2 receptor (*Amhr2*)-*Cre* is a knock-in model that expresses *Cre* in the uterine mesenchyme-derived cells only, except for a subset of cells on the mesometrial side [15]. The uterine epithelium and endothelial cells were excluded from gene deletion. Lactoferrin (*Ltf*) promoter-regulated *Cre* mice achieve floxed gene deletion only in the uterine epithelium [16]. Progesterone receptor (*Pgr*)-*Cre* is a knock-in model where *Cre* replaces 1 copy of *Pgr* [17]. This model shows the widest range of *Cre* expression in almost all cells present

in the uterus, including endothelial cells [18] and various immune cells [19,20].

Our laboratory previously produced uterine tissue-specific deletion models of floxed *Atg7* (*Atg7^{fl/fl}*) with the aforementioned *Cre* models. Initial surveys searching for the site of high SQSTM1 levels in all three models revealed an interesting phenomenon. As we had already reported that hormone deprivation achieved by ovariectomy (OVX) turns on autophagy in the uterus [21], we surmised that OVX of these *Cre* mice would lead to SQSTM1 accumulation in cells where autophagy is active. Therefore, we compared the status of SQSTM1 accumulation in OVX mice in all three models. *Amhr2-Cre/Atg7^{fl/fl}* mice showed high SQSTM1 accumulation in the uterine stroma and myometrium. As expected, some cells in the mesometrial side did not show SQSTM1 accumulation, as *Atg7* was retained in that area (Figure 1A). This result indicate that autophagy is generally active in these mesenchymal cells. *Pgr-Cre/Atg7^{fl/fl}* mice were expected to remove floxed *Atg7* in all uterine cells including the epithelium. However, *Pgr-Cre/Atg7^{fl/fl}* uteri showed high SQSTM1 accumulation in the stroma and myometrium, but limited accumulation in the epithelium (Figure 1B). This result suggests that the uterine epithelium does not depend on autophagy as much as the uterine mesenchyme. This notion was further demonstrated in the *Ltf-Cre/Atg7^{fl/fl}* uteri, where SQSTM1 was almost undetectable in the *Atg7*-deleted uterine epithelium. A small population of luminal epithelial cells showed some puncta, as indicated by an arrow in Figure 1C. From these initial surveys, it became evident that the uterine mesenchyme heavily depends on autophagic clearance.

Uterine vessel microenvironment demands autophagy

During reproductive cycles and pregnancy, progesterone and estrogen regulate the proliferation and differentiation of cells in a tissue-specific manner. Such cyclic changes produce diverse molecules with different functions, and dynamic turnover of these molecules is expected to occur to facilitate clearance. What are the pathophysiological outcomes of uterine cells in OVX mice when the autophagic process is entirely blocked? This question was addressed through the use of *Amhr2-Cre/Atg7^{fl/fl}* mice, where the need for dynamic autophagic flux was demonstrated by high SQSTM1 accumulation in the uterine mesenchyme [22].

In various systems, the autophagic rate can increase beyond a basal rate when cells face certain changes within themselves or their surrounding environment, such as inflammation, viral infection, stress, hypoxia, or deprivation of nutrients [4,6,23]. Conditions affecting the autophagic rate vary greatly depending on the cell or tissue type. As a highly hormone-responsive organ, the uterus responds to

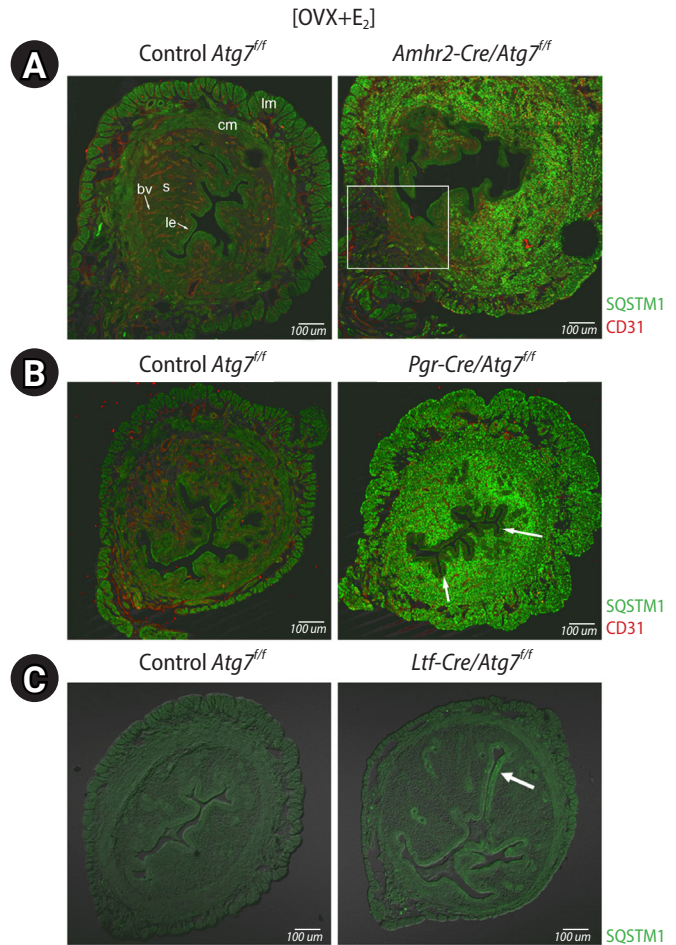


Figure 1. SQSTM1 accumulation in uterine-specific *Atg7* deletion models after ovariectomy. Approximately 8- to 10-week-old mice were ovariectomized and rested for 2 weeks for hormone clearance. They received an injection of 17β -estradiol (E_2) 24 hours before euthanasia. Uterine cross-sections were subjected to immunofluorescence staining with anti-SQSTM1 antibody. SQSTM1 signals are shown as green puncta in cells with autophagic deficit. Scale bar=100 μ m. lm, longitudinal muscle; cm, circular muscle; bv, blood vessel; s, stroma; le, luminal epithelium. (A) *Amhr2-Cre/Atg7^{fl/fl}* mice. Since the *Amhr2* promoter is not active in some mesenchymal cells in the mesometrial side (white rectangle area), *Atg7* is retained here. High SQSTM1 accumulation is shown in the stroma and myometrium, suggesting that autophagy is in demand in these cells. CD31, a marker of endothelial cells, was co-stained red. (B) *Pgr-Cre/Atg7^{fl/fl}* mice. The *Pgr* promoter is active throughout the uterus. SQSTM1 accumulation is shown in the stroma and myometrium, but the epithelium does not show distinct accumulation. Some cells in the luminal epithelium show weak SQSTM1 accumulation (arrows). This suggests that the demand for autophagic turnover is much lower in the uterine epithelium than in the mesenchyme. (C) *Ltf-Cre/Atg7^{fl/fl}* mice. The *Ltf-Cre* promoter is active in the uterine epithelium. Autophagic deficit is expected in the epithelium, but SQSTM1 accumulation is only weakly shown in some areas of the luminal epithelium (arrow).

OVX and increases autophagy in all major uterine cell types [21]. Systemic deprivation of steroid hormones also induces autophagy in other organs, such as the kidneys and prostate [24]. In *Amhr2-Cre/Atg7^{ff}* mice, accumulation of SQSTM1 in the uterine mesenchyme begins to be noticeable within 3 days after OVX (unpublished observation). In random-cycling *Amhr2-Cre/Atg7^{ff}* mice, SQSTM1 puncta begin to show in the mesenchyme of 4-week-old uteri at a much lower intensity than in OVX *Amhr2-Cre/Atg7^{ff}* mice [22]. Therefore, in the mouse uterus, hormone deprivation increases the autophagic rate, especially in the stroma and myometrium [21], as evidenced by high SQSTM1 accumulation in these tissues in OVX *Amhr2-Cre/Atg7^{ff}* mice. When steroid hormone levels are adequate during the reproductive cycle and pregnancy in intact mice, drastic increases in the autophagic rate probably do not occur.

The phenotype of the *Amhr2-Cre/Atg7^{ff}* model was further scrutinized to identify factors generally targeted by autophagy. *Amhr2-Cre/Atg7^{ff}* mice were found to be fertile, producing pups comparable to those of control mice. One outstanding characteristic of *Amhr2-Cre/Atg7^{ff}* uteri was that their stromal regions showed exaggerated edema [22]. Water imbibition in the uterus was significantly higher in the *Amhr2-Cre/Atg7^{ff}* uteri than in the control uteri, suggesting that the *Amhr2-Cre/Atg7^{ff}* uterine vessels are leakier and hyperpermeable. The uterine blood vessels were found to be more relaxed and leakier than those of control mice based on the expression levels of the endothelial junctional proteins [22]. Nitric oxide (NO) is a strong vasorelaxant produced by NO synthases (NOS). Among the three forms of NOS, NOS1 is present at notably increased levels in *Amhr2-Cre/Atg7^{ff}* uteri [22]. Based on these strong vascular phenotypes, it was surmised that autophagy is in strong demand for the maintenance of dynamic uterine vascularity.

Phenotype hints at the targets of uterine autophagy: vasoactive factors

The uterine mesenchymal cell populations can be roughly separated from the thick myometrium and uterine epithelium using the conventional method of uterine stromal cell (USC) preparations [25]. This method yields a heterogeneous cell population containing fibroblasts, smooth muscle cells, endothelial cells, immune cells, and vascular pericytes [25]. The vascular phenotype in *Amhr2-Cre/Atg7^{ff}* uteri prompted us to focus on the dysregulation of vasoactive factors in isolated USCs [22].

Vascularity is governed by various vasoactive factors. Vascular endothelial growth factor A (VEGFA) is an essential vasoactive factor in the uterus [18,26] that increases vascular permeability and dictates adult tissue angiogenesis in the mouse uterus under hormonal effects [2]. VEGFA was indeed identified as one of the proteins accumu-

lated in *Amhr2-Cre/Atg7^{ff}* USCs. VEGFA sits at the top of the vasoactive regulatory pathway by regulating the function of other vascular factors. One of the deciphered pathways involves increasing the production of NO in the vessel microenvironment, thereby causing vessel relaxation and leakiness [27]. NO does this by nitrosylating the seminal endothelial junction stabilizing the protein β -catenin, leading to the disintegration of the endothelial barrier [28]. In *Amhr2-Cre/Atg7^{ff}* uteri, several observations have aligned to indicate the potential mechanism of uterine hyperpermeability: autophagic deficit leads to VEGFA overaccumulation, which, in turn, produces a greater amount of NO and, consequently, the disintegration of the endothelial barrier. The notion that NO is the mediator of hyperpermeability was demonstrated by the use of the NOS inhibitor, N-nitroarginine methyl ester (L-NAME). L-NAME administration to OVX *Amhr2-Cre/Atg7^{ff}* mice decreased stromal edema and restored the levels of β -catenin.

Physiological interventions: compensation of hyperpermeability in the absence of autophagy

While these results summarize what happens in the uterine mesenchyme in the absence of autophagy, other changes in *Amhr2-Cre/Atg7^{ff}* uteri have demonstrated remarkable compensatory changes to counterbalance the hyperpermeability phenotype [22]. The USC populations from *Amhr2-Cre/Atg7^{ff}* uteri consistently had a significantly greater number of cells than USCs from control mice. Among many cell populations, the melanoma cell adhesion molecule (MCAM/CD146)-positive population showed the most dramatic increase in USCs from *Amhr2-Cre/Atg7^{ff}* uteri compared to control USCs. MCAM is a broad mesenchymal marker [29,30] that is important for the maintenance of vascular permeability in the blood-brain barrier [31]. Therefore, an increased MCAM+ population in *Amhr2-Cre/Atg7^{ff}* uteri with leaky vessels could be an attempt to fortify vessels to alleviate the hyperpermeability phenotype.

Endothelin-1 (EDN1) is a potent vasoconstrictor expressed in many tissues, including the uterus [32]. In *Amhr2-Cre/Atg7^{ff}* USCs, the expression level of Edn1 mRNA is significantly decreased, whereas its protein levels are much higher than that in control USCs [22]. The heightened EDN1 levels would normally suggest a vasoconstriction phenotype, but vessels in *Amhr2-Cre/Atg7^{ff}* uteri remain relaxed. Therefore, EDN1 accumulation appears to be a compensatory mechanism to reduce overly relaxed vessels. However, in *Amhr2-Cre/Atg7^{ff}* uteri, overrepresented VEGF signaling appears to override EDN1 action.

Conclusion

The uterus, a dynamic organ with many functions, is constantly influenced by factors produced both outside of and within itself. The uterus undergoes cyclic changes in cell proliferation and differentiation, stromal edema, angiogenesis, and regeneration; however, it is resilient to all these changes. It is plausible that bulk degradation by autophagy is required for quality control in this versatile tissue. Compared to the uterine mesenchyme, the epithelium seems almost quiescent with respect to autophagic activation. Further investigation is warranted to reveal the mechanism of protein quality control in the uterine epithelium. In mice, gene-manipulated models generally provide the most direct evidence for gene function. In humans, where no such models are available, uterine and placental tissues obtained during various procedures have been used as the primary source for autophagy research. Review articles summarizing the potential role of endometrial or placental autophagy are recommended for further reading [33,34].

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Impact of imatinib or dasatinib coadministration on *in vitro* preantral follicle development and oocyte acquisition in cyclophosphamide-treated mice

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Objective: We investigated the impact of tyrosine kinase inhibitor (imatinib or dasatinib) coadministration with cyclophosphamide (Cp) on preantral follicle development in an *in vitro* mouse model.

Methods: Seventy-three female BDF1 mice were allocated into four experimental groups: group A, saline; group B, Cp (25 mg/kg); group C, Cp (25 mg/kg) and imatinib (7.5 mg/kg); and group D, Cp (25 mg/kg) and dasatinib (7.5 mg/kg). Preantral follicles were isolated and cultured *in vitro* up to 12 days. Final oocyte acquisition and spindle integrity of metaphase II (MII) oocytes were assessed. Levels of 17 β -estradiol and anti-Müllerian hormone (AMH) in the final spent media were measured by enzyme-linked immunosorbent assays, and the mRNA levels of *Star*, *Sod1*, *Mapk3*, and *Casp3* in the final follicular cells were quantified by real-time polymerase chain reaction.

Results: The percentage of MII oocytes per initiated follicle, the proportion of MII oocytes with normal spindles, and the 17 β -estradiol level were similar in all four groups. The median AMH level in group B (7.74 ng/mL) was significantly lower than that in group A (10.84 ng/mL). However, the median AMH levels in group C (9.96 ng/mL) and group D (9.71 ng/mL) were similar to that in group A. The mRNA expression levels of *Star*, *Sod1*, *Mapk3*, and *Casp3* were similar in all four groups.

Conclusion: Coadministration of imatinib or dasatinib with Cp could preserve AMH production capacity in this *in vitro* mice preantral follicle culture model, and it did not affect MII oocyte acquisition.

Keywords: Cyclophosphamide; Dasatinib; Imatinib; Oocyte; Tyrosine kinase

Introduction

Administration of cyclophosphamide (Cp) to female cancer pa-

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tients may severely impair ovarian function or lead to premature ovarian failure [1-4]. The mechanisms of Cp-induced ovarian follicular damage are not fully understood, although a proposed mechanism is the apoptotic pathway that occurs in response to intracellular DNA damage [5-7]. Cp-induced intracellular apoptosis frequently occurs in growing follicles in the ovary, especially in proliferating granulosa cells [8]. In response to intracellular DNA damage caused by Cp, intracellular c-Abl is strongly activated, and cell death is subsequently induced through the TAp63 pathway [9-11]. The c-Abl protein is fused to the breakpoint cluster region (BCR) protein to form the breakpoint cluster region-Abelson murine leukemia viral oncogene 1 (BCR-ABL) complex, which acts as an oncogenic tyrosine ki-

nase, thus leading to cell deformation and leukemic disease.

Tyrosine kinase inhibitors, such as imatinib or dasatinib, are used clinically to treat chronic myeloid leukemia and acute lymphoblastic leukemia. Tyrosine kinase inhibitors inhibit c-Abl or the BCR-ABL complex. The inhibitory effect of dasatinib on BCR-ABL complex is 324 times stronger than that of imatinib, and dasatinib is therefore used in patients with resistance to imatinib [12].

Simultaneous administration of imatinib with cisplatin in mice has been reported to be effective in preserving ovarian follicles [10,11]. In those reports, it has been proposed that imatinib could inhibit the c-Abl-TAp63 pathway, thereby reducing cisplatin-induced follicular damage in the mouse ovary. Furthermore, coadministration of imatinib in Cp-treated mice has been reported to increase the oocyte yield, fertilization rate, and the embryo developmental rate [13]. However, there have been no reports regarding the ovoprotective role of dasatinib. In this study, we investigated the impact of imatinib or dasatinib coadministration on *in vitro* preantral follicle development and oocyte acquisition in Cp-treated mice.

Methods

1. *In vitro* culture of mice preantral follicles

Female 7- to 8-week-old BDF1 mice (Orient Co., Seoul, Korea) were maintained under 12-hour light and a 12-hour dark conditions at 23°C and fed ad libitum. All experiments were conducted ethically with the approval of the Institute of Animal Care and Use Committee of the Seoul National University Bundang Hospital (IACUC No. BA-1903-267-014-04).

After 1 week of adaptation, mice were divided into 4 groups and then treated with an intraperitoneal injection of each agent. In group A, which served as a control group, 0.1 mL of saline was injected once. In group B, Cp (25 mg/kg body weight) (Cp monohydrate; Cat. no. 29875, Sigma-Aldrich, St. Louis, MO, USA) was injected once. In group C, Cp (25 mg/kg) and imatinib (7.5 mg/kg; Enzo Life Sciences, Farmingdale, NY, USA) was injected once. In group D, Cp (25 mg/kg) and dasatinib (7.5 mg/kg; Enzo Life Sciences) was injected once. An imatinib or dasatinib stock solution was obtained by dissolving imatinib or dasatinib powder in phosphate-buffered saline (PBS). Three days later, the mice were killed by cervical dislocation, and the bilateral ovaries were collected in 1 mL of L-15 medium (WelGENE, Daegu, Korea) supplemented with 0.4% bovine serum albumin (BSA; Sigma-Aldrich).

Intact preantral follicles were mechanically isolated and cultured in 96-well plates (BD BioCoat; BD Falcon, Franklin Lakes, NJ, USA) at 37°C in 5% CO₂ for 10 days in a growth medium. The growth medium was composed of alpha-minimum essential medium (WelGENE), 5% fetal bovine serum (Gibco, Paisley, UK), 10 mIU/mL recombinant

follicle-stimulating hormone (Merck-Serono, Geneva, Switzerland), 1% insulin-transferrin-selenium mixture (Sigma-Aldrich), and 1% penicillin-streptomycin mixture (Sigma-Aldrich). Every 3–4 days, the medium was changed, and follicle survival and formation of the antrum were assessed. Oocytes were considered to be dead if they were not surrounded by granulosa cells or if the granulosa cells appeared to be dark and fragmented, and the follicle decreased in size.

After 10 days of culture in the growth medium, the follicles were transferred to maturation medium and cultured for 16 hours at 37°C in 5% CO₂. The maturation medium was prepared by adding 1.5 IU/mL human chorionic gonadotropin (Merck-Serono), and 5 ng/mL recombinant mouse epidermal growth factor (Sigma-Aldrich) to the growth medium. The oocytes were then harvested either from spontaneously ruptured or non-ruptured follicles. The surrounding cumulus cells were removed by treating them with 0.3% hyaluronidase (Sigma-Aldrich) and gentle pipetting. Oocytes were classified as metaphase II (MII), metaphase I (MI), germinal vesicle, dead, or degenerated. If a polar body was present in the perivitelline space, the oocytes were regarded as MII oocytes. Fragmented or shrunken oocytes were classified as degenerated oocytes. Figure 1 shows mouse preantral follicles and various developmental stages of follicles during *in vitro* culture, as well as in the finally obtained oocytes.

2. Meiotic spindle integrity

The spindle integrity of the MII oocytes was assessed using previously described methods [14]. The MII oocytes were washed three times with 1% BSA in PBS for 5 minutes and then fixed with 4% paraformaldehyde for 1 hour at room temperature (RT). After washing twice with 1% BSA in PBS, permeabilization was performed with 0.25% Triton X-100 in PBS for 10 minutes at RT. After washing twice with 1% BSA in PBS, blocking was performed with 3% BSA in PBS for 1 hour at RT and then washing twice with 1% BSA in PBS. A primary antibody for α -tubulin (Cell Signaling, Danvers, MA, USA) diluted in 1% BSA (1:100) was added and incubated overnight at 4°C. After washing three times with 1% BSA in PBS, a secondary antibody (Alexa Fluor 488 goat anti-rabbit immunoglobulin G; Invitrogen, Carlsbad, CA, USA) diluted in 1% BSA (1:100) was added for 1 hour at RT in the dark. After washing three times with 1% BSA in PBS, the oocytes were air-dried on a silane-coated slide (DAKO, Glostrup, Denmark). The slide was counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and examined using a confocal microscope (LSM 710; Carl Zeiss, Oberkochen, Germany). A typical barrel-shaped microtubule structure between both poles with centrally aligned chromosomes was considered normal (Figure 2).

3. Measurement of hormones in the final spent media

In each experimental group, the final spent media, in which five

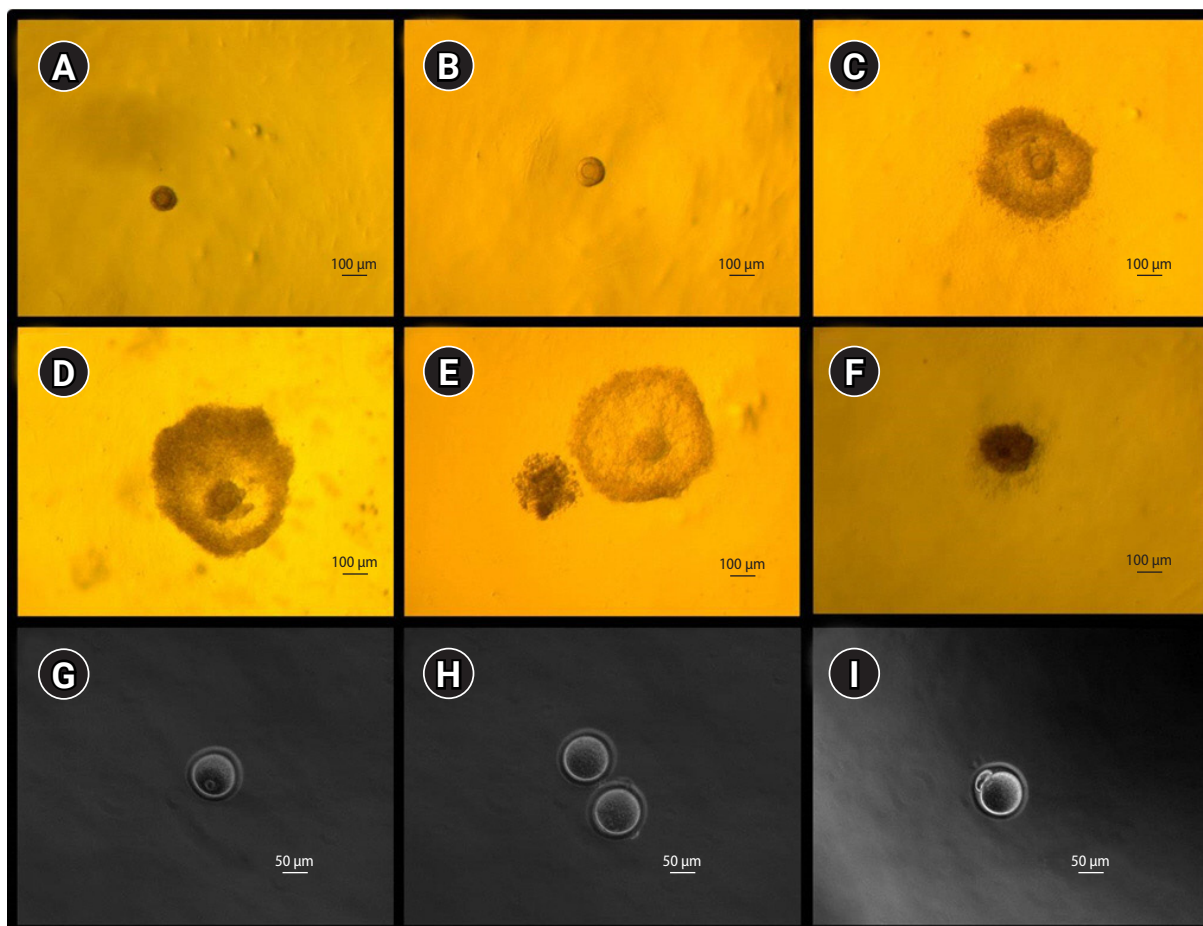


Figure 1. Microphotographs showing mice preantral follicles, *in vitro* growing follicles (A-F; $\times 60$) and the resultant oocytes (G-I; $\times 200$). (A) Preantral follicle at day 0, (B) growing follicle at day 4, (C) growing follicle at day 8, (D) antral follicle at day 10, (E) ruptured (ovulated) follicle at day 11, (F) dead follicle at day 8, (G) germinal vesicle oocyte, (H) two metaphase I oocytes, and (I) metaphase II oocyte.

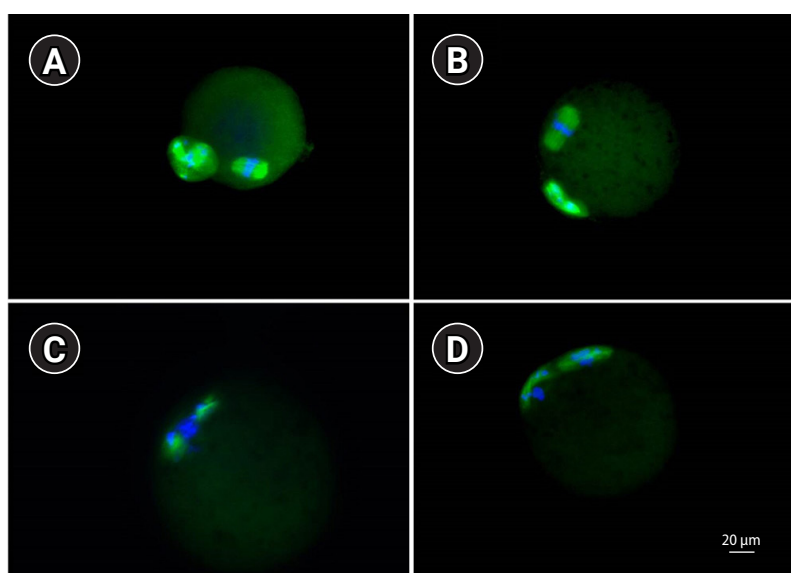


Figure 2. Representative confocal microphotographs showing meiotic spindle organization and chromosome alignment in metaphase II oocytes ($\times 400$). (A, B) Normal metaphase II, (C, D) abnormal metaphase II.

ruptured follicles were present, were pooled and then frozen at -80°C . After thawing, the levels of 17β -estradiol and anti-Müllerian hormone (AMH) were measured by commercially available enzyme-linked immunosorbent assay kits. The limits of sensitivity for 17β -estradiol (Enzo Life Sciences) and AMH (Ansh Labs, Webster, TX, USA) were 10 pg/mL and 0.01 ng/mL, respectively.

