CERM Clinical and Experimental Reproductive Medicine

Vol. 50 • No. 4 • December 2023

pages 213-298

CERM

Clinical and Experimental Reproductive Medicine

pISSN 2233-8233
eISSN 2233-8241

Volume 50, Number 4, December 2023

www.eCERM.org

Official Journal of
Korean Society for Reproductive Medicine
Korean Society for Assisted Reproduction
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Korean Society for Fertility Preservation
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1. Data on file. IQVIA Market Data Analysis. Dec 2021; 2. Manzi L, et al. Int. J. Mol. Sci. 2022;23(12):6762

KR-GON-00119 Sep 2022

CERM
Clinical and Experimental Reproductive Medicine

pISSN 2233-8233
eISSN 2233-8241

Aims and Scope

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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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President: Soo Woong Kim, M.D., PhD.
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Printing by M2PI
#805, 26 Sangwon 1-gil, Seongdong-gu, Seoul 04779, Korea
E-mail: support@m2-pi.com Tel: +82-2-6966-4930 Fax: +82-2-6966-4945

♾ This paper meets the requirements of KS X ISO 9706, ISO 9706-1994 and ANSI/NISO Z39.48-1992 (Permanence of Paper).

pISSN 2233-8233 • eISSN 2233-8241

Editorial Board

CERM
Clinical and Experimental Reproductive Medicine

pISSN 2233-8233 • eISSN 2233-8241

Volume 50, Number 4, December 2023

CERM
Clinical and Experimental Reproductive Medicine

REVIEW ARTICLES

ORIGINAL ARTICLES

REVIEW ARTICLE
https://doi.org/10.5653/cerm.2023.06016
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):213-222

CERM

New strategies for germ cell cryopreservation: Cryoinjury modulation

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Cryopreservation is an option for the preservation of pre- or post-pubertal female or male fertility. This technique not only is beneficial for human clinical applications, but also plays a crucial role in the breeding of livestock and endangered species. Unfortunately, frozen germ cells, including oocytes, sperm, embryos, and spermatogonial stem cells, are subject to cryoinjury. As a result, various cryoprotective agents and freezing techniques have been developed to mitigate this damage. Despite extensive research aimed at reducing apoptotic cell death during freezing, a low survival rate and impaired cell function are still observed after freeze-thawing. In recent decades, several cell death pathways other than apoptosis have been identified. However, the relationship between these pathways and cryoinjury is not yet fully understood, although necroptosis and autophagy appear to be linked to cryoinjury. Therefore, gaining a deeper understanding of the molecular mechanisms of cryoinjury could aid in the development of new strategies to enhance the effectiveness of the freezing of reproductive tissues. In this review, we focus on the pathways through which cryoinjury leads to cell death and propose novel approaches to enhance freezing efficacy based on signaling molecules.

Keywords: Apoptosis; Autophagy; Cryoinjury; Cryopreservation; Fertility preservation; Necroptosis

Introduction

Fertility preservation refers to the techniques used to preserve germ cells or reproductive tissue, thereby enabling the future production of biological offspring. Cryopreservation is an option for preserving the fertility of both pre- and post-pubertal male and female patients. This technique relies on a variety of cryoprotective agents (CPAs), which can be either non-permeable or permeable, along with methods such as slow freezing, vitrification, and freeze-drying [1,2]. These freezing techniques not only are beneficial for human clinical applications, but also play a crucial role in the breeding of livestock and endangered species.

Unfortunately, frozen germ cells are subject to cryoinjury, which can result from the excessive generation of reactive oxygen species (ROS), apoptosis, and cold shock during freezing. As a result, various CPAs and freezing methods have emerged to minimize the extent of cryoinjury [3-6]. In the process of developing these solutions, most research has been dedicated to preventing cell death induced by cryoinjury, which is primarily caused by apoptosis. Despite considerable research effort aimed at minimizing apoptotic cell death during freezing, a low survival rate or compromised function of cells after freeze-thawing is often still observed. This suggests that additional cell death pathways may be involved in cryoinjury.

Unsurprisingly, various cell death pathways (including necroptosis, autophagy-dependent cell death [ADCD], NETosis, ferroptosis, parthanatos, and the mitochondrial permeability transition pore) have been discovered in recent decades in addition to apoptosis, which is the most widely recognized cell death pathway [7]. While necroptosis and autophagy have been associated with cryoinjury, our under-

Received: March 19, 2023 · Revised: June 29, 2023 · Accepted: July 17, 2023
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*This work was supported by the Basic Science Research Program, which is facilitated through the National Research Foundation of Korea (NRF), under the grant number NRF-2018R1A6A1A03025159, in the Republic of Korea and the Chung-Ang University research grant in 2023.

standing of the contributions of these pathways is still incomplete [8-11]. Consequently, gaining insight into the molecular mechanisms of cryoinjury could offer new strategies to enhance freezing efficacy.

The compounds identified through the study of cryoinjury pathways may serve not only as cryoprotectants but also as supplements to culture media following thawing. Cryoinjury is primarily understood to occur during the freezing process; however, research indicates that damage continues to occur after thawing, particularly within the first 24 hours of post-thaw culture. This suggests the necessity for careful rescue of frozen cells and tissues, even post-thawing [12]. To meet this need, researchers have sought to improve cell survival and restore cell function using agents such as apoptosis inhibitors and antioxidants before freezing or after thawing to prevent cryoinjury, yielding positive results for freeze-thawed cells [13-16]. As previously suggested, the clear identification of cryoinjury pathways will facilitate the development of new strategies to be used alongside existing techniques.

Understanding the fundamental pathways of cryoinjury is crucial for manipulating cryoinjury at a molecular level and enhancing fertility preservation strategies through improved freezing efficacy. In this review, we focus on the pathways through which cryoinjury induces cell death and propose novel approaches to enhance the freezing efficacy of reproductive tissues, based on signaling molecules.

Cell death pathways

In recent decades, our understanding of various cell death pathways has substantially improved. These pathways include the well-known apoptosis, necroptosis, and necrosis, as well as other pathways such as ADCD, NETosis, ferroptosis, parthanatos, and the mitochondrial permeability transition pore [7]. This review primarily focuses on apoptosis, necroptosis, and ADCD. These pathways have

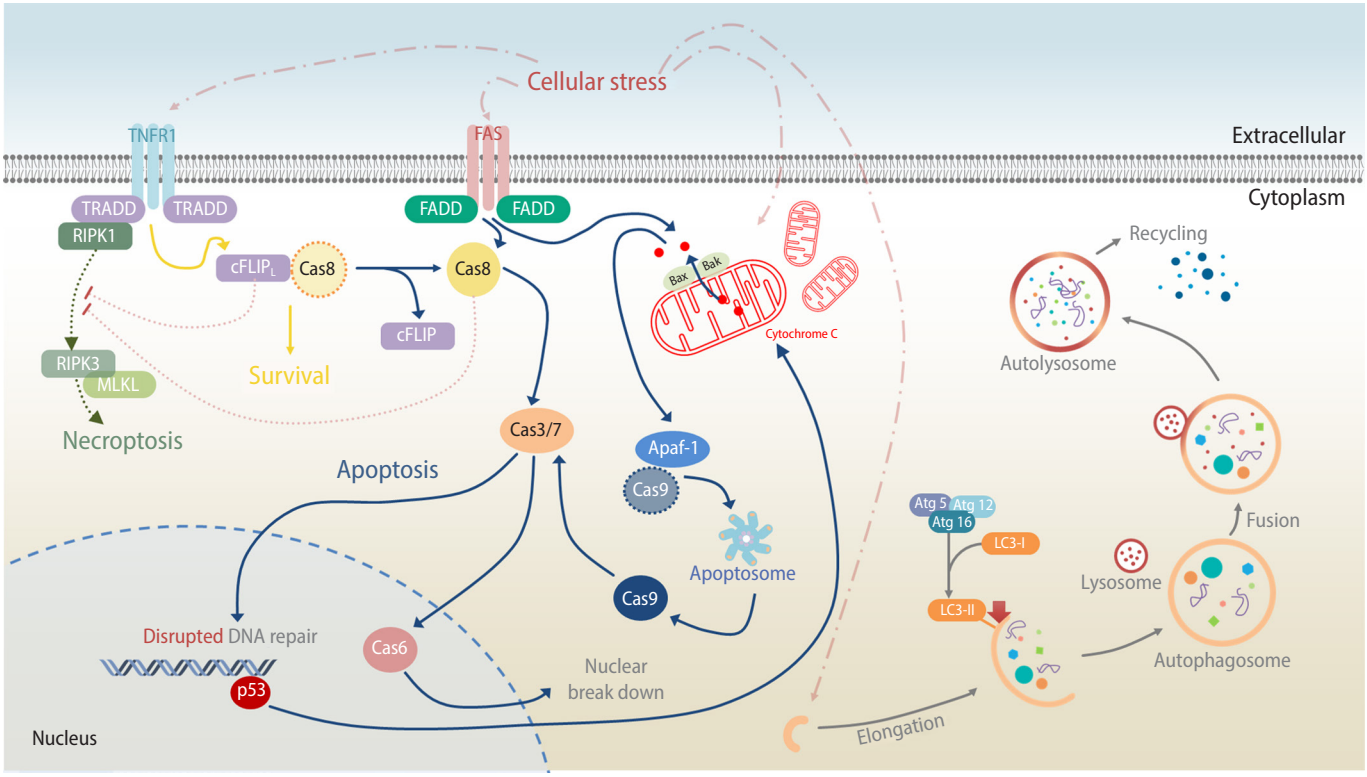

Figure 1. Apoptosis, necroptosis, and autophagy. In extrinsic apoptosis, activated Fas-associated protein with death domain (FADD) interacts with procaspase-8, resulting in the formation of cleaved caspase. Intrinsic apoptosis involves the release of cytochrome C from the mitochondria, which forms apoptosomes and allows procaspase-9 to be cleaved. These cleaved caspases eventually follow a common apoptosis execution pathway. Necroptosis is triggered when the activation of caspase-8 is compromised. The maturation of the autophagosome involves the conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3)-I to LC3-II through the conjugation of autophagies (ATGs). These then fuse with the lysosome to degrade intracellular content and recycle the components. TNFR1, tumor necrosis factor receptor 1; TRADD, TNFR1-associated death domain protein; RIPK, receptor-interacting protein kinase; cFLIP$_L$, cellular FLICE (FADD [Fas-associated death domain]-like IL-1β–converting enzyme)-like inhibitory protein long form; Cas, caspase; MLKL, mixed lineage kinase domain-like protein; Apaf-1, apoptotic protease activating factor-1.

https://doi.org/10.5653/cerm.2023.06016

been studied in relation to their roles in germ cell cryopreservation (Figure 1).

1. Apoptosis

Apoptosis, or programmed cell death, is a crucial process in maintaining tissue homeostasis. It contributes to cell turnover, embryonic development, and the functioning of the reproductive system [17,18]. During apoptosis, cells undergo a series of morphological changes, such as cell shrinkage and the fragmentation of cells, organelles, and DNA into apoptotic bodies [19,20]. This process can be initiated by two primary pathways. The extrinsic pathway is activated by cell-surface death receptors, such as tumor necrosis factor (TNF) receptor 1, Fas, and death receptors, which interact with their respective ligands, including TNF-α, Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In contrast, intrinsic apoptosis is initiated when pro-apoptotic proteins from the B-cell lymphoma 2 (BCL-2) family permeabilize the outer mitochondrial membrane within cells [21]. Both apoptotic pathways lead to the activation of a family of proteases and caspases. In the extrinsic pathway, the activation of cell-surface death receptors results in the recruitment of the adaptor protein Fas-associated protein with death domain (FADD). The activated FADD then interacts with procaspases 8 and 10 to form cleaved caspase, marking the onset of apoptosis. In the intrinsic pathway, the activation of BCL-2 proteins leads to the permeabilization of the outer mitochondrial membrane, a step often referred to as the point of no return. This allows for the release of cytochrome C into the cytoplasm. Once in the cytoplasm, cytochrome C forms apoptosomes with apoptotic protease activating factor 1. These apoptosomes enable the cleavage of procaspase-9. The cleaved caspases 8, 10, and/or 9 then proceed along a common apoptosis execution path, which involves the activation of caspase-3 and/or caspase-7 [21].

2. Necroptosis

Necroptosis is a regulated, caspase-independent form of cell death that serves as an alternative pathway to bypass resistance to apoptosis. Cells undergoing necroptosis exhibit necrotic characteristics, such as swelling and rupture of the cellular membrane. This pathway can be initiated by death receptors, toll-like receptors, TNF, Z-DNA binding protein 1, or certain viral infections, particularly when the activation of caspase-8 is compromised [22-24]. While necroptotic cell death can be triggered by pro-inflammation or as an alternative to apoptosis, our focus is on the former, given that necroptosis induced by cryoinjury is seldom associated with viral infection or inflammation. Furthermore, apoptosis and necroptosis are closely regulated by each other, with caspase-8 playing a pivotal role as a mediator of both apoptotic and necroptotic pathways. This is because caspase-8 not only regulates apoptosis but also serves as a key component of the ripoptosome [25]. In the absence of caspase-8 activity, receptor-interacting protein kinase (RIPK) 1 autophosphorylates RIPK3, leading to the formation of the ripoptosome. The RIPK1/RIPK3 complex then phosphorylates mixed lineage kinase domain-like protein (MLKL), resulting in the creation of the necrosome. This structure triggers cell death by destabilizing mitochondria, perturbing the membrane, and causing cell lysis [26-28].

3. Autophagy: a double-edged sword

Autophagy is a process in which cellular components such as mitochondria, organelles, and endoplasmic reticula are degraded by lysosomes, allowing them to be recycled and used in cellular function during periods of stress or starvation. This makes autophagy a crucial player in maintaining cellular homeostasis. The autophagy process can be broken down into five stages: (1) initiation; (2) nucleation and phagophore formation; (3) phagophore expansion; (4) fusion with the lysosome; and (5) degradation [29]. The primary regulators of autophagy are the mammalian target of rapamycin, which functions as an inhibitor, and adenosine monophosphate-activated kinase, which acts as an activator. The initiation of autophagy triggers the regulation of nucleation by various protein complexes, including autophagy-related proteins. Subsequently, phagophores marked with microtubule-associated protein 1A/1B-light chain 3 (LC3) mature into autophagosomes. These autophagosomes then fuse with lysosomes to create autolysosomes, where the cargo is degraded [30].

While autophagy is typically viewed as a survival mechanism, its deregulation has also been linked to a non-apoptotic form of cell death known as either ADCD or autophagic cell death [7,31-33]. Morphologically, ADCD is characterized by the extensive presence of autophagic components in the cytoplasm, devoid of other forms of programmed cell death. This process can be halted either pharmacologically or genetically [31,33]. ADCD can occur via an autophagic flux-dependent or flux-independent pathway. The former, known as autosis, is a non-apoptotic form of cell death that is autophagy- dependent and results from excessive or uncontrolled autophagy levels [34]. The latter is triggered by glycosylceramidase beta 1 and leads to autophagic cell death [35].

Cryoinjury-induced cell death in germ cell freezing

Cryopreservation is a method used to preserve the fertility of both male and female patients, whether pre- or post-pubertal. However, the process of freezing and thawing often exposes cells or tissues to various forms of stress, including extensive ROS generation, oxidative stress, apoptosis, and cold shock. These factors can reduce the efficacy of freezing [3-6]. Additionally, CPAs, which are necessary for bio-

logical freezing, can have adverse effects on frozen cells or tissues, such as osmotic stress or toxicity [4,36]. In this context, it is crucial to understand the molecular mechanisms of cryoinjury that occur during freezing in order to overcome these limitations and improve the efficacy of freezing. Therefore, this review is focused on the mechanisms of cell death induced by cryoinjury, including apoptosis, necroptosis, and ADCD, in the context of germ cell cryopreservation (Table 1).

Cryoinjury-induced apoptosis has been observed in a broad range of cell types, including oocytes, ovarian tissue, spermatozoa, testicular tissue, and spermatogonial stem cells (SSCs). This phenomenon has been reported across various species, such as mice, cattle, pigs, and humans, and through different methods, namely slow freezing and vitrification. In the vitrification of bovine oocytes, it was discovered that the oocytes degenerated during subsequent culture, as evidenced by signs of apoptosis like DNA fragmentation and caspase-3 activation [37]. Similarly, an increase in apoptotic levels was noted in murine metaphase II (MII) oocytes and porcine oocytes post-vitrification [38,39]. More specifically, in 2015, Dai et al. [40] demonstrated that porcine MII oocytes experienced apoptosis due to the generation of ROS, the upregulation of intrinsic apoptotic pathways, and impaired mitochondrial function following vitrification. Numerous studies have also shown that cryopreservation is linked to apoptosis in sperm freezing, with a higher activation of pan-caspases in frozen spermatozoa compared to fresh ones [41-45]. One study revealed that vitrified prepubertal mouse testicular tissue exhibited a high degree of apoptosis [46], and slow-frozen porcine testicular tissue was separately associated with elevated levels of apoptosis [47]. Furthermore, several studies have determined that the freezing efficiency of SSCs, regardless of the species, was reduced due to apoptosis when slow freezing was used [48-50].

As previously noted, apoptosis is not the sole process accountable for cryoinjury that results in cell death during freezing, just as additional cell death pathways exist beyond apoptosis [51,52]. Unfortunately, unlike apoptosis, which is recognized as a cause of cell death during freezing, the mechanisms of cryoinjury-induced necroptosis

Table 1. Cryoinjury-induced cell death mechanisms in germ cell freezing

Pathway	Cell type	Freezing method	Cryoinjury	Reference
Apoptosis	Bovine oocyte	Vitrification	(↑) DNA fragmentation	[37]
			(↑) Caspase-3 activation	
	Murine MII oocytes	Vitrification	(↑) Apoptotic level	[38]
	Porcine oocytes	Vitrification	(↑) Apoptotic level	[39]
	Porcine MII oocytes	Vitrification	(↑) ROS generation	[40]
			(↑) Intrinsic apoptotic pathways	
			(↑) Impaired mitochondria	
	Bull sperm	LN_2 vapor procedure	(↑) Pan-caspase activity	[41]
	Human sperm	LN_2 vapor procedure	(↑) Pan-caspase activity	[42]
	Human sperm	LN_2 vapor procedure	(↑) DNA fragmentation	[44]
			(↑) Caspase-3 activation	
	Murine prepubertal testicular tissue	Vitrification	(↑) Apoptotic level	[46]
	Porcine prepubertal testicular tissue	Slow freezing	(↑) Early apoptosis	[47]
	Murine SSCs	Slow freezing	(↑) Early apoptosis	[48]
	Bovine prepubertal SSCs	Slow freezing	(↑) Cytochrome C release	[49]
Necroptosis	Murine SSCs	Slow freezing	(↑) RIP1 expression	[10]
	Ovarian tissue	Vitrification	Higher intact follicle ratio in the necrostatin-1–treated groups	[11]
	Murine oocytes	Vitrification	Non-significant difference of necroptosis-associated genes due to unique localization patterns of pMLKL and pRIPK1 in oocyte	[54]
Autophagy	Stallion sperm	Stored at 5 ℃	(↑) LC3B expression	[8]
	Murine immature oocytes	Vitrification	(↑) Beclin-1 expression	[55]
			(↑) Number of GFP-LC3 puncta	
	Murine oocytes	Vitrification	(↑) Beclin-1 expression	[56]
	Murine SSCs	Slow freezing	(↑) Excessive autophagy	[9]
			(↑) Beclin-1, ATG7, p53, LC3II/I, and Lamp2 expression	

MII, metaphase II; ROS, reactive oxygen species; LN_2, liquid nitrogen; SSC, spermatogonial stem cell; RIP1, receptor-interacting protein 1; pMLKL, phosphate mixed lineage kinase domain-like protein; pRIPK1, phosphate receptor-interacting protein kinase 1; LC3, microtubule-associated protein 1A/1B-light chain 3; GFP, green fluorescent protein; ATG7, autophagy-related 7.

https://doi.org/10.5653/cerm.2023.06016

or ADCD remain largely unknown. Recent research has indicated that the inhibition of necroptosis in SSCs is crucial for maintaining male fertility during gonadotoxic treatment [53]. In this context, necroptosis could be a contributing factor to cryoinjury resulting in cell death. This hypothesis is backed by studies that have reported a cryoprotective effect when necrostatin-1 (a receptor-interacting protein [RIP] 1-targeted inhibitor of necroptosis) was employed as a CPA in SSC or ovarian tissue freezing [10,11]. However, another study indicated that the inclusion of necrostatin-1 decreased the survival rate of vitrified oocytes, suggesting that the positioning of pMLKL and pRIPK is unique in mouse oocytes and that necroptosis plays a role in preserving oocyte integrity post-vitrification [54].

The role of autophagy, a crucial process for cellular homeostasis, in determining cell survival or death following freeze-thawing remains a topic of debate. Gallardo Bolanos et al. [8] posited that autophagy aids in the survival of spermatozoa during cooled storage. In a similar vein, Gao et al. [55] demonstrated that inhibiting autophagy triggered intrinsic apoptosis in immature oocytes during the vitrification-warming process, while Bang et al. [56] depicted autophagic activation as a natural adaptive response to cold stress in vitrified-warmed oocytes. Conversely, Jung et al. [9] suggested that autophagy could contribute to cryoinjury during SSC cryopreservation, indicating that excessive autophagy could result in cell death after freeze-thawing.

New strategies for germ cell freezing

While numerous modifications to freezing protocols (such as alterations to CPAs, freezing rate, cell concentration, and storage temperature) have been explored to preserve pre- or post-pubertal female or male fertility, the molecular mechanisms underlying cryoinjury in germ cell freezing remain unclear. As previously noted, the majority of research has focused on apoptosis, with few studies investigating necroptosis or autophagy. This lack of understanding will impede the development of innovative techniques to enhance cryoprotective efficacy until we fully comprehend the pathways involved in cryoinjury. Consequently, emerging strategies for germ cell freezing are intended to modulate target signaling molecules based on a more comprehensive understanding of cryoinjury pathways; this moves beyond current freezing strategies that focus solely on apoptosis, as well as the development of new CPAs. Furthermore, the primary objective is to target and inhibit various signaling molecules in the cell death cascades that trigger apoptosis, necroptosis, and autophagy, not only during freezing but also before and after thawing. In Table 2, we provide a summary of the targeted CPAs used in germ cell freezing.

1. Targeting CPAs

Apoptosis can be initiated by two distinct pathways: the extrinsic and intrinsic pathways. These pathways can be activated during low-temperature freezing, as evidenced by the protective role of specific cysteinase inhibitors (for example, Z-Val-Ala-Asp-(OMe)-fluoromethylketone [Z-VAD-FMK], a pan-caspase inhibitor; Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone [Z-IETD-FMK], a caspase-8 inhibitor; and Z-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethylketone [Z-LEHD-FMK], a caspase-9 inhibitor) when used as CPAs in germ cell freezing. Ha et al. [57] revealed that the proliferation capacity of SSCs was increased in frozen SSCs treated with a mixture of 200 mM trehalose and 15 μM Z-VAD-FMK. This treatment also reduced early apoptosis after thawing through pan-caspase inhibition. In a similar vein, Niu et al. [58] reported a decrease in caspase activity and early apoptosis in vitrified porcine oocytes treated with Z-IETD-FMK and Z-LEHD-FMK. In 2020, Colombo et al. [59] also discovered that Z-VAD-FMK reduced DNA damage and caspase activity in vitrified

Table 2. Functions of cryoprotective agents

Target	Component	Function
Apoptosis inhibitor	Z-VAD-FMK	Pan-caspase inhibitor
	Z-IETD-FMK	Caspase-8 inhibitor
	Z-LEHD-FMK	Caspase-9 inhibitor
	Bongkrekic acid	mPTP opening inhibitor
Antioxidant	Coenzyme Q10	Increases the production of key antioxidants
	Melatonin	Mitochondrial-targeted antioxidant
	Vitamin C	Scavenger of free radicals
	Vitamin E	Lipophilic chain-breaking antioxidant
	MnTBAP	Cell-permeable superoxide dismutase mimetic
	Pentoxifylline	Phosphodiesterase inhibitor
	NAC	Reduces various radicals by donating 1 electron
	Leptin	Supports antioxidant enzyme activity
	Selenium	Antioxidant to break down peroxides
	Y-27643	ROCK inhibitor
Necroptosis inhibitor	Necrostatin-1	RIPK inhibitor
Autophagy inhibitor	3-methylademine	PI3K inhibitor
	Melatonin	Inhibits autophagosome formation

Z-VAD-FMK, Z-Val-Ala-Asp-(OMe)-fluoromethylketone; Z-IETD-FMK, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone; Z-LEHD-FMK, Z-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethylketone; mPTP, mitochondrial permeability transition pore; MnTBAP, manganese (III) tetrakis (4–69 benzoic acid) porphyrin; NAC, N-acetyl-L-cysteine; ROCK, Rho-associated coiled-coil containing kinases; RIPK, receptor-interacting protein kinase; PI3K, type III phosphatidylinositol 3-kinase.

oocytes. These findings suggest that inhibiting apoptotic proteolytic activation allows frozen germ cells to minimize cell death induced by cryoinjury. This conclusion is consistent with the findings of Jung et al. [60], who showed that extrinsic apoptosis, which involves the proteolytic activation of caspase-8, is increased in frozen SSCs. Therefore, Z-IETD-FMK can significantly enhance the cryoprotective efficacy of SSCs by suppressing caspase-8 activity during freezing. Furthermore, bongkrekic acid, an inhibitor of mitochondrial permeability transition pore opening, has been shown to improve the freezing efficacy of stallion sperm. This is achieved by reducing caspase activity and increasing the mitochondrial membrane potential [61].

Antioxidants are often used as CPAs to reduce apoptosis, as excessive ROS generation during freezing can cause cell death or damage upon thawing. For instance, vitrification of bovine oocytes with 50 μM of coenzyme Q10, an antioxidant, significantly improved oocyte survival and mitigated premature cortical granule (CG) exocytosis, thereby preserving the CG migration pattern [62]. Similarly, freezing SSCs with 100 μM of melatonin, a mitochondria-targeted antioxidant, greatly enhanced survival, reduced ROS production, and decreased intrinsic apoptosis [63]. Vitamin C, known for its potent antioxidant properties due to its strong reducing power, has been found useful in preserving DNA integrity, sperm motility, acrosome membranes, and plasma after freezing bovine sperm with vitamin C [64]. Vitamin E, a lipophilic chain-breaking antioxidant, has also been effectively used as a CPA for the freezing of ram sperm [65-67]. Moreover, resveratrol, a powerful non-flavonoid antioxidant, has shown beneficial effects on post-thaw sperm survival, fertility, and quality by inhibiting ROS production [68-70]. In addition, previous research has suggested that an increase in intracellular TNF-α after freezing is implicated in TNF-α–mediated necroptotic cell death [53]. The activation of the RIP complex also induces ROS production in the mitochondria and mediates plasma membrane rupture [71]. As such, the necroptosis inhibitor necrostatin-1 has shown beneficial effects on the proliferation rate while reducing necroptosis and apoptosis after thawing in SSC cryopreservation; it exerts these impacts by reducing ROS generation, apoptosis, and RIP1 activity [10]. The addition of pifithrin μ (a p53 inhibitor) or 3-methyladenine, which blocks autophagosome formation by inhibiting type III phosphatidylinositol 3-kinase, has been found to enhance cryoprotective effects and help maintain normal stem cell characteristics after thawing during SSC freezing [9]. Mitophagy, a selective autophagy that removes aged and damaged mitochondria, is also activated in frozen oocytes to improve survival by eliminating damaged mitochondria [55]. In 2020, Feng et al. [72] demonstrated that melatonin inhibits autophagosome formation and regulates the levels of autophagy-related proteins, thereby reducing cryoinjury-induced excessive autophagy in goat SSC freezing.

Interestingly, autophagy does not consistently safeguard frozen germ cells; in fact, excessive autophagy can lead to cryoinjury-induced cell death [73]. Furthermore, while each pathway regulator may have cryoprotective effects resembling those of CPAs, the intricate nature of these pathways prevents complete rescue from cryoinjury-induced cell death. This is due to our limited understanding of the complex interplay among these pathways. This underscores the need for further molecular mechanistic studies to enhance our comprehensive understanding of cryoinjury-induced cell death.

2. Pre-freeze or post-thaw care

Efforts have been made to develop effective CPAs to prevent cryoinjury during the freezing process. However, these CPAs only partially mitigate cryoinjury, as damage continues to occur even after thawing. This highlights the necessity for careful handling and recovery of frozen cells and tissues post-thaw [12]. In this context, Hwang and Hochi [14] discovered that a brief post-thaw culture of oocytes with Y-27643, a Rho-associated protein kinase inhibitor, prevented the formation of multiple asters. This formation is a key factor limiting the embryonic potential of vitrified-warmed bovine oocytes [14]. Additionally, Girka et al. [13] found that treatment with 5 and 10 μM Y-27632 effectively improved spindle rescue following vitrification-warming. In 2018, Pero et al. [74] proposed that the use of 20 mM Z-VAD-FMK during *in vitro* culture post-thawing could partially reduce cryoinjury-induced apoptosis by suppressing active caspase-3. Pagano et al. [16] demonstrated that treating with 100 μM Z-VAD-FMK enhanced the mass motility of bovine sperm prior to freezing and maintained sperm membrane integrity post-thawing. Finally, Jung et al. [15] reported that supplementing post-thaw media with 200 μM Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (Z-DEVD-FMK) enhanced the proliferation potential of frozen SSCs, safeguarding them against ROS generation and apoptosis following cryo-thawing.

Numerous studies have demonstrated that incorporating antioxidants into media during *in vitro* maturation prior to freezing can enhance embryo development in various species, including mice [75,76], sheep [77], and cattle [62,78]. Furthermore, a range of antioxidants, such as selenium, manganese (III) tetrakis (4–69 benzoic acid) porphyrin (MnTBAP), pentoxifylline, N-acetyl-L-cysteine (NAC), and leptin, have been found to mitigate oxidative stress related to cryoinjury-induced damage when added to the *in vitro* culture media used in sperm freezing. Gavella and Lipovac [79] found that pre-incubating human sperm with 3.7 or 10 mM pentoxifylline, a phosphodiesterase inhibitor that also protects against lipid peroxidation by hydrogen peroxide, for 30 minutes at 37 °C reduced oxidative stress and enhanced fertility potential post-thawing. Oeda et al. [80] reported that incubating human semen with 1 mg/mL NAC for

https://doi.org/10.5653/cerm.2023.06016

20 minutes at room temperature significantly lowered ROS levels and improved sperm motility. Boroujeni et al. [81] found that rinsing with 5 μg/mL selenium after thawing can enhance human sperm parameters and survival. Fontoura et al. [82] found that adding leptin, a hormone that regulates food intake behavior, to human sperm prior to freezing supports antioxidant enzyme activity and provides additional antioxidant protection. Additionally, Treulen et al. [83] demonstrated that pre-incubating with MnTBAP, a cell-permeable superoxide dismutase mimetic, for 4 hours at 38 °C improved stallion sperm motility and reduced oxidative stress, while maintaining normal fertilization potential post-thawing.

While the addition of antioxidants or apoptosis inhibitors to *in vitro* culture media may help reduce oxidative stress, the use of these compounds in freezing is not always effective. Prolonged exposure or high concentrations can disrupt the appropriate physiological balance, as apoptosis is instrumental in removing damaged cells in adults and regulating embryonic development [84]. Similarly, excessive antioxidant supplementation may disrupt the oxidation-reduction balance, resulting in reductive stress [85]. However, the apoptosis or oxidative stress induced by cryoinjury often results in considerably more additional apoptotic/oxidative stress, which affects cell survival [86]. Therefore, the inclusion of antioxidants or apoptosis inhibitors could be beneficial in alleviating cell death caused by cryoinjury, provided optimization and functional tests for fertility are conducted.

Conclusion

Cryopreservation of germ cells, reproductive tissue, and SSCs is an essential technique for preserving the fertility of patients with infertility or adolescents diagnosed with cancer. The primary objective of this technique is to ensure high viability and normal function post-freezing. Despite concerted efforts, current cryopreservation methods for reproductive tissues lack a comprehensive understanding of the underlying mechanisms of cryoinjury. Cryoinjury appears to be implicated in various cell death pathways, not just apoptosis, which has been the primary focus of research to date. However, the pathways involved in cryoinjury are not yet fully understood. As such, it is imperative to conduct in-depth investigations into the potential processes and pathways that lead to cryoinjury during freezing. We propose that future research should expand to include various cell death mechanisms associated with cryoinjury. Furthermore, we suggest that this research should incorporate both foundational and recent findings to enhance freezing efficacy by modulating signaling molecules and to devise new solutions that can be applied before freezing and after thawing.

Conflict of interest

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

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Conceptualization: SEJ, BYR. Data curation: SEJ. Funding acquisition: BYR. Project administration: BYR. Visualization: SEJ. Writing-original draft: SEJ. Writing-review & editing: SEJ, BYR.

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https://doi.org/10.5653/cerm.2023.06016

REVIEW ARTICLE
https://doi.org/10.5653/cerm.2023.06128
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):223-229

CERM

Dienogest in endometriosis treatment: A narrative literature review

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Endometriosis is characterized by the implantation of endometrial cells outside the uterus. This hormone-dependent disease is highly prevalent among women of reproductive age. Clinical symptoms of endometriosis include dysmenorrhea, pelvic pain, and infertility, which can negatively impact the overall quality of life of those affected. The medical treatment of endometriosis serves as an important therapeutic option, aimed at alleviating pain associated with the condition and suppressing the growth of endometriotic lesions. As such, it is employed as an adjuvant therapy following surgery or an empirical treatment after the clinical diagnosis of endometriosis. Dienogest, a fourth-generation progestin, has received approval for the treatment of endometriosis in many countries. A growing body of evidence has demonstrated its efficacy in managing endometriosis-associated pain, preventing symptoms, and reducing lesion recurrence. In this review, we examine the clinical efficacy, safety, and tolerability of dienogest in treating endometriosis. We also provide updated findings, drawing from clinical studies that focus on the long-term use of this medication in patients with endometriosis.

Keywords: Dienogest; Endometriosis; Medical therapy; Progestins

Introduction

Endometriosis, defined as the ectopic implantation of endometrial cells, is a common gynecological disease affecting 10% to 15% of women of reproductive age [1]. This condition often results in painful symptoms such as dysmenorrhea, noncyclic chronic pelvic pain, and dyspareunia, and it is frequently linked with infertility. Studies report that the prevalence of endometriosis ranges from 25% to 50% among women with infertility [2].

The etiopathogenesis of endometriosis continues to be a subject of debate and is not fully understood. Several theories have been proposed to explain its origin, including retrograde menstruation, lymphatic spread, coelomic metaplasia, Müllerian remnants, and stem cell recruitment [3-8]. However, none of these theories fully account for the various types of endometriosis. Endometriosis is known for its high recurrence rate and often follows a chronic clinical course, even after surgical intervention. As a result, medical treatment is typically employed as an adjuvant therapy following surgery. In recent years, a consensus has emerged that medical therapy can be proposed as a primary empirical treatment following a clinical diagnosis of endometriosis. Furthermore, long-term medical treatment is widely considered necessary for managing endometriosis-associated pain and preventing recurrence [9-13].

Currently, the medical treatment options for endometriosis include progestins, oral contraceptive pills, gonadotropin-releasing hormone (GnRH) agonists, hormone-releasing intrauterine devices, and subdermal implants. Among these, progestins have emerged as one of the most important options worldwide [11,14,15]. Dienogest (DNG) is a fourth-generation progestin that is orally active and exhibits highly selective binding to the progesterone receptor [16]. It has received approval for the treatment of endometriosis in numerous countries [14,17-19]. In this literature review, we discuss the clinical efficacy, safety profile, and tolerability of DNG in patients with endometriosis, as well as recent evidence regarding its long-term use.

Received: May 12, 2023 · Revised: July 13, 2023 · Accepted: August 9, 2023
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Mechanisms of action of DNG in endometriosis

DNG is a derivative of 19-nortestosterone, distinguished from other progestins of the same derivation by the presence of a cyanomethyl group rather than an ethynyl group at position 17α [20]. Pharmacologically, DNG primarily exerts a localized effect on endometriotic lesions, demonstrating minimal angiogenic, estrogenic, glucocorticoid, or mineralocorticoid activity. It also exhibits both anovulatory and antiproliferative effects [9,21,22]. Studies have shown that DNG moderately inhibits the secretion of gonadotropins, leading to a reduction in the endogenous production of estradiol. This suppression induces decidualization of the ectopic endometrium, with subsequent atrophy [20,23]. Furthermore, DNG inhibits the proliferation of endometrial cells by modulating the expression of matrix metalloproteinases and aromatase, which are involved in the ectopic endometrial response to endogenous estrogen [24,25]. The anti-angiogenic and anti-inflammatory properties of DNG, which are relevant to the reduction of endometriotic lesions, have been observed in both *in vivo* and *in vitro* studies involving eutopic or ectopic endometrial cells [26-28].

Therapeutic efficacy of DNG in placebo-controlled trials

The therapeutic efficacy, safety, and tolerability of oral DNG in the treatment of endometriosis have been studied in clinical trials (Table 1). In a phase 3, placebo-controlled, randomized, double-blind, multicenter study, the efficacy and safety of DNG were assessed within a Chinese population [29]. DNG (2 mg) was administered once daily to 255 women, aged 18 to 45 years, who had been laparoscopically diagnosed with endometriosis and had an endometriosis-associated pelvic pain (EAPP) score of at least 30 mm on a 0- to 100-mm visual analog scale (VAS). The primary efficacy variable was the absolute change in EAPP score from baseline to week 24, while secondary efficacy variables included the intake of supportive analgesic medication (SAM). After 24 weeks of treatment, the mean reduction in EAPP score was significantly greater in the women treated with DNG compared to those who had received the placebo. Women in the DNG group also reported a decrease in SAM intake (specifically, in the number of 200 mg ibuprofen tablets taken in the previous 4 weeks), from an average of 1.5 at the start of the study to 0.5 at week 24. The women in the placebo group reported a slight increase in SAM intake, from a mean of 1.7 to 1.9.

