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An update of the effects of metal nanoparticles on female reproduction

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ABSTRACT

Nanoparticles (NPs) can have adverse effects on human health, and, in particular, on female reproductive performances. Literature data have shown that NPs can enter the ovary, uterus, and placenta, causing dramatic effects on fetus health. However, NPs can also be used for therapeutic purposes and can be promising tools to preserve fertility. Here, we briefly summarize the toxic effects on female fertility exerted by a particular class of NPs, the metal NPs, because of their wide diffusion. At the same time, we highlighted the attempts to use them as new molecular approaches for ovarian cancer and infertility.

KEYWORDS

Nanoparticles, reproductive toxicity, cancer, biomedical applications.

Introduction

Nanomaterials (NMs) are defined by European legislation as “an insoluble or bio-persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to ≤ 100 nm”^[1]. NMs can originate from natural events or be synthesized intentionally for a wide range of applications like electronics, cosmetics, pharmacology and target therapies^[2]. Their small size and elemental composition determine their biological properties, as smaller NMs can be internalized by cells, leading to positive or negative effects^[3,4].

Their composition influences absorption and bioaccumulation into cells. Lipophilic NMs can cross cell membranes by diffusion, while hydrophilic NMs need active transport^[5]. The shape is also relevant. In fact, NMs can have regular shapes (spheres, filaments, fibers, rods, cylinders...) or, in the case of naturally occurring ones, they present irregular morphologies because they are subjected to different environmental stresses (e.g. salinity, UVs, heat and biological degradation)^[6,7]. These properties can be modified by adding other components on their surface; for instance, the presence of bioconjugates can increase cellular absorption by recognizing specific receptors or stimulating endocytosis or macropinocytosis^[7-9].

Environmental NMs can bind other contaminants causing, the so-called “Trojan effect”, which consists of double exposure to NMs and one or more bonded molecules leading to cumulative effects compared to single exposure^[10]. The Trojan effect can be used in clinical practice, and for this reason the materials used must be stable, biocompatible, and selectively directed to specific sites in the body after systemic administration. An example is the treatment of intrahepatic cholangiocarcinoma (ICC)^[11] via nanoparticles (NPs), usually with a size between 10 and 500 nm, able to carry the chemother-

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apic compound paclitaxel (PTX) and a siRNA able to inhibit cPKM, a miRNA upregulated in this cancer^[11]. The siRNA/PTX co-loaded nanosystems are able to penetrate tumor tissues by releasing both molecules, encouraging paclitaxel sensitization and inhibiting ICC proliferation and metastasis *in vivo*. In contrast, the co-exposure to NMs and other compounds can be ineffective or reduce the total effect. For example, the co-exposure to ozone and silica NMs did not increase the genotoxic damage compared to the single exposure, confirming that the interactions between NMs and compounds result in different effects based on the specific chemical properties^[12,13].

NPs are one of the most spread types of NMs both in medical applications and as environmental pollutants. They are defined as “zero dimensional nanomaterials”: they have all their dimensions in the nanoscale range and “the lengths of the longest and the shortest axes of the nano-object do not differ significantly”^[14]. The increasing use of products containing NPs and their potential health risks due to occupational exposure is contributing to the growing public health concern about trans-generational effects because reproductive organs of both sexes are sensitive^[15].

Metal NPs (MNPs) are of great interest because their physical properties make them potential candidates for efficient biomedical applications. However, data on their toxic potential is still needed before their clinical usage.

Their distinctive feature is a metal core made of inorgan-

ic metal or metal oxide, typically encased in an organic, inorganic, or metal oxide-based shell. Silver (Ag), gold (Au), zinc oxide (ZnO), titanium dioxide (TiO₂), copper oxide (CuO), cerium dioxide (CeO₂) and iron oxide (FeO) are the MNPs most frequently utilized in industry, medicine, food, and agriculture because their small size facilitates infiltration through biological membranes that are usually impermeable to other macromolecules. Among them, TiO₂ NPs are mainly employed for biomedical applications, such as bioimaging and the production of medical devices [16].

The use of MNPs significantly impacts female fertility and fetal development because of the fixed, non-renewable quantity of oocytes in mammalian ovaries at birth and long-term fetal exposure [17]. Moreover, the two-fold increase in ovarian and uterine MNP content that occurs during mouse ovulation in comparison with the other stages of estrus cycle suggests that MNPs accumulation is substantially conditioned by the menstrual cycle [18]. MNPs double face switching from therapeutic effects to adverse outcomes is reported in Figure 1.

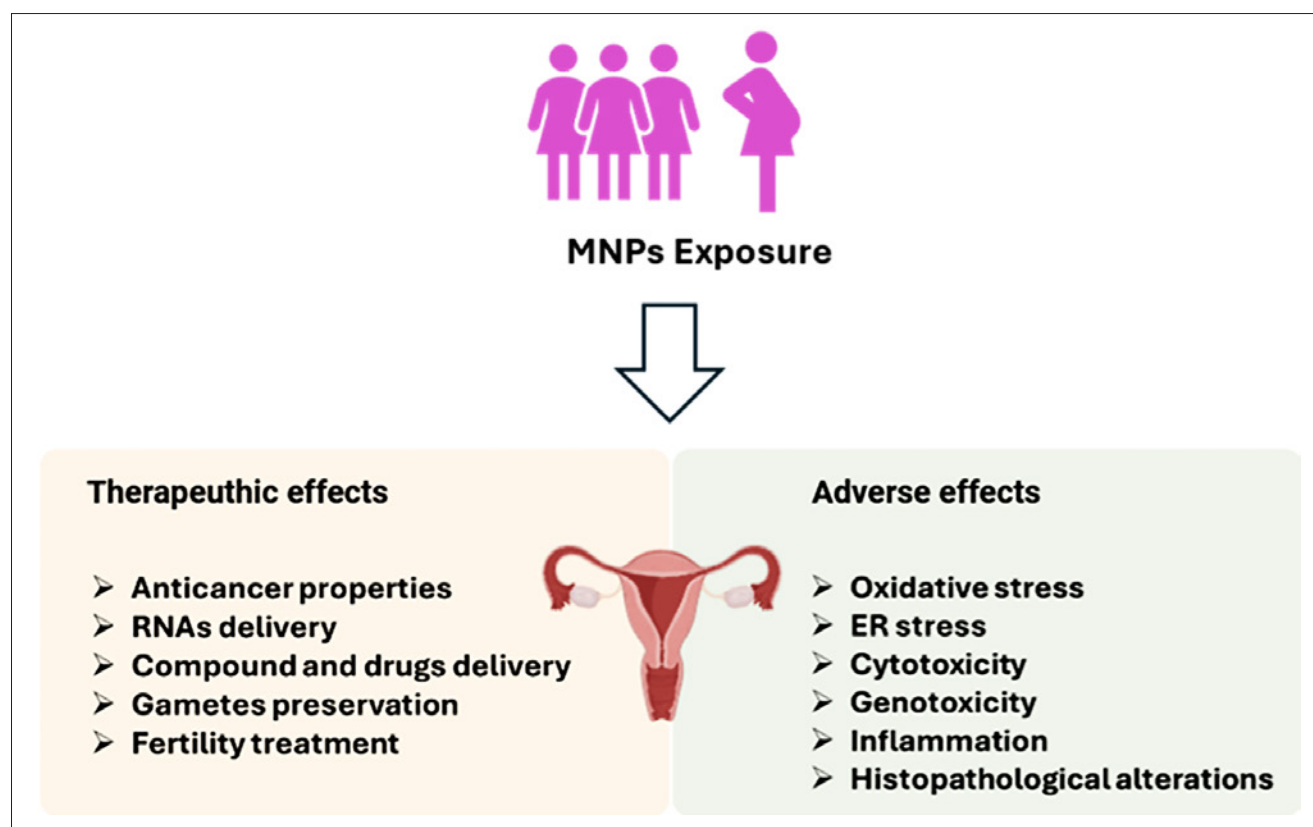
The toxicity of MNPs on the ovary

TiO₂ NPs or nTiO₂ and ZnONPs or nZnO are widely used because they are components of toothpaste and food packaging. The release of zinc ions seems to be the main cause of toxicity, but more studies are needed to define the mechanisms [19]. In mouse fetal ovaries, ZnO NP exposure (0.2–0.8 mg/kg for

2 days) increased DNA double-strand breaks with induction of apoptosis and reduction of the ovarian follicle reservoir [15,20]. In a recent study, ZnO NPs tested on *Zebrafish* caused a toxic effect not only on oocytes and ovaries but also on global fertility [21] because a long-term exposure caused a significant reduction of the gonad somatic index (GSI=gonad weight/body weight X 100), probably determined by vitellogenesis suppression. ZnO NP exposure maintained primary oocyte dormancy and caused the death of the few growing oocytes before ovulation, suggesting cellular stress as indicated by p53 and caspase 3 overexpression. Apoptotic induction was confirmed also in rats administered with ZnO NPs for 21 days (i.p., 100 mg/kg and 200 mg/kg) that underwent massive follicle and ovarian tissue degeneration together with Bax, Bcl, Caspase 3, and Caspase 9 overexpression [22]. Hamster Ovarian Cells (CHO-K1) were also used to study ZnO NP effects *in vitro*, and a reduced cell growth and overexpression of γ -H2AX and ATM were recorded [23].

TiO₂ NPs are usually employed in sunscreens and cosmetics; therefore, their daily usage opens up to questions related to human hazards. Despite the wide range of applications, few studies were found in the literature on their toxic effects on female fertility. It has been demonstrated that these MNPs are able to induce inflammation and oxidative stress in ovaries with consequent follicle degeneration [15]. More recently, Karimipour *et al.* [24] demonstrated that exposed female mice (100 mg/kg/daily TiO₂ NPs solution orally for 5 weeks) underwent extensive histopathological changes in their ovaries, characterized by ovarian cyst formation and disruption of follicular

Figure 1 Metal nanoparticles (MNPs) are characterized by the double ability of inducing toxic effects or co-adjuvating therapeutic strategies. In the second case, the adverse effects can be used and directed against abnormal cells such as in the case of cancer.



development likely due to high levels of malondyaldehyde and estrogens. Moreover, the number of pregnancies and births in these treated animals was significantly lower than in the control due to reduced fertilization rate and pre-implantation embryo development. Considering the relevance of TiO₂NPs in cosmetics and the frequency of their usage, more studies should be necessary to ascertain their reproductive toxicity.

Au NPs are considered jolly in clinical practice because of the lack of negative consequences following their exposure^[15] and their potent antimicrobial activity^[25]. Au NP inert properties do not apply to all living systems. In buffalo granulosa cells, they act as endocrine disruptors^[26] and can enhance metabolic mortality. Conversely, both in the presence and absence of these MNPs, CHO cells exposed to radiofrequency radiations had a decreased capacity to form colonies^[27]. Additionally, compared to untreated cells, the amount of reactive oxygen species (ROS) in gold-treated CHO cells was significantly lower. Ag NPs are widely used as preservatives in cosmetics and food production^[28]. Despite this, their reproductive toxicity has been ascertained.

Recently, the effects of oral Ag NP treatment on thyroid hormone production and ovarian steroidogenesis of Hy-Line Brown hens were examined by Katarzyńska-Banasik *et al.*^[29]. The results demonstrated altered ovarian steroidogenesis because Ag NPs targeted pre-hierarchical follicles more than preovulatory ones. In order to investigate the effects of Ag NPs on ovarian functions, hormone levels (FSH, LH, progesterone, estrogen) were assessed in rats administered with increasing doses of this chemical (12.5, 25 and 50 mg/kg; 20–30 nm) for 10–30 days. After 10 days, estrogen level significantly increased at all used concentrations, but after 20 days, it dropped in the groups treated with the higher concentrations. After 30 days, there was a noticeable decrease in estrogen levels at all dosages and a rise in ovarian weight at 50 mg/kg^[30].

In conclusion, Au NPs have been demonstrated to be valid carriers for treating cancer co-adjuvating antitumoral therapies without adding toxic effects. On the contrary, Ag NPs influenced hormone levels in rats exposed for 1 month indicating their bioactive properties. While gold is considered the best MNPs for clinical applications because it does not react with biological molecules, the other MNPs need more attention to specifically determine their biological interactions.

The toxicity of MNPs on placenta

Studies to understand the impact of MNPs on placenta are complex and hardly performed on human tissues^[31]. Exposure during fetal life can damage the placenta and impair the development of the embryo. Dugershaw-Kurzer *et al.*^[32] showed that a single exposure of human placental explants to physiologically relevant concentrations of engineered (SiO₂ NPs, TiO₂ NPs) or environmental (diesel exhaust particles, DEPs) NPs resulted in extensive deregulation of placental signalling, evidenced by the release of altered extracellular vesicle (EV)-associated proteins and a variety of secreted inflammatory, vascular, and endocrine placental components. Furthermore, explants exposed to these NPs released EVs with strong antiangiogenic and an-

ti-vasculogenic properties, affecting mainly early neuro-developmental processes. By identifying vascular morphogenesis as a sensitive consequence of the indirect developmental toxicity of various MNPs, these findings highlighted the potential of metal oxide NPs and DEPs for extensive interference with the placental secretome^[32].

The benefits of MNPs

Metal NPs are beneficial in treating pathologies related to female fertility and urogenital organs^[15]. New applications have been found, and the field is rapidly increasing the range of diseases treatable with this kind of NPs.

Au NPs, based on their inert characteristics, are ironically the golden standard in the field. Their most practical feature is their ability to multi-functionalize by attaching molecules that offer the necessary activity. Because there are so many functional groups that have an affinity for gold, like carboxyls, thiols, and amines, their surface chemistry is quite simple to alter^[33]. Nguyen *et al.*^[33] reviewed several clinical trials that used Au NPs as delivery systems to cytokines, chemotherapies and RNAs used to treat tumors proving Au NP's efficacy. Kumar *et al.*^[34] used Au NPs to deliver drugs and as a diagnostic tool. Specifically, using inductively coupled plasma (ICP) and MARS spectral photon-counting CT imaging (MARS SP-CCT), the authors demonstrated the selective and efficient uptake of 18 nm and 80 nm sized gold nanoparticles by SKOV3 ovarian cancer (OC) cells *in vitro*. The conjugation of luteinizing hormone-releasing hormone (LHRH) to the surface of NPs was validated by chemical analysis using FTIR spectroscopy. LHRH-conjugated gold NPs were utilized for selective *in vivo* and *in vitro* OC targeting. Wagstaff *et al.*^[35] used carboxylate surface-modified Au NPs bounded with cisplatin as an anti-cancer system on ovarian endometrioid adenocarcinoma cells (A2780). High concentrations of gold-coated NPs with cisplatin (6–12 mM for 24 hours) induced cell death in tumoral cells resistant to cisplatin. Indeed, the presence of the MNPs rescued cisplatin resistance to the same extent as sensitive cells. These results showed how gold-coated NPs can influence the activity of a compound, allowing the modification of therapy based on the patient's needs.

Combining of different compounds with FeO NPs is a new strategy to exploit their cytotoxicity and redirect it to tumoral cells. Wang *et al.*^[36] used EVs as mimetic nanovesicles encapsulating FeO NPs and β -Lapachone (Lapa) to increase oxidative stress to induce cell death. Excellent biocompatibility was demonstrated by ovarian epithelial cells through serial extrusion, and magnetic guiding was used to improve endocytosis in OC cells. MNPs can act not only as vectors but also directly react with biological targets. The ROS-inducing effect was confirmed on the ovaries of *P. reticulata* where 0.3 mg/L of FeO NPs induced oxidative stress, inflammation and histological alterations^[37]. Therefore, the adverse effects of MNPs must be characterized and optimized to be used efficiently against cancerous cells. El-Beltagy *et al.*^[38] demonstrated that Zn NPs can alleviate the damage induced by lipopolysaccharide (LPS, bacterial endotoxin) on rat placenta (20 mg/kg orally from

Conclusions

The two sides of NM usage highlight the need of studies to characterize them both and to determine the best application for each of them^[42]. Reducing environmental pollution and improving our understanding of how NPs enter the human body to alter cell metabolism are essential to minimizing their harmful side effects. In addition, creating NPs that can target particular organs or cells will provide a non-invasive means of successfully treating a variety of human diseases and genetic abnormalities, particularly those affecting embryos or oocytes. The creation of novel therapies for pathological reproductive disorders and the most common pregnancy difficulties is therefore necessary and crucial for the health of women. In order to improve MNP stability and targeted distribution in the human body, as well as to enable their penetration into cells, several coatings that can lessen harmful effects, such as albumin, polyethylene glycol, and aspartic acid, are being researched. Technologies also shift toward novel chemical forms, such as lipid NPs, from inorganic and organic particles. Vaccines are one of the more recent uses^[43]. Therefore, it becomes crucial to distinguish between the applications of nanomedicine and nanotoxicology. We can enhance potential therapeutic uses by figuring out where the line is drawn between biomedical use and side effects. Only by integrating prevention and diagnostic testing, we will be able to enhance both sides of this coin (Figure 2).

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Overview of fertility preservation for oncology patients and advanced medicinal product therapy application

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ABSTRACT

Recent advances in cancer therapy have significantly increased survival rates among pediatric and adolescent patients, highlighting the need to address long-term effects such as infertility caused by gonadotoxic treatments. Ovarian tissue cryopreservation (OTC) has emerged as a vital fertility preservation strategy, particularly for prepubertal patients or those requiring immediate chemotherapy. While oocyte and embryo cryopreservation remain the gold standard for postpubertal women, OTC offers the only viable option for prepubertal patients and those with hormone-sensitive cancers. Despite over 200 reported live births following ovarian tissue Autotransplantation (OTT), clinical success is still limited by post-transplant ischemic injury and potential malignant cell reintroduction. To mitigate these risks and improve graft viability, recent bioengineering approaches focus on enhancing graft revascularization through the use of endothelial cells (ECs) and biocompatible 3D scaffolds. In a novel study by Spazzapan *et al.*, ECs isolated from ovarian tissue were expanded in a GMP-compliant bioreactor and combined with a collagen-based dermal scaffold (Integra) to form an advanced therapeutic medicinal product (ATMP). This construct was tested in a murine model and showed enhanced vascularization of the graft, suggesting a promising strategy to improve ovarian graft function.