4. Real-time quantitative polymerase chain reaction

The final follicular cells from 20 ruptured follicles were pooled (after isolation of oocytes) and then frozen at -80°C . After thawing, total RNA was extracted using the Dynabeads method (Dynabeads mRNA DIRECT kit; Ambion, Oslo, Norway), and cDNAs were synthesized using a PrimeScript first strand cDNA Synthesis Kit (Takara, Kusatsu, Japan) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) was performed using a StepOne-Plus real-time PCR system with TaqMan probes (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was conducted in a 20- μL reaction volume containing 10 μL of Applied Biosystems TaqMan Universal PCR Master Mix I (Cat. no. 4427788), 2 μL of cDNA, and 6 μL of RNase-free water. Specific primers were purchased from Integrated DNA (Coraville, IA, USA). Primer sequences and their product sizes, accession number, annealing temperature, and total PCR cycles of each gene are listed in Table 1.

In this study, the mRNA level of steroidogenic acute regulatory protein (*Star*; a marker of luteinization), superoxide dismutase 1 (*Sod1*; a marker of oxidative stress), mitogen-activated protein kinase 3 (*Mapk3*; a marker of cell growth), and caspase 3 (*Casp3*; a marker of apoptosis) were quantitatively measured. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control. *Star* transports cholesterol from the outer mitochondrial membrane to the inner membrane and converts it into pregnenolone, which regulates steroid hormone synthesis, especially during the luteal phase [15]. *Sod1* is present in the cytoplasm and catalyzes the disproportionation of superoxide into hydrogen peroxide and dioxygen by binding to copper or zinc ions [16]. *Mapk3* serves as a signaling molecule and involves cell growth. In the granulosa cells of preovulatory follicles, it is activated by a luteinizing hormone (LH) surge and plays a role in the LH-induced oocyte resumption of meiosis, ovulation, and luteinization [17]. *Casp3* is the last enzyme in the cell death process and is a marker of apoptosis [10,18].

In each experimental group, at least five repeats were conducted. Real-time quantitative PCR was repeated five times for each repeat, and the values were averaged. For each experiment, five different readouts were obtained for each gene of interest. The measured values were obtained as the cycle threshold (Ct) at a constant fluorescence intensity. The level of each transcript was inversely related to the observed Ct value. The relative expression (R) levels of the genes were normalized to that of *Gapdh* as an internal control. The ΔCt value was calculated as follows: the Ct of the target gene minus the Ct of *Gapdh*. The $\Delta\Delta\text{Ct}$ value was calculated as ΔCt minus the mean value of each group. To determine the fold change for each gene, the relative gene expression of the four treatment groups was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method as previously described [19].

In each experimental group, at least five repeats were conducted. Real-time quantitative PCR was repeated five times for each repeat, and the values were averaged. For each experiment, five different readouts were obtained for each gene of interest. The measured values were obtained as the cycle threshold (Ct) at a constant fluorescence intensity. The level of each transcript was inversely related to the observed Ct value. The relative expression (R) levels of the genes were normalized to that of *Gapdh* as an internal control. The ΔCt value was calculated as follows: the Ct of the target gene minus the Ct of *Gapdh*. The $\Delta\Delta\text{Ct}$ value was calculated as ΔCt minus the mean value of each group. To determine the fold change for each gene, the relative gene expression of the four treatment groups was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method as previously described [19].

5. Statistical analysis

Statistical analyses were performed using IBM SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA), and *p*-values < 0.05 were considered to indicate statistical significance. The Fisher exact test was used to compare the proportions among the groups. Numerical data were compared using the Kruskal-Wallis test. If the value was significant, the Mann-Whitney *U*-test with the Bonferroni correction was used for further analysis.

Results

The detailed outcomes of *in vitro* growth of preantral follicles and

Table 1. Primer sequences and their conditions for real-time PCR

Gene	Primer sequence	Product size (bp)	Accession number	Annealing temperature ($^{\circ}\text{C}$)	Cycle
<i>Star</i>	F: CCTCCAAGCGAAACACCTT	109	NM_011485	60	40
	R: GGCATACTCAACAACCAGGAA				
<i>Sod1</i>	F: GTCCTTTCCAGCAGTCACAT	146	NM_011434	60	40
	R: GGTTCACGTCATCAGTATG				
<i>Mapk3</i>	F: TCCATGAGGTCTGAACAATG	112	NM_011952	60	40
	R: TCCAGATCTTGCTGCGATTC				
<i>Casp3</i>	F: GGAAGTGGATGAACCACGAC	124	NM_009810	60	40
	R: GACTGATGAGGAGATGGCTTG				
<i>Gapdh</i>	F: GTGGAGTCATACTGGAACATGTAG	150	NM_008084	60	40
	R: AATGGTGAAGGTCGGTGTG				

PCR, polymerase chain reaction; *Star*, steroidogenic acute regulatory protein; *Sod1*, superoxide dismutase 1; *Mapk3*, mitogen-activated protein kinase 3; *Casp3*, caspase 3; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

the percentage of MII oocytes with normal spindles in group A (saline), B (Cp), C (Cp and imatinib), and D (Cp and dasatinib) are presented in Table 2. There were no significant differences in the follicle survival rate, antrum formation rate, rupture rate, and total oocyte acquisition rate per initiated follicle among the four groups. The percentage of MII oocytes per initiated follicle and the proportion of MII oocytes with normal spindles were also similar in all four groups.

The median level of 17 β -estradiol in the final spent media was similar among the four groups (Table 3). Nonetheless, the median AMH level in group B (7.74 ng/mL) was significantly lower than that in group A (10.84 ng/mL). However, the median AMH levels in group C (9.96 ng/mL) and group D (9.71 ng/mL) were similar to that in group A. The relative mRNA levels of *Star*, *Sod1*, *Mapk3*, and *Casp3* in the final follicular cells were similar in all four groups (Figure 3).

Discussion

We investigated the impact of imatinib or dasatinib coadministration with Cp on preantral follicle development in an *in vitro* mouse model. In the groups that received imatinib or dasatinib in addition to Cp, follicle survival, antrum formation, spontaneous follicular rupture, acquisition of total and MII oocytes, and the proportion of MII oocytes with normal spindles were all similar to the Cp-only group. Thus, imatinib or dasatinib coadministration with Cp might not af-

fect *in vitro* mice preantral follicle development and healthy oocyte acquisition. However, imatinib or dasatinib coadministration with Cp could preserve AMH levels in the final spent media. This indicates that imatinib or dasatinib coadministration with Cp could help to preserve AMH production capacity in ruptured follicles *in vitro*.

In the present study, administration of Cp (25 mg/kg) reduced AMH levels but preserved estradiol levels in the final spent media. Generally, AMH is produced principally in primary/preantral follicles, whereas estradiol is mainly produced in antral/mature follicles. Harvested preantral follicles might be initially damaged by Cp administration, and this damage might reduce AMH production capacity, which was maintained during the 12-day culture period. However, estradiol is produced mainly in secondary/mature follicles; therefore, *in vitro* ruptured follicles in the Cp-treated group might produce estradiol to a similar degree to the group that did not receive Cp treatment.

It has been reported that primordial follicles are most sensitive to Cp treatment, followed by antral and growing follicles [20]. In that report, although growing follicles were sometimes damaged by Cp treatment, those follicles were found to be the least sensitive to Cp; they recovered well, and thus could produce estradiol. A further investigation would be necessary to verify the preservation of primordial/preantral follicles and their ability to produce AMH after administration of Cp with imatinib or dasatinib at the ovarian level.

Table 2. Outcomes of *in vitro* growth of mice preantral follicles under four treatment conditions

Variable	Saline	Cp	Cp+imatinib	Cp+dasatinib
No. of mice	15	16	23	19
No. of preantral follicles initiated	100	145	143	145
No. of follicles survived at day 10 (% per initiated follicle)	97 (97.0)	131 (90.3)	132 (92.3)	132 (91.0)
No. of follicles with antrum formation (% per initiated follicle)	75 (75.0)	91 (62.8)	99 (69.2)	100 (67.0)
No. of follicles with spontaneous rupture (% per initiated follicle)	67 (67.0)	84 (57.9)	84 (58.7)	85 (58.6)
No. of total oocytes (% per initiated follicle)	67 (67.0)	82 (56.6)	83 (58.0)	83 (57.2)
No. of degenerated oocytes	0	0	0	0
No. of GV oocytes	34	44	60	51
No. of GVBD oocytes	20	28	14	20
No. of MII oocytes (% per initiated follicle)	13 (13.0)	10 (6.9)	9 (6.3)	12 (8.3)
Proportion of MII with normal spindle, n (%)	6/7 (85.7)	6/9 (66.7)	7/9 (77.8)	9/12 (75.0)

Cp, cyclophosphamide; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II.

Table 3. Hormone level in the final spent media after *in vitro* growth of mice preantral follicles under four treatment conditions

Variable	Saline	Cp	Cp+imatinib	Cp+dasatinib	<i>p</i> -value ^{a)}
Repeat	18	20	20	20	
17 β -estradiol (pg/mL)	366 (234–600)	483 (191–772)	331 (230–612)	481 (160–727)	0.995
AMH (ng/mL)	10.84 ^{b)} (9.52–12.54)	7.74 ^{c)} (6.23–9.06)	9.96 (7.97–12.10)	9.71 (8.59–12.65)	0.003, < 0.001 ^{b)-c)}

Values are presented as median (interquartile range).

Cp, cyclophosphamide; AMH, anti-Müllerian hormone.

^{a)}Kruskal-Wallis test.

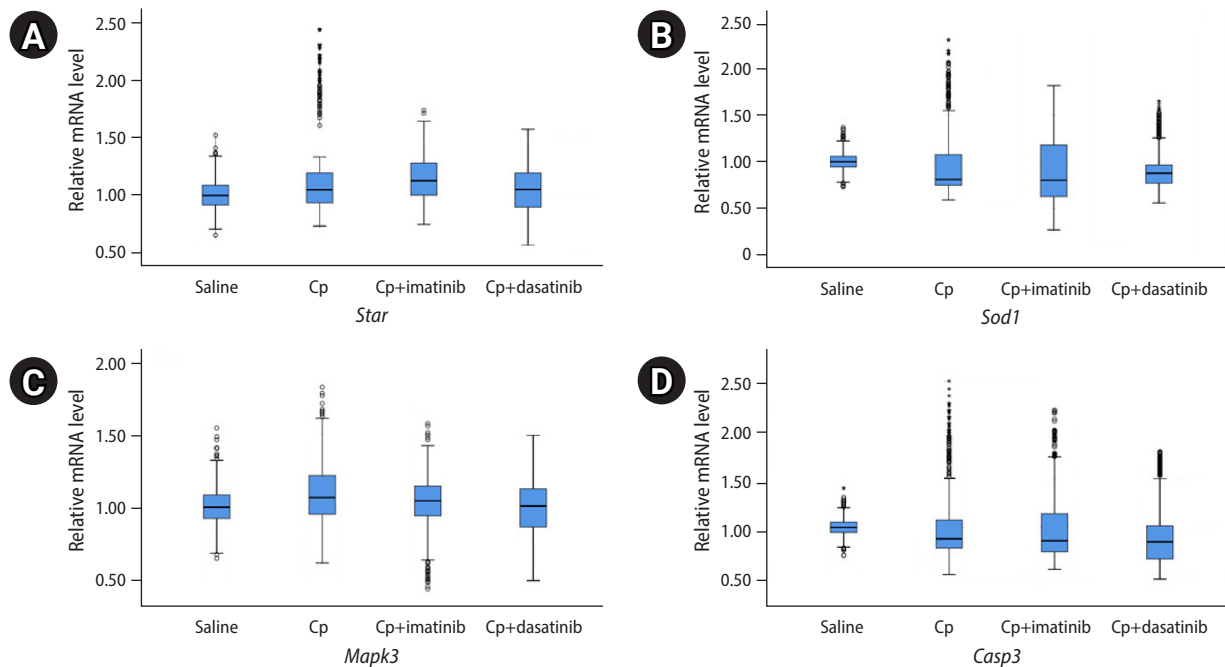


Figure 3. Relative mRNA levels of four genes through real-time polymerase chain reaction. The mRNAs were extracted from the final follicular cells after 12 days of culture of the preantral follicles. The preantral follicles were harvested 3 days after intraperitoneal injections of 0.1 mL of saline, cyclophosphamide (Cp; 25 mg/kg), Cp (25 mg/kg)+imatinib (7.5 mg/kg), and Cp (25 mg/kg)+dasatinib (7.5 mg/kg). (A) Steroidogenic acute regulatory protein (*Star*), (B) superoxide dismutase 1 (*Sod1*), (C) mitogen-activated protein kinase 3 (*Mapk3*), (D) caspase 3 (*Casp3*).

It is known that AMH suppresses primordial follicle recruitment, thereby maintaining the dormancy of the ovarian follicle pool; in contrast, the loss of AMH activates primordial follicle recruitment and finally depletes the primordial follicle pool [21]. Our finding that AMH production was preserved in mice that received imatinib or dasatinib coadministration with Cp suggests that imatinib or dasatinib may protect fertility. Imatinib has been reported to play a role in preserving mouse primordial follicles after cisplatin treatment [11,22]. Since imatinib is not a specific inhibitor of tyrosine kinase, dasatinib—as a more specific and potent compound—could play a role as a fertoprotective agent, such as amifostine, ceramide-1-phosphate, or mammalian target of rapamycin inhibitors (e.g., everolimus and rapamycin) [23,24].

A mouse experiment recently demonstrated that imatinib itself has no impact on folliculogenesis. However, it remains generally unknown whether tyrosine kinase inhibitors could modulate the function of ovarian follicles [25]. Since tyrosine kinase inhibitors affect the signaling of c-kit, platelet-derived growth factor receptor, and c-Src, which are also key regulators in the ovary, the direct effect of imatinib or dasatinib on folliculogenesis should be investigated [26].

In the present study, we investigated mouse ovarian follicle development 3 days after exposure to a single dose of imatinib or dasatinib with Cp. However, a further study will be needed to verify the impact of various durations of exposure or various doses of imatinib or

dasatinib with Cp. Moreover, further research is required on the impact of imatinib or dasatinib with Cp on ovarian or embryo-related parameters. In conclusion, coadministration of imatinib or dasatinib with Cp did not affect *in vitro* preantral follicle development and healthy oocyte acquisition in mice, but could preserve AMH production capacity in the final ruptured follicles. Our findings suggest that imatinib and dasatinib might have a fertoprotective role by preserving AMH production.

Conflict of interest

Byung Chul Jee has been an editor of Journal of Clinical and Experimental Reproductive Medicine since 2018; however, he was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflict of interest relevant to this article was reported.

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Sperm chromatin structure assay versus sperm chromatin dispersion kits: Technical repeatability and choice of assisted reproductive technology procedure

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Objective: The sperm DNA fragmentation index (DFI) guides the clinician's choice of an appropriate assisted reproductive technology (ART) procedure. The DFI can be determined using commercially available methodologies, including sperm chromatin dispersion (SCD) kits and sperm chromatin structure assay (SCSA). Currently, when DFI is evaluated using SCD kits, the result is analyzed in reference to the SCSA-derived threshold for the choice of an ART procedure. In this study, we compared DFI values obtained using SCSA with those obtained using SCD and determined whether the difference affects the choice of ART procedure.

Methods: We compared SCSA to two SCD kits, CANfrag (n=36) and Halosperm (n=31), to assess the DFI values obtained, the correlations between tests, the technical repeatability, and the impact of DFI on the choice of ART.

Results: We obtained higher median DFI values using SCD kits than when using SCSA, and this difference was significant for the CANfrag kit ($p < 0.001$). The SCD kits had significantly higher coefficients of variation than SCSA ($p < 0.001$). *In vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) would be chosen for a significantly higher proportion of patients if a decision were made based on DFI derived from SCD rather than DFI determined using SCSA ($p = 0.003$).

Conclusion: Our results indicate that SCD kit-specific thresholds should be established in order to avoid the unnecessary use of IVF/ICSI based on sperm DNA damage for the management of infertility. Appropriate measures should be taken to mitigate the increased variability inherent to the methods used in these tests.

Keywords: Assisted reproductive technology; DNA fragmentation; Male infertility; Sperm

Introduction

Successful pregnancy depends on several factors, including the integrity of the sperm chromatin, which is represented by the sperm

DNA fragmentation index (DFI). An elevated DFI is inconducive to both fertilization and pregnancy [1-8]. Clinicians therefore recognize the value of DFI in the evaluation of male infertility in couples with recurrent pregnancy failure and in the choice of an appropriate assisted reproductive technology (ART) procedure. A number of methodologies are currently available to assess DFI. Two of these detection methods are based on the denaturing capacity of sperm chromatin: sperm chromatin structure assay (SCSA) and sperm chromatin dispersion (SCD) kits.

SCSA, as the gold standard for the assessment of sperm DNA fragmentation, consists of a fixed flow cytometry protocol, requires a

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proprietary software program (SCSAsoft; SCSA Diagnostics, Brookings, SD, USA) and produces a highly repeatable measure of DFI [9]. SCD kits are technician-dependent light microscope tests that measure 50–500 sperm per sample to provide a DFI based on the presence or absence of a dispersion halo around the fragmented or non-fragmented sperm, respectively [10]. Several SCD kits are available and serve as inexpensive alternatives to SCSA.

The proprietors of SCSA have classified statistical categories of sperm fertility potential based on DFI, with < 15% considered to indicate excellent to good fertility potential, 15%–25% good to fair fertility potential, > 25%–50% fair to poor fertility potential, and > 50% very poor integrity. The probability of a successful pregnancy outcome sharply declines with a DFI > 25% when female factor infertility is excluded [7,11,12], and the suggested clinical intervention when DFI is > 25% is *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) rather than *in vivo* or intrauterine insemination [13–15]. Similar to SCSA, SCD has been indicated in published studies to have a predictive threshold between 20% and 27% for infertile men [16–19]. A threshold above 17%–18% has been found to affect the fertilization outcome [18,20]. This cutoff is specific to one SCD kit, Halosperm, and is not a global threshold that is applicable across all SCD kits. Additionally, no general consensus exists regarding the threshold above which a certain ART procedure should be selected based on DFI from determined with a particular SCD kit, and the current standard is to use SCSA thresholds. To add another layer of complexity, studies comparing DFI generated by SCSA and SCD have indicated both concordance between the 2 tests and discordance, such as a higher DFI obtained when an SCD kit is utilized [16,17,21,22].

In this study, we compared SCSA to the two most commonly used SCD kits (CANfrag and Halosperm) with regard to the DFI values obtained and the technical repeatability. Our goal was to determine whether the values generated from each of these kits would be similar in the same patient. We also investigated whether the type of kit used and the DFI generated affects the type of ART procedure chosen for the patient.

Methods

1. Patients and study design

A total of 41 male patients (age range, 27–45 years) were enrolled in this study, which was performed at the Andrology Centre in Coimbatore City, Tamil Nadu, India. Semen samples were collected from each patient after informed consent was obtained. All procedures were performed according to Institutional Review Board policy. All patients underwent serology assessment for viral or bacterial infections, including tests for human immunodeficiency virus (HIV)-1 and 2, hepatitis B surface antigen, hepatitis C virus, and other standard

laboratory tests for sexually transmitted infections, before undergoing the SCSA or SCD tests. Prior to sample collection, patients were asked to adhere to an ejaculatory abstinence regime spanning 24–48 hours. The semen samples were collected via masturbation into a sterile wide-mouthed calibrated container. After liquefaction for 1 hour at room temperature, 200–500 μ L of the raw semen was aliquoted into cryovials without cryoprotectant and flash-frozen in liquid nitrogen. Samples were analyzed fresh or frozen/thawed. Two SCD kits (CANfrag and Halosperm) and SCSA were chosen for the DFI analyses. A CANfrag kit was utilized on 36 patient samples and a Halosperm kit on 31 samples, and all 41 patients were assessed via SCSA (Figure 1). The DFI value, the correlation of DFI between the different assays, the coefficient of variation (CV) between technical replicates, and the impact on the choice of ART procedure were compared among SCSA and the specific kits (Figure 1). All reagents were purchased from Sigma-Aldrich (Millipore-Sigma, St. Louis, MO, USA) unless otherwise noted.

2. SCSA test protocol

Individual semen samples, stored in liquid nitrogen tanks (-196°C), were thawed in a 37°C water bath and then immediately placed on crushed ice. An aliquot of raw semen was transferred to a solution of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA; pH 7.4) at 4°C to yield a final concentration of approximately $1-2 \times 10^6$ sperm/mL. A total of 200 μ L of this sperm suspension was admixed with 400 μ L of a solution containing 0.08 N HCl, 0.15 M NaCl, and 0.1% (v:v) Triton X-100 at 4°C . Importantly, the HCl was diluted from a commercial solution of 2.0 N HCl. After 30 seconds, sperm were stained by adding 1.2 mL of staining solution containing 6 $\mu\text{g}/\text{mL}$ acridine orange (AO, chromatographically purified; Polysciences, Warrington,

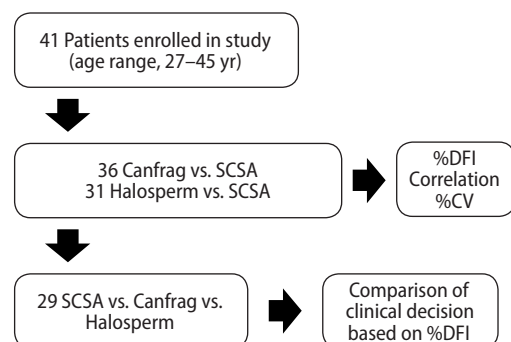


Figure 1. Flowchart of study design. A total of 41 patients were enrolled in the study, all of whom were assessed via sperm chromatin structure assay (SCSA). Subsets of patients were also compared using CANfrag (n=36) or Halosperm (n=31) sperm chromatin dispersion kits as indicated. The DNA fragmentation index (DFI), correlation, % coefficient of variation (CV), and impact of the derived DFI on the clinical decision were assessed.

PA, USA), 0.2 M Na₂PO₄, 0.1 M citric acid (pH 6.0), 1 mM EDTA, and 0.15 M NaCl to yield an AO:DNA-P molar ratio of ≥ 2 [23]. The acid-/AO-stained sample was placed in a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) sample chamber, and sample flow was initiated to bring the sheath flow and sample flow to equilibrium within 2 minutes. Subsequently, 5,000 sperm were analyzed at an event rate of 100–250 events/sec. If the event rate exceeded 250 events/sec, a new sample was prepared to ensure full equilibrium between the AO dye and the sperm. The flow cytometer was calibrated with a reference sample at the start of sample analysis, and the same reference sample was analyzed after every five test samples to calibrate the instrument. Each test sample was analyzed in duplicate, and replicates of the data were utilized to determine the percentage of sperm with measurably increased red fluorescence (sperm with fragmented DNA as determined using SCSAsoft). If a $> 10\%$ difference in DFI was observed between the raw X and Y means in the replicates, the sample was repeated. The standard deviations between the replicates were calculated.

3. CANfrag

Low-melting-point agarose, which was pre-provided in a microcentrifuge tube, was placed in a float in boiling water (90°C–100°C) for 5 minutes and then transferred to a 37°C water bath for equilibration. An aliquot of fresh or flash-frozen semen sample was added to this tube of melted agarose in order to achieve a final sperm concentration of 15–20 million/mL. A volume of 150 μL of this agarose-semen sample mixture was pipetted onto the provided slide, covered with a coverslip (25 mm \times 50 mm), and allowed to solidify at 4°C for 5 minutes. The coverslip was carefully removed, and the slide was kept on a horizontal staining tray and immersed in 1 mL of acid denaturant solution for 7 minutes. After this period, the excess acid solution was drained, 1 mL of lysis buffer was added, and the slide was incubated at room temperature for 10 minutes. The slide was washed gently with 20 mL of distilled water to remove the lysis buffer, and sequential dehydration steps were performed using dehydrating solutions 1, 2, and 3 (provided in the kit). The slides were air-dried and stained using the staining solution provided with the kit (working stain prepared: 300 μL of stain+100 μL of dilution buffer), 250 μL of which was used to stain each slide. The slides were incubated for 5 minutes, air-dried, and examined under $\times 400$ magnification using a light microscope (CH20i; Olympus, Tokyo, Japan). Each slide was scored for 200 spermatozoa by three lab technicians. Sperm cells with absent or small halos ($\leq 1/3$ of the head width) were counted as sperm with fragmented DNA; otherwise, they were considered normal sperm. The means and standard deviations were calculated.

4. Halosperm

Low-melting-point agarose (1%), which was pre-provided in a microcentrifuge tube, was placed in a float in boiling water (90°C–100°C) for 5 minutes and then transferred to a 37°C water bath for equilibration. An aliquot of fresh or flash-frozen semen sample was added to this tube of melted agarose in order to achieve a final sperm concentration of 5–10 million/mL. A drop of 10–15 μL of this agarose-semen sample mixture was pipetted onto the pre-treated slide and covered with a coverslip (18 \times 18 mm or 22 \times 22 mm), the edges of which were pressed gently to obtain an uniform distribution of the gel on the slide. The slide was then incubated at 4°C for 5 minutes. The coverslips were carefully removed, and the slides were immediately placed on a horizontal staining tray. An acid denaturant solution was freshly prepared (80 μL of the acid denaturant+10 mL of distilled water, provided in the kit), added to the gel, and allowed to react for 7 minutes. On completion, the slides were placed on another tray of lysis buffer and incubated at room temperature for 25 minutes. The slides were washed with abundant distilled water to completely remove the lysis solution and were then incubated for 5 minutes. Following this, the slides were sequentially dehydrated using 70%, 90%, and 100% ethanol (Changshu Hongsheng Fine Chemical, Jiangsu, China) for 2 minutes each, respectively. The slides were air-dried and stained using Diff-Quik Stain (Cell Life Ref: CL06; Cell Life, Visakhapatnam, India). First, azure A (eosin-red) stain was added and incubated for 7 minutes, and azure B (nigrosin-blue) stain was added and incubated for 7 minutes. The slides were incubated for an additional 5 minutes, air-dried, and examined under $\times 400$ magnification using a light microscope (CH20i, Olympus). Each slide was scored for 500 spermatozoa by three lab technicians. Sperm cells with absent or small halos ($\leq 1/3$ of the head width) were counted as sperm with fragmented DNA; otherwise, they were considered normal sperm. The means and standard deviations were calculated.