In another randomized, double-blind, multicenter study, the effectiveness of oral DNG (2 mg once daily) over a 12-week period was compared to that of a placebo [30]. This study enrolled 198 patients (aged 18 to 45 years) with laparoscopically confirmed endometriosis, approximately 70% of whom had stage III or IV disease. EAPP was evaluated using the VAS score. The mean reductions in VAS score from baseline to week 12 in the full analysis set were 27.4 mm for the DNG group and 15.1 mm for the placebo group. This constituted a significant score difference of 12.3 mm, favoring DNG. In terms of numerical data, a higher percentage of patients receiving DNG were rated as "much improved/very much improved" on the clinician-rated Clinical Global Impressions improvement scale (52.9% vs. 22.9%). Similarly, a greater proportion of patients gave responses of "highly satisfied/very highly satisfied" on a patient-rated overall satisfaction scale (43.1% vs. 20.8%) compared to those receiving the placebo.

In a 12-week placebo-controlled trial [30], an open-label extension study was conducted to assess the long-term efficacy and safety of DNG. This study recruited 168 women, of whom 91% completed the 53-week extension, resulting in a total study duration of 65 weeks. This research was conducted in parallel with two other studies [31]. Over the course of DNG treatment, the EAPP score decreased steadily, dropping from 57±17 at the baseline of the placebo-controlled study to 12±11 at the conclusion of treatment. Abnormal bleeding profiles were observed, including irregular bleeding (22%), infrequent bleeding (24%), and amenorrhea (28%). Despite these findings, only two patients discontinued treatment due to bleeding irregularities.

Therapeutic efficacy of DNG compared with GnRH agonists

Several studies have been conducted to compare the therapeutic efficacy of DNG with that of GnRH agonists, such as leuprorelin, buserelin, and triptorelin. One randomized parallel clinical trial involved 59 patients with endometriosis who were administered DNG (1 mg twice daily), along with 61 patients who were given decapeptyl (3.75 mg via intramuscular injection every 28 days), for a period of 16 weeks. The results showed comparable efficacy between the two treatments [32]. In another randomized, open-label, multicenter study, oral DNG (2 mg once daily; n=90) was administered for 24 weeks and was found to be noninferior to intramuscular leuprorelin (3.75 mg every 4 weeks; n=96) in reducing EAPP score from baseline. This was assessed using VAS scores in the per-protocol set [33]. The proportions of patients in the DNG and leuprorelin groups who experienced an improvement in pelvic pain from baseline were 97% and 96%, respectively.

In a separate randomized, double-blind, multicenter study conducted in Japan, oral DNG (1 mg twice daily; n=129) and intranasal buserelin acetate spray (300 mg three times daily; n=125) were administered for 24 weeks. Both treatments demonstrated broadly comparable efficacies for all symptoms, including lower abdominal

https://doi.org/10.5653/cerm.2023.06128

Table 1. Included randomized clinical studies on the efficacy and safety of DNG compared with placebo or GnRH agonists

Study	Design	Participants	Duration (wk)	Intervention	Main outcomes
Placebo-controlled study					
Lang et al. (2018) [29]	Placebo-controlled, randomized, double-blind, multicenter phase 3 study	255 Chinese women (aged 18–45 years) who reported VAS score ≥ 30 mm	24	129 Women with placebo and 126 with DNG 2 mg/day	DNG was superior to placebo in reducing EAPP and was safe and well tolerated in Chinese women with endometriosis.
Strowitzki et al. (2010) [30]	Randomized, double-blind, placebo-controlled, multicenter trial	198 Women (aged 18–45 years) who reported VAS score ≥ 30 mm	12	96 Women with placebo and 102 with DNG 2 mg/day	In the full analysis set, the mean reductions in VAS score between baseline and week 12 were 27.4 mm and 15.1 mm in the DNG and placebo groups, respectively; a significant score difference of 12.3 mm was observed in favor of DNG ($p < 0.0001$).
DNG vs. GnRH agonist					
Cosson et al. (2002) [32]	Multicenter, open, randomized, parallel-group clinical trial	142 Women (aged 18–40 years) with grade 2, 3, or 4 endometriosis at initial laparoscopy	16	59 Women with DNG 1 mg/day and 61 with decapeptyl 3.75 mg IM every 4 weeks	VAS scores were comparable in both groups, and efficacy was not significantly different between the two groups.
Strowitzki et al. (2010) [33]	Randomized, multicenter, open-label trial	Women (aged 18–45 years) with histologically proven endometriosis	24	124 Women with DNG 2 mg/day and 128 with leuprolide acetate 3.75 mg IM every 4 weeks	DNG demonstrated equivalent efficacy to leuprolide at the standard dose in relieving the pain associated with endometriosis, while offering advantages in terms of safety and tolerability.
Harada et al. (2009) [22]	Phase III, randomized, double-blind, multicenter, controlled trial	271 Patients (aged ≥ 20 years) with endometriosis diagnosed via surgery or imaging analysis	24	137 Women with DNG 2 mg/day and 134 with intranasal buserelin acetate 900 μg/day	Pre- to post-treatment changes in the scores of five subjective symptoms during non-menstruation (lower abdominal pain, lumbago, defecation pain, dyspareunia, and pain on internal examination) and two objective findings (induration in the pouch of Douglas and limited uterine mobility) were measured. DNG reduced the scores of all symptoms and findings at the end of treatment. The mean changes in the scores of all symptoms and findings, except induration in the pouch of Douglas, were comparable to those obtained with buserelin acetate. The reduction in BMD during DNG treatment was significantly lower than that during buserelin acetate treatment.
Ceccaroni et al. (2021) [34]	Prospective randomized controlled trial	146 Women (aged 18–45 years), laparoscopic eradication of rASRM stage III–IV DIE with bowel and parametrial surgery	24	65 Women with DNG 2 mg/day and 81 with triptorelin or leuprorelin 3.75 mg IM every 4 weeks	Both DNG and GnRH agonists were associated with a highly significant reduction of pain at 6 and 30 months, without any significant difference ($p < 0.001$). Regarding treatment tolerability, a more satisfactory profile was reported with DNG ($p = 0.026$). No difference was found in terms of clinical relapse, imaging relapse, or live births.

DNG, dienogest; GnRH, gonadotropin-releasing hormone; VAS, visual analog scale; EAPP, endometriosis-associated pelvic pain; IM, intramuscular; BMD, bone mineral density; rASRM, revised American Society for Reproductive Medicine; DIE, deep infiltrating endometriosis.

pain, lumbago, dyschezia, and dyspareunia, from baseline to the end of the treatment period [22]. However, the DNG group reported a higher frequency of irregular genital bleeding and a lower loss of bone mineral density (BMD) compared to the buserelin acetate group. In a separate prospective randomized controlled trial, 146 patients who had undergone laparoscopic surgery for deep infiltrating endometriosis were enrolled. These participants were randomized to receive either 2 mg/day of DNG or either triptorelin or leuprorelin every 4 weeks for a duration of 6 months [34]. Both groups exhibited a significant reduction in pain at the 6- and 30-month marks. No differences were observed in clinical relapse, imaging relapse, or live births between the two treatment regimens. However, the DNG group reported a more satisfactory profile.

Efficacy of postoperative DNG on recurrence of endometriosis

A meta-analysis of 10 studies evaluated the risk of endometriosis recurrence in women who received DNG following surgery [35]. Recurrence in this study was defined based on radiographic evidence of endometrioma via ultrasound or magnetic resonance imaging; patient-reported recurrence of symptoms such as pelvic pain, dysmenorrhea, dyspareunia, or noncyclic pelvic pain after conservative endometriosis surgery with DNG treatment; and findings from second-look laparoscopy. The recurrence rate was then compared to that of control participants. The use of DNG postoperatively was found to reduce the risk of endometriosis recurrence. The incidence rate of endometriosis recurrence in patients treated with DNG was 2 per 100 women over an average follow-up period of 29 months (95% confidence interval [CI], 1.43 to 3.11). This was in contrast to the recurrence rate of 29 per 100 women who were under expectant management over an average follow-up period of 36 months (95% CI, 25.66 to 31.74). The likelihood of recurrence was significantly reduced with the postoperative administration of DNG (log odds, −1.96; 95% CI, −2.53 to −1.38; $p<0.001$).

Another recent meta-analysis of 11 studies examined disease recurrence in patients who received DNG maintenance treatment compared with those who received other medications, including the levonorgestrel-releasing intrauterine system and GnRH analogs, as well as those who did not receive any treatment [36]. The recurrence rate for patients on DNG maintenance was found to be lower than that for patients who received no treatment, while demonstrating comparable efficacy to other treatments in preventing disease recurrence. Therefore, DNG is suggested as a viable maintenance treatment for patients with endometriosis, with the aim of reducing the rate of disease recurrence following conservative surgery [36].

Long-term use of DNG and adverse effects

Several studies have reported a broad range of systemic adverse effects (AEs) during DNG treatment. A pooled analysis of four randomized clinical trials evaluated the safety and tolerability of DNG in treating endometriosis. This analysis involved 332 women who were administered 2 mg of DNG for a period of 12 to 65 weeks [37]. The most frequently reported AEs were headaches (9%), breast discomfort (5.4%), depressive mood (5.1%), and acne (5.1%). In another study that examined DNG treatment over a duration of up to 65 weeks, the most common AEs (occurring in more than 5% of cases) were headache, nasopharyngitis, and breast discomfort. Despite these AEs, DNG was generally well tolerated and exhibited an overall favorable safety profile [31].

A retrospective multicenter study conducted in Korea analyzed the long-term efficacy and safety of postoperative DNG (2 mg) in 514 women. The average duration of DNG administration was 72.2±5.2 weeks, with a range of 48 to 164 weeks [38]. The most frequently observed AEs were bleeding-related events such as amenorrhea (29%; 149/514 cases) and abnormal uterine bleeding (AUB) (6.4%; 33/514 cases). Notably, the most common reason for DNG discontinuation in this study was not related to AEs, but to physician discretion (>60%). This may be attributed to many physicians' awareness of a previous study that extended the use of DNG to 65 weeks, leading to hesitation in prescribing the medication beyond this period. Cycle irregularity, or AUB, is one of the most common AEs during DNG treatment. This is typically more frequent during the first few weeks of medication, after which it tends to decrease with continued use [19,29,39-42]. Changes in bleeding patterns associated with DNG are generally well tolerated and are unlikely to be the primary reason for DNG discontinuation [14,37].

The Visanne Post-approval Observational Study, the largest real-world, non-interventional study on this topic, investigated the safety of DNG and other hormonal treatments for endometriosis in routine clinical practice [43]. This study included more than 25,000 women who were initiating new treatments for endometriosis, including DNG (2 mg) and other hormonal medications. These women were recruited from 1,000 centers across six European countries and were monitored for 7 years. The primary outcomes evaluated were anemia and either *de novo* or clinically worsening depression. The findings indicated that the hazard ratio for anemia was 1.1 (95% CI, 0.4 to 2.6) for DNG in comparison to "other approved endometriosis treatment (OAED)," and 1.3 (95% CI, 0.7 to 2.4) for DNG when compared with options "not approved but frequently used for endometriosis treatment (NAED)" [44]. The adjusted hazard ratios for new or worsening depression were 1.8 (95% CI, 0.3 to 9.4) for DNG compared to OAED and 1.5 (95% CI, 0.8 to 2.8) for DNG

https://doi.org/10.5653/cerm.2023.06128

compared to NAED. The study identified no safety concerns related to anemia for DNG users. Although a slight increase in the risk of depression cannot be excluded, this may be attributable to the baseline severity of endometriosis or unidentified country-specific confounding variables.

The EffectiveNess of VISanne in Improving quality of life in Asian wOmen with eNdometriosis (ENVISIOeN) study, a prospective non-interventional study conducted in six Asian countries, evaluated health-related quality of life in a real-world setting [40]. This study included 887 patients who received DNG following a clinical or surgical diagnosis of endometriosis. Data were gathered for up to 24 months after initiation of DNG. The study findings indicated that DNG consistently enhanced EAPP from 6 to 24 months and improved health-related quality of life. This improvement was measured using the Endometriosis Health Profile-3 tool in women who had received either a clinical or a surgical diagnosis of endometriosis.

DNG has been found to inhibit ovulation and moderately suppress the production of estradiol, with the average systemic E2 concentration remaining at 39 pg/mL following treatment with 2 mg of DNG [45]. This raises potential concerns about the long-term use of DNG and its potential negative impact on bone health. In a 52-week trial involving 135 patients who were administered 2 mg of DNG daily, the BMD at the lumbar spine decreased by −1.6% at 24 weeks and −1.7% at 52 weeks [41]. However, no cumulative decrease was observed. A separate study investigated changes in BMD in 60 patients who were given DNG (2 mg/day) following surgery for endometrioma (mean duration of DNG treatment, 18.6 months) [46]. The BMD at the lumbar spine significantly decreased after the first 6 months (−2.2%) and 1 year (−2.7%) compared to baseline measurements. Similarly, the BMD at the femoral neck also significantly decreased after 1 year (−2.8%). For the 24 women who were administered DNG for 2 years or more, the BMD values after 2 years were comparable to those recorded after 1 year at both the lumbar spine and femoral neck sites.

In a retrospective study, researchers examined changes in BMD among 44 patients who used DNG for 3 years. The study found that BMD in the lumbar spine and femur decreased by −4.4% and −3.6%, respectively, compared to baseline measurements [47]. The researchers found that bone loss primarily occurred in the lumbar spine during the 1st year of treatment, and this loss gradually lessened over the course of the treatment period. These findings should be considered when advising patients on appropriate preventive measures [47]. Another study noted that bone loss associated with DNG had partially recovered by 6 months after the cessation of treatment [48]. Current evidence indicates a reduction in BMD during DNG treatment in adolescents and women of reproductive age. However, the clinical significance of this finding—specifically, whether this decrease in BMD is progressive and accumulates over the duration of DNG treatment, as well as whether it increases the risk of fractures later in life—requires further investigation.

Conclusion

For many years, the primary use of medical therapy for endometriosis has been as an adjunct or maintenance treatment following surgical diagnosis and intervention. The surgical management of endometriosis offers several benefits, including the removal of lesions with histological confirmation and anatomical restoration, and it can alleviate pain symptoms associated with endometriosis. However, a potential risk exists of diminished ovarian function due to surgical trauma, which could adversely impact fertility, especially in women planning to conceive. Consequently, a patient-tailored therapeutic approach, involving either surgical or medical treatment for endometriosis, should be meticulously evaluated. This evaluation should take into account various factors including age, the nature and severity of symptoms, future plans for pregnancy, and the condition of the ovarian reserve.

A recent paradigm shift has occurred towards considering empirical medical treatment following the clinical diagnosis of endometriosis [10,11,49]. Current guidelines, along with expert opinions, endorse progestin as the first-line medical option for the treatment of endometriosis. DNG offers benefits including comparable efficacy to GnRH agonists in reducing endometriosis-related pain and fewer side effects due to hypoestrogenism. Therefore, its long-term use is feasible and supported by accumulated data [11,49-51].

Endometriosis is a chronic condition that can cause persistent symptoms, such as pelvic pain, disease progression, or recurrence throughout a person's lifetime. Consequently, emphasis has been placed on the necessity for long-term medical treatment to alleviate endometriosis-related symptoms and prevent recurrence. During treatment with DNG, some AEs may occur, including changes in bleeding patterns and various hormone-related symptoms (such as weight gain, mood changes, and androgenic effects). However, these are generally well tolerated and are not primary causes of treatment discontinuation. More importantly, DNG-related AEs do not overshadow the established benefits of DNG in managing endometriosis symptoms and reducing the risk of recurrence.

Conflict of interest

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

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https://doi.org/10.5653/cerm.2023.06128

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ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06149
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):230-243

CERM

Retinoic acid loaded with chitosan nanoparticles improves spermatogenesis in scrotal hyperthermia in mice

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Objective: High temperatures can trigger cellular oxidative stress and disrupt spermatogenesis, potentially leading to male infertility. We investigated the effects of retinoic acid (RA), chitosan nanoparticles (CHNPs), and retinoic acid loaded with chitosan nanoparticles (RACHNPs) on spermatogenesis in mice induced by scrotal hyperthermia (Hyp).

Methods: Thirty mice (weighing 25 to 30 g) were divided into five experimental groups of six mice each. The groups were as follows: control, Hyp induced by a water bath (43 °C for 30 minutes/day for 5 weeks), Hyp+RA (2 mg/kg/day), Hyp+CHNPs (2 mg/kg/72 hours), and Hyp+RACHNPs (4 mg/kg/72 hours). The mice were treated for 35 days. After the experimental treatments, the animals were euthanized. Sperm samples were collected for analysis of sperm parameters, and blood serum was isolated for testosterone measurement. Testis samples were also collected for histopathology assessment, reactive oxygen species (ROS) evaluation, and RNA extraction, which was done to compare the expression levels of the *bax*, *bcl2*, *p53*, *Fas*, and *FasL* genes among groups. Additionally, immunohistochemical staining was performed.

Results: Treatment with RACHNPs significantly increased stereological parameters such as testicular volume, seminiferous tubule length, and testicular cell count. Additionally, it increased testosterone concentration and improved sperm parameters. We observed significant decreases in ROS production and caspase-3 immunostaining in the RACHNP group. Moreover, the expression levels of *bax*, *p53*, *Fas*, and *FasL* significantly decreased in the groups treated with RACHNPs and RA.

Conclusion: RACHNPs can be considered a potent antioxidative and antiapoptotic agent for therapeutic strategies in reproductive and regenerative medicine.

Keywords: Azoospermia; Chitosan; Hyperthermia; Nanoparticles; Retinoic acid; Scrotum; Spermatogenesis

Received: May 16, 2023 · Revised: June 22, 2023 · Accepted: July 3, 2023
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*This article was financially supported by the Research Department of the Kermanshah University of Medical Sciences School of Medicine (Grant No: 4010057).

Introduction

Spermatogenesis and fertility require a testicular temperature that is approximately 2 to 4 °C below the core body temperature [1], as research has demonstrated that scrotal hyperthermia (Hyp) can harm sperm and testicular germ cells [2]. Several lifestyle factors, including clothing [3], laptop use [4], microwave oven exposure [5], mobile phone use [6], clinical diseases such as cryptorchidism and varicocele [7,8], and occupational hazards can disrupt testicular temperature [3]. Elevated testicular temperature can result in a signifi-

cant but reversible decrease in spermatogenesis and total sperm count, potentially leading to azoospermia. This is due to an increased loss of germ cells, reduced expression of proliferating cell nuclear antigen, and an imbalance between oxidative stress and antioxidant capacity [9-11]. Various treatments aimed at enhancing spermatogenesis have been investigated, including the use of vitamin C and/or vitamin E along with other antioxidants. These treatments have demonstrated promising results in improving semen quality [12,13].

Retinoic acid (RA), a derivative of vitamin A, is instrumental in regulating cell proliferation and differentiation in the development of organs such as the eye, heart, pancreas, and testis. RA is required for signaling and transcriptional control during both male and female development. It interacts with receptor proteins that influence the transcription of several genes vital for these processes [14,15]. A diet deficient in vitamin A can induce infertility in feeding rats, resulting in the inability to produce viable sperm; this underscores the importance of RA in spermatogenesis. However, the administration of exogenous RA can restore spermatogenesis in these animals [16]. The importance of substances such as RA has been proposed for many spermatogenic processes, including the differentiation of spermatogonia [17], the establishment of the blood-testis barrier, and the release of mature spermatids into the lumen [18,19].

Vitamin A is a hydrophobic compound that can easily become inactive or rapidly degrade in aqueous systems. It is poorly soluble in water and has low stability in the presence of oxidants, light, heat, temperature, and moisture. These factors limit its clinical application. However, the stability and dispersibility of vitamin A can be increased by incorporating it into a carrier with beneficial physical and chemical properties [20]. Recent advancements in nanotechnology have broadened the potential medical applications of such carriers [21,22]. Nanoparticles (NPs) have biological effects that may enhance male fertility by improving the quality of sperm cells *in vivo* or *in vitro*. However, the effects of NPs at the cellular and tissue levels are not fully understood [23]. Chitosan, a derivative of chitin, is the second most abundant biomass on earth. It has been used as a delivery system for proteins, nutraceuticals, genes, growth factors, and drugs due to its low toxicity, biocompatibility, biodegradability, mucoadhesion, and low production cost [24-26]. Chitosan nanoparticles (CHNPs), which are positively charged at low pH levels, can form polyelectrolytes in solution by associating with anions [27]. CHNPs containing RA may positively impact sperm parameters and testicular tissue characteristics. In the current study, we investigated the use of retinoic acid loaded with chitosan nanoparticles (RACHNPs), an effective technique for the slow release of vitamin A, to promote the proliferation and differentiation of spermatogonia and sperm parameters in mice subjected to Hyp.

Methods

1. Synthesis and characterization of CHNPs

The CHNPs were synthesized using a method similar to the process detailed in our prior research [28]. In brief, 0.9 mg/mL of medium-molecular-weight chitosan (50,000 to 190,000, Sigma-Aldrich) was mixed into 5 mL of 0.5% acetic acid. This mixture underwent continuous magnetic stirring at room temperature for 24 hours. The pH of the chitosan solution was adjusted to 5.5 using a 2 M NaOH solution. Separately, polyanion sodium tripolyphosphate (TPP) (Sigma-Aldrich) was dissolved in deionized water to attain a final concentration of 0.25 mg/mL. Then, 2 mL of TPP solution was added to the chitosan solution at a rate of 0.2 mL/min while stirring the mixture for 1 hour to form CHNPs. These CHNPs were obtained after freeze-drying for 24 hours. The average particle size was determined using dynamic light scattering (SZ-100Z; HORIBA Jobin Yvon), and the surface charge of the NPs was measured with a zeta potential analyzer (HORIBA Jobin Yvon).

2. Preparation of RACHNPs

Initially, 25 mg of CHNP powder was dispersed in a 5-mL mixture of deionized water and alcohol in equal proportions. Separately, RA was dissolved to a concentration of 20 mg/mL in dimethyl formamide (DMF). This solution was then added dropwise to the pre-prepared CHNP suspension (5 mL) under the action of a magnetic stirrer operating at 150 rpm. This was done for 1 hour at room temperature in a dark environment. The resulting solution was then placed on a shaker for 24 hours. Subsequently, drug loaded NPs were dialyzed using a dialysis membrane with a cut-off value of 12 to 14 kDa (D9402; Sigma-Aldrich), to completely remove non-reactive and oxidant substances. The dialyzed sample was centrifuged at 10,000 rpm for 1 hour, and the resulting precipitate was placed in a freeze dryer to dry for 24 hours. The powder obtained was used to perform Fourier-transform infrared spectroscopy (FTIR) testing, to calculate the amount of drug loaded into the CHNPs, and to assess drug release.

To quantify the loaded drug, a specific weight of RACHNPs was dissolved in 1 mL of DMF and agitated on a shaker. The mixture was then centrifuged at 4,000 rpm for 5 minutes, and the optical absorption of the supernatant was measured using a spectrophotometer (BioTek Synergy HTX; Agilent Technologies Inc.). For this study, a standard curve for RA was established by preparing a series of RA concentrations in DMF, measured at a wavelength of 352 nm. The loading capacity (LC %) was then calculated using the equation derived from the standard curve:

Equation 1. LC% = Wf/Wn × 100

(Wn = weight of NPs; Wf = concentration of loaded retinoic acid in the NPs)

3. Fourier-transform infrared spectroscopy

The chemical structures of RA, CHNPs, and RACHNPs were analyzed using FTIR in the wavenumber range of 4,000 to 400 cm^{-1} resolution (Thermo-Avatar; Thermo Fisher Scientific).

4. Examination of RA release from CHNPs

The drug release investigation was adapted from previous research [29]. To evaluate the release of RA from the RACHNPs, 2.5 mg of RACHNPs was incubated in 500 µL of phosphate-buffered saline (PBS; pH = 7.4) at 37 °C. The suspension was then centrifuged at 10,000 rpm for 30 minutes at regular intervals of 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, 60, 72, 84, 96, 108, and 120 hours. At specific time points, a 400-µL sample of PBS solution was collected and its absorbance measured using a spectrophotometer (BioTek Synergy HTX) at 352 nm. After each measurement, the sample was replaced with fresh PBS. Finally, the cumulative percentage of RA released from the CHNPs at different time intervals was calculated, using the equation derived from the standard curve.

5. Animal study

This study adhered to the guidelines and regulations established by the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.AEC.1400.024). Thirty healthy adult male Naval Medical Research Institute mice, weighing 25 to 30 g, were selected and housed under standard laboratory conditions. The animals were randomly assigned to five groups of six mice each. The first group served as the control, while the second (Hyp) was exposed to Hyp without any treatment. A third group (the RA group) received an intraperitoneal (IP) injection of RA (2 mg/kg), as per previous studies [30], following 35 days of Hyp. A fourth (the CHNP group) received an IP injection of CHNPs (2.5 mg/kg/72 h) after 35 days of Hyp. Finally, the RACHNP group received an IP injection of RACHNPs (2 mg/kg/72 hours) after 35 days of Hyp.

6. Hyperthermia induction

The animals underwent 30-minute heat shocks for 35 days. Before the daily induction of hyperthermia, each animal was anesthetized using IP injections of ketamine (100 mg/kg) and xylazine (5 mg/kg). Its scrotum and hind legs were then immersed in a water bath maintained at 43 °C [31].

7. Semen analysis

After a 70-day hyperthermia induction period, sperm samples were collected from the epididymal tail 24 hours after administration of NPs and RA. These samples were then transferred to 1 mL of Ham's F-10 medium (Product No. N6635; Sigma-Aldrich) and incubated for 20 minutes at 37 °C. Subsequently, 10 µL of the sample was placed on a slide, and sperm motility was examined under an inverted microscope. The sperm count was then determined by counting the chambers of the hemocytometer. For viability and morphology analyses, the sperm smear was placed on a slide, air-dried at room temperature, fixed in methyl alcohol, and stained with eosin-nigrosin.

8. Serum testosterone measurement

With the animals under general anesthesia, blood samples were collected from the heart. These samples were then centrifuged at 6,000 × *g* for 5 minutes at 4 °C before being stored at −80 °C for future use. We measured the serum levels of testosterone in the mice using a mouse-specific enzyme-linked immunosorbent assay kit (catalog no. CSB- E11162r).

9. Tissue preparation

Before the final phase of the experiment, the animals were given a lethal dose of xylazine hydrochloride and ketamine to induce anesthesia. Tissue samples were collected from the testes and preserved in Bouin solution for 48 hours. Next, the samples were fixed in a tissue processor and subsequently embedded in paraffin. Stereological methods were employed to cut serial sections (5 µm thick for volume estimation and 25 µm thick for number estimation) using a microtome (Leica RM2125 RTS; Leica Biosystems). Each sample consisted of 10 parts between 1 and 10 randomly selected using the systematic uniform random sampling method. Hematoxylin and eosin staining (Sigma) was utilized. Notably, morphological distinctions were used to identify the cells of the testis. Leydig cells, which are polyhedral and have a round nucleus and eosinophilic cytoplasm, can be found in the interstitial tissue between seminiferous tubules. Sertoli cells, which are rare and large, may be located toward the base of the epithelium. Dome-shaped spermatogonia can be found at the epithelial basement membrane, and their nuclei may be either dark or bright. Primary spermatocytes, the largest cells of the seminiferous epithelium, can be found in the germinal epithelium. Round spermatids are visible towards the lumen.

10. Total volumes of the testis

The total testicular volume (in mm^3) was determined using equation 2, and a projection microscope was developed at the Stereology Research Center according to the Cavalieri principle.

Equation 2. $V = \sum P \times a/p \times t$

https://doi.org/10.5653/cerm.2023.06149

∑P represents the total number of points counted. The quantity a/p denotes the probe point area divided by the magnification factor, while t indicates the tissue section separation (or thickness of sections) [32,33].

11. Length and density of seminiferous tubules

The length and density of the seminiferous tubules were estimated using the following formula:

Equation 3. $Lv = (2\sum Q)/(\sum p \times a/f)$

where ΣQ represents the total number of seminiferous tubules, ΣP denotes the total number of points superimposed on the testis tissue, and a/f indicates the area per frame. The total length of the seminiferous tubules was estimated by multiplying the length density (Lv) by the total volume of the testis [32,33].

12. Number of testicular cells

An unbiased counting frame and an optical dissecting technique were used to determine the total number of testicular cells. Each image of a testis segment was overlaid with a neutral counting frame. We mounted a microcator (Heidenhain) on the microscope stage to take z-axis measurements. The height of the dissector was defined as the thickness of the section, subtracting 20% from both the top and bottom. The distinct morphology of the testicular cells aided in their identification. Leydig cells, which can be found in the interstitial tissue, are polyhedral and possess eosinophilic cytoplasm. The Sertoli cells of the seminiferous tubule have a small, oval, pale nucleus located at their base. Spermatogonia are dome-shaped cells with either dark or light, spherical nuclei, situated at the base of the seminiferous epithelium. Primary spermatocytes, the largest cells of the seminiferous epithelium, are found in the center of the germinal epithelium. Spermatids have a rounded appearance on the luminal side. We used equation 4 to estimate the density of testicular cells (Nv).

Equation 4. $Nv = [\sum Q/(\sum P \times a/f \times h) \times t/BA]$

where h represents the height of the dissector, t denotes the actual section thickness measured with the micrometer (here, approximately 20 µm), BA indicates the tissue section thickness as determined by cutting the sections at 25 µm, ΣQ denotes the number of counted cells, a/f indicates the area per frame, and ΣP represents the number of counting frames for all the fields. The total testicular cell count was calculated using equation 5:

Equation 5. $N\_((total)) = Nv \times V\_((final))$

where Nv represents the testicular cell density and V denotes the testicular volume.

13. Measurement of reactive oxygen species

We utilized a modified fluorometric technique to measure reactive oxygen species (ROS) in the testis, with 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) serving as both a ROS and a cellular marker of oxidative stress. Specifically, 50 mg of testis tissue was homogenized in 600 µL of 0.1 M PBS (pH 7.4) and pre-incubated with DCFH-DA (20 µM) at 37 °C. This process allowed the probe to integrate into membrane-bound vesicles and facilitated the cleavage of the diacetate group by esterases. The conversion of DCFH into the fluorescent product 2',7'-dichlorofluorescein (DCF) was recorded using a DTX 880 multimodal detector fluorescent spectrophotometer (Beckman Coulter). This was done with excitation at 485 nm and emission at 535 nm after a 30-minute incubation period. Protein concentrations were measured following sample collection from each well, and data were reported as the percentage of the maximum fluorescence.

14. Immunohistochemical staining

The testicular tissues were first fixed, then embedded in paraffin wax. These tissues were sectioned using a microtome to a thickness of 5 µm. The tissue slices were subsequently deparaffinized and rinsed with xylene to remove the paraffin. They were then dehydrated through a series of graded ethanol baths to displace the water, effectively rehydrating them in an alcohol gradient. Blocks of 10% hydrogen peroxide were placed over the sections and incubated for 10 minutes in darkness. The sections were then incubated with the primary antibody against caspase-3 (catalog number CPP324-1-18, dilution 1:100) for 50 minutes at room temperature. The slides were further incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G for 40 minutes at room temperature. After this, diaminobenzidine tetrahydrochloride chromogen was added for 10 minutes, and the slides were washed three times in buffer. The final step involved counterstaining with hematoxylin for nuclear staining.

15. Quantitative real-time PCR findings

In this experiment, total RNA was isolated from mouse testicles using a high-purity RNA isolation kit (Invitrogen). After collection, the RNA samples were treated with DNase I enzyme. Next, the total purity of the extracted RNA was determined using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific), measuring absorbance at 260 and 280 nm. Complementary DNA (cDNA) was synthesized with a cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. To quantify relative gene expression, quantitative re-

al-time polymerase chain reaction (qRT-PCR; TaqMan) was utilized with the QuantiTect SYBR green master mix no. RUX RT-PCR kit (21H0601).

Primers for *bax*, *bcl2*, *p53*, *FAS*, and *FasL* were designed using Oligo 6 software (Molecular Biology Insights Inc.) and are shown in Table 1. Subsequently, the efficacy of these PCR primers was evaluated using the Primer-Blast tool from the National Center for Biotechnology Information, which can be accessed at www.ncbi.nlm.nih.gov/tools/primer-blast (Table 1).

16. Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc.). The data are displayed as the mean ± standard error (SE). To analyze the data, we used one-way analysis of variance and the Tukey *post hoc* test for multiple comparisons. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

1. Synthesis and characterization of CHNPs and RACHNPs: size and zeta potential

CHNPs were produced using the ionic gelation method. The formation of CHNPs occurred through electrostatic interaction between the positively charged amine groups in chitosan and the negatively charged sodium TPP. Table 2 presents the dynamic light scattering results indicating the sizes of the purified CHNPs and RACHNPs. The mean size of the CHNPs was 228.20 ± 22.78 nm, while the RACHNPs had a mean size of 324 ± 29.9 nm. Consequently, the NPs synthesized in this study can be considered suitable as a nanocarrier for RA delivery. The zeta potentials of the CHNPs and RACHNPs were found to be 32.65 and 10.8 MV, respectively.

2. Fourier-transform infrared spectroscopy

To determine the chemical structures of RA, CHNPs, and RACHNPs, we generated FTIR spectra, as depicted in Figure 1A. The FTIR absorption spectrum of chitosan revealed the peaks of the amine and carbonyl groups of the O=C-NHR of chitosan at 15,780 and 16,380 cm^{-1}, respectively. Furthermore, the FTIR peak ranging from 3,500 to 3,300 cm^{-1} was attributed to the O-H group of chitosan. The FTIR spectrum for RA indicated the presence of absorption bands of the carbonyl group at 1,685 cm^{-1} and aliphatic alkanes at approximately 2,900 cm^{-1}. In the RACHNP spectra, an additional absorption band appearing at 1,704 cm^{-1} demonstrates the conjugation between RA and CHNPs. Lastly, the presence of other reference bands of RA and CHNPs indicates the formation of an RACHNP copolymer, resulting from the interaction between the RA carbonyl group and the chitosan amino group.

3. Loading capacity of CHNPs

First, the standard curve of ultraviolet absorbance was calculated for RA. Then, the equation relating the optical density (OD) and the concentration (C) of RA was determined, as displayed below:

$$OD = 0.0106C + 0.0403 \ (R^2 = 0.9888)$$

Based on the equation, the LC% of RA for RACHNPs was 50.97% (Figure 1B).

Table 2. Size and zeta potential of CHNP and RACHNP

Sample	Size ± SD (nm)	Zeta potential (mV)
CHNP	228 ± 22.78	32.6
RACHNP	324 ± 29.9	10.8

CHNP, chitosan nanoparticle; RACHNP, retinoic acid loaded with chitosan nanoparticle; SD, standard deviation.

Table 1. Primers used

Genes	Primer sequences	Product length (nt)	TM (° C)
Bax	F: GGATGATTGCTGACGTGGAC R: CCCCAGTTGAAGTTGCCATC	91	58
Bcl2	F: TCGCAGAGATGTCCAGTCAG R: CACCCCATCCCTGAAGAGTT	92	58
P53	F: ACCCTGGCACCTACAATGAA R: TGGAAGGAAAGTAGGCCCTG	102	58
Fas	F: ACCTCAGTTCCAGCCATGAA R: TGCTGGCAAAGAGAACACAC	111	60
FasL	F: GAACCGCTCTGATCTCTGGA R: GCTGGTTGTTGCAAGACTGA	117	60

nt, nucleotide; TM, melting temperature; F, forward; R, reverse.

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4. *In vitro* release of RA from CHNPs

Figure 1C illustrates the cumulative release of RA from RACHNPs over a span of 120 hours. The RA release rate was 59.3% within this period, with approximately half of this release taking place in the initial 60 hours. This is attributed to the quantity of RA absorbed on the CHNP surface. The release rate then proceeded more slowly over the subsequent hours, which is associated with the amount of RA embedded in the CHNP matrix.

5. Effects of RA, CHNPs, and RACHNPs on sperm parameters

The total number of spermatozoa was significantly lower in the Hyp group than in the RA, CHNP, and RACHNP groups ($p < 0.001$ for all). The total numbers of spermatozoa in the RA, CHNP, and Hyp groups were significantly lower than in the control group ($p < 0.001$), but the sperm count in the RACHNP group did not significantly differ from that in the control group. A significant difference was observed between the RACHNP and both the CHNP and RA groups. The data also showed a higher rate of improvement in the group treated with RACHNPs compared to the RA and CHNP groups (Figure 2A). Relative to the control group, sperm viability was significantly lower in the Hyp group ($p < 0.001$), but no significant difference was observed between the Hyp and CHNP groups. The proportion of viable sperm in the RA and RACHNP groups was statistically higher than in the Hyp group ($p < 0.001$ for both). Sperm viability in the RA group was significantly lower than in the control group ($p < 0.001$), but no significant difference was found between the RACHNP and control groups (Figure 2B). The study results showed a statistically significant decrease in sperm motility in the Hyp group compared to the control group ($p < 0.001$). Statistically significant differences were also observed in sperm motility between the CHNP, RA, and RACHNP groups and the Hyp group ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). The groups treated with CHNPs, RA, and RACHNPs showed greater increases in sperm motility relative to the Hyp group, but no significant difference was noted between the RACHNP and control groups (Figure 2C). Sperm morphology in the Hyp group was significantly poorer than in the RA, RACHNP, and control groups ($p < 0.001$). A significant difference in sperm morphology was observed between the RACHNP and CHNP groups, but no significant difference was found between the RACHNP, RA, and control groups (Figure 2D).