These innovative approaches offer hope for improving fertility outcomes in young cancer survivors, although further clinical validation is needed before application in human trials.

KEYWORDS

Oncofertility preservation, OTT, ATMPs.

Introduction

Fertility preservation for cancer patients has significantly increased long-term survival for children and adolescents with malignancy, due to recent advancements in cancer treatment and diagnostic techniques^[1].

Ovarian dysfunction can disrupt secondary sexual characteristics development and trigger menopausal symptoms^[2]. Approximately 90% of estradiol comes from pre-ovulatory follicles, crucial in endocrine function. While hormone replacement therapy (HRT) is common, determining the ideal dose and frequency is challenging compared to natural hormone production and long-term HRT use is associated with increased risks of hormone-dependent cancers, stroke, and cardiovascular disease^[3].

Fertility preservation for cancer survivors

The American Society of Clinical Oncology categorizes chemotherapy agents by their ovarian toxicity. Adjuvant polychemotherapy for breast cancer is considered the most gonadotoxic, especially alkylating agents and platinum compounds.

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These drugs can damage primordial and growing follicles by disrupting DNA replication and cellular division, leading to cell apoptosis. The administration of these drugs for six months, in women under the age of 40, may lead to irreversible ovarian failure and infertility, reducing the quality of life^[2,4]. Though fertility preservation options for females have developed more slowly than for males, several techniques can be used before cancer treatment to reduce the risk of ovarian failure, including GnRH analogues to temporarily suppress ovarian function and surgical ovarian transposition, before radiotherapy^[4]. The efficacy of these interventions remains limited. In terms of effective fertility preservation, cryopreservation of oocytes or embryos is the most successful approach, but it may not be appropriate in women with cancer, due to time constraints and specific conditions such as hormone-responsive cancers.

Moreover, patients with the high-risk BRCA1 and BRCA2 mutations may have lower baseline fertility, resulting in fewer eggs retrieved after hormonal stimulation ^[5]. For pre-pubertal patients needing immediate chemotherapy, ovarian tissue cryopreservation (OTC) represents the only viable fertility preservation option. After cure, follicles can be isolated, embedded in artificial ovaries and auto-transplanted back into the body to restore hormone levels and enable natural conception. Endocrine function recovery is well established, and live birth rates are increasing ^[6,7].

Indications for ovarian tissue cryopreservation

Ovarian auto-transplantation (OTT) foresees slicing the ovarian tissue excised into cortical strips and cryopreserving it utilizing slow freezing or vitrification techniques. If more follicles are needed, fragment size or sample number could be adapted. After treatment, provided there is no potential risk of malignant cell transmission, the ovarian tissue may be grafted onto the ovarian medulla or specially designed peritoneal pockets. If a risk of malignant cell transmission exists, ovarian follicles can be extracted from the thawed tissue and utilized to construct a transplantable artificial ovary.

In particular, OTC treatments may pose risks for patients diagnosed with colon, gastric, and endometrial cancers, known to preferentially metastasize to the ovaries; in addition, the OTC procedure would not be advisable if the primitive oncological condition involves ovarian tissue. Therefore, it is mandatory to screen for metastasis into the ovary before offering it ^[8].

Procedures for ovarian cryopreservation and transplantation

The objective of OTC is to preserve primordial follicles, located in the ovarian cortex. The most common approach involves cortical grafting rather than whole-ovary microsurgical transplantation. Currently, two primary techniques exist for human ovarian tissue cryopreservation: slow freezing and vitrification. Both present challenges. The primary concern with slow freezing is the potential formation of ice crystals, which can occur within the cytoplasm and result in cellular damage. Conversely, the issue with vitrification is the toxic effect that cryoprotectants may have on metabolically active cells after the tissue is thawed ^[9,10].

Thawed cortex strips may be subjected to either orthotopic transplantation in ovarian medulla, peritoneal window, and serosa of the fallopian tubes or heterotopic transplantation in subcutaneous areas, such as the arm, abdomen, or back. Generally, orthotopic transplantation is preferred, as this method allows for spontaneous pregnancies and not only hormone replacement, as heterotopic transplants do ^[11].

The inaugural case of live birth resulting from the auto-transplantation of cryopreserved human ovarian fragments was documented in 2004, and, to date, more than 200 births have been reported after the autotransplantation of frozen ovarian tissue.

Past and present of OTT

Although the principal scientific societies in Reproductive Medicine have recognized OTT as standard care and no longer an experimental procedure, its implementation in clinical practice continues to pose several challenges ^[12].

The primary challenges include the tissue damage resulting from cryopreservation procedures and the issue of ischemic/reperfusion injury that occurs due to the initial absence of vascular support. Moreover, the potential for transplant reinfection stemming from tumor contamination remains an unresolved concern ^[13-15].

To avoid the risk of reintroducing cancer cells during OTT, researchers are endeavoring to develop ovarian grafts, such as an in vitro culture system for primordial follicles and the artificial ovary, that involve the isolation and culture of follicles within a three-dimensional scaffold that facilitates follicle survival and development. To enhance the efficacy of the artificial ovary, it is proposed that growth factors, stromal, endothelial and supporting cells, may be incorporated into a 3D supporting matrix that degrades with time, to promote hormone production and improve vascularization ^[16]. The artificial ovary would also be a unique research tool to investigate folliculogenesis and to provide a better understanding of how some diseases affect fertility in women ^[17].

Bioengineering approaches to increase the revascularization

Several research groups focused their attention on the ischemic-reperfusion damage. When the ovarian tissue is transplanted, it is essential that the blood vessels regenerate and connect themselves with the host tissue, to provide oxygen and nutrients for the integration of the graft and to prevent ischemic injury to the follicles after grafting.

Angiogenesis is fundamental for ovarian functionality because it supports follicle maturation, and VEGF is the main angiogenic factor, promoting cell proliferation, migration and vascular permeability ^[18]. The antral follicle is a small avascular structure that, starting from a diameter of 200 micrometers, in approximately 10 days reaches 10 mm of diameter. It is easy to understand that in this process, the perfusion is essential.

Research has demonstrated that the hypoxic phase following reimplantation persists for at least 5 to 7 days, with functional, perfused blood vessels becoming observable only after 7 to 10 days (Figure 1) ^[19,20]. This ischemic period following OTT can result in the loss of over 50% of follicles. Ovarian transplants may yield a reproductive functionality period ranging from approximately 4 to 5 years, with instances extending beyond 10 years. One of the factors influencing this duration is the quantity of viable follicles. Consequently, there is a pressing need to develop innovative strategies aimed at minimizing the hypoxic interval.

Numerous studies have been conducted to develop a strategy for reducing ischemic and hypoxic damage in auto-transplanted ovarian tissue. Certain research groups have explored the utilization of mesenchymal stem cells to support OTT.

Figure 1 Schematic representation of the ischemic/reperfusion time of ovarian tissue auto-transplantation (modified by Ref. 19 & 20).

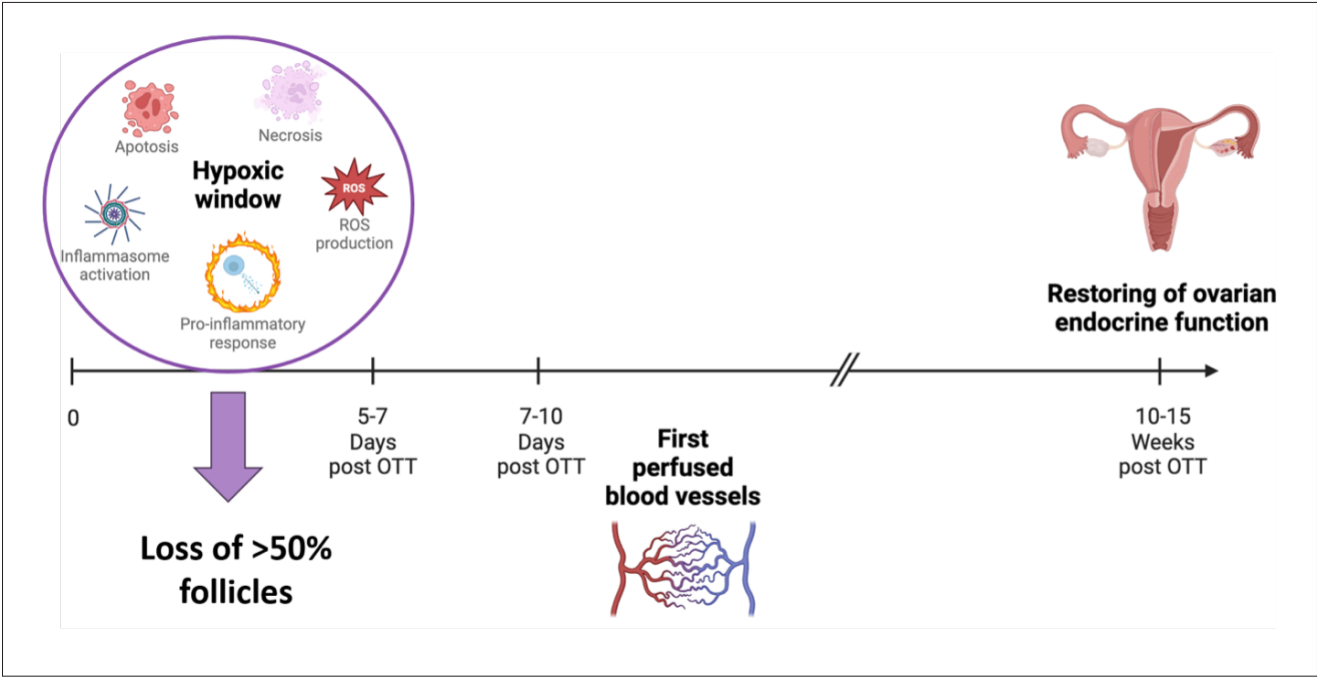


Table 1 General characteristics of enrolled women.

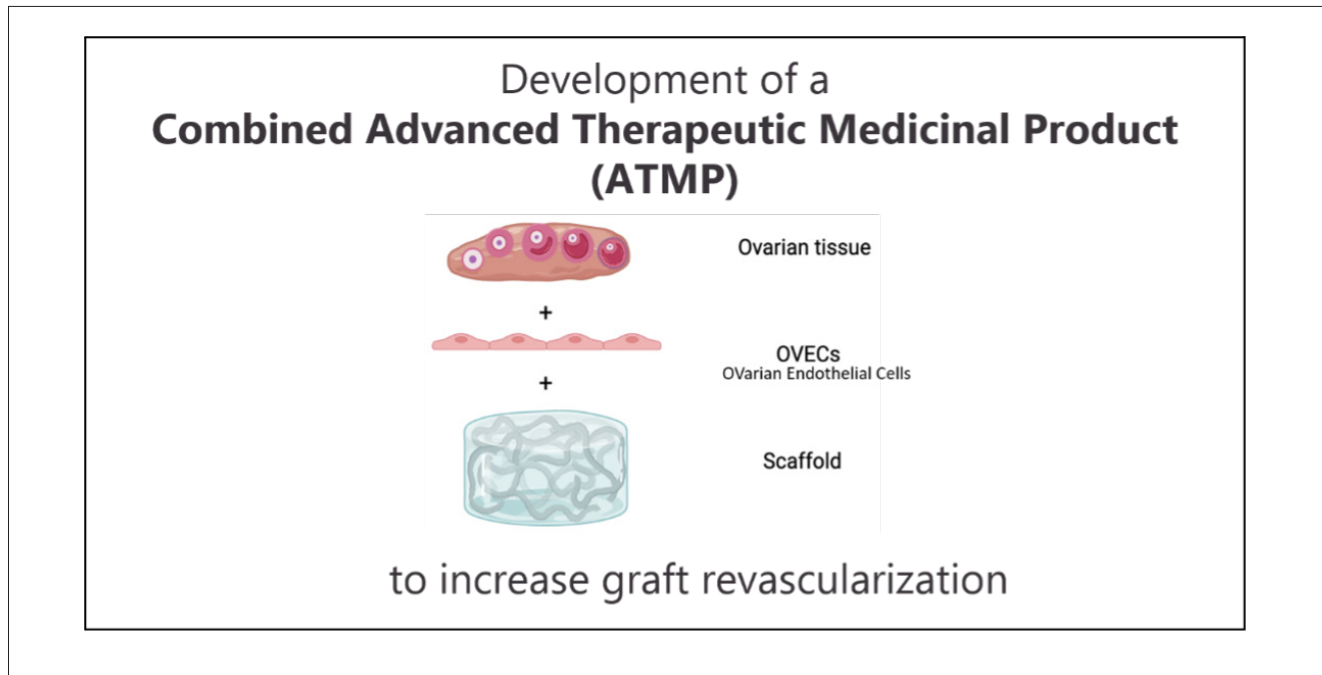
GROWTH FACTORS	REFERENCES	SPECIES
VEGF	Shikanov A, et al. Tissue Eng Part A. 2011	mouse
bFGF	Gao IM, et al. Hum Reprod. 2013	mouse
HORMONES		
AMH		
HMG	WangY, et al. Biomed Online. 2012	mouse
ANTIOXIDANTS		
N-acetylcysteine	Olesen HØ, et al. Hum Reprod. 2021	mouse
Vitamine C	Nugent D, et al. J Reprod Fertil. 1998	human
STEM CELLS		
Adipose-derived stem cells	Manavella DD, et al. Hum Reprod. 2018	mouse
Bone marrow	Herraiz S, et al. Fertil Steril. 2018	human
Amniotic fluid mesenchymal stem cells	Utu T et al. Int J Med Sci. 2012	mouse

Given that the presence of graft-resident endothelium is crucial for tissue recovery post-transplantation, incorporating an autologous source of endothelial cells (ECs) into the grafts prior to transplantation—a technique known as inosculation—may present a promising strategy for reducing the duration of ischemic and hypoxic conditions (Table 1).

In the context of revascularization, studies on the application of 3D scaffolds are well-established, and there are two main bioengineering approaches: the activation of the scaffold with systems able to release growth factors or chemokines that stimulate angiogenesis, or the inosculation, the pre-seeding of EC onto the scaffold to induce the formation of novel vascular

networks prior to the implantation ^[21].

In a recent study published by Spazzapan *et al.*, the research group opted for the second methodology, based on prior studies concerning revascularization in wound healing (Figure 2) ^[22, 23]. It has been observed that the pre-seeding of endothelial cells into a dermal substitute, already utilized in plastic surgery, demonstrated a significant enhancement in revascularization and restoration of skin integrity. Consequently, we have decided to implement a similar approach, aiming to develop a combined Advanced Therapeutic Medicinal Product (ATMP) by joining ovarian slices with endothelial cells isolated from the ovary and inosculated in a 3D scaffold.

Figure 2 Graphical abstract of the innovative approach for vascularization improvement of ovarian auto-transplantation proposed by Spazzapan et al.

ATMPs represent a new class of medicines based on gene therapy, somatic cell therapy, and tissue engineering [24]. ATMPs fall under the regulatory framework of biological products, which defines the legal basis for their development. The European Medicines Agency (EMA) classifies ATMPs into four main types: I) Gene therapy, which contains genes that have a therapeutic effect (GTMP); II) Somatic-cell Therapy, which contains cells manipulated to change their biological role (sCTMP); III) Tissue-engineered medicines in which cells or tissues are modified to repair or regenerate human tissue (TEP); IV) Combined ATMPs, with one or more medical devices, combined with cells or tissues.

ATMPs comply with EMA legislation at different stages of their development, including Clinical Practice (GCP), Good Manufacturing Practice and requirements.

GLP define the procedures and principles with which laboratory research for non-clinical tests is planned, conducted, controlled, recorded and reported.

GCP are guidelines for clinical trials that must be followed by all parties involved in clinical trials, to guarantee the protection of the subjects included and to ensure that the data produced have been obtained according to quality procedures and are therefore reliable.

Finally, GMP defines the requirements that must be met during the development, production and control phases of medicinal products to guarantee their pharmaceutical quality and their safety and efficacy.

In the study by Spazzapan *et al.* [25], the ovarian samples were obtained from patients with benign gynecological diseases or with gender dysphoria undergoing ovariectomy/adnexectomy. Firstly, they set up a protocol to isolate endothelial cells from ovarian tissues (OVECs), obtaining a small number of pure endothelial cells, then expanded in an automated bioreactor named NANT-XL (VivaBiocell, Italy), which performs the

process in a GMP-compliant manner [26].

Secondly, the scaffold to support the seeding and the implantation of the EC was chosen, combined with the ovarian tissue. They analyzed the capability of the cells to adhere and proliferate on different dermal substitutes already used in plastic surgery. The best scaffold was Integra, a 3D matrix composed of bovine collagen and shark chondroitin sulphate.

Finally, an in vivo model of OTT was set up: the ovary slices were defrosted and grafted subcutaneously with Integra in the back of a SCID mouse, in the presence or in the absence of ECs. After 14 days, the human ovarian tissue was excised from the mouse dermis and the vascularization and viability of the human tissue were investigated. Observations indicated that OVEC supplementation significantly boosts graft revascularization, offering a promising future for fertility preservation strategies.

Conclusions

Scientific research has made significant strides in diagnosis and treatment of pediatric cancers, making childhood neoplasm, in most cases, curable. Long-term survival for children and adolescents with malignancy has exceeded 80% over the past decade [27-29].

The challenge remains improving quality of life after chemotherapy or radiotherapy. Sexual dysfunction after cancer treatment is highly prevalent, affecting up to 90% of female patients [30], and include low libido, genitourinary syndrome of menopause, dyspareunia, erectile dysfunction (ED), hypogonadism, body image concerns, and impacts on intimate relationships. A comprehensive, patient-centered approach to sexual health can help improve outcomes and overall well-being for cancer survivors.

Cryopreserved ovarian tissue autotransplantation has offered real hope for restoring fertility after fertility-destroying treatments for prepubertal patients. However, this procedure still has a low success rate due to the oxygen deficit that occurs in post-transplant tissue not yet connected to the local vasculature. This condition causes a state of stress that leads to significant egg loss and a drastic reduction in the functionality of the transplanted tissue.