5. Statistical analysis

The CV was calculated for the triplicate readings from SCD and the duplicate readings from SCSA using the formula ($[\text{standard deviation}/\text{mean}] \times 100$). The DFI value was used as an indicator of sperm quality, namely excellent ($< 15\%$), good to fair (15%–25%), fair to poor ($> 25\%$ –50%) and very poor ($> 50\%$). The specific statistical tests used to determine significant differences ($p < 0.05$) are mentioned in the respective parts of the Results section.

Results

1. Higher DFI in SCD kits than in SCSA

The DFI derived from SCSA was significantly lower than the DFI derived using the CANfrag SCD kit (Wilcoxon test, $p < 0.001$) (Table 1).

Additionally, in the correlation analysis between SCSA and the SCD kits, both SCD kits had a strong significant correlation with the SCSA kit (Spearman rank tests, *p*-values in Table 1). Of the patients tested, 29 had their sperm analyzed using all three tests. Two-way analysis of variance (ANOVA) revealed that DFI was the lowest when determined using SCSA and was slightly greater when determined using either Halosperm or CANfrag (Figure 2). The median DFI levels were 19.3%, 24.7%, and 29% for SCSA, Halosperm and CANfrag, respectively, and the level associated with CANfrag was significantly higher than those for SCSA or Halosperm (two-way ANOVA, *p* < 0.0001) (Figure 2).

2. Technical repeatability of SCSA was superior to that of SCD kits

We calculated the CVs of the replicates for each patient to determine the technical repeatability of the tests. The CVs ranged from 0%–18% (mean, 4.7%) for SCSA, 2%–54% (mean, 24.2%) for CANfrag and 7%–62% (mean, 27.2%) for Halosperm. The CV associated with SCSA was significantly lower than the CVs associated with CANfrag or Halosperm (repeated-measures ANOVA and Bonferroni post-hoc test, *p* < 0.001). When an arbitrary cut-off value of 10% was set for CV, we observed that 30 of 36 patients (83.3%) for CANfrag, 29 of

31 (93.5%) for Halosperm, and four of 41 (9.7%) for SCSA would require repetition of the test. The mean DFI, along with its 95% confidence interval, was calculated for each test (Figure 3). Outside lines closer to the mean indicate higher repeatability of the test.

3. Fewer patients chosen for IVF/ICSI when SCSA was utilized

Since DFI informs the choice of ART procedure, we compared DFI and the subsequent ART procedure chosen in 29 patients when SCSA, Halosperm or CANfrag was used. A heatmap representing the classification of the sperm into four categories of sperm potential showed a striking difference in the number of patients presenting with a higher DFI and lower fertility potential when an SCD kit was used (Figure 4A). The clinical management plan for each of these pa-

Table 1. Comparison of DFI and correlation of SCSA to SCD kits

Test	No. of patients	Median DFI SCSA vs. SCD	Correlation to SCSA (Spearman rho)
CANfrag	36	19.8 vs. 27.4 ^{a)}	0.71 ^{a)}
Halosperm	31	19.3 vs. 24.3	0.62 ^{a)}

The DFI was higher with CANfrag and Halosperm than with SCSA, with a significant difference noted for CANfrag (per the Wilcoxon test). All tests correlated with SCSA test values.

DFI, DNA fragmentation index; SCSA, sperm chromatin structure assay; SCD, sperm dispersion kit.

^{a)}*p* < 0.001.

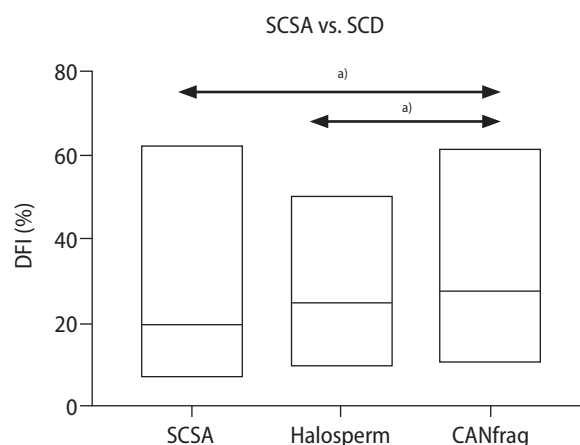


Figure 2. Lower DNA fragmentation index (DFI) values obtained with sperm chromatin structure assay (SCSA) than with sperm chromatin dispersion (SCD) kits. The graph indicates the DFI with the line within the box set at the median in 29 semen samples analyzed using SCSA (median DFI, 19.3%), Halosperm (median DFI, 24.7%) or CANfrag (median DFI, 29%). The DFI of SCSA and Halosperm was significantly lower than CANfrag. ^{a)}*p* < 0.001, statistically significant.

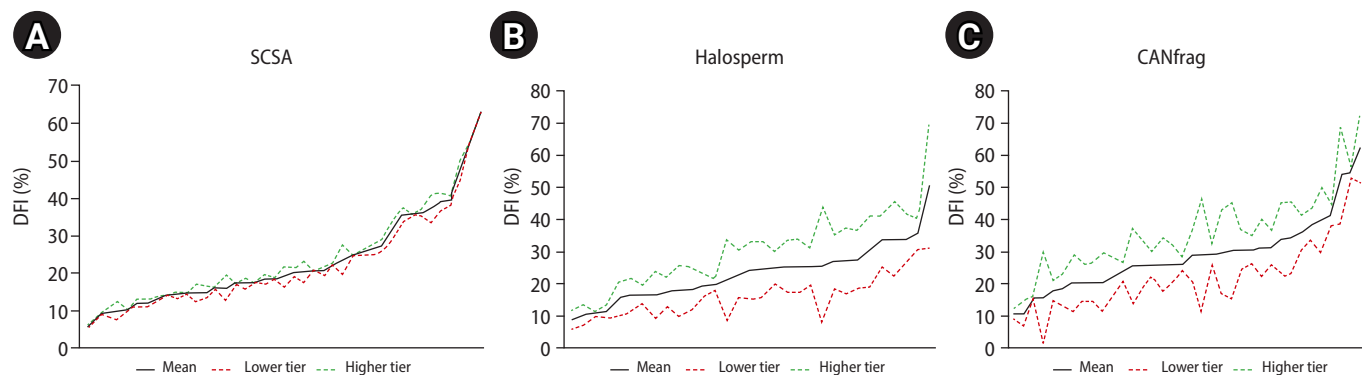


Figure 3. Technical repeatability of the test. The graph indicates the mean DNA fragmentation index (DFI; black line) and the 95% confidence interval (green, upper limit; red, lower limit) of the different tests. (A) Sperm chromatin structure assay (SCSA). (B) Halosperm. (C) CANfrag.

tients was further analyzed. This comparison revealed that if the SCSA testing method had been used for assessing sperm damage, and 25% DFI was set as the cutoff above which IVF/ICSI was selected instead of *in vivo* or intrauterine insemination, clinicians would have selected only nine of 29 patients for IVF/ICSI when using SCSA as opposed to 14 of 29 and 22 of 29 when using Halosperm and CANfrag, respectively (chi-square test, $p = 0.003$) (Figure 4B).

Discussion

In this proof-of-concept report, we established that commercially available SCD kits showed higher DFI values than SCSA for the same patient, were technically less reliable, and resulted in the ART approach of IVF/ICSI being chosen for more patients. This is the first study of its kind that not only included the analysis of DFI across two different methodologies (SCSA and SCD) but also employed the use of various SCD kits on the same patient sample, highlighting the high subjectivity and variability of clinical DFI values for the same pa-

tient across various testing methods.

In SCSA, the use of flow cytometry is employed to determine the levels of intact native double-stranded DNA and fragmented single-stranded DNA. One of the major advantages of flow cytometric assessments is the number of cells analyzed; in this case, a minimum of 5,000 individual sperm cells are analyzed in duplicate. The use of a proprietary software program to convert the data into a DFI value removes the ambiguity of an operator-dependent method and provides a robustness that is lacking in other methodologies. In comparison, the SCD kit methods involve the analysis of 100–500 cells in select fields, depends on the experience of technicians, and provides an incomprehensive representation of the DNA integrity of the complete sample. It is imperative to choose an appropriate method with strict guidelines when using sperm DNA integrity reports to inform the clinical management of infertile couples.

It is currently challenging for researchers and clinicians to compare DFI derived from different assays across diverse studies to assess the risk of male factor infertility. In our results, a higher DFI was recorded

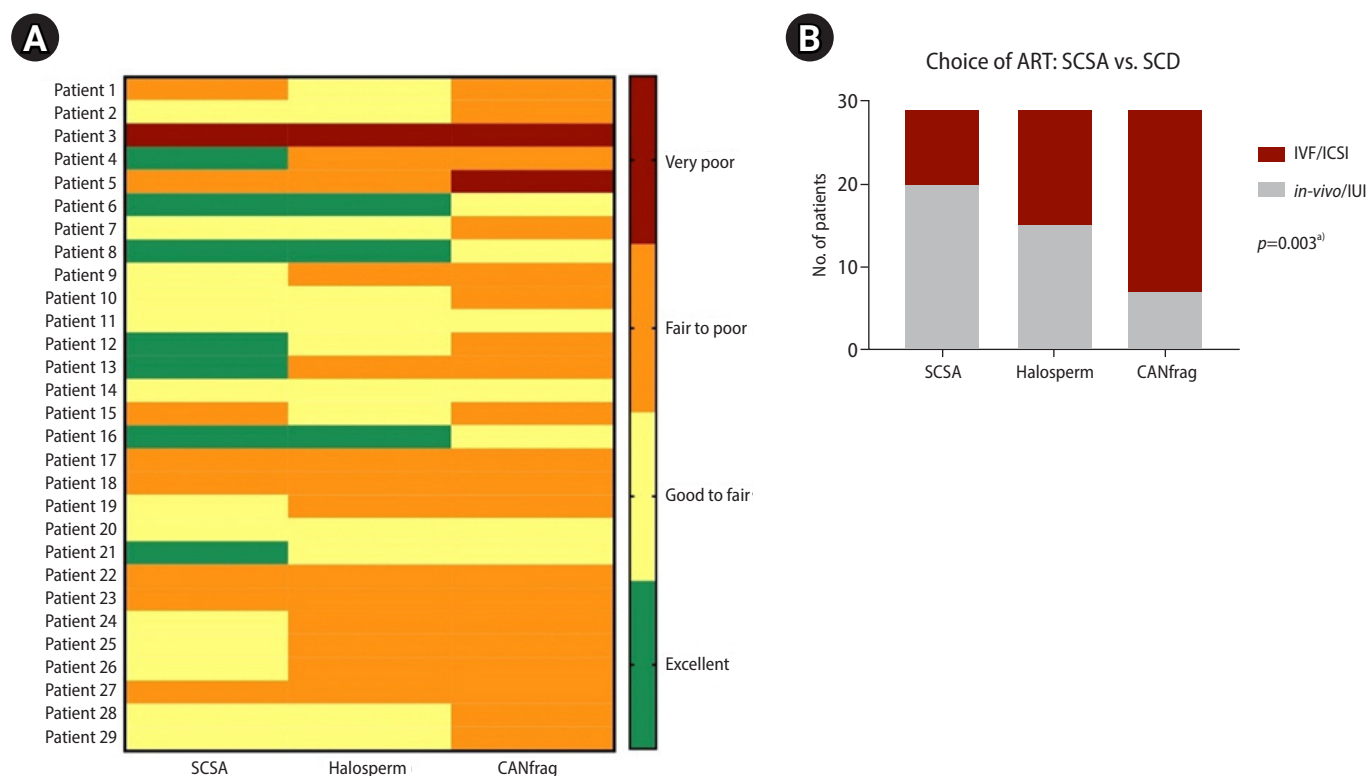


Figure 4. (A) Differences in categorizing sperm potential. A heat map of 29 patients with the representative DNA fragmentation index (DFI) for a patient when sperm chromatin structure assay (SCSA), Halosperm, or CANfrag were used. The color-coding represents the categories of sperm potential established for SCSA (green, <15% DFI, indicating excellent fertility potential; yellow, 15%–25% DFI, indicating good to fair fertility potential; orange, >25%–50% DFI, indicating fair to poor fertility potential; red, >50% DFI, indicating very poor integrity). (B) Clinical management is affected by the type of assay utilized to measure the DFI. The bars provide a visual representation of the number of patients selected for *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) or *in vivo*/intrauterine fertilization (*in vivo*/IUI) when SCSA, Halosperm, or CANfrag kits were used. The chi-square test revealed a statistically significant difference in the type of assisted reproductive technology (ART) procedure chosen based on the assay ($p = 0.003$). SCD, sperm chromatin dispersion. ^aStatistically significant.

when using a SCD kit than when using SCSA, and a similar result has been found in other studies [20,24,25]. The results regarding CV and technical repeatability appear to be contentious; the Halosperm pioneer laboratory reported a CV of 6%–12% [10], which is much lower than our observed values (2%–54% for CANfrag and 7%–62% for Halosperm). The results of our study also suggest that SCSA produced reliable results with duplicates in > 90% of cases, while using an SCD test produced reliable results in only < 17% of cases. A limitation of this study is the lack of clinical data and the need for validation in a much larger patient population with known clinical outcomes. Regardless, our manuscript brings into focus the subjective nature of SCD and emphasizes that it is unrealistic to expect technicians of varying levels of experience to be sufficiently skilled to assess DFI accurately. The availability of several SCD test kits also confounds the accurate interpretation of results [26]. Ideally, the proprietors of SCD kits should put forth a cut-off value specific to each kit, as in the case of SCSA (i.e., > 10% DFI between technical replicates), above which the test needs to be repeated. This is currently unaddressed, and no guidelines are available for use by inexperienced technicians or labs.

The majority of andrology laboratories that use SCD test kits currently use the available SCSA thresholds to determine sperm quality and inform subsequent clinical management. Our analyses of the impact of DFI derived from SCD versus that derived from SCSA indicated that when SCD kits are used, a significantly higher number of patients would be categorized as having poor-quality sperm, and subsequently, a higher number of patients would be selected to undergo IVF/ICSI, creating ambiguity in the clinical setting. Given that a higher DFI is known to be derived from SCD kits, it is critical to utilize threshold values specific to the patient population or infertility center for the preferred SCD kit in order to maximize the potential derived from each of these tests, as suggested by Ribas-Maynou et al. [20]. This approach allows refinement of the choice of ART procedure based on SCD results and avoids the unwarranted use of ICSI for the clinical management of male factor infertility. As in the case of technical repeatability, thresholds to simplify patient management are also needed. This is achievable only when a larger number of patients with known clinical outcomes are compared with regard to DFI values obtained using a specific SCD test and the existing gold-standard assay (SCSA). Additionally, a threshold value must be defined not only for male factor infertility but also to identify a cutoff DFI above which an increased risk of miscarriage or pregnancy failure is observed.

In conclusion, our report establishes that SCSA is reliable with respect to technical repeatability and provides a more streamlined approach for the management of infertile couples. Parameters must be established for a chosen SCD test instead of utilizing the thresholds

set for SCSA for the clinical management of infertile couples.

Conflict of interest

Vidya Laxme B, Silviya Stephen, Ramyashree Devaraj, and Tara Mahendran are employees of the Andrology Center in Coimbatore, India. No other potential conflict of interest relevant to this article was reported.

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Author contributions

Conceptualization: TM. Data curation: VLB, SS, RD, TM. Formal analysis: SM, RPB. Methodology: VLB, SS, RD, TM. Project administration: TM. Visualization: TM, SM. Writing—original draft: TM, SM. Writing—review & editing: all authors.

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Effect of evaporation-induced osmotic changes in culture media in a dry-type incubator on clinical outcomes in *in vitro* fertilization-embryo transfer cycles

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Objective: This study investigated whether adding outer-well medium to inhibit osmotic changes in culture media in a dry-type incubator improved the clinical outcomes of *in vitro* fertilization-embryo transfer (IVF-ET) cycles.

Methods: In culture dishes, the osmotic changes in media (20 μ L)-covered oil with or without outer-well medium (humid or dry culture conditions, respectively) were compared after 3 days of incubation in a dry-type incubator. One-step (Origio) and G1/G2 (Vitrolife) media were used.

Results: The osmotic changes in the dry culture condition (308 mOsm) were higher than in the humid culture conditions (285–290 mOsm) after 3 days of incubation. In day 3 IVF-ET cycles, although the pregnancy rate did not significantly differ between the dry (46.2%) and humid culture (51.0%) groups, the rates of abortion and ongoing pregnancy were significantly better in the humid culture group (1.5% and 49.5%, respectively) than in the dry culture group (8.3% and 37.8%, respectively, $p < 0.05$). In day 5 IVF-ET cycles, the abortion rate was significantly lower in the humid culture group (2.2%) than in the dry culture group (25.0%, $p < 0.01$), but no statistically significant difference was observed in the rates of clinical and ongoing pregnancy between the dry (50.0% and 25.0%, respectively) and humid culture groups (59.5% and 57.3%, respectively) because of the small number of cycles.

Conclusion: Hyperosmotic changes in media occurred in a dry-type incubator by evaporation, although the medium was covered with oil. These osmotic changes were efficiently inhibited by supplementation of outer-well medium, which resulted in improved pregnancy outcomes.

Keywords: Clinical outcomes; Dry-type incubator; Evaporation; Osmotic change; Outer-well medium

Introduction

Even brief exposure of preimplantation mouse embryos to high-os-

molality culture medium (> 300 mOsm/kg) in the absence of osmolytes resulted in impaired development [1-3]. The detrimental effect of hyperosmolality has also been reported in the development of various mammalian embryos, including murine [4-6], rat [7], porcine [8,9], and bovine [10,11] embryos. Moreover, *in vitro* two-cell blocks of mouse embryos were significantly alleviated by culturing them in low-osmolality (250 mOsm) medium compared to high-osmolality (> 300 mOsm) medium [12]. Biggers et al. [3] reported that when the NaCl concentration was increased in the medium, the intracellular Na^+/K^+ ratio dramatically increased, which was detrimen-

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tal to mouse embryo development. The detrimental effects of hyperosmolality occur by triggering cell shrinkage, oxidative stress, protein carbonylation, mitochondrial depolarization, DNA damage, cell cycle arrest, and apoptosis [13,14].

From a different point of view, an increase in extracellular osmolality can promote water flux out of the cell, triggering cell shrinkage and intracellular dehydration [15]. Intracellular water loss interferes with many cellular functions, including DNA synthesis and repair, transcription, protein translation and degradation, and mitochondrial function. As a result, cell cycle progression and cell proliferation are arrested [13]. Dry-type incubators are now widely used instead of humid-type incubators due to the development of infrared CO₂ sensors [16] and the low possibility of microorganism overgrowth [17]. In addition, dry-type incubators have smaller culture chambers than humid-type incubators, is advantageous in terms of the short recovery time for gas and temperature after the door is opened. However, concerns about the possible change in the osmolality of the medium by evaporation remain a reason why clinicians may hesitate to use a dry-type incubator. Although overlying culture dishes with oil could inhibit the shift in the osmolality of the medium, whether it completely eliminates the change in osmolality remains controversial [18]. Recently, human embryos cultured in dry-type incubators showed significantly lower implantation and clinical pregnancy rates than those cultured in humid-type incubators [18].

The present study was performed to compare osmotic changes in culture media covered in oil in various types of culture dishes, and to investigate whether a beneficial effect on clinical outcomes in *in vitro* fertilization-embryo transfer (IVF-ET) cycles could be obtained by supplementation with outer-well medium to inhibit osmotic changes in culture media in a dry-type incubator.

Methods

This retrospective study was approved by the Institutional Review Board of Mamapapa and Baby Clinic (IRB No. 2019-10-01), and was conducted from August 2018 to August 2019.

1. Patients

In total, 796 IVF-ET cycles in 673 patients were analyzed in the present study. Twelve patients who underwent their first 3-day IVF-ET cycles using a cell culture dish, but failed to show implantation or ongoing pregnancy, completed their second IVF-ET cycles using a GPS dish to compare the clinical outcomes between cell culture and GPS dish cycles. After a comparison of these 12 patients, we changed the culture medium from 1-Step medium to G1/G2 medium; the remaining 772 IVF-ET cycles in 661 patients were per-

formed using G1/G2 medium. Of these cycles, 168 IVF-ET cycles (156 day 3 IVF-ET cycles + 12 day 5 IVF-ET cycles) in 159 patients used cell culture dishes, while 628 IVF-ET cycles (539 day 3 IVF-ET cycles + 89 day 5 IVF-ET cycles) in 514 patients used GPS dishes. In the early stage of the study, we found that using the GPS dish had a beneficial effect on clinical outcomes, and after confirming a significant improvement in pregnancy outcomes in the GPS dish group, we completely changed to GPS dishes. The ultimate goal of our studies is to improve the pregnancy rate in IVF cycles; therefore, we could no longer use the cell culture dishes. This change resulted in the difference of the number of cycles between the cell culture and GPS dish groups.

2. Ovarian stimulation and oocyte aspiration

Controlled stimulation for IVF cycles was performed with a mild stimulation protocol using a combination of a gonadotropin-releasing hormone (GnRH) antagonist and gonadotropins. Patients received 150 IU of recombinant follicle-stimulating hormone (Gonal-F; Merck Serono, Darmstadt, Germany) alone as a daily injection from cycle day 3 until the day when human chorionic gonadotropin (hCG) was administered. The GnRH antagonist (Cetrotide, Merck Serono) was initiated on the day when the leading follicle reached a diameter of 14 mm. Ovarian follicular development was monitored by transvaginal ultrasonography. When the leading follicles reached ≥ 18 mm in maximum diameter, as detected by sonography, ovulation was induced by injecting 250 μ g of hCG (Ovidrel, Merck Serono). Oocyte retrieval was performed using 20-gauge ovum aspiration needles (Cook Medical, Bloomington, IN, USA) under standard transvaginal ultrasound guidance 35–36 hours after hCG administration. The luteal phase was supported by progesterone injection or vaginal gel (Crinone, Merck Serono). A serum β -hCG test was performed about 2 weeks after oocyte retrieval. Clinical pregnancy was confirmed by the visualization of a gestational sac. Ongoing pregnancy was defined as a pregnancy that was maintained for over 20 weeks of gestation.

3. Embryo culture *in vitro*

One-Step (Origio, Malov, Denmark) and G1/G2 media (Vitrolife, Göteborg, Sweden) were employed in IVF-ET cycles. The culture dishes for IVF and embryo culture were prepared and incubated in a humid-type incubator (HERAcell 150i; Thermo Scientific, Waltham, MA, USA) overnight to achieve an optimal pH of 7.2–7.3. Fertilized oocytes were individually cultured in 20- μ L drops of the culture medium covered in oil for 3–5 days until transfer, in 6.0% CO₂, 5% O₂ and 89.0% N₂, in a dry-type incubator (Miri; ESCO, New Haven, CT, USA). The embryos were cultured in a cell culture dish without outer-well medium (dry culture condition) or in a μ -drop GPS dish with out-

er-well medium (humid culture condition). In day 5 IVF-ET cycles of the cell culture dish and GPS dish groups, dish change was performed on day 3 by transferring the embryos to a new culture dish prepared on day 2, to inhibit osmotic changes induced by evaporation and to serve as a new culture medium.

4. Estimation of osmolality changes in media

Osmotic changes in micro-drops (20 µL) of medium covered in oil and with or without outer-well medium supplementation were compared in the following types of culture dishes: cell culture (Corning Inc., Corning, NY, USA), µ-droplet culture (Vitrolife), microwell culture (DNP, Kashiwa, Japan), and µ-drop GPS (LifeGlobal, Brussels, Belgium) (Figure 1). Ham's F-10 (Gibco, Grand Island, NY, USA) and G1 (Vitrolife) were used to investigate changes in osmolality. The basic osmolality of the media was about 280 and 275 mOsm/kg, respectively. The osmolality of media was estimated with a micro-osmometer (Advanced, Norwood, MA, USA), after 3 days of incubation in the dry-type incubator.

5. Embryo sequential scoring

The quality of embryos was daily evaluated and scored according to the developmental stage and speed, as well as the shape of blastomeres and degree of fragmentation (Figure 2). The embryo grades and scores were as follows: grade 1 (+5 points), no fragmentation

with equal-sized blastomeres; grade 2 (+4 points), < 10% fragmentation with equal-sized blastomeres or no fragmentation with unequal-sized blastomeres; grade 3 (+3 points), 10% ≤ fragmentation < 25%; grade 4 (+2 points), 25% ≤ fragmentation < 50%; grade 5 (+1 point), ≥ 50% fragmentation; arrested embryos (0 point). When an embryo showed a normal developmental speed and stage, an additional point was given (+1 point), such as two-cell cleavage on day 1, four-cell stage on day 2, eight-cell stage on day 3, or compaction on day 4. It has been reported that fast cleavage speed on day 4 showed an association with high aneuploidy and low blastocyst formation rates. Moreover, in our accumulated data, the pregnancy rate of embryos with a normal cleavage speed was higher than high-speed embryos or low-speed embryos. The quality of blastocysts were evaluated separately in terms of the inner cell mass and trophectoderm; each cell type was classified as grade A to C, and points were given according to the grades, as follows; A (+3 points), B (+2 points) and C (+1 point). The embryos with the highest cumulative scores were selected for transfer.

6. Statistical analysis

Statistical analysis was performed with SPSS ver. 11.0 (SPSS Inc., Chicago, IL, USA). Means and standard deviations were calculated for all variables. The Student *t*-test was employed to analyze differences in patients' age, endometrial thickness, number of oocytes retrieved, scores of embryos, and number of embryos transferred between the dry and humid culture groups. Differences in clinical outcomes between the two groups were analyzed by the chi-square test, and *p*-values < 0.05 were considered to indicate statistical significance.