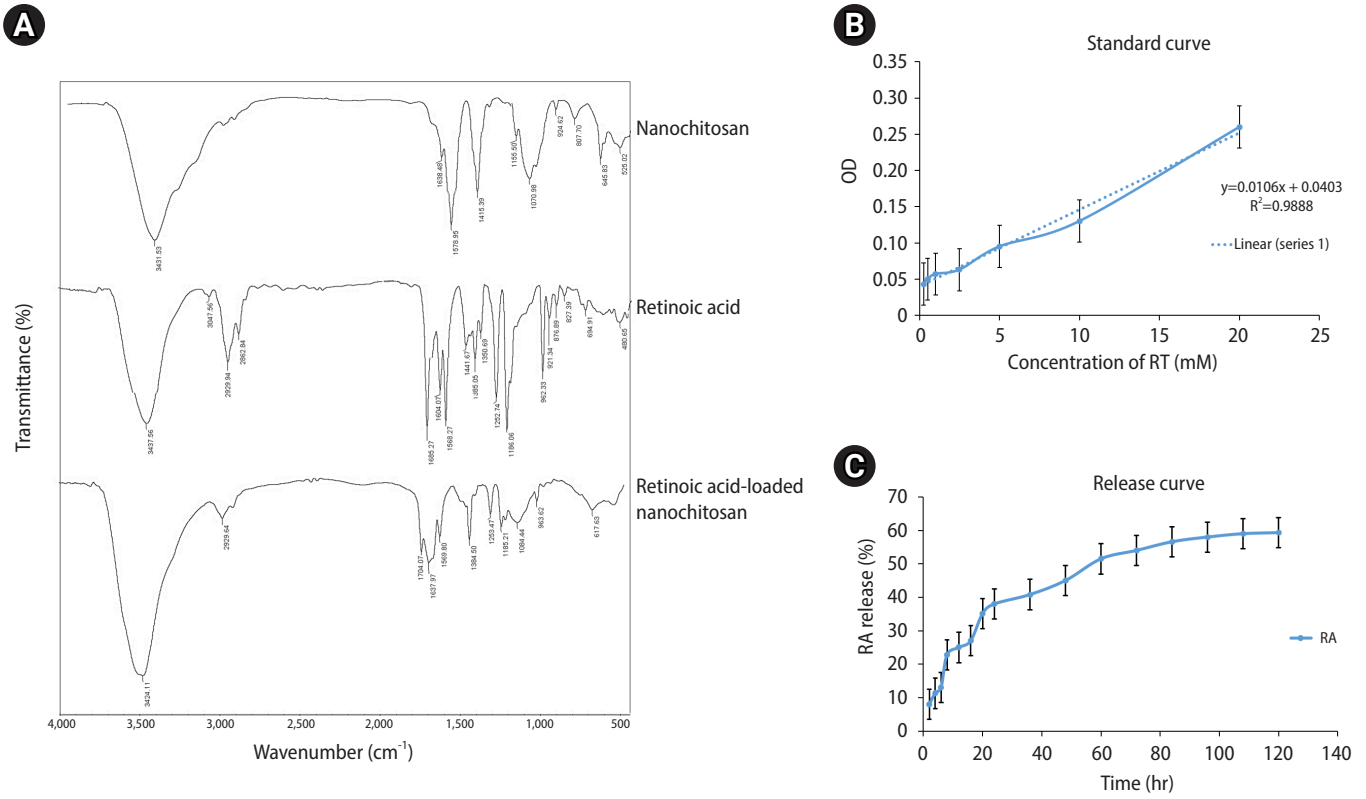

Figure 1. (A) Fourier-transform infrared spectroscopy analysis of chitosan nanoparticles, retinoic acid, and retinoic acid loaded chitosan nanoparticles. (B) Standard curve of retinoic acid ultraviolet absorbance. (C) *In vitro* release profile of retinoic acid from chitosan nanoparticles. OD, optical density; RA, retinoic acid.

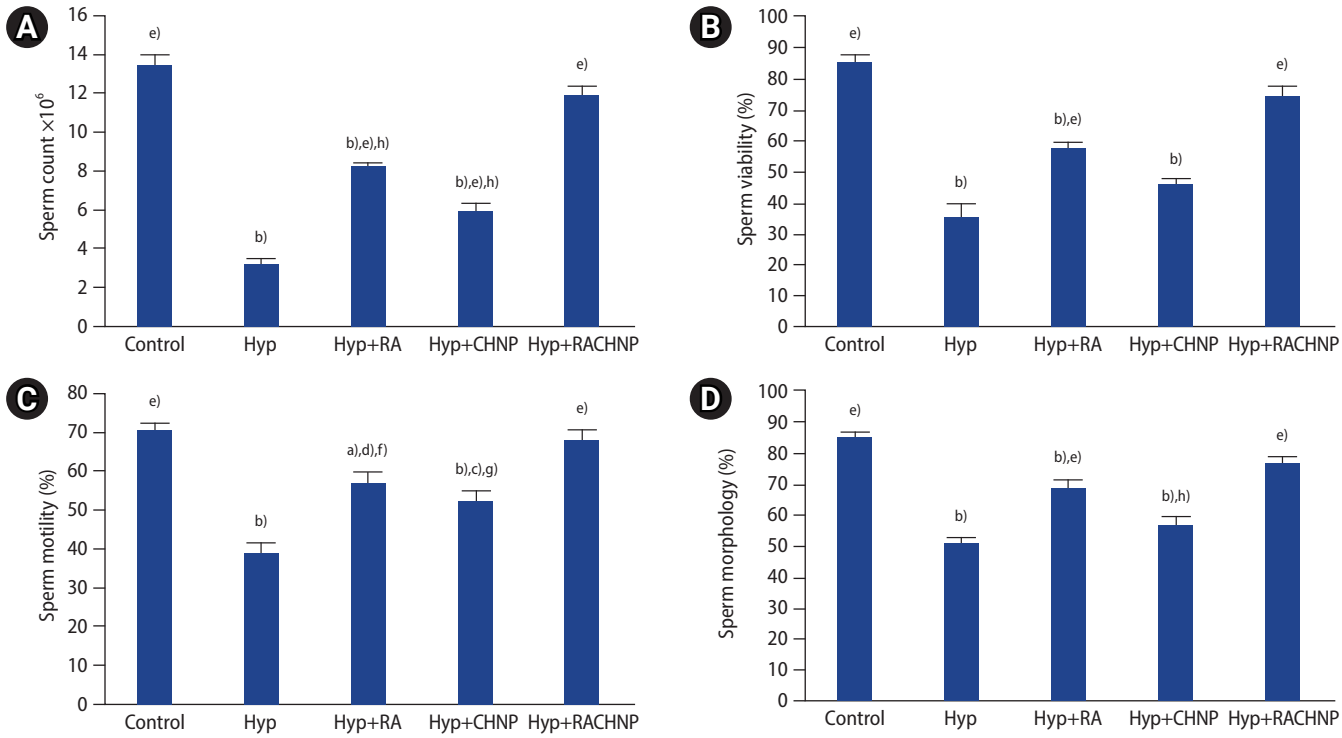

Figure 2. Effects of retinoic acid (RA), chitosan nanoparticle (CHNP), and retinoic acid loaded with chitosan nanoparticle (RACHNP) administration on sperm parameters. (A) Sperm count. (B) Sperm morphology. (C) Sperm motility. (D) Sperm viability. Data are presented as mean±standard error. Hyp, scrotal hyperthermia. [a]$p<0.01$ and [b]$p<0.001$ compared to the control group; [c]$p<0.05$, [d]$p<0.01$, and [e]$p<0.001$ compared to the Hyp group; [f]$p<0.05$, [g]$p<0.01$, and [h]$p<0.001$ compared to the RACHNP group.

6. Effects of RA, CHNPs, and RACHNPs on serum testosterone levels

The hormone assay revealed a decrease in serum testosterone concentration in animals subjected to Hyp, compared to both the control and RACHNP groups ($p < 0.001$, $p < 0.05$). Our findings indicated no significant difference in serum testosterone levels between the RA and CHNP groups and the Hyp group. However, a significant difference was observed between the RACHNP group and the control group ($p < 0.05$). Additionally, significant differences were found when comparing the RACHNP group to both the CHNP and RA groups ($p < 0.01$ and $p < 0.05$, respectively) (Figure 3A).

7. Effects of RA, CHNPs, and RACHNPs on ROS measurement

The data revealed significant increases in ROS levels in the Hyp group when compared to the RACHNPs, RA, and control groups ($p < 0.001$, $p < 0.001$, $p < 0.001$) based on testis tissue measurements. Furthermore, the results indicated that ROS levels were notably lower in the RACHNP and RA groups than in the Hyp group ($p < 0.001$). However, no significant difference was present between the Hyp and CHNP groups. The ROS measurements revealed a statistically significant difference between the RA and control groups ($p < 0.05$), but no such difference was found between the RACHNP and control groups. Additionally, our results showed a significant difference between the RACHNP therapy group and the CHNP group, but no significant difference was observed between the RACHNP and RA groups (Figure 3B).

8. Effects of RA, CHNPs, and RACHNPs on stereological parameters

Stereological test results revealed a significantly reduced testicular volume in the Hyp group compared to the control group ($p < 0.001$). While the RACHNP treatment group showed a statistically significant increase in testicular volume compared to the Hyp group ($p < 0.001$), no difference was observed between the Hyp group and the RA or CHNP treatment groups. Similarly, no significant change in testicular volume was detected between the RACHNP and control groups (Figures 4 and 5A).

The findings revealed a significantly lower count of spermatogonia in the Hyp group compared to both the control and RACHNP groups ($p < 0.001$ for both). No statistically significant difference was found in the number of spermatogonia between either the RA or CHNP groups and the Hyp group. Although RACHNP treatment significantly increased the count of spermatogonia, it did not reach the level observed in the control group (Figure 5B).

Stereological data revealed a significant decrease in the number of

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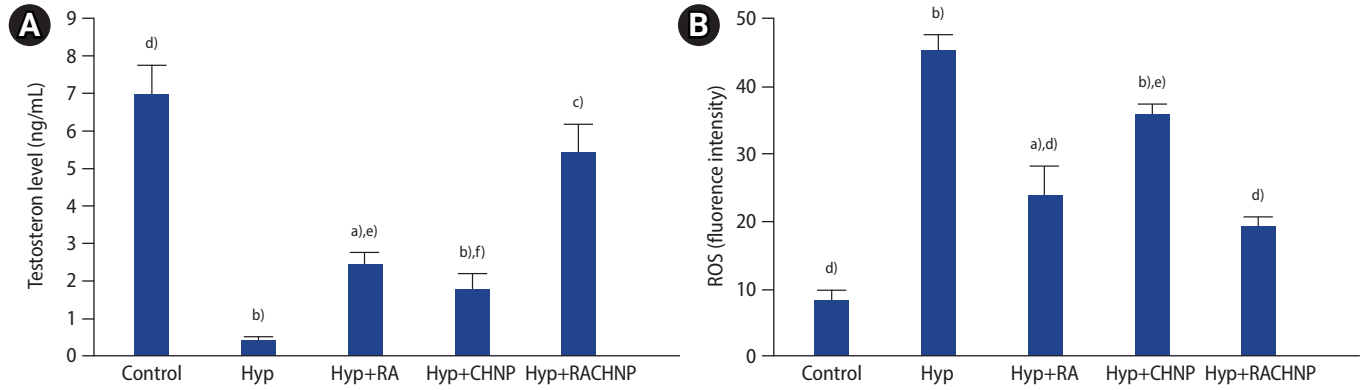

Figure 3. Effects of retinoic acid (RA), chitosan nanoparticle (CHNP), and retinoic acid loaded with chitosan nanoparticle (RACHNP) administration on: (A) testosterone hormone levels across groups. (B) Reactive oxygen species (ROS) level across groups (n=6). Data are presented as mean±standard error. Hyp, scrotal hyperthermia. [a)]$p<0.01$ and [b)]$p<0.001$ compared to the control group; [c)]$p<0.01$ and [d)]$p<0.001$ compared to the Hyp group; [e)]$p<0.05$ and [f)]$p<0.01$ compared to the RACHNP group.

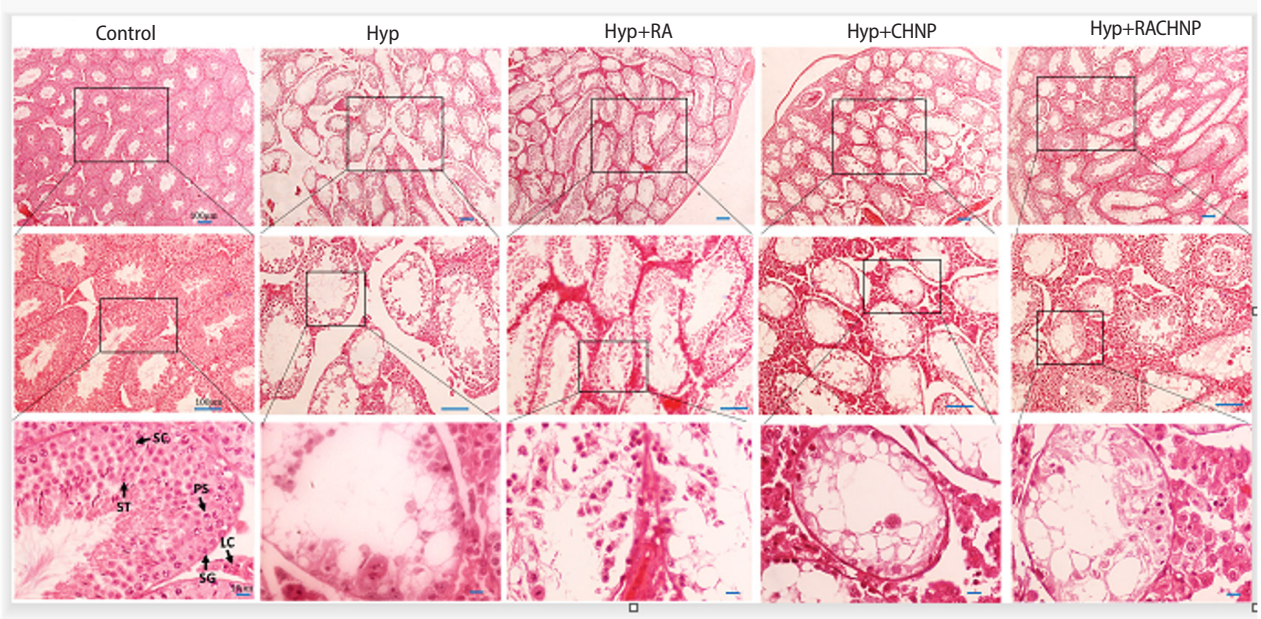

Figure 4. Photomicrograph of the testis stained with hematoxylin and eosin (×4, ×10, and ×40) in the study groups. Histological findings showed normal spermatogenesis in the control group. Testicular tissue showed impaired spermatogenesis with degeneration of seminiferous tubules in the scrotal hyperthermia (Hyp) group and improvement in the retinoic acid loaded with chitosan nanoparticle (RACHNP) group. RA, retinoic acid; CHNP, chitosan nanoparticle; SC, Sertoli cell; ST, spermatid; PS, primary spermatocyte; LC, Leydig cell; SG, spermatogonia.

primary spermatocytes in the Hyp group compared to the control and RACHNP groups ($p < 0.001$). However, the number of primary spermatocytes in the Hyp group did not significantly differ from those in the RA and CHNP groups. Interestingly, the RACHNP group exhibited a notable increase in the number of primary spermatocytes relative to the RA and CHNP groups ($p < 0.001$ for both). These results suggest that RACHNPs significantly mitigated the detrimental effects of heat stress (Figure 5C). The data also indicated a significant decrease in the number of round spermatids in the testis tissue of the Hyp group compared to the control and RACHNP groups ($p < 0.001$ for both). While the RACHNP group showed a notable increase in the number of round spermatids, this increase was not statistically significant relative to the control group (Figure 5D).

The findings indicate a significant decrease in the number of Leydig cells in the Hyp group when compared to both the RACHNP ($p < 0.001$) and control ($p < 0.001$) groups. Conversely, the RACHNP

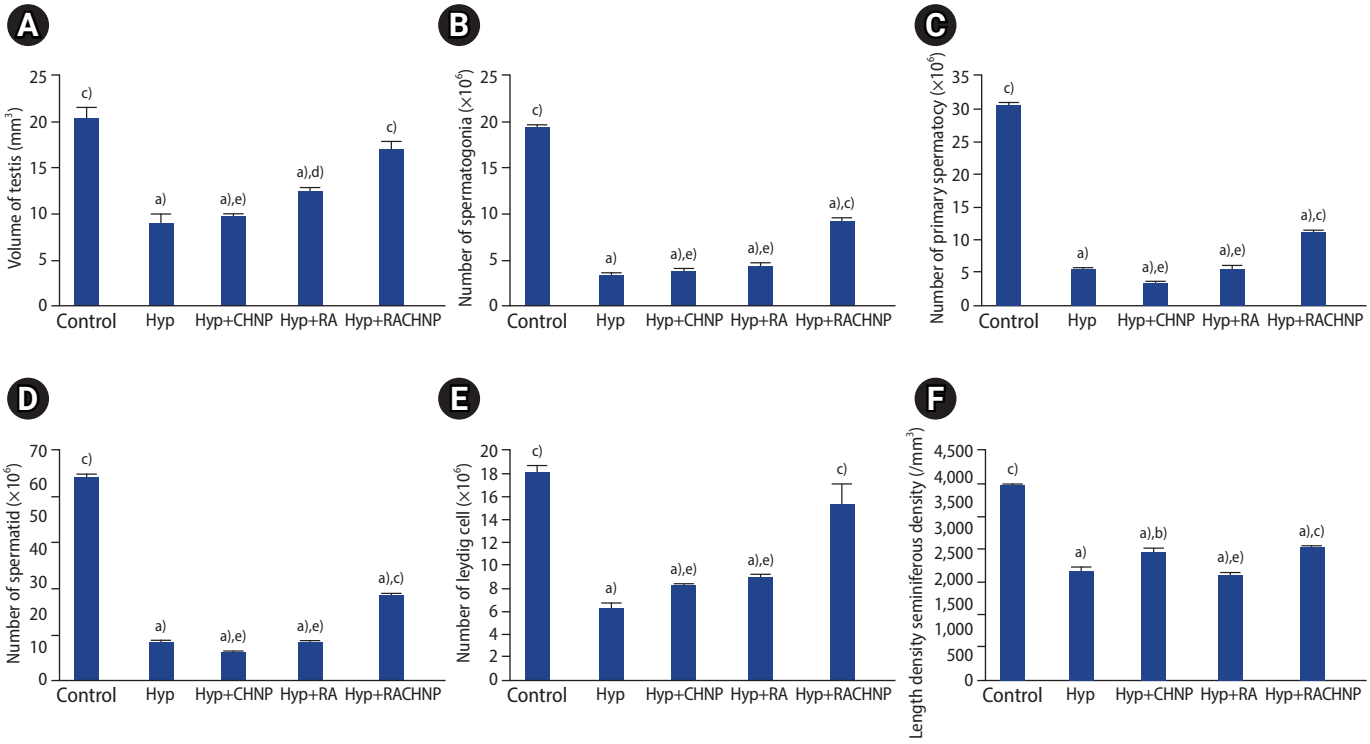

Figure 5. Effects of retinoic acid (RA), chitosan nanoparticle (CHNP), and retinoic acid loaded with chitosan nanoparticle (RACHNP) administration on stereological parameters: (A) testis volume, (B) number of spermatogonia, (C) number of primary spermatocytes, (D) number of spermatids, (E) number of Leydig cells, and (F) seminiferous tubule length in scrotal hyperthermia (Hyp)-induced mice (n=6). Data are presented as mean±standard error. [a]$p<0.0001$ compared to the control group; [b]$p<0.01$ and [c]$p<0.001$ compared to the Hyp group; [d]$p<0.01$ and [e]$p<0.001$ compared to the RACHNP group.

treatment group demonstrated an increase in the number of Leydig cells, with no significant difference observed between the RACHNP and control groups (Figure 5E).

The length of the seminiferous tubules was significantly shorter in the Hyp group compared to the control, RACHNP, and RA groups ($p<0.001$, $p<0.001$, and $p<0.01$, respectively). Relative to the Hyp group, an increase was observed in the length of the seminiferous tubules after the mice were administered RA and RACHNPs (Figure 5F). The statistical data indicated that the total count of Sertoli cells remained consistent across all study groups. The Sertoli cells demonstrated resilience to the rising temperature within the testis.

9. Effects of RA, CHNPs, and RACHNPs on immunohistochemical staining

The percentage of apoptotic factor immunoreactivity in testicular cells was assessed using immunohistochemical (IHC) staining. Figure 6 illustrates the caspase-3 immunoreactivity in testicular tissues after the induction of Hyp. A significant increase in caspase-3 immunoreactivity was observed in the Hyp group compared to the control ($p<0.001$), RA ($p<0.01$), RACHNP ($p<0.001$), and CHNP ($p<0.01$) groups. Additionally, a significant increase in apoptosis was noted in the RA and CHNP groups compared to the control group ($p<0.01$ for both). However, no significant difference was found between the RACHNP and control groups. In this study, a significant difference was noted between the RACHNP group and both RA and CHNP groups ($p<0.05$ for both) (Figure 6).

10. Effects of RA, CHNPs, and RACHNPs on qRT-PCR findings

The relative messenger RNA (mRNA) expression levels of *Bax*, *bcl2*, *p53*, *Fas*, and *FasL* were normalized to the control and quantified across different groups. As indicated in Table 3, the Hyp group exhibited a significant increase in the relative mRNA expression levels of *p53*, *Fas*, and *FasL* compared to the control group ($p<0.001$, $p<0.05$, and $p<0.01$, respectively) (Table 3). The statistical analysis revealed that the expression level of *Bax* was elevated in the Hyp group compared to both the RA and RACHNP groups ($p<0.05$ for both). The expression of *p53* was also higher in the Hyp group compared to the control, RA, CHNP, and RACHNP groups ($p<0.001$ for all). Similarly, *Fas* expression was increased in the Hyp group compared to the control, CHNP, RA, and RACHNP groups ($p<0.05$, $p<0.05$, $p<0.01$, and $p<0.01$, respectively). *FasL* expression was also elevated in the Hyp group compared to the control, CHNP, RA,

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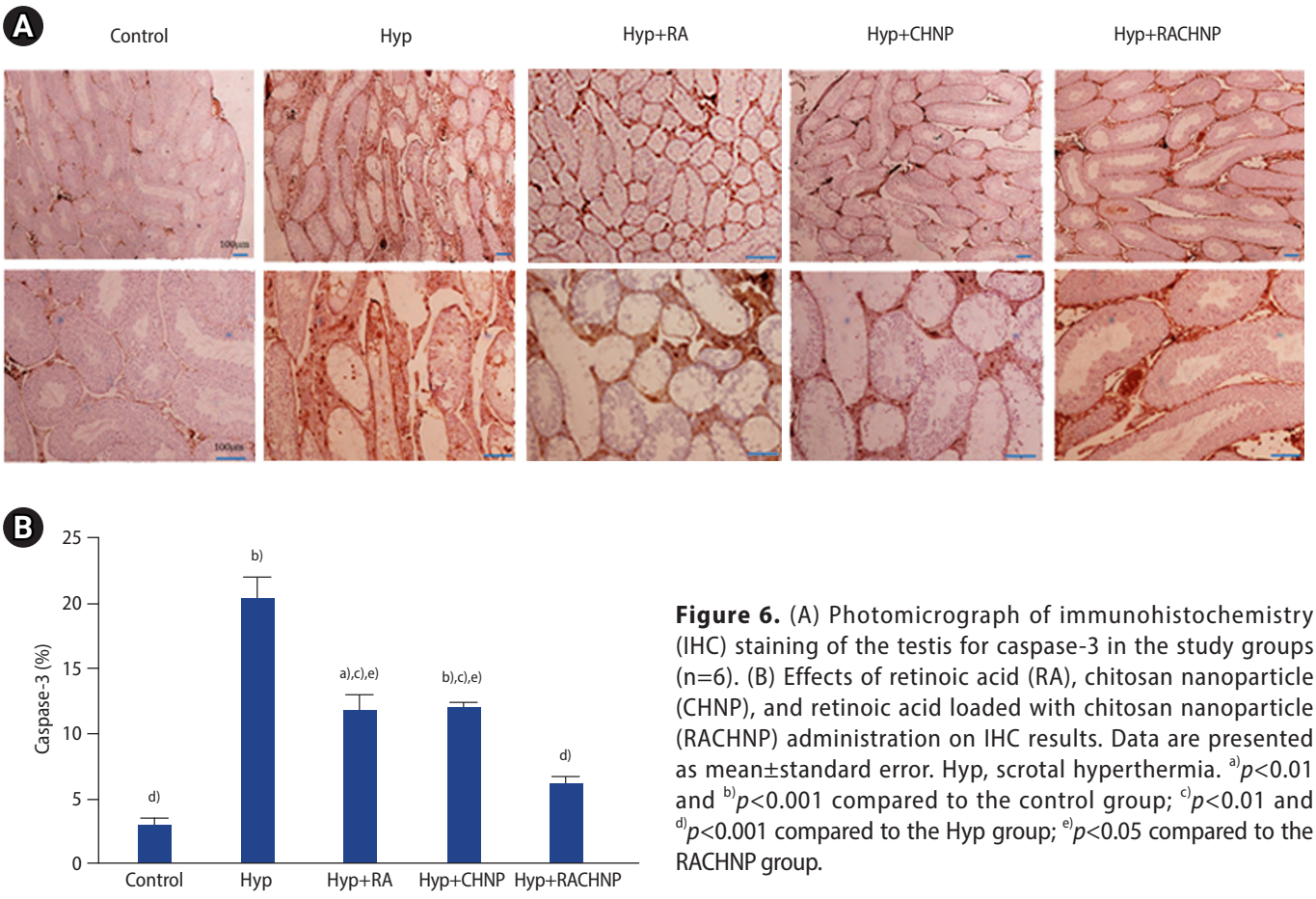

Figure 6. (A) Photomicrograph of immunohistochemistry (IHC) staining of the testis for caspase-3 in the study groups (n=6). (B) Effects of retinoic acid (RA), chitosan nanoparticle (CHNP), and retinoic acid loaded with chitosan nanoparticle (RACHNP) administration on IHC results. Data are presented as mean±standard error. Hyp, scrotal hyperthermia. [a)]$p<0.01$ and [b)]$p<0.001$ compared to the control group; [c)]$p<0.01$ and [d)]$p<0.001$ compared to the Hyp group; [e)]$p<0.05$ compared to the RACHNP group.

Table 3. mRNA expression levels of *Bax, p53, Fas, FasL*, and *Bcl2* in the testis across groups

Group	*P53*	*Bax*	*Fas*	*FasL*	*Bcl2*
Control	1±0.00[f)]	1±0.00	1±0.00[d)]	1±0.00[e)]	1±.00[f)]
Hyp	7.53±0.305[c)]	3.44±0.80	4.15±0.54[a)]	4.47±0.54[b),f)]	0.005±0.00015[c)]
CHNP	0.18±0.12[f)]	1.25±0.76	1.03±0.71[d)]	0.34±0.17[f)]	0.015±0.003[c)]
RA	0.015±0.001[a),f)]	0.25±0.05[d)]	0.17±0.009[e)]	0.098±0.022[f)]	0.105±0.050[c)]
RACHNP	0.012±0.005[a),f)]	0.012±0.007[d)]	0.05±0.04[e)]	0.040±0.0048[f)]	0.118±0.023[c)]

Values are presented as mean±standard error.
mRNA, messenger RNA; Hyp, scrotal hyperthermia; CHNP, chitosan nanoparticle; RA, retinoic acid; RACHNP, retinoic acid loaded with chitosan nanoparticle.
[a)]$p<0.05$, [b)]$p<0.01$, and [c)]$p<0.001$ in comparison to the control group; [d)]$p<0.05$, [e)]p<0.01, and [f)]$p<0.001$ compared to the Hyp group.

and RACHNP groups ($p < 0.01$, $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). In contrast, the mRNA expression level of *bcl2* was reduced in the Hyp group compared to the control group ($p < 0.001$). A significant difference was found in the expression level of *bcl2* between the control group and all other groups ($p < 0.001$) (Table 3).

Discussion

In line with previous studies on the effects of heat stress on spermatogenesis [11,34], our research found that Hyp leads to reproductive toxicity and azoospermia in animals. The sperm parameter analysis showed significant improvements in sperm count, motility, viability, morphology, and serum testosterone levels, along with a noticeable decrease in ROS production levels in animals treated with RACHNPs. Additionally, substantial improvements were observed in testis volume, seminiferous tubule length, and testicular cell count in the group that received RACHNPs. A reduction in the caspase-3 protein expression level was also noted in animals treated with RACHNPs. This study presents the first evidence of the increased effectiveness of RACHNPs in alleviating testicular damage caused by

hyperthermia in mice.

CHNP formulations appear to be an ideal slow release mechanism for drugs with unfavorable kinetics, such as RA. Formulations with a high RA load often display a delayed drug release profile. Techniques for delivering RA help to overcome challenges such as poor solubility in water, a short half-life of only a few hours, and adverse side effects like dryness, headaches, and hypertriglyceridemia when administered in high doses [35,36]. Studies have shown that considerable plasma levels of RACHNPs persist for a longer duration following IP delivery compared to administration without RA. RA is a free radical scavenger with antioxidant properties. Both laboratory and animal studies have demonstrated that RACHNPs are more effective than RA-free formulations in enhancing the antioxidant effects of RA [37].

The histological analysis revealed that Hyp led to a decrease in the count of spermatogonia, primary spermatocytes, and round spermatids. It also caused atrophic changes in the seminiferous tubules, including the degeneration, reduction, and disorganization of germ cells. Additionally, Leydig cells in the interstitial tissues underwent degeneration, developed small to large vacuoles, and displayed irregular gaps; additionally, a significant decrease in testicular volume was observed [3,11,38]. However, the injection of RACHNPs restored the spermatogenic processes, testis weight, testicular histoarchitecture, and pathophysiological parameters in the testes affected by Hyp. Neither CHNPs nor the absence of RA yielded positive results in the testicular cells of the heat-stressed group, suggesting that RACHNPs could rectify the spermatogenesis abnormalities induced by Hyp. The present study showed that RACHNPs significantly enhanced the proliferation of germ cells, resulting in increases in the number of spermatogonia, primary spermatocytes, and round spermatids. Consistent with previous studies, long-term Hyp inflicted severe damage on the testis tissue. The antioxidative properties of RACHNPs and their protective effects on mitochondria may be the key factors in counteracting the effects of heat stress and restoring spermatogenesis following RACHNP consumption.

Heat stress, or hyperthermia, can negatively impact the function of Leydig cells and the production of testosterone. This aligns with previous research that has shown a dramatic decrease in serum testosterone levels due to heat stress [39,40]. However, treatment with RACHNPs can counteract the reproductive damage caused by heat exposure in male mice. This treatment regulates Leydig cell steroidogenesis, leading to an increase in testosterone levels. The observed increase in testosterone levels in mice treated with RACHNPs underscores its potential to mitigate the reproductive toxicity caused by heat stress. Consequently, RACHNPs enhance spermatogenesis and reduce testicular dysfunction arising from heat exposure.

IHC analysis was performed to detect caspase-3 and illustrate apoptosis in the seminiferous tubules. Hyp heightened the immunoreactivity of sperm cells to caspase-3, suggesting that oxidative stress induced by hyperthermia could result in cell death. However, the application of RA, CHNPs, and RACHNPs mitigated the apoptotic damage instigated by hyperthermia. Prior research has indicated that CHNP may aid in alleviating testicular damage by diminishing antiapoptotic activity [41]. Meanwhile, RA, functioning as an antioxidant, can neutralize free radicals and impede apoptosis to safeguard spermatogenic cells [42,43]. In the current study, mice treated with RACHNPs did not exhibit significant differences from the control group, while mice treated with RA and CHNPs demonstrated notable differences compared to the control group.

Interestingly, treatment with RACHNPs led to a decrease in the localization of the caspase-3 immunoreactive protein, which suggests a reduction in apoptotic cells. This observation implies that RACHNPs have a strong antioxidant activity and are more effective at scavenging free radicals than RA alone. Furthermore, RACHNPs may employ a unique method of cellular internalization, triggering a more potent antiapoptotic response than that elicited by RA alone. The diminished efficacy of RA, when compared to RACHNPs, could be attributed to its short half-life and swift conversion to an inactive metabolite. In contrast, RACHNPs exhibit a longer lifespan and increased effectiveness.

Previous research has demonstrated that elevated temperatures significantly increase the production of caspase-3 mRNA and the amount of cleaved caspase-3 protein [44]. However, the application of RA therapy has been found to significantly reduce the increase in cleaved caspase-3 protein levels and caspase-3 mRNA expression caused by heat stress [45]. These results imply that RACHNPs could potentially inhibit the caspase-3–mediated apoptosis pathway, thereby reducing cell death. This conclusion aligns with our own research findings [46]. Caspase-3, a common executor of cell death, is crucial for the induction of apoptosis by heat stress.

Research has established a strong link between oxidative stress caused by ROS and both death receptor-mediated and mitochondrial-mediated apoptosis. Numerous studies suggest that ROS contributes to heat-induced apoptosis by encouraging ROS accumulation. Our research suggests that heat significantly increases the buildup of ROS in testicular cells. However, treatment with RA and RACHNPs notably decreases the increase in ROS^+ cell count and ROS concentration triggered by thermal stress. We observed that mice treated with RACHNPs showed no significant differences from the control group, while the RA and CHNP groups displayed considerable differences compared to the control group. This could be due to the antioxidant properties of RA and CHNPs.

Rao et al. [47] found that RA enhanced the activity of superoxide dismutase (SOD) and decreased the production of malondialdehyde (MDA) in rats, thereby mitigating the effects of hepatic ischemia/reperfusion injury. Similarly, Khafaga and El-Sayed [45] discovered

https://doi.org/10.5653/cerm.2023.06149

that all-*trans*-retinoic acid (ATRA) lessened the amount of MDA and alleviated doxorubicin-induced cardiac oxidative damage in rats by promoting the activities of antioxidant enzymes such as glutathione peroxidase, SOD, and catalase (CAT). A previous study revealed that RA treatment significantly elevated the activities of SOD, CAT, and total antioxidant capacity, while also reducing MDA levels. That study also examined the cellular expression of genes associated with antioxidants, finding that RA diminished oxidative stress related to cell damage by improving antioxidant capacity [48]. RACHNPs, known for their antioxidant properties, have the potential to actively inhibit apoptosis in spermatogenic cells and perform free radical scavenging functions.

Furthermore, several apoptotic signaling pathways, including the mitogen-activated protein kinase (MAPK) and p53 pathways, have been associated with ROS. To determine whether the P38MAPK signaling pathway plays a role in the mechanism by which ATRA reduces cell apoptosis, researchers used Western blot analysis to assess the level of P38MAPK phosphorylation. Prior research has shown that damaged cells display a significant increase in P38MAPK phosphorylation. The findings of this study suggest that ATRA effectively mitigates cellular damage caused by oxidative stress and ROS by inhibiting the P38MAPK signaling pathway. However, further research is needed to definitively confirm whether ATRA inhibits the ROS-mediated P38MAPK signaling pathway, thereby reducing cellular damage and apoptosis [47]. Antioxidant supplements, such as RA and RACHNPs, could potentially shield against the detrimental effects of high ROS production caused by testicular hyperthermia. The primary ROS species, including superoxide anion, hydroxyl radical, and hydrogen peroxide, may be absorbed by RACHNPs via their amino group chelation sites. The positive charges present in the RACHNP complex may allow it to neutralize negatively charged radicals, such as superoxide and hydroxyl.

We examined the relative mRNA expression levels of the *bax*, *p53*, *Fas*, and *FasL* genes in testicular tissue due to the extensive cell death observed at 43 °C. Our findings revealed that mice subjected to high temperatures exhibited increased transcription levels of these genes compared to the control group. Furthermore, we found that animals subjected to Hyp demonstrated reduced relative levels of *bcl2* mRNA expression. Following heat treatment, we observed evidence of the upregulation of the *p53*, *Fas*, and *FasL* proteins, along with the downregulation of *bcl2*.

We observed that mice treated with RA and RACHNPs exhibited reduced apoptosis when exposed to heat. Our findings indicated that the expression levels of apoptotic genes in the RA and RACHNP treatment groups were lower than those in the control group. This could be attributed to the increased expression of the *hsp90* and *hsp70* genes during hyperthermia. These genes possess antiapoptotic properties, which are further enhanced by the antiapoptotic effects of RA and RACHNP [49,50].

In conclusion, the results of this study indicate that the RACHNP formulation is biocompatible and shows superior effectiveness in reducing Hyp in male mice, compared to free RA. The protective impact of this formulation on spermatogenesis underscores the potential of CHNPs as an effective nanocarrier for RA delivery. The increased therapeutic effectiveness of RACHNPs could be due to their enhanced absorption and sustained-release properties, as previously discussed. Therefore, the RACHNP formulation could serve as a promising alternative to traditional RA formulations and CHNP-based treatments for male infertility. These findings are likely to stimulate further research into the protective role of RACHNPs in male reproductive health.

Conflict of interest

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

Acknowledgments

This article is derived from a thesis authored by Fatemeh Mazini PhD, a student of anatomical sciences at the School of Medicine, Kermanshah University of Medical Science, Kermanshah, Iran (Registration no. 4010057).