Recent studies have demonstrated that the addition of autologous cells derived from blood vessels, inserted into biocompatible matrices during transplantation, represents a promising strategy for enhancing graft revascularisation and improving the supply of oxygen and nutrients. The scientists proposed applying integrated bioengineering and cell therapy strategies to the ovarian autotransplant procedure, adding blood vessel cells isolated from the ovarian tissue itself to the tissue to speed up post-graft revascularization and reduce the ischemic window.

The protocol, developed by Spazzapan *et al.*, although very promising, has so far only been evaluated in animal models and will need further validation before entering a clinical trial.

The results achieved give hope for further development of the study, which will hopefully soon benefit young lives already sorely challenged by cancer.

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Reproductive outcome of single dose of corifollitropin-alfa comparing with recombinant FSH in advanced age women: a retrospective cohort study

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ABSTRACT

Objectives: to evaluate reproductive outcomes of advanced age women candidate for IVF/ICSI stimulated with a single course of corifollitropin-alfa (CF α). **Methods:** An observational retrospective study was carried out at IVF unit of Federico II University from December 2012 to December 2015 comparing reproductive outcomes of 50 advanced age women stimulated with a single course of CF α (Group A) vs 100 matched controls where stimulation was performed with recombinant FSH (Group B). The following inclusion criteria were adopted: age ≥ 35 years; Body Mass Index $> 19 \text{ Kg/m}^2$ and $< 30 \text{ Kg/m}^2$; basal Follicle Stimulating Hormone (FSH) $< 12 \text{ IU/L}$; menstrual cycles ranging from 24–35 days (intraindividual variability 3 days); hysteroscopic evidence of a normal uterine cavity. Woman affected by polycystic ovarian syndrome, genetic, immunologic and endocrinological disorders have been excluded. CF α was administered as a single subcutaneous injection, during the early follicular phase of the menstrual cycle in control group, starting dose of r-FSH was established evaluating BMI, age and ovarian reserve in terms of antral follicle count and was subsequently adjusted in function of ovarian response during follicular monitoring. **Results:** Both groups showed similar anthropometric, hormonal parameters. Duration and diagnosis of infertility were also comparable among groups. No statistical differences were observed between groups in terms of BPR and CPR. A higher number of follicles $> 15 \text{ mm}$ was detected in Group A compared with Group B. **Conclusions:** According to our preliminary findings, in a subgroup of advanced age women, stimulation with a single dose of CF α is sufficient to achieve similar reproductive results compared to recombinant FSH. Larger trials in future could clarify which kind of women may benefit from this feasible approach.

KEYWORDS

ART, corifollitropin-alfa, recombinant-FSH, ovulation induction.

Introduction

The number of older patients turning to assisted reproductive technology (ART) is increasing worldwide ^[1,2]. However, the success of ART is significantly hampered by the age of the patients, and it is becoming increasingly important to optimise treatment outcomes for these types of women ^[3,4]. Female age is a critical factor of the POSEIDON classification of low prognosis patients because it directly affects oocyte quality and embryo ploidy ^[5]. The incidence of low-prognosis patients who come to a fertility Centre may vary between clinics and countries, but about 47% of patients undergoing assisted reproductive technology (ART) fall into one of the POSEIDON categories ^[6]. Cumulative delivery rate was lower in the older population (POSEIDON Groups 2 and 4) than in the younger population (POSEIDON Groups 1 and 3) ^[7,8]. Currently, the evidence concerning treatment strategies for POSEIDON

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is limited, but apparently, these patients could be treated more efficiently ^[9,10]. Although the medical management of low-prognosis patients remains incredibly challenging, recent reports suggest that specific interventions might be beneficial in relevant subpopulations. In predicted poor-responder patients, Corifollitropin-alfa (CF α) was found to be useful due to the rapid increase in FSH levels, which in turn could facilitate follicle recruitment and subsequent growth ^[11,12].

CF α is a long-acting follicle-stimulating hormone analog that was developed by fusing the C-terminal peptide of the β -subunit of human chorionic gonadotropin (hCG) to human follicle-stimulating hormone (FSH). This drug has a different pharmacokinetic profile with a plasma half-life of approximately 65 hours and in about one third of women, who underwent a single injection in the first phase of the menstrual cycle (cycle days 2 or 3), triggering was possible without further injections of exogenous FSH [13-15].

Moreover, CF α is not inferior to daily rFSH injections in expected normo-responder patients undergoing IVF/ICSI treatment cycles, leading to similar success rates and risks of OHSS [16]. Barrenetxea *et al.* reported that CF α represented a cheaper option compared with the daily administration of ≥ 200 IU rFSH when the 7 days of controlled ovarian stimulation were considered [17]. Nevertheless, data regarding this effect on advanced age women is still scanty. The aim of the present study is to evaluate the reproductive outcome of a single dose of CF α in advanced age women.

Materials and methods

Study design

This cohort study analysed records of 1500 women attending in vitro fertilization (IVF) at the IVF Unit of University “Federico II”, Naples, Italy, from December 2012 to December 2015. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guideline [18]. All patients selected have signed an appropriate informed consent form at initial consultation, which allows for the scientific purpose of medical record if they remain confidential and identity protected. Patients who consecutively perform IVF and fulfilled the inclusion and exclusion criteria illustrated in Table 1 were included in the study [19,20]. Advanced maternal age was defined according to POSEIDON criteria [11]. Patients were enrolled using a consecutive sampling method. No matching procedures were applied during the recruitment process. All eligible patients who met the inclusion criteria and presented during the study period were consecutively included, regardless of age or other baseline characteristics. The population study (age range: 36-45 years) was divided into two Groups:

- Group A ($n = 50$): women who received a single subcutaneous injection of 150 UI of CF α , during the early follicular phase

of the menstrual cycle (cycle days 2 or 3) [13,21];

- Group B ($n = 100$): women who received recombinant FSH on the same period. Starting dose of r-FSH was established evaluating BMI, age and ovarian reserve in terms of antral follicle count and was subsequently adjusted in function of ovarian response during follicular monitoring [22].

Clinical and ultrasonographic evaluation

For each patient we collected: age, BMI Kg/m², menstrual cycle characteristics, last menstrual period, basal FSH and estradiol levels, diagnosis and duration of infertility. Ongoing medications were also registered. The ultrasound examinations were executed using a Voluson E6 device (GE Healthcare, Zipf, Austria) with a transvaginal probe.

They were all performed within the fifth day of OS. Antral follicle count was measured by classic 2D ultrasound approach (AFC) using trans-vaginal pelvic ultrasound (multifrequency vaginal probe 5.0–7.5 MHz) according to Broekmans *et al.* criteria [23].

IVF protocol

Patients in Group A received a single subcutaneous injection of 150 UI of CF α (Elonva, Merck Sharp & Dohme Limited, UK) during the early follicular phase of the menstrual cycle (cycle days 2 or 3), while patients in Group B received daily recombinant FSH (Gonal F, Merck Serono Europe Limited, UK) on the same period. Starting dose of r-FSH was established evaluating BMI, age and ovarian reserve in terms of antral follicle count and was subsequently adjusted in function of ovarian response during follicular monitoring. Pituitary suppression was obtained with antagonist protocol in all patients included. A dose of 0.25 mg/day of the GnRH-ant Cetrorelix (Cetrotide, Merck Serono Europe Limited, UK) was started from day 6-7 until the day of the exogenous human Chorionic Gonadotropin hCG administration. In women with at least two follicles of mean diameter >17 mm, 10.000 IU of hCG (Gonasi HP, IBSA Farmaceutici Italia Srl, Lodi, Italy) or triptorelin 0.2 mg (Fertipeptil, Ferring S.p.A., Milano, Italy) administered as ovulation inducer, the latter to be preferred in the presence of more than 20 follicles during ovarian stimulation and estradiol levels above 3,500 pg/mL [24-26].

Oocyte retrieval (PU) was performed by ultrasound-guided transvaginal aspiration 34–36 h after the trigger. Oocytes were collected in human tubal fluid and placed in culture medium immediately after the screening. Conventional IVF or IC-SI were performed according to the laboratory's routine procedures and then oocytes were cultured in 25 mL pre-equilibrated microdroplets under the oil at 37°C, under 6% CO₂. After 16-18 hours the fertilization was checked and fertilized oocytes were placed in cleavage medium. Embryo development was evaluated directly on Day 3. Embryos were transferred to blastocyst medium on Day 3 and cultured at 37°C, under 6% CO₂ and 5% O₂. Ultrasound-guided Embryo transfer (ET) was performed within 5 days of oocyte yield. Luteal phase support was prescribed vaginally in the form of 400 mg micronized progesterone (Prometrium, Rottapharm S.p.A., Milan, Italy) twice daily, from the day of oocyte retrieval until the day of the pregnancy test.

Table 1 Study protocol selection criteria.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Age > 35 years	Presence of ovarian neoformations at the time of OS
FSH < 12 IU/L	Body Mass Index (BMI) <18 and >30 Kg/m ²
Regular menstrual cycle (every 25-35 days)	Polycystic ovary syndrome [19]
Hysteroscopic evidence of a normal uterine cavity	Endocrinopathies (disorders of the hypothalamic-pituitary axis, dysthyroidism or hyperprolactinaemia)
No previous ovarian surgery	Diabetes type I e II
	Severe male factor [20]

Outcomes

The primary endpoint was the clinical pregnancy rate (CPR) according to ICMART glossary (a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy). Secondary endpoints were: number of follicles >15 mm at trigger, number of total oocytes retrieved and number of mature oocytes (metaphase II oocytes), mature oocytes percentage, number of embryos transferred, estradiol (E2) levels at 5th day and at peak of OS, duration of stimulation, total dose of gonadotropins, biochemical pregnancy rate (BPR) per aspirated cycle (a pregnancy diagnosed only by the detection of beta hCG in serum or urine)^[27]. The final outcome of interest (live birth rate, LBR) could not be fully assessed, due to the short duration of follow-up.

Statistical analysis

Reproductive outcomes were compared between the CF α treated group (Study Group) and Control group. Continuous variables are expressed in terms of mean $\pm$ SD. Categorical variables are expressed in terms of frequency and percentage. The two-sided t-test for independent samples was used to assess inter-group differences, whereas the Chi-square test was adopted to verify differences in terms of categorical data between groups. Results were analyzed using the statistical package SPSS 22 for Windows (Statistical Package for the Social Sciences, IBM, New York). A p-value <0.05 was considered statistically significant.

Results

A total of 150 women fulfilled the inclusion criteria: 50 in Group A and 100 in Group B (Figure 1). Demographic and infertility history data are summarized in Table 2.

The mean age and the BMI of the women enrolled in the study were comparable in the two groups. The two groups were also comparable regarding ovarian reserve (AFC), basal FSH

and infertility duration and diagnosis. IVF outcomes are summarized in Table 3. All patients were triggered with 10000 UI hCG.

Pregnancy outcomes (BPR and CPR) were similar in both groups. Duration of stimulation was shorter in Group A compared with Group B (8.66 ± 1.6 vs 10.04 ± 2.3 ; $p = 0.001$). All hormonal variables analysed during OS showed comparable values between groups except for estradiol measured on day 5 which was statistically higher in group where CF α was administrated (582.9 ± 413.9 vs 391.1 ± 246 ; $p < 0.05$). A significant increased number of follicles >15 mm diameter was detected in patients stimulated with CF α (8.3 ± 4.1 vs 6.7 ± 3.8 ; $p = 0.04$). No difference regarding number of total oocytes retrieved, number of mature oocyte and number of embryos transferred was observed. No cases of OHSS were reported in the two study groups.

Discussion

The present study reported comparable reproductive results among older women stimulated with a single dose of CF α or with r-FSH. The BPR and CPR did not appear to differ between the groups. No differences were observed in terms of number of oocytes retrieved, MII oocytes and embryos transferred. Except for two cases, CF α was administered at a dosage of 150 μ g, which seems the most appropriate to achieve adequate follicle recruitment without further supplementation. The costs of stimulations significantly decreased in the study group treated with r-FSH. Furthermore, there were no differences between the two groups in terms of OHSS cases.

Our study reported a significant increased number of follicles >15 mm diameter in study group. This is consistent with previous data showing an increased number of follicles > 14 mm^[28]. The desire to identify indicators of ovarian function and response, and the awareness that the number of retrieved oocytes does not always reflect ovarian potential, led to the development of ovarian sensitivity indicators.

Figure 1 Standard for reporting observational trials flow chart.

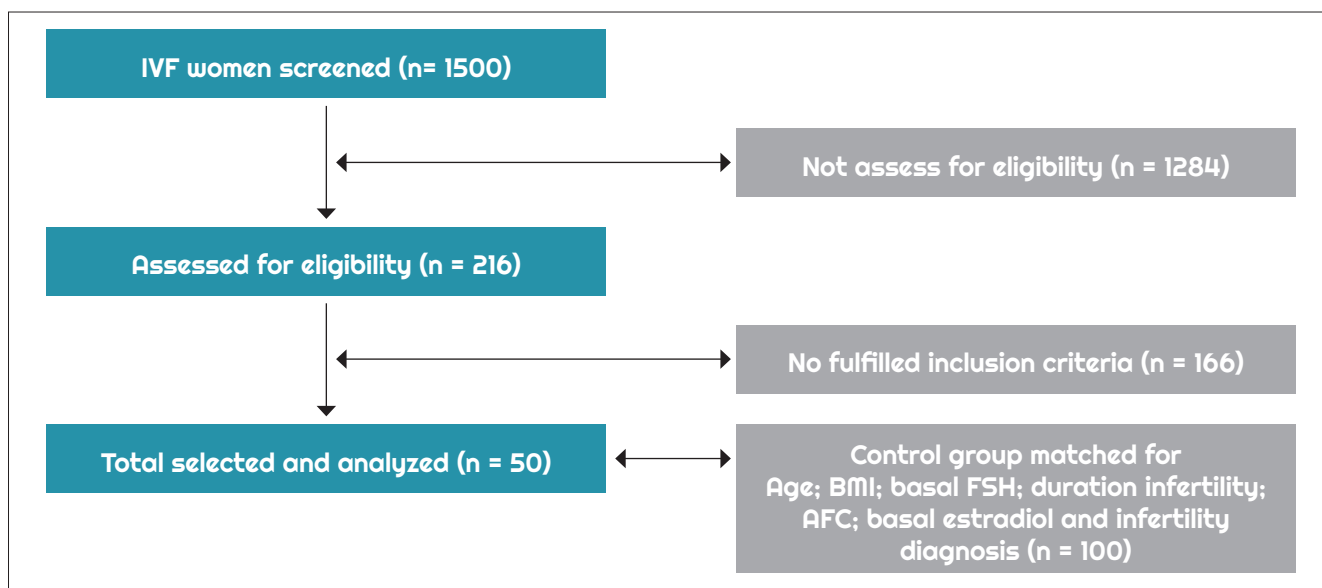


Table 2 General characteristics of enrolled women.

	GROUP A (N = 50)	GROUP B (N = 100)	P-VALUE
Age (years)	39.2 ± 2.8	39.3 ± 2.4	0.29
BMI (Kg/m ²)	24.5 ± 4.1	24.1 ± 5.1	0.73
FSH basal (IU/L)	7.73 ± 3.2	8.2 ± 3.3	0.24
Duration of infertility (years)	5.04 ± 3.6	4.8 ± 2.9	0.17
AFC	7.9 ± 3.5	8.4 ± 4.6	0.49
Basal estradiol (pg/mL)	61.75 ± 45.0	51.36 ± 32.8	0.11
DIAGNOSIS (%)			
Ovulatory	13/50 (26%)	27/100 (27%)	1
Male factor	16/50 (32%)	29/100 (29%)	0.85
Tubal	3/50 (6%)	11/100 (11%)	0.49
Unexplained	11/50 (22%)	20/100 (20%)	0.94
Endometriosis	1/50 (2%)	3/100 (3%)	1

Continuous data are reported as mean ± SD. Categorical data are reported as %.

Table 3 Ovarian stimulations outcomes, embryos and pregnancies between the two study groups.

	GROUP A (N = 50)	GROUP B (N = 100)	P-VALUE
Total gonadotropin dose (IU)	145 ± 15.1	2317.2 ± 903.4	<0.001
Duration of ovarian stimulation (days)	8.66 ± 1.6	10.04 ± 2.3	0.001
E2 at 5 th day (pg/mL)	706.22 ± 492.98	320.69 ± 234.05	<0.001
E2 at peak (pg/mL)	1387.49 ± 741.80	1176.42 ± 745.79	0.65
N. Follicles > 15mm	8.3 ± 4.1	6.7 ± 3.86	0.04
N. oocytes retrieved	6.4 ± 3.8	5.3 ± 3.7	0.28
N. MII oocytes	4.8 ± 3.0	4.05 ± 2.5	0.11
N. embryo transferred	2.2 ± 0.79	2.1 ± 0.82	0.48
BPR	10 (20%)	25 (25%)	0.63
CPR	6 (12%)	12 (12%)	1

Continuous data are reported as mean ± SD. Categorical data are reported as %. NS: no statistical significance.