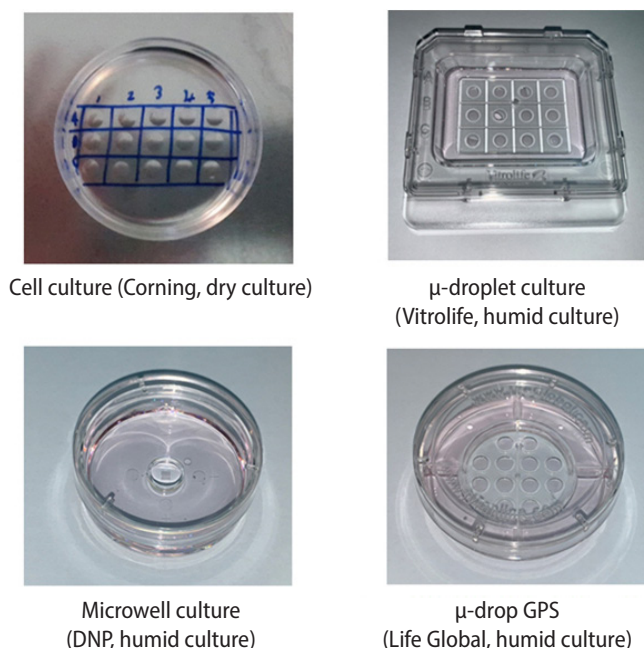


Figure 1. Various culture dishes were used to compare osmotic changes of µ-drop medium covered in oil and with (humid culture) or without outer well medium (dry culture) after 3 days of incubation in a dry-type incubator.

Embryo quality	Day 1	Day 2	Day 3	Day 4	Day 5
2-Cell cleavage	+1				
Grade 1		+5	+5		
Grade 2		+4	+4		
Grade 3		+3	+3		
Grade 4		+2	+2		
Grade 5		+1	+1		
Arrest		0	0	0	0
4-Cell stage		+1			
8-Cell stage			+1		
Compaction				+1	
Expanded					+3
Expanding					+2
Early					+1
AA (ICM/trophectoderm)					+3
AB, BA					+2
BB					+1

Figure 2. Embryo sequential scoring for evaluation of embryo quality. ICM, inner cell mass.

Results

1. Osmotic changes in media according to the type of culture dish after 3 days of incubation in a dry-type incubator

Osmotic changes in 20- μ L droplets of Ham's F-10 (280 mOsm) and G1 (275 mOsm) media according to the various types of culture dishes were compared after 3 days of incubation in the dry-type incubator (Table 1). The osmolality of Ham's F-10 in the cell culture dishes (301.1 mOsm) in the dry-type incubator was higher than that (286.1 mOsm) in the humid-type incubator, although the dishes were covered in 6 mL of oil. Compared to the cell culture dishes with dry culture conditions (without outer-well medium, 301.1 mOsm), the humid culture conditions (with outer-well medium) using the μ -droplet culture (290.4 mOsm), Microwell culture (285.3 mOsm) and μ -drop GPS (287.2 mOsm) dishes showed lower osmolality. The osmolality of the G1 medium in the cell culture dishes (293.7 mOsm) and μ -drop GPS dishes without outer-well medium (293.6 mOsm) was

higher than that in the μ -drop GPS dishes with outer-well medium (285.0 mOsm) after 3 days of incubation in the dry-type incubator. Although there was no difference in the osmotic change between the GPS dish and the other dishes supplemented with outer well medium, we selected the μ -drop GPS dishes to use for human embryo culture because of their suitability for our culture system.

2. Clinical outcomes of the first (cell culture dish, dry culture) and second day 3 IVF-ET cycles (GPS dish, humid culture) in the same 12 patients using 1-Step (Origio) medium

As shown in Table 1, supplementation of outer-well medium (humid culture condition) was efficient for maintaining the osmolality of media in the dry-type incubator. To investigate the possible beneficial effects of the humid culture condition, in the same 12 patients, the first IVF-ET cycle was performed using the cell culture dishes and the second IVF-ET cycle was performed using the GPS dishes (Table 2).

There were no significant differences in the characteristics of the

Table 1. Osmotic changes in media according to the type of culture dishes after 3 days of incubation in a dry type incubator

Culture dish	Incubator type	Media (mOsm/kg)	Cover oil (mL)	Outer well media (mL)	Osmolality (mOsm/kg)
Cell culture (Corning)	Humid	Ham's F-10 (280)	6	0	286.1
Cell culture (Corning)	Dry	Ham's F-10 (280)	6	0	301.1
μ -droplet culture (Vitrolife)	Dry	Ham's F-10 (280)	0.8	0.9	290.4
Microwell culture (DNP)	Dry	Ham's F-10 (280)	0.03	2	285.3
μ -drop GPS (LifeGlobal)	Dry	Ham's F-10 (280)	2	4	287.2
Cell culture (Corning)	Dry	G1 (275)	6	0	293.7
μ -drop GPS (LifeGlobal)	Dry	G1 (275)	2	0	293.6
μ -drop GPS (LifeGlobal)	Dry	G1 (275)	2	4	285.0

Humid, HERAcell 150i incubator; Dry, ESCO Miri R incubator.

Table 2. Clinical outcomes of the first (cell culture dish, dry culture) and second (GPS dish, humid culture) day 3 IVF-ET cycles in the same 12 patients using 1-step (Origio) medium

1-step medium/day 3 ET (dry-type incubator)	First IVF cycle (cell culture dish)			Second IVF cycle (GPS dish)			p-value
	ICSI	cIVF	Total	ICSI	cIVF	Total	
No. of IVF cycles	8	4	12	9	3	12	
Mean age (yr)	40.1	35	38.4 \pm 4.9	40.3	33.3	38.5 \pm 4.9	0.467
Mean endometrium thickness (mm)	9.9	9.3	9.7 \pm 1.7	9.8	11.1	10.1 \pm 2.1	0.300
Mean no. of oocytes aspirated	4.5	7.7	5.5 \pm 3.9	5.4	6	5.5 \pm 2.6	0.500
2PN oocytes (%)	26/36 (72.2)	17/31 (54.8)	43/67 (71.6)	29/49 (59.1)	14/18 (77.7)	43/67 (74.1)	0.924
Mean no. of embryos transferred	1.7	2.0	1.8 \pm 0.3	2.0	2.0	2.0 \pm 0.0	0.076
Mean score of embryos transferred	7.3	8.3	7.6 \pm 2.2	6.4	8.0	6.8 \pm 2.7	0.211
Clinical pregnancy cycle (%)	3 (37.5)	1 (25.0)	4 (30.7)	4 (44.4)	2 (66.6)	6 (50.0)	0.678
Abortion cycle (%)	3 (37.5)	1 (25.0)	4 (30.7)	0	0	0	0.100
Ongoing pregnancy (%)	0	0	0	4 (44.4)	2 (66.6)	6 (50.0) ^{a)}	0.018

Values are presented as mean \pm standard deviation or number (%).

Dry culture, without outer well medium; Humid culture, with outer well medium; IVF, *in vitro* fertilization; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; cIVF, conventional IVF; PN, pronuclei.

^{a)} $p < 0.05$.

same 12 patients between the first and second IVF-ET cycles, because the second IVF-ET cycles were performed within 6 months after the completion of the first IVF cycles. Although the rates of clinical pregnancy and abortion (50.0% and 0%) in the second IVF-ET cycles were more favorable than those (30.7% and 30.7%, respectively) in the first IVF-ET cycles, the difference was not statistically significant. However, the ongoing pregnancy rate (50.0%) of the second IVF-ET cycles was significantly higher than the rate (0%, $p < 0.05$) of the first IVF-ET cycles. When the two IVF cycle groups were subdivided into intracytoplasmic sperm injection (ICSI) and conventional IVF cycle groups, no differences were found in the characteristics of patients and clinical outcomes between the subgroups of the first and second IVF-ET cycle groups.

3. Clinical outcomes of day 3 IVF-ET cycles in the cell culture dish (dry culture) and GPS dish (humid culture) groups using G1/G2 medium (Vitrolife)

In the day 3 IVF-ET cycles, there were no significant differences in the mean age (36.9 ± 4.4 and 37.3 ± 4.2), endometrial thickness (10.6 ± 2.4 and 10.4 ± 2.0 mm), score of transferred embryos (9.1 ± 2.3 and 8.9 ± 2.4) and fertilization rate (67.4 and 69.4%) between the dry (156 cycles) and humid culture (539 cycles) groups (Table 3). However, the numbers of oocytes retrieved (8.4 ± 4.4) and transferred embryos (2.0 ± 0.4) in the dry culture group were significantly higher than those in the humid culture group (7.3 ± 4.0 and 1.9 ± 0.3 , respectively, $p < 0.05$). Nevertheless, the rates of abortion and ongoing pregnancy in the humid culture group (1.5% and 49.5%) were significantly more favorable than those in the dry culture group (8.3% and 37.8%, respectively, $p < 0.01$), although there was no significant

difference in the clinical pregnancy rate between the dry (46.2%) and humid culture (51.0%) groups. When the two culture groups were subdivided into ICSI and conventional IVF cycle groups, the characteristics of patients and clinical outcomes of the subgroups showed a similar pattern to the total IVF cycles between the two culture groups.

4. Clinical outcomes of day 5 IVF-ET cycles in the cell culture dish (dry culture) and GPS dish (humid culture) groups using G1/G2 medium

In the day 5 IVF-ET cycles, there were also no differences in the mean age (36.1 ± 3.9 and 36.3 ± 3.4 years, respectively), endometrial thickness (10.8 ± 1.7 and 10.6 ± 2.1 mm, respectively), the number (1.0 ± 0.0 and 1.0 ± 0.2 , respectively) and score (16.5 ± 1.8 and 15.3 ± 3.0 , respectively) of embryos transferred, or the fertilization rate (77.7% and 76.2%, respectively) between the dry (12 cycles) and humid culture (89 cycles) groups (Table 4). However, the number of oocytes aspirated (12.3 ± 4.7) in the dry culture group was significantly higher than that in the humid culture group (9.9 ± 3.4 , $p < 0.05$). The abortion rate in the humid culture group (2.2%) was significantly lower than that in the dry culture group (25.0%, $p < 0.01$), but no statistically significant difference was observed in the rates of clinical and ongoing pregnancy between the dry (50% and 25.0%) and humid culture groups (59.5% and 57.3%, respectively), most likely due to the small number of cycles in the dry culture group. When the day 5 IVF-ET cycles in the two culture groups were also subdivided into ICSI and conventional IVF cycle groups, the characteristics of patients and clinical outcomes of the subgroups showed a similar trend to the total IVF cycles between the two cul-

Table 3. Clinical outcomes of day 3 IVF-ET cycles in cell culture dish (dry culture) and GPS dish (humid culture) groups using G1/G2 medium (Vitrolife)

G1/G2 media/day 3 ET (dry-type incubator)	Cell culture dish (dry culture)			GPS dish (humid culture)			p-value
	ICSI	cIVF	Total	ICSI	cIVF	Total	
No. of IVF cycles	82	74	156	236	303	539	
Mean age (yr)	37.3	36.6	36.9 ± 4.4	38.2	36.5	37.3 ± 4.2	0.434
Mean endometrium thickness (mm)	10.6	10.6	10.6 ± 2.4	14.2	10.5	10.4 ± 2.0	0.354
Mean no. of oocytes aspirated	7.9 ^{a)}	8.9 ^{a)}	8.4 ± 4.4 ^{a)}	6.4	8.0	7.3 ± 4.0	0.003
2PN oocytes (%)	411 (63.1)	471 (71.5)	882 (67.4)	992 (65.7)	1,737 (71.7)	2,729 (69.4)	0.177
Mean no. of embryos transferred	1.9 ^{a)}	2.0	2.0 ± 0.4 ^{a)}	1.8	2.0	1.9 ± 0.3	0.013
Mean score of embryos transferred	8.8	9.4	9.1 ± 2.3	8.4	9.2	8.9 ± 2.4	0.273
Clinical pregnancy cycle (%)	36 (43.9)	36 (48.6)	72 (46.2)	103 (43.6)	172 (56.7)	275 (51.0)	0.337
Abortion cycle (%)	7 (8.5) ^{a)}	6 (8.1) ^{a)}	13 (8.3) ^{a)}	3 (1.3)	5 (1.7)	8 (1.5)	<0.001
Ongoing pregnancy (%)	29 (35.3)	30 (40.5)	59 (37.8)	100 (42.3)	167 (55.1) ^{a)}	267 (49.5) ^{a)}	0.012

Values are presented as mean \pm standard deviation or number (%).

IVF, *in vitro* fertilization; ET, embryo transfer; Dry culture, without outer well medium; Humid culture, with outer well medium; ICSI, intracytoplasmic sperm injection; cIVF, conventional IVF; PN, pronuclei.

^{a)} $p < 0.05$.

ture groups.

Discussion

When the osmolality of the medium increases above a certain threshold, embryo development is compromised [1,5] and apoptosis is increased [19]. Hyperosmolality (> 300 mOsm) was shown to have a detrimental effect on preimplantation mouse embryo development in previous studies [4-6]. However, paradoxically, the osmolality of oviductal fluid is about 340 mOsm [2,20], which is high enough to impair mouse embryo development *in vitro*. However, unlike *in vitro*, mouse zygotes have no difficulty developing *in vivo*, which suggests that an unknown mechanism may exist that helps them overcome the detrimental effect of hyperosmolality *in vivo* [1].

One possible mechanism is the use of various amino acids as organic osmolytes by embryos. The addition of various organic osmolytes, including taurine [21-24], hypotaurine [25], and glutamine [26], were beneficial for embryo culture. Glycine has also been shown to protect mouse [3] and rabbit embryos [27] against the effects of high NaCl levels. Indeed, over 70% of zygotes developed to the blastocyst stage when 1 mM glutamine was present at 310 mOsm, but only about 10% did so in the absence of glutamine [1]. At 310 mOsm of KSOM medium in the absence of glycine, over 80% of outbred mouse zygotes were arrested at the two-cell stage. However, in the presence of glycine, 60% of the zygotes developed to the blastocyst stage [12]. Furthermore, more blastocysts formed when bovine zygotes were cultured in a 247 mOsm medium (34.6%) than in a 286 mOsm medium (17.0%) in the presence of 1 mM glycine [28]. The beneficial effect of organic osmolytes can be explained by previous

findings that amino acids, including osmolytes, are present in oviductal and uterine fluid [29-31] and carry out various physiological functions in the preimplantation embryo, including ATP production [32], ammonium detoxification [33,34] and maintaining the redox balance [35].

Baltz and Tartia [36] suggested a possible answer for the question “Why did lower osmolality support embryo development in culture?” They proposed that the osmolality in the *in vivo* environment of early preimplantation embryos may be lower than in blood plasma (280 mOsm). This possibility conflicts with previous reports that the osmolality of oviductal fluid is about 340 mOsm [2,20]. Although the oviduct is the major *in vivo* environment for early-stage embryos, the follicle can be seen as the birthplace of the oocyte and embryo. In mice, a large amount of follicular fluid moves into the ampulla of the oviduct with all ovulated oocytes via the ovarian bursa. In human, immediately after ovulation, a small amount of follicular fluid flows weakly from the ovarian wall to the ampulla of the oviduct to help the sliding movement of the oocyte. This means that follicular fluid may play an important role in determining the oviductal environment for early-stage embryos. In our preliminary test (data not published), we compared the osmolality of human follicular fluid and blood serum. Interestingly, the osmolality of follicular fluid (275.3 mOsm) was lower than that of serum (282.0 mOsm). There are still no reports about the osmolality of human oviductal fluid, so further research on the osmolality of the *in vivo* environment of human preimplantation embryos is required. Nevertheless, interestingly, current commercial culture media already have lower osmolality (260-270 mOsm) than the older generation of media (280-295 mOsm) [36].

In the present study, after 3 days of incubation, even under an oil

Table 4. Clinical outcomes of day 5 IVF-ET cycles in cell culture dish (dry culture) and GPS dish (humid culture) groups using G1/G2 medium (Vitrolife)

G1/G2 media/day 5 ET (dry-type incubator)	Cell culture dish (dry culture)			GPS dish (humid culture)			p-value
	ICSI	cIVF	Total	ICSI	cIVF	Total	
No. of IVF cycles	5	7	12	28	61	89	
Mean age (yr)	36.6	35.7	36.1 ± 3.9	36.8	36	36.3 ± 3.4	0.846
Mean endometrium thickness (mm)	9.84	11.5	10.8 ± 1.7	10.8	10.5	10.6 ± 2.1	0.701
Mean no. of oocytes aspirated	12.8 ^{a)}	12	12.3 ± 4.7	9	10.2	9.9 ± 3.4	0.026
2PN oocyte (%)	51 (79.6)	64 (76.1)	115 (77.7)	190 (75.3)	480 (76.5)	670 (76.2)	0.773
Mean no. of embryos transferred	1.0	1.0	1.0 ± 0.0	1.0	1.0	1.0 ± 0.2	0.459
Mean score of embryos transferred	16.4	16.6	16.5 ± 1.8	14.5	15.6	15.3 ± 3.0	0.168
Clinical pregnancy cycle (%)	3 (60.0)	3 (42.8)	6 (50.0)	14 (50.0)	39 (63.9)	53 (59.5)	0.750
Abortion cycle (%)	1 (20.0)	2 (28.5)	3 (25.0) ^{a)}	0	2 (3.3)	2 (2.2)	0.006
Ongoing pregnancy (%)	2 (40.0)	1 (14.2)	3 (25.0)	14 (50.0)	37 (60.6)	51 (57.3)	0.072

Values are presented as mean ± standard deviation or number (%).

IVF, *in vitro* fertilization; ET, embryo transfer; Dry culture, without outer well medium; Humid culture, with outer well medium; ICSI, intracytoplasmic sperm injection; cIVF, conventional IVF; PN, pronuclei.

^{a)}p < 0.05.

layer, the osmolality of media in the dry culture condition (301.1 mOsm) was higher than that of media in the humid culture condition (285.3–290.4 mOsm) in the dry-type incubator. Gasperin et al. [37] also reported that in the absence of an oil layer, the addition of water in the four-well dish central hole reduced the osmolality of medium (294 mOsm) after 24 hours of incubation compared to the osmolality of control dishes without water (305 mOsm) in a humid-type incubator. These results indicate that osmolality can increase in both dry- and humid-type incubators. Therefore, both an oil layer and extra medium or water supplementation are essential for maintaining the osmolality of the medium by inhibiting evaporation in *in vitro* culture conditions.

In the present study, in day 5 ET cycles, the rates of clinical and ongoing pregnancy in the humid culture group were higher than the rates in the dry culture group. Similarly, no meaningful difference was found in the clinical and ongoing pregnancy rates of the humid culture groups in day 5 ET cycles (57% and 52%) between the previous [18] and the present study (59.5% and 57.3%), even though there was a large difference in the mean age of female patients between the two studies (28 vs. 36 years, respectively). The first possible reason is medium renewal. We performed medium renewal by exchanging G1 to G2 medium on day 3, but Fawzy et al. [18] did not perform medium renewal for 5 days. Culture for 5 days without medium renewal could degrade the organic osmolytes, and this degradation may augment the negative influence of this osmolality shift on embryo development. In addition, medium renewal may not only reduce the accumulation of metabolites such as ammonium, but could also supply fresh nutrients for embryo development. The second possible reason relates to the humid culture condition. In the present study, for the preparation of the humid culture condition, we added 6 mL of medium into the outer well of GPS culture dishes (LifeGlobal), while Fawzy et al. [18] used petri dishes containing 10 mL of water. In the preliminary experiment, we also used petri dishes containing water, but this was not efficient to inhibit the increase of osmolality in the medium compared to direct medium supplementation in the outer well of the culture dish.

Fawzy et al. [18] reported that the blastulation rate of human embryos was significantly higher in the humid culture group (73%) than in the dry culture group (51%, $p < 0.05$). Gasperin et al. [37] reported a similar trend, according to which the blastocyst rate of bovine embryos was higher in the humid culture group (29.7%, $p < 0.05$) than in the dry culture group (16.2%). Unlike the above results, in the present study, there was no significant difference in the blastulation rate between the dry (56.5%) and humid culture (55.5%) groups. Of particular note, the blastulation rate of the humid culture group (55.5%) was markedly lower than the rate (73%) reported in the other study [18]. This difference may have resulted from the large gap in

the mean age of female patients between the other study (28 years) and the present study (36 years). The age of female patients is widely considered as a critical factor for determining the quality of oocytes, the grade of embryos, and the success of pregnancy in IVF-ET cycles. Therefore, the relatively low blastulation rate in the humid culture group in the present study may be explained by the relatively old age of patients compared to the previous study [18].

There was no significant difference in the development of early-stage mouse embryos cultured between the high-osmolality (310–330 mOsm) and low-osmolality (270–290 mOsm) media. However, in the development of late-stage embryos, the embryos cultured in the low-osmolality medium showed a significantly higher blastocyst formation rate than those cultured in the high-osmolality medium [6]. A similar result was observed in porcine embryos cultured *in vitro* [8]. These results suggest that late-stage embryos are more susceptible to osmotic changes than early-stage embryos. In the present study, a similar pattern in response to osmotic changes in human embryos was observed, although we did not observe a significant difference in the blastocyst formation rate between the high-osmolality (56.5%, dry culture) and low-osmolality (55.5%, humid culture) culture groups. However, in day 3 ET cycles, involving relatively early-stage embryos compared to day 5 ET cycles, the transferred embryos showed a small difference in pregnancy outcomes between the high- and low-osmolality culture groups. The differences in the rates of pregnancy, abortion, and ongoing pregnancy were 4.8%, 6.8%, and 11.7%, respectively. In contrast, in day 5 ET cycles, the differences in these rates in the late-stage embryos were 9.5%, 22.8%, and 32.3%, respectively. This result is consistent with the previous finding that late-stage embryos are more susceptible to osmotic changes [6,8].

When a cell shrinks in hyperosmotic conditions, inorganic ions (Na^+ , K^+) are accumulated via osmotically regulated ion transporters [38]. However, in this case, cells accumulate organic osmolytes intracellularly to replace a portion of the inorganic ions [39–41]. High ionic strength disrupts cell functions, while in contrast, a high concentration of organic osmolytes is not toxic [39,42]. The beneficial action of organic osmolytes was proven by a report that in a hyperosmotic medium (310 mOsm), the blastocyst formation rate of mouse embryos cultured in the presence of 1 mM glycine was significantly more favorable than that of embryos cultured in the absence of glycine [12].

We used G1/G2 medium (Vitrolife), which contains 135–145 μM glycine [43], a concentration that is much lower than the effective concentration of 1 mM in previous studies [12,28]. In the present study, the osmolality of G1 medium was 275 mOsm, which increased to 293.7 and 285.0 mOsm in the dry and humid culture conditions, respectively, after 3 days of incubation. The better clinical outcomes

in the humid culture condition than the dry culture condition might have resulted from the low osmolality of the medium in the humid culture condition. Due to the maintenance of low osmolality in the culture medium, glycine probably did not have the chance to act as an organic osmolyte. Instead, the higher osmotic change in the dry culture condition might have increased the concentration of intracellular ionic osmolytes, which are detrimental for embryo development. Moreover, a portion of the ionic osmolytes could not be replaced completely with organic osmolytes because of the low concentration of glycine [43].

In conclusion, the dry culture condition showed a higher osmotic change in the medium than the humid culture condition, even though the media were covered in oil. Hyperosmotic changes showed a detrimental effect on clinical outcomes in human IVF-ET cycles. This hyperosmotic stress could be alleviated by supplementation with outer-well medium to maintain the optimal osmolality of medium, which resulted in the improvement of clinical outcomes in IVF-ET cycles. We are preparing a follow-up study to investigate relationships between the osmolality of culture media and specific patterns of gene expression in porcine embryos.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Reanalysis of discarded blastocysts for autosomal aneuploidy after sex selection in cleavage-stage embryos

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Objective: The goal of the present study was to investigate the rate of chromosomal aneuploidies in surplus embryos after sex determination at the cleavage stage. Then, the same chromosomal aneuploidies were evaluated in blastocysts after extended culture.

Methods: Sixty-eight surplus embryos were biopsied at the cleavage stage and incubated for an additional 3 days to allow them to reach the blastocyst stage. The embryos were reanalyzed via fluorescence *in situ* hybridization (FISH) to examine eight chromosomes (13, 15, 16, 18, 21, 22, X, and Y) in both cleavage-stage embryos and blastocysts.

Results: Although the total abnormality rate was lower in blastocysts (32.35%) than in cleavage-stage embryos (45.58%), the difference was not significant ($p=0.113$). However, when we restricted the analysis to autosomal abnormalities, we observed a significant difference in the abnormality rate between the cleavage-stage embryos (44.11%) and the blastocysts (17.64%, $p=0.008$). A higher rate of sex chromosomal abnormalities was also observed in cleavage-stage embryos (29.4%) than in blastocysts (14.70%, $p=0.038$).

Conclusion: The data indicated that embryo biopsy should be conducted at the blastocyst stage rather than the cleavage stage. The results also emphasized that examination of common chromosomal aneuploidies apart from sex selection cycles can be conducted in the blastocyst stage with the FISH method.

Keywords: Blastocyst; Embryo; Fluorescence *in situ* hybridization; Trophoctoderm biopsy

Introduction

The assessment of morphology alone is not an adequate method

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of differentiating euploid embryos from aneuploid embryos to facilitate a viable pregnancy and a live birth [1,2]. Therefore, an alternative approach is required for precise embryo selection; this approach should include genetic and cytogenetic assessments following morphological investigation for the detection of aneuploidy [1]. Chromosomal abnormalities can be independent of the morphological features of the embryo. These abnormalities are widespread during meiosis and mitosis early in embryonic development, and they result in aneuploidies, such as mosaicism, in embryos [3]. Chromosomal instability causes a marked decline in the success rate of conception [4]. Genotype determination using cytogenetic techniques accompanied by assessment of the morphology of the embryo has been

demonstrated to significantly improve both implantation and live birth rates [5]. The prevalence of aneuploidy has been reported to be greater than 50% of preimplantation embryos in *in vitro* fertilization programs [3,6-9]. Aneuploidy is a pivotal genetic condition that can lead to implantation failure, miscarriage, and congenital abnormalities and impacts the success rates of assisted reproductive technology programs [10]. It has been reported that, in cases of advanced maternal age, almost 40% of aneuploid embryos with chromosomal abnormalities can reach the blastocyst stage with similar morphological appearance to euploid embryos [1,7].