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ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06436
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):244-252

CERM

Enhancement of preimplantation mouse embryo development with optimized *in vitro* culture dish via stabilization of medium osmolarity

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Objective: We evaluated the efficacy of the newly developed optimized *in vitro* culture (OIVC) dish for cultivating preimplantation mouse embryos. This dish minimizes the need for mineral oil and incorporates microwells, providing a stable culture environment and enabling independent monitoring of individual embryos.
Methods: Mouse pronuclear (PN) zygotes and two-cell-stage embryos were collected at 18 and 46 hours after human chorionic gonadotropin injection, respectively. These were cultured for 120 hours using potassium simplex optimized medium (KSOM) to reach the blastocyst stage. The embryos were randomly allocated into three groups, each cultured in one of three dishes: a 60-mm culture dish, a microdrop dish, and an OIVC dish that we developed.
Results: The OIVC dish effectively maintained the osmolarity of the KSOM culture medium over a 5-day period using only 2 mL of mineral oil. This contrasts with the significant osmolarity increase observed in the 60-mm culture dish. Additionally, the OIVC dish exhibited higher blastulation rates from two-cell embryos (100%) relative to the other dish types. Moreover, blastocysts derived from both PN zygotes and two-cell embryos in the OIVC dish group demonstrated significantly elevated mean cell numbers.
Conclusion: Use of the OIVC dish markedly increased the number of cells in blastocysts derived from the *in vitro* culture of preimplantation mouse embryos. The capacity of this dish to maintain medium osmolarity with minimal mineral oil usage represents a breakthrough that may advance embryo culture techniques for various mammals, including human *in vitro* fertilization and embryo transfer programs.

Keywords: Blastulation; Cell count; In vitro culture; Mineral oil; Optimized in vitro culture dish; Osmolarity

Introduction

Human assisted reproductive technology (ART) has progressed to include advanced methods, including *in vitro* fertilization (IVF) and *in vitro* culture techniques for mammalian embryos. *in vitro* embryo development requires the consideration of numerous factors that differ substantially from those *in vivo* [1]. The success of these cultures, particularly when applied to sensitive preimplantation embryos, largely depends on the stability of the culture environment. This includes factors such as osmolarity, temperature, and gas concentrations [1]. Traditional oil-drop culture methods employ mineral oil overlays to prevent the evaporation and maintain the osmolarity of the culture medium [2]. However, the excessive use of mineral oil can

Received: September 1, 2023 · Revised: October 19, 2023 · Accepted: November 6, 2023

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*This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare of the Republic of Korea (grant numbers: HI21C135003 and HI21C1639).

pose challenges, including high costs, variability in oil quality, and potential contamination [2].

For decades, communal culture of multiple embryos was a widely used method in *in vitro* embryo culture due to its numerous advantages [3,4]. In essence, cultured embryos thrive when grouped together. The communal culture method has been shown to positively impact blastocyst rates and subsequent *in vivo* development. The primary mechanisms believed to enhance development in group culture include accommodation, communication, and protection [5-7]. Accommodation refers to the capacity of embryos to adapt to their surroundings. In communal culture, embryos encounter a more varied environment, potentially fostering greater resilience [8]. Communication involves the exchange of signals between embryos, which may aid in synchronizing their development and ensuring their progression at a similar pace [9,10]. Finally, protection indicates that embryos in communal culture are less susceptible to damage from environmental stressors than are individual embryos. This is attributed to the surrounding embryos, which help serve as a shield against harmful factors [11-18].

However, the current approach to embryo culture has evolved, with individual culture now being the preferred method among laboratories [18]. This shift is largely because most methods of identifying embryos with the highest developmental competence necessitate individual follow-up screenings [19]. Furthermore, with the rising average patient age and the reduced intensity of stimulation in mild protocols, the number of zygotes may be decreased to the point that group effects are not present. This suggests that the advantages of group culture, such as nutrient and growth factor sharing, may not be as pronounced for older patients or those undergoing mild stimulation [20]. The presence of degenerated or dead embryos can also impede the development of healthy ones, as these can release harmful chemicals into the culture medium, potentially damaging the developing embryos [21].

Oil droplet culture is a widely used method for culturing individual preimplantation embryos in both mice and humans. This technique can help minimize potentially harmful changes in osmotic pressure and pH by preventing the evaporation of the medium. Furthermore, the oil layer can inhibit the dispersion of essential nutrients and growth factors away from the embryo. However, the specifics of this method are not yet fully understood. For instance, the required volume of medium is not well established and may differ based on the laboratory and culture conditions [22]. Additionally, the high surface-to-volume ratio of the droplets could potentially result in nutrient loss and the accumulation of toxic waste.

The well-of-the-well (WOW) system is a novel embryo culture technology designed to enhance embryo health and improve pregnancy rates by facilitating individual embryo culture. Originally developed for bovine embryo culture [23], the WOW system has since been adapted for use with other animal embryos. It has been demonstrated to promote embryo growth, improve quality, and minimize damage [24]. Furthermore, the WOW system has been found to augment both embryo fertilization rates and pregnancy rates in human ART programs [25].

The objective of this study was to assess the effectiveness of the newly developed optimized *in vitro* culture (OIVC) dish for cultivating preimplantation mouse embryos. The performance of the OIVC dish was assessed based on its capacity to maintain osmolarity and developmental outcomes in mouse pronuclear (PN) zygotes and two-cell-stage embryos. The results were compared to other commercially available culture dishes that employ the conventional oil-drop culture method.

Methods

1. Animals and hormonal stimulation

This study received approval from the Eulji University Institutional Animal Care and Use Committee (No. EUIACUC 21-23).

The protocol for superovulating mice was outlined by Park et al. [26]. In brief, female BDF (mouse strain) or ICR (mouse strain) mice aged between 6 and 9 weeks were superovulated using intraperitoneal injections of 5 IU of pregnant mare's serum gonadotropin (Prospec). After 48 hours, these mice received an additional injection of 5 IU of human chorionic gonadotropin (hCG; Prospec). Then, each superovulated mouse was individually mated with a fertile male BDF or ICR mouse.

2. Embryo collection and conditions for *in vitro* culture

Approximately 18 to 19 hours after hCG injection and mating, BDF female mice with a confirmed vaginal plug were euthanized via cervical dislocation. Subsequently, cumulus-enclosed one-cell embryos (PN zygotes) were collected from the oviductal ampullae. These zygotes were then denuded via 1 minute of incubation with 0.1% hyaluronidase (Sigma-Aldrich) in phosphate-buffered saline (PBS; Welgene). Following this, the zygotes were pooled and washed three times in potassium simplex optimized medium (KSOM, MR-121; Sigma-Aldrich) supplemented with 0.4% bovine serum albumin (BSA; Sigma-Aldrich).

Two-cell-stage embryos were obtained from ICR female mice through oviduct flushing, approximately 42 to 44 hours after hCG injection. Healthy zygotes and two-cell-stage embryos were individually cultured in a droplet of KSOM medium containing 0.4% BSA, which was covered with light mineral oil (9305; FujiFilm Irvine Scientific). This was done using three distinct types of culture dishes. The embryos were randomly assigned and cultured in three groups, each

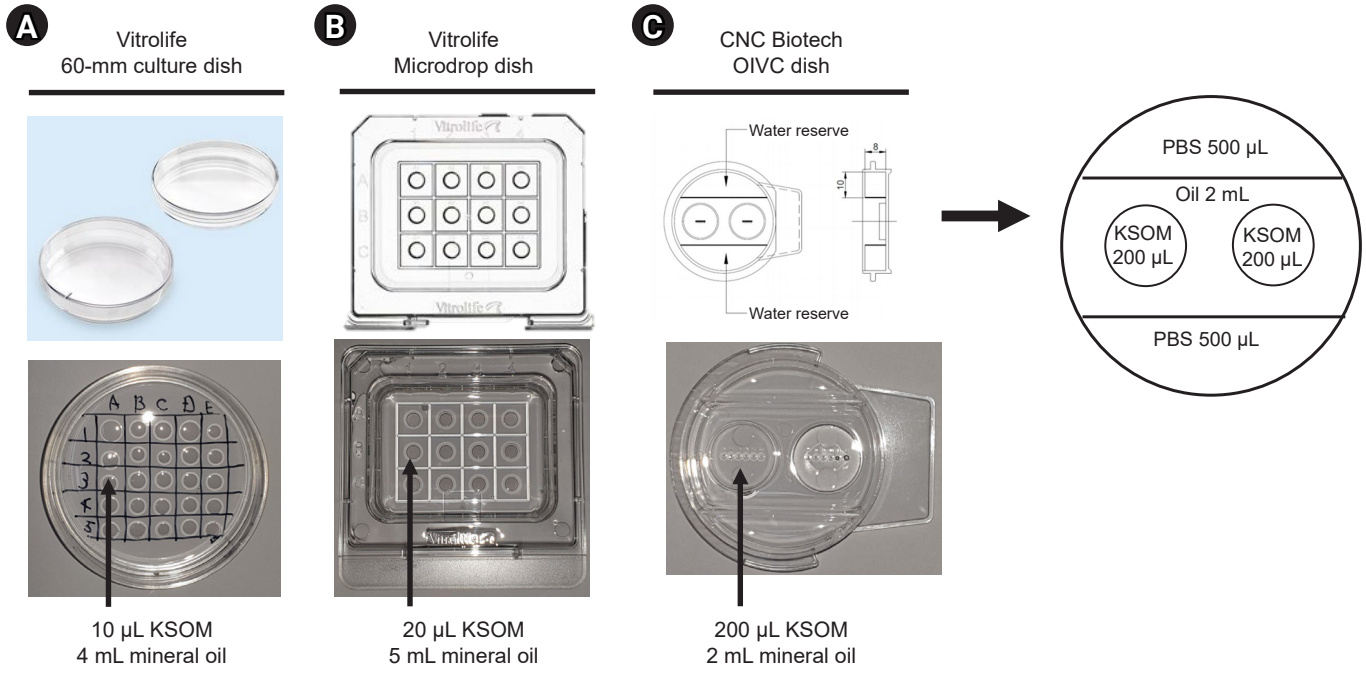

Figure 1. Dishes utilized in this study. (A) A 60-mm culture dish (Vitrolife, 16002) containing 10-μL drops of potassium simplex optimized medium (KSOM), overlaid with 4 mL of mineral oil. (B) A microdrop dish (Vitrolife, 16003) with 20-μL drops of KSOM, overlaid with 5 mL of mineral oil. (C) An optimized *in vitro* culture (OIVC) dish (CNC Biotech) featuring six small wells, each containing 200 μL of KSOM overlaid with 2 mL of mineral oil. PBS, phosphate-buffered saline.

corresponding to a different dish type as illustrated in Figure 1: (1) a 60-mm culture dish (16002; Vitrolife) with 10-μL droplets of KSOM overlaying 4 mL of mineral oil; (2) a microdrop dish (16003; Vitrolife) with 20-μL droplets of KSOM overlaying 5 mL of mineral oil; and (3) an OIVC dish (CNC Biotech) featuring six small wells, each containing 200 μL of KSOM overlaying 2 mL of mineral oil. The mouse embryos were cultured *in vitro* in a forma direct heat CO_2 Incubator (Thermo Fisher Scientific), a device with regulatory approval, under standard embryonic culture conditions at 37 ℃ with 5% CO_2.

3. Osmolarity measurement in three types of dishes over 5 days of *in vitro* culture

The osmolarity of the media was measured using the freezing-point depression method with an automatic osmometer (Micro Osmometer 3300; Advanced Instruments), in accordance with the manufacturer's instructions. After 5 days of *in vitro* culture, the culture media from each of the three types of dishes were collected and measured at least three times each.

4. Evaluation of blastulation rate and cell numbers in blastocysts

The cleavage rate from the PN zygote to the two-cell stage was determined 24 hours after PN zygote collection. The blastulation rates of PN zygotes and two-cell-stage embryos were ascertained 96 and 72 hours after embryo collection, respectively. Following observation, the blastocysts were fixed with 4% paraformaldehyde. The nuclei of the blastocysts were then stained and counted using a solution of 10 mg/mL bisbenzimide (Hoechst 33342; Sigma-Aldrich) under a fluorescence microscope (AX-70; Olympus).

5. Statistical analysis

All experiments were performed at least in triplicate. Depending on the data format, group comparisons were made using either the chi-square test or one-way analysis of variance. The Tukey honestly significant difference *post hoc* test was employed for all group comparisons. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

1. Changes in osmolarity in three types of dishes over 5 days of *in vitro* culture

The osmolarity of the KSOM culture media in the OIVC dish, covered with only 2 mL of mineral oil, was effectively maintained over a 5-day *in vitro* culture period. This contrasted with the 60-mm culture dish, which displayed a significant increase in osmolarity ($p<0.05$) (Figure 2). More specifically, the osmolarity in the OIVC dish was con-

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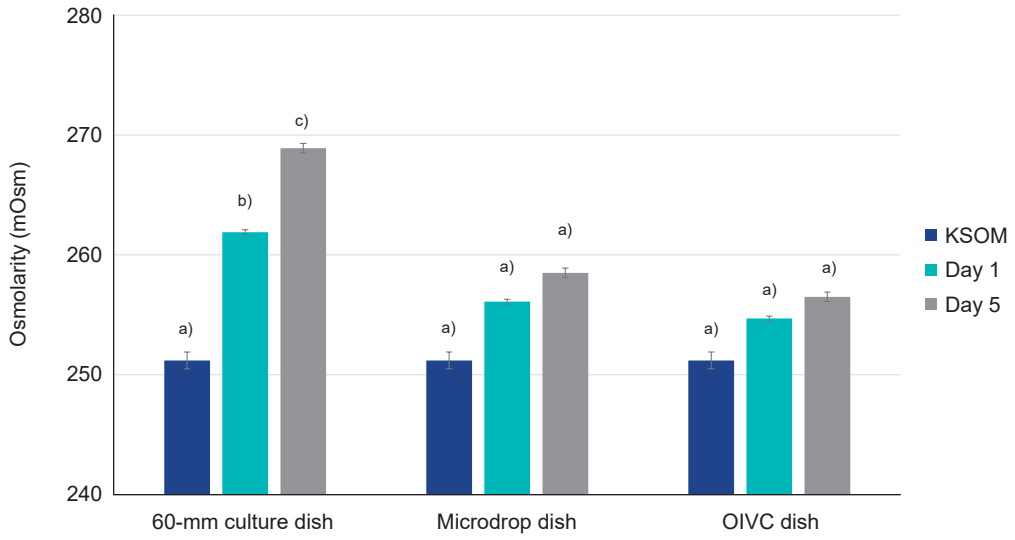

Figure 2. Osmolarity of potassium simplex optimized medium (KSOM) culture media in three types of dishes following 5 days of *in vitro* culture. OIVC, optimized *in vitro* culture. One-way analysis of variance was used, along with the Tukey multiple comparison test, to compare groups [a), b)], and [c)] (p<0.05).

sistently maintained over the 5 days of *in vitro* culture, ranging from 251.2±0.7 to 256.5±0.4 mOsm. However, the osmolarity of the KSOM culture media in the 60-mm culture dish significantly increased from 251.2±0.7 to 258.9±0.4 mOsm over the same period (p<0.05) (Table 1).

2. Preimplantation development of PN zygotes in three types of dishes

The rates of mouse embryonic development were evaluated at intervals of 24 hours (corresponding to development to the two-cell stage) and 96 hours (corresponding to development to the blastocyst stage). These results are displayed in Table 2, Figure 3. The cleavage rate was significantly higher in the 60-mm culture dish compared to the microdrop dish (p<0.05), but it was comparable between the 60-mm dish and the OIVC dish. Similar patterns were observed in the blastulation rates across groups. However, the total cell counts of blastocysts were significantly higher in the OIVC dish (70.6±1.9) relative to both the 60-mm culture dish (46.1±2.0) and the microdrop dish (47.4±1.9; p<0.05) (Table 2). The cells of the blastocysts were stained with bisbenzimide and examined under a fluorescent microscope, as shown in Figure 4.

3. Preimplantation development of two-cell embryos in three types of dishes

The rates of mouse embryonic development were evaluated after culturing from the two-cell stage to the blastocyst stage, and these results are displayed in Table 3. The OIVC dish demonstrated high blastulation rates, although no significant differences were observed among the groups. The blastulation rates were 100% for the OIVC dish, 95.7% for the microdrop dish, and 95.8% for the 60-mm culture dish. However, the total cell counts of the blastocysts were significantly higher in the OIVC dish (84.5±2.0) compared to the 60-mm culture dish (71.1±2.1) and the microdrop dish (77.3±2.6; p<0.05) (Table 3).

Table 1. Osmolarity of KSOM culture media in three types of dishes following 5 days of *in vitro* culture

Group (volume)	Osmolarity (mOsm)		
	KSOM	Day 1	Day 5
60-mm culture dish	251.2±0.7[a)]	261.9±0.2[b)]	268.9±0.4[c)]
Microdrop dish	251.2±0.7[a)]	256.1±0.4[a)]	258.5±0.4[a)]
OIVC dish	251.2±0.7[a)]	254.7±0.3[a)]	256.5±0.4[a)]

Values are presented as mean±standard error of the mean.
KSOM, potassium simplex optimized medium; OIVC, optimized *in vitro* culture.
One-way analysis of variance and Tukey's multiple comparison test, [a)] vs. [b)] vs. [c)], p<0.05.

Discussion

Numerous embryologists have pursued research aimed at enhancing the success rate of human embryonic development and ART. In a series of prior studies, the impact of fertilization methods on morphological events and abnormal division during embryonic

Table 2. Assessment of mouse embryonic development rates from zygotes following culture every 24 hours (corresponding to development to the two-cell stage) and 96 hours (corresponding to development to the blastocyst stage)

Variable	60-mm culture dish	Microdrop dish	OIVC dish
Cleavage rate at 48 hr post-hCG (day 2)	86.0[a] (86/100)	71.0[b] (66/93)	82.2[a),b)] (97/118)
Blastocyst/1-cell rate at 120 hr post-hCG (day 5)	80.0 (80/100)	68.8 (64/93)	76.3 (90/118)
Blastocyst/2-cell rate at 120 hr post-hCG (day 5)	93.0 (80/86)	97.0 (64/66)	92.8 (90/97)
Total cell in blastocysts	46.1 ± 2.0 (n = 62)	47.4 ± 1.9 (n = 61)	70.6 ± 1.9[c] (n = 87)

Values are presented as percentage (number/total number) or mean±standard error of the mean.
OIVC, optimized *in vitro* culture; hCG, human chorionic gonadotropin.
The rate was tested by chi-square test, [a] vs. [b], $p<0.05$; One-way analysis of variance, Tukey's multiple comparison test, [c] $p<0.05$.

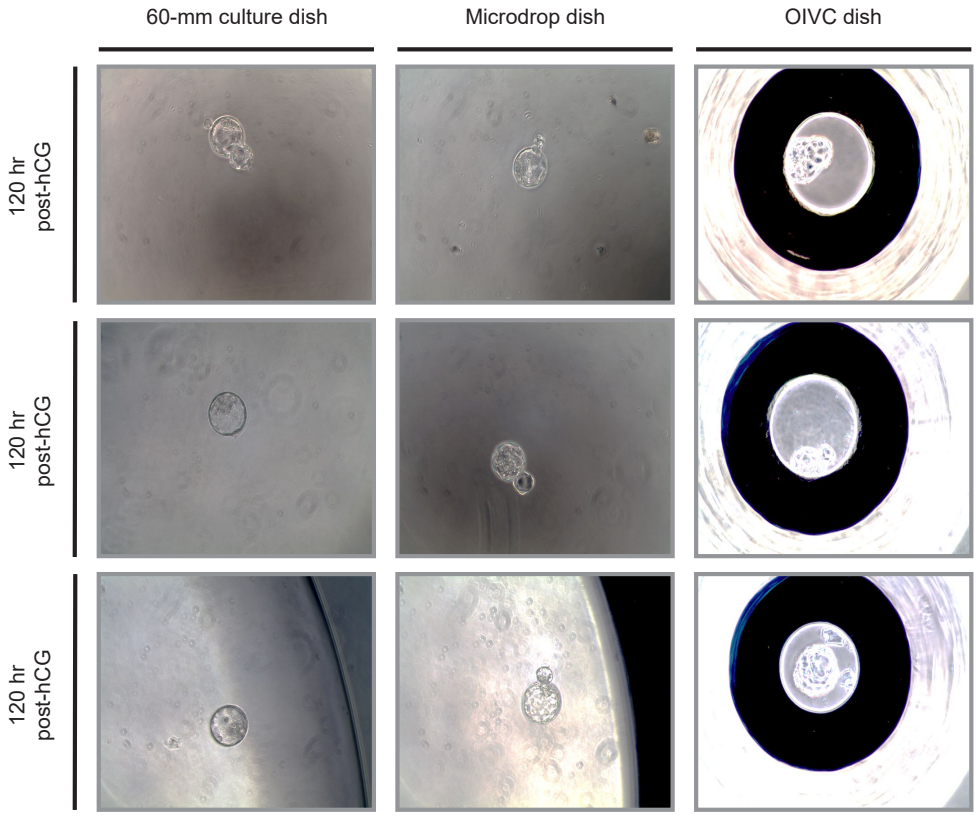

Figure 3. Mouse embryonic development rates after culture were assessed at intervals of 24 hours, which corresponds to development to the two-cell stage, and 96 hours, which corresponds to development to the blastocyst stage. OIVC, optimized *in vitro* culture; hCG, human chorionic gonadotropin.

development was examined. The findings indicated differences up to the six-cell stages between embryos derived from IVF and those obtained from intracytoplasmic sperm injection [27]. Another study delved into embryonic development, morphology, and clinical outcomes, revealing higher success rates in regularly developed blastocysts as opposed to those with morphokinetic variables [28]. In a study investigating the effects of oxygen, a novel gas-supplied incubator was utilized, and dynamic oxygen concentrations were observed to improve mouse embryonic development [29]. Additionally, we conducted a review on non-invasive embryonic quality evaluation using time-lapse imaging, artificial intelligence, molecular markers, and nucleic acid analysis. The review underscored the necessity for robust clinical trials to integrate and validate various methods for comprehensive evaluation [30]. In the present study, we introduced a newly designed culture dish for the *in vitro* culture of preimplantation mammalian embryos, aimed at increasing the success rate of embryonic development.

The OIVC dish is a novel design aimed at reducing the use of mineral oil and maintaining the osmolarity of the culture medium more effectively than other dishes used for *in vitro* preimplantation em-

https://doi.org/10.5653/cerm.2023.06436

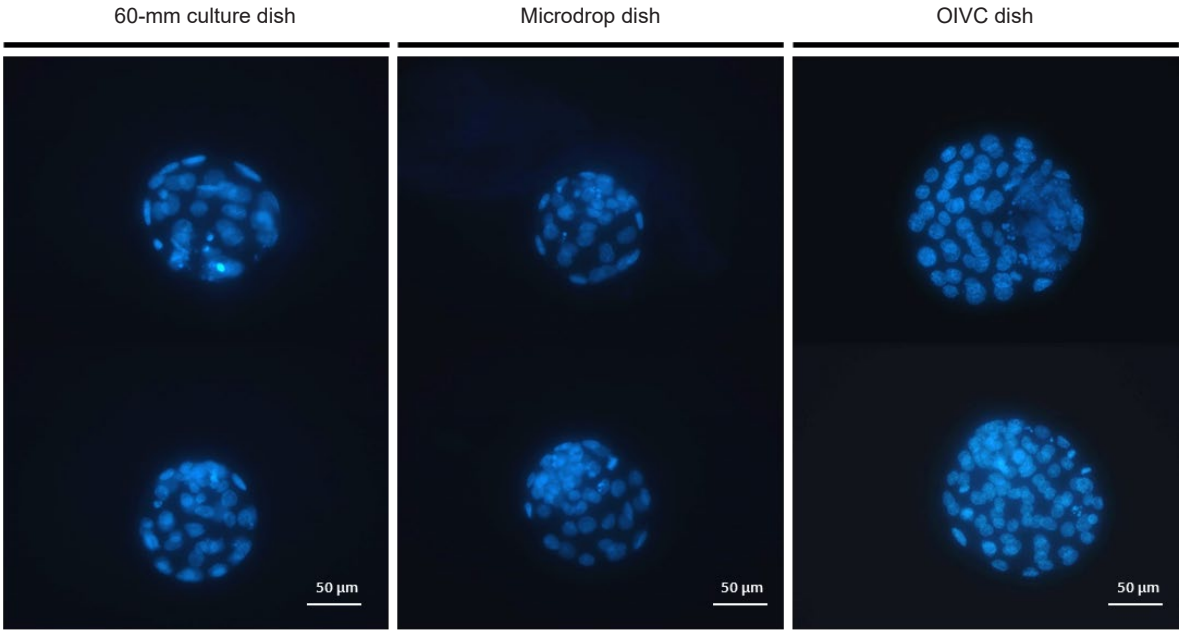

Figure 4. Total blastocyst cell counts were significantly higher in the OIVC dish (70.6±1.9) compared to both the 60-mm culture dish (46.1±2.0) and the microdrop dish (47.4±1.9) (×400 magnification). OIVC, optimized *in vitro* culture.

Table 3. Assessment of mouse embryonic development rates following culture from the two-cell stage to the blastocyst stage

Variable	60-mm culture dish	Microdrop dish	OIVC dish
No. of 2-cell embryos retrieved (ea)	95	94	151
No. of blastocyst on 120 hr post-hCG (ea)	91	90	151
Blastocyst formation rate on 120 hr post-hCG (%)	95.8	95.7	100
Total cell in blastocysts	71.1 ± 2.1[a] (n = 63)	77.3 ± 2.6[a),b)] (n = 43)	84.5 ± 2.0[b)] (n = 100)

Values are presented as mean±standard error of the mean unless otherwise indicated.
OIVC, optimized *in vitro* culture; hCG, human chorionic gonadotropin.
One-way analysis of variance and Tukey's multiple comparison test, [a)] vs. [b)], $p<0.05$.

bryo culture. In fact, the OIVC dish demonstrated a 50% reduction in mineral oil usage compared to a standard 60-mm culture dish and a 60% reduction compared to a microdrop dish. This study revealed that the use of the OIVC dish yielded substantial improvements in blastulation rates and the number of cells in blastocysts from *in vitro* culture of mouse PN and two-cell-stage embryos.

Mineral oil plays a crucial role in the *in vitro* culture of preimplantation embryos, providing protection against osmotic stress by preventing medium evaporation [25]. However, the drawbacks of using mineral oil are well-documented. Variations in quality and the presence of potential pollutants can lead to inconsistent results and an increased risk of infection [20,31]. Osmolarity, a measure of the concentration of dissolved particles in a solution, is a critical factor in cell proliferation and embryo development *in vitro*. If the osmolarity of the culture medium becomes too high or too low, the embryos may be harmed. Numerous studies have underscored the importance of maintaining the osmolarity in embryo culture media, with research conducted on mouse, porcine, bovine, and human models.

Prior research has also highlighted the role of osmolarity in various contexts. For instance, when preserving rabbit embryos, it was found that maintaining osmolarity within a specific range (285 to 340 mOsm) was crucial for effective preservation. Any deviation from this range resulted in structural changes to the embryos [32]. In the context of embryo culture, adjustments to osmolarity can aid in overcoming the two-cell block [33]. This can be achieved by fine-tuning the components of the culture medium, such as sodium chloride (NaCl), potassium chloride, and glucose, indicating that osmolarity is not the sole factor influencing embryo development [34]. Similarly, the development of mouse zygotes is also influenced by osmolarity. Elevated NaCl concentrations can impede development, whereas the presence of glutamine/ betaine can offer protection against this effect [35]. The development of bovine embryos is likewise linked to

NaCl concentration, with specific ranges promoting development. Adjustments to osmolarity can influence different stages of development, highlighting its importance [36]. In the case of porcine nuclear transfer and IVF embryos, early manipulation of osmolarity can impact development and gene expression, potentially enhancing growth and reducing apoptosis [37]. In summary, osmolarity has emerged as a key factor in these studies, influencing various aspects of embryo development, preservation, and gene expression.

Maintaining osmotic balance is crucial for optimal embryonic development [38]. Substantial changes in the osmotic pressure of the culture, which is designed to be suitable for embryo development during *in vitro* culture, can induce physiological stress and impact the morphology, viability, and implantation potential of the embryo [39]. The OIVC plate offers an advantage in this regard, as it can consistently maintain osmotic pressure while minimizing the use of mineral oil, making it highly durable. By inhibiting excessive evaporation of the culture, it ensures the maintenance of appropriate osmotic pressure, thereby creating a stable environment that mitigates stress risk and improves the prospects of embryonic survival and implantation.

From a pragmatic perspective, decreasing the usage of mineral oil results in cost savings, particularly in high-volume IVF clinics where mineral oil is a costly reagent. Furthermore, minimizing the handling of mineral oil can enhance workflow efficiency, streamlining the processes within the IVF clinic.

The integration of time-lapse systems into the *in vitro* culture of preimplantation embryos represents a key advancement, enabling continuous monitoring of embryonic development without the need for manual evaluation [40]. The potential to combine this time-lapse technology with the OIVC dish could offer several benefits. First, continuous non-invasive monitoring using the OIVC dish could facilitate uninterrupted observation of individual embryos, providing valuable data on crucial developmental milestones such as the timing of cell division, which is indicative of embryonic viability [41]. Second, time-lapse monitoring can enhance the accuracy of embryo selection by identifying subtle morphological changes and anomalies that may be missed during fixed-time evaluations [42]. Third, an optimized culture environment, achieved by maintaining osmotic pressure and minimizing the use of mineral oil, ensures that time-lapse records are captured under consistent conditions that are conducive to optimal embryonic development during *in vitro* culture. These synergistic benefits not only bridge the gap between research and clinical embryology, but also have the potential to revolutionize our understanding of early mammalian development *in vitro*. While the present findings are focused on mouse embryos, they could considerably improve embryonic culture practices by balancing cost-effectiveness with optimal conditions.

Adding to its value, the OIVC dish incorporates innovative embryonic culture techniques. These methods improve embryonic health and pregnancy rates, while also being cost-effective and environmentally stable. Furthermore, they facilitate research into early mammalian development through integration with time-lapse systems. The dish provides a stable microwell-based culture environment, minimizing the use of mineral oils. This commitment to media stability, individualized culture, and embryo tracking begins with individualized cultural concepts such as WOW systems. The effectiveness of OIVC plates is demonstrated through the evaluation of osmotic retention and developmental outcomes in mouse PN junctions and two-cell-stage embryos, showing equivalent effectiveness to standard methods [33]. Additionally, the capacity to culture individual embryos offers the potential for optimized embryonic selection for quality evaluation and metastasis.

The OIVC dish, a new innovation within the WOW system, was designed for the *in vitro* culture of mammalian preimplantation embryos, including those from mice and humans. This cutting-edge dish reduces the reliance on mineral oil and includes microwells, which provide a stable culture medium environment and allow for the individual monitoring of each embryo. Moreover, it enables differential cultivation between groups by partitioning the microwells into two sections, facilitating a direct comparison of the conditions within each group. The dish also incorporates double microwells, which allow for both individual and group cultivation by linking the groups to the culture medium. The OIVC dish was designed to effectively prevent oil and media evaporation during *in vitro* culture. This is achieved by placing supplemented culture medium or other non-toxic solutions, such as PBS or distilled water, on the sides of the dish to maintain osmotic pressure. This innovative design concept was applied to the development of a new OIVC plate.

In conclusion, our research demonstrates that the OIVC dish yielded significant improvements in blastulation rates and the number of cells in blastocysts during the *in vitro* culture of mouse PN and two-cell-stage embryos. Its capacity to maintain media osmolarity while reducing the use of mineral oil represents a breakthrough with the potential to enhance embryo culture techniques, including those relevant to human IVF and embryo transfer programs. Further research is necessary to confirm these results, evaluate long-term safety and effectiveness, and investigate the potential for a revolutionary change in the embryonic culture system.

Conflict of interest

Jin Hyun Jun is an editorial board member of the journal, but he was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts.

https://doi.org/10.5653/cerm.2023.06436

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https://doi.org/10.5653/cerm.2023.06436

ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06219
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):253-261

CERM

Polymorphisms and expression levels of *TNP2*, *SYCP3*, and *AZFa* genes in patients with azoospermia

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Objective: Azoospermia (the total absence of sperm in the ejaculate) affects approximately 10% of infertile males. Despite diagnostic advances, azoospermia remains the most challenging issue associated with infertility treatment. Our study evaluated transition nuclear protein 2 (*TNP2*) and synaptonemal complex protein 3 (*SYCP3*) polymorphisms, azoospermia factor a (*AZFa*) microdeletion, and gene expression levels in 100 patients with azoospermia.

Methods: We investigated a *TNP2* single-nucleotide polymorphism through polymerase chain reaction (PCR) restriction fragment length polymorphism analysis using a particular endonuclease. An allele-specific PCR assay for *SYCP3* was performed utilizing two forward primers and a common reverse primer in two PCR reactions. Based on the European Academy of Andrology guidelines, *AZFa* microdeletions were evaluated by multiplex PCR. *TNP2*, *SYCP3*, and the *AZFa* region main gene (DEAD-box helicase 3 and Y-linked [*DDX3Y*]) expression levels were assessed via quantitative PCR, and receiver operating characteristic curve analysis was used to determine the diagnostic capability of these genes.

Results: The *TNP2* genotyping and allelic frequency in infertile males did not differ significantly from fertile volunteers. In participants with azoospermia, the allelic frequency of the *SYCP3* mutant allele (C allele) was significantly altered. Deletion of sY84 and sY86 was discovered in patients with azoospermia and oligozoospermia. Moreover, *SYCP3* and *DDX3Y* showed decreased expression levels in the azoospermia group, and they exhibited potential as biomarkers for diagnosing azoospermia (area under the curve, 0.722 and 0.720, respectively).

Conclusion: These results suggest that reduced *SYCP3* and *DDX3Y* mRNA expression profiles in testicular tissue are associated with a higher likelihood of retrieving spermatozoa in individuals with azoospermia. The homozygous genotype TT of the *SYCP3* polymorphism was significantly associated with azoospermia.

Keywords: Azoospermia; Gene expression; Polymerase chain reaction; Polymorphism

Introduction

It is a public health concern that 7% of males experience fertility issues at some point in their lives. Male infertility is a complicated condition that, by international consensus, is defined by the failure to achieve a clinical pregnancy following 12 months of regular, unprotected sexual activity. Male factor infertility is present in 45% to 50% of couples affected by infertility [1].

A lack of sperm in the ejaculate is known as azoospermia [2]. Azoospermia always results in infertility, although the term itself does not describe the underlying causation. Global estimates indicate that up to 10% of infertile men and one out of every 100 males of reproductive age have azoospermia [3]. The two broad categories are obstructive azoospermia (OA) and nonobstructive azoospermia (NOA) [4]. This distinction has clinical significance because it impacts patient care and recovery [3]. Notably, NOA refers to an inherent testicular abnormality induced by several diseases that can significantly impact sperm production [5]. Primary testicular failure affecting spermatogenic cells is known as spermatogenic failure, and hypogonadotropic hypogonadism (HH) is a condition that results in a severe

Received: May 27, 2023 · Revised: July 7, 2023 · Accepted: July 12, 2023
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spermatogenic deficiency in patients with NOA [6].

OA, in contrast, is caused by a mechanical obstruction along the reproductive tract, specifically the vas deferens, epididymis, or ejaculatory duct [7]. Unlike NOA, spermatogenesis is preserved in patients with OA, and reconstructive operations and sperm retrieval are frequently very successful [2]. Abnormalities in sex chromosomes and a growing number of uncommon detrimental variations in genes essential for spermatogenesis are two genetic factors that contribute to NOA [8]. According to World Health Organization (WHO) guidelines, the term "azoospermia" is used when there are no sperm in the ejaculate, and "oligozoospermia" is used when the sperm concentration is <15 million sperm/mL of ejaculate (i.e., below the lower reference limit). There are three levels of oligozoospermia: mild (10 to 15 million sperm/ mL), moderate (5 to 10 million sperm/mL), and severe (<5 million sperm/mL) [9,10].

The transition nuclear protein 2 (*TNP2*) and protamine 1 (*PRM1*) genes are both found on chromosome 16 (16p13.3), where they form a multigenic cluster with the protamine 2 (*PRM2*) gene [11]. *TNP1* and *TNP2*, which have only one copy each, encode TP1 and TP2 proteins [12]. The two protamine genes and *TNP2* have a close connection, suggesting they were formed through gene duplication and may still exhibit shared activities. During the stages of spermiogenesis, 90% of the chromatin-basic proteins are made up of TNPs [13]. At later stages of spermiogenesis, TNPs are shown to be essential for the replacement by protamine and sperm DNA condensation [14].

Synaptonemal complex protein 3 (*SYCP3*), on the 12q23.2 chromosome, is a crucial indicator of meiotic germ cell division throughout spermatogenesis. Some unexplained instances of azoospermia are thought to be caused by abnormalities in human synaptonemal complex development; thus, *SYCP3* may be a tool for predicting the course of human spermatogenesis, particularly in infertile men [15,16]. Furthermore, it is thought that *SYCP3* is mainly expressed in germ cells [15], and it is a testis-specific gene that functions as the central component of the lateral elements of the synaptonemal complex, which controls synapsis, sister chromatid cohesion, DNA binding to the chromatid axis, and recombination [17].

Moreover, the long arm of the Y chromosome contains the azoospermia factor (AZF) region. It is broken down into three subregions: AZFa, AZFb, and AZFc, and plays a crucial part in the genetics of male infertility [18]. Genes important to spermatogenesis and testicular development can be found in these locations. Microdeletions in this region cause male infertility and spermatogenetic abnormalities. The human Y chromosome is essential for the development and function of male germ cells as well as the determination of the human sex [19]. The proximal long arm of the Y chromosome (Yq) is where the *AZFa* subregion is found [20]. The primary gene of the *AZFa* area is DEAD-box helicase 3 and Y-linked (*DDX3Y*), which has been linked to the advancement of premeiotic germ cells and is expressed in the testis. This suggests that suppressing *DDX3Y* expression contributes to the loss of prenatal germ cells and to infertility [21,22]. The sY84 and sY86 markers are the first choice for sequence-tagged site (STS) primers in the *AZFa* region, their deletions suggest a very high probability of complete deletion of AZF, and NOA is frequently caused by complete deletion of the *AZFa* region [23]. Our study evaluated *TNP2* and *SYCP3* gene polymorphisms, *AZFa* microdeletions, and their gene expression levels in 100 patients with azoospermia.