Several tools have been proposed to measure ovarian sensitivity [9,29]. Genro *et al.* [30] proposed the Follicle output rate (FORT) that is calculated as the ratio between the number of pre-ovulatory follicles obtained in response to OS with FSH administration and the pre-existing pool of small antral follicles [31]. The ovarian sensitivity index (OSI) measures the ration between cumu-lative gonadotropin and the number of retrieved oocytes [32]. More recently, POSEIDON group proposed follicle oocytes index (FOI) that consists in the ratio between the total number of oocytes collected at the end of OS, and the number of antral follicles available at the start of stimulation [29]. The study of these indicators in response to the use of CF α could provide useful information. Mardešić *et al.* investigated the reproductive outcomes in women aged between 18 and 36 years

stimulated with a single dose of CF α and found no difference [33]. So far, data regarding the effect of CF α among advanced age patients are still scanty. The latest metanalysis reported no differences in normal and poor-responder patients in pregnancy outcomes and risks of serious adverse events or cycle cancellations [16]. The total number of oocytes, MII oocytes, and embryos obtained were significantly higher in patients stimulated with CF α than r-FSH. In agreement with previous data, a single dose of corifollitropin alfa for the first 7 days of ovarian stimulation is a generally well-tolerated treatment and as effective as the daily effect of rFSH in women over 35 years of age, with no differences in pregnancy rate, live birth rate and oocyte recovery [34-36]. In our study, the mean number of follicles >15 mm was significantly higher in the study group. However, no

statistically significant differences in the number of retrieved and mature oocytes were found between the groups. Unlike these two RCTs, in our study no supplementation with r-FSH from day 8 was administrated. Our results confirmed that the shorter duration of stimulation seemed to no impact on reproductive outcome even in advanced age women. Many attempts have been made to identify effective protocols in patients with a poor prognosis. It has been reported that minimal ovarian stimulation is a cost-effective alternative to conventional protocols in both POSEIDON groups 2 and 4 [37-39]. Moreover, several adjuvant injections and the treatments have been studied to improve the response with conflicting results [28,40]. As for the psychological stress due to IVF treatments, the identification of a simpler COS regimen reduces the duration of stimulation, the number of gonadotropin drop-out rate [41,42]. Recently, Messaoud *et al.* found that in a national context of full coverage of all infertility treatments, advanced maternal age was a reason of the high rate of early drop-out [43]. In this context, the use of a single dose of CF α could improve treatment efficiency, with a significant reduction in costs and time. It is important to emphasise how the introduction of regimens with few injections, perhaps with PPOS protocols, can reduce patients' stress while maintaining the same effectiveness.

The limitations of our study are the small sample size, the retrospective design and the use of a consecutive enrolment strategy without matching procedure. Although this approach reflects routine clinical practice and facilitates patient recruitment, it may introduce potential confounding due to differences in baseline characteristics. However, a comparative analysis of baseline variables between the study groups revealed no significant differences, suggesting that the cohorts were comparable at baseline. This reduces, but does not entirely eliminate, the risk of balance bias between the groups. The short-term follow-up did not make it possible to assess LBR and this reduces the strength of the study. Due to the small sample size, a subgroup analysis was not performed between patients in groups 2 and 3 according to POSEIDON, which would have allowed a comparison according to ovarian reserve. Therefore, we are not able to provide the precise profile of the woman of advanced age that is able to sustain the entire stimulation only with CF α [6,44]. On Clinicaltrials.gov, four unpublished RCTs (NCT02254928, NCT03816670, NCT02046655, NCT03177538) examined the role of CF α in poor-responder, the last of these using the POSEIDON classification (TEMPER Study).

Conclusions

Our observations suggest that single injection of CF α is a well-tolerated and convenient option even in advanced age patients and in some case appeared to be sufficient for ovulation triggering. Larger trials in future could clarify which kind of women may benefit from this strategy which in turn could offer the same results but with more elevated compliance and feasibility.

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Gonadotropins: reversing azoospermia in hypo-hypo

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ABSTRACT

Purpose: This study evaluates the effectiveness of gonadotropin therapy in inducing spermatogenesis and improving semen parameters in azoospermic males with hypogonadotropic hypogonadism (HH), distinguishing between pre-pubertal and post-pubertal onset forms. Additionally, it compares responses in congenital versus acquired HH within the pre-pubertal group. **Materials and methods:** Seventeen azoospermic males with HH (FSH <2 IU/L) were included. Nine had pre-pubertal onset HH, and eight had post-pubertal onset HH. Semen analysis was conducted at baseline and after 3, 6, and 9 months of gonadotropin therapy (FSH 150 IU, three times/week; hCG 2000 IU, once/week). **Results:** After gonadotropin therapy, 88.9% of patients in the pre-pubertal group resumed spermatogenesis, with response rates of 22.2% at 3, 55.5% at 6, and 11.1% at 9 months. In the post-pubertal group, 87.5% achieved spermatogenesis, with 62.5% responding at 3 months, 12.5% at 6 and 9 months. Post-pubertal patients showed greater semen parameter improvements, with lower atypical forms ($p = 0.032$) and higher sperm concentration ($p = 0.012$), total count ($p = 0.004$), and motility ($p = 0.013$). Among pre-pubertal patients, acquired HH cases had a better response (100% vs. 75% in congenital HH). **Conclusion:** Gonadotropin therapy effectively induces spermatogenesis in HH patients, with high response rates. Post-pubertal onset cases respond faster and exhibit superior semen quality. Acquired HH cases in the pre-pubertal group show better outcomes than congenital cases, underscoring the need for individualized treatment based on HH etiology and onset timing.

KEYWORDS

Hypogonadotropic hypogonadism, gonadotropin therapy, male infertility, spermatogenesis, male reproductive health.

Introduction

Hypogonadotropic hypogonadism (HH) represents a primary endocrine disorder in which inadequate hypothalamic or pituitary stimulation leads to deficient gonadotropin secretion, resulting in impaired testicular function and subsequent infertility^[1]. This condition results in low levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone, often causing azoospermia or severe oligozoospermia^[1-3].

The pathophysiology of HH-related infertility varies based on the onset timing of the disorder, typically classified as either pre-pubertal or post-pubertal^[1]. In congenital cases, such as Kallmann syndrome, and in some acquired pre-pubertal cases (e.g., due to cranial trauma or pituitary surgery), the testes may remain underdeveloped, leading to smaller testicular volume and a prolonged or muted response to gonadotropin therapy^[4-6]. Conversely, post-pubertal HH patients often retain some testicular function and may show more robust responses to hormonal stimulation, with potentially shorter times to achieve clinically meaningful sperm concentrations^[6-8].

Hormonal stimulation is crucial for spermatogenic initiation or restoration, as FSH and LH are essential for Sertoli and Leydig cell function, respectively^[6,9-12]. Gonadotropin therapy, including human or recombinant FSH, in association with human chorionic gonadotropin (hCG) has become a cornerstone in treating

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infertility in men with HH, aiming to stimulate spermatogenesis by mimicking natural endocrine signals^[11,13,14]. Studies have demonstrated that combined gonadotropin therapy can restore spermatogenesis in approximately 80–90% of patients, though sperm concentration typically remains below normative fertile thresholds^[7,9]. Despite these positive outcomes, response rates vary due to several factors such as age, underlying etiology, and prior androgen exposure. The latter significantly influence the likelihood of restoring spermatogenesis, as prior exposure can induce testicular fibrosis and reduce Sertoli cell responsiveness^[6,8]. Additionally, semen parameter improvements, including sperm motility and morphology, have been linked to optimal FSH dosing and individual receptor polymorphisms^[6,10]. This study aims to analyze the treatment response and semen profile of males with HH undergoing gonadotropin therapy, comparing outcomes between pre-pubertal and post-pubertal onset.

By examining these distinct profiles, the study seeks to identify factors predictive of successful spermatogenesis restoration, refine therapeutic protocols, and improve prognostic accuracy for infertility management in HH patients.

Materials and methods

Study population

A total of 17 azoospermic patients with baseline FSH levels <2 IU/L consecutively referred to our Andrology Unit for fertility assessment between January 2015 and July 2019 were included in the study. Among them, 9 had pre-pubertal onset HH due to conditions such as Kallmann syndrome with anosmia/hyposmia, isolated hypogonadotropic hypogonadism (IHH), craniopharyngioma, or panhypopituitarism, while 8 had post-pubertal onset HH, including cases of panhypopituitarism secondary to GH-secreting adenoma or prolactinoma, selective IHH, and Cooley’s anemia (Tables 1 and 2).

Semen analysis

Standard semen analysis was conducted at the Andrology Unit of Sant’Andrea Hospital. For all patients, semen samples were collected and analyzed under baseline conditions (following testosterone washout) and at subsequent intervals of 3, 6, and 9 months. Semen examination was performed in accordance with the 2010 World Health Organization (WHO) guidelines [14]. All patients underwent semen cryopreservation after the achievement of spermatogenesis.

Treatment protocol

For patients receiving testosterone replacement therapy at the time of study enrollment, semen analysis was conducted following a minimum 3-month washout period from testosterone administration. The treatment regimen for all subjects consisted of the following: subcutaneous administration of FSH at 150 IU, three times per week, combined with subcutaneous administration of hCG at 2000 IU, once per week. At the 3-month evaluation, non-responders were given a new treatment cycle of 3 months.

Table 1 Characteristics of patients with pre-pubertal onset hypogonadotropic hypogonadism.

DIAGNOSIS	AGE AT DIAGNOSIS	PREVIOUS GONADOTROPIN TREATMENT	CURRENT TREATMENT	AGE IN STUDY
Selective IHH	12	yes	testosterone	34
Kallmann syndrome (anosmia)	14	–	testosterone	41
Selective IHH	12	–	testosterone	36
Selective IHH	12	–	testosterone	35
Panhypopituitarism	11	–	testosterone	34
Kallmann syndrome (hyposmia)	14	–	testosterone	31
Kallmann syndrome (hyposmia)	16	–	–	20
Craniopharyngioma	2	–	testosterone	33
Kallmann syndrome (hyposmia)	16	yes	testosterone	23

IHH = Isolated Hypogonadotropic Hypogonadism

Table 2 Characteristics of patients with post-pubertal onset hypogonadotropic hypogonadism.

DIAGNOSIS	AGE AT DIAGNOSIS	PREVIOUS GONADOTROPIN TREATMENT	CURRENT TREATMENT	AGE IN STUDY
Panhypopituitarism (GH-secreting adenoma)	19	–	–	19
Panhypopituitarism (Prolactinoma)	24	–	testosterone	32
Selective IHH	22	yes	testosterone	31
Selective IHH	20	–	testosterone	25
Selective IHH	28	–	testosterone	34
Cooley’s anemia	24	–	testosterone	28
Cooley’s anemia	25	–	testosterone	44
Selective IHH	45	–	–	45

IHH = Isolated Hypogonadotropic Hypogonadism; GH = Growth Hormone

Statistical analysis

Statistical analyses were performed using mean and standard deviation (SD) metrics. Frequency differences were assessed using the chi-squared test, and unpaired data were analyzed using Student's t-test. Statistical significance was established at a p-value < 0.05.

Results

Pre-pubertal hypogonadotropic hypogonadism

The mean age at diagnosis for patients with pre-pubertal HH was 12.1 ± 4.2 years, with a range between 2 and 16 years. At diagnosis, the mean FSH level was 0.6 ± 0.3 IU/L. By the time of treatment initiation, the mean age had increased to 31.9 ± 6.5 years, spanning from 20 to 41 years.

In this group, 8 out of 9 patients (88.9%) exhibited resumption of spermatogenesis following gonadotropin therapy. Spermatogenic activity resumed in 2 patients (22.2%) after three months of treatment, in 5 additional patients (55.5%) after six months, and in the final patient (11.1%) by nine months. Table 3 reports specific semen values.

Post-pubertal hypogonadotropic hypogonadism

The mean age at diagnosis for patients with post-pubertal HH was 25.9 ± 8.2 , with a range of 19 to 45 years. At diagnosis, the mean FSH level was 1.1 ± 0.4 IU/L. At the time of treatment initiation, the mean age was 32.3 ± 8.9 years, with a range from 19 to 45 years. Spermatogenesis resumed in 7 out of 8 patients

(87.5%) in the post-pubertal onset group. This group demonstrated a progressive response over the treatment period, with 5 patients (62.5%) achieving spermatogenesis within the first three months. An additional patient (12.5%) showed resumed spermatogenic activity by the six-month mark, and the final patient (12.5%) by nine months. Table 4 reports specific semen values.

Comparison between pre-pubertal and post-pubertal onset hypogonadotropic hypogonadism

Both groups demonstrated high response rates to gonadotropin therapy, with 88.9% of pre-pubertal onset and 87.5% of post-pubertal onset patients resuming spermatogenesis. However, the post-pubertal onset group showed a quicker response, with 62.5% achieving spermatogenesis by three months compared to 22.2% in the pre-pubertal onset group.

The analysis of semen parameters revealed significant improvements in the post-pubertal onset group following gonadotropin stimulation. After three months of treatment, the proportion of atypical sperm forms was significantly reduced ($p = 0.032$) (Table 3). By six months, patients in this group exhibited significant increases in sperm concentration ($p = 0.012$), total sperm count ($p = 0.004$), and motility ($p = 0.013$) (Table 4; Figure 1).

Comparison between congenital and acquired hypogonadotropic hypogonadism in pre-pubertal onset patients

Within the pre-pubertal onset HH group, spermatogenesis resumption was compared between congenital and acquired

Table 3 Comparison of semen parameters between the two groups after 3 months of gonadotropin treatment.

PARAMETER	PRE-PUBERTAL HH	POST-PUBERTAL HH	P-VALUE
PH	7.51 ± 0.11	7.50 ± 0.14	0.871
Volume (ml)	0.8 ± 0.8	1.0 ± 0.6	0.324
Concentration (n x10 ⁶ /ml)	0.9 ± 0.1	4.6 ± 2.8	0.066
Total ejaculate (n x10 ⁶)	0.3 ± 0.1	5.0 ± 4.0	0.091
Total motility (%)	3.0 ± 0	12.4 ± 7.2	0.071
Atypical forms (%)	97.5 ± 0.7	87.8 ± 5.4	0.032

HH = Hypogonadotropic Hypogonadism

Table 4 Comparison of semen parameters between the two groups after 6 months of gonadotropin treatment.

PARAMETER	PRE-PUBERTAL HH	POST-PUBERTAL HH	P-VALUE
PH	7.49 ± 0.11	7.55 ± 0.16	0.553
Volume (ml)	1.1 ± 0.8	1.4 ± 1.2	0.277
Concentration (n x10 ⁶ /ml)	1.4 ± 1.0	10.8 ± 9.6	0.012
Total ejaculate (n x10 ⁶)	1.7 ± 2.2	9.6 ± 6.2	0.004
Total motility (%)	6.0 ± 2.8	18.3 ± 11.1	0.013
Atypical forms (%)	95.6 ± 3.6	87.0 ± 6.1	0.004

HH = Hypogonadotropic Hypogonadism

Figure 1 Pre-pubertal and post-pubertal response to gonadotropin therapy.

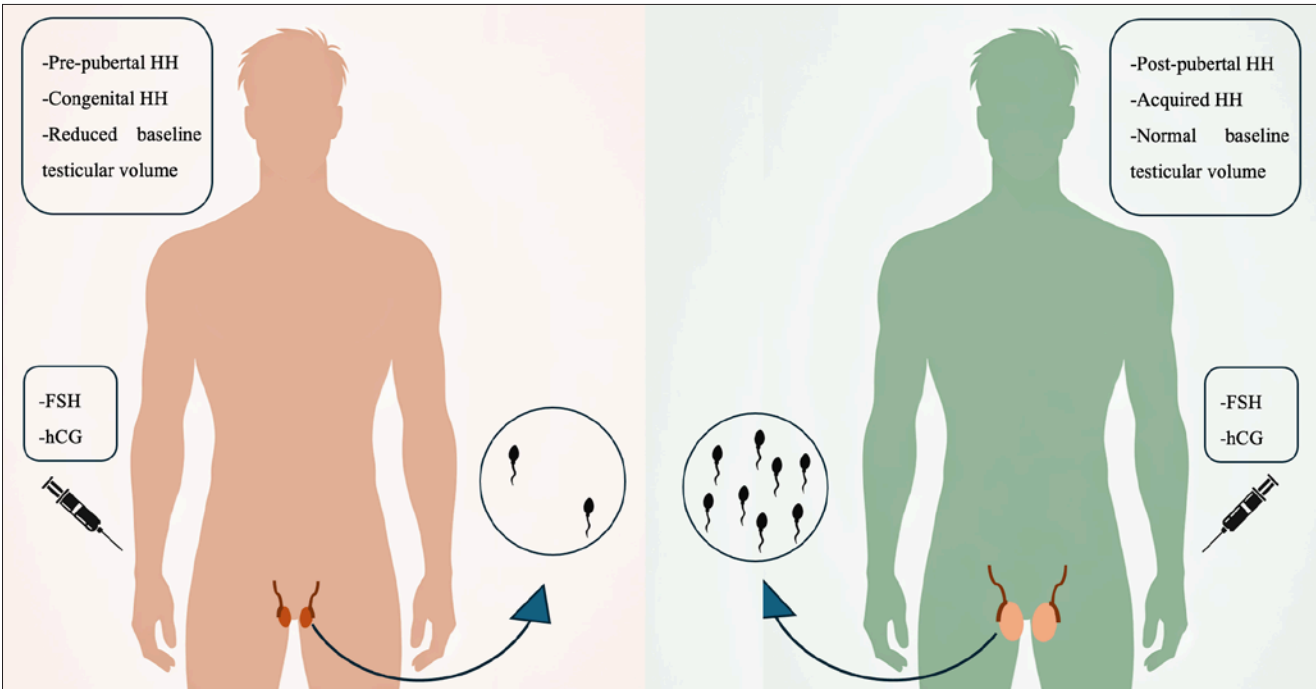


Table 5 Semen parameters of HH after 3 and 6 months of gonadotropin treatment.

DIAGNOSIS	SEMEN PARAMETERS AFTER 3 MONTHS				SEMEN PARAMETERS AFTER 6 MONTHS			
	VOLUME (ml)	CONCENTRATION (N X10 ⁶ /ml)	TOTAL MOTILITY (%)	ATYPICAL FORMS (%)	VOLUME (ml)	CONCENTRATION (N X10 ⁶ /ml)	TOTAL MOTILITY (%)	ATYPICAL FORMS (%)
Kallmann syndrome (anosmia)	0.4	Azoospermia	–	–	0.4	Azoo	–	–
Kallmann syndrome (hyposmia)	0.5	Azoospermia	–	–	0.4	0.1	–	100
Kallmann syndrome (hyposmia)	1.9	Azoospermia	–	–	2.2	3	5	94
Kallmann syndrome (hyposmia)	0.6	Azoospermia	–	–	0.9	1.35	10	95
Selective IHH	0.4	Azoospermia	–	–	0.6	1	3	96
Selective IHH	0.3	0.8	3	97	0.9	2	7	91
Selective IHH	0.4	0.9	3	98	0.8	1.5	8	92
Panhypopituitarism	0.4	Azoospermia	–	–	0.6	0.5	3	96
Craniopharyngioma	2.5	Azoospermia	–	–	2.7	–	–	100
HH = Hypogonadotropic Hypogonadism								

cases. Among patients with acquired HH (including selective IHH, panhypopituitarism, and craniopharyngioma), 2 out of 5 (40%) showed spermatogenic activity by three months. By six months, all 5 patients (100%) with acquired HH had resumed spermatogenesis. In contrast, none of the patients with congenital HH (Kallmann syndrome) exhibited spermatogenesis at the three-month mark, but by six months, 3 out of 4 patients (75%) had resumed spermatogenesis (Table 5).