Several studies have shown that multiple chromosomes (13, 15, 16, 18, 21, 22, X, and Y) are involved in the high rates of aneuploidies in cleavage-stage embryos. These aneuploidies include trisomies that cause spontaneous abortion, along with those in pregnancies carried to term in the form of trisomic syndrome [11,12]. In addition, mosaicism increases the risk for intrauterine fetal demise or uniparental disomy [13,14]. Some studies have declared that mosaic embryos self-correct via a different aneuploidy rescue mechanism from non-mosaic aneuploid embryos. Following such a mechanism, the aneuploid cells are excluded and do not participate in the embryo's cytogenetic status [1,2,4]. Recently, Fesahat et al. [15] reported a significant difference in the aneuploidy rates between sex chromosomes and autosomal chromosomes in human cleavage-stage embryos. However, according to a previous study, biopsies are only performed at the blastocyst stage in about 7% of cycles, up from less than 1% previously [16]. Almost 67% of biopsies were performed at the cleavage stage using fluorescence *in situ* hybridization (FISH).

The objective of the present study was to determine the common abnormalities (aneuploidies) in embryos resulting from intracytoplasmic sperm injection (ICSI) in two steps. The first step was the evaluation of autosomal abnormalities in biopsies taken for sex selection in the second round of FISH. The second step was the evaluation of autosomal abnormalities in blastocysts generated from surplus embryos from a sex selection program.

Methods

The study was approved by the independent Ethics Committee at the Yazd Reproductive Sciences Institute (IR.SSU.REC.1396.20). Written informed consent was obtained from all participants in the study. All experiments were performed in accordance with relevant guidelines and regulations.

1. Participants and sample collection

Sixty-eight surplus embryos from 37 young couples (aged 25–35 years) with proven fertility were subjected to sex chromosome analysis. The surplus embryos were analyzed in two sequential rounds. The first round included rewashed sex selection slides from surplus embryos, which developed to reach the blastocyst stage. Next, the surplus embryos were assessed in the blastocyst stage (Figure 1).

This work was done at Avicenna Infertility Center in Tehran between August and November 2018. The inclusion criteria were surplus embryos from sex selection that had reached the blastocyst stage, normal ejaculation according to 2010 World Health Organization criteria, and women < 40 years old without a history of recurrent pregnancy loss or genetic disorders. All participants had at least one child.

2. Semen preparation for ICSI

Semen was prepared using the swim-up method in accordance with World Health Organization criteria. The ICSI procedure was carried out as previously described [17,18].

3. Fertilization and embryo selection

After ICSI, the injected oocytes were cultured in 20- μ L drops of cleavage medium (ORIGIO Sequential Cleav medium; Origio, Måløv, Denmark) covered with paraffin oil. Fertilization was verified 16–18 hours after ICSI. Then, the fertilized oocytes were cultured in cleav-

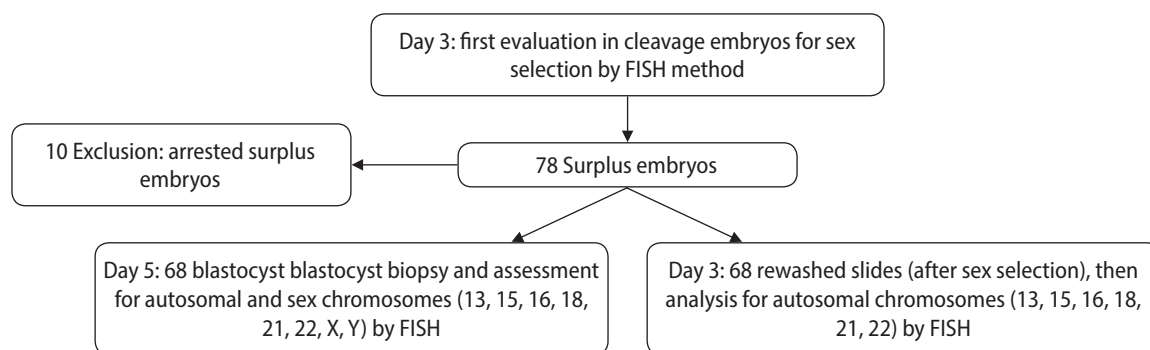


Figure 1. Flowchart of the chromosomal assessment of embryos in the cleavage and blastocyst stages via fluorescence *in situ* hybridization (FISH).

age medium for 48 hours, and embryonic development was assessed according to the categorization system proposed by Gardner [19,20]. On day 3, the cleavage-stage embryo biopsy was performed for the genetic analysis via FISH. Genetic testing was carried out via laser biopsy on 2 blastomeres from each top-quality embryo that had at least 7 blastomeres available for sex selection analysis [19]. After the blastomeres were lysed and fixed on 2 different glass slides. The nuclei were analyzed using the FISH method (Figure 1) [7].

4. Rewashing slides from cleavage-stage embryos

Reanalysis slides from surplus embryos were subjected to sex selection for autosomal chromosomes (13, 15, 16, 21, and 22) via the FISH method. The procedure included washing in phosphate-buffered saline, fading of the previous probe, and a subsequent dehydration series in ethanol. Finally, the slides were hybridized with a second set of probes. Two slides were applied for the chromosomal X series (13, 18, 21, and X) (Cytocell; Oxford Gene Technology, Oxfordshire, UK) and Y series (15, 16, 22, and Y) centromeric probes (MetaSystems GmbH, Altlußheim, Germany). Then, the slides were assessed using a fluorescent microscope (Olympus BX51 and Genetics GSL-10 with BX61; Olympus, Tokyo, Japan). The installation filter sets on the microscope were triple-band filters (aqua, orange, and green) and single-band pass filters (red, green, and aqua). Images were captured at $\times 60$ or $\times 100$ magnification using imaging software (Spectral Instruments Imaging, Tucson, AZ, USA).

5. Blastocyst grading and biopsy

The blastocysts were graded according to the categorization system proposed by Gardner [19,20]. On day 3, the blastocyst biopsy of the surplus embryos was conducted for sex selection with assessment of the autosomal and sex chromosomes (13, 15, 16, 18, 21, 22, X, and Y) via the FISH method. Blastocyst biopsy of trophectoderm (TE) cells was performed on day 5 after fertilization from hatching blastocysts [21]. For the biopsy, each embryo was placed in 5 μL of blast medium (ORIGIO Sequential Blast) under mineral oil in a separate dish. The blast medium was warmed in an incubator with carbon dioxide. The hatching blastocyst was left in the blast medium instead of Ca^{2+} -free medium for the biopsy. This prevented the loosening of the adhesion junction between cells and avoided mixing of TE and inner cell mass (ICM) cells. TE cells were gently captured by a hooked (30°) micropipette. Then, 5–20 TE cells were cutoff with a noncontact 2.3- μm laser (ZILOS-tk; Hamilton Thorne, Beverly, MA, USA) in the process of laser-assisted hatching. The blastocyst was then released from the holding micropipette. Finally, the hook micropipette was very gently rubbed against the holding micropipette to facilitate the release of biopsied cells from the hook micropipette.

6. Slide preparations for FISH

The separated TE cells were placed in droplets of hypotonic medium that had already been warmed in the incubator with carbon dioxide for 10–30 minutes. The duration of incubation depended on the number of TE cells being biopsied. Then, swelling cell samples were transferred into a scored circle on a coated slide and fixed with cold Carnoy solution (methanol:acetic acid, 3:1). Finally, the slides were left to air-dry at room temperature.

The slides prepared from each embryo for the FISH testing for the chromosome X series (13, 18, 21, and X; Oxford Gene Technology) and Y series (15, 16, 22, and Y) centromeric probes (MetaSystems, Altlußheim, Germany) were checked with fluorescent microscopy. The FISH procedure was accomplished according to modified instructions (Oxford Gene Technology).

7. Statistical analysis

SPSS ver. 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The data were categorical and were expressed as percentages. The chi-square and Fisher exact tests were used for data analysis as appropriate. The p -values ≤ 0.05 were considered to indicate statistical significance.

Results

1. FISH results in two stages

A significant rescue in autosomal aneuploidy was observed in blastocysts relative to cleavage-stage embryos (17.64% vs. 44.11%, respectively; $p = 0.008$). Significant self-correction of sex abnormalities was also observed in blastocysts relative to cleavage-stage embryos (14.70% vs. 29.4%, respectively; $p = 0.038$). In addition, significant differences were seen in the aneuploidy rates in cleavage-stage embryos compared to blastocysts in chromosomes 13 (20.4% vs. 0%, respectively; $p < 0.001$), 21 (20.5% vs. 0%, respectively; $p < 0.001$), 16 (26.4% vs. 11.76%, respectively; $p = 0.029$), and Y (30.8% vs. 8.82%, respectively; $p = 0.001$). The rate of chaotic aneuploidy in blastocysts was 13.23%, the frequency of polyploidy was 7.35%, and the rate of mosaicism in TE cells was 14.70% (Figure 2). Overall, no significant differences were found between the total abnormality of embryos in the cleavage and blastocyst stages (45.58% vs. 32.35%, respectively; $p = 0.113$). The numbers of abnormalities in each group are also presented separately in Table 1.

Discussion

The aim of the current study was to estimate the rates of autosomal aneuploidies in surplus cleavage-stage embryos generated from young, fertile women admitted to a sex selection program. More-

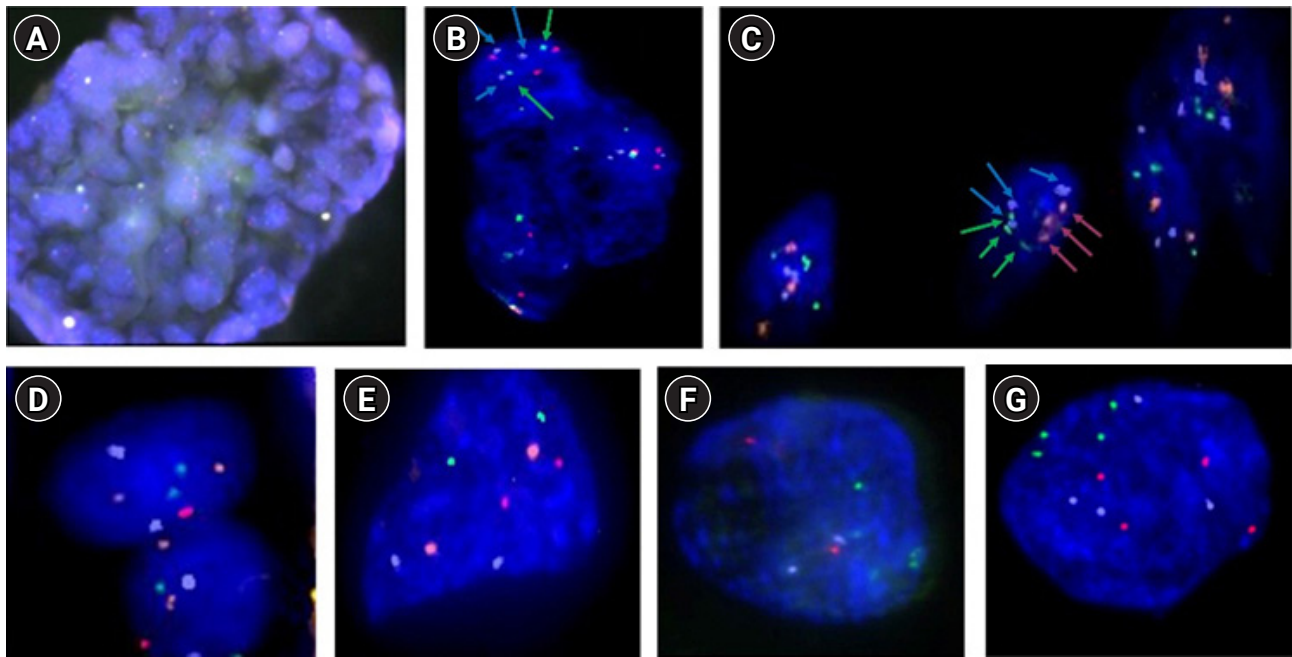


Figure 2. The probes from the X series, which include green (chromosome 13), blue (16), red (21), and orange (X). The probes from the Y series include green (chromosome 15), blue (16), red (22), and orange (Y). (A) Trophoblast (TE) cells from a hatching blastocyst, with approximately 40 cells biopsied at $\times 20$ magnification. (B) Mosaicism of TE cells for the Y series at $\times 60$ magnification. Green: chromosome 15 (2), blue: chromosome 16 (3), red: chromosome 22 (3), orange: Y chromosome (0), green: chromosome 15 (2), blue: chromosome 16 (2), red: chromosome 22 (2), orange: Y chromosome (0). (C) Triploid and chaotic TE cells with the probe Y series at $\times 60$ magnification. Green: chromosome 15 (3), blue: chromosome 16 (3), red: chromosome 22 (3), orange: Y chromosome (0). (D) Normal cells for the X series from TE cells at $\times 60$ magnification. Green: chromosome 13 (2), blue: chromosome 18 (2), red: chromosome 21 (2), orange: X chromosome (2). (E) X series for a euploid blastomere at $\times 100$ magnification. One blastomere from an embryo in the cleavage stage. Green: chromosome 13 (2), blue: chromosome 18 (2), red: chromosome 21 (2), orange: X chromosome (2). (F) Y series for a euploid blastomere at $\times 100$ magnification. Green: chromosome 15 (2), blue: chromosome 16 (2), red: chromosome 22 (2), orange: Y chromosome (2). (G) Tetraploid blastomere from the X series at $\times 100$ magnification. Green: chromosome 13 (4), blue: chromosome 18 (4), red: chromosome 21 (4).

over, the rates of autosomal aneuploidies were estimated in surplus embryos that were further cultured to the blastocyst stage. The study design was unique, as embryo biopsy was performed twice—at the cleavage and the blastocyst stage—in the same embryo. In the present study, the total abnormality rate of the blastocysts was not significantly lower than at the cleavage stage. However, when we restricted our analysis to autosomal abnormalities, the abnormality rate of the blastocysts became significantly lower than at the cleavage stage. The rate of sex chromosome abnormality in the blastocysts was also significantly lower than at the cleavage stage. A reduction in aneuploidy from the cleavage to the blastocyst stage has been reported by others [22]. According to the available data, this reduction is due to the selection of normal cells in embryonic development [23]. However, the insignificant decrease in the abnormality rate of embryos that were examined at the blastocyst stage in the present study may be related to the small study population and differences in the techniques used.

Numerous studies have shown that different mechanisms are in-

involved in the process of abnormality correction. These include multipolar division for trisomic rescue, fragment resorption, endoreduplication in cases of uniparental disomy for monosomic errors, blastomere exclusion via selection against highly aneuploid blastomeres to overcome chromosomal instability during the morula-blastocyst transition, encapsulation into micronuclei, elimination via cellular fragmentation, apoptosis of aneuploid cells, migration of euploid cells within the ICM, and migration of the aneuploid population to the blastocyst TE cells [1,2,4,24]. In addition, we reported that chaotic aneuploidy had a frequency of 13.23% in the blastocysts. One reason for chaotic aneuploidy is multipolar division [4]. Additionally, we reported a 14.70% rate of mosaicism in the blastocysts. The women in our study were 25–35 years old. However, Daughtry and Chavez [1] reported that mosaicism was prevalent in cases with advanced maternal age. Furthermore, we reported a 14.70% rate of sex chromosome abnormality as determined via FISH. In contrast, Liang et al. [25] reported a 6.5% rate of sex chromosome abnormality as determined using oligonucleotide DNA microarray analysis in blastocysts.

Table 1. FISH results from two embryonic stages (cleavage and blastocyst)

Chromosome	FISH (washing slides) in cleavage-stage embryos (n = 68)	FISH in blastocysts (n = 68)	p-value
13	14 (20.4)	0	< 0.001 ^{a)}
18	5 (22)	1 (1.47)	0.208 ^{a)}
21	14 (20.5)	0	< 0.001 ^{a)}
X	19 (27.9)	15 (22.05)	0.428 ^{b)}
15	13 (19.1)	8 (11.76)	0.235 ^{b)}
16	18 (26.4)	8 (11.76)	0.029 ^{b)}
22	11 (16.1)	7 (10.29)	0.312 ^{b)}
Y	21 (30.8)	6 (8.82)	0.001 ^{b)}
Autosomal abnormality	30 (44.11)	12 (17.64)	0.008 ^{b)}
Sex abnormality	20 (29.4)	10 (14.70)	0.038 ^{b)}
Total abnormal	31 (45.58)	22 (32.35)	0.113 ^{b)}
Total normal	37 (54.41)	46 (67.64)	0.114 ^{b)}
Chaotic		9 (13.23)	
Polyploid		5 (7.35)	
Mosaic		10 (14.70)	

Values are presented as number (%).

FISH, fluorescence *in situ* hybridization.

Statistically significant as determined with ^{a)}Fisher exact test, ^{b)}chi-square test.

Interestingly, we did not observe any abnormalities of chromosome 21, while Liang et al. [25] reported a 5% rate of chromosome 21 abnormality in blastocysts. Our findings showed that 29.4% of embryos at the cleavage stage exhibited aneuploidy of a sex chromosome versus 14.70% in the blastocyst stage. Zakharova et al. [14] declared that 44% of the embryos at the cleavage stage exhibited aneuploidy of a sex chromosome.

Kort et al. [23] also reported a lower rate of nuclear abnormalities in the blastocyst stage (5%) than in cleavage-stage embryos (16%), but the population of that study was larger than that of the present study. In our study, the total rate of abnormalities in the blastocyst stage was 32.35%. The genetic condition of blastocyst is more stable than the cleavage stage because genomic errors occur in different steps in oocytes, meiosis I and II [7], and fertilization, and about 50% of errors happen in post-fertilization mitotic division, causing mosaicism [26,27]. Furthermore, Lagalla et al. [2] reported that mitotic anomalies could become amended in the blastocyst stage. Barbash-Hazan et al. [22] also reported that some embryos have the potential to normalize their chromosomal status to proceed to the blastocyst stage [1]. Similar to previous studies, we found that the morphological characteristics of embryos did not exactly align with the chromosomal content, because 22 of 68 high-quality embryos had chromosomal abnormalities [15], whereas all of the aneuploid embryos had reached the blastocyst stage similar to the euploid embryos.

In this study, biopsy was performed at both the cleavage and blastocyst stages. Performing biopsy on ICM cells in addition to TE cells

would facilitate a better understanding of the genetic correlation; however, that is considered an unethical procedure in a clinical setting. Munne and colleagues reported that the genetics of TE were different from ICM cells [28]. The main limitation of the FISH method is its capacity to analyze only a limited number of chromosomes [29,30]. Other flaws of FISH include signal overlap, hybridization failure, the lack of individual hybridization, the interpretation of results in the contiguous signals, the capacity to examine only one-third of the chromosomes, and the ambiguity of results for the other chromosomes [7]. Nowadays, newer technologies, such as comparative genomic hybridization microarray and next-generation sequencing, can be used to increase the accuracy of diagnosis of embryonic chromosomal abnormalities. However, these methods are not available in the majority of laboratories worldwide and are not cost-effective [31]. Therefore, the FISH method can be still considered a useful tool for identification of common chromosomal errors in embryos at both cleavage and blastocyst stages [31].

Another nuance that should be mentioned is that mosaic embryos may produce different results dependent on the population biopsied [2]. Therefore, we should be careful when making decisions about the presence of false or true mosaicism in blastocysts [1]. According to our results and previous reports, no doubt should exist that biopsy at the blastocyst stage is superior to biopsy at the cleavage stage. Sahin et al. [7] concluded that morula embryos had many abnormalities and that self-correction is disabled at this stage. Blastocyst biopsy facilitates the presence of a greater number of cells and a more informed genetic decision, such as in the contexts of mosaicism and

chaotic aneuploidy in embryos [13]. Embryos can perform self-rescue by one of the mechanisms previously mentioned [1,2,4,24]. In the blastocyst, the embryo represents the first visible stage of differentiation, past the primary steps of cell differentiation. Therefore, embryos are known to have a notable implantation potential and a lower rate of aneuploidy compared to the cleavage stage [14,31]. Despite all of the mentioned advantages, less than 1% to 7% of biopsies are done in the blastocyst stage [16].

In conclusion, we recommend embryo biopsy at the TE stage rather than the cleavage stage in sex selection programs. The results of this study also emphasize that the examination of common chromosomal aneuploidies apart from sex selection cycles can be conducted in the blastocyst stage with the FISH method.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Dual trigger in normally-responding assisted reproductive technology patients increases the number of top-quality embryos

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Objective: The feasibility of a gonadotropin-releasing hormone agonist (GnRHa) trigger in normal responders is still a matter of debate. The aim of this study was to compare the number of mature oocytes, the number of good-quality embryos, and the live birth rate in normal responders triggered by GnRHa alone, GnRHa and human chorionic gonadotropin (hCG; a dual trigger), and hCG alone.

Methods: A retrospective cohort study was conducted at the infertility clinic of a university hospital. Data from 200 normal responders who underwent controlled ovarian hyperstimulation and intracytoplasmic sperm injection with a GnRH antagonist protocol between January 2016 and January 2017 were reviewed. The first study group consisted of patients with cycles triggered by GnRHa alone. The second study group consisted of patients with cycles triggered by both GnRHa and low-dose hCG (a dual trigger). The control group consisted of patients with cycles triggered by hCG alone.

Results: The groups were comparable in terms of demographics and cycle characteristics. The numbers of total oocytes retrieved and metaphase II oocytes were similar between the groups. The total numbers of top-quality embryos were 3.2 ± 2.9 in the GnRHa group, 4.4 ± 3.2 in the dual-trigger group, and 2.9 ± 2.1 in the hCG group ($p=0.014$). The live birth rates were 21.4%, 30.5%, and 28.2% in those groups, respectively ($p=0.126$).

Conclusion: In normal responders, a dual-trigger approach appears superior to an hCG trigger alone with regard to the number of top-quality embryos produced. However, no clinical benefit was apparent in terms of live birth rates.

Keywords: Assisted reproductive technology; Dual trigger; Final oocyte maturation; GnRH agonist; Normal response

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Introduction

Mature oocytes are necessary for oocyte retrieval and fertilization, and final oocyte maturation is the key step in assisted reproductive technology (ART) cycles. Human chorionic gonadotropin (hCG) has been used for final oocyte maturation for many years and has been demonstrated to promote an increased pregnancy rate. However, the administration of hCG for final oocyte maturation results in supraphysiologic steroid levels in the luteal phase due to its long half-life and is consequently associated with an increased risk of ovarian hyperstimulation syndrome (OHSS) [1]. In addition, it has been sug-

gested that an hCG trigger may have negative impacts on endometrial receptivity and embryo quality [2].

At the end of the last century, gonadotropin-releasing hormone agonists (GnRHAs) were introduced to promote final oocyte maturation in GnRH antagonist cycles [3,4]. GnRHa has been documented to induce endogenous luteinizing hormone (LH) and follicle-stimulating hormone (FSH) surges similar to the natural mid-cycle LH surge with a shorter duration and smaller amplitude than are associated with the administration of exogenous hCG, which may help to reduce the risk of OHSS [3,5]. The use of a GnRHa trigger may have possible benefits, including the induction of both an FSH and an LH surge and the possibility of retrieving more metaphase II (MII) oocytes than is expected with an hCG trigger [2,3,5]. Previously, the major drawback of a GnRHa trigger was LH depletion and the withdrawal of LH support of the corpus luteum, reflected by a reduced live birth rate and an increased miscarriage rate [2,5]. However, this problem has been overcome with intensive luteal phase support (LPS) and luteal-phase hCG administration [6].

As a result of developments in LPS, the concept of a GnRHa trigger has been fully established during the last decade in hyper-responder patients [6,7]. However, insufficient evidence is available regarding the impact of a GnRHa or a dual trigger on normal responders. A recent observational study reported cycle outcomes following a GnRHa trigger with exclusive hCG support in a progesterone-free luteal phase, and the results were comparable to those of cycles triggered by hCG [8]. This study was rare in that it supported a GnRHa trigger as a valid alternative in normal responders. However, a GnRH trigger is not yet a standard of care, and its feasibility in normal responders needs to be clarified.

The aim of the present study was to investigate whether differences were present in the number of oocytes collected, the number of top-quality embryos, and the live birth rate in normal responders triggered by GnRHa alone, GnRHa and hCG (a dual trigger), and hCG alone.

Methods

In the present retrospective cohort study, data from normal responders who underwent intracytoplasmic sperm injection following a cycle downregulated by a GnRH antagonist at a university-based infertility clinic between January 2016 and January 2017 were reviewed. The study was approved by the Ethical Committee of Ankara University School of Medicine (No. 08-341-16). In total, 214 patients who underwent intracytoplasmic sperm injection cycles with an antagonist protocol involving a starting dose of 225 IU per day during the study period were selected from the hospital database. The inclusion criteria were the female patient being 18–40

years of age, a baseline FSH level of 3–15 IU/L, a baseline LH level above 3 IU/L, a normal response to ovarian stimulation, and (as mentioned above) a starting dose of gonadotropin stimulation of 225 IU/day. Cases of female factor infertility (tubal factor, pelvic adhesions, or mild endometriosis), mild male factor infertility, and unexplained infertility were included in the analyses. A normal response was defined as the aspiration of 6–14 oocytes and a maximum oestradiol level lower than 2,500 pg/mL. The exclusion criteria were secondary infertility, a body mass index over 30 kg/m², poor or hyper-response to ovarian stimulation, severe male oligozoospermia or azoospermia, the presence of any untreated thyroid dysfunction or hyperprolactinaemia, the presence of uterine abnormality, and the avoidance of fresh embryo transfer (freeze-all). After application of the inclusion and exclusion criteria, 200 patients were found to be eligible for analysis. Eleven patients who underwent a freeze-all cycle and three patients with abnormal thyroid function tests were excluded. For all women, only the first fresh cycles were included in the analyses.