Methods

1. Study participants

Patients with azoospermia, aged 28 to 40 years, were chosen for the examination of promoter region polymorphism in the *TNP2* and *SYCP3* genes and microdeletions in *AZFa*. All patients received a thorough andrological examination, which included a medical history and physical examination, semen analysis, scrotal ultrasonography, and hormone analysis. In addition, every patient was examined to determine whether their karyotype was normal (46XY). A comprehensive medical history and physical examination were conducted for the controls, and all were fertile and had normal children.

In total, 100 men were selected for gene expression analysis as patient group after undergoing bilateral microdissection testicular sperm extraction (mTESE) to obtain spermatozoa for intracytoplasmic sperm injection. Karyotyping and Y chromosomal microdeletion analyses and the blood levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were assessed before surgery. The patients were not on hormone treatment and had primary infertility. Mean±standard deviation values for the serum LH, FSH, and testosterone levels of participants are illustrated in Table 1. None of the 100 male controls had a history of mTESE or cryptorchidism. The study excluded patients with cystic fibrosis, chromosomal

Table 1. Serum LH, FSH, and testosterone levels in infertile males and healthy individuals (controls)

Group	LH (mIU/mL)[a]	FSH (mIU/mL)[b]	Testosterone (ng/mL)[c]
Azoospermia	12.20±1.19	23.36±2.17	2.84±0.38
Oligozoospermia	10.93±1.00	17.02±2.55	2.86±0.45
Severe oligozoospermia	10.94±1.07	17.68±2.26	2.93±0.39
Controls	8.06±0.58	9.47±0.71	4.47±0.29

Values are presented as mean±standard deviation.
LH, luteinizing hormone; FSH, follicle-stimulating hormone.
[a]Normal testosterone level=3–10 ng/mL; [b]Normal LH=2–12 mIU/mL; [c]Normal FSH=1–12 mIU/mL.

https://doi.org/10.5653/cerm.2023.06219

abnormalities, and Y chromosome microdeletion. The control group consisted of 100 men with normal spermatogenesis (>15.0×10^6 sperm/mL) according to the WHO criteria and confirmed fertility.

2. Sampling and DNA extraction

The research was approved by the local ethics committee (NO. 52/422370/1) and all subjects provided written informed consent. Blood samples from patients with azoospermia were collected to analyze gene polymorphisms and mutations. The salting-out DNA extraction technique was used to extract DNA samples from total blood samples.

To evaluate gene expression levels, frozen testis tissue was homogenized. Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen) according to the manufacturer's protocol and stored at −80 °C. In-solution DNase digestion was used to eliminate DNA contamination. The concentration and purity of RNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and confirmed by agarose gel electrophoresis. Template cDNA was synthesized from 1 μg of total extracted RNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) simultaneously with oligo-dT and random hexamer primers for each reaction in an Eppendorf Mastercycler Gradient (Eppendorf AG).

3. Restriction fragment length polymorphism genotyping

Polymerase chain reaction (PCR) amplification was performed using primer pairs (Table 2). PCR fragments were amplified as a 473bp fragment from the *TNP2* gene, and the PCR conditions for *TNP2* were as follows: 32 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 1 minute, and extension at 72 °C for 30 seconds. The restriction fragment length polymorphism method was used for genotyping the *TNP2* rs2857758 allele. The *EarI* restriction endonuclease enzyme was used to detect the CTCTTC sequence. The digestion reaction was carried out for 2 hours at 37 °C, and it digested 473-bp PCR products with altered G alleles into 353-bp and 120-bp fragments. The DNA fragments were isolated using 1.5% agarose gel electrophoresis and visualized in a gel documentation system by safe staining.

Table 2. Specific polymerase chain reaction primers, their sequence, and amplicon size

Gene	Primer name	Primer sequence (5´-3´)	Amplicon size (bp)
TNP2	F	GTGGTTGGTGGATGAATTGGTTAG	473
	R	TTCTCCTTTGGGTGAAACACGCAG	
SYCP3	F1 (T allele)	ATGTTGCAAAAAAAAATTATGATGGAAGCT	286
	F2 (C allele)	ATGTTGCAAAAAAAAATTATGATGGAAGCC	
	R	TTGCTGCTGCTGTTTCATG	
AZFa	sY84F	AGAAGGGTCTGAAAGCAGGT	326
	sY84R	GCCTACTACCTGGAGGCT TC	
AZFa	sY86F	GTGACACACAGACTATGCTTC	320
	sY86R	ACACACAGAGGGACAACCCT	

bp, base pairs/length; *TNP2*, transition nuclear protein 2; *SYCP*, synaptonemal complex protein 3; *AZF*, azoospermia factor.

4. Allele-specific PCR

The presence of the T657C mutation in the *SYCP3* gene was evaluated with the allele-specific (AS) PCR (ASPCR) technique. Two forward primers with variations in their 3' nucleotides were designed so that each was specific for one of the two variants (Table 2). They were combined with a common reverse primer into two PCR reactions. A 25-μL reaction mixture comprising 100 ng of DNA; 40 pmol of primers (*SYCP3* F1, F2, and *SYCP3* R); and 12.5 μL of 2x Taq PCR Master Mix was utilized for amplification. An initial melting phase of 94 °C for 5 minutes was followed by 35 cycles of 94 °C for 30 seconds, an annealing temperature starting at 60 °C for 30 seconds and 72 °C for 30 minutes for extension, and a final elongation step of 5 minutes at 72 °C. A gel documentation system was used to visualize the amplified DNA on 1.5% agarose gel with safe staining. The C and T alleles produced 286-bp bands due to the AS primers' reactions.

5. Multiplex PCR

Multiplex PCR was employed according to the standard protocol for analysis of the AZF region of the Y chromosome. The *AZFa* subregion was analyzed using STS primers (Table 1). The European Academy of Andrology recommends these STS primers, which can identify 90% of the deletions in AZF loci (sY84, sY86 for the *AZFa* region). A total volume of 25L was used for the PCR amplification, including 100 to 200 ng of human genomic DNA as a template, 2.5 mM deoxynucleotide triphosphates (2.5 mM each of 2'-deoxythymidine-5'-triphosphate [dTTP], 2'-deoxycytosine-5'-triphosphate [dCTP], 2'-deoxyguanosine-5'-triphosphate [dGTP], 2'-deoxyadenosine-5'-triphosphate [dATP]), oligonucleotide primers (0.1 to 2.0 μmol/L each of the forward and reverse primers), 10x Taq DNA polymerase assay buffer (Tris with 15 mM $MgCl_2$), and 3 U of Taq DNA polymerase.

The conditions for thermocycling were standardized for the *AZFa* subregions, utilizing a TC-512 gradient thermocycler. PCR was performed on the samples in 35 cycles at 94 °C for 30 seconds, 53 °C for 45 seconds, and 72 °C for 60 seconds. Initial denaturation was performed at 94 °C for 5 minutes, followed by a final extension at 72 °C for 10 minutes. The PCR amplified products were stained with safe staining and identified on 2% agarose gel electrophoresis. Before loading the samples into the wells of the agarose gel, samples were mixed with gel loading dye in a ratio of 1:1.

6. Quantitative real-time PCR

Expression analysis was carried out by quantitative real-time PCR (qRT-PCR) on cDNA libraries using primers specific for *TNP2*, *SYCP3*, *DDX37* (located within the AZFa region), and the housekeeping gene β-actin (Table 3). The expression of β-actin was examined as an internal control for RNA isolation and RT-PCR efficiency. Initial denaturation for RT-PCR started at 95 ℃ for 8 minutes, followed by 40 cycles of denaturation at 95 ℃ for 10 seconds, annealing at 60 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds. To avoid primer dimer amplification, the melting curve was created by raising the temperature from 72 to 95 ℃. The qPCR was performed in triplicate on 48-well plates in the StepOne-Plus RT-PCR System (Thermo Fisher Scientific/Applied Biosystems) using 1.0 μL of produced cDNA, 10 μL of the SYBR Green qPCR Master Mix (Applied Biosystems ABI/PE), and 7.0 μL of DNase/RNase-free water. One microliter of designed primers was used for the gene expression profile. The average cycles to threshold (C_T) value was used for further analysis, and all RT-PCR cycles included non-template (cDNA) controls to rule out contamination. The comparative C_T method was used to analyze relative gene expression ($2^{-\Delta\Delta CT}$). The $2^{-\Delta\Delta CT}$ parameter displays the expression level (expressed as a fold change) with respect to the housekeeping gene.

7. Statistical analysis

The chi-square test was used to compare allele and genotype frequencies between case and control groups. Statistical tests of significance and chi-square analysis were performed using R programming version 4.0.5 (R Foundation for Statistical Computing). Differences in the allelic and genotypic frequencies of rs2857758 for *TNP2* and rs769825641 for *SYCP3* between case and control groups were evaluated using the chi-square test with an odds ratio to express the risk of single-nucleotide polymorphisms (SNPs) for the disease of interest. The Fisher exact test was used for statistical testing when the allelic count was less than 5.

The gene expression results were verified as the mean±standard error of mean. The Mann-Whitney *U* test was used for analysis of non-normally distributed data. Statistical analysis was implemented using Prism 6 (GraphPad Software) and *p*-values <0.05 were deemed statistically significant. Furthermore, receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were employed to assess the potential of these genes as diagnostic biomarkers.

Table 3. The quantitative real-time polymerase chain reaction primer sequences and their product length

Gene	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)
TNP2	F	CGCCATTGCCAAACCTTCAG	144
	R	AGTGTTGGGACTCTGGCTCT	
SYCP3	F	GCGGTGTGTTTCAGTCAGGA	82
	R	CACAGACGGCTTCCCAGATT	
DDX3Y	F	AATGGTCACTGCGCCTAACA	112
	R	CATGACTCATCCCTTTGACAAGC	
β-Actin	F	AGAGCTACGAGCTGCCTGAC	184
	R	AGCACTGTGTTGGCGTACAG	

bp, base pairs/length; *TNP2*, transition nuclear protein 2; *SYCP3*, synaptonemal complex protein 3; *DDX3Y*, DEAD-box helicase 3 and Y-linked.

Results

1. Gene polymorphisms

The genotype frequencies for the rs2857758 SNP of the *TNP2* gene on chromosome 16 (16p13.13) were 87% for T/T, 10% for T/G, and 3% for G/G in sterile males, whereas the corresponding frequencies among the control group were 76%, 15%, and 9%, respectively (Figure 1). The altered allele frequency was higher among normal controls than in the case group. However, no significant difference (p=0.053) was found between infertile and fertile males (Table 3).

SYCP3 rs769825641 polymorphism, located on 12q23.2, was genotyped using ASPCR. Two PCR reactions were performed, one with forward primer 1 for the T allele and the other with forward primer 2 for the C allele, run side by side on an agarose gel. A 286-bp band indicated the presence of the specific allele. We observed that 167 (83.5%) cases and controls were homozygous TT, 27 (13.5%) were heterozygous TC, and six (3%) were homozygous CC. The genotyping frequency difference between the case and control groups was

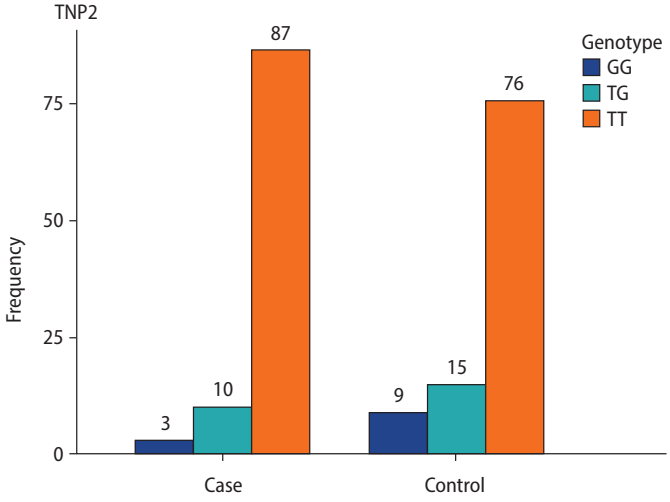

Figure 1. Genotype frequency of the transition nuclear protein 2 (*TNP2*) rs2857758 gene.

https://doi.org/10.5653/cerm.2023.06219

Table 4. Genotype and allelic frequency of *TNP2* rs2857758 and *SYCP3* rs769825641 in patients with azoospermia and a control group

Gene	Case (n = 100)	Control (n = 100)	*p*-value	OR (95% CI)
TNP2				
TT	87	76	0.093	1
TG	10	15		1.71 (0.728–4.04)
GG	3	9		3.43 (0.897–13.1)
G allele (additive)	8%	16.5%	0.053	0.531 (0.290–0.970)
(TG/GG) dominant	13	24		0.473 (0.225–0.993)
TT/TG	97	91		1
Recessive	3	9		0.312 (0.082–1.19)
SYCP3				
TT	77	90	0.04	1
TC	18	9		0.42 (0.18–1.0)
CC	5	1		0.17 (0.19–1.4)
C allele (additive)	14%	0.5%	0.006	2.7 (1.2–6.4)
(TC/CC) dominant	23	10		2.3 (1.0–5.3)
TT/TC	95	99		1
Recessive	5	1		0.19 (0.02–1.6)

TNP2, transition nuclear protein 2; *SYCP3*, synaptonemal complex protein 3; OR, odds ratio; CI, confidence interval.

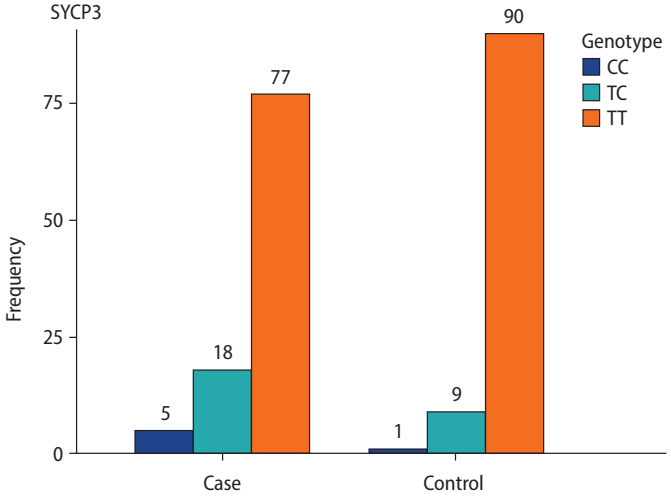

Figure 2. Genotype frequency of the synaptonemal complex protein 3 (*SYCP3*) rs769825641 gene.

statistically significant (p=0.04). Additionally, the frequency of mutant alleles was higher in the sterile men than in the fertile male volunteers (Table 4, Figure 2).

To validate the deletions in the AZFa region, the PCR reactions were repeated three times. The number of samples with deletions and their proportions are presented in Table 5. Deletions in the AZFa region were not found in patients with severe oligozoospermia. Among the 100 samples tested for the sY84 marker, four exhibited deletions in the *AZFa* region, and four showed deletions for the sY86 marker.

Table 5. Detection of microdeletions in the AZFa region with the sY84 and sY86 markers

	Total number	Microdeletion in AZFa (sY86)	Microdeletion in AZFa (sY84)
Azoospermia	33	2	3
Severe oligozoospermia	19	0	0
Oligozoospermia	48	2	1

AZF, azoospermia factor.

2. Expression analysis

The cDNA libraries from testis tissue were screened by PCR for the *TNP2*, *SYCP3*, and *DDX3Y* genes with their specific primers. Based on the Mann-Whitney *U* test, *SYCP3* and *DDX3Y* showed altered expression in the testis tissue of infertile patients compared to fertile male controls. In contrast, no significant alteration was observed for *TNP2* expression among the infertile males as compared to controls (Figure 3).

3. Biomarker potential

ROC curve analyses were carried out to assess the diagnostic value of the *TNP2*, *SYCP3*, and *DDX3Y* genes for azoospermia. The results showed that *SYCP3* and *DDX3Y* were potential biomarkers for azoospermia in fertile males, with AUCs of 0.772 and 0.720, respectively. However, *TNP2* (AUC=0.568) showed no biomarker potential for diagnosing azoospermia (Figure 4).

4. Hormonal analysis

The analysis of hormone levels indicated that both LH and FSH

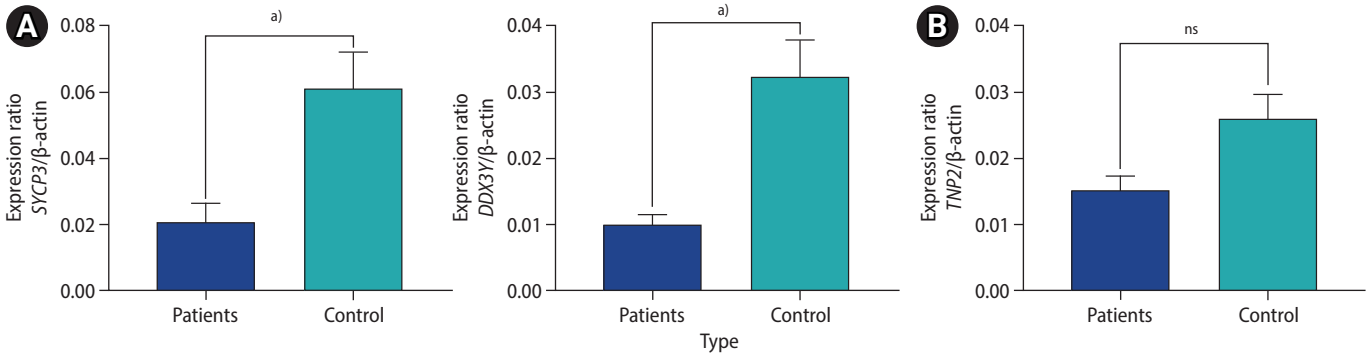

Figure 3. Gene expression levels in infertile patients compared to healthy subjects (controls). (A) synaptonemal complex protein 3 (*SYCP3*) and DEAD-box helicase 3 and Y-linked (*DDX3Y*) expression levels were significantly lower in patients than in controls (p<0.0001). (B) Transition nuclear protein 2 (*TNP2*) showed no significant alteration of expression in patients (p=0.095). ns, not significant. [a]p<0.0001.

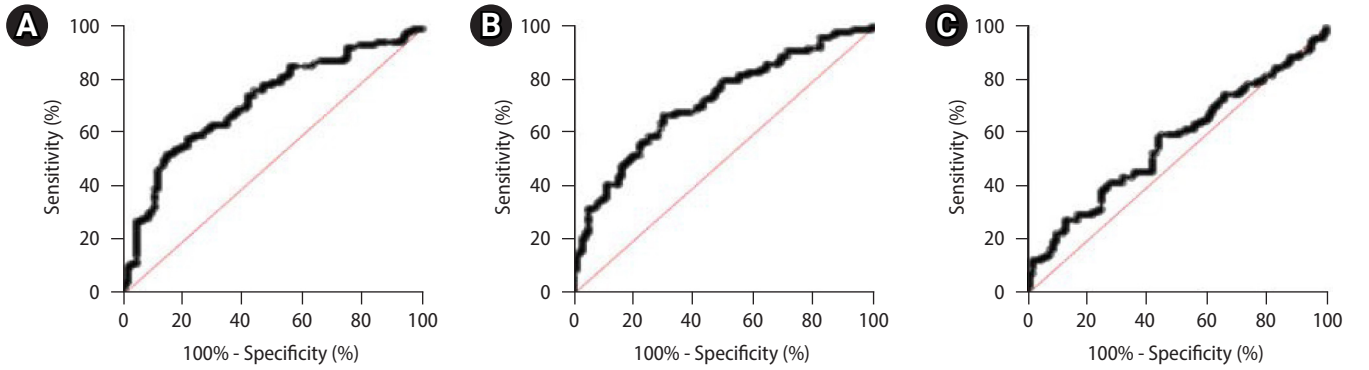

Figure 4. Receiver operating characteristic curve analysis of: (A) synaptonemal complex protein 3 (*SYCP3*) with an area under the curve (AUC) of 0.722 (95% confidence interval [CI], 0.651 to 0.793; sensitivity=70%, specificity=61%), (B) DEAD-box helicase 3 and Y-linked (*DDX3Y*) with an AUC of 0.720 (95% CI, 0.649 to 0.790; sensitivity=67%, specificity=70%), and (C) transition nuclear protein 2 (*TNP2*) with an AUC of 0.568 (95% CI, 0.488 to 0.647; sensitivity=58%, specificity=56%).

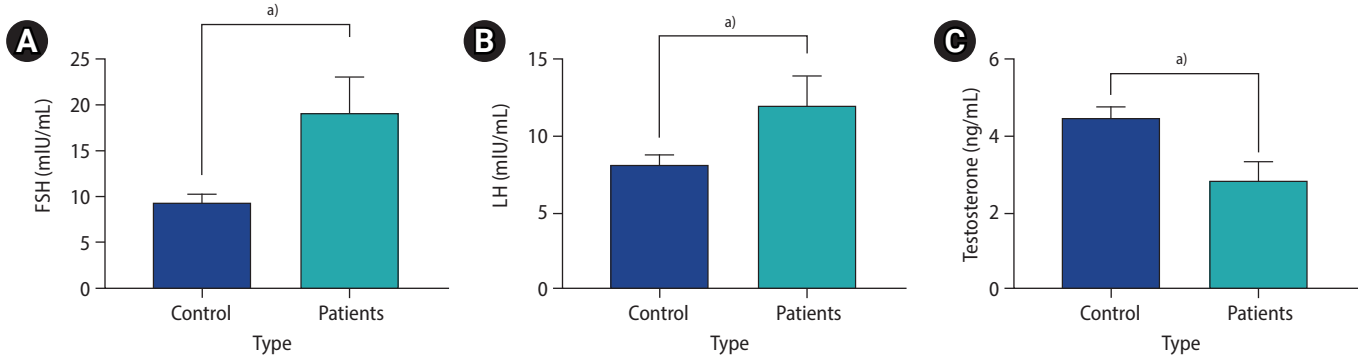

Figure 5. (A, B) Serum gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) and (C) testosterone levels were significantly different (p<0.001) in infertile patients when compared to healthy male controls. [a]p<0.0001.

https://doi.org/10.5653/cerm.2023.06219

levels were significantly lower in infertile patients than in healthy controls ($p<0.001$). In addition, testosterone levels were significantly lower ($p<0.001$) (Figure 5).

Discussion

Azoospermia, which affects roughly 1% of males, contributes significantly to male infertility [24]. Over the past 40 years, the diagnostic tools for identifying azoospermia have included standard tests for Y chromosome microdeletions, karyotype analysis, and a few monogenic investigations. Since the widespread adoption of whole-exome sequencing, increasingly many novel candidate genes associated with azoospermia have been identified, particularly in congenital HH [25].

The *TNP* gene is essential for maintaining the integrity of sperm DNA [8]. In chromatin remodeling and DNA compaction, *TNP* and *PRM* work together. In the postmeiotic phase of spermatogenesis, chromatin remodeling occurs as nucleosomal histones are initially replaced by TNPs (TNP1 and TNP2) and protamines (*PRM1* and *PRM2*) to facilitate extensive chromatin condensation. Any disruption in the transition of either *TNP1* or *TNP2* may result in elevated oxidative stress, DNA strand breakage, and ultimately azoospermia due to activation of the apoptotic pathway [26]. In addition, it was discovered that mir-122a can reduce *TNP2* expression during spermatogenesis. Arefnia et al. [27] demonstrated that the purpose of this miRNA is to inhibit gene expression by degrading the transcription of this gene.

One study concluded that the mRNA expression profile of *TNP2* and the hormonal parameters in men with azoospermia were significantly associated with the successful retrieval of spermatozoa. Amjad et al. [26] concluded that the probability of spermatozoa retrieval in patients with azoospermia is elevated when the mRNA expression profile of *TNP2* is increased. Heidari et al. [28] discovered that males with the CC genotype of the g.IVS1-26G>C SNP in the *TNP2* gene have a greater chance of developing varicocele than men without this genotype.

The relative expression level of *TNP2* in a study by Amor et al. [11] demonstrated a strong positive correlation with sperm count, progressive motility, and normal sperm morphology. They also suggested that smoking modifies the mRNA expression level of *TNP2*, which in turn alters normal sperm function [11].

In a study of genes associated with the VASA protein and protein-protein interactions, *TNP2* and *SYCP3* were identified as important genes causing infertility. Amirian et al. [17] observed that VASA and its interacting hub proteins may provide insights into the pathogenesis of aberrant germ cells and infertility. According to their findings, the *TNP2* gene expression level was reduced in infertile cells compared to normal cells, while the *SYCP3* gene expression level was increased [17].

Expression of the meiosis marker *SYCP3* has been observed in spermatocytes [29]. According to Dhulqarnain et al. [15], osteocalcin (OCN) increased the expression of *SYCP3* and enhanced spermatogenesis. Undercarboxylated OCN functions as a hormone, promoting insulin production, β-cell proliferation, and fertility [15]. Rybina et al. [30] suggested that one of the genetic causes of recurrent miscarriage is T657C polymorphism in the *SYCP3* mutation of the synaptonemal complex.

A prevalent genetic cause of male infertility is Y chromosome microdeletion in the three subregions of AZF. The occurrence of co-microdeletions is also possible in these subregions. Y chromosomal microdeletions have been identified in a varying number of infertile males around the world [31]. If AZF microdeletions are found in a patient's primary screening, further investigation is advised in the *AZFa* subregion [19,32]. Two genes, including ubiquitin specific peptidase 9 Y-linked (*USP9Y*) and *DBY*, also known as *DDX3Y*, are present in AZFa. Even in the earliest stages of development, it was significant to note that no *DDX3Y* expression was seen outside the tubules, indicating that the expression was only found in germ cells [33].

Y chromosomal microdeletions were found in a high percentage of infertile patients. Therefore, thorough evaluation of infertile males by semen analysis, hormonal assessment, and when necessary, karyotype analysis and physical examination may identify patients for whom a Y chromosome microdeletion study is required and also help to keep costs down [34]. Further studies are needed to confirm our results, and additional investigations are required to detect the effects of other polymorphisms and AZF region microdeletions on azoospermia.

This study investigated the genotype and allele frequencies of some gene polymorphisms (*TNP2* rs2857758 and *SYCP3* rs769825641) and microdeletions in the *AZFa* region to detect their associations with azoospermia in sterile males. The best control samples are individuals with confirmed fertility. However, in this study the control subjects had never been referred to infertility facilities for mTESE surgery; thus, we chose individuals with normal spermatogenesis samples as the control group.

Although we found no significant association between *TNP2* rs2857758 polymorphism and azoospermia, the homozygous genotype TT for *SYCP3* rs769825641 polymorphism was significantly associated with the incidence of azoospermia. Furthermore, analysis of Y chromosomal microdeletions can help clinicians counsel infertile patients before planning assisted reproductive techniques. The deletion of the AZFa region results in Sertoli cells, and there was an 8% deletion in the AZFa region using sY84 and sY86 markers.

In the current investigation, the gene expression profile was also

examined. Significant downregulation of *SYCP3* and *DDX3Y* expression was identified in the testis tissue of infertile males. In contrast, *TNP2* downregulation was not statistically significant in patients with azoospermia compared to the control group. Moreover, ROC curve results suggested that *SYCP3* and *DDX3Y* could be effective biomarkers for the diagnosis of azoospermia.

Conflict of interest

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

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ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06100
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):262-269

CERM

Effects of a short abstinence period on sperm quality in oligozoospermic men

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Objective: The aim of this study was to compare semen parameters and sperm DNA fragmentation (SDF) and explore the relationship between semen parameters and SDF between 2 and 7 days of abstinence and a short abstinence period (within 4 hours) in oligozoospermic infertile patients.
Methods: Two semen samples were collected from infertile oligozoospermic men (n=34) after an abstinence period of 2 to 7 days and within 4 hours, respectively. Sperm parameters were compared between the two abstinence duration groups, including semen volume, sperm concentration, total sperm count, sperm motility, total motile sperm count (TMSC), morphology, and SDF.
Results: The semen volume, concentration, and total sperm count were significantly decreased after 4 hours of abstinence than after 2 to 7 days of abstinence, with median differences of 1.2 mL (p<0.001), 2×10^6/mL (p=0.011), and 9.6×10^6/ejaculation (p<0.001), respectively. TMSC was significantly lower after a short abstinence, with a median difference of 4.24×10^6/ejaculate (p<0.001). However, there were no significance differences in the percentage of motility, the SDF, and the percentage of sperm with normal morphology. Interestingly, volume, concentration, total sperm count, sperm motility, and SDF, but not TMSC, exhibited significant linear correlations between the two abstinence groups in univariate regression analysis, except for TMSC.
Conclusion: In oligozoospermic men, the volume, concentration, and total sperm count were significantly lower after a short abstinence period, but without adverse effects on sperm motility and SDF.

Keywords: Oligozoospermic; Semen parameter; Short abstinence; Sperm DNA fragmentation

Introduction

Male factor infertility is the sole cause of infertility in approximately 20% of infertile couples and an essential contributing factor in another 20% to 40% of couples with reproductive failure [1]. In the initial evaluation, at least one proper semen analysis should be performed. If an abnormality is found, another semen analysis should be performed after at least 4 weeks [2]. Oligozoospermia is the most common abnormality and is typically a crucial contributing factor in men classified as infertile [3]. To solve infertility problems, assisted reproductive technologies (ARTs), such as intrauterine insemination (IUI), *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI), have been developed. These procedures require sperm preparation to concentrate motile spermatozoa. Progressive motility and the total motile sperm count (TMSC) are the initial sperm characteristics most closely related to pregnancy. IUI is an effective therapy for male factor infertility when initial sperm motility is ≥30%, and the TMSC is ≥5×10^6 per ejaculate [4]. IVF is preferable to IUI when the initial values are lower because of its higher effectiveness and cost-effectiveness. ICSI generally serves as a robust bypass procedure instead of a first-line treatment, and it has dramatically improved the fertility prospects in patients with oligozoospermia [5]. However, standard semen analysis may not completely provide all information to evaluate male fertility status, as 15% of patients with male factor

Received: April 26, 2023 · Revised: July 8, 2023 · Accepted: July 25, 2023
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*The study was financially supported by the Research Fund of the Faculty of Medicine Ramathibodi Hospital.

infertility were found to have normal semen analysis results [6]. Therefore, additional tests should be performed in addition to using the results of the semen analysis alone. Extensive research has been conducted during the past decade is the integrity of sperm DNA. Several studies have shown that sperm DNA fragmentation (SDF) had a statistically significant negative impact on the chance of pregnancy [7].

Human sperm is produced in the seminiferous tubules and stored in the epididymis for future release. The epididymal transit time has been estimated to range from 2 to 11 days. Variation is influenced by the frequency of ejaculation [8]. During epididymal transit and storage, spermatozoa are significantly exposed to reactive oxygen species (ROS) [9]. Prolonged exposure to ROS results in an alteration of sperm function and fertilizing capacity. They also affect the sperm genome, causing high frequencies of single- and double-strand DNA breaks [10]. The World Health Organization (WHO) recommends 2 to 7 days of abstinence before collection for standard semen analysis [11]. However, recent studies have suggested that shorter abstinence is associated with improved ART outcomes [12-16]. A large cohort study in normozoospermic subfertile men showed that longer abstinence was associated with increased ejaculate volume, concentration, total sperm count, and TMSC. However, in oligozoospermic men, longer abstinence time was not associated with improvements in semen parameters, except for ejaculate volume [17]. Prolonged sexual abstinence can have a negative impact on motility, viability, and SDF [18]. However, a significant increase in total and progressive motility was observed after a short abstinence period of 4 hours [19]. One day of abstinence improved sperm quality by protecting against ROS damage and a higher total seminal antioxidant capacity [20]. There is a lack of consensus on the exact impact of the abstinence period on the conventional and functional parameters of sperm. Thus, the effect of sexual abstinence on sperm parameters is still debatable [18,21-23].

The association between short abstinence and semen quality in oligozoospermic men has been evaluated in some studies, but the results have remained inconclusive. Therefore, we conducted a prospective experimental study in oligozoospermic men to compare sperm parameters and SDF in semen samples after 2 to 7 days of abstinence and within 4 hours of abstinence and explore the relationship between semen parameters and SDF between the two abstinence periods.

Methods

1. Study population and sample size

This single center prospective experimental study enrolled 42 oligozoospermic men aged 20 to 45 years with infertility problems visiting the Assisted Reproductive Technology Clinic, Ramathibodi Hospital, Bangkok, Thailand, from February 2021 to February 2022. The study was approved by the Committee on Human Rights Related to Research Involving Human Subjects at Ramathibodi Hospital, Mahidol University (MURA 2020/1895). Written informed consent was obtained from all patients.

2. Inclusion criteria

Men who met all inclusion criteria were invited to participate in the study.

(1) Men aged 20 to 45 years with infertility that had lasted for more than 1 year at the time of screening.
(2) At least one previous semen analysis result with a sperm concentration less than 15×10^6/mL with an abstinence period of 2 to 7 days.
(3) A semen volume of at least 1.5 mL per ejaculation at the screening.
(4) No history of previous testicular/penile surgery or vasectomy.
(5) No history of cancer, no history of radiation therapy, or chemotherapy.

Volunteers who met all inclusion criteria were asked to abstain for 2 to 7 days to collect study samples. Furthermore, the study sample collection had to be collected for at least 4 weeks from the previous semen analysis. Semen and SDF analyses were performed from semen after the abstinence for 2 to 7 days (referred to as the standard abstinence sample or sample A) and from semen collected within a 4-hour interval from sample A (referred to as the short abstinence sample or sample B) (Figure 1).

3. Exclusion criteria

The volunteers were excluded from the study analysis if they had one of the following.

(1) Sperm concentration $\geq15\times10^6$/mL when repeated at least 4 weeks after a previous semen analysis.
(2) Inability to ejaculate by masturbation.
(3) Azoospermia from sample A.

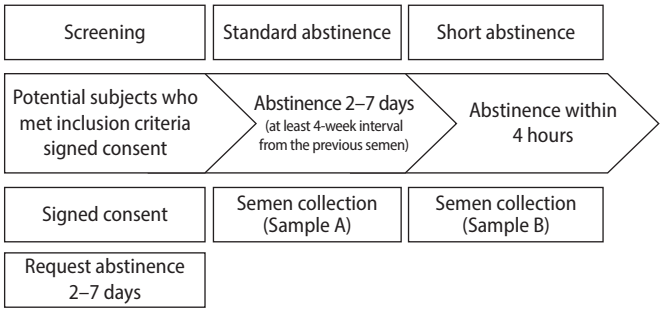

Figure 1. Study flowchart.

4. Semen collection and analysis procedure

Semen samples were collected from the volunteers by masturbation and provided routine information on the duration of abstinence to technicians. After liquefaction, the semen volume and pH were measured. Sperm motility analysis was performed on a 10-μL drop on a glass slide with a 22×22 mm coverslip for two replicates. Sperm motility was analyzed with phase-contrast optics at ×200 magnification with an eyepiece reticle. At least 200 sperm were evaluated in each replicate to determine the percentage of different motility categories according to the WHO 2010 guideline [11].

Sperm concentration measurement was performed by diluting a 50-μL semen sample with fixative. Sperm counting was then performed using an improved Neubauer hemocytometer. At least 200 spermatozoa per replicate were counted and compared whether the difference is acceptable or not according to WHO 2010 standard. If so, proceed to calculate the concentration in spermatozoa per mL; if not, prepare a new dilution.

Sperm morphology was evaluated by eosin/methylene blue staining (Dip Quick Stain; SE Synergist) using bright field optics at ×1,000 magnification with an oil immersion microscope based on Kruger's strict criteria.

5. SDF assessment using a TUNEL assay and flow cytometry

SDF was evaluated using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay with an APO-DIRECT Kit (BD Biosciences), as described by Sharma et al. [24]. Spermatozoa within the propidium iodide (PI)/RNase solution were analyzed by flow cytometry (BD FACSVerse Flow Cytometer). The output data were imported and analyzed using BD FACSuit software with a 488 nm argon laser as the light source. Two dyes were used: PI, which stains total DNA, and fluorescein isothiocyanate (FITC-dUTP), which stains broken DNA. PI fluoresces at about 623 nm and FITC at 520 nm. The results were expressed as the percentage of SDF. The Supplementary Figure 1 shows the measurement of SDF.

6. Statistical analysis

Statistical analyses were performed using STATA Statistics for Windows version 14.0 (StataCorp LLC). For continuous variables, the mean with standard deviation (SD) or median with interquartile range (IQR) were used for data presentation as appropriate. Frequencies with percentages were used to describe categorical data. The paired t-test or Wilcoxon signed-rank test was used to compare continuous data from the short and 2- to 7-day periods of abstinence. Linear regression analysis explored the linear relationship between parameters with both abstinence periods. The level of statistical significance was set at $p<0.05$.

Results

1. Baseline characteristics of semen parameters at screening

Forty-two men with at least one previous examination indicating oligozoospermia who met the study inclusion were invited and consented to participate in the study. Eight volunteers were excluded from the study analysis because sample A showed a sperm concentration ≥15×10^6/mL. Therefore, 34 volunteers were included in the study (Figure 2).

The mean±SD age of the oligozoospermic men in this study was 37.08±5.05 years, with the median of semen volume, concentration, and total sperm count of 2.75 mL (IQR, 1.6 to 3.4), 6×10^6/mL (IQR, 3 to 10), and 13.6 ×10^6/ejaculate (IQR, 7.25 to 26.6), respectively. The mean±SD of total motility was 46.64%±15.62%. The baseline characteristics of the semen parameters are shown in Table 1.