Although the differences were not statistically significant, semen parameters, including sperm concentration and motility,

tended to be higher in patients with acquired HH compared to those with congenital HH after both three and six months of treatment (Table 5).

Discussion

This study highlights the effectiveness of gonadotropin therapy in inducing spermatogenesis and improving semen parameters in males with HH, confirming its central role in the

management of HH-related infertility. The observed response rates (88.9% in the pre-pubertal onset group and 87.5% in the post-pubertal onset group) align closely with previously reported success rates of gonadotropin treatment. These findings strengthen the evidence base for gonadotropins, particularly hCG and r-FSH, as the cornerstone of treatment for this condition [6,10,15,16].

The differences in response timing between pre-pubertal and post-pubertal onset HH patients provide critical insights into the pathophysiology and therapeutic management of the condition. Post-pubertal onset patients achieved spermatogenesis more quickly, with 62.5% responding within three months compared to only 22.2% in the pre-pubertal onset group. This faster response may be attributed to the residual testicular function often preserved in post-pubertal onset cases, where testicular development and spermatogenic potential were established prior to the onset of hypogonadism. These findings are consistent with the notion that testicular volume and baseline functionality are key predictors of therapeutic success, as larger and more developed testes are more responsive to exogenous gonadotropins [6,17]. Furthermore, the preservation of Sertoli and Leydig cell activity in post-pubertal onset HH may facilitate quicker activation of the hypothalamic-pituitary-gonadal axis upon initiation of therapy [11,13].

In contrast, pre-pubertal onset HH, particularly in congenital cases such as Kallmann syndrome, often presents a more challenging clinical scenario. These patients frequently exhibit delayed or incomplete testicular maturation, necessitating prolonged gonadotropin therapy to initiate and sustain spermatogenesis. The lower initial testicular volume and potential genetic alterations associated with congenital forms of HH may further limit the responsiveness to treatment [17,18]. Despite these challenges, the 88.9% success rate in restoring spermatogenesis in pre-pubertal onset HH patients underscores the potential of prolonged gonadotropin therapy, even in cases with significant baseline impairments.

The observed improvements in semen quality, particularly among post-pubertal onset HH patients, further highlight the efficacy of gonadotropin therapy. Significant increases in sperm concentration, total sperm count, and motility, as well as reductions in atypical sperm forms, were noted after three to six months of treatment in post-pubertal HH compared with pre-pubertal onset. These findings align with existing literature emphasizing the role of FSH in germ cell development and the enhancement of sperm morphology and motility [10].

Another significant finding of this study is the comparison between congenital and acquired HH within the pre-pubertal onset group. Patients with acquired HH demonstrated a more favorable response, with 100% achieving spermatogenesis by six months compared to 75% in congenital HH. This discrepancy likely reflects differences in baseline testicular architecture and the absence of inherent genetic defects in acquired cases. Acquired HH, often secondary to pituitary tumors or trauma, may preserve testicular integrity, allowing for a more robust response to gonadotropins [8]. Conversely, congenital HH may involve intrinsic impairments in the hypothalamic-pituitary-gonadal axis or gonadal tissue itself, which can delay or attenuate

the therapeutic response [5,8,11].

Additionally, the potential influence of prior testosterone therapy, administered to the majority of patients before gonadotropin treatment, was considered. Although previous androgen exposure has been associated with reduced testicular responsiveness due to suppression of Sertoli cell activity, no significant differences in spermatogenic outcomes were observed in our sample. However, given the small sample size and the fact that most patients had received androgens, a statistically robust comparison between exposed and unexposed individuals was not feasible and should be addressed in future studies.

The clinical implications of these findings could be various. Firstly, they underscore the importance of early diagnosis and prompt initiation of gonadotropin therapy, particularly in congenital HH, where prolonged stimulation may be required to achieve optimal results. The timing of intervention is especially critical in adolescent and young adult patients, as early therapy can mitigate the long-term consequences of delayed testicular maturation and irreversible damage [6,17]. Secondly, the study highlights the need for individualized treatment protocols tailored to the etiology and onset timing of HH. Incorporating baseline assessments such as testicular volume, inhibin B levels, and genetic testing into clinical decision-making could enhance treatment planning and predict therapeutic outcomes [7].

This study presents some limitations, including the small sample size, lack of complete hormonal profiling due to prior androgen exposure, and absence of long-term follow-up beyond 9 months.

In conclusion, this study highlights the efficacy of gonadotropin therapy in managing HH-associated infertility, demonstrating high response rates and significant improvements in semen parameters across both pre-pubertal and post-pubertal onset groups. While post-pubertal onset cases benefit from faster responses, pre-pubertal onset and congenital HH patients show promising outcomes with sustained therapy. These findings underscore the need for tailored treatment strategies, early intervention, and further research into optimizing therapeutic regimens to maximize fertility potential in this population.

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Sperm handling during ART: effect of concentration and two different temperatures on sperm quality

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ABSTRACT

Human semen may have a variable degree of sperm DNA fragmentation. Moreover, sperm handling during ART can increase the basal sperm DNA fragmentation and this has been correlated to oxidative stress (OS). As sperm DNA fragmentation and OS may result in poor fertilization, embryo development and even exert long term effects on newborns, it is fundamental to understand the factors that can add a iatrogenic damage to sperm during ART. The main aims of the present study were to assess the effect of two different temperatures, room temperature and 37°C, on motility, viability, oxidative DNA damage and DNA fragmentation on human spermatozoa selected or not with swim up. Moreover, we evaluated if sperm concentration affects the motility, viability and DNA fragmentation of human sperm. Sperm macroscopic and microscopic features were analyzed in 25 patients. Experiment I: semen samples incubated 1h at RT or 37°C. Experiment II: swim up samples incubated 3h at RT or 37°C. Experiment III: washed samples incubated 3h at 37°C at different sperm concentrations (RANGE: 1, 100-75; 2, 50-35; 3, 25-15; 4, 10-5 x10⁶). Our data indicate that viability and DNA integrity of semen or swim-up samples were better preserved at RT versus 37°C. Oxidative DNA damage was higher in semen storage at 37°C than semen storage at RT, but no significant differences were observed in swim-up samples. Washed sperm were better preserved at low concentration..

KEYWORDS

Handling, spermatozoa, temperature, oxidative stress, concentration.

Introduction

Semen analysis is the first step to identify male infertility. Standardized methods of semen analysis allow accurate assessment of sperm quality. Clinical ART procedures are performed on pre-treated/manipulated gametes, to enhance and facilitate fertilization process, obtained through a variety of *in vitro* manipulations, such as sperm washing, cryopreservation, different methods of selection, exposure to different temperature and oxygen pressure.

Such manipulative techniques may compromise important features of gamete integrity, structure and function. Sperm handling during ART can increase the basal sperm DNA fragmentation and this has been correlated to oxidative stress (OS) [1]. As sperm DNA fragmentation and OS may result in poor fertilization, embryo development and even exert long term effects on newborns, it is fundamental to understand factors that can add a iatrogenic damage to sperm during ART [2-5]. Gametes and embryos are natural sources of free radicals, but, when manipulated *in vitro* during ART, they run the risk of generating and being exposed to supraphysiological levels of ROS, causing a significant impact on IVF outcome [6].

Moreover, high levels of ROS occur in about 40–88% of sperm samples from infertile men [7-9]. A high level of ROS and decreased levels of antioxidants can reduce sperm motility,

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ty, DNA integrity, and viability, and increase midpiece defects (Wright *et al.*, 2014 and Pasqualotto *et al.*, 2000). Poor DNA integrity is correlated with lower pregnancy rates, irregular pre-implantation development, early loss of pregnancy, and increased disease rates in offspring conceived through ART [10-12].

Main aims of the present study were to assess the effect of two different temperatures, room temperature and 37°C, on motility, viability, oxidative DNA damage and DNA fragmentation on human spermatozoa selected or not with swim up. Moreover, we evaluated if sperm concentration affects the motility, viability and DNA fragmentation of human sperm.

Materials and methods

Chemicals

Sydney IVF Gamete Buffer was from Cook Medical (Ire-

land). Paraformaldehyde, Triton X100, sodium citrate, Hoechst 33342 and DL-Dithiothreitol were from Sigma Chemical Company (Milan, Italy). In Situ Cell Death Detection Kit, Fluorescein and DNase I were from Roche Diagnostics (Milan, Italy), Live/dead Fixable Far Red Dead Cell Stain Kit (L10120) from Molecular Probes (Monza, Italy) and Oxy DNA Kit Fluorometric were from Calbiochem.

EXPERIMENT I

All this study was approved by our Institutional Review Board. Semen samples were collected, following written informed consent, from 13 patients (23–30 years of age). Samples were analyzed according to criteria of WHO for concentration and motility through Sperm Class Analyzer (SCA Microptic S.L. Barcelona, Spain), on a heated stage at 37°C. Ten normospermic, two oligospermic and one asthenospermic patients were included. Samples were divided into aliquots and treated as detailed below.

Effects of temperature on total and progressive sperm motility

In all experiments, one aliquot was used as control and other two aliquots incubated for 1h at room temperature (1H RT) or 37°C (1H 37°C). Samples were loaded into a Makler chamber and analyzed with a Nikon TE 2000 inverted microscope connected to a Basler Vision Technology A312 fc camera with a positive phase contrast 10X objective by SCA. Spermatozoa were evaluated for total and progressive motility and curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), with the following software settings: frame rates: 25 frames/s, number of frames: 10 frames/object, velocity limit for slow spermatozoa: 10 $\mu\text{m/s}$, velocity limit for moderately motile spermatozoa: 15 $\mu\text{m/s}$, velocity limit for rapid spermatozoa 35 $\mu\text{m/s}$, minimal linearity 50%, straightness for progressive fast spermatozoa 80%.

Sperm viability

Viability was detected using Live/Dead Fixable Far Red Dead Cell Stain Kit, based on the reaction of a fluorescent reactive dye with cellular amines. It permeates compromised membranes of necrotic cells and reacts with free amines both in interior and on cell surface, resulting in intense fluorescent staining. In contrast, only cell-surface amines of viable cells are available to react with dye, resulting in relatively dim staining. The difference in intensity is typically greater than 50-fold. Control, 1H RT and 1H 37°C samples were washed in Gamete Buffer at 650 g for 10 min and pellets suspended in 1ml DPBS. According to the manufacturer, samples were incubated with 1 μL of reconstituted fluorescent reactive dye for 30 min on ice protected from light. Then samples were washed in DBPS at 650 g for 10 min and pellets re-suspended in fresh Gamete Buffer for TUNEL assay.

Sperm DNA fragmentation

DNA fragmentation was analyzed by TUNEL assay through In Situ Cell Death Detection Kit, Fluorescein. Control, 1H RT and 1H 37°C samples were incubated with 2mM DL-Dithiothreitol (DTT) for 45 min at RT. DTT is a small molecular mass reducing

agent, which relaxes chromatin structure of mammalian spermatozoa by breaking disulphide bridges between adjacent protamine molecules. After incubation, samples were washed in PBS at 650 g for 10 min and pellets fixed in 4% paraformaldehyde in PBS for 30 min at RT. Then samples were washed three times in PBS through centrifugation at 650 g for 10 min, smeared on glass slides and air dried. Samples were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at 4°C, washed in PBS and then incubated with TUNEL reaction mixture according to the manufacturer, for 1 h in the dark at 37°C. At the end of incubation, slides were washed in PBS as above, labelled with Hoechst 33342 10 $\mu\text{g/ml}$ for 7 min at RT, washed again in PBS, mounted and observed. During each procedure, negative and positive controls were prepared by omission of TdT in the reaction mixture or by pretreatment with 1 mg/ml DNase I (Roche Diagnostics) for 10 min at RT. Samples were observed at a Leica TCS SP5 confocal microscope (Leica, Milan, Italy) fitted with a 405nm laser for hoechst, 488 nm laser for fluoresceine and 633nm laser for far red. Hoechst was detected at 415-625 nm, fluoresceine at 498-640 nm and far red at 643-800 nm. At least 200 spermatozoa were analyzed in each sample.

Oxidative DNA damage

Oxidative DNA damage was detected by OxyDNA Assay Kit Fluorometric. Control, 1H RT and 1H 37°C samples were incubated for 1H at RT or 37°C; after incubation, samples were washed in PBS at 650 g for 10 min and pellets were fixed in 4% paraformaldehyde in PBS for 30 min at RT. Then samples were washed three times in PBS through centrifugation at 650 g for 10 min, smeared and air dried. Samples were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at 4°C, washed in PBS three times for 10 min and then incubated with Oxy reaction mixture according to the manufacturer, for 1 h in the dark at RT. The probe is specific for 8-oxoguanosine that is formed during free radical damage to DNA and is a sensitive and specific indicator of oxidative DNA damage. At the end of incubation, slides were washed in PBS as above, labelled with Hoechst 33342 10 $\mu\text{g/ml}$ for 7 min at RT, washed again in PBS, mounted and observed at a Leica TCS SP5 confocal microscope (Leica, Milan, Italy) fitted with a 405nm laser for Hoechst and 488 nm laser for fluoresceine. At least 200 spermatozoa were analyzed in each sample.

EXPERIMENT II

Effects of temperature on sperm total and progressive motility, viability, oxidative DNA damage and DNA fragmentation after swim-up procedure

Semen samples were collected from 7 patients (27–35 years of age). In all experiments, one aliquot was used as control and other two aliquots treated for swim up procedure for 1h at 37°C and then incubated for 3h at RT (3H PSU RT) or 37°C (3H PSU 37°C). At the end, all samples were analyzed as before.

EXPERIMENT III

Effects of concentration on sperm total and progressive motility, viability and DNA fragmentation.

Semen samples were collected from 7 patients (25–35 years of age). In all experiments, one aliquot was used as control and

other aliquots incubated for 3h at 37°C. Serial dilutions were performing comply with concentration ranges:

- Range 1 = 100 mln – 70 mln
- Range 2 = 50 mln – 35 mln
- Range 3 = 25 mln – 15 mln
- Range 4 = 10 mln – 5 mln

Following which, all samples were analyzed as previously described.

Statistical analysis

The data are presented as the mean $\pm$ SD. Overall, analysis was performed by Fisher's exact test or by the estimate model of ANOVA followed by the Tukey's honestly significant difference test for pairwise comparisons when overall significance was detected.

Results

EXPERIMENT I

Sperm kinetics

Data showed no significant differences in kinetics in all samples.

Viability, DNA damage and DNA fragmentation

After 1h of incubation there was a significant different viability in 1H RT compared with CONTROL (72.7% vs 75.75 %) while extremely significant differences were between 1H 37°C compared with Time 0 and 1H RT samples (Time 0 75.7%; 1HRT 72.7%; 1H 37°C 58.6%). Even extremely significant differences in percentage of spermatozoa characterized by oxidative damage were between 1H 37°C compared with Time 0 and 1H RT samples (Time 0 15.8%; 1H RT 15%; 1H 37°C 27.6%). Furthermore, data showed no significant differences in DNA fragmentation between Time 0 and 1H RT samples (9.4% vs 11.7%) but the percentage of spermatozoa with fragmented DNA was significantly increased in 1H 37°C compared with Time 0 and 1H RT samples (Time 0 9.4%; 1H RT 11.7%; 1H 37°C 21.3%). Moreover, data showed that percentage of TUNEL-positive live spermatozoa wasn't different between

Time 0 and 1H RT samples but it was significantly increased in 1H 37°C samples (Time 0 4.38%; 1H RT 5%; 1H 37°C 10.35%) (Figure 1).

EXPERIMENT II

Sperm kinetics

Data showed no significant differences in kinetics in all samples.

Viability and DNA fragmentation

Viability data showed no significant differences between samples. Moreover, extremely significant differences in percentage of spermatozoa characterized by oxidative damage were observed between 3H PSW 37°C and 3H PSW RT compared with Time 0 (Time 0 PSW 2.4%; 3H PSW RT 5.5%; 3H PSW 37°C 6.7 %).

Furthermore, percentage of spermatozoa with fragmented DNA significantly increased in 3H PSW 37°C compared with Time 0 PSW and 3H PSW RT samples (Time 0 PSW 6.4%; 3H PSW RT 9%; 3H PSW 37°C 15.3%). Moreover, data showed that the percentage of TUNEL-positive live spermatozoa significantly increased in 3H PSW 37°C compared with Time 0 PSW and 3H PSW RT (Time 0 PSW 3.9%; 3H PSW RT 5.7 %; 3H PSW 37°C 11.8%) (Figure 2).

EXPERIMENT III

Sperm kinetics

Data showed no significant differences in sperm kinetics in all samples.

Viability and DNA fragmentation

Viability data showed no significant differences between the samples. Furthermore, percentage of spermatozoa with fragmented DNA and percentage of TUNEL-positive live spermatozoa significantly increases in RANGE 1 sample compared with all other samples.

There is a dependent concentration effect. TUNEL-positive total (Time 0 7%; RANGE 1 15%; RANGE 2 11.1%; RANGE 3 8.7%; RANGE 4 6.9%). TUNEL-positive live (Time 0 3%; RANGE 1 6%; RANGE 2 5.5%; RANGE 3 4.7%; RANGE 4 2.5%) (Figure 3).

Figure 1 Effect of temperature on sperm viability, DNA fragmentation and oxidative DNA damage #: significant difference compared to control ($p < 0.05$); ##: very significant difference compared to control ($p < 0.01$); **: very significant difference related to comparative sample ($p < 0.01$).