For eligible participants, all data on ovarian stimulation and clinical outcomes were extracted from the database, and patients were divided into three groups according to the trigger method. The first study group consisted of patients with cycles triggered by GnRHa alone. An injection of 0.2 mg triptorelin acetate (Gonapeptyl 0.1 mg; Ferring, Istanbul, Turkey) or 1 mg (20 units in a tuberculin syringe of 5 mg/mL injectable solution) leuprolide acetate (Lucrin 5 mg; Abbott, Istanbul, Turkey) was administered for final oocyte maturation in the GnRHa group. The second study group consisted of patients with cycles triggered by both GnRHa and hCG (the dual-trigger group). Those patients received an additional 1,500-IU hCG injection for final oocyte maturation (Pregnyl 5,000 IU; MSD, Oss, the Netherlands). The control group consisted of patients with cycles triggered by 10,000 IU hCG alone (the hCG group). The method of final oocyte maturation was based on the primary physician's preference. Additionally, as we previously reported that triptorelin acetate and leuprolide acetate have similar effects on final oocyte maturation, we included both drugs in the study [7].

Ovarian stimulation was carried out with recombinant FSH (Gonal-F; Merck-Serono, Istanbul, Turkey) beginning on the second day of the menstrual cycle with a fixed starting dose of 225 IU/day when the antral follicle count was < 12 per ovary. The dose was adjusted individually according to the ovarian response. A GnRH antagonist (Cetrotide, Merck-Serono) was introduced at a dose of 0.25 mg/day on the 6th day (a fixed antagonist protocol) and continued to be administered throughout ovarian stimulation. When at least two follicles reached 18 mm or three follicles reached 17 mm in diameter, an agent was administered to trigger final oocyte maturation. Transvaginal ultrasonography-guided oocyte retrieval was performed

35–36 hours after final oocyte maturation. Embryo transfer was performed on the third day of oocyte retrieval. A maximum of two embryos were transferred under ultrasound guidance in accordance with national embryo transfer regulations.

All women received 90 mg/day of vaginal micronized progesterone (Crinone 8% gel, Merck-Serono) for LPS from the day of oocyte collection until the pregnancy test performed 12 days after ET, and women with a positive pregnancy test continued this LPS regimen until 10 weeks of gestation. In addition, 1500 IU of hCG was administered on the day of oocyte retrieval for LPS in the GnRHa group.

Morphologically, top-quality embryos were those with the following characteristics: four or five blastomeres on day 2 and at least seven blastomeres on day 3, an absence of multinucleated blastomeres, and < 20% fragmentation on days 2 and 3 after fertilization [9]. A positive pregnancy test was defined based on serum β-hCG levels measured 2 weeks after embryo transfer. Clinical pregnancy was defined as a pregnancy diagnosed via ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy. The primary outcome measures were the live birth rate, the number of good-quality embryos, and the number of mature oocytes.

1. Statistical analysis

Data analyses were performed using IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA). Samples were tested with the Shapiro-Wilk test to determine the normality of the distribution. Based on the results, parametric tests were preferred. The continuous variables were compared using one-way analysis of variance. The categorical variables were compared using the chi-square test or Fisher exact test, as appropriate. A *p*-value of < 0.05 was considered to indicate statistical significance. When a statistically significant difference was present, a post-hoc analysis was performed between each pair of groups to identify the source of the statistical significance.

Results

The groups were comparable in baseline and demographic parameters, including age, body mass index, duration of infertility, cause of infertility, and baseline hormonal status (Table 1). All patients were normal responders from whom between 6 and 14 oocytes were collected.

The total dose of gonadotropins used, the duration of ovarian stimulation, number of oocytes collected, number of MII oocytes, and fertilization rate were comparable among the groups (Table 2). The number of transferred embryos was 1.1 ± 0.7 in the GnRHa group, 1.2 ± 0.6 in the dual-trigger group, and 1.2 ± 0.5 in the hCG group. The total number of top-quality embryos was 3.2 ± 2.9 in the GnRHa group, 4.4 ± 3.2 in the dual-trigger group, and 2.9 ± 2.1 in the hCG group (*p* = 0.014). The statistical significance stemmed from the difference between the dual-trigger and hCG groups (*p* = 0.011).

The clinical pregnancy and miscarriage rates were similar among the groups. The live birth rates per started cycle in the GnRHa, dual-trigger, and hCG trigger groups were 21.4%, 30.5%, and 28.2%, respectively (*p* = 0.126) (Table 2). No cases of OHSS were found in any of the groups.

Discussion

The present retrospective cohort study was conducted to assess the impact of the trigger of final oocyte maturation in antagonist co-treated ART cycles in normal responders. Three trigger methods were compared: a GnRHa trigger, an hCG trigger, and a dual trigger involving GnRHa and hCG. According to the results, the dual-trigger method resulted in a significantly higher number of top-quality embryos than the hCG trigger. However, the live birth rates were comparable among the groups. Although the live birth rate was lower in

Table 1. Demographic and baseline characteristics of the study groups

Variable	GnRHa (n = 56)	Dual trigger (n = 59)	hCG (n = 85)	<i>p</i> -value
Age (yr)	30.6 ± 4.7	31.4 ± 4.2	32.1 ± 4.5	0.188
Body mass index (kg/m ²)	23.5 ± 4.2	24.8 ± 3.5	24.9 ± 3.7	0.166
Basal E ₂ (pg/mL)	42.2 ± 2.14	53.4 ± 3.8	48.7 ± 2.02	0.092
Baseline FSH (IU/mL)	8.2 ± 4.4	75 ± 4.5	82 ± 3.7	0.571
Baseline LH (IU/mL)	4.7 ± 2.7	4.4 ± 2.3	4.4 ± 2.1	0.761
Duration of infertility (yr)	6.4 ± 3.5	6.1 ± 2.8	5.8 ± 2.4	0.508
Cause of infertility				0.062
Female factor	9 (16.1)	15 (25.4)	20 (23.5)	
Male factor	31 (55.4)	17 (28.8)	38 (44.7)	
Unexplained	16 (16.1)	27 (16.1)	27 (16.1)	

Values are presented as mean ± standard deviation or number (%).

GnRHa, gonadotropin-releasing hormone agonist; hCG, human chorionic gonadotropin; E₂, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

the GnRHa-trigger group than in the other groups, this difference was not statistically significant.

hCG has been used in routine ART cycles for final oocyte maturation. Usually, a bolus of 5,000–10,000 IU of hCG is administered to promote final oocyte maturation and ovulation. hCG primarily binds LH receptors to facilitate oocyte maturation. The sustained luteotropic effect of hCG (owing to its long half-life) supports the luteal phase but also increases the risk of OHSS [1]. Although both LH and FSH surge during natural cycles to trigger ovulation, hCG lacks FSH receptor activity. Since FSH induces LH receptor formation on granulosa cells to promote oocyte maturation and cumulus expansion, an hCG trigger does not fully resemble natural oocyte maturation and ovulation [10]. Unlike hCG, a GnRHa trigger results in both LH and FSH surges [11]. Hence, the result is more similar to natural ovulation. In addition, a GnRHa trigger can also activate the GnRH receptors on granulosa cells, which may regulate ovulation [12]. Previously, the most important drawback of a GnRHa trigger was the associated low clinical pregnancy and high miscarriage rates [13,14]. Then, the Copenhagen Workshop group suggested that the luteolytic properties of GnRHa were effective in preventing OHSS, but also result in lower success rates [15]. The LH surge following a GnRHa trigger peaks more rapidly and has a shorter duration than the LH surge in a natural cycle [16]. This short surge results in oocyte maturation and ovulation but is not sufficient to support the corpus luteum [17]. This suggestion has led to the utilization of new LPS strategies, such as intensive luteal support, adjuvant low-dose hCG at the same time as GnRHa administration (dual trigger) or on the day of oocyte retrieval, and adjuvant very-low-dose hCG in the luteal phase [18–21]. Because of these strategies, the pregnancy rates have become comparable to

those obtained using an hCG trigger, and OHSS rates have decreased significantly [18,22].

A GnRHa trigger has become one of the preferred strategies to avoid OHSS in hyper-responder patients worldwide. However, it also confers additional benefits to ART cycles. Griffin et al. [10] reported the results of patients with a history of a >25% proportion of immature oocytes retrieved in a prior IVF cycle who were treated with a GnRHa trigger in the subsequent cycle. The authors reported a significantly increased rate of oocyte maturation following a GnRHa trigger (75%) compared to an hCG trigger (38.5%) [10]. In another study, Kim et al. [23] randomly assigned normal responders to dual-trigger and hCG-trigger groups. They found significant improvements in the implantation rate (24.7% vs. 14.9%), clinical pregnancy rate (53.3% vs. 33.3%), and live birth rate (50% vs. 30%) when a dual-trigger approach was used [23]. The suggested benefits of a dual trigger are improvements in endometrial receptivity and pregnancy rate.

In the present study, we demonstrated a significantly greater number of top-quality embryos in normal responders treated with a dual-trigger approach compared to an hCG trigger alone, but not to a GnRHa trigger alone. However, we failed to show a significant difference among the groups regarding live birth rates. While the difference was not statistically significant, the live birth rate in the GnRHa trigger group was remarkably lower than in the dual- and hCG-trigger groups (21.4% vs. 30.5% vs. 28.2%, respectively). The mean numbers of oocytes retrieved and MII oocytes were comparable among the groups in our study. However, the total number of MII oocytes tended to be greater in the dual-trigger group than in the other groups. Hence, the results of this study should be interpreted with

Table 2. Comparison of cycle characteristics and outcome parameters between the study groups

Variable	GnRHa (n = 56)	Dual trigger (n = 59)	hCG (n = 85)	p-value
Duration of stimulation (day)	9.4 ± 1.3	9.5 ± 2.4	9.3 ± 1.7	0.913
Total dose of gonadotropins (IU)	2,442 ± 975	2,390 ± 1,049	2,725 ± 932	0.086
Oestradiol levels on the day of trigger (pg/mL)	1,640 ± 1,205	1,916 ± 1,128	1,406 ± 928	0.026 ^{a)}
Number of retrieved oocytes	7.4 ± 4.9	9.2 ± 5.3	7.6 ± 4.5	0.087
Number of MII oocytes	6.2 ± 4.2	7.2 ± 4.7	5.6 ± 3.7	0.095
Fertilization rate (%)	69 ± 42	70 ± 33	62 ± 29	0.500
Number of top-quality embryos	3.2 ± 2.9	4.4 ± 3.2	2.9 ± 2.1	0.014 ^{b)}
Number of transferred embryos	1.1 ± 0.7	1.2 ± 0.6	1.2 ± 0.5	0.291
Endometrial thickness on the day of embryo transfer (mm)	10.4 ± 1.7	10.4 ± 1.8	10.7 ± 1.6	0.490
Positive pregnancy test	15 (26.8)	20 (33.9)	31 (36.5)	0.141
Clinical pregnancy	13 (23.2)	20 (33.9)	26 (30.6)	0.112
Miscarriage	1 (1.8)	2 (3.4)	2 (2.4)	0.148
Live birth	12 (21.4)	18 (30.5)	24 (28.2)	0.126

Values are presented as mean ± standard deviation or number (%).

GnRHa, gonadotropin-releasing hormone agonist; hCG, human chorionic gonadotropin; MII, metaphase II.

Significant difference between the dual-trigger and hCG groups: ^{a)} $p < 0.001$, ^{b)} $p = 0.011$.

caution, as the number of top-quality embryos could be affected by the number of MII oocytes. Additionally, the greater number of MII oocytes could result from the type of trigger, as well the higher peak oestradiol levels in the dual-trigger group.

Recently, in a retrospective cohort study, Zhou et al. [24] demonstrated that a dual trigger results in greater numbers of two-pro-nuclear embryos, embryos available, and high-quality embryos. They also reported nonsignificant trends towards higher implantation, clinical pregnancy, and live delivery rates in the dual-trigger group compared to the hCG-trigger group [24]. In another recent study, Beck-Fruchter et al. [8] compared the results following a GnRH α trigger and an hCG trigger in normal responders. The authors reported similar outcomes regarding the number of oocytes, oocyte maturation rate, implantation rate, and live birth rate [8]. Our results aligned with the results of these two recent studies in that a dual trigger was superior to an hCG trigger alone. However, we showed no significant advantage of a dual trigger over a GnRH α trigger alone in normal responders with regard to the number of top-quality embryos.

The most important strength of our study was its comparison of three different types of ovulation triggers. To the best of our knowledge, this is the first study that included a comparison between a dual trigger and a GnRH α trigger in normal responders. The systematic exploration of individual parameters may also add credence to our observations. The main limitations of the present study were its retrospective nature and small sample size. For instance, the non-significant differences among the dual-trigger group and the other groups regarding oocyte numbers, MII numbers, and (in particular) live birth rates may be reflections of the small sample size. Considering the relatively low live birth rate found in the GnRH α -trigger group, one could assume that a significant difference might be present if a larger cohort were used. However, when interpreting the results, the smaller number of patients and higher peak oestradiol levels in the dual-trigger group should be noted as limitations of this study. Also, the non-randomized case selection makes our study unlikely to be involved in future meta-analyses. Another limitation of our study was the absence of frozen-thawed cycles in the analyses. It is plausible that the inclusion of such cycles would result in better outcomes. Finally, the lack of a sample size calculation should also be noted as a limitation of this study.

In conclusion, in terms of the number of top-quality embryos in normal responders, a dual-trigger approach seems superior to an hCG trigger alone, but not superior to a GnRH α trigger alone. However, no clinical benefit seems to exist in terms of live birth rates. Future randomized controlled trials in large cohorts and meta-analyses are needed to clarify the exact impact of the trigger of final oocyte maturation in normal responders undergoing ART cycles.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Effect of a dual trigger on oocyte maturation in young women with decreased ovarian reserve for the purpose of elective oocyte cryopreservation

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Objective: The aim of this study was to determine whether co-administration of a gonadotropin-releasing hormone (GnRH) agonist and human chorionic gonadotropin (hCG) for final oocyte maturation improved mature oocyte cryopreservation outcomes in young women with decreased ovarian reserve (DOR) compared with hCG alone.

Methods: Between January 2016 and August 2019, controlled ovarian stimulation (COS) cycles in women (aged ≤ 35 years, anti-Müllerian hormone [AMH] < 1.2 ng/mL) who underwent elective oocyte cryopreservation for fertility preservation were retrospectively analyzed.

Results: A total of 76 COS cycles were triggered with a GnRH agonist and hCG (the dual group) or hCG alone (the hCG group). The mean age and serum AMH levels were comparable between the two groups. The duration of stimulation, total dose of follicle-stimulating hormone used, and total number of oocytes retrieved were similar. However, the number of mature oocytes retrieved and the oocyte maturation rate were significantly higher in the dual group than in the hCG group ($p=0.010$ and $p<0.001$). After controlling for confounders, the dual-trigger method remained a significant factor related to the number of mature oocytes retrieved ($p=0.016$).

Conclusion: We showed improved mature oocyte collection and maturation rate with the dual triggering of oocyte maturation in young women with DOR. A dual trigger appears to be more beneficial than hCG alone in terms of mature oocyte cryopreservation for young women with DOR.

Keywords: Dual trigger; GnRH agonist; Fertility preservation; Oocyte; Ovarian reserve

Introduction

Fertility preservation (FP) techniques consist of oocyte, embryo, and oocyte tissue cryopreservation that can prolong the ability to conceive. The cryopreservation of oocytes matured *in vivo* that were obtained in controlled ovarian stimulation (COS) cycles has generat-

ed many successful results [1,2]. Mature oocyte cryopreservation can now be successfully offered as part of FP programs for patients looking to preserve fertility for medical and social reasons. Thus, this is no longer only an experimental concept [3]. However, the usefulness of cryopreservation of collected immature oocytes before and after *in vitro* maturation (IVM) is still debatable [4]. Therefore, it is still important for FP to collect a large number of mature oocytes.

Women with decreased ovarian reserve (DOR) are at risk of losing their reproductive ability due to the reduced number and quality of oocytes in the ovary [5,6]. Young women with DOR may be candidates for elective oocyte cryopreservation for FP before ovarian failure. However, previous COS studies were conducted with regards to the *in vitro* fertilization (IVF) treatment of infertile older women with DOR or young women without DOR (oocyte donors). There is a lack

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of data on obtaining a large number of mature oocytes in young women with DOR.

Meanwhile, one of the most critical steps during COS is the triggering of final oocyte maturation. Human chorionic gonadotropin (hCG) is usually used as a surrogate for the luteinizing hormone surge for final oocyte maturation, resumption of meiosis, and luteinization of the granulosa cells [7]. To reduce the risk of ovarian hyperstimulation syndrome (OHSS), the substitution of hCG with a gonadotropin-releasing hormone (GnRH) agonist was proposed for IVF high responders [8]. However, adverse effects on clinical pregnancy [9] resulted in the emergence of the concept of a dual trigger with a GnRH agonist along with a low dose of hCG [10]. In addition to IVF high responders, the dual-trigger approach has advantages for normal responders in terms of implantation, oocyte collection yield, and oocyte maturation [11]. Furthermore, the dual-trigger approach has been found to improve the oocyte maturation rate in patients with prior low oocyte maturation rates [12]. These benefits have been attributed to the GnRH agonist induced mid-cycle follicle-stimulating hormone (FSH) surge believed to promote oocyte nuclear maturation and cumulus expansion [13,14]. Results regarding the number of mature oocytes or oocyte maturation rates following a dual trigger compared with hCG alone in poor responders are inconsistent [15,16]. Thus, the aim of this study was to investigate whether a dual trigger with a GnRH agonist along with hCG for final oocyte maturation improved mature oocyte cryopreservation outcomes in young women with DOR compared with hCG alone.

Methods

This study was conducted retrospectively with approval from the Institutional Review Board of CHA Gangnam Medical Center (IRB No. GCI-19-44). Due to the retrospective design, the requirement for informed consent was waived. This retrospective study included women 35 years old and younger who underwent elective oocyte cryopreservation for FP due to DOR. DOR was defined when the serum anti-Müllerian hormone (AMH) level was lower than 1.2 ng/mL at the time of COS initiation [17]. AMH concentrations were measured using an Elecsys AMH immunoassay (Roche Diagnostics, Mannheim, Germany). A total of 122 GnRH antagonist COS cycles performed between January 2016 and August 2019 at a CHA Gangnam Medical Center were selected. The exclusion criteria were women on more than their second cycle, a natural cycle, mild stimulation with oral medication, and retrieval failure. After exclusion, a total of 76 COS cycles were included in the final analysis. The study population was divided into two groups based on the trigger method: a dual trigger with a GnRH agonist and hCG (the dual group, n = 40) and a trigger of hCG alone (the hCG group, n = 36). Figure 1 depicts the study pop-

ulation. We compared the number of mature oocytes and oocyte maturation rates of the two groups and investigated factors associated with the number of mature oocytes retrieved to determine the effect of each triggering method while controlling for confounding factors.

Baseline hormone tests and pelvic ultrasound examinations were performed on day 2 or 3 of the menstrual cycle. COS commenced on the 3rd day of the menstrual cycle using recombinant FSH (rFSH; Gonal-F, Merck-Serono, Darmstadt, Germany; Follitrope, LG Life Sciences, Seoul, Korea) and/or highly purified human menopausal gonadotropin (hMG; IVF-M HP, LG Life Sciences; Menopur, Ferring, Saint-Prex, Switzerland). Patient response was monitored during the COS cycle with serial transvaginal ultrasound examinations, and the gonadotropin dose was adjusted. A GnRH antagonist (Cetrotide, Merck-Serono; Orgalutran, MSD Pharmaceuticals, Courbevoie, France) was added when the leading follicle reached 14 mm in diameter. When at least one of the follicles reached 18 mm in diameter, 250 µg of recombinant hCG (Ovidrel, Merck-Serono) or coadministration of 0.2 mg of a GnRH agonist (Decapeptyl; Ipsen Pharma, Barcelona, Spain) with 250 µg of hCG was administered for final oocyte maturation. The choice of triggering method was made at the physicians' discretion.

Under transvaginal ultrasound guidance, oocyte retrieval was performed 35 to 36 hours after dual or hCG trigger. Oocyte maturity was evaluated using microscopy, and the mature oocyte count was assessed. Mature oocytes had a single polar body with an expanded cumulus-corona. Immature oocytes were cultured for IVM. The oocyte maturation rate was calculated by dividing the number of mature oocytes by the number of total oocytes. After excluding degenerative oocytes, the oocytes were cryopreserved using the vitrifica-

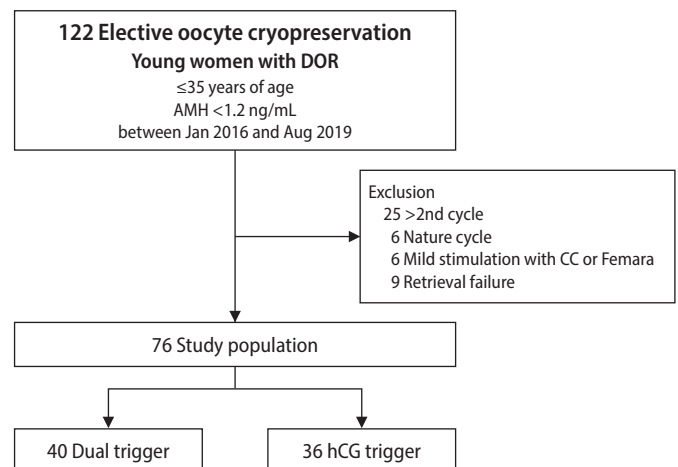


Figure 1. Flow diagram depicting the selection of the study population. DOR, decreased ovarian reserve; AMH, anti-Müllerian hormone; CC, clomiphene citrate; hCG, human chorionic gonadotropin.

tion method.

All statistical analyses were performed using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). Continuous variables are presented as mean ± standard deviation, and categorical variables are presented as numbers with percentages. Differences between the two groups were analyzed using the Student *t*-test or the Pearson chi-square test. Furthermore, we used univariate analysis to identify factors related to the number of mature oocytes retrieved. Multivariate analysis was performed to examine the adjusted effect of related factors. Subgroup analysis according to the COS regimen was performed using the Mann-Whitney test, and *p*-values < 0.05 were considered to indicate statistical significance.

Results

The clinical characteristics of the study subjects are summarized in Table 1. No significant differences were observed in age or ovarian reserve (determined by AMH levels and basal FSH). Approximately one-third of the women had a history of ovarian surgery. Table 2 represents the ovarian stimulation and laboratory outcomes. The duration of stimulation, the total dose of FSH, and the estradiol level on the triggering day were similar. The total FSH dose by the number of oocytes retrieved was comparable between the two groups. However, the total FSH dose by the number of mature oocytes retrieved was significantly lower in the dual group than in the hCG group (634 ± 480 IU vs. 982 ± 748 IU, respectively; *p*=0.030). Although the number of total oocytes retrieved was statistically similar (5.3 ± 3.5

Table 1. Clinical characteristics of the study subjects

Variable	Dual trigger (n = 40)	hCG trigger (n = 36)	<i>p</i> -value
Age (yr)	30.9 ± 4.2	29.6 ± 4.0	0.174
Body mass index (kg/m ²)	20.0 ± 2.3	20.4 ± 2.6	0.361
Basal AMH (ng/mL)	0.64 ± 0.35	0.57 ± 0.36	0.413
Basal FSH (IU/L)	14.6 ± 16.0	13.9 ± 14.5	0.863
Basal LH (mIU/mL)	8.5 ± 10.4	5.5 ± 3.0	0.248
Basal TSH (μIU/mL)	1.7 ± 1.0	1.4 ± 0.6	0.143
Basal prolactin (ng/mL)	19.3 ± 24.5	17.4 ± 14.4	0.754
Basal estradiol (pg/mL)	57.8 ± 45.9	52.4 ± 20.3	0.586
Basal AFC	6.2 ± 3.3	5.4 ± 2.6	0.281
Previous ovarian surgery	35.0 (14/40)	33.3 (12/36)	0.878
USG diagnosis of ovarian cyst	10.0 (4/40)	22.2 (8/36)	0.145

Values are presented as mean ± standard deviation or percent (number). Basal values were measured at day 2 or 3 of the menstrual cycle. hCG, human chorionic gonadotropin; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; AFC, antral follicle count; USG, ultrasonography.

Table 2. Stimulation and laboratory results of the study subjects

Variable	Dual trigger (n = 40)	hCG trigger (n = 36)	<i>p</i> -value
Gonadotropin combination			0.628
rFSH only (%)	50.0 (20/40)	55.6 (20/36)	
rFSH+hMG (%)	50.0 (20/40)	44.4 (16/36)	
Duration of stimulation (day)	7.3 ± 2.1	7.7 ± 2.0	0.440
Total dose of FSH used (IU)	1,658 ± 642	1,874 ± 829	0.207
FSH dose/oocyte	457 ± 389	536 ± 490	0.439
FSH dose/mature oocyte	634 ± 480	982 ± 748	0.030
Estradiol on triggering day (pg/mL)	1,007 ± 597	981 ± 548	0.875
Number of total oocytes retrieved	5.3 ± 3.5	5.0 ± 2.7	0.655
Number of mature oocytes retrieved	3.7 ± 2.7	2.3 ± 1.7	0.010
Degenerative oocyte rate	0.5 (1/213)	1.7 (3/180)	0.336
Oocyte maturation rate	68.5 (146/213)	45.6 (82/180)	< 0.001

Values are presented as percent (number) or mean ± standard deviation. hCG, human chorionic gonadotropin; rFSH, recombinant follicle-stimulating hormone; hMG, human menopausal gonadotrophin; FSH, follicle-stimulating hormone.

vs. 5.0 ± 2.7 , $p=0.655$), the number of mature oocytes retrieved was significantly higher in the dual group than in the hCG group (3.7 ± 2.7 vs. 2.3 ± 1.7 , respectively; $p=0.010$). Furthermore, the oocyte maturation rate was significantly higher in the dual group than in the hCG group (68.5% vs. 45.6%, respectively; $p < 0.001$). OHSS did not occur in either group.

Univariate correlation analysis was used to determine factors significantly related to the number of mature oocytes retrieved: basal AMH ($p=0.041$), basal FSH ($p=0.014$), basal antral follicle count (AFC) ($p < 0.001$), and the dual trigger versus the hCG trigger method ($p=0.011$). In the multivariate analysis, basal AFC ($p < 0.001$) and the use of the dual trigger method ($p=0.016$) remained significant factors (Table 3).

Discussion

The present study showed an increased number of mature oocytes retrieved and higher oocyte maturation rates with the use of a dual trigger in young women with DOR undergoing GnRH antagonist-downregulated COS cycles. A dual trigger in elective oocyte cryopreservation for young women with DOR is a viable treatment to cryopreserve a large number of mature oocytes in FP. Single women could achieve reproductive autonomy via elective oocyte cryopreservation. Currently, women with DOR consider elective oocyte cryopreservation before compromising their fertility. Young women with DOR have fewer oocytes retrieved, but the possibility of a high-quality embryo and clinical pregnancy are higher once the oocytes are acquired [18]. Although young women with low AMH levels are challenging subjects for oocyte retrieval due to their poor response to COS [19], they should be counseled about efficient oocyte cryopreservation plans. Efficient oocyte cryopreservation could diminish the pressure on young women to have a child or select a

partner at a certain time.