2. Comparison of semen parameters and SDF between 2 and 7 days and short abstinence

Semen volume, concentration, and total sperm count were significantly lower after the short abstinence period than after 2 to 7 days of abstinence. The median differences in the volume, concentration, and total sperm count after the short abstinence period were 1.2 mL ($p<0.001$), 2×10^6/mL ($p=0.011$), and 9.6×10^6/ejaculate ($p<0.001$), respectively. The percentage of non-progressive motility was significantly higher after the short abstinence period ($p<0.036$), while

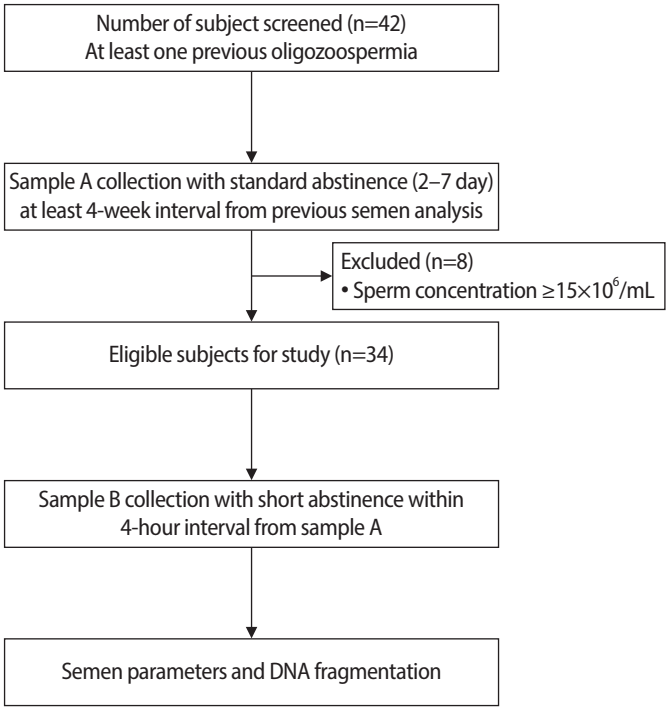

Figure 2. Participant flowchart.

https://doi.org/10.5653/cerm.2023.06100

there was no difference in the percentage of immotile sperm (p=0.694). The TMSC was significantly lower after the short abstinence period than after 2 to 7 days of abstinence, with a median difference of 4.24×10^6/ejaculate (p<0.001). There were no significant differences in the percentage of total motility, the percentage of SDF, and the percentage of normal morphology (Table 2).

3. Relationship of semen parameters and SDF between 2 and 7 days and short abstinence

Univariate regression showed significant linear relationships of volume, concentration, total sperm count, sperm motility, and SDF between the samples from 2 to 7 days of abstinence and those from the short abstinence period, except for TMSC (Figure 3). The Supplementary Figure 2 shows the SDF results of each semens.

Table 1. Baseline characteristics of semen parameters at screening (n=34)

Baseline characteristic	Value
Age (yr)	37.08 ± 5.05
Abstinence (day)	3 (2–3)
Volume (mL)	2.75 (1.6–3.4)
Concentration (10^6/mL)	6 (3–10)
Total sperm count (10^6/ejaculate)	13.6 (7.25–26.6)
Motility (%)	46.64 ± 15.62
Progressive (%)	34.79 ± 17.79
Non-progressive (%)	10 (5–14)
Immotile (%)	53.50 ± 15.63
Total motile sperm count (10^6/ejaculate)	5.57 (3–11.2)
Normal morphology	
1%	30 (88.24)
2%	4 (11.76)

Values are presented as mean±standard deviation, median (interquartile range), or number (%).

Discussion

In this study, in oligozoospermic men, a short abstinence period (within 4 hours) was associated with lower semen volume, concentration, and total sperm count than the values observed after 2 to 7 days of abstinence. The median differences in semen volume, concentration, and total sperm count in short abstinence were 1.2 mL, 2×10^6/mL, and 9.60×10^6/ejaculate, respectively. Furthermore, there were significant linear relationships in volume, concentration, and total count between the two abstinence periods. Hence, repeated semen collection within 4 hours is likely to provide comparable or lower results in terms of semen volume, sperm count, and sperm motility. Most previous studies in normozoospermic men found that shorter abstinence resulted in lower volume, concentration, and total sperm count [17,19,21,25,26]. However, studies in men with oligozoospermia have inconsistent regarding whether shorter abstinence decreases or increases semen volume, concentration, and total count [15,26,27]. Studies in infertile men have shown that the duration of abstinence had a statistically significant favorable influence on semen volume, sperm concentration, and total sperm count [13,14,18]. The results of the present study appeared to be consistent with most studies in normozoospermic and infertile samples, showing that shorter abstinence resulted in lower semen volume, concen-

Table 2. Comparison of semen parameters and SDF between the standard and short abstinence periods (n=34)

Parameter	Abstinence		Difference	p-value
	Standard (2–7 days) (Sample A)	Short (within 4 hours) (Sample B)		
Abstinence (hr)	66.24 ± 18.72	2.63 ± 0.50		
Volume (mL)	3 (2 to 3.8)	1.45 (1 to 2.1)	–1.2 (–1.90 to –0.90)[d]	< 0.001[a,b]
Concentration (10^6/mL)	7.45 (2.20 to 12.4)	3.68 (1.6 to 8.3)	–2 (–5.07 to 0.25)[d]	0.011[a,b]
Total sperm count (10^6/ejaculate)	21.36 (8.14 to 27.39)	5.04 (1.86 to 12.1)	–9.60 (–21.30 to –3.36)[d]	< 0.001[a,b]
Motility (%)	43.73 ± 15.96	44.64 ± 16.39	0.90 (–5.24 to 7.06)[e]	0.766[c]
Progressive (%)	32.92 ± 14.34	30.75 ± 12.30	–2.17 (–7.70 to 3.35)[e]	0.430[c]
Non-progressive (%)	9.6 (6 to 15)	13.5 (8 to 20)	2.5 (–2 to 11)[d]	0.036[a,b]
Immotile (%)	56.26 ± 15.96	55.06 ± 15.98	–1.20 (–7.37 to 4.96)[e]	0.694[c]
Total motile sperm count (10^6/ejaculate)	8.74 (3 to 13.20)	2.13 (0.60 to 5.55)	–4.24 (–9.39 to –0.72)[d]	< 0.001[a,b]
SDF (%)	40.26 ± 16.64	41.64 ± 16.89	0.93 (–3.50 to 5.38)[e]	0.670[c]
Normal morphology				
1%	34 (100)	34 (100)		

Values are presented as mean±standard deviation, median (interquartile range), or number (%).
SDF, sperm DNA fragmentation.
[a]The p-value was calculated by Wilcoxon signed-rank test; [b]Significant; [c]p-value was calculated by the paired t-test; [d]Median (interquartile range); [e]Mean (95% confidence interval).

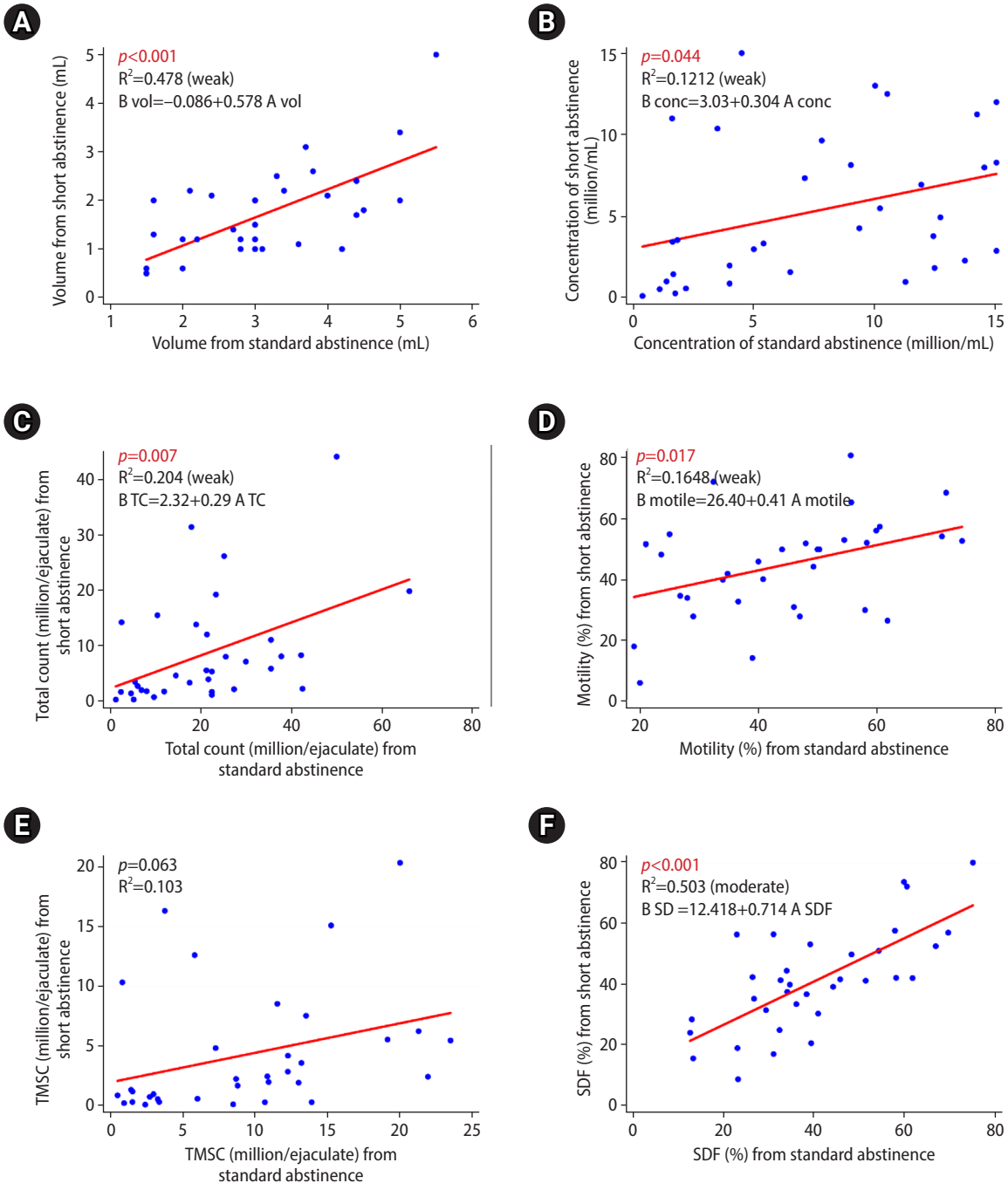

Figure 3. Scatter plot and linear regression line of semen parameters: (A) volume, (B) concentration, (C) total count, (D) motility, (E) total motile sperm count (TMSC), and (F) sperm DNA fragmentation (SDF) between standard and short abstinence periods.

tration, and total sperm count. The reduction in sperm concentration and total count in the short abstinence ejaculate may be due to the lack of time to transfer spermatozoa from the more proximal sections of the epididymis to the cauda and vas deferens, as well as semen volume, the majority of which is produced in the seminal vesicles and prostate gland.

This study showed that the percentage of non-progressive motility was significantly higher in the short abstinence group, while there was no significant difference in the percentage of immotile sperm. The TMSC was significantly lower in the short abstinence group than after 2 to 7 days of abstinence, with a median difference of 4.24×10^6/ejaculate. Univariate regression also showed significant linear rela-

https://doi.org/10.5653/cerm.2023.06100

tionships of sperm motility between 2 and 7 days and short abstinence, but no linear relationship of TMSC was detected. In terms of motility, the results of the present study differ from those of most previous studies. Previous studies in normozoospermic men showed a significant decrease in the percentage of sperm motility on days 11 to 14 of sexual abstinence compared with abstinence of fewer than 11 days [23]. In other words, shorter abstinence was associated with better sperm motility. Short abstinence (4 hours) was associated with significantly higher values of the total and progressive motility and velocity parameters [19]. Short abstinence (2 hours) showed higher velocity, progressiveness, and hyperactivation [25]. In addition, 1 day of abstinence showed better sperm motility than 4 days of abstinence [28]. However, another study found no difference in total motility and TMSC between 3–5 days and 18–30 hours of sexual abstinence [29]. Previous studies of patients with oligozoospermia or oligoasthenoteratozoospermia showed better motility with shorter abstinence periods [15,26,27]. A maximum mean sperm motility of 30.3% was observed after 1 day of abstinence [23]. Short abstinence (40 minutes) improved progressive grade A motility [27]. The sperm motility and TMSC of the second ejaculation (after 30 to 60 minutes of abstinence) were significantly higher than those of the first ejaculation (after 3 to 5 days) [15]. Sexual abstinence of less than 24 hours showed the highest mean percentage of progressively motile sperm [26]. The significant increase in non-progressive motility after a short abstinence period from this study is inconsistent with the findings of most previous studies. In oligozoospermic men, the sperm transport time through the epididymis was three times longer than in normozoospermic men. In addition, it has been suggested that some patients with supposed idiopathic testicular failure might have a partial obstruction [23]. Therefore, spermatozoa may be exposed to a high level of ROS for a more extended period in the genital tract, which might have a deleterious effect on sperm motility. However, there were remarkable variations between the duration of the short abstinence periods and the control group among the previous studies compared to our study.

This study included only oligozoospermic men; all samples from the 2 to 7 days and short abstinence periods had a 1% proportion of normal morphology according to Kruger's strict criteria. A large study in 1,621 normozoospermic samples revealed no difference in morphology according to abstinence time [26]. Most studies in men with oligozoospermia have shown an increase in normal morphology with short abstinence [15,23,27]. However, a study in 416 oligozoospermic samples did not find a significant difference in the percentage of normal sperm morphology during different abstinence times. Nonetheless, an abstinence period of less than 24 hours was associated with the highest mean percentage of normal sperm morphology [26]. This study could not compare morphology because all oligozoospermic samples had 1% proportions of normal morphology. A high level of SDF should cause this result in all samples; furthermore, we analyzed a small number of samples. The percentage of normal sperm morphology showed a significant negative correlation with the percentage of SDF [30].

There was no significant difference in the percentage of SDF in this present study between the short abstinence period and 2 to 7 days of abstinence. Most studies in normozoospermic men showed an improvement in SDF with short abstinence periods [18,21,22,29,31]. However, a study of 100 normozoospermic men found no differences in SDF between an abstinence period of 4 days and 4 hours [19]. A study that included both normozoospermic and oligozoospermic men showed lower SDF after a short abstinence period. However, the study did not perform a subgroup analysis for each group [31]. The results from the present study in terms of SDF are inconsistent with previous study results. Different methodologies for detecting SDF (e.g., TUNEL assay, the comet assay, the sperm chromatin structure assay, and the sperm chromatin dispersion test) may also be responsible for these contradictory findings. Since the etiology of SDF is multi-factorial, involving intrinsic and/or extrinsic factors [32], a persistently high level of SDF despite short abstinence in this study might reflect severe damage to spermatozoa due to problems with the sperm chromatin packaging process during spermatogenesis.

1. Strengths

Most previous studies investigating the influence of ejaculatory abstinence on semen parameters were retrospective and based on the results of a single semen analysis. In this study, we conducted prospective research on semen parameters in the same participants who had confirmed oligozoospermia by repeating the semen analysis after at least 4 weeks to avoid enrolling individuals with significant discrepancies in sperm concentration.

There may have been substantial variation in sperm counting chambers and counting techniques in many studies, which could have led to significant overestimations or underestimations of sperm motility and concentration, especially in oligozoospermic samples [11,33]. Therefore, we performed semen analysis based on the standard WHO 2010 guideline [11], which provides the most accurate results. We excluded men with a semen volume of less than 1.5 mL/ejaculate associated with ductal obstruction or incomplete samples. The TUNEL assay, a direct assay to assess DNA fragmentation, was used with flow cytometry; this method provides objective and accurate results with minimal interobserver variability, can be performed on a few sperm, and has a high level of experimental repeatability.

2. Limitations

The results of this study were obtained from a single center and

with a relatively low number of men. In addition, the participants in this study were 45 years old or younger. Therefore, the results could not be generalized to oligozoospermic men >45 years of age. Furthermore, we did not have data on ROS testing and other functional semen parameters. Additionally, sperm parameters, as intermediate outcomes for infertility treatment, may not reflex the pregnancy outcomes.

3. Conclusions

This study in oligozoospermic men showed that concentration, volume, total count, and TMSC after a short abstinence period were significantly lower than after 2 to 7 days of abstinence. There were no significant differences in the percentage of total sperm motility and SDF between 4 hours and 2–7 days of abstinence. Significant linear relationships were found for volume, concentration, total sperm count, sperm motility, and SDF, but not TMSC, between both groups in the univariate regression analysis. A short abstinence period did not have a negative effect on sperm quality, as represented by sperm motility and SDF. The IVF cycle for oligozoospermic men can be improved by consecutive semen collection within 4 hours in addition to the first semen sample in some circumstances.

Conflict of interest

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

Acknowledgments

We thank the entire Ramathibodi IVF Laboratory staff for their technical assistance.

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Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2023.06100.

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ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06030
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):270-276

CERM

Clinical and laboratory factors associated with the presence of dysmorphic oocytes in intracytoplasmic sperm injection cycles

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Objective: This study investigated the clinical and laboratory factors associated with the presence of dysmorphic oocytes in intracytoplasmic sperm injection (ICSI) cycles.
Methods: The study involved 200 ICSI cycles, performed from 2020 to 2021, that yielded at least one mature oocyte. Clinical characteristics and ovarian stimulation methods were compared between 68 cycles with at least one dysmorphic oocyte (the dysmorphic group) and 132 cycles with normal-form oocytes only (the non-dysmorphic group). Dysmorphic oocytes were characterized by dark cytoplasm, cytoplasmic granularity, cytoplasmic vacuoles, refractile bodies in the cytoplasm, smooth endoplasmic reticulum in the cytoplasm, an oval shape, an abnormal zona pellucida, a large perivitelline space, debris in the perivitelline space, or an abnormal polar body.
Results: The ages of the women, indications for *in vitro* fertilization, serum anti-Müllerian hormone levels, and rates of current ovarian endometrioma were similar between the dysmorphic and non-dysmorphic groups. In both groups, the three ovarian stimulation regimens, two types of pituitary suppression, and total gonadotropin dose were employed similarly. However, the dual-trigger method was used more frequently in the dysmorphic group (67.6% vs. 50%, p=0.024). The dysmorphic group contained significantly more immature oocytes and exhibited significantly lower oocyte maturity (50% vs. 66.7%, p=0.001) than the non-dysmorphic cycles. Within the dysmorphic group, significantly lower oocyte maturity was found in the cycles using a dual-trigger, but not in those with a human chorionic gonadotropin trigger.
Conclusion: ICSI cycles with dysmorphic oocytes are closely associated with reduced oocyte maturity. This association was observed exclusively in dual-trigger cycles.

Keywords: Assisted reproductive techniques; Intracytoplasmic sperm injections; In vitro oocyte maturation;Oocyte retrieval

Introduction

Obtaining good-quality metaphase II oocytes is an essential prerequisite for human *in vitro* fertilization (IVF) programs. Several criteria are used to determine the quality of a mature oocyte. These include the compactness and thickness of the cumulus-oocyte complex; the brightness of the cytoplasm; the granularity and clustering of organelles within the cytoplasm; the polar body (PB) shape, size, and appearance; the thickness and structure of the zona pellucida (ZP); the size and granulation of the perivitelline space (PVS); and the location and refraction of meiotic spindles [1,2]. Typically, a mature oocyte with clear or moderately granular cytoplasm, a narrow PVS, a normal PB shape, and a colorless and birefringent ZP is considered to be of good-quality [3].

Dysmorphic oocytes can be categorized based on various characteristics, including a dark cytoplasm, cytoplasmic granularity, cytoplasmic vacuoles, refractile bodies in the cytoplasm, the presence of smooth endoplasmic reticulum (SER) in the cytoplasm, an oval

Received: March 20, 2023 · Revised: June 15, 2023 · Accepted: July 10, 2023
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shape, an abnormal ZP, a large PVS, debris in the PVS, or an abnormal PB [4]. While some oocytes display only one type of dysmorphism, others may present with two or more abnormalities.

In our previous report, we noted a 58% (58 of 100) incidence of dysmorphic oocytes in 35 intracytoplasmic sperm injection (ICSI) cycles, each of which yielded at least one dysmorphic oocyte. However, when considering all 154 ICSI cycles, including those that produced only oocytes of normal-form, the incidence rate dropped to 10.7% (58 of 541) [4].

Numerous studies have revealed that fertilization and embryonic development are comparable between dysmorphic and normal-form oocytes [2,5,6]. Separate research has indicated that the morphology of the first PB does not negatively impact embryo development [7,8].

However, in other studies, oocytes with dysmorphic characteristics have been found to exhibit a lower fertilization rate compared to normal-form oocytes [9-12]. In a study by Rienzi et al. [12], the presence of vacuoles, an abnormal first PB, and a large PVS were associated with a decreased fertilization rate. Regarding embryo development and quality, the presence of SER clusters, a large PVS, and shape abnormalities are considered poor prognostic factors [13].

We previously reported that dysmorphic oocytes exhibited a significantly lower fertilization and cleavage rate, even with ICSI applied. However, these oocytes demonstrated a comparable rate of producing top- or good-quality embryos to that of normal-form oocytes [4]. Our report also indicated that oocytes with dark cytoplasm, abnormal PBs, or cytoplasmic vacuoles had a favorable prognosis, as evidenced by the percentage of top-quality embryos produced [4].

The origin of morphological abnormalities in oocytes remains largely unknown, but it is likely multifactorial. Intrinsic factors, such as age and genetic defects, as well as extrinsic factors, such as the ovarian stimulation protocol or handling procedures following oocyte retrieval, have been suggested [14].

Several studies have been conducted on the conditions associated with higher retrieval of dysmorphic oocytes. In one prospective study, the rate of dysmorphic oocytes was found to be similar between a group with two or fewer immature oocytes and a group with more than two immature oocytes. However, a wider PVS was more commonly observed in the group with two or fewer immature oocytes [15].

A retrospective study revealed that the serum anti-Müllerian hormone (AMH) level was inversely associated with cytoplasm granulation, abnormally amorphous oocytes, extended PVS, granulated PVS, fragmented PB, and oocyte morphology score as represented by the average oocyte quality index (AOQI) [16]. The AOQI was established by Sigala et al. [17] in 2015. This index is calculated by counting the number of abnormalities in oocyte morphology across seven categories: cytoplasmic granularity, irregular shape or thickened ZP, presence of intracytoplasmic vacuoles, materials in the PVS, anomalies of the first PB, large PVS, and oocyte shape. The index is then calculated as the ratio of the total number of abnormalities to the number of metaphase II oocytes [17].

In another retrospective study, the AOQI was found to be similar between women with and without endometriosis. However, two specific abnormalities—abnormal oocyte shape and intracytoplasmic vacuoles—were observed more frequently in women with endometriosis [18].

The objective of this study was to examine whether the presence of dysmorphic oocytes in ICSI cycles is associated with various clinical and laboratory factors. The clinical factors considered included the age of the woman, serum AMH level, diagnosis of endometriosis, dose or type of gonadotropin used, pituitary suppression methods, and triggering agents. The laboratory factors considered include the number and maturity of oocytes.

Methods

1. Study participants

We conducted a retrospective review of data from 200 ICSI cycles, involving 121 women, carried out at Seoul National University Bundang Hospital between 2020 and 2022. The selection criteria included: (1) the retrieval of at least one mature oocyte; (2) the use of recombinant follicle-stimulating hormone (FSH), urinary human menopausal gonadotropin, or a combination of both as the ovarian stimulation agent (excluding follitropin delta, mild stimulation, a combination of gonadotropins and oral agents, or a natural cycle); (3) the use of a gonadotropin-releasing hormone (GnRH) agonist or a GnRH antagonist for pituitary suppression; and (4) the use of human chorionic gonadotropin (hCG) or a combination of hCG and a GnRH agonist (that is, a dual method) for the final trigger. The Institutional Review Board of Seoul National University Bundang Hospital (B-2302-808-102) granted approval for this study. Written informed consent by the patients was waived due to a retrospective nature of our study.

An oocyte was classified as dysmorphic if it exhibited any of the following characteristics: dark cytoplasm, cytoplasmic granularity, cytoplasmic vacuoles, refractile bodies within the cytoplasm, SER in the cytoplasm, an oval shape, an abnormal ZP, a large PVS, debris within the PVS, or an abnormal PB. This classification is consistent with our previous report [4].

In 68 ICSI cycles, at least one dysmorphic oocyte was obtained; this was considered the dysmorphic group. In contrast, in 132 cycles, no dysmorphic oocytes were found (that is, only normal-form oocytes were present; this was considered the non-dysmorphic group).

Data regarding each woman, including age, body mass index, indications for IVF, endometriosis diagnosis, current presence of ovarian endometrioma, and serum AMH level, were gathered via chart review. Serum AMH levels were measured using fully automated AMH assays (Beckman Coulter or Roche Diagnostics).

2. Ovarian stimulation and oocyte retrieval

Ovarian stimulation was performed using one of the following regimens: recombinant FSH (Gonal-f, Merck-Serono; or GONADOPIN-NF, Donga-ST); highly purified urinary FSH (IVF-M; LG Chem); recombinant FSH in combination with any urinary gonadotropins (IVF-M or Menopur; Ferring).

Cycles stimulated with follitropin delta, recombinant FSH in conjunction with recombinant luteinizing hormone (Pergoveris; Merck-Serono), mild stimulation, any combination of gonadotropins, and any oral agents (including aromatase inhibitors or clomiphene citrate) were excluded. Cycles stimulated in combination with growth hormone were also excluded. Pituitary suppression was achieved using either a daily GnRH agonist long protocol (Decapeptyl; Ferring) or a flexible daily GnRH antagonist protocol (Cetrotide, Merck-Serono; or Ganirelix, LG Chem).

When the leading follicle reached a diameter of 18 to 19 mm, a final trigger was administered using either 250 μg of recombinant hCG (Ovidrel; Merck-Serono) or a combination of recombinant hCG and GnRH agonist (Decapeptyl 0.2 mg), also known as dual triggering. Oocytes were then retrieved 35 to 36 hours later. The oocytes were denuded using 85 IU/mL hyaluronidase (Cook) and mechanical pipetting. An oocyte was defined as mature if the first PB was present and the germinal vesicle was absent. In contrast, an oocyte was considered immature if it either contained a germinal vesicle or lacked both a germinal vesicle and the first PB.

3. Data analysis

Statistical analysis was performed using SPSS version 26.0 (IBM Corp). All variables were presented as the median (interquartile range), and the Mann-Whitney *U* test was employed to compare medians. The Pearson chi-square test or the Fisher exact test was used to compare proportions. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

Age, body mass index, indications for IVF, serum AMH levels, and the proportions of participants with serum AMH ≤1.0 ng/mL were similar between the dysmorphic and non-dysmorphic groups (Table 1). The proportions of endometriosis as an indication for IVF and the current presence of ovarian endometrioma were also similar between groups.

In both groups, the three types of ovarian stimulation agents (recombinant FSH, highly purified urinary FSH, and recombinant FSH in combination with any urinary gonadotropins) were used similarly. The total dose of gonadotropins was also comparable between groups.

The use of a GnRH antagonist for pituitary suppression was predominant in both groups (97.1% for the dysmorphic group vs. 94.7% for the non-dysmorphic group). However, the dual-trigger method was more frequently used in the dysmorphic group (67.6% vs. 50%, p=0.024).

While the total numbers of oocytes and mature oocytes were similar between the two groups, the dysmorphic group had a significantly higher number of immature oocytes, resulting in a significantly lower oocyte maturity rate (50% vs. 66.7%, p=0.001). In fact, the percentage of cycles with more than two immature oocytes was significantly higher in the dysmorphic group (48.5% vs. 31.8%, p=0.030).

Because the dual-trigger method was more frequently used in the dysmorphic group, we compared various clinical characteristics and ovarian stimulation outcomes between the cycles with an hCG-trigger (88 cycles) and those with a dual-trigger (112 cycles). As illustrated in Table 2, the dual-trigger group exhibited characteristics consistent with diminished ovarian reserve and/or poor ovarian response. Specifically, the women in this group were older and had lower serum AMH levels relative to the hCG-trigger group. Interestingly, despite higher gonadotropin usage in the dual-trigger group, both the serum estradiol level at trigger and the total number of oocytes were lower. Recombinant FSH was used less frequently in this group, while GnRH antagonist suppression was used more often. However, the number of immature oocytes was similar between the hCG-trigger and dual-trigger groups, as was the proportion of mature oocytes (60% vs. 62.5%, respectively). Notably, the proportion of cycles with dysmorphic oocytes present was significantly higher in the dual-trigger group (41.1% vs. 25%, p=0.024).

Given that the more frequent use of a dual-trigger in the dysmorphic group may act as a confounding factor, we compared the outcomes of ovarian stimulation between the dysmorphic and non-dysmorphic groups considering the trigger method used (Table 3). In the cycles with an hCG-trigger, the numbers of total, mature, and immature oocytes, as well as the oocyte maturity, were similar between the dysmorphic and non-dysmorphic groups. However, among the cycles with a dual-trigger, the dysmorphic group had a significantly higher number of immature oocytes and a significantly lower proportion of mature oocytes (50% vs. 66.7%, p<0.001). In fact, the percentage of cycles with more than two immature oocytes was significantly higher in the dysmorphic group (52.2% vs. 22.7%, p=0.002).

https://doi.org/10.5653/cerm.2023.06030

Table 1. Clinical characteristics and ovarian stimulation outcomes of dysmorphic and non-dysmorphic groups

Characteristic	Dysmorphic group (68 cycles)	Non-dysmorphic group (132 cycles)	*p*-value
Age (yr)	37 (34–39.8)	36.5 (34–40)	0.431
Body mass index (kg/m^2)	22.2 (20–24.4)	22.7 (21.1–26.1)	0.084
Serum AMH level (ng/mL)	1.4 (0.8–2.5)	1.4 (0.6–3.1)	0.468
No. of women with serum AMH < 1.0 ng/mL	28 (41.2)	50 (37.9)	0.451
Indication for *in vitro* fertilization			0.308
Male	4 (5.9)	7 (5.3)	
Tubal	3 (4.4)	10 (7.6)	
Ovulatory	4 (5.9)	3 (2.3)	
Endometriosis	23 (33.8)	34 (25.8)	
Unexplained	34 (50)	73 (55.3)	
Uterine	0	5 (3.8)	
Current endometrioma	12 (17.9)	17 (12.9)	0.396
Agent(s) for ovarian stimulation			0.072
Recombinant FSH	77 (58.3)	43 (63.2)	
Urinary FSH	49 (37.1)	17 (25)	
Recombinant+urinary FSH	6 (4.5)	8 (11.8)	
Dose of gonadotropins (IU)	2,400 (2,100–3,000)	2,400 (2,100–2,700)	0.184
Pituitary suppression			0.721
GnRH agonist, long	2 (2.9)	7 (5.3)	
GnRH antagonist	66 (97.1)	125 (94.7)	
Triggering method			0.024
hCG	22 (32.4)	66 (50)	
hCG+GnRH agonist (dual)	46 (67.6)	66 (50)	
Serum estradiol at trigger (pg/mL)	911 (586–1,870)	1,312 (671–2,050)	0.071
Serum progesterone at trigger (ng/mL)	0.6 (0.3–0.9)	0.6 (0.3–0.9)	0.372
No. of total oocytes	5 (3–8.8)	4 (2–8.8)	0.336
No. of mature oocytes	2 (1–4)	3 (1–5)	0.073
No. of immature oocytes	2 (1–4)	2 (1–3)	0.020
Oocyte maturity (%)	50 (33.3–66.7)	66.7 (50–77.1)	0.001
No. of cycles with immature oocytes ≤ 2	35 (51.5)	90 (68.2)	0.030
No. of cycles with immature oocytes > 2	33 (48.5)	42 (31.8)	

Values are presented as median (interquartile range) or number (%). Statistical significance ($p<0.05$) was determined using the Mann-Whitney *U* test for continuous variables and the Pearson chi-square test or Fisher exact test for nominal variables.
AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin.

Discussion

In this study, the dysmorphic group exhibited a significantly higher number of immature oocytes and significantly lower oocyte maturity than the non-dysmorphic group. The proportion of cycles with more than two immature oocytes was also significantly higher in the dysmorphic group. These findings suggest that the overall inferior quality of the oocyte pool could impact the acquisition of dysmorphic oocytes.

The dual-trigger method was utilized more often in the dysmorphic group in the present study. As a result, our initial comparison focused on the various clinical characteristics and outcomes of ovarian stimulation between the hCG-trigger group (88 cycles) and the dual-trigger group (112 cycles). Subsequently, we compared the outcomes of ovarian stimulation between the dysmorphic and the non-dysmorphic groups by the trigger method.

We discovered that the dual-trigger group exhibited peculiar characteristics consistent with reduced ovarian reserve and/or poor ovarian response. This is believed to reflect the physician's preference for a dual-trigger when conducting ovarian stimulation in women with diminished ovarian reserve or anticipated poor ovarian response. However, the quantity of immature oocytes and the level of oocyte maturity were comparable between the hCG-trigger and dual-trigger groups.

In a prior study, dual triggering resulted in a significantly higher number of mature oocytes and greater oocyte maturity than hCG-only triggering in young women with diminished ovarian reserve undergoing elective oocyte cryopreservation [19]. However, in

Table 2. Clinical characteristics and ovarian stimulation outcomes by trigger method

Characteristic	hCG-trigger (88 cycles)	Dual-trigger (112 cycles)	*p*-value
Age (yr)	36 (33–38.8)	37.5 (35–41)	0.005
Body mass index (kg/m^2)	22.3 (20.8–25.4)	22.6 (20–25.4)	0.299
Serum AMH level (ng/mL)	1.7 (1–3.3)	1.1 (0.6–2.5)	< 0.001
No. of women with serum AMH < 1.0 ng/mL	23 (26.1)	55 (49.1)	< 0.001
Indication for *in vitro* fertilization			0.034
Male	6 (6.8)	5 (4.5)	0.540
Tubal	9 (10.2)	4 (3.6)	0.082
Ovulatory	5 (5.7)	2 (1.8)	0.244
Endometriosis	26 (29.5)	31 (27.7)	0.875
Unexplained	38 (43.2)	69 (61.6)	0.011
Uterine	4 (4.5)	1 (0.9)	0.171
Current endometrioma	17 (19.3)	12 (10.7)	0.106
Agent(s) for ovarian stimulation			< 0.001
Recombinant FSH	65 (73.9)	55 (49.1)	< 0.001
Urinary FSH	23 (26.1)	43 (38.4)	0.071
Recombinant+urinary FSH	0	14 (12.5)	< 0.001
Dose of gonadotropins (IU)	2,400 (1,800–2,700)	2,400 (2,100–3,000)	< 0.001
Pituitary suppression			0.011
GnRH agonist, long	8 (9.1)	1 (0.9)	
GnRH antagonist	80 (90.9)	111 (99.1)	
Serum estradiol at trigger (pg/mL)	1,735 (739–2,259)	867 (638–1,851)	0.009
Serum progesterone at trigger (ng/mL)	0.6 (0.4–1.1)	0.5 (0.3–0.8)	0.021
No. of total oocytes	6 (3–9)	4 (2–8)	0.040
No. of mature oocytes	3 (2–5)	2 (1–4)	0.075
No. of immature oocytes	2 (1–4)	2 (1–3.8)	0.162
Oocyte maturity (%)	60 (50–75)	62.5 (42–75)	0.492
No. of cycles with immature oocytes ≤ 2	52 (59.1)	73 (65.2)	0.382
No. of cycles with immature oocytes > 2	36 (40.9)	39 (34.8)	
No. of cycles yielding dysmorphic oocyte	22 (25)	46 (41.1)	0.024

Values are presented as median (interquartile range) or number (%). Statistical significance ($p<0.05$) was determined using the Mann-Whitney *U* test for continuous variables and the Pearson chi-square test or Fisher exact test for nominal variables.
hCG, human chorionic gonadotropin; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone.

previous research conducted by our team, we found that dual triggering yielded a similar number of oocytes and comparable oocyte maturity to hCG-only triggering in women with various malignancies or endometrioma who also underwent elective oocyte cryopreservation [20]. A systematic review and meta-analysis additionally reported similar numbers of oocytes and oocyte maturity proportions between hCG-only and dual triggers [21]. In the present study, we likewise found a similar number of immature oocytes and similar oocyte maturity between hCG-only and dual-trigger cycles. While the aim of this study was not to evaluate the effectiveness of dual triggering, our findings suggest that a dual-trigger is not associated with lower oocyte maturity.

However, the proportion of cycles featuring dysmorphic oocytes was significantly higher in the group that underwent dual triggering. Thus, the dual-trigger method could be a contributing factor in the acquisition of dysmorphic oocytes.

Furthermore, in the dysmorphic group, a significantly higher number of immature oocytes or lower oocyte maturity was found, but this was observed only in the dual-trigger group and not in the hCG-trigger group.

Collectively, we postulated that the dual-trigger method may contribute to a greater acquisition of dysmorphic oocytes. Furthermore, we found a close association between the presence of dysmorphic oocytes and lower oocyte maturity in ICSI cycles utilizing dual triggering.