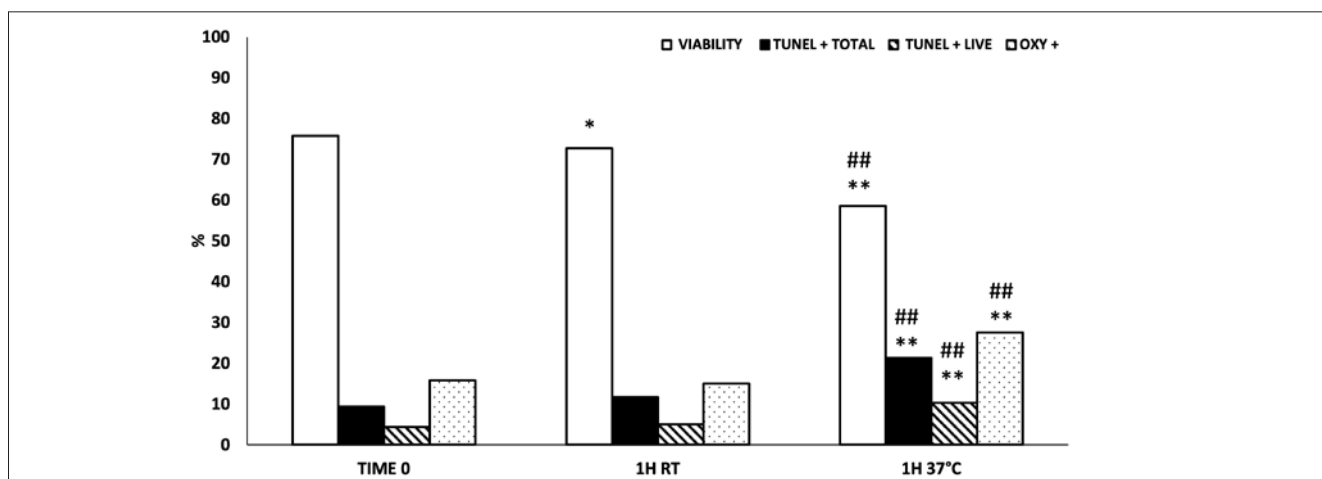


Figure 2 Effect of temperature on selected sperm viability, DNA fragmentation and oxidative DNA damage #: significant difference compared to control ($p < 0.05$); ##: very significant difference compared to control ($p < 0.01$); **: very significant difference related to comparative sample ($p < 0.01$).

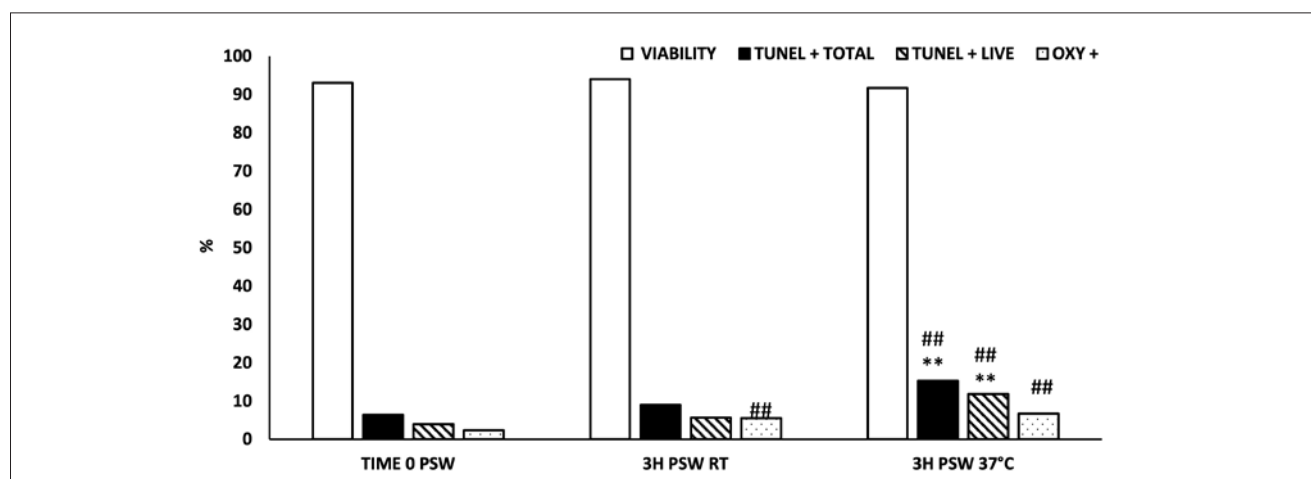
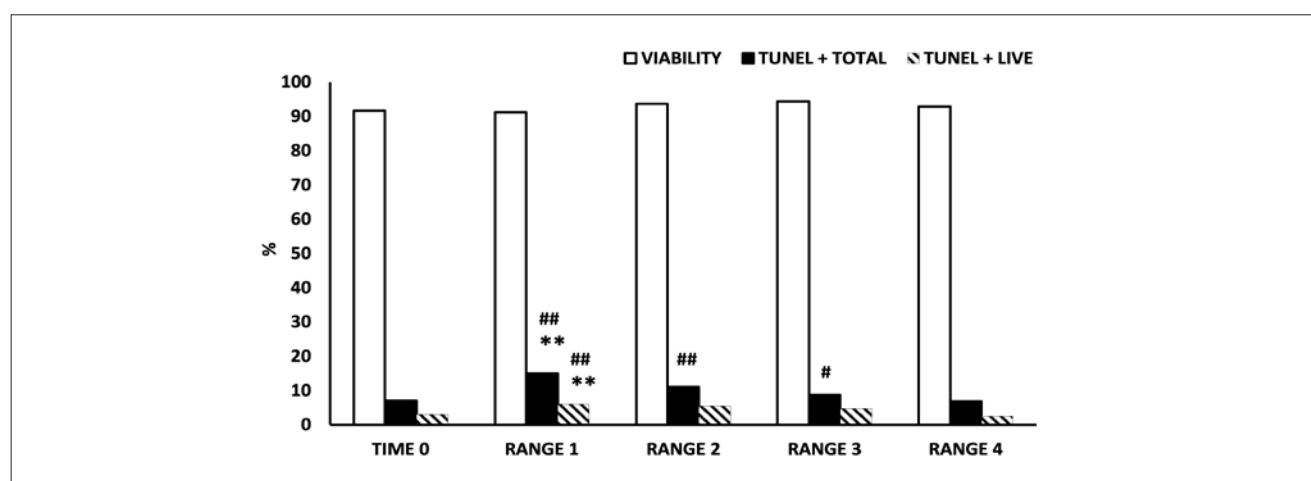


Figure 3 Effect of concentration on sperm viability, DNA fragmentation and oxidative DNA damage #: significant difference compared to control ($p < 0.05$); ##: very significant difference compared to control ($p < 0.01$); **: very significant difference related to comparative sample ($p < 0.01$).



Discussion

During ART techniques gametes and embryos are in an *in vitro* environment where reactive oxygen species build-up leading to OS^[13], that is among the chief causes of defective gametes or poorly-developing embryos in ART. Its negative impact may be amplified both from the lack of natural antioxidants and the presence of multiple manipulating steps such as centrifugation or heat stress that are potential sources of ROS^[5,6,14].

This condition can lead to decrease sperm concentration, motility, number of morphologically normal sperm^[15-17] and increase DNA fragmentation^[18]. A recent article related sperm nuclear vacuolization and incubation at 37°C^[19]. It has been shown that temperature of storage affects DNA fragmentation in different species, inducing an increase of DNA fragmentation^[20]. In mice, even short incubations at a low (4°C) or high (37°C) temperature, reduces the motility and membrane integrity of sperm^[21].

The purpose of this study was to investigate the changes in sperm-quality parameters after *in vitro* sperm incubation at RT

versus 37°C on human spermatozoa selected or not with swim up. Moreover, we evaluated the effect of culture in different sperm concentration. Sperm kinetics, viability, oxidative DNA damage and DNA fragmentation were considered.

Results showed a significantly better preservation of sperm quality when samples were incubated at RT compared with 37°C for both spermatozoa populations and better spermatozoa preservation in a low concentration culture condition.

Incubation of human spermatozoa at RT and 37°C does not influence kinetics, in according to Hellpi *et al.* 2015. Instead, viability decreases significantly at 37°C compared with control and with RT samples according to Knox *et al.* 2015. It is assumed that spermatozoa, incubated at lower temperatures, adopt a resting state, which allows them to preserve their energy^[22]. In 2009, Gallup proposed “the activation hypothesis” as the rise in temperature when spermatozoa enter the female reproductive tract could act as a trigger for the activation of spermatozoa, making them hyperactive^[23].

Oxidative DNA damage and DNA fragmentation increase significantly in 37°C sample compared with control and with RT sample according to Aitken *et al.* 2014^[14,24], therefore we

could conclude that most DNA damage in spermatozoa is oxidatively induced [25-27]. The study by Dalzell *et al.* showed that DNA fragmentation in spermatozoa increases after 4 h of incubation at 37°C. In addition, it was reported a significant increase in spermatozoa with de-condensed chromatin upon *in vitro* incubation at 37°C for 24 h. In our experiments the percentage of live spermatozoa with fragmented DNA increases at 37°C.

In the second part of experiments, we incubated selected spermatozoa by swim-up for 3h at RT and 37°C. The difference of temperature does not influence kinetics, viability and oxidative DNA damage but affects DNA fragmentation. Moreover, storage at 37°C increases percentage of live spermatozoa with fragmented DNA in according with Thijssen *et al.* and Schwarz *et al.* [19,22]. It means that temperature affects genomic integrity also in selected spermatozoa. Genomic spermatozoa damage affects embryo development and causes consequences in newborn [10], so the presence of TUNEL-positive live spermatozoa is alarming.

In the last experiment we incubated washed samples at different sperm concentrations (RANGE: 1, 100-75; 2, 50-35; 3, 25-15; 4, 10-5 x10⁶). It does not influence kinetics while DNA fragmentation and percentage of live spermatozoa with DNA fragmented increases in a concentration dependent manner.

Conclusion and future proposal

Taken together, our data demonstrated that lower temperatures and sperm concentrations better preserve sperm DNA integrity. Our results have potential practical implications for optimizing laboratory protocols during ART procedures, especially semen preparation. However, spermatozoa fertilization capacity or ability to support embryo development have not yet been investigated. We are aimed to include such endpoints in future work to add significant translational value to our experimental data.

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Deep learning enhances Day 3 embryo evaluation: validation of new software in IVF laboratories

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ABSTRACT

Introduction: During day 3 of development, embryos are classified according to morphological parameters: symmetry of cell divisions, fragmentation degree, division rate, number of cells. However, this practice is subject to intra and inter-operator variability. The need for more objective and standardised tools has stimulated interest in artificial intelligence (AI). New Software (Vitrolife's iDA v2.0) algorithm is an AI system designed to evaluate embryo image. **Materials and methods:** This single-center retrospective study analyzed 1,418 embryos from 245 IVF cycles (own and donor oocytes, with or without PGT) with the use of Embryoscope® system (Vitrolife, Denmark). **Results:** Higher iDAScore on day 3 are significantly linked to increased blastocyst formation (5.46 ± 1.44 vs. 3.91 ± 1.51 ; $p < 0.001$). Blastocyst formation rates increased across higher iDAScores: ≤ 3.1 (15.7%), 3.2–4.6 (36.6%), 4.7–6.1 (60.6%), and ≥ 6.2 (74.1%) ($p < 0.01$). Elevated iDAScores on day 3 also correlated with better inner cell mass (ICM) and trophectoderm (TE) quality. A moderate positive correlation was observed between Day 3 iDAScores and Day 5/6 (Pearson's $r = 0.52$, $p < 0.001$), with higher Day 3 scores predicting higher Day 5/6 scores. **Conclusion:** This suggest that Day 3 iDAScore may be as a promising predictor of blastocyst formation and later embryo quality, offering more objective and standardized laboratory assessments.

KEYWORDS

Embryo selection, artificial intelligence (AI), iDAScore v2.

Introduction

On the third day of embryonic development, embryos are typically assessed based on morphological features such as the symmetry of cell division, the number of blastomeres, the degree of fragmentation, and the timing of cleavage. These parameters are used to predict the developmental potential of embryos and guide clinical decisions in in vitro fertilization (IVF) procedures^[1]. However, this evaluation is often subjective and largely influenced by the embryologist's experience and interpretation, leading to considerable intra- and inter-operator variability that can compromise the consistency and reliability of embryo selection^[2].

To reduce variability and enhance objectivity in embryo evaluation, recent years have seen a growing interest in the application of artificial intelligence (AI) in this field. AI-based systems have the capacity to process extensive datasets and recognize intricate morphokinetic and morphological patterns that may not be easily discernible through traditional grading methods^[3]. One such technological advancement is time-lapse imaging (TLI), which allows for continuous, non-invasive monitoring of embryo development within stable incubator conditions, thereby minimizing environmental disturbances^[4]. However, despite the integration of TLI, embryo assessment

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still relies heavily on manual annotations and subjective interpretation. As a result, considerable inter- and intra-observer variability remains, especially when embryologists apply conventional morphological criteria or interpret TLI-derived developmental events^[5]. This underscores the need for more standardized and automated evaluation tools to support consistent and reproducible clinical decision-making.

One such system is the iDAScore v2.0 algorithm developed by Vitrolife, which uses deep learning to analyze time-lapse videos of embryos. The algorithm has been trained on thousands of embryos with known clinical outcomes, allowing it to assign a score that reflects the likelihood of implantation and live birth^[6]. The first version of the algorithm was trained specifically for blastocyst transfer^[7].

Multiple external validation studies, involving embryos selected using standard clinical protocols, have demonstrated that iDAScore correlates with blastocyst morphology and mor-

phokinetics^[8,9], as well as with ploidy status^[9].

This study explores the application of the iDAScore v2.0 algorithm in evaluating embryos on Day 3, comparing its assessments with conventional morphological evaluation, particularly in terms of subsequent blastocyst formation and quality. The aim is to determine whether AI can support more consistent and standardized embryo selection at the cleavage stage in clinical practice.

Materials and methods

This single-center retrospective study analyzed 1,418 embryos from 245 IVF cycles (own and donor oocytes, with or without PGT) conducted over 12 consecutive months.

The data collected included the day 3 iDA value, selection of the day 3 embryo by senior embryologists, the day 5 or 6 iDA value, embryo fate, PGT outcomes (euploidy and aneuploidy rates), and blastocyst quality.

A total of 1418 embryos, on day three were analysed and followed up to blastocyst stage.

During day three of development iDA v2.0 values were taken and the evaluation was made by the senior embryologist.

During day five the blastocysts were graded according to ASEBIR morphological criteria, based on quality: highest (ICM+TE grade A), high (ICM+TE grade A/B), medium (ICM+TE grade B), low (ICM or TE grade C/D).

Images of embryos were obtained using EmbryoScope® time-lapse systems (Vitrolife, Denmark).

Data analysis was conducted using SPSS software (Statistical Package for the Social Sciences, IBM, Armonk, NY, USA). A *p*-value of < 0.01 is considered statistically significant. A one-way analysis was conducted to compare the iDAScore D3 between two groups defined by the blastocyst formation variable. First, descriptive statistics were obtained for each group, analyzing the mean, standard deviation, standard error, and confidence intervals.

Subsequently, a one-way analysis of variance (ANOVA) was applied to determine whether there were statistically significant differences in the iDAScore D3 between the groups defined by blastocyst formation.

Additionally, the iDAScore D3 was recoded into quartiles to create an ordinal variable with categories grouping the score values. Cross-tabulations were performed between this grouped variable and blastocyst formation to assess the association between these variables using the chi-square test, including percentages within each group to facilitate interpretation.

Furthermore, a one-way ANOVA was conducted with blastocyst formation as the dependent variable and the iDAScore D3 quartiles as the factor, accompanied by post hoc tests (Bonferroni) to identify specific differences between quartile groups. Embryos were stratified into quartiles according to their iDA Score values, as follows: Q1: iDA Score ≤ 3.1 (376 embryos) Q2: iDA Score 4.3–4.6 (350 embryos) Q3: iDA Score 4.7–6.1 (343 embryos) Q4: iDA Score ≥ 6.2 (343 embryos).

A filter was also applied to analyze only cases with positive blastocyst formation, and the relationship between iDAScore D3 and other variables—inner cell mass quality, trophectoderm

quality, and euploidy rate—was examined again using ANOVA and descriptive analyses.

Finally, a Pearson correlation analysis was performed to evaluate the relationship between iDAScore values on different days of embryonic development (D3, D5, and D6). To further investigate the association between Day 3 iDA Score and embryo ploidy status, embryos were stratified into quartiles according to their iDA Score values, as follows: Q1: iDA Score ≤ 4.40 (73 embryos) Q2: iDA Score 4.41–5.75 (71 embryos) Q3: iDA Score 5.76–6.70 (76 embryos) Q4: iDA Score ≥ 6.7 (68 embryos).

Results

Higher iDAScore on Day 3 are significantly linked to increased blastocyst formation (5.46 ± 1.44 vs. 3.91 ± 1.51 ; $p < 0.001$). The numbers in parentheses represent the mean ($\pm$) standard deviation of the iDAScore on Day 3 for embryos that progressed to blastocyst versus those that did not. Embryo selection was evaluated using the iDAScore v2.0 model, which assigns scores from 1 to 9.99. Blastocyst formation rates increased significantly across higher iDAScores: ≤3.1 (15.7%), 3.2–4.6 (36.6%), 4.7–6.1 (60.6%), and ≥6.2 (74.1%) ($p < 0.01$) (Figure 1). Elevated iDAScores on day 3 correlated with better inner cell mass (ICM) and trophectoderm (TE) quality. ICM grade A embryos had the highest mean score (5.99 ± 1.18), compared to grade B (5.2 ± 1.46) and C (4.58 ± 1.63) ($p < 0.01$). Similarly, TE grade A embryos scored (6.03 ± 1.3), compared to B (5.39 ± 1.43) and C (4.81 ± 1.62) ($p < 0.01$). A moderate positive correlation was observed between iDAScores on Day 3 and Day 5/6 (Pearson's $r = 0.52$, $p < 0.001$), with higher Day 3 scores predicting higher Day 5/6 scores. Indeed, it could be expected that embryos with higher scores on Day 3 would be associated with higher scores on Day 5, as embryos with better morphological quality on Day 3 tend to maintain better quality on Day 5. Although higher Day 3 scores were correlated to increased euploidy rates, the association was not statistically significant ($p = 0.26$). For each quartile, the proportion of euploid embryos was calculated (Q1= 53.40%; Q2=63.40%; Q3= 64.50%; Q4=70.4%). A progressive increase in euploidy rates was observed across quartiles, ranging from 53.4% in Q1 to 70.6% in Q4. This trend supports a positive correlation between higher iDA Score values and the probability of embryo euploidy (Figure 2).