In DOR patients, results regarding the number of mature oocytes following dual trigger compared with a trigger of hCG alone remain inconsistent. Zhang et al. [15] concurred that a dual-trigger approach was beneficial in women with a mean age of 36 years. However, Lin et al. [16] showed no difference in mature oocyte outcomes in women (mean age, 38 years) with DOR undergoing a dual-trigger treatment method. Our study was performed in relatively young women; therefore, a dual-trigger method could be more effective in younger women.

Women undergoing FP want to eventually have a healthy child with their cryopreserved mature oocytes. Although we cannot provide results for embryo quality according to the trigger method, the reproductive outcomes of oocytes treated with a dual-trigger approach do not seem to be compromised. Thorne et al. [20] recently reported that the embryo aneuploidy rate was similar in patients stimulated with a GnRH agonist trigger and patients administered an hCG trigger. Embryo development and quality appear to be unaffected in GnRH agonist trigger cycles [21]. Moreover, a meta-analysis revealed that patients treated with a dual-trigger approach had significantly more good-quality embryos than patients treated with an hCG trigger [22].

The endogenous gonadotropin surge released by the administration of GnRH agonist, similar to the natural cycle surge, is considered more physiological than the hCG trigger. The importance of a mid-cycle FSH surge induced by a GnRH agonist has been determined in several animal and molecular biology studies. Animal studies have confirmed the importance of FSH in the upregulation of LH receptor sites formation in granulosa cells [23,24]. FSH also plays a crucial role in promoting the resumption of oocyte meiosis [25,26] and the expansion of cumulus cells [13,27]. Furthermore, the expression of messenger RNA of reproduction-related genes such as am-

Table 3. Correlation analysis of factors related to the number of mature oocytes retrieved

Variable	Univariate analysis		Multivariate analysis	
	B	p-value	B	p-value
Female age (yr)	-0.025	0.709		
Body mass index (kg/m ²)	-0.203	0.075		
Basal AMH (ng/mL)	1.607	0.041	0.071	0.937
Basal FSH (IU/L)	-0.050	0.014	-0.030	0.263
Basal AFC	0.396	< 0.001	0.374	< 0.001
Gonadotropin combination, rFSH+ hMG (vs. rFSH)	-0.897	0.102		
Duration of stimulation (day)	0.064	0.64		
Total dose of FSH used (IU)	0.001	0.412		
Triggering method, dual trigger (vs. hCG trigger)	1.372	0.011	1.36	0.016

Basal values were measured on day 2 or 3 of the menstrual cycle.

B, correlation coefficient; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count; rFSH, recombinant FSH; hMG, human menopausal gonadotropin; hCG, human chorionic gonadotropin.

phiregulin and epiregulin was found to be higher in granulosa cells after a dual-trigger treatment compared to an hCG trigger [28]. Amphiregulin and epiregulin are epidermal growth factor receptor ligands that have been reported to play essential roles in cumulus expansion, oocyte maturation, and meiosis resumption [29,30].

When planning FP, the potential complications and cost of oocyte retrieval should be taken into consideration. In our study, no OHSS or other complications occurred in either group. Although the total dose of FSH used was similar, the efficiency in terms of FSH dose per retrieved mature oocyte was much better with the dual trigger. Further study is needed to evaluate whether the dual-trigger approach is more cost-effective in FP.

We subanalyzed the data according to the gonadotropin combinations (rFSH only or rFSH+hMG; data not shown in the results). The oocyte maturation rates were significantly higher in the dual-trigger group regardless of the COS regimen (rFSH only, 69.5% vs. 45.8%, $p < 0.001$; rFSH with hMG, 67.4% vs. 45.0%, $p = 0.006$). The number of mature oocytes retrieved was higher with the dual-trigger approach, but the difference was not statistically significant due to the small numbers involved (rFSH only: 4.1 ± 2.6 vs. 2.8 ± 1.9 , $p = 0.127$; rFSH with hMG: 3.2 ± 2.8 vs. 1.7 ± 1.4 , $p = 0.089$). The dual-trigger approach for final oocyte maturation seemed to improve mature oocyte retrieval regardless of the combination of gonadotropins used.

This study had a few limitations, such as the small number of study subjects and the retrospective nature of the study. Moreover, only the result of mature oocyte cryopreservation was reported; the results after cryopreservation of *in vitro* matured oocytes were not presented. To the best of our knowledge, this is the first study comparing a dual trigger to an hCG-only trigger in elective oocyte cryopreservation for young women with DOR. The beneficial effect of the dual trigger with a GnRH agonist and hCG on obtaining mature oocytes enabled more mature oocyte cryopreservation in young women with DOR. Future prospective randomized controlled studies are required to validate our findings.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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The impact of post-warming culture duration on clinical outcomes of vitrified-warmed single blastocyst transfer cycles

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Objective: The objective of the study was to compare the effects of long-term and short-term embryo culture to assess whether there is a correlation between culture duration and clinical outcomes.

Methods: Embryos were divided into two study groups depending on whether their post-warming culture period was long-term (20–24 hours) or short-term (2–4 hours). Embryo morphology was analyzed with a time-lapse monitoring device to estimate the appropriate timing and parameters for evaluating embryos with high implantation potency in both groups. Propensity score matching was performed to adjust the confounding factors across groups. The grades of embryos and blastocoels, morphokinetic parameters, implantation rate, and ongoing pregnancy rate were compared.

Results: No significant differences were observed in the implantation rate or ongoing pregnancy rate between the two groups (long-term culture group vs. short-term culture group: 56.3% vs. 67.9%, $p=0.182$; 47.3% vs. 53.6%, $p=0.513$). After warming, there were more expanded and hatching/hatched blastocysts in the long-term culture group than in the short-term culture group, but there was no significant between-group difference in embryo grade. Regarding pregnancy outcomes, the time to complete blastocyst re-expansion after warming is shorter in women who became pregnant than in those who did not in both culture groups (long-term: 2.19 ± 0.63 vs. 4.11 ± 0.81 hours, $p=0.003$; short-term: 1.17 ± 0.29 vs. 1.94 ± 0.76 hours, $p=0.018$, respectively).

Conclusion: The outcomes of short-term culture and long-term culture were not significantly different in vitrified-warmed blastocyst transfer. Regardless of the post-warming culture time, the degree of blastocyst re-expansion 3–4 hours after warming is an important marker for embryo selection.

Keywords: Blastocyst; Clinical outcomes; Post-warming culture duration; Vitrification

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Introduction

Elective single embryo transfer has been widely adopted to reduce the risk of maternal/neonatal complications and multiple births with *in vitro* fertilization. Accordingly, the need to cryopreserve surplus blastocysts after embryo transfer is increasing. In addition, with the recent trend to take steps to reduce the risk of ovarian hyperstimulation syndrome and to transfer embryos in a more physiologic environment, cryopreservation has become a common strategy. Therefore, interest has been growing in the proper selection criteria for high-quality warmed embryos [1].

In vitrified-warmed blastocyst transfer (VBT), complete blastomere survival and mitotic resumption during warming are generally considered to be the most important factors affecting pregnancy outcomes. It has been assumed that a sufficient warming time may be required for resumption of cell proliferation and development [2]. However, the effect of culture duration on pregnancy outcomes in VBT remains controversial. One study suggested that a short-term culture period of 2–5 hours was associated with more favorable clinical outcomes, although there was no statistically significant difference in mitotic division between the two groups. However, another study showed that the post-warming culture duration (1 hour vs. 18 hours) was not relevant for the implantation rate (IR) and live birth rate when evaluating high-quality vitrified-warmed blastocysts [3,4]. Ebner et al. [5] performed a morphological analysis of the post-warming process of re-expansion and development with a time-lapse monitoring device. The study demonstrated that the completion of re-expansion took 2.70 ± 1.20 hours on average, suggesting that a sufficient time (i.e., not too short) may be needed to evaluate embryo development.

The purpose of the study was to compare the clinical outcomes of warmed blastocysts after long-term culture (20–24 hours) or short-term culture (2–4 hours). The morphological parameters of embryo re-expansion were also analyzed with a time-lapse monitoring device to estimate the appropriate timing and parameters for evaluating embryos and predicting the implantation potency of post-warmed embryos.

Methods

This study was approved by the Institutional Review Board of CHA Gangnam Medical Center (IRB No. GCI-19-27). Patients' informed consent was not needed due to the nature of retrospective study.

1. Participants

This retrospective study was conducted using the medical records of patients who underwent VBT procedures from March 2017 to December 2018 at the Fertility Center of CHA Gangnam Medical Center. Patients in whom a single blastocyst was transferred, regardless of the number of thawed embryos, were selected for the analysis. Cycles with double blastocysts were excluded due to the uncertainty of the associations with embryo quality when one of the two transferred embryos was implanted. Cycles were excluded if procedural difficulties were encountered or if patients had a thin endometrium (< 8 mm). Cycles were divided according to whether the culture period was long-term (20–24 hours) or short-term (2–4 hours).

2. Blastocyst vitrification and warming

For vitrification, blastocysts were first equilibrated in a mixture of HEPES medium (SAGE Quinn's-HEPES; CooperSurgical, Trumbull, CT, USA) and 20% HSA (SAGE, CooperSurgical) supplemented with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). For the final equilibration, 15% EG, 15% DMSO and 0.5 M sucrose were used. Each blastocyst was loaded onto a gold electron microscopic (EM) grid (EM Grid; SPI Supplies, West Chester, PA, USA). For the warming process, the EM grid containing the blastocyst was sequentially transferred to culture dishes containing HEPES medium and 0.5 M, 0.25 M, 0.125 M, and 0.0 M sucrose at intervals of 2.5 minutes, with 20% human serum albumin (SAGE BioPharma). After warming, the blastocyst was washed with blastocyst medium (Cook Medical, Bloomington, IN, USA) at 37°C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂ and then cultured.

3. Embryo grading

Blastocyst morphology was evaluated according to the degree of blastocoel expansion and inner cell mass (ICM) and trophectoderm (TE) morphology. The blastocoel expansion grade was categorized into five groups: early, the blastocoel filling < 50% of the non-expanded embryo; mid, the blastocoel filling > 50% of the embryo; expanded, full blastocyst, cavity completely filling the embryo; hatching, a hatching blastocyst; and hatched, a blastocyst that has completely hatched out of the zona pellucida. ICM morphology was graded as follows: A, many tightly packed cells; B, several loosely grouped cells; and C, very few cells. TE morphology was graded following the same logic: A, many cells creating a cohesive epithelium; B, few cells forming a loose epithelium; and C, very few large cells. Embryo grading was performed using the modified Gardner blastocyst grading system: excellent (E: expanded AA, hatching AA, hatched AA), good (G: early AA, mid AA, expanded AB or BA, hatching AB or BA, hatched AB, BA), average (A: early AB, BB, or BA; mid AB, BA, or BB; expanded BB; hatching BB; hatched BB), and poor (P: early AC, BC, CA, CB, or CC; mid BC, CA, CB, or CC; expanded BC, CB, or CC; hatching BC, CB, or CC; hatched BC, CB, or CC).

4. Time-lapse monitoring of blastocysts after warming

Immediately after warming, blastocysts were cultured in a time-lapse system (Embryoscope; Vitrolife, Göteborg, Sweden). The time-lapse video system captures images at intervals of 10 minutes followed by annotation. For the vitrified-warmed blastocysts, the blastocoel re-expansion time was analyzed in terms of tRE (start of re-expansion) and tCRE (completion of re-expansion), which were recorded and compared between the groups.

5. VBT cycle

All patients underwent endometrial preparation through either hormone replacement therapy or natural cycles. Patients with regular ovulation were treated using natural cycles. Luteal support was started using Crinone vaginal gel 8% (Merck Serono, Geneva, Switzerland) or vaginal Utrogestan (600 mg; Han Hwa Pharmaceuticals, Seoul, Korea) after confirmation of ovulation. In hormone replacement therapy cycles, oral estrogen (6 mg/day) was commenced on the 3rd day of the menstrual cycle until endometrial thickness reached at least 8 mm. Luteal support was applied for 5 days before VBT.

6. Assessed outcome variables

The primary outcomes of the study were clinical pregnancy outcomes, including the IR, clinical pregnancy rate (CPR), and ongoing pregnancy rate (OPR). The IR was calculated as the percentage of embryos that successfully underwent implantation compared to the number of embryos transferred. Clinical pregnancy was defined as the presence of a fetal heartbeat on ultrasonography. An ongoing pregnancy was defined based on a positive fetal heartbeat at 11 weeks or more of gestation confirmed on ultrasonography. Miscarriage was defined as a pregnancy that did not continue until it was classified as an ongoing pregnancy. Any pregnancy where the embryo implanted and developed outside the uterine endometrial cavity was defined as an ectopic pregnancy.

7. Statistical analysis

Logistic regression was used to estimate propensity scores for constructing a propensity score model. Potential confounders to be adjusted were included as variables, such as patient characteristics (female partner's age, paternal age, body mass index, anti-Müllerian hormone levels, infertility duration, number of previous attempts), fresh cycle variables (intracytoplasmic sperm injection, retrieved oocyte numbers) and endometrial thickness at embryo transfer. For propensity score matching, 2:1 nearest neighbor matching was performed without replacement.

The statistical analysis was performed using the Student *t*-test or Mann-Whitney *U*-test for continuous variables and the chi-square test for categorical variables. The level of significance was defined as $p < 0.05$. Baseline characteristics of the groups were presented as the mean \pm standard deviation or number (percentile). Statistical analysis was done using IBM SPSS ver. 23.0 (IBM Corp., Armonk, NY, USA).

Results

1. Patient characteristics

During the study period, a total of 369 single VBT cycles were in-

cluded: 304 in the long-term group and 65 in the short-term group. After propensity score matching, 108 cycles in the long-term group and 56 in the short-term group were analyzed. Patients' demographic characteristics did not significantly differ between the groups. An analysis of previous fresh *in vitro* fertilization cycles and subsequent VBT cycle parameters revealed no differences in the number of retrieved oocytes, the number of fertilized oocytes, and the number of vitrified blastocysts in both groups. Both groups also showed similar characteristics on the day of VBT (Table 1). More embryos were vitrified on day 5 than on day 6, and the cryo-survival rate was 96% (Table 2).

2. Embryo morphological evaluation

The evaluation of the morphological quality of the warmed blastocysts at the time of freezing and at the time of embryo transfer is summarized in Table 2. When evaluating the quality of embryos at the time of vitrification, there were no significant differences in embryo status between the two groups according to blastocyst grade, blastocoel expansion grade, ICM grade, or TE grade. After warming, the embryo quality also remained similar in terms of the grade of blastocysts, ICM and TE. The embryos before vitrification were in the early, mid, and expanded states. However, there was a significant difference between the degree of expansion of the two groups after warming (hatching and hatched embryos, long-term vs. short-term: 83.9% vs. 57.1%, $p < 0.001$).

3. Morphokinetic analysis using a time-lapse monitoring system

The warming embryos were observed through an embryoscope for morphokinetic analysis to evaluate the extent of blastocoel expansion over time. The long-term culture group comprised 58 blastocysts, and the short-term culture group contained 31 blastocysts. There were no embryos that did not start or complete re-expansion during the incubation time in either group.

Regardless of culture duration, the embryos in patients who became pregnant started re-expansion significantly more quickly than embryos in those who did not become pregnant (tRE: long-term group, 0.56 ± 0.18 vs. 1.57 ± 0.41 , $p = 0.021$; short-term group, 0.37 ± 0.09 vs. 0.81 ± 0.18 ; $p = 0.041$, respectively) (Figure 1A). tCRE was also faster in patients who became pregnant than in those who did not regardless of culture duration (tCRE: long-term group, 2.19 ± 0.63 vs. 4.11 ± 0.81 hours, $p = 0.003$; short-term group, 1.17 ± 0.29 vs. 1.94 ± 0.76 hours, $p = 0.018$, respectively) (Figure 1B). In patients who became pregnant, the duration for tCRE in the long-term culture group was 0.4–4.7 hours, while that for short-term culture was 0.6–2.7 hours, indicating that all embryos underwent re-expansion within 4 hours.

Table 1. Characteristics of previous fresh IVF cycles and vitrified-warming blastocyst transfer cycle in the two different post-warmed culture period groups

Variable	Long-term culture (20–24 hr, n = 112)	Short-term culture (2–4 hr, n = 56)	p-value
Maternal age (yr)	33.8 ± 2.8	33.7 ± 3.2	0.773
Paternal age (yr)	37.2 ± 4.3	37.5 ± 4.5	0.612
BMI (kg/m ²)	20.8 ± 2.3	21.5 ± 2.9	0.077
Infertility duration (yr)	3.2 ± 2.1	2.8 ± 2.0	0.508
Previous IVF attempts (n)	1.4 ± 0.9	1.4 ± 0.7	0.513
AMH (ng/mL)	4.3 ± 3.5	4.6 ± 3.3	0.605
Basal E ₂ (mIU/mL)	48.3 ± 19.3	48.0 ± 17.5	0.929
Basal FSH (mIU/mL)	7.6 ± 2.8	7.2 ± 2.2	0.318
Etiology of infertility (%)			0.084
Female	32 (28.6)	15 (26.8)	
Male	12 (10.7)	9 (16.1)	
Combine	41 (36.6)	17 (30.3)	
Unexplained	27 (24.1)	15 (26.7)	
E ₂ on hCG trigger (pg/mL)	3,061.9 ± 1,770.5	3,145.1 ± 1,988.4	0.788
Number of retrieved oocytes	16.6 ± 8.7	17.4 ± 8.2	0.533
ICSI (%)	70 (62.5)	36 (64.3)	0.734
Number of fertilizations	11.0 ± 5.6	12.0 ± 5.3	0.264
Number of freezing blastocysts	3.7 ± 2.8	3.6 ± 2.4	0.871
Protocol Endometrial preparation (%)			0.876
Natural	75 (67.0)	37 (66.1)	
HRT	37 (33.0)	19 (33.9)	
Endometrial thickness at transfer (mm)	10.0 ± 1.7	10.2 ± 1.7	0.455

Values are presented as mean ± standard deviation or number (%).

IVF, *in vitro* fertilization; BMI, body mass index; AMH, anti-Müllerian hormone; E₂, estradiol; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; ICSI, intra-cytoplasmic sperm injection; HRT, hormone replacement therapy.

4. Clinical outcomes

The clinical outcomes of both post-warming culture groups are compared in Table 3. The IR and CPR per embryo transferred were similar in the long-term culture group and the short-term culture group (56.3% vs. 67.9%, $p = 0.182$ and 53.6% vs. 60.7%, $p = 0.413$, respectively). There was no significant difference in the OPR per embryo transferred between the two groups (47.3% vs. 53.6%, $p = 0.513$). The miscarriage rate in both groups was comparable ($p = 0.627$).

Discussion

The results of this study showed that the clinical outcomes, including the IR, pregnancy rate, and miscarriage rate were similar in the long-term culture group and the short-term culture group. Extended culture time was associated with a higher degree of blastocoel re-expansion just before embryo transfer, but there was no significant difference in the clinical results. Furthermore, through a morphokinetic analysis of vitrified-warmed embryo re-expansion, we demonstrated

that the speed of re-expansion was a significant post-warming morphological predictor of clinical pregnancy outcomes in both groups.

The culture time interval after warming may serve as an important factor that affects the quality of the embryo. In the fresh embryo transfer cycles, the implantation potential of embryos has usually been evaluated based on the embryos' morphology, such as the ICM/TE grade and the degree of blastocoel expansion. However, in VBT cycles, the predictive power of blastocyst morphology is unclear, because the structure of the embryo shrinks through vitrification and then undergoes re-expansion after warming. Some previous reports suggested that long-term culture after warming may increase the degree of blastocoel expansion and increase the chance of selecting an embryo with high implantation potential [3,6]. Du et al. [6] showed that a higher proportion of blastocysts were re-expanded after long-term (20 hours) culture compared with blastocysts with short-term (4 hours) culture (80% vs. 36%), and the IR of embryos cultured for 20 hours was significantly higher.

However, regardless of how the environment of embryo culture aims to mimic Fallopian tube and intrauterine conditions, the influ-

Table 2. Characteristics of blastocysts in each group at the time of vitrification and warming

Variable	(Blastocyst) grades at the time of vitrification		p-value	(Blastocyst) grades at the time of warming ^{a)}		p-value
	Long-term culture (20–24 hr, n = 112)	Short-term culture (2–4 hr, n = 56)		Long-term culture (20–24 hr, n = 112)	Short-term culture (2–4 hr, n = 56)	
Blastocyst grade (%)			0.344			0.795
Excellent	16 (14.3)	8 (14.3)		22 (19.6)	9 (16.1)	
Good	6 (5.4)	1 (1.8)		1 (0.9)	0	
Average	67 (59.8)	36 (64.3)		59 (52.7)	31 (55.4)	
Poor	23 (20.5)	11 (19.6)		30 (26.8)	16 (28.6)	
Blastocoel grade (%)			0.691			0.001
Early	7 (6.3)	2 (3.6)		1 (0.9)	1 (1.8)	
Mid	29 (25.9)	14 (25.0)		1 (0.9)	4 (7.1)	
Expanded	76 (67.9)	40 (71.4)		16 (14.3)	19 (33.9)	
Hatching	0	0		69 (61.6)	25 (44.6)	
Hatched	0	0		25 (22.3)	7 (12.5)	
ICM grade (%)			0.951			0.860
A	20 (17.9)	10 (17.9)		23 (20.5)	10 (17.9)	
B	85 (75.9)	43 (76.8)		77 (68.8)	39 (69.6)	
C	7 (6.3)	3 (5.4)		12 (10.7)	7 (12.5)	
TE grade (%)			0.958			0.800
A	19 (17.0)	9 (16.1)		22 (19.6)	9 (16.1)	
B	73 (65.2)	37 (66.1)		61 (54.5)	31 (55.4)	
C	20 (17.9)	10 (17.9)		29 (25.9)	16 (28.6)	
Day of freezing (%)			0.694			
5 Day	87 (77.7)	44 (80.0)				
6 Day	25 (22.3)	11 (20.0)				
Cryo-survival rate (%)				96.5 ± 12.7	96.1 ± 14.2	0.884

Values are presented as number (%) or mean ± standard deviation.

ICM, inner cell mass; TE, trophoctoderm.

^{a)}Grade of embryos at the time of embryo transfer after warming.

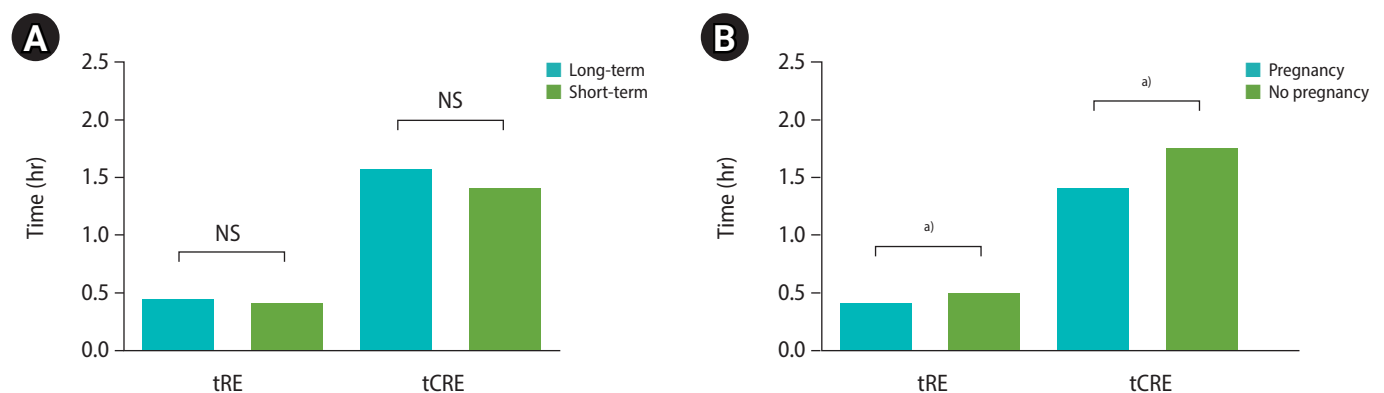


Figure 1. Morphological analysis of blastocoel re-expansion after warming according to ongoing pregnancy in both culture groups with a time-lapse monitoring device. (A) Comparison of the time of the start of re-expansion (tRE) in both culture duration groups. (B) Comparison of the time of the completion of re-expansion (tCRE) in both culture duration groups. Long-term group: 20–24 hours; Short-term group: 2–4 hours; Pregnancy: ongoing pregnancy, defined as the presence of a fetal heartbeat on ultrasonography at 11 weeks or more of gestation. NS, not significant. ^{a)} $p < 0.05$.

Table 3. Clinical outcomes in the two different post-warming culture period groups

Variable	Long-term culture (20–24 hr, n = 112)	Short-term culture (2–4 hr, n = 56)	p-value
Implantation (%)	63 (56.3)	38 (67.9)	0.182
Clinical pregnancy (%)	60 (53.6)	34 (60.7)	0.413
Ongoing pregnancy (%)	53 (47.3)	30 (53.6)	0.513
Miscarriage (%)	8 (7.1)	3 (5.3)	0.627
Ectopic pregnancy (%)	0	0	

Values are presented as number (%).

ence of stress over culture time always needs to be considered [7]. One study found that reducing the post-thaw culture duration decreased culture-related stress and preserved embryonic developmental potential [8]. Furthermore, as improved vitrification and warming skills have reduced the frequency of embryos undergoing lysis or failing to re-expand, the need for extended culture to deselect blastocysts with the low development potential would have been reduced. Indeed, our study demonstrated that the culture time was not significantly associated with the IR or clinical outcomes. This finding is consistent with results of a previous prospective randomized study showing no association between the culture duration (18 hours vs. 1 hour) and clinical outcomes in the vitrification setting (IR, 38.0% vs. 36.0%; $p=0.87$) [3].