In a prior study, the oocyte morphology score, represented by AOQI, was found to be comparable between women with and without endometriosis [18]. We also observed that the proportion of endometriosis as an indication for IVF, as well as the current presence of ovarian endometrioma, were similar between the dysmorphic and

https://doi.org/10.5653/cerm.2023.06030

Table 3. Ovarian stimulation outcomes compared between dysmorphic and non-dysmorphic groups by trigger method

Variable	hCG-trigger			Dual-trigger		
	Dysmorphic group (22 cycles)	Non-dysmorphic group (66 cycles)	*p*-value	Dysmorphic group (46 cycles)	Non-dysmorphic group (66 cycles)	*p*-value
No. of total oocytes	5 (3–8.3)	6 (3–10)	0.236	5 (2.8–9)	4 (2–7.3)	0.082
No. of mature oocytes	2 (1.8–4)	3 (2–6)	0.077	2 (1–4)	2 (1–4.3)	0.312
No. of immature oocytes	2 (0.8–4)	2 (1–4)	0.444	3 (1–4)	1 (1–2)	0.001
Oocyte maturity (%)	55.6 (40–86.4)	61.3 (50–75)	0.350	50 (33.3–66.7)	66.7 (50–86)	< 0.001
No. of cycles with immature oocytes ≤ 2	13 (59.1)	39 (59.1)	1.000	22 (47.8)	51 (77.3)	0.002
No. of cycles with immature oocytes > 2	9 (40.9)	27 (40.9)		24 (52.2)	15 (22.7)	

Values are presented as median (interquartile range) or number (%). Statistical significance ($p<0.05$) was determined using the Mann-Whitney *U* test for continuous variables and the Pearson chi-square test or Fisher exact test for nominal variables.
hCG, human chorionic gonadotropin.

non-dysmorphic groups. Therefore, endometriosis may not be a considerable factor in the acquisition of dysmorphic oocytes.

A previous study indicated an inverse relationship between serum AMH level and oocyte morphology score, as represented by AOQI [16]. We did not evaluate the oocyte morphology score, so a direct comparison with our results was not possible. However, both the serum AMH levels and the proportion of women with diminished ovarian reserve were similar between the dysmorphic and non-dysmorphic groups. Therefore, it can be inferred that diminished ovarian reserve is not a contributing factor to the acquisition of dysmorphic oocytes.

In conclusion, we identified a close association between the presence of dysmorphic oocytes and lower oocyte maturity, particularly when dual triggering was used. Furthermore, this association between dysmorphic oocytes and lower oocyte maturity was observed only in ICSI cycles employing the dual-trigger method.

Conflict of interest

Byung Chul Jee has served as editor-in-chief of *Clinical and Experimental Reproductive Medicine* since 2018. However, he was not involved in the selection, evaluation, or decision-making process for the peer review of this article. No other potential conflicts of interest related to this article have been reported.

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https://doi.org/10.5653/cerm.2023.06030

ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06184
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):277-284

CERM

Factors affecting the ongoing pregnancy rate in women with repeated implantation failure undergoing an endometrial receptivity array

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Objective: In this retrospective study, we analyzed factors influencing the ongoing pregnancy rate (PR) in women with repeated implantation failure (RIF) undergoing embryo transfer with endometrial receptivity array (ERA).
Methods: Eighty-three consecutive personalized embryo transfers (pETs) with ERA, from 54 women with RIF, were selected from June 2020 to April 2022. Vitrified blastocyst transfer was timed based on ERA results.
Results: The ongoing PR per pET was 33.7%. Using ERA, the endometrium was identified as pre-receptive in 26 cycles, early receptive in 25 cycles, receptive in 31 cycles, and late receptive in one cycle. With cycles categorized into three receptivity phases (pre-receptive, early receptive, or receptive), no significant differences were found in the clinical PR (27.3%, 55.6%, and 40%, respectively) or ongoing PR (9.1%, 55.6%, and 40%, respectively) after a single blastocyst transfer. Similarly, no significant differences were observed in the clinical PR or ongoing PR after the transfer of two or more blastocysts. Among women with ongoing pregnancy relative to those without, age at first pET was significantly lower (35 years vs. 39 years, p=0.001), while blastocyst score (23 vs. 18, p=0.012) and the proportion of blastocyst scores >18 (71.4% vs. 38.9%, p=0.005) were significantly higher. In multiple logistic regression analysis, the woman's age (odds ratio [OR], 0.814; 95% confidence interval [CI], 0.706 to 0.940; p=0.005) and blastocyst score >18 (OR, 3.052; 95% CI, 1.075 to 8.665; p=0.036) were identified as significant factors influencing ongoing pregnancy.
Conclusion: In pET with ERA, ongoing pregnancy was closely associated with woman's age and blastocyst quality.

Keywords: Blastocyst; Embryo transfer; Endometrium; Pregnancy; Receptivity

Introduction

Repeated implantation failure (RIF) is typically defined as the inability to become pregnant after three or more embryo transfer (ET) cycles, despite the use of good-quality embryos. A receptive endometrium is crucial for successful implantation, and cross-communication between the endometrium and the embryo is also required. However, even euploid and morphologically good-quality blastocysts fail to implant in approximately one-third of transfer cases [1]. The endometrium must be in a receptive or acceptable state at the time of embryo implantation, a period known as the window of implantation (WOI), which typically occurs during the mid-luteal phase. If transfer is not conducted during the WOI, RIF may arise due to inadequate communication between the endometrium and the embryo.

Consequently, endeavors have been made to determine the timing of the receptive phase of the endometrium. A variety of "omics" techniques have been established to evaluate markers for DNA (genomics), messenger RNA (transcriptomics), and protein (proteomics) in the human endometrium during the WOI [2].

A previous study established the transcriptomic profiling of the endometrium throughout the menstrual cycle, including the WOI [3].

Received: May 24, 2023 · Revised: July 17, 2023 · Accepted: July 25, 2023
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The findings from these profiling analyses led to the development and patenting of the endometrial receptivity array (ERA) by Igenomix (Spain) in 2009. The ERA involves the analysis of 236 implantation-related DNA markers using next-generation sequencing techniques. A prediction program, based on the analysis of accumulated data, then indicates the appropriate timing for ET.

In artificial cycles supplemented with estradiol and progesterone treatment (EPT), an endometrial sample is obtained on day Progesterone (P)+5, at which point an ERA test is requested. Then, blastocysts are harvested and subsequently cryopreserved. These cryopreserved blastocysts are later transferred under the guidance of EPT, with the timing of this transfer determined based on the ERA results.

The ERA provides information on endometrial receptivity, allowing the endometrium to be categorized as receptive, non-receptive, early receptive, late receptive, pre-receptive, or post-receptive. The term "receptive" indicates that the endometrium is within the WOI at P+5 days, suitable for blastocyst transfer. "Early receptive" or "pre-receptive" signifies that the endometrium has not yet reached the WOI, suggesting that blastocyst transfer should occur slightly later than P+5 days. In contrast, "late receptive" or "post-receptive" indicates that the endometrium has passed the WOI, and the blastocyst transfer should thus take place slightly earlier than P+5 days.

The ERA offers insights into the optimal timing for blastocyst transfer for individual patients. Consequently, ET that is guided by the results of the ERA test is often referred to as personalized embryo transfer (pET).

In 2020, a multicenter randomized controlled trial examining the utility of pET using ERA was published [4]. The study involved women aged 37 years or younger, who were randomly assigned to three groups: pET (80 women), frozen ET (82 women), and fresh ET (94 women). Following the first ET, the clinical pregnancy rates (PRs) were 72.5%, 54.3% (p=0.01), and 58.5% (p=0.057), respectively. The live birth rates (LBRs) were 56.2%, 42.4% (p=0.09), and 45.7% (p=0.17), respectively. The cumulative LBRs within a 12-month period were 71.2%, 55.4% (p=0.04), and 48.9% (p=0.003), respectively, indicating a significant improvement in the pET group. However, if the LBR from the first ET is considered the most crucial evidence of efficacy, pET with ERA could be deemed ineffective.

A retrospective study published in 2021 enrolled women who were undergoing their first ET with a euploid embryo. The LBR demonstrated no significant difference, with a rate of 56.5% recorded in 147 pET cycles compared to 56.6% in 81 standard frozen ET cycles [5]. Another retrospective study from 2021 revealed no significant difference in LBR (49.6% in 133 pET cycles vs. 54.9% in 353 control cycles) [6].

A recent randomized clinical trial indicated that the ERA did not improve the ongoing PR from single euploid ET in an unselected population [7]. In that report, the clinical PR and LBR were 68.8% and 58.5%, respectively, in the pET group (n=381) and 72.8% and 61.9%, respectively, in the control group (n=386, p>0.05 for all). The studies mentioned above were not specifically targeted at patients with RIF. To date, very few studies have examined the efficacy of pET among such patients.

In patients with RIF, a multicenter retrospective study published in 2020 demonstrated no significant benefit of pET with ERA [8]. In that study, clinical *in vitro* fertilization (IVF) outcomes were compared among groups treated with preimplantation genetic testing for aneuploidy (PGT-A), ERA, PGT-A+ERA, and standard frozen ET (i.e., neither test). Among 2,110 patients with moderate RIF, defined as implantation failure after the transfer of three embryos, only the PGT-A group exhibited a significantly improved implantation rate and ongoing PR relative to the standard ET group. In 488 patients with severe RIF (defined as implantation failure after the transfer of five embryos), neither PGT-A nor ERA had a meaningful impact. In a subsequent study involving 255 patients with a single previous failed transfer, pET with ERA did not improve pregnancy outcomes when compared with non-pET controls, which included both fresh ET and frozen ET groups [9].

Similarly, another study demonstrated no significant improvement in clinical outcomes associated with pET using ERA [10]. That research indicated that neither the combination of PGT-A and ERA nor ERA alone improved the clinical PR compared to standard frozen ET in women with RIF. Treatment with PGT-A alone was associated with a statistically higher likelihood of achieving clinical pregnancy than frozen ET.

However, a separate retrospective study carried out in China demonstrated that pET with ERA yielded a significantly higher ongoing PR and implantation rate than standard frozen ET (p<0.01) among 281 patients with RIF [11]. Therefore, the utility of pET with ERA requires further clarification for both non-RIF and RIF populations. In a systematic review encompassing eight studies, Arian et al. [12] reported that the ongoing PR and LBR in the ERA group were comparable to those in the non-ERA group. This observation applied to groups with two prior unsuccessful ET attempts as well as those with more than two such attempts [12].

Conversely, it remains unclear which factors affect the establishment of ongoing pregnancy in patients with RIF receiving pET using ERA. The objective of this study was to identify and analyze the factors that impact ongoing pregnancy in this patient population.

Methods

An ERA test under EPT was performed at a single center (the iORA clinic). This study involved 54 women with RIF, defined as three or

https://doi.org/10.5653/cerm.2023.06184

more previous failed ETs. Based on the ERA results, 83 consecutive pETs were performed. All vitrified blastocyst transfer cycles took place between June 2020 and April 2022. The study received approval from the Institutional Review Board (IRB) at Seoul National University Bundang Hospital (IRB No. B-2301-802-101). Written informed consent by the patients was waived due to the retrospective nature of our study.

The mean number of previous failed ETs, despite the use of good-quality embryos, was seven cycles (range, 3 to 16). The mean age of the women at the time of the first pET was 37.7 years (range, 28 to 46). Any woman with a thin endometrium, measuring less than 7 mm, was excluded from the study.

In the cycle for endometrial biopsies, all women received artificial EPT. This involved the daily administration of 6 to 8 mg of estradiol valerate (Progynova; Bayer), which was initiated on the third or fourth day of the menstrual cycle. Once an endometrial thickness of more than 7 mm was achieved, a daily intramuscular injection of 100 mg progesterone (Sugest; Watson Laboratories Inc. or Taiyu P; Jaytech Biogen) was introduced. Following EPT, endometrial biopsies were conducted at P+5 days using a 5-mm silastic catheter, and the samples were subsequently stored at −4 °C. These samples were then sent to Igenomix-Korea for ERA testing, and the results were obtained after 2 to 3 weeks.

For blastocyst formation, ovarian stimulation was performed using recombinant follicle-stimulating hormone (FSH) (Gonal-F; Merck-Serono), recombinant FSH with recombinant luteinizing hormone (Pergoveris; Merck-Serono), or purified human menopausal gonadotropin (Menopur; Ferring). Depending on the situation, pituitary suppression was achieved using either a flexible gonadotropin-releasing hormone (GnRH) antagonist protocol or a luteal long GnRH agonist protocol. Once ultrasound monitoring revealed the presence of two or more follicles ≥18 mm in diameter, 250 μg of recombinant human chorionic gonadotropin (hCG) (Ovidrel; Merck-Serono) was administered. Oocyte retrieval was performed 36 to 38 hours after hCG injection. Mature oocytes were fertilized using the conventional method, split insemination, or intracytoplasmic sperm injection, as indicated. The blastocysts were obtained through sequential culture up to day 7; ultimately, 88 blastocysts were obtained on day 5, 51 on day 6, and three on day 7.

For blastocyst vitrification, sequential equilibrium solution (ES) and vitrification solution (VS) were used with a Cryo-Top device (Kitazato). Initially, the blastocysts were suspended in the ES, which contained 7.5% ethylene glycol (EG; Sigma-Aldrich) and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in basic medium (Global for Fertilization; Life Global). This suspension was maintained for 5 minutes at room temperature (RT). Subsequently, the blastocysts were transferred to the VS, which contained 15% EG, 15% DMSO, and 0.5 mol/L sucrose (Sigma-Aldrich) in a basic medium. This step was carried out for 45 to 60 seconds at RT. Following this, the blastocysts were loaded into the Cryo-Top and immediately submerged in liquid nitrogen for storage.

For blastocyst warming, the Cryo-Top was directly immersed in a warming solution at 37 °C, which contained 1.0 mol/L sucrose in basic medium, for 1 minute. The blastocysts were then immediately transferred to dilution solutions, which contained 0.5 and 0.25 mol/L sucrose in basic medium. These were serially incubated at RT for 3 minutes each, then washed twice with the basic medium. The warmed blastocysts were transferred to the culture medium (Sydney IVF Medium; Cook Medical), cultured until transfer at 37 °C, and kept in a humidified air environment with 5% CO_2.

For vitrified-warmed blastocyst transfer, all women received the same artificial EPT as that administered for endometrial biopsies. The endometrial thickness was consistently above 7 mm. Blastocysts were transferred based on the timing suggested by the ERA test results (Table 1). All blastocysts were warmed for 4 to 16 hours prior to transfer.

At the time of transfer, blastocysts were graded based on the developmental stage, quality of the inner cell mass, and quality of the trophectoderm [13]. We utilized a straightforward formula, developed at our center, to calculate the blastocyst score: (development score)×(inner cell mass or trophectoderm score). The development score was assigned as follows: early expanded blastocyst=1; middle expanded blastocyst=2; expanded blastocyst=3.5; hatching blasto-

Table 1. Timing of blastocyst transfer based on ERA results

Results of ERA	The time of blastocyst transfer	No. of cycles	The average no. of blastocyst transferred	Days of blastocyst-forming		
				Day 5	Day 6	Day 7
Pre-receptive	P+6 days	26	1.6±0.6	31	11	-
Early receptive	P+133 hours	25	1.8±0.7	24	19	1
Receptive	P+117 to P+123 hours	31	1.7±0.6	31	21	2
Late receptive	P+110 hours	1	2	2	-	-

Values are presented as mean±standard deviation.
ERA, endometrial receptivity array; P, progesterone.

cyst=5; and hatched blastocyst=6. The scores for the inner cell mass or trophectoderm were separately assigned based on a 4-grade scale, as follows: A=4; B=3; C=2; and D=1. For example, an expanded blastocyst with partial hatching and a grade B inner cell mass or trophectoderm received a score of 15. If two or more blastocysts were present, the total blastocyst score was calculated as the sum of the individual scores. Top-quality blastocysts were those that were expanded, hatching, or hatched, with assigned grades of A or B.

Serum hCG levels were measured at P+14 days. Pregnancy outcomes were categorized as clinical pregnancy, miscarriage, or ectopic pregnancy. A clinical pregnancy was defined by the identification of at least one gestational sac exhibiting a fetal heartbeat. Miscarriage was defined as the termination of a clinical pregnancy prior to the 12th week of gestation, and an ongoing pregnancy was defined as a clinical pregnancy that continued past 12 gestational weeks. The implantation rate was determined by dividing the number of gestational sacs by the number of transferred embryos.

Statistical analysis was performed using SPSS version 25 (IBM Corp.). All data were presented as the median and interquartile range. The Mann-Whitney *U* test was employed to compare numerical data between two groups. For comparisons among three groups, the Kruskal-Wallis test was utilized. The chi-square test or Fisher exact test was used to compare proportions between two groups. Multiple logistic regression analyses were performed to identify parameters influencing clinical or ongoing pregnancy. Receiver operating characteristic (ROC) curve analysis was conducted to determine cutoff values for specific parameters. A $p<0.05$ was considered to indicate statistical significance.

Results

According to the ERA results for the 54 patients, 22 were categorized as receptive, 15 as pre-receptive, 16 as early receptive, and one as late receptive. As such, 59.3% of women with RIF exhibited a displaced WOI. None of the women were classified as post-receptive.

Among the 54 women studied, a total of 83 consecutive pETs were performed, with a range of 1 to 6 procedures per individual. The mean number of pETs performed per woman was 1.6±1.0. In the analysis of IVF cycle characteristics and pregnancy outcomes, each pET cycle was treated as an independent event.

The overall clinical PR per pET was found to be 44.6% (37 of 83). Nine miscarriages occurred; therefore, the miscarriage rate per clinical pregnancy was 24.3% (nine of 37). Overall, 28 ongoing pregnancies were confirmed, yielding an ongoing PR per pET of 33.7% (28 of 83).

For the 83 pET cycles examined, Table 1 details the receptivity phase, the timing of blastocyst transfer, the average number of blastocysts transferred, and the number of days required for blastocyst formation. A total of 142 vitrified-warmed blastocysts were transferred across these cycles: in 30 cycles, one blastocyst was transferred; in 48 cycles, two were transferred; in four cycles, three were transferred; and in one cycle, four were transferred. Table 2 presents the clinical PR and ongoing PR, by receptivity phase, for cycles in which a single blastocyst was transferred. No significant differences were observed in the cycle characteristics or the clinical outcomes of IVF. Table 3 presents the clinical PR, ongoing PR, and implantation rate by receptivity phase for cycles in which at least two blastocysts were transferred. Similarly, no significant differences were noted in

Table 2. Pregnancy outcomes in 30 pET cycles in which one blastocyst was transferred

Variable	Pre-receptive (11 cycles)	Early receptive (9 cycles)	Receptive (10 cycles)	*p*-value
Woman's age at the time of first pET (yr)	41 (32-42)	34 (32.5–38)	37 (34–41)	0.262
No. of previous failed cycles	7 (5–8)	6 (4.5–7.5)	7 (5.8–8.5)	0.425
Days of blastocyst-forming				0.897
Day 5	9	7	7	
Day 6	2	2	3	
Day 7	0	0	0	
Blastocyst score at transfer	15 (7–18)	18 (6.8–18)	14 (9.3–21)	0.714
Interval from endometrial biopsy to pET (day)	52 (48–244)	54 (28–64)	49 (22–94)	0.353
Clinical pregnancy	3	5	4	
Miscarriage	2	0	0	
Ectopic pregnancy	1	0	0	
Clinical pregnancy rate per pET (%)	27.3	55.6	40	0.539
Ongoing pregnancy rate per pET (%)	9.1	55.6	40	0.129

Values are presented as median (interquartile range).
pET, personalized embryo transfer.

https://doi.org/10.5653/cerm.2023.06184

Table 3. Pregnancy outcomes in 52 pET cycles in which two or more blastocysts were transferred (excluding one cycle with late receptivity)

Variable	Pre-receptive (15 cycles)	Early receptive (16 cycles)	Receptive (21 cycles)	*p*-value
Woman's age at the time of first pET (yr)	41 (34–42)	37 (35–40.3)	37 (35–41)	0.544
No. of previous failed cycles	8 (6–10)	6 (4.3–8)	7 (4.5–8.5)	0.066
Days of blastocyst-forming				0.604
Day 5	22	17	24	
Day 6	9	17	18	
Day 7	0	1	2	
No. of blastocyst transferred	2 (2–2)	2 (2–2)	2 (2–2)	0.927
Total blastocyst score at transfer	22 (10.5–40)	23.8 (17.3–40.5)	24 (14.5–31.5)	0.337
Interval from endometrial biopsy to pET (day)	83 (51–237)	83 (27–161)	60 (27–149)	0.364
Clinical pregnancy	9	7	10	
Miscarriage	3	2	3	
Ectopic pregnancy	0	0	0	
Clinical pregnancy rate per pET (%)	60	43.8	52.4	0.505
Ongoing pregnancy rate per pET (%)	40	31.3	33.3	0.706
Implantation rate per embryo (%)	29	20	25	0.344

Values are presented as median (interquartile range).
pET, personalized embryo transfer.

the cycle characteristics or clinical IVF outcomes.

Among the 83 pET cycles, the total blastocyst score ranged from 3.5 to 48. Table 4 presents a comparison of seven parameters between those with and without clinical pregnancy, as well as between those with and without ongoing pregnancy. These parameters include the woman's age at first pET, the number of previous failed cycles, the proportion of cycles considered receptive, the number of blastocysts transferred, the total blastocyst score at the time of transfer, the proportion of total blastocyst scores at transfer that exceeded 18.0, and the interval from the endometrial biopsy to pET.

For clinical pregnancy, two factors—the total blastocyst score at transfer and the proportion of total blastocyst scores at transfer exceeding 18.0—exhibited significant differences between the two groups. Regarding ongoing pregnancy, three factors—the woman's age at first pET, total blastocyst score at transfer, and proportion of the total blastocyst scores at transfer surpassing 18.0—differed significantly between the groups.

As demonstrated in Table 5, multivariate logistic regression analysis indicated that a total blastocyst score greater than 18.0 at the time of transfer was the only factor significantly predicting clinical pregnancy. Furthermore, two factors—the woman's age at the time of the initial pET and a total blastocyst score exceeding 18.0 at transfer—were identified as significant factors for predicting ongoing pregnancy.

As shown in Table 6, the ongoing PR was significantly higher in the group for which the woman's age at first pET was under 34.5 years and the total blastocyst score at transfer exceeded 18.0, compared to the other three groups.

Discussion

In the present study, we found that 59.3% (32/54) of women with RIF exhibited a displaced (that is, non-receptive) WOI. This incidence is relatively high when compared to other studies. In previous research, the incidence of displaced WOI among women with RIF has been reported as 25.9% [14], 27.5% [15], 24% [16], 17.7% [17], 41.1% [18], and 47.4% [19]. The higher incidence in our study could reflect the fact that the RIF group examined had a relatively high number of previous failed cycles (mean, 7.0). Similarly, Jia et al. [11] reported a higher incidence of displaced WOI (65%), comparable to our study, in a group with a mean of 5.8 previous failed cycles.

Bellver et al. [20] found that WOI displacement was much more common among women with obesity than among non-obese women (25.3% vs. 9.7%). This suggests that the displacement of the WOI may be dependent on the body mass index. Ota et al. [21] additionally proposed that chronic endometritis could potentially influence WOI displacement.

In the present study, involving women with relatively high-order RIF, the overall ongoing PR per pET was 33.7%. The clinical and ongoing PR, as well as the implantation rates, were similar regardless of whether one blastocyst or two or more blastocysts were transferred (Tables 2 and 3). This suggests that pET with ERA effectively informed the appropriate timing for ET.

Previous research has shown comparable clinical IVF outcomes between the receptive and non-receptive phases. For instance, a study by Mahajan [15] found that the ongoing PR was 42% (20 of 48) in women classified as receptive and 44.5% (eight of 18) in those classified as non-receptive, with the number of previous failed cycles

Table 4. Comparison of seven parameters based on clinical pregnancy and ongoing pregnancy statuses

Parameter	Clinical pregnancy (39 cycles)	No clinical pregnancy (44 cycles)	*p*-value	Ongoing pregnancy (28 cycles)	Not ongoing pregnancy (55 cycles)	*p*-value
Woman's age at the time of first pET (yr)	36 (34–40)	37.5 (35–41)	0.072	35 (33–38)	39 (36–41)	0.001
No. of previous failed cycles	6 (5–9)	7 (5–8.8)	0.8	6 (5–8)	7 (5–9)	0.455
Proportion of "receptive" cycle (%)	38.5	37.2	1	39.3	37	0.814
No. of blastocysts transferred	2 (1–2)	2 (1–2)	0.307	2 (1–2)	2 (1–2)	0.877
Total blastocyst score at transfer	22 (18–35)	16.3 (7–24)	0.001	23 (18–38.8)	18 (10–24)	0.012
Proportion of total blastocyst score at transfer > 18.0 (%)	69.2	32.6	0.001	71.4	38.9	0.005
Interval from endometrial biopsy to pET (day)	54	65	0.565	66	54	0.500

Values are presented as median (interquartile range).
pET, personalized embryo transfer.

Table 5. Multiple logistic regression analysis of factors influencing clinical or ongoing pregnancy

Parameter	B	Standard error	Odd ratio	95% CI
Clinical pregnancy				
Total blastocyst score at transfer	0.33	0.032	1.033	0.971–1.100
Total blastocyst score at transfer > 18.0	1.573	0.474	4.821	1.902–12.220[a]
Ongoing pregnancy				
Woman's age at the time of first pET	–0.205	0.073	0.814	0.706–0.940[a]
Total blastocyst score at transfer	0.015	0.031	1.015	0.955–1.079
Total blastocyst score at transfer > 18.0	1.116	0.532	3.052	1.075–8.665[a]

CI, confidence interval; pET, personalized embryo transfer.
[a] $p<0.05$.

Table 6. Clinical and ongoing pregnancy rates based on the cutoff values of two parameters

Group	Woman's age at the time of first pET (yr)	Total blastocyst score at transfer	Clinical pregnancy rate	Ongoing pregnancy rate
1	< 34.5	> 18.0	90.9 (10/11)	90.9 (10/11)
2	< 34.5	≤ 18.0	40.0 (4/10)[a]	30.0 (3/10)[a]
3	≥ 34.5	> 18.0	56.7 (17/30)	33.3 (10/30)[a]
4	≥ 34.5	≤ 18.0	25.0 (8/32)[a]	15.6 (5/32)[a]

Values are presented as percentage (number).
pET, personalized embryo transfer.
[a] $p<0.05$ when compared with Group 1.

ranging from 2 to 6. Similarly, a study by Hashimoto et al. [16] reported an LBR of 23.7% (14 of 59) in receptive patients and 16.7% (3 of 18) in the non-receptive group, with mean numbers of previous failed cycles of 7.1 and 7.8, respectively. In a study by Patel et al. [17], the ongoing PR was 32.4% (24 of 74) in the receptive and 63.6% (7 of 11) in the non-receptive group, with average numbers of previous failed cycles of 3.6 and 4.0, respectively. Two additional studies have also reported comparable clinical IVF outcomes between the receptive and non-receptive phases [18,19].

In the present study, the likelihood of ongoing pregnancy was negatively associated with the woman's age and positively associated with the quality of the frozen blastocyst. These two parameters are widely recognized as predictors of successful pregnancy in conventional frozen ET cycles. Our team has also previously reported that a high-quality frozen blastocyst score at transfer (≥38.3) is a significant factor associated with clinical pregnancy [22]. Our current findings suggest that even in women with RIF undergoing pET using ERA, the probability of pregnancy remains associated with the quality of the frozen blastocyst.

Recently, ongoing debate has been held over the limitations of the ERA [23]. In their review article, Ben Rafael [24] described the ERA as an "unproven technology." Despite this, few reports have addressed the efficacy of the ERA in women with RIF [8-11]. This underscores the urgent need for additional research on this topic. Our findings suggest that the age of the woman and the quality of the blastocyst should be considered as potential confounding factors in any re-

https://doi.org/10.5653/cerm.2023.06184

search conducted on the efficacy of the ERA.

Furthermore, the combination of the ERA and endometrial immune profiling has been reported to potentially hold more clinical value than using either the ERA or immune profiling independently in women with RIF [25]. Therefore, this adjunctive strategy should also be considered when utilizing the ERA in women with RIF.

WOI displacement is an endometrial cause of embryo implantation failure, particularly in women with RIF. Consequently, a need exists for a more accurate tool to assess endometrial receptivity, beyond the conventional ERA. This tool would guide the optimal timing of ET with greater precision. Efforts to develop such a tool are ongoing. In a retrospective study conducted in Japan, a new endometrial receptivity test, known as ERPeakSM, was used in women with RIF. The findings indicated that the clinical PR (37.7% vs. 20.0%) and LBR (29.9% vs. 9.7%) were significantly higher in the pET group compared to the non-pET patients [26]. Furthermore, a study from China reported that an RNA-Seq-based endometrial receptivity test tool, which uses transcriptomic biomarkers, was effective in improving the clinical PR in women with RIF [27].

The primary limitations of this study stem from its retrospective nature. Additionally, the study was conducted with a small population from a single clinic. Despite these limitations, the strength of this study lies in its uniqueness; it is one of the few studies that have investigated the factors influencing clinical and ongoing PRs in women with RIF undergoing the ERA.

While the ERA may be viewed as an unproven technology, it can also be seen as a worthwhile option for women with intractable RIF. Furthermore, given the scarcity of studies addressing the efficacy of the ERA in women with RIF, a need exists for additional well-designed studies to confirm the clinical value of the ERA and identify specific populations that may benefit from this technology.

Conflict of interest

Byung Chul Jee has served as an editor of *Clinical and Experimental Reproductive Medicine* since 2018. However, he did not participate in the selection, evaluation, or decision-making process concerning the peer reviewers of this article. No further potential conflicts of interest pertinent to this article have been disclosed.

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https://doi.org/10.5653/cerm.2023.06184

ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06086
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):285-291

CERM

Beneficial effects of intraovarian injection of platelet-rich plasma in women with poor ovarian response

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Objective: Infertility can result from a diminished ovarian reserve, but a potential remedy exists in the form of platelet-rich plasma (PRP) administration. This treatment involves both biological factors and tissue trauma mechanisms, which stimulate folliculogenesis, making it a promising and effective strategy. We assessed the impact of direct PRP injections into the ovaries on the fertility outcomes of women classified as poor responders.

Methods: A quasi-experimental study was conducted from April 2021 to December 2022, focusing on patients classified as POSEIDON grade 3 or 4. PRP injections were administered into both ovaries. After 3 months, data were collected on anti-Müllerian hormone (AMH) level, follicle-stimulating hormone (FSH) level, and the numbers of oocytes, mature oocytes, and good-quality embryos following ovarian stimulation. We then compared the data from before and after PRP injection.

Results: This study included 50 women, with a mean of 39 years (interquartile range [IQR], 35 to 43) and 4 years (IQR, 2 to 6) for age and infertility duration, respectively. FSH levels decreased after treatment, while AMH levels and the numbers of oocytes, metaphase II oocytes, and high-quality embryos increased. However, only the increase in high-quality embryos was significant. The pregnancy and spontaneous pregnancy rates were 20% and 14%, respectively. Notably, women with secondary infertility exhibited a significantly higher pregnancy rate than those with primary infertility.

Conclusion: Ample evidence suggests that PRP can enhance ovarian function. However, further studies are needed to identify the appropriate candidates for this procedure, establish the optimal PRP preparation method, and standardize the procedure for its adjuvant use in assisted reproductive technology cycles.

Keywords: Assisted reproductive technology; Infertility; Oocytes; Ovary; Platelet-rich plasma; Pregnancy

Introduction

As a woman ages, her likelihood of conceiving naturally diminishes due to the depletion of her ovarian oocyte reserve. Ovarian insufficiency occurs when the number of oocytes drops below a critical level. This decline begins subtly around the age of 32 years and accelerates noticeably after the age of 37 years [1].

As an individual grows older, fertility typically decreases due to a variety of factors [2]. These include an increased probability of various issues that reduce fertility, a heightened risk of spontaneous abortion, and an elevated risk of aneuploidy [3].

Patient-Oriented Strategy Encompassing IndividualizeD Oocyte Number (POSEIDON) is a recently established classification system. It is particularly appropriate for patients with diminished ovarian reserves or those who demonstrate a suboptimal response to exogenous gonadotropins. Four groups have been established in this system based on both qualitative and quantitative parameters, includ-

Received: April 22, 2023 · Revised: June 23, 2023 · Accepted: July 19, 2023
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ing age, antral follicle count, and anti-Müllerian hormone (AMH) level. Patients in POSEIDON classes 3 or 4 have a low functional ovarian reserve [4].

Ongoing research is dedicated to finding an effective solution for the decline or loss of ovarian reserve. This has resulted in the adoption of several strategies aimed at optimizing ovarian function, including intraovarian platelet-rich plasma (PRP) infusion; stem cell injection to the ovaries; the use of antioxidant supplements; and the application of dehydroepiandrosterone, testosterone supplements, and growth hormones as adjuvants in ovarian stimulation [5,6].

PRP has been employed as an experimental treatment for several years. Initially used for experimental purposes, its clinical application was first seen in other medical fields such as dermatology, orthopedics, and plastic surgery [7]. The concept of utilizing PRP to enhance ovarian function was first proposed in Greece. PRP is obtained by centrifuging peripheral blood to extract platelets [8].

Platelet concentrate is composed of more than 700 proteins, including growth factors, immunomodulators, hormones, and other biologically active proteins. Recent research has indicated that the injection of these factors can stimulate angiogenesis, anabolic processes, cell migration, cell differentiation, and proliferation in targeted tissues [9]. Additionally, PRP has been found to influence mitochondrial activity and reduce oxidative stress [10,11].

Aside from biological factors, it is believed that trauma can also influence PRP by disrupting the Hippo pathway. This pathway includes the oncogenic Yes-associated protein/transcriptional co-activator with PDZ binding motif (YAP/TAZ) system. When activated, this system promotes follicular growth. Mechanical factors play a crucial role in regulating this system. When tensile forces within the cytoplasm increase, the YAP/TAZ system becomes activated. In contrast, a decrease in tensile forces inhibits this system. However, it seems improbable that the insertion of a 17-G needle could cause sufficient damage to the ovary to disrupt the Hippo pathway [12].

The present study aimed to expand on the promising results of previous research involving substantial sample sizes. In it, we explored the effects of injecting PRP into the ovaries of individuals with poor response rates.

Methods

The quasi-experimental study took place in the infertility ward of Shariati Hospital, located in Tehran, Iran, from April 2021 to December 2022. This study employed a before and after design to assess the effects of ovarian PRP injections on patients exhibiting poor ovarian response.

This study included patients who received ovarian PRP within a defined period and were classified as POSEIDON 3 or 4. This classification implies that they had an AMH level below 1.1 ng/mL, fewer than five to seven antral follicles, or a history of cycle cancellation due to insufficient follicular growth or the retrieval of fewer than three oocytes.

Patients who had a partner with male factor infertility or a history of amenorrhea lasting more than 6 months were excluded from the study. In this study, the criteria for selecting patients for intraovarian PRP injections varied based on their previous *in vitro* fertilization (IVF) history. Patients who had previously undergone an IVF cycle were included to form a comparison group. Conversely, patients who had not previously undergone an IVF cycle were included based on the outcomes of the IVF cycle following intraovarian PRP injection.

We collected data from both hospital records and patients' medical histories. Approval for the study was granted by the Ethics Committee of Shahid Beheshti University of Medical Sciences (approval number: IR. SBMU. SME. REC.1401.081). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Informed consent was obtained from each participant.

Regarding PRP injection, the PRP concentrate was administered during the initial follicular phase, specifically between days 3 and 5 of the menstrual cycle. In all cases, the PRP solution was freshly prepared on the day of the injection. This was done by drawing venous blood from the forearm, typically from the medial vein [13].

A collection kit was utilized according to the manufacturer's instructions (ROOYA GEN PRP kit [Co. SN: 312569]; Arya Mabna Tashkhis), facilitating the collection of 80 mL of blood. The injection was administered using a 17-G single-lumen needle, under the guidance of transvaginal ultrasound.

The physician injected 4 mL of PRP into each ovarian parenchyma, approaching the ovary at a safe distance from the vascular pedicle to prevent hemorrhagic accidents. A single physician performed all sonographic evaluations using a Mindray sonography machine (Mindray) equipped with a 4 to 9 MHz vaginal probe. During the PRP procedure, patients were under conscious sedation anesthesia.

We examined the levels of AMH and follicle-stimulating hormone (FSH), along with the quantities of oocytes, mature oocytes, and good-quality embryos in IVF cycles before and after 3 months of PRP injection. For patients who had undergone multiple IVF cycles before PRP treatment, the comparison was made between their most recent cycle and their first cycle following 3 months of treatment. However, for patients who had not experienced any IVF cycles prior to treatment, only their first cycle post-treatment was considered.

1. Data analysis

The values were calculated using the mean±standard deviation and the median (interquartile range [IQR]). To compare parametric

https://doi.org/10.5653/cerm.2023.06086

and non-parametric variables before and after the intervention, we respectively utilized the paired *t*-test and the Wilcoxon signed-rank test.

To ascertain the correlation between successful pregnancy and various factors, including age, secondary infertility, previous AMH levels, and the duration of infertility, we computed odds ratios (ORs). To mitigate any confounding effects, we calculated adjusted ORs along with their corresponding 95% confidence intervals for variables that exhibited *p*-values <0.3 in multiple models.

The data were analyzed using SPSS version 24 (IBM Corp.), with two-tailed tests applied at a significance level of $p \leq 0.05$. Univariate logistic regression was performed to examine the relationships between age, secondary infertility, prior AMH levels, duration of infertility, and successful pregnancy outcomes. Subsequently, variables with a significance level of <0.3 in the univariate analysis were incorporated into the multiple logistic regression model. ORs, along with 95% confidence intervals, were reported for each variable in the model.

Results

Table 1 presents the basic characteristics and clinical information of women with poor ovarian response undergoing PRP therapy. The study involved 50 women referred to Shariati Hospital. The median of age and infertility duration were 39 years (IQR, 35 to 43) and 4 years (IQR, 2 to 6), respectively. The mean±standard deviation of body mass index were 25.3±2.9 kg/m^2. The median duration of infertility was 4 years, with an IQR of 2 to 6 years. Table 1 also provides information about the women's obstetric history. Our data revealed that 44% of the women had a history of pregnancy, 26% had a history of live births, 64% had primary infertility, and 22% had a history of abortion. Among those with prior abortion, 20% had experienced this in the first trimester and 6% in the second trimester. Furthermore, 70% had undergone oocyte retrieval prior to PRP treatment.