Conclusion

This study project showed that iDAScore v2 represents a valid predictive tool for blastocyst formation and quality. The results of the analysis indicate a clear correlation between the score assigned to embryos on day three and their development to the blastocyst stage, suggesting that this system may support and help embryologist to be more consistent and efficient during embryo evaluation and selection.

Indeed, a high iDAScore v2 score on day three is significantly correlated not only with the probability of blastocyst

Figure 1 Blastocyst formation rates increased significantly across higher iDA score groups: $\leq 3,1$ (15,7%), 3,2–4,6 (36,6%), 4,7–6,1 (60,6%), and $\geq 6,2$ (74,1%) ($p < 0,01$).

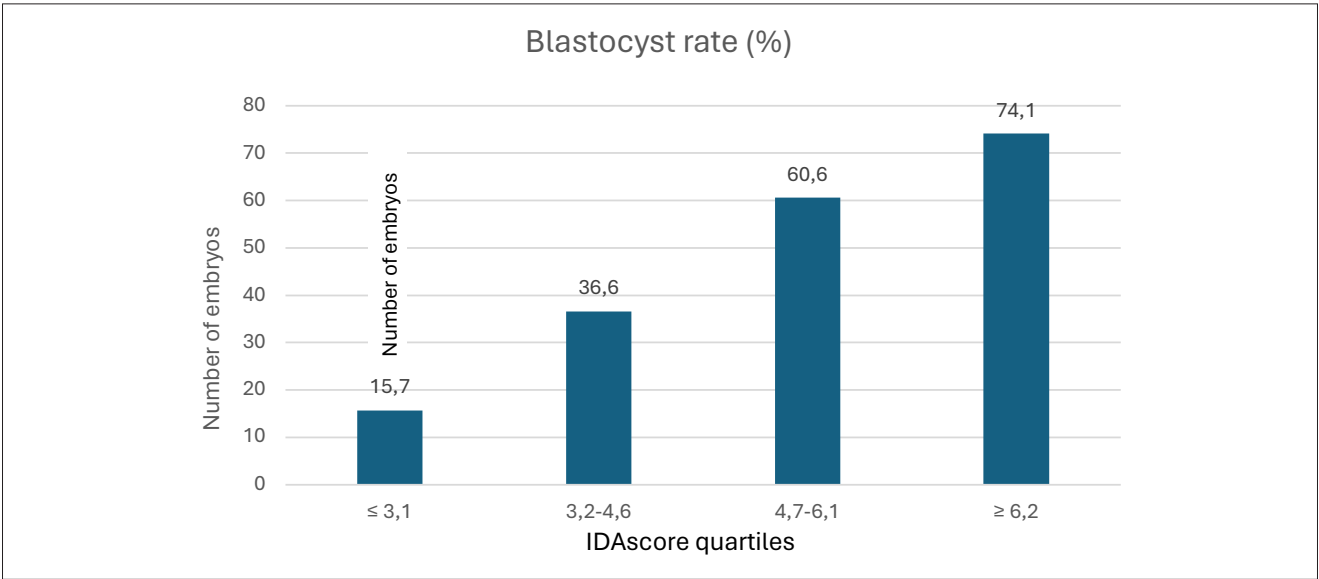
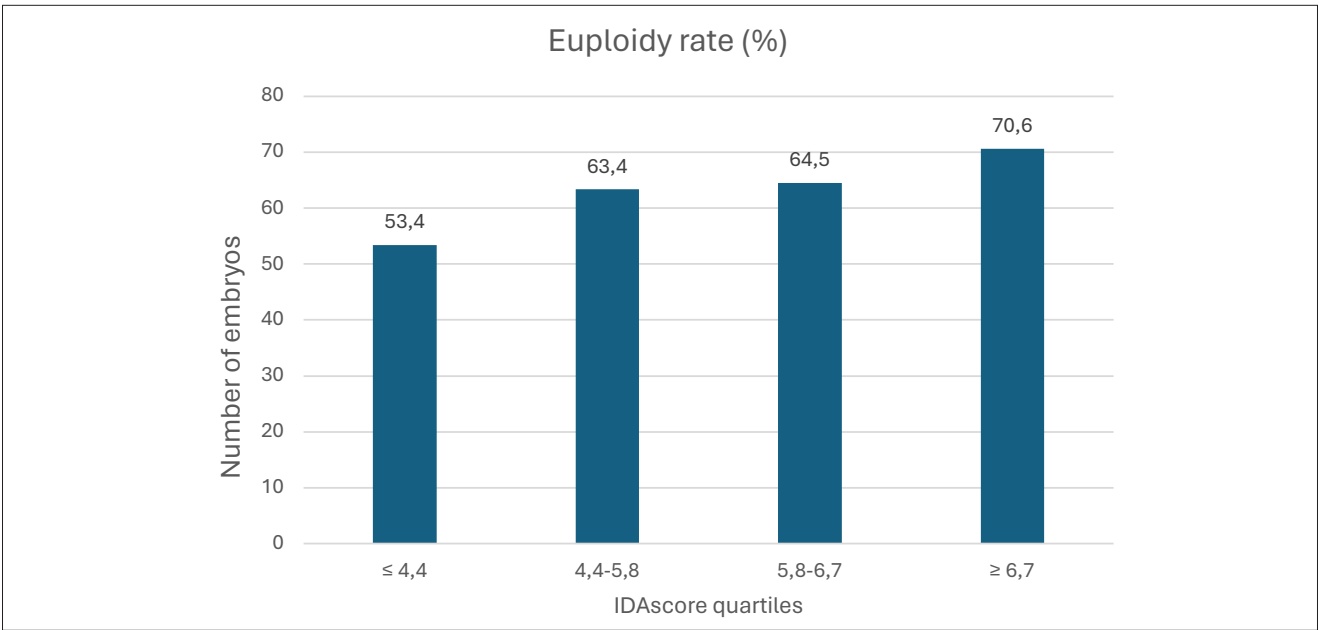


Figure 2 Euploid rate increased across higher iDA score groups: $\leq 4,4$ (53,40%), 4-4-5,8 (63,40 %), 5,8-6,7 (64,50 %), and $\geq 6,7$ (70,60%) ($p < 0,026$).



formation, but also with its morphological quality, as measured by internal cell mass and trophoectoderm. This aspect is of fundamental importance, since the quality of the blastocyst is closely linked to its implantation potential and, consequently, to the likelihood of successful assisted reproductions.

These findings are in line with the general assumption that embryos with higher morphological quality on Day 3 are more likely to develop into high-quality blastocysts. Indeed, it could be expected that embryos with higher scores on Day 3 would be associated with higher scores on Day 5, as embryos with better morphology at earlier stages tend to maintain their developmental potential over time. However, this is not always the case. Previous studies have reported that some embryos initially classified as poor quality on Day 3 may still reach the

blastocyst stage and even result in clinical pregnancy, whereas not all good-quality early-stage embryos necessarily maintain high-quality parameters at later stages ^[10].

Recent literature specifically evaluating iDA Score v2.0 has provided supportive evidence for its clinical utility in embryo assessment. Studies have demonstrated that higher scores are significantly associated with increased rates of blastocyst formation, better morphological quality, and even higher likelihood of euploidy ^[11]. In addition, iDA Score v2.0 has been validated for use across multiple developmental stages (day 2, day 3, and blastocyst), confirming its applicability as a general-purpose scoring system ^[12]. These findings confirm the potential of iDA Score v2.0 as a reliable, early, and non-invasive tool that enhances embryo selection by supporting reproducible

and standardized clinical decisions.

To confirm and extend the validity of the results, it would be useful to carry out studies on a larger scale, involving multiple centres and more diverse selection criteria, given the mono-centric nature of the present study.

The implementation of iDAScore v2 in clinical practice could therefore improve the standardisation of embryo selection criteria, helping to optimise IVF treatment success rates. The automation of the evaluation process, based on a large dataset of embryo images, also allows the exploitation of artificial intelligence to continuously refine the predictive criteria by increasing the effectiveness of assisted reproduction treatments.

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The role of key performance indicators in IVF laboratory

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ABSTRACT

Purpose: The purpose of this study, conducted at the “Centro Scienze della Natalità” of San Raffaele Scientific Institute, is to assess the quality of monthly results through laboratory KPI (Key Performance Indicators) analysis. To achieve this, the Shewhart control chart, is a statistical tool used to monitor data variability over time and detect deviations from the mean. **Methods:** Specific KPIs were selected because they are functional for monitoring and verifying the performance of both the IVF laboratory and the processes preceding oocyte pick-up, such as ovarian stimulation. The selected KPIs: metaphase II oocyte rate (MII rate), immature oocyte rate (GV rate), fertilization rate, the three pronuclei zygote rate (3PN rate) and blastulation rate. Shewhart control charts were used to track variability in these KPIs and detect deviations from the mean, with data collected monthly. **Results:** The analysis of KPIs showed that the IVF laboratory maintained high-quality standards and consistently met its performance objectives. The MII rate, GV rate, fertilization rate, 3PN rate, and blastulation rate were all within acceptable limits, and fluctuations were monitored to identify and address any critical issues. The results indicate that the laboratory is continuously improving its processes, effectively resolving issues, and enhancing its overall performance. **Conclusions:** The use of KPI monitoring through Shewhart control charts is essential for maintaining high-quality standards in IVF laboratories. This study demonstrated that the systematic tracking of KPIs not only supports the identification of areas for improvement but also ensures continuous optimization of laboratory performance and patient care.

KEYWORDS

IVF laboratory, performance indicator, KPI, quality control, laboratory performance.

Introduction

The Quality Management System (QMS) is of fundamental importance to ensure effective and efficient management of the In Vitro Fertilization (IVF) laboratory. Since gametes and embryos are manipulated within it, it is important to ensure that all procedures are standardized and constantly monitored. The quality management system includes several procedures aimed at ensuring that the IVF laboratory functions properly.

In particular, quality management involves the integration of several activities:

- **Quality Control (QC):** This process involves monitoring a series of activities aimed at verifying that the laboratory is functioning correctly (for example, daily recording of incubator temperatures, CO₂, and O₂ levels).
- **Quality Assurance (QA):** is a program used to ensure the quality of a laboratory, which considers the entire process and aims to identify any problems or errors to correct them and improve the effectiveness of the laboratory as a whole.
- **Quality Improvement (QI):** continually focuses on increasing the effectiveness and efficiency of an IVF laboratory to improve its performance^[1].

Another crucial tool for assessing and improving the quality

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of healthcare and work performed within a clinical laboratory is the monitoring of Key Performance Indicators (KPIs). They allow for the objective measurement of the level of effectiveness, safety, equity, timeliness, and efficiency of the care provided and activities carried out by the laboratory, as well as monitoring compliance with the quality requirements defined by ISO 15189:2012 standards^[2]. KPIs are therefore a fundamental tool within the QMS, allowing for the identification of strengths and areas for improvement of the laboratory and the adoption of necessary corrective actions to ensure the quality of the service provided to patients^[3]. Performance evaluation criteria are also essential for assessing the introduction of new techniques or processes, establishing minimum competency standards, monitoring current performance within a quality management system, benchmarking, and improving overall quality. In general, the

results of analyzing a series of KPIs can provide a suitable overview of the most important phases within the IVF laboratory process ^[4]. Each laboratory should therefore develop its own set of KPIs based on the organization and processes of the laboratory, and develop a systematic, transparent, and consistent approach to data collection and analysis and KPI calculation ^[3]. A group of experts from the ESHRE Special Interest Group of Embryology and the Alpha scientists in reproductive medicine created a list of twelve key performance indicators (KPIs) for in vitro fertilization (IVF) laboratories for fresh IVF and ICSI cycles in 2017. The goal of these KPIs is to provide a framework for monitoring the performance of ART laboratories ^[5]. KPIs can generally refer to three main areas: structures, processes, and outcomes ^[6].

Structural KPIs

Structural indicators are measures used to evaluate the quality of the environment in which treatments are carried out. These indicators take into account both physical aspects, such as the structures and equipment used, and the human resources involved, such as the number of operators, their skills, and qualifications ^[7]. The monitoring process, which allows for the identification of deviations to be able to act if they exceed certain limits, plays an important role in avoiding negative consequences and maintaining optimal performance ^[8]. Another important parameter concerns the number of instruments within the IVF laboratory, which should be appropriate to the workload ^[3]. It is also necessary to consider the appropriate number of operators in an in vitro fertilization laboratory, as an excessive workload is highly risky. High workload and insufficient staff could lead to potential errors in procedures, omissions in the witnessing process, or incompatibilities ^[9].

Process KPIs

Process indicators are metrics used to evaluate the quality of work performed in a laboratory. They measure the efficiency of the process, timeliness, and safety in performing operations. Process indicators are preferred over outcome KPIs because they are easier to interpret. Outcome KPIs, such as pregnancy rates, can be influenced by multiple external and internal factors in the laboratory that are uncontrollable. In contrast, process indicators measure the quality of the work process itself and therefore can be used as a tool to improve the efficiency and quality of work performed by the laboratory ^[10].

Outcome KPIs

Outcome indicators are used to evaluate how effective the care provided to a patient is. However, their interpretation can be influenced by many external factors, so they cannot be used as the sole criterion to evaluate the quality of professionals and facilities. For this reason, when outcome KPIs are adopted, data collection must be standardized and results must be corrected for confounding factors (such as different patient populations) ^[10].

Methods

This study was conducted at the Birth Sciences Center of the San Raffaele Scientific Institute in Milan, in the medical-

ly assisted reproduction laboratory. The objective of the study was to implement and verify the efficiency of a quality control system based on the analysis of selected KPIs. This was a study, in which data were collected and analyzed periodically between January and December 2022. The couples who underwent in vitro fertilization (ICSI) cycles between January 2022 and December 2022 were considered for the analysis and gave their written consent for the anonymous use of collected data for research and statistical evaluation purposes. To induce superovulation, patients were administered hormonal therapy using GnRH agonists and antagonists. Oocytes were retrieved about 35 hours after the hCG injection. The cumulus-oocyte complexes (COCs) collected were washed using a solution containing HEPES and HSA, which is a culture medium called Quinn's Advantage, enriched with human serum albumin at 100 mg/ml in a 5% physiological saline solution. After a three-hour incubation period, mature oocytes were denuded by removing the surrounding cells through using hyaluronidase and mechanical removal with decreasing diameter pipettes and subsequently fertilized using the ICSI technique. Those fertilized with the IVF technique were not denuded. For embryonic culture, plates containing micro-drops of a culture medium called Continuous Single Culture-NX were used, integrated with a 10% serum substitute supplement, and the plates were covered with oil. All oocytes were checked about 18 hours after insemination or ICSI to verify the presence of two equally sized pronuclei in a central position, indicative of correct fertilization. After collecting the embryos, evaluations were made on their quality and quantity to decide whether to perform embryo transfer on the third or fifth day.

The "freeze all" technique, which consists of cryopreserving all obtained embryos through vitrification, was chosen if the patient was unable to undergo embryo transfer due to the risk of ovarian hyperstimulation or other issues. This choice aims to preserve the embryos and increase the probability of success in the next cycle. The electronic medical record management software Meditex is used at this center for the digital registration of patient data.

This system allows for the secure recording of medical information such as medical history, laboratory tests, diagnoses, treatments, and personal patient information. The software has been designed to assist healthcare professionals in managing patient information more efficiently, reducing record-keeping errors, and ensuring patient data privacy and security. The use of an electronic medical record system also facilitates statistical analysis of patient data. The software allows for the collection and storage of large amounts of data, which makes data processing for analysis easier.

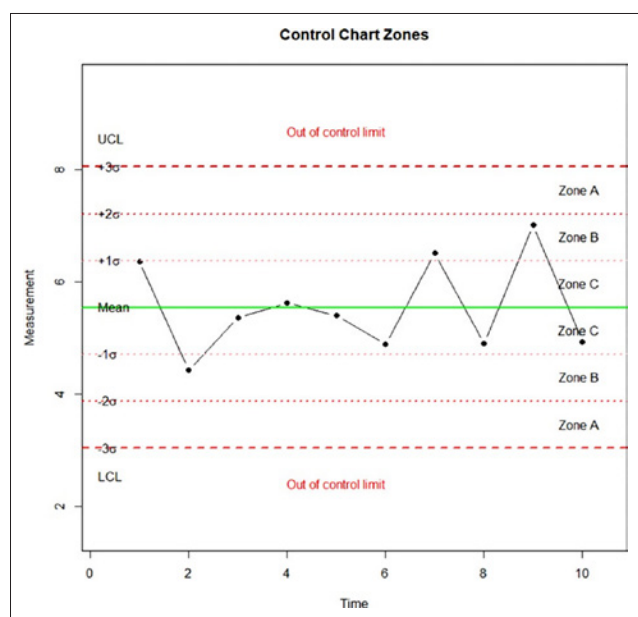
KPI selection and calculation

In this study, specific KPIs were selected as they are functional for monitoring and verifying the performance of both the IVF laboratory and the processes preceding the oocyte pick-up, such as ovarian stimulation. The selected KPIs are metaphase II (MII) oocyte rate, immature oocyte rate (GV)fertilization rate, the three pronuclei (3PN) rate, blastulation rate.

Table 1 Selected Key Performance Indicators and their calculation formulas.