The proportion of hatching/hatched blastocysts was significantly higher in the long-term culture group (84.8% vs. 53.8%), but all post-warmed embryos were re-expanded at the point of the embryo transfer regardless of the culture period. Ahlstrom et al. [9] assessed cultured embryos after warming for up to 6 hours at short intervals (20–30 seconds), and showed that 2 hours was sufficient to assess the warmed blastocysts. That study suggested that the degree of re-expansion is a direct response after warming, and reported that after 2 hours, time had little effect on the degree of re-expansion [9]. Shu et al. [10] also reported that 75.9% of thawed blastocysts re-expanded after 3 to 4 hours in post-thaw culture. When we analyzed the start and completion time of re-expansion with a time-lapse monitoring device, 90% of embryos started re-expansion immediately after warming and completed re-expansion within an average of 1.4–3.5 hours.

Lin et al. [11] also showed that the blastocyst re-expansion time was not related to the total culture time, but was determined to be a significant indicator of clinical pregnancy outcomes. Other studies also reported that the speed of blastocoel re-expansion during warming significantly predicted clinical outcomes [6,9]. Because of vitrification and cryoprotectant toxicity, less damaged blastocysts with a lower percentage of cell loss tend to undergo an increased degree of re-expansion after warming. In our study, the start time and the completion time of blastocyst re-expansion were found to be significantly faster in patients who became pregnant in both the

short-term and long-term culture groups. In the patients who became pregnant, re-expansion began within an average of 0.3–0.5 hours and was completed within 1.1–2.1 hours, approximately twice as fast as in the patients who did not become pregnant. The blastocyst re-expansion mechanism is unclear, but its main process is regulated by the velocity of water flux and resealing of the TE [12]. Under cryodamage, impaired function of Na^+/K^+ -ATPase and water transport mechanisms lead to lesser and slower re-expansion [13]. Therefore, less damaged blastocysts showed faster re-expansion, and the speed of re-expansion may be a promising marker of cellular viability and developmental competency [14].

In order to reduce the potential bias of re-expansion speed on culture time, a subgroup analysis was performed only on embryos that completed re-expansion by 4 hours. This subgroup analysis also showed that the culture time after warming was irrelevant for blastocyst implantation potential, and that the speed of re-expansion was correlated with clinical outcomes, as previously discussed (data not shown). Therefore, long-term culture was considered to have no advantage for selecting embryos with a low implantation potential. At the same time, the extended culture period did not show any detrimental effects on the embryo.

In this study, it was shown the choice of long-term or short-term culture time did not have a significant effect on clinical outcomes. However, the times of start and completion of blastocyst re-expansion can predict the implantation potential, and they showed significance as parameters for selecting appropriate embryos for transfer. Therefore, evaluating the speed of embryo re-expansion 3–4 hours after warming may help to decide which embryo to transfer without waiting until embryos develop to the hatching or hatched stages. This study is limited by its retrospective design and small sample size. Therefore, these findings should be confirmed through prospective randomized controlled studies with larger samples to achieve statistically well-powered results.

In conclusion, the outcomes of short-term culture and long-term culture were not significantly different in VBT cycles. Embryo transfer timing after warming may be determined by optimizing each laboratory's work flow. Regardless of post-warming culture time, the degree of blastocyst re-expansion 3–4 hours after warming is a valuable

marker for embryo selection in warmed embryo transfer cycles.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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A spontaneous pregnancy and live birth in a woman with primary infertility following the excision of an ovarian adrenal rest tumor: A rare case

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Adrenal rest tumors are a rare extra-adrenal complication of congenital adrenal hyperplasia (CAH) in women although they are more commonly found in the testes of male patients with CAH. An ovarian adrenal rest tumor (OART) may coexist with CAH or imitate its symptoms without CAH. In this case report, we present the case of a woman with OART without CAH, whose main complaint was infertility and who had a baby after successful surgical treatment.

Keywords: Infertility; Laparoscopy; Ovarian adrenal rest tumor

Introduction

Ovarian adrenal rest tumors (OARTs) occur in a very small proportion (< 0.1%) of women with congenital adrenal hyperplasia (CAH), and are extremely rare in women without CAH [1], although adrenal rest tumors are relatively common in the testes of men with CAH [2]. According to the literature, these tumors imitate CAH symptoms (e.g., hirsutism, clitoromegaly, oligo-amenorrhea, and, rarely, pelvic masses) [3]. Herein, we describe the first known case of OART in a woman without CAH presenting with primary infertility.

Case report

This is a case report, and the patient and her relatives were informed in detail and their written consents were obtained.

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A 34-year-old nulliparous woman presented to our *in vitro* fertilization (IVF) center because of an inability to conceive for 2 years. While taking her medical history, the patient reported that her menstrual cycle had started at the age of 13, and was regular until the age of 19. Upon her first examination, the patient had been diagnosed with polycystic ovary syndrome due to her severe hirsutism and secondary amenorrhea (two of the three Rotterdam 2013 criteria). Therefore, she used oral contraceptive (OC) pills for 12 years. Her menstrual cycle improved while taking OC medication, and her hirsutism partially improved. She discontinued OC treatment after marriage because of a desire to have a child.

Two months after marriage, her irregular menses recurred. She presented to a local hospital with this complaint. Upon evaluation, her basal serum 17-hydroxyprogesterone (17-OHP) level was 20 ng/mL, which was higher than normal, leading the clinicians to consider the possibility of CAH. Therefore, dexamethasone therapy (0.5 mg/day) was started. Several ovulation inductions (with an aromatase inhibitor and timed coitus for six cycles) and an IVF cycle were performed with the goal of conception after the 6th month of her marriage. During IVF therapy, the gonadotropin (recombinant follicle-stimulating hormone [FSH]) dose used was 300 IU/day for 9 days and the endometrial thickness was 6.4 mm on trigger day. From

the two collected oocytes, one mature oocyte was obtained and fertilized. The embryo was transferred on day 3, but she failed to achieve a pregnancy, even though her husband was healthy and his semen analysis was normal.

When she was transferred to us, we performed a physical examination of the patient. We assessed hirsutism using the Ferriman-Gallwey score, with a score of 22. There were also signs of virilism, including thickening of the vocal cords, alopecia, and clitoromegaly. The results of the hormone assay indicated that the patient's 17-OHP level remained high (86 ng/mL), despite dexamethasone therapy. Her dehydroepiandrosterone sulfate, free testosterone, and total testosterone levels were 385.9 µg/dL, 5.5 pg/mL, and 416.2 ng/dL, respectively. Serum FSH, luteinizing hormone, thyroid-stimulating hormone, free T4, prolactin, and cortisol levels were normal. Transvaginal ultrasonography showed a normal uterus size, with a thin endometrium (4 mm) and diminished ovarian reserve. The right ovary measured 40×35×32 mm in size and had one antral follicle, and

the left ovary measured 29×25×25 mm in size and had three antral follicles. A 34×30×29 mm solid mass was observed in the right ovary (Figure 1). Tumor markers (i.e., CA 125, CA 15.3, alpha-fetoprotein, and carcinoembryonic antigen) were found to be within normal limits. Since she had an ovarian mass and a high serum 17-OHP level, we suspected an adrenal rest tumor and ordered an abdominal and pelvic magnetic resonance imaging scan. It showed a 42×32-mm hyperintense right ovarian solid mass with a normal left ovary; however, the bilateral adrenal glands were of normal size and morphology (Figure 2). We planned laparoscopy for a definitive diagnosis of the right ovarian mass. Laparoscopic examination of the pelvic cavity showed a yellowish solid 4-cm mass on the right ovary. The mass was removed in an endoscopic bag without rupture (Figure 3), and sent for an immediate pathological frozen section, which revealed a benign tumor. Then it was sent for histological examination. Macroscopically, it was a yellow-colored tumor measuring nearly 4 cm, with a smooth and well-limited surface. Microscopically, almost all of the

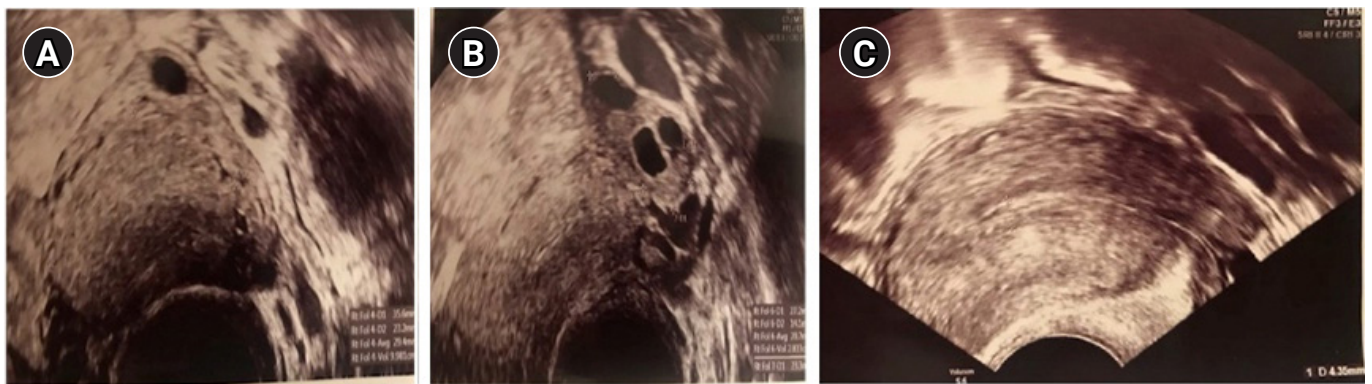


Figure 1. Ultrasonographic appearance of the right ovarian adrenal rest tumor demonstrating an echogenic border (A), the left ovary (B), and thin endometrium (C).

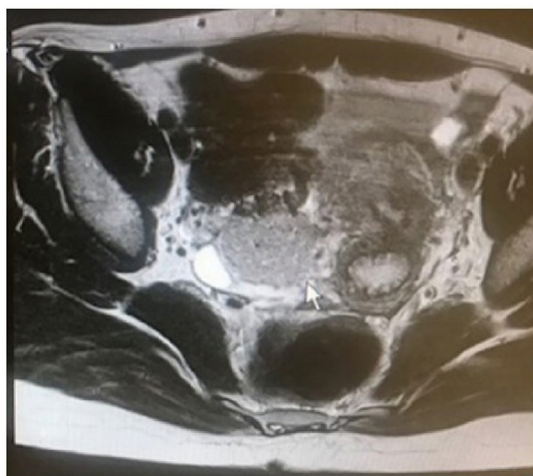


Figure 2. Magnetic resonance imaging showed a 42×32-mm hyperintense right ovarian solid mass in the pelvic cavity.

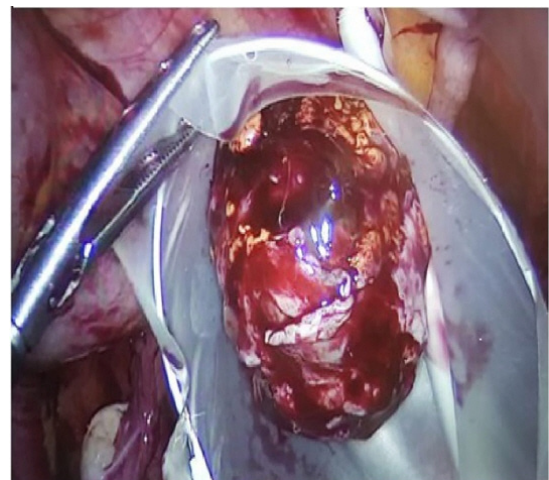


Figure 3. Laparoscopic removal of the yellowish solid mass in an endoscopic bag.

ovarian parenchyma was occupied by the tumor lesion, as shown by large cells with small nuclei without mitotic activity, nuclear atypia, and Reinke crystalloids. Hemorrhagic or necrotic areas were absent in the sample. Immunohistochemical analysis revealed diffuse staining for calretinin, inhibin, vimentin, and melan-A (Figure 4). The final histopathological findings confirmed the diagnosis of OART.

One week after the operation, the patient's serum 17-OHP level decreased dramatically (1.82 ng/mL). Therefore, we suspected CAH, and consulted with an endocrinologist to carry out an adrenocorticotropic hormone (ACTH) simulation (Synacthen) test for the definitive diagnosis of CAH. In the ACTH simulation test, the 17-OHP levels were 0.21 ng/mL, 0.54 ng/mL and 0.60 ng/mL at 0, 30, and 60 minutes, respectively. Based on these findings, the diagnosis of CAH was excluded. We also conducted a genetic test to investigate the possible presence of a *CYP21A2* gene mutation, the test result was negative. We offered detailed genetic tests for the other enzyme deficiencies involved in CAH, but the patient did not agree to undergo the recommended tests.

Postoperatively, the patient's menstrual cycles became regular and signs of hyperandrogenism disappeared. Furthermore, 4 months after the operation, she spontaneously became pregnant and then gave birth to a healthy infant at term.

Discussion

Adrenal rest tumors are benign, but may produce excessive 17-OHP and androgens. These tumors are commonly found in the testes of male patients, and their prevalence has been reported to be as high as 95% in men with CAH. In contrast, OARTs are less common in women with CAH (<0.1%) [1]. In a literature search, we found that

Yilmaz-Agladioglu et al. [3] reported the first case of OART in a girl without CAH. They presented a 13-year-old female patient who was diagnosed with non-classical CAH at 6 years of age due to premature pubarche, and received hydrocortisone therapy for 6 years. The patient was further investigated because hormonal control was unsuccessful (high levels of 17-OHP and total testosterone) while under high-dose steroid therapy, and she was diagnosed with a steroid cell ovarian tumor, similar to our case. In our case, signs of hyperandrogenism, disordered menstruation, and infertility were the main complaints of the patient, whereas, in their case, the patient's complaint was limited to signs of hyperandrogenism. This discrepancy reflects the fact that the patients were at different reproductive stages. Surgical removal of the ovarian mass resulted in clinical improvements in both cases. To the best of our knowledge, our patient is second case in which OART was seen in a woman without CAH.

The mechanism of ART formation still has not yet been fully elucidated. Kim et al. [4] mentioned that these tumors may arise from ectopic migration of residual adrenal cortex cells into the developing gonads because of the connection between the adrenal glands and gonads during embryonic development. Claahsen-van der Grinten et al. [5] suggested that defective primary sex cord regression, including all aberrant adrenal cells, during gonad development may be a causative factor in OART development.

In the literature, it has been shown that OARTs were related with distinct enzyme deficiencies associated with CAH, such as 21-hydroxylase, 11-hydroxylase, and 3- β hydroxysteroid dehydrogenase [6]. In our case, beside the negative results of Synacthen and genetic (*CYP21A2* gene) tests, improvement of the patient's clinical complaints (menstrual disturbances and hyperandrogenism signs, even including infertility) after the operation, supported exclusion of the diagnosis of CAH.

The diagnosis of OART masses by conventional imaging may be difficult because of the rarity of OART and sometimes small size of these tumors. Chen et al. [7] recommended examination of the ovaries and excision of any suspicious OART masses in female CAH patients who respond poorly to hormone therapy. In our case, we also suspected OART because the patient was unresponsive to hormone therapy and because of the patient's ongoing complaints; additionally, the patient stated that this ovarian mass had been present for a long time, but it was not considered important since it was small.

Testicular adrenal rest tumors may be an important cause of infertility in men, as they lead to obstruction of the seminiferous tubules, azoospermia and gonadal dysfunction [8]. Disruption in the hypothalamic-pituitary-ovarian axis due to excessive androgens and excessive progesterone secretion in OART patients can cause infertility. Androgens can also directly inhibit folliculogenesis by having a negative effect on aromatase activity in granulosa cells. Additionally,

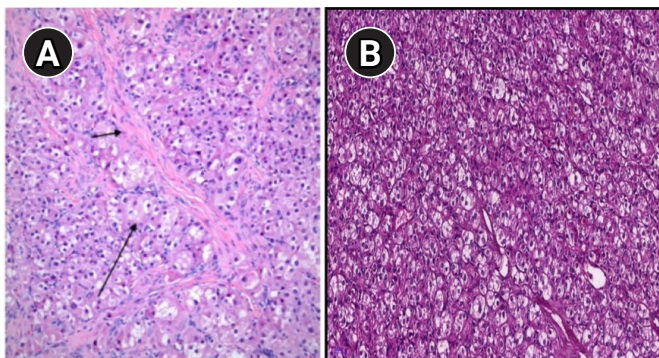


Figure 4. (A) Microscopic appearance of the tumor cells showed a uniform size, polygonal shape, abundant eosinophilic and vacuolated cytoplasm, and round nuclei with small nucleoli (long arrow). Acellular hyaline areas were observed among clusters of tumor cells (short arrow). (B) Mitotic activity was low. Crystalloids of Reinke were not identified (H&E, $\times 200$).

progesterone hypersecretion in OARTs can affect the quality of cervical mucus and sperm penetration, accelerate endometrial maturation, reduce endometrial receptivity, decrease implantation, and exert negative effects on oocyte quality [9].

To the best of our knowledge, this is the first known case of OART causing infertility. There are three principal aspects that should be noted: first, this was an extremely rare case; second, the patient's infertility and other hyperandrogenism-related complaints were successfully treated; and third, it was possible to exclude the diagnosis of CAH, which requires excessive steroid use and repeated treatments for infertility. Increased levels of androgen hormones by OARTs may lead to infertility by damaging the hypothalamo-hypophyseal axis. The appropriate diagnosis and treatment of these cases can prevent both excessive steroid use and recurrent treatments for infertility. The patient described herein conceived spontaneously following the excision of an OART.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; (3) final Enacted: December 31, 1974 Revised: November 31, 2018 ii approval of the version to be published; and (4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of it are appropriately investigated and resolved. Every author should meet all 4 of these conditions. Copyright assignment must also be completed by every author.

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5. Process for scientific misconduct

When the journal faces suspected cases of research and publication misconduct such as redundant (duplicate) publication, plagiarism, fraudulent or fabricated data, changes in authorship, an undisclosed conflict of interest, ethical problems with a submitted manuscript, a reviewer who has appropriated an author's idea or data, complaints against editors, and so on, the resolution process will be completed following the procedures outlined in the flowchart provided by the COPE (<http://publicationethics.org/resources/flowcharts>). The discussion and decision on the suspected cases will be carried out by the Editorial Board.

6. Conflict-of-interest statement

The corresponding author must inform the editor of any potential conflicts of interest that could influence the authors' interpretation of the data. Examples of potential conflicts of interest are financial support from or connections to pharmaceutical companies, political pressure from interest groups, and academically related issues. In particular, all sources of funding applicable to the study should be explicitly stated.

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Cases that require editorial expressions of concern or retraction shall

follow the COPE flowcharts (<http://publicationethics.org/resources/flowcharts>). If a correction is required, the procedure to provide the correction will follow the ICMJE Recommendation (<http://www.icmje.org/recommendations/browse/publishing-and-editorialissues/corrections-and-version-control.html>).

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For clarification on result accuracy and reproducibility of the results, raw data or analysis data will be deposited to a public repository or *CERM* homepage after acceptance of the manuscript. Therefore, submission of the raw data or analysis data is mandatory. If the data is already a public one, its URL site or sources should be disclosed. If data cannot be publicized, it can be negotiated with the editor. If there are any inquiries on depositing data, authors should contact the Editorial Office for more information.

5. Clinical data sharing policy

This journal follows the data sharing policy described in "Data Sharing Statements for Clinical Trials: A Requirement of the International Committee of Medical Journal Editors" (<https://doi.org/10.3346/jkms.2017.32.7.1051>). As of January 1, 2019 manuscripts submitted to *CERM* that report the results of clinical trials must contain a data sharing statement. Clinical trials that begin enrolling participants on or after January 1, 2019 must include a data sharing plan in the trial's registration. The ICMJE's policy regarding trial registration is explained at <https://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html>. If the data sharing plan changes after registration this information should be reflected in the statement submitted and

published with the manuscript, as well as being updated in the registry record.

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- Invited review articles provide a concise review of a subject of importance to researchers written by an invited expert in reproductive medical science.
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- Case reports deal with clinical cases of medical interest or innovation.
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- Letter to editor includes a reader's comment on an article published in *CERM* and a reply from the authors.

VI. PREPARATION OF MANUSCRIPTS

1. General guideline

- The main document with manuscript text and tables should be prepared with an MS-word or RTF format. The manuscript should be written in 11-point font with double-line spacing on A4 (21.0×29.7 cm) or letter (8.5×11.0 in) sized paper with 2.5 cm (1.0 in) margins.
- All manuscript pages are to be numbered at the upper right corner consecutively, beginning with the title page as page 1.
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- Submit each figure as individual files separate from the manuscript. Do not insert figures into the text document. Figures should be in tiff, tif, jpg, jpeg files. Do not submit your manuscript or figures as pdf files.
- For specific study designs, such as randomized control studies, studies of diagnostic accuracy, meta-analyses, observational studies, and non-randomized studies, authors are encouraged to also consult the reporting guidelines relevant to their specific research design. A good source of reporting guidelines is the EQUATOR Network (<https://www.equator-network.org/>) and the NLM (https://www.nlm.nih.gov/services/research_report_guide.html).
- Drug and chemical names should be stated in standard chemical or generic nomenclature.
- Description of genes or related structures in a manuscript should include the names and official symbols provided by the US National Center for Biotechnology Information (NCBI) or the HUGO Gene Nomenclature Committee.
- Standard metric units are used for describing length, height, weight, and volume. The unit of temperature is given in degree Celsius (°C). Specifically, use 'sec', 'min', 'hr', 'day', 'wk', 'mo', and 'yr' for time units. All others units of measure should be presented according to the International System (SI) of Units. All units must be preceded by one space except percentage (%), temperature (°C), and angle (°).
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Manuscripts will not be acceptable for publication unless they meet the following editorial requirements. Manuscripts includes (1) Title page, (2) Structured abstract and Keywords, (3) Introduction, (4) Methods, (5) Results, (6) Discussion, (7) Acknowledgments, (8) References, (9) Tables,

and (10) Figure legends. Each component should begin on a new page in the following sequence. Manuscripts should be no longer than 5,000 words and the combined numbers of tables and figures should be no more than 10 items.

1) Title page

- Provide running title (a maximum of 50 spaces and letters), manuscript title, the full name of author and the author's institutional affiliation(s). For different institution, use the sequential Arabic number (1, 2, 3...) in superscript ahead of institution.
- All persons designated as authors should be qualified for authorship (See the part of ETHICS IN PUBLISHING). Each author should have participated sufficiently in the work to take public responsibility for the content.
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- All funding, other financial support, and material support for the work, if it exists, should be clearly identified in the conflict of interest statement. If no conflicts of interest exist for any of the authors, this should be noted.
- Include presentation history at a meeting.

2) **Structured abstract and keywords:** The abstract should present the Objective, Methods, Results, and Conclusion. The abstract should also emphasize new and important aspects of the study or observation and tract may not exceed 250 words. Below the abstract, provide up to 10 keywords that will assist indexers in crossindexing the article. For selecting keywords, refer to the MeSH database (<https://www.ncbi.nlm.nih.gov/mesh>).

3) **Introduction:** Briefly describe the purpose of the investigation, including relevant background information.

4) **Methods:** Describe the research plan, the materials (or subjects), and the methods used, in that order. Explain in detail how the disease was confirmed and how subjectivity in observations was controlled. When experimental methodology is the main issue of the paper, describe the process in detail so as to recreate the experiment as closely as possible. The sources of the apparatus or reagents used should be given along with the source location (name of company, city, and country). Ensure correct use of the terms sex (when reporting biological factors) and gender (identity, psychosocial or cultural factors), and, unless inappropriate, report the sex and/or gender of study participants, the sex of animals or cells, and describe the methods used to determine sex and gender. If the study was done involving an exclusive population, for example in only one sex, authors should justify why, except in

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Examples of authors’ contributions are as follows:

Conceptualization: BCJ. Data curation: DL. Formal analysis: YIA. Funding acquisition: JHA. Methodology: BCJ. Project administration: MYP. Visualization: MHC. Writing – original draft: DL. Writing – review & editing: BCJ.

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Examples of references

(1) Journal article

Kim SG, Kim YY, Park JY, Kwak SJ, Yoo CS, Park IH, et al. Early fragment removal on in vitro fertilization day 2 significantly improves the subsequent development and clinical outcomes of fragmented human embryos. *Clin Exp Reprod Med* 2018;45:122-8.

(2) Website

American Society for Reproductive Medicine. Headlines in reproductive medicine [Internet]. Birmingham: American Society for Reproductive Medicine; 2010 [cited 2018 Jan 10]. Available from: <http://www.asrm.org/headlines/>.

(3) Book

Suikkari AM. Use of in vitro maturation in a clinical setting. In: Gardner DK, Weissmaan A, Howles CM, Shoham Z, editors. *Textbook of assisted reproductive technologies*. 3rd ed. London: Informa Healthcare; 2009. p. 155-62.

(4) In press

Yang XL, Chen F, Yang XY, Du GH, Xu Y. Low molecular weight heparin does not reduce miscarriages in non-thrombophilic IVF/ICSI-treated women. *Acta Obstet Gynecol Scand* 2018 Oct 14 [Epub]. <https://doi.org/10.1111/aogs.13483>.

11) **Tables:** Tables should be typed double-spaced on separate pages within manuscript, and they should be titled and numbered in Arabic numerals (not Roman numerals) in the order of their first citation in the text. Give each column a short heading. Place explanatory matter in footnotes, not in the heading. For footnotes vi use the following symbols, in this sequence: a), b), c), d), e), f) in superscript. Do not use internal vertical lines.

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Review article will be requested by the editors. Review articles are generally prepared in the same format as original articles, but the details of manuscript format may be flexible according to the contents. The manuscripts are limited to 5,000 words of text and includes 250- word summary in the place of unstructured abstract.

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Case reports should be succinct, informative, and limited to 2,000 words of text (including Title page, 150-word Case report summary, Introduction, Case, Discussion, References, Table, and Figure legend).

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6. Letter to the editor

This section of the journal is set aside for critical comments directed to a specific article that has recently been published in the journal. Letters should be brief (500 words), double-spaced, and limited to a maximum

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VII. AUTHOR'S MANUSCRIPT CHECKLIST

1. Double-spaced typing with 11-point font using MS-Word or RTF format.
2. Sequence of Title page, Structured abstract and keywords, Introduction, Methods, Results, Discussion, Acknowledgments, References, Tables, and Figure legends. All pages should be numbered consecutively starting from the title page.
3. Title page with running title, manuscript title, author's full name, and institution, address for correspondence.
4. Abstract in format within 250 words, and keywords as in MeSH.
5. References listed in proper format. Check that all references listed in the references section are cited in the text and vice versa.
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IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

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