Table 1. Basic characteristics and other clinical information of poor-responder women

Variable	Value
Age (yr)	39 (35–43)
BMI (kg/m^2)	25.3±2.9
Duration of infertility (yr)	4 (2–6)
History of pregnancy	22 (44)
History of live birth	13 (26)
History of abortion	11 (22)
History of first trimester abortion	10 (20)
History of second trimester abortion	3 (6)
Primary infertility	32 (64)
History of oocyte retrieval before PRP	35 (70)

Values are presented as median (interquartile range), mean±standard deviation, or number (%).
BMI, body mass index; PRP, platelet-rich plasma.

Table 2 presents a comparison of hormone levels and fertility outcomes in women with poor response before and after PRP injection. Prior to PRP injection, the median AMH level was 0.4±0.6 ng/mL (IQR, 0.2 to 0.6), and the median FSH level was 9.1±16.5 mIU/mL (IQR, 8.5 to 17). Following PRP injection, the median AMH level rose to 0.5±0.70 ng/mL (IQR, 0.2 to 1), while the median FSH level decreased to 6±14.09 mIU/mL (IQR, 6 to 15). Statistically significant differences were observed for FSH, but not for AMH (p=0.004 and p=0.48, respectively). The mean number of oocytes retrieved increased from 3.86±2.23 to 5±4.33 after PRP injection, but this difference was not statistically significant (p=0.08). The median number of metaphase II oocytes similarly rose from 2 (IQR, 1.5 to 2.5) to 3.5 (IQR, 3 to 4.5), but the increase was not statistically significant (p=0.08). However, the median number of embryos obtained after PRP injection differed significantly from the number obtained prior to injection (p=0.05). The percentage of patients experiencing abnormal uterine bleeding decreased from 10% to 8% following PRP injection, but this decrease was not statistically significant.

The results revealed that 20% of women with poor response who received PRP injections became pregnant, with 14% experiencing

Table 2. Hormone levels and fertility outcomes before and after PRP injection in poor-responder women

Variable	Before PRP injection	After PRP injection	*p*-value
AMH (ng/mL)	0.4±0.6 (0.2–0.8)	0.5±0.7 (0.2–0.9)	0.48[a]
FSH (mIU/mL)	9.1±16.50 (8.5–17)	6±14.09 (6–15)	0.004[b]
Number of oocytes	3.86±2.23	5±4.33	0.08[a]
Number of MII oocytes	2 (2)	3.5 (4)	0.08[a]
Number of embryos	1 (2)	1 (4)	0.05[a]
Abnormal uterine bleeding (%)	10	8	0.05

Values are presented as mean±standard deviation (interquartile range) or frequency (median).
PRP, platelet-rich plasma; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; MII, metaphase II.
[a]Wilcoxon signed-rank test; [b]Paired-sample t-test.

Table 3. Post-platelet-rich plasma outcomes in poor-responder women

Variable	Value
Pregnancy rate	10 (20)
Spontaneous pregnancy	7 (14)
Abortion	3 (6)
Multiple pregnancy	2 (4)
Duration to spontaneous pregnancy (mo)	7.6 (3–12)

Values are presented as number (%) or median (interquartile range).

spontaneous pregnancy (Table 3). However, we observed instances of abortion in 6% of cases and multiple pregnancies in 4% of cases. The time frame for achieving spontaneous pregnancy varied between 3 and 12 months, with a median duration of 7.6 months.

After controlling for confounding variables, we found that an increase in age among poor responders decreased the likelihood of successful pregnancy by 17% (Table 4). The data revealed that age was not significantly associated with successful pregnancy in either unadjusted or adjusted analysis (p=0.28 and p=0.13, respectively). Secondary infertility, however, was significantly related to successful pregnancy in both unadjusted (OR, 13.33; p=0.003) and adjusted (OR, 8.57; p=0.023) analyses. In women with secondary infertility, the odds of successful pregnancy were 8.57 times greater than among those with primary infertility.

The pre-intervention AMH level did not show a significant association with successful pregnancy in either unadjusted or adjusted analyses. However, an increase in the AMH level prior to the intervention did correlate with a 3.64-fold increase in the likelihood of successful pregnancy. The duration of infertility did not significantly correlate with successful pregnancy in the unadjusted analysis (OR, 0.88; p=0.28).

Discussion

The existence of oogonial stem cells presents a new possibility for treating age-related fertility decline and pathological conditions such as premature ovarian failure. Ovarian rejuvenation may be achieved with this approach. A recent study investigated the potential effects of intraovarian injections of autologous PRP on ovarian rejuvenation [14]. The study involved 50 patients, aged 27 to 40 years, who were anticipated to have a poor response to ovarian stimulation and were categorized as POSEIDON 3 or 4. Additionally, 64% of these patients had a history of ovarian retrieval.

Several reports have demonstrated that women with diminished ovarian reserve or premature ovarian failure experience improvements in follicular count, hormone levels, and successful pregnancy outcomes after undergoing intraovarian PRP treatment [15,16]. Our research corroborates these findings, indicating that PRP positively impacts ovarian function. This is evidenced by increased levels of AMH, decreased levels of FSH, and increases in the numbers of both oocytes and mature oocytes.

The most noteworthy result is the improvement in oocyte quality, which led to the development of high-quality embryos, regardless of any alterations in the ovarian reserve or oocyte count. This topic has attracted considerable attention. In a study conducted by Merhi and Mouanness [17], the application of PRP to the ovaries of infertile women who had previously experienced failed IVF cycles yielded relatively higher embryo euploidy rates. The localized paracrine effect of growth factors present in PRP could potentially improve meiotic aberrations in human oocytes, thereby enhancing euploidy rates [17]. Several *in vitro* and experimental studies have demonstrated the beneficial effects of PRP. Hosseini et al. [18] conducted research to examine the impact of PRP on the growth and viability of both fresh and vitrified-thawed ovarian follicles.

Several case series and studies have documented pregnancies in women diagnosed with premature ovarian insufficiency (POI) after receiving ovarian PRP injections, either through IVF or spontaneously [13,19]. Consistent with these findings, our study revealed that of the 10 pregnancies observed, seven (70%) occurred spontaneously, while three (30%) were the result of IVF. It is widely accepted that women with POI have a 5% to 10% chance of conceiving naturally without any fertility intervention [20]. However, our research indicates a higher probability, agreeing with the findings of other studies. Interestingly, we found that the pregnancy success rate was significantly higher in individuals experiencing secondary infertility. Our study did not specifically distinguish between primary and secondary infertility in the analysis. Nevertheless, the finding that secondary infertility was associated with a significantly higher OR for successful pregnancy indicates that additional factors may contribute to lower success rates in patients with primary infertility. This suggests that primary infertility, defined as the inability to conceive after 1 year of regular unprotected intercourse in couples without previous live births, may have unique characteristics that our study did not fully reveal.

Aflatoonian et al. [21] reported a satisfactory pregnancy rate of 47% among women who had previously exhibited a poor response,

Table 4. Association between clinical factors and successful pregnancy (unadjusted and adjusted analysis) in poor-responder women

Variable	Unadjusted analysis			Adjusted analysis		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Age	0.9	0.75–1.08	0.28	0.83	0.66–1.05	0.13
Secondary infertility	13.33	2.38–74.40	0.003	8.57	1.35–54.15	0.023
AMH before intervention	0.88	0.10–7.30	0.31	3.64	0.21–62.05	0.37
Duration of infertility	0.88	0.70–1.11	0.28	-	-	-

OR, odds ratio; CI, confidence interval; AMH, anti-Müllerian hormone.

https://doi.org/10.5653/cerm.2023.06086

with 50% of these pregnancies resulting in the birth of a live baby. Notably, all pregnancies occurred naturally after PRP administration [21].

Our study population was characterized by its heterogeneity due to the inclusion of patients with diverse IVF backgrounds and treatment histories. We particularly acknowledge a subgroup of patients who had never previously undergone oocyte retrieval, representing a unique subset within our cohort. The observed success rate of 30% observed in this subgroup underscores the potential impact of previous treatment experience on outcomes.

In our study, all clinical and spontaneous pregnancies occurred within 1 year in patients who had undergone ovarian stimulation cycles within 90 days of receiving a PRP injection. Typically, under physiological conditions, the progression of primary follicles to pre-antral follicles takes approximately 120 days [22]. Furthermore, the initiation of the transition from primary to pre-antral follicles could explain the delayed effect of PRP, which was observed 2 to 3 months post-injection, even though the infused cytokines had already broken down. However, many aspects of the paracrine control of folliculogenesis and the contents of PRP remain unclear [9,23].

Although PRP has shown promising results in many cases, a universally accepted method for its preparation remains elusive, as do clear guidelines for identifying suitable candidates for PRP therapy. This absence of standardization could account for the diverse results seen across studies as well as the differing mechanisms of PRP's effects.

Several methods exist for the processing of whole blood to create PRP, which may involve variations in centrifugation speed and duration, separation techniques (like mechanical or manual pipettes), and the application of activators after preparation. These techniques differ across studies.

Various injection techniques also exist, such as the cortex, medulla, and intraperitoneal methods. Injections can be administered through single or multiple sites, and they can be carried out using either laparoscopic or vaginal routes [9].

Our patients were categorized as poor responders; however, their mean FSH level was 9 mIU/mL. Only three patients had an FSH level exceeding 25 mIU/mL, and none surpassed 40 mIU/mL. Furthermore, only 10% of patients experienced irregular menstruation due to the perimenopausal period. In one previous study, Barad et al. [24] examined 80 consecutive patients with poor ovarian reserve, aged between 28 and 54 years. Poor ovarian reserve was defined as an AMH level below 1.1 ng/mL, an FSH level above 12 mIU/mL, or at least one previous IVF cycle yielding three oocytes within a year.

The study followed women for 1 year following an intraovarian PRP procedure, which entailed the injection of 1.5 mL of PRP into the ovarian cortex, averaging 12 injections per ovary. Despite this, the study findings suggested no statistically significant advantages associated with the intraovarian PRP treatment. Among all the patients, only two (4.7%) experienced ongoing pregnancies [24].

In our analysis, we included cases with identified causes of infertility, such as male factor infertility, tubal factor infertility, and uterine abnormalities. Notably, these factors can influence both the number of embryos and pregnancy rates. While we did not specifically stratify the results based on individual causes of infertility, we maintain that incorporating these cases enhances the overall heterogeneity of our study population. This approach offers a relatively stronger representation of real-world clinical scenarios.

When interpreting the results of our study, it is crucial to recognize the statistical limitations that arise from the relatively small sample size. The inclusion of a limited number of participants could potentially impact the statistical power and generalizability of the findings.

To date, no serious adverse effects associated with ovarian PRP injections, such as vascular injury, organ perforation, infection, abscess formation, or oocyte tissue necrosis, have been reported. Similarly, no adverse effects were observed in the present cohort. Nevertheless, it is important to acknowledge that the long-term effects of this procedure remain unknown. Additionally, a theoretical risk is associated with administering highly concentrated growth factors to tissues, which could potentially induce malignant transformation [9].

In conclusion, for those who have previously been pregnant and are now experiencing infertility primarily due to age-related fertility decline, PRP presents a viable approach to enhance ovarian function and improve response to ovarian stimulation. This could potentially yield successful pregnancy outcomes.

The most notable impact of PRP appears to be on the quality of oocytes and the subsequent embryos. This effect is believed to stem from alterations in the ovarian microenvironment, which enhance angiogenesis and mitigate oxidative and inflammatory effects. Numerous studies have also demonstrated that PRP can stimulate folliculogenesis, resulting in an increased number of follicles and an enhancement in ovarian reserve.

Overall, referring to PRP administration as "ovarian rejuvenation" may not be fully accurate, as any observed benefits might be only temporary. Although this method has not yet been recognized as a standard treatment option for poor responders, the current research indicates that it could be a viable alternative. This is supported by the promising results observed in other studies, particularly in a statistically significant number of poor-responder patients who underwent ovarian PRP treatment.

Conflict of interest

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

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ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2023.06366
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2023;50(4):292-298

CERM

The effects of berberine on ischemia-reperfusion injuries in an experimental model of ovarian torsion

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Objective: Ovarian torsion is a gynecological disorder that causes ischemia-reperfusion injuries in the ovary. Our study investigated berberine's short- and long-term effects on ovarian ischemia-reperfusion injuries.
Methods: This study included 28 Wistar albino female rats weighing 180 to 220 g, which were divided into four groups: sham (S), torsion/detorsion (T/D), torsion/ detorsion+single dose berberine (T/D+Bb), and torsion/detorsion+15 days berberine (T/D+15Bb). The torsion and detorsion model was applied in all non-sham groups. In the T/D+Bb group, a single dose of berberine was administered, while in the T/D+15Bb group, berberine was administered over a period of 15 days. After the rats were euthanized, their ovaries were excised. The left ovaries were used for histopathologic evaluation, which included ovarian injury scoring and follicle count, while the right ovaries were used for biochemical analyses (tissue transforming growth factor-β [TGF-β] and alpha-smooth muscle actin [α-SMA] levels).
Results: The histopathologic evaluation scores for the ovaries were significantly lower in the T/D+B group ($p<0.05$) and the T/D+15B group ($p<0.005$) than in the T/D group. The follicle counts in the T/D group were lower than those in both the sham and treated groups ($p<0.005$). The TGF-β levels were significantly lower in the T/D+15B group ($p<0.005$), whereas the α-SMA levels did not show a significant difference.
Conclusion: Both short- and long-term berberine use could potentially have therapeutic effects on ovarian torsion. Long-term berberine use exhibited anti-inflammatory effects by reducing TGF-β levels, thereby preventing ischemia-reperfusion injuries. Therefore, we suggest that long-term berberine use could be beneficial for ovarian torsion.

Keywords: Berberine; Ischemia reperfusion injury; Ovarian follicle; Ovarian torsion; Transforming growth factor beta

Introduction

Adnexal torsion is defined as rotation of the ovary and fallopian tube around their own axis. This condition is primarily observed during the reproductive period and is considered a gynecological emergency due to its potential for serious complications. The adnexa, composed of the fallopian tubes and ovaries, may experience torsion either together or independently [1]. The etiology of the ischemic injury that can result from torsion is believed to be oxidative stress and inflammation caused by reactive oxygen metabolites in the surrounding environment. This phase, known as the ischemic injury period, is primarily associated with ovarian injury due to hypoxia. Once the torsion is alleviated, the reperfusion injury process commences, characterized by the production of reactive oxygen and nitrogen compounds. The total tissue damage is thought to be the cumulative result of injuries caused by both ischemia and reperfusion. Therefore, successful prevention of reperfusion injury can significantly enhance the effectiveness of ischemia treatment [2].

Free oxygen radicals are produced during ischemia and reperfusion, leading to oxidative stress [2]. It is believed that ischemia and free oxygen radicals, resulting from torsion, are the primary causes of diminished ovarian reserve. These injuries can potentially lead to follicular cell degeneration in the ovary, vascular congestion, hemorrhage, inflammation, and tissue loss, as evidenced by histopatholog-

Received: July 18, 2023 · Revised: August 29, 2023 · Accepted: August 31, 2023
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ical findings [3]. From this perspective, antioxidant agents have been explored in the literature as potential adjuncts to surgical treatment for torsion [4-8].

Berberine is a non-basic, herbal quaternary benzylisoquinoline alkaloid with a well-documented history in Ayurveda and Chinese medicine [9]. Its active components include berberine, berbamine, and palmatine [10]. Today, berberine is also produced through chemical synthesis. The chloride or sulfate salt of berberine is typically used for clinical applications. Berberine has been utilized for at least 3,000 years in Ayurveda and Chinese medicine, largely due to its potent antimicrobial, antiprotozoal, and antidiarrheal properties [11]. Moreover, clinical research over time has revealed that berberine possesses a broad range of pharmacological effects. Numerous studies suggest that it exhibits significant antioxidant, anti-inflammatory, antiarrhythmic, antihypertensive, anticancer, antihyperglycemic, analgesic, antidepressant, anxiolytic, neuroprotective, and hypolipidemic activities [12-14]. Additionally, the nephroprotective [15], hepatoprotective [16,17], cardioprotective [18], and cerebroprotective [14] effects of berberine have been demonstrated in various studies. Recently, there has been an emphasis on the potential therapeutic use of berberine in ischemia-reperfusion (I/R) injury of different organs, given its notable antioxidant effect and tissue injury prevention properties. For instance, the protective effects of berberine administration have been observed in renal and testicular I/R injury in rats [19,20].

Delayed diagnosis and treatment of ovarian torsion can lead to irreversible damage to the ovaries, potentially resulting in infertility in women [21]. Recent research indicates that simply detorsing the ovary does not fully address fertility issues [22,23]. Alongside detorsion, antioxidant agents have been employed to safeguard ovarian reserves. Given this context, we aimed to determine the effects of the short-term and long-term use of berberine, which has been shown to have antioxidant effects in different studies, on ovarian I/R injuries.

Methods

Ethics approval for this study was granted by the ethics committee of Dokuz Eylul University (Protocol No. 27/2021). The research was carried out at the Experimental Animal Laboratory of Dokuz Eylul University in 2022. The subjects of the study were 28 Wistar albino female rats, each 8 weeks old and weighing between 180 and 220 g. Throughout the experimental period, the rats were maintained under standard environmental conditions (21±2 °C) and given regular access to water and food.

At the beginning of the study, vaginal smears were performed to standardize the effects of sex hormones, and only rats in estrus were included. The rats were then divided into four groups (n=28) in a random manner, as follows: The sham (S) group (n=7) involved a procedure where the abdomen was opened and closed after 1 minute, with saline being applied throughout the experiment. The torsion/detorsion (T/D) group (n=7), involved the creation of a torsion and detorsion model, with saline being applied throughout the experiment. The T/D+single dose berberine (T/D+Bb) group (n=7) underwent the establishment of a torsion and detorsion model, followed by the administration of a single dose of berberine (barium chloride, Sigma-Aldrich; cas.no:633-65-8) at 200 mg/kg intraperitoneally 30 minutes prior to detorsion [19]. No further treatment was applied post-detorsion. The T/D+15 days berberine (T/D+15Bb) group (n=7) involved the establishment of a torsion and detorsion model, followed by the administration of 150 mg/kg/day berberine via oral gavage for 15 consecutive days post-detorsion [20,24].

1. Establishment of an ovarian torsion and detorsion model in rats and berberine administration

A midline incision measuring approximately 2.5 to 3 cm was made in the lower abdomen of anesthetized rats. The ovaries were located by tracing the uterine horns. In the non-sham groups, the right and left ovarian tissue of the rats was rotated 360° clockwise and secured with vascular clamps. Following a 3-hour waiting period, detorsion was performed [25,26]. The first dose of berberine, at 200 mg/kg, was administered intraperitoneally 30 minutes prior to detorsion [19]. Subsequently, berberine was administered orally at a dosage of 150 mg/kg/day for 15 consecutive days post-detorsion [20,24]. After 15 days, all subjects were euthanized. The right and left ovaries were then removed. The left ovaries were preserved in formalin for histological examination, while the right ovaries were set aside for biochemical analysis.

2. Histopathologic evaluation

The left ovaries were preserved using formalin, dehydrated through varying concentrations of alcohol, and then embedded in paraffin. The maximum number of 4 µm thick sections were extracted from the ovaries and stained with hematoxylin and eosin. The histopathologic evaluation of ovarian tissue involved counting follicles and scoring injuries. The averages of primary, primordial, atretic secondary, and tertiary follicles were computed [27]. For injury scoring, parameters such as inflammation, vascular congestion, hemorrhage, and follicular cell degeneration were assessed. The specimens were semiquantitatively scored by examining a minimum of five microscopic fields. A scale from 0 to 3 was utilized for each parameter (0 for absent, 1 for mild, 2 for moderate, and 3 for severe) for each sample [28,29].

3. Biochemical evaluation

A biochemical evaluation was conducted by measuring the levels of transforming growth factor-beta1 (TGF-β) and smooth muscle actin (α-SMA). We utilized enzyme-linked immunosorbent assays to assess the levels of TGF-β and α-SMA, using products from BTLAB (Bioassay Technology Laboratory) (catalog numbers E1688Ra and E2330Ra).

4. Statistical analysis

SPSS version 26.0 (IBM Corp.) was employed for data analysis. The mean±standard deviation of the data were calculated for the analysis. The Kruskal-Wallis test was adopted to investigate the difference between the groups, and the Mann-Whitney *U* test was employed to identify from which group the difference originated.

Results

The findings from the histopathologic evaluation are depicted in Figure 1. In the S group, no pathologic changes were noted. The T/D group, however, exhibited significantly higher scores for follicular degeneration, vascular congestion, edema, hemorrhage, and inflammation ($p<0.005$) compared to the other groups (S, T/D+Bb, T/D+15Bb). No significant differences were observed between the T/D+Bb group, the sham group, and the T/D+15Bb group. Similarly, no significant difference was detected between the T/D+15Bb group and the T/D+Bb group. The histopathologic scores are detailed in

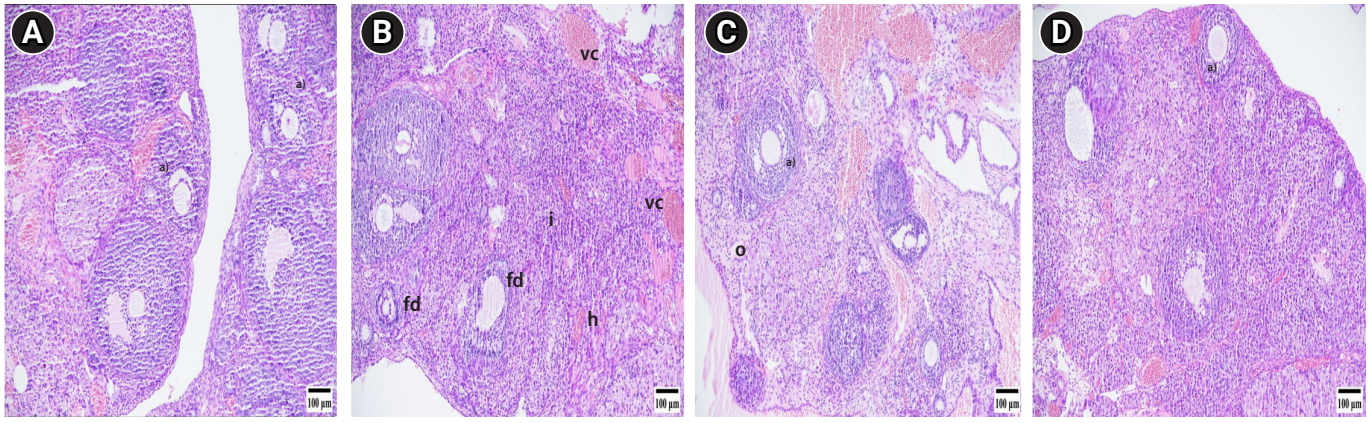

Figure 1. Photomicrographs of ovarian tissue. (A) Sham group, (B) torsion/detorsion (T/D) group, (C) T/D+berberine (Bb) group, and (D) T/D+15Bb group (hematoxylin and eosin stain, scale bar: 100 μm). [a)]Follicles at different stages of development, vascular congestion (vc), hemorrhage (h), edema (o), follicular degeneration (fd), inflammation (i).

Table 1. Histopathological scores of the ovaries between groups, follicular cell degeneration, vascular congestion, hemorrhage, and inflammation parameters[a)]

Variable	Sham group	T/D group	T/D+Bb group	T/D+15Bb group
Follicular degeneration	0.3±0.5	2.8±0.3[b)c)]	1.4±0.5[d)e)]	1.1±0.3[b)f)]
Vascular congestion	0.7±0.7	2.8±0.4[b)c)]	2.2±0.9[d)e)]	1.1±0.3[b)f)]
Hemorrhage	0.4±0.5	2.7±0.5[b)c)]	1.6±0.5[d)e)]	1.5±0.5[d)f)]
Inflammatory cell	0.7±0.7	2.7±0.4[b)c)]	1.4±0.5[d)e)]	1.4±0.6[b)f)]
Primordial follicles	6.1±0.9	2.1±1[b)c)]	3.5±0.9[d)e)]	5.5±1[b)f)]
Primary follicles	4.5±1	1.8±0.6[b)c)]	3.8±0.7[d)e)]	5.1±0.6[b)f)]
Secondary follicles	3.8±1	1.4±0.5[b)c)]	3.4±1[d)e)]	4.8±0.8[b)f)]
Tertiary follicles	2.4±0.5	1.5±0.5[c)d)]	3.1±0.6[d)e)]	3.2±0.7[b)f)]
TGF-β1 (ng/L)	64.3±21	437±368[b)c)]	128.8±56.3	58.3±27.1[b)f)]
α-SMA (ng/mL)	34.3±15.6	30.2±11.9	28.9±7.5	26±4.9

Values are presented as mean±standard error of the mean. Primordial, primary, secondary, tertiary, and atretic follicles were counted, and their averages were calculated. The results of biochemical evaluation, TGF-β, and α-SMA levels in tissue.
T/D, torsion/detorsion; Bb, berberine; TGF-β, transforming growth factor-β; α-SMA, alpha-smooth muscle actin.
[a)]0: none, 1: mild, 2: moderate, 3: severe; [b)]$p<0.005$; [c)]Group Sham vs. group T/D; [d)]$p<0.05$; [e)]group T/D vs. group T/D+Bb; [f)]group T/D vs. group T/D+15Bb (n=7 for each group).

https://doi.org/10.5653/cerm.2023.06366

Table 1.

The T/D group exhibited significantly fewer primordial, primary, secondary, and tertiary follicles compared to the other groups (S, T/D+Bb, T/D+15Bb) (p<0.005). No significant difference was found between the T/D+Bb group, the sham group, and the T/D+15Bb group. Similarly, no significant difference was noted between the T/D+Bb group and the T/D+15Bb group. The follicle counts are detailed in Table 1.

Figure 2 illustrates the biochemical results of TGF-β and α-SMA levels in tissue. A statistically significant increase in the TGF-β level was observed in the tissue of the T/D group (p<0.005) compared to the other groups (S, T/D+Bb, T/D+15Bb). However, there was no significant difference between the T/D+15Bb group and the sham group. Similarly, no significant difference was found between the T/D+Bb group and the T/D group. The α-SMA levels in the tissue did not significantly differ among the groups (p>0.05).

Discussion

Recent studies involving rats have suggested that detorsion alone does not resolve fertility issues associated with ovarian torsion. Prior to the detorsion procedure, anti-inflammatory and antioxidant agents were employed to safeguard ovarian reserves. However, none of the drugs utilized in these experimental studies have been clinically approved for use in cases of ovarian torsion. Consequently, there is an ongoing search in the literature for safe and effective drugs that can be administered parenterally in humans during the brief interval between torsion and detorsion surgery [22]. Topcu et al. [30] administered a single dose of metformin prior to detorsion of the torsioned ovary. They observed a decrease in ovarian destruction, suggesting a positive contribution to treatment [30]. However, when Topcu et al. [31] administered a single dose of amiodarone before detorsion of the torsioned ovary, they did not observe a similar decrease in ovarian destruction. Other studies have recommended the long-term use of antioxidants, rather than a single dose [27,28,32]. In their research, Karakas et al. [28] demonstrated that administering metformin for 14 days protected the ovarian reserve in an ovarian T/D model. Kalyoncu et al. [32] suggested that administering octreotide shortly before and 7 days after detorsion is the most effective method for preserving the ovarian reserve in the ovarian T/D model. Our study examined the impact of a single 200 mg/kg dose of berberine administered before detorsion, and a 150 mg/kg dose of berberine given for 15 days after detorsion, on ovarian I/R injury. We aimed to compare the short-term and long-term effects of berberine administration. A high dose and peritoneal administration were chosen for immediate effects in single dose berberine administration. To observe the long-term effects, a 15-day period and medium doses were chosen for oral administration. The dosages used were determined based on a review of the literature [19,20], and it has been demonstrated that the lethal dose of oral berberine in rats exceeds 2,000 mg/kg [33].

Torsion, a condition that can affect various organs, leads to vascular congestion, edema, and hemorrhage. When torsion is corrected, or detorsed, it can result in I/R injuries. During this process, antioxidants are introduced to mitigate tissue damage caused by increased inflammation and the release of free oxygen radicals. Consequently, numerous studies have explored various pharmacological agents to prevent I/R injuries. Berberine has been identified as a potential treatment in several of these studies. For instance, Kazaz et al. [19] administered 200 mg/kg of berberine intraperitoneally 30 minutes prior to detorsion in a study of testicular I/R injury in rats, and observed protective effects. Kumas et al. [20] developed a bilateral I/R model in diabetic rats and administered varying doses of berberine (50, 100, and 150 mg/kg) over a 14-day period. They observed thera-

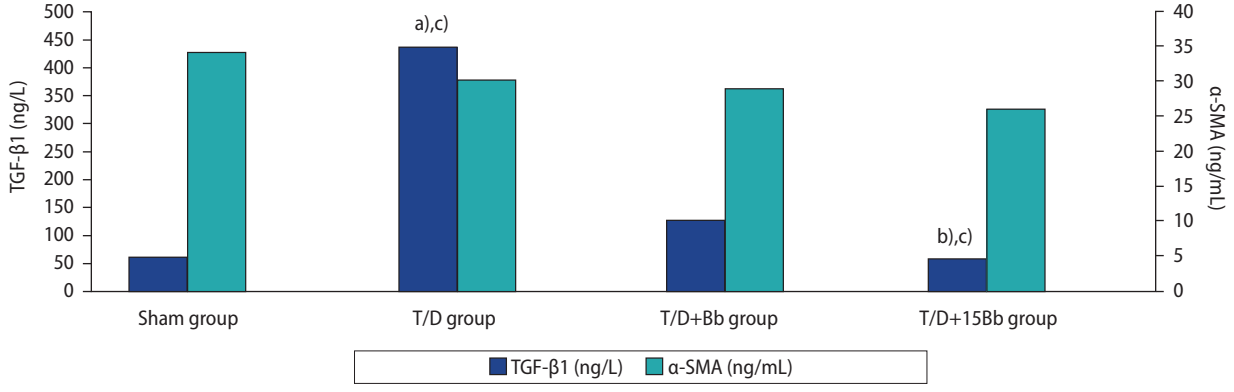

Figure 2. Comparison of tissue transforming growth factor-β (TGF-β) and alpha-smooth muscle actin (α-SMA) levels. T/D, torsion/detorsion; Bb, berberine. [a]Group Sham vs. group T/D; [b]group T/D vs. group T/D+15Bb; [c]p<0.005.

peutic effects in the groups that received 100 and 150 mg/kg doses of berberine [20]. In our study, we administered a single dose of 200 and 150 mg/kg of berberine over a 15-day period. Our histopathological evaluation, which included assessments of inflammation, follicular cell degeneration, vascular congestion, and hemorrhage, revealed reductions in the effects of I/R injuries.

Numerous pathological studies have detailed tissue damage resulting from ovarian torsion and ovarian lipid peroxidation, as well as cell death and inflammation [4-8]. Ilgen et al. [34] demonstrated that follicular degeneration, inflammatory cell infiltration, and vascular congestion were significantly elevated in the torsion group. Uzun et al. [35] conducted a comparison between ovarian torsion lasting 8 and 24 hours, finding that follicular degeneration and inflammation were more pronounced in the torsion lasting 24 hours. Consequently, the interval between torsion and detorsion surgery should be kept as brief as possible. The duration of the detorsion is thought to be extended when a conservative treatment option is pursued.

In their study, Wang et al. [36] administered berberine at doses of 100 and 200 mg/kg to rats with polycystic ovarian syndrome. They reported that this treatment could stimulate the development of antral follicles and induce ovulation [36]. In a separate study, rats with ulcerative colitis were treated with berberine. The results indicated that berberine exhibited anti-inflammatory properties by suppressing cytokines (interleukin 3 [IL-3], IL-7, IL-11, TGF-β, and tumor necrosis factor α [TNF-α]), as well as inhibiting apoptosis. These effects were found to provide a protective benefit against ulcerative colitis [37]. In our study, we corroborated the anti-inflammatory properties of berberine. Our findings showed that berberine increased the number of antral follicles, reduced inflammation as per histopathologic evaluations, and lowered TGF-β levels in the tissue.

The follicle reserve serves as an indicator of fertility. There is ongoing therapeutic research aimed at protecting the follicle reserve, which is negatively impacted by T/D injury. Eken et al. [38] observed a statistically significant reduction in the count of primordial, preantral, and antral follicles in studies related to T/D injury. They also reported a substantial increase in follicle numbers following treatment with etanercept [38]. In a separate study, berberine was administered both *in vivo* and *in vitro* in a model of premature ovarian failure. The results indicated that berberine enhanced ovarian reserve capacity and regulated ovarian hormone secretion [39]. Our study showed that 15-day supplementation of berberine increased the follicle reserve, which had been diminished due to T/D injury.

Inflammation is another pathophysiological mechanism involved in I/R injury, characterized by excessive oxidative stress resulting from reperfusion following ischemia [40]. For instance, Nayki et al. [41] demonstrated that rutin possesses antioxidative (evidenced by a decrease in malondialdehyde and cyclooxygenase activity) and anti-inflammatory (indicated by a decrease in TNF-α and IL-1β) properties against ovarian I/R injury in rats. They administered rutin 1 hour prior to detorsion. Histopathological findings revealed hemorrhage, dilated blood vessels, and degenerated follicles in the I/R group, while the rutin+I/R group exhibited nearly normal morphology [41]. In a separate study, Sagsoz et al. [4] investigated the effects of vitamin C, mannitol, and verapamil on I/R injury in rat ovaries. They observed moderate hemorrhage, edema, and loss of cohesion in the ischemia group, with more severe pathological findings in the I/R group. However, they found that vitamin C and mannitol effectively reduced I/R injuries [4]. In yet another study, it was found that rosmarinic acid modulated inflammation and prevented ovarian damage following I/R injuries [40].

During the inflammatory process, the release of platelet-derived growth factor, epidermal growth factor, and TGF-β stimulates the formation of granulation tissue, which is characterized by the presence of α-SMA-expressing myofibroblasts. TGF-β has the potential to induce myofibroblasts to overproduce extracellular matrix, which could lead to the formation of scars. If myofibroblast activity becomes excessive, it can result in fibrosis and organ dysfunction [42,43]. In the T/D group, a higher level of TGF-β was indicative of increased inflammation. Conversely, a lower level of TGF-β in the T/D+15Bb group suggested that berberine has an anti-inflammatory effect. The lack of significant differences between the T/D and T/D+Bb groups was interpreted as an indication of the weak anti-inflammatory effect of a single dose of berberine. Zhu et al. [37] demonstrated in their study that berberine possesses anti-inflammatory properties in an ulcerative colitis model. Our study further corroborates this anti-inflammatory effect. The levels of α-SMA did not significantly differ between the groups, leading us to interpret these findings as indicative of no impact on fibrosis formation in the T/D model.

In conclusion, we have demonstrated that both short- and long-term use of berberine could potentially have therapeutic effects on ovarian I/R injury. We suggest that the anti-inflammatory properties of berberine, along with its impact on follicle reserve, may aid in preserving fertility. We are confident that our study will provide a significant contribution to existing literature regarding the treatment of ovarian I/R injury. Further research will be required to determine the applicability of this agent in human subjects.

Conflict of interest

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

https://doi.org/10.5653/cerm.2023.06366

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https://doi.org/10.5653/cerm.2023.06366

INSTRUCTIONS TO AUTHORS

CERM

Enacted: December 31, 1974
Revised: November 31, 2018

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The areas of interest or the scopes of CERM are as follows;
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- Submission items include a manuscript, table (s), and figure (s). Send also Author's Signature Form and Copyright Transfer Form (These files can be found at the journal's website) as jpg or pdf files. Revised manuscripts should also be accompanied by a response note.
- 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).
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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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- 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 obvious cases (e.g., prostate cancer). Authors should define how they determined race or ethnicity and justify their relevance. If needed, include information on the IRB/IACUC approval and informed consent. Methods of statistical analysis and criteria for statistical significance should be described.

5) **Results:** The results should be presented in logical sequence in the text, tables, and illustrations. Do not repeat in the text all data in the tables or figures, but describe important points and trends.

6) **Discussion:** Observations pertaining to the results of research and other related materials should be interpreted for your readers. Emphasize new and important observations; do not merely repeat the contents in the Introduction or Results. Explain the meaning of the observed opinion along with its limits, and within the limits of the research results connect the conclusion to the purpose of the research.

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Examples of authors' contributions are as follows:
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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/ICSItreated 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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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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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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All manuscripts will be evaluated by two peer reviewers who are selected by the editors. The acceptance criteria for all papers are based on the quality and originality of the research and its clinical and scientific significance. An initial decision will normally be made within 4 weeks of receipt of a manuscript, and the reviewers' comments are sent to the cor-

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IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

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After the paper has been accepted for publication, the author(s) should submit the final version of the manuscript for review. The names and affiliations of the authors should be double-checked to omit any spelling errors, and if the originally submitted image files were of poor resolution, higher resolution image files should be submitted at this time. Color images must be created as CMYK files. The electronic original should be sent for review with appropriate labeling and arrows. The EPS, TIFF, Adobe Photoshop (PSD), JPEG, and PPT formats are preferred for submission of digital files of photographic images. Symbols (e.g., circles, triangles, squares), letters (e.g., words, abbreviations), and numbers should be large enough to be legible on reduction to the journal's column widths. All of the symbols that are used must be defined in the figure caption. If the symbols are too complex to appear in the caption, they should appear on the illustration itself, within the area of the graph or diagram, not to the side of the illustration. If references, tables, or figures are moved, added, or deleted during the revision process, they should be renumbered to reflect such changes in order that all tables, references, and figures are cited in numeric order.

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