KPIs	CALCULATION
MII rate	$\frac{\text{No. of MII oocytes}}{\text{No. of retrieved oocytes}}$
GV rate	$\frac{\text{No. of GV oocytes}}{\text{No. of retrieved oocytes}}$
Fertilization rate	$\frac{\text{No. of fertilized oocytes}}{\text{No. of oocytes injected}}$
3PN rate	$\frac{\text{No. of 3PN zygotes}}{\text{No. of oocytes injected}}$
Blastulation rate	$\frac{\text{No. of blastocysts}}{\text{No. of fertilized oocytes}}$

Figure 1 Representative diagram of a control chart. The X-axis represents the time at which the data were collected. The Y-axis indicates the corresponding measurement values at those times. Three standard deviations corresponding to 1σ , 2σ , and 3σ were calculated and used for the analysis of the data. Centerline: corresponds to the mean (refers to a period preceding the one under analysis) of the measurement under analysis. Zone A: corresponds to the zone from two sigmas to three sigmas above/below the center line. Zone B: corresponds to the zone from one sigma to two sigmas above/below the center line. Zone C: corresponds to the area from the center line to one sigma above/below the center line. UCL (Upper Control Limit): corresponds to the maximum tolerance above the mean. LCL (Lower Control Limit): corresponds to the maximum tolerance below the mean. (Adapted from <https://asq.org/quality-resources/control-chart>)^[12].



Statistical analyses were performed using R software (RStudio Team, 2022), and data visualization was achieved through the ggplot2 package^[11]. Shewhart control charts were used to track KPI trends, with reference means derived from a 13-month historical dataset. To perform KPI analysis, the Statistical Process Control (SPC) method was used, employing Shewhart control charts. Control charts are graphical tools for displaying the performance of a process over time, using statistical techniques to aid interpretation.

Several types of control charts have been described in the literature, each designed to monitor specific process characteristics. Through analysis of the data obtained via the control chart, it was possible to evaluate the laboratory's performance and identify any variations or anomalies to intervene promptly. The Stewart control chart used for this study reports a "control mean" related to the previous 13 months, "warning limits" set at ± 2 standard deviations, and "control limits" set at ± 3 standard deviations. Three standard deviations corresponding to 1σ , 2σ , and 3σ were calculated and used for the analysis of data, which spanned from December 2020 to December 2021. For data analysis and a graphical representation, the R software (RStudio Team, 2022) was used.

This is a programming language that provides a wide range of functionalities for data processing, including data manipulation, statistical modeling, data analysis, machine learning, and data visualization. In particular, the ggplot2 package^[11] was used for graphical representation.

Results

In an ideal condition, an indicator should always remain within the control limits. The oscillation between the lower and upper control limits is physiological and is due to patient variability. However, we can distinguish four different scenarios:

1. The indicator exceeds the lower control limit, and immediate action is necessary to identify the problem and consequently find a solution.
2. The indicator exceeds the lower warning limit, and again, immediate action is necessary to identify the problem and find a solution.
3. The indicator exceeds the lower warning limit, and action is necessary to determine if there is a real problem.
4. The indicator drops below the warning limit three consecutive times without exceeding the control limit, and action is necessary to determine if a problem may arise.
5. The indicator exceeds the upper control limit, and the reason for this occurrence should be defined^[1]

For this study, a population with the characteristics of average age of women, number of retrieved oocytes, and number of MII oocytes reported in Table 2 was used. For a more detailed view, the characteristics of the population during the study period (January-December 2022).

Mean and standard deviation were calculated during the period from January to December 2022.

The followings are the results obtained from the statistical analysis and the consequent interpretation according to the rules of Shewhart control chart reading for the five selected KPIs

Table 2 Characterization of the population under examination (average age of patients, number of retrieved oocytes, and number of MII oocytes).

2022			
	AGE	RETRIEVED OOCYTES	MI OOCYTES
Mean (ds)	37 (4,2)	8 (6,5)	5 (5,3)

MII rate

Significant fluctuations were observed between January and April 2022, with respective values of 72.4%, 71.5%, 74.2%, and 70.9%, all below the 78.5% reference value; notably, February and April values fell below the lower control limit (LCL) (Figure 2A).

These deviations indicate potential procedural inconsistencies or variations in ovarian stimulation protocols. One of the possible causes of this trend could be the entry of a new operator in the laboratory who performs the procedures^[13]. Beyond the two points that show more significant deviations, an oscillating trend can also be noticed in the other months under examination, according to the rules of interpretation of the control chart. In fact, the absence of 8 control points in the zone closest to the mean (zone C) is evident. This could indicate that the process has undergone a change and may require further investigation or corrections to ensure that the results remain within acceptable control limits^[13].

GV rate

The rate of GV (immature oocytes) is a useful indicator to evaluate the effectiveness of the ovarian stimulation protocol and the quality of the collected oocytes in an ART laboratory. If the rate of immature oocytes is too high, it may be necessary to rethink the ovarian stimulation protocol or make changes to improve oocyte maturation. The GV rate remained stable within control limits, with minor oscillations potentially linked to changes in culture media batches^[1] (Figure 2B).

Fertilization rate

The rate of fertilized oocytes is a highly effective indicator, as it provides information on both gamete quality and operator competence. The fertilization of oocytes involves a complex series of events that require the growth, development, and cytoplasmic maturation of microinjected oocytes with sperm^[14]. The fertilization rate remained consistently high and aligned with the reference mean, demonstrating stable laboratory procedures and operator competency^[14] (Figure 2C).

3PN rate

The presence of three or more pronuclei within a fertilized oocyte indicates abnormal fertilization, which can be caused by the failure of the second polar body to separate or the presence of multiple sperm.

Polyspermy, the presence of multiple sperm attempting to fertilize the same oocyte, can be due to the immaturity of the oocyte, its excessive maturity, or the presence of a high concentration of sperm in the insemination sample^[15]. A high 3PN rate may be a signal of problems in the in vitro fertilization procedure, such as a condition of environmental stress within the laboratory^[3] (Figure 2D).

Blastulation rate

The blastulation rate is an indicator that measures the percentage of normally fertilized oocytes that develop into mature blastocysts within a specific time (116 ± 2 hours). This KPI provides an assessment of the culture system's ability to support the formation of viable embryos, as only healthy embryos capable of developing properly can form a blastocyst. It is important to note that this KPI focuses only on the formation of blastocysts and does not take into account the stage or quality of the blastocyst itself.

Periodic fluctuations were noted in the blastulation rate, though all values remained within control limits. This suggests that the culture environment supports consistent blastocyst formation, although minor variations may warrant further investigation^[16] (Figure 2E).

Monthly mean monitoring (from January 2022 to December 2022) for five indicators: (A) MII rate, (B) GV rate, (C) fertilization rate, (D) 3PN rate, and (E) blastulation rate. Control limits and centerlines are based on the reference mean and standard deviation calculated from the previous 13 months.

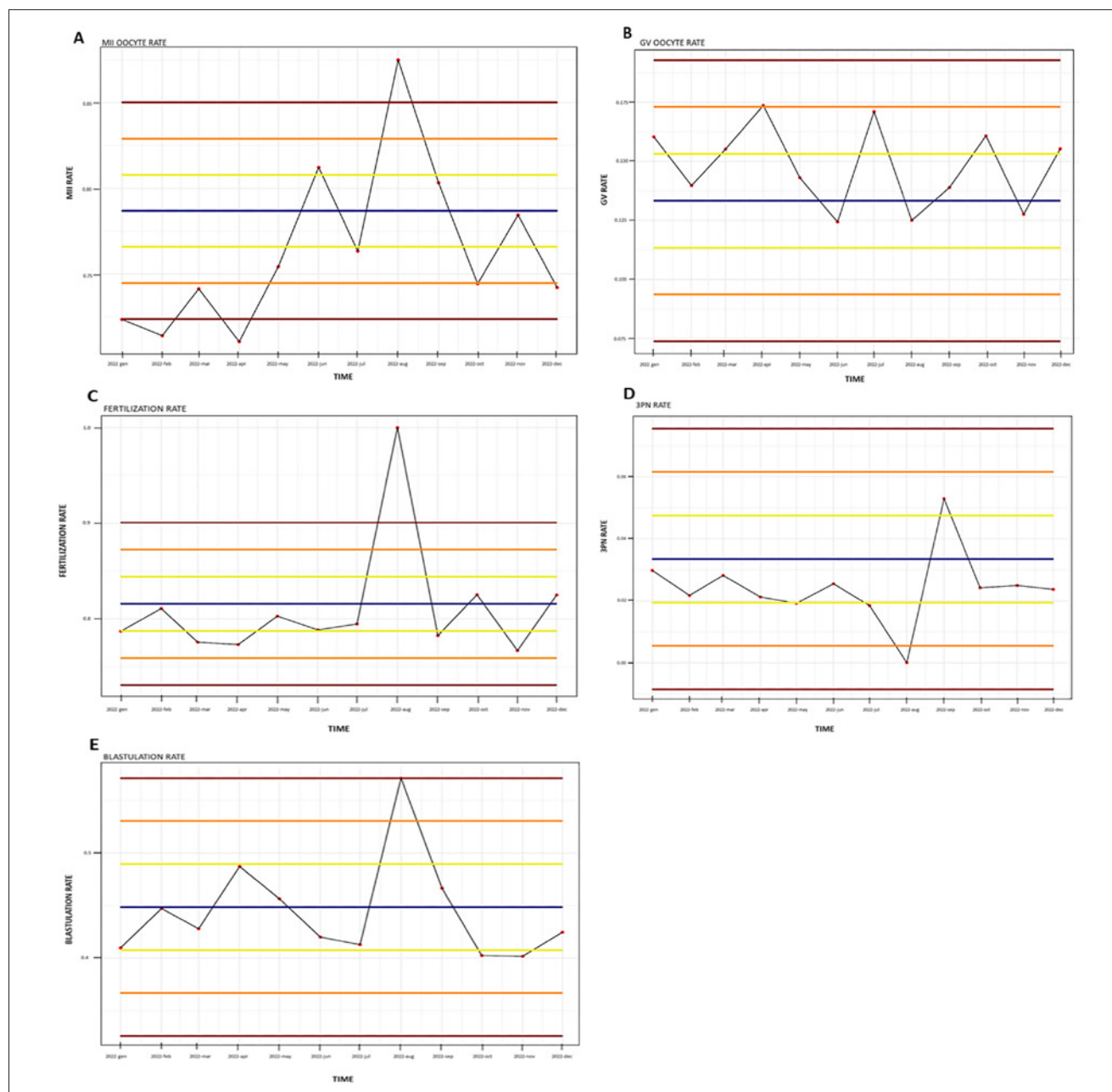
Discussion

The majority of KPIs remained stable, reflecting high laboratory standards. However, fluctuations in the MII rate suggest that ovarian stimulation protocols, laboratory workflow, or environmental factors may influence oocyte maturation^[9]. The importance of KPI monitoring in quality management is underscored by these findings, as early detection of trends allows for timely corrective actions^[8]. Shewhart control charts proved to be a valuable tool for monitoring IVF laboratory performance, providing insights into process stability and potential areas for improvement^[17]. Unlike outcome KPIs, which are influenced by external patient factors, process KPIs offer a more accurate reflection of laboratory performance and can guide continuous improvement initiatives. Future studies could expand the KPI set to include additional parameters such as embryo implantation rates and clinical pregnancy outcomes, further enhancing quality assessment in ART laboratories. Moreover, integrating advanced data analytics and artificial intelligence could improve predictive capabilities and optimize laboratory workflows.

A recent study conducted by Sergei Sergeev and Iuliia Diakova, titled "Advanced KPI Framework for IVF Pregnancy Prediction Models in IVF Protocols" claimed that deep neural networks can be used successfully for the development in the area of assisted reproduction techniques. They presented an innovative analytical approach that combines KPIs and machine learning models in order to improve the prediction of the clinical pregnancy outcome^[18].

Conclusion

Continuous monitoring of KPIs allows for the timely identification of any problems and prompt adoption of corrective

Figure 2 Shewhart control charts for monitoring various laboratory indicators.

measures. When there is a variation in the clinical results of an in vitro fertilization clinic, monitoring the laboratory's KPIs can answer the question, "Is everything going well inside the laboratory?" for that specific period. If every process inside the IVF laboratory is constantly monitored using its own set of KPIs and all KPIs fall within control limits during the considered period, then the cause may be found elsewhere and sometimes can be attributed to the patient population [19]. The present study has demonstrated the importance of KPIs within an IVF laboratory. The analysis of the five selected KPIs (fertilization rate, 3PN rate, blastulation rate, MII oocytes rate, and GV oocytes rate) allowed for monitoring of the laboratory's performance and providing useful information to make strategic decisions. Ideally, every laboratory should develop its set of KPIs selected based on both its setting and the processes performed

within the laboratory [3]. However, it is advisable to avoid using too many indicators, as their number should be manageable to collect, process, and analyze data efficiently [1].

The results obtained through the analysis of control charts related to KPIs have shown a regular and consistent trend for most of the selected indicators (GV oocytes rate, fertilization rate, 3PN rate, blastulation rate), compared to the previous year's average. Only the MII oocytes rate showed an irregular trend and a deviation from the previous year's average. However, this fluctuation may have multiple causes that may concern the population of patients undergoing the cycle, so it is important to investigate the cause of the periodic fluctuation of the data.

Based on the analysis of the selected KPIs, it can be stated that the IVF laboratory is maintaining high standards of quality

ty, continuously improving its performance, and achieving set goals. Moreover, the analyzed KPIs have allowed the identification of any critical issues, monitoring of the effectiveness of the solutions adopted to address them, and promotion of us improvement. Therefore, the implementation of a KPI monitoring system is crucial for the success of an IVF laboratory and to ensure high-quality services for patients.

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Blastocyst re-expansion and implantation in FER

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ABSTRACT

Purpose: This study aimed to assess the feasibility and value of blastocyst re-expansion timing as a predictor of embryo implantation in Frozen Embryo Replacement (FER). **Methods:** One hundred and sixty-four patients submitted to 205 single FER were enrolled in the period between September 2021 and April 2023 at the Physiopathology of Reproduction and IVF Unit, Hospital del Delta, AUSL Ferrara, Italy. Blastocysts re-expansion timing has been retrospectively correlated to the implantation. **Results:** The results indicated a significantly higher implantation rate for fully re-expanded blastocysts compared to non-re-expanded ones 4 hours post-warming. The exact timing of re-expansion did not demonstrate a significant correlation with implantation potential, whereas the completion of re-expansion within 4 hours post-warming did. The quality of the pre-vitrification Inner Cell Mass (ICM) and Trophectoderm (TE) also significantly influenced implantation potential, reinforcing the importance of these pre-vitrification factors. **Conclusions:** This study highlights the significance of pre-vitrification ICM and TE quality and the complete re-expansion of blastocysts within 4 hours post-warming as strong predictors of embryo implantation in frozen embryo-transfer cycles. These findings offer valuable insights for clinical practice in optimizing the success of FER.

KEYWORDS

Blastocyst re-expansion; vitrification; implantation potential; FER.

Introduction

Embryo cryopreservation has significantly changed the management of infertility treatment during the last two decades. Specifically, vitrification at blastocyst stage has improved the cumulative pregnancy rate reducing the multiple gestational risk by the Single-Embryo Transfer (SET) policy^[1]. Moreover, blastocyst vitrification has increased the number of IVF cycles associated to Preimplantation Genetic Testing (PGT) as well as the utilization of freeze-all strategy for various indications^[1-3]. Despite the continuous increase of Frozen Embryo Replacement (FER), the variables affecting the implantation potential of vitrified blastocysts still need to be thoroughly characterized. Pre-vitrification blastocoele expansion, Inner Cell Mass (ICM) and Trophectoderm (TE) evaluation represent a well-established approach to estimate embryo viability and could predict the clinical outcomes^[4,5]. Some researchers have found that the degree of post-warming blastocyst re-expansion and cell survival correlated with clinical outcomes^[6-8], while others have not^[5,9,10]. Blastocysts experienced multiple morphological changes in the process of vitrification and warming. Observations in animal models suggest that the quick temperature increase during warming could disintegrate the TE cells junctions, leading to the collapse of the blastocoele. This

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makes the blastocyst evaluation difficult right after warming. During post-warming culture, the TE junctions, Na⁺/K⁺-ATPase pump and aquaporins could be gradually restored, leading to the blastocyst re-expansion by the transition of the water across the TE^[11]. This allows a more accurate post-warming blastocyst evaluation. Given these observations, some investigators recommend assessing blastocyst survival and viability within 2–4 hours post-warming assuming that the faster is the blastocyst re-expansion, the higher could be the implantation potential^[6,8,9,12]. Recent data seems to support this hypothesis, showing that the timing of blastocyst re-expansion post-warming is significantly associated to implantation potential, independently to other factors including embryo euploidy^[13]. The present study was undertaken to evaluate the feasibility and value of blastocyst re-expansion timing as predictor of embryo implantation in FER.

Methods

Ovarian stimulation, oocytes insemination and blastocyst vitrification

In this retrospective study, we included all single FER between September 2021 and April 2023 at the Physiopathology of Reproduction and IVF Unit, Hospital del Delta, AUSL Ferrara, Italy. The study was approved by the local ethical committee (protocol number 76/2022/Oss/AUSLFe). One hundred and sixty-four patients were included in the study. All the patients submitted to Controlled Ovarian Stimulation (COS) had normal menstrual cycles that lasted 26–34 days, had normal body weight (Body Mass Index [BMI] of 18–28 kg/m²), and had not been on endocrine treatment (including gonadotropins and oral contraception) in the 3 months before the study. They had a normal uterus and ovaries and an antral follicle count between 5 and 20 on day 1 of gonadotropin administration. Serum Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), estradiol and progesterone were checked on day 2–3 of all stimulation cycles as baseline hormone profile. The patients began injections of recombinant FSH (rFSH, Ovaleap; Theramex, Milano, Italy) since day 2–3 of menstruation, with daily dose of 150–350 IU adjusted according to individual conditions. For pituitary suppression, the patients received GnRH antagonist (ganirelix; Fyremadel, Ferring, Milano, Italy) 0.25 mg/day subcutaneously since the dominant follicles reached the diameter of 12–14 mm. The serum LH, estradiol and progesterone levels as well as number and size of follicles were monitored every 1–2 days, starting from stimulation day 5 until the day of hCG injection. When two dominant follicles reached diameter of 18 mm, the final oocyte maturation was induced with hCG (Ovitrelle, Merck-Serono, Geneva, Switzerland) 250 micrograms. Then, oocyte retrieval was performed 34–36 h later. Mature oocytes were inseminated by ICSI with fresh ejaculated spermatozoa and incubated in G-TL™ continuous culture medium (Vitrolife, Sweden) under 37°C, 5% CO₂, 6% O₂ for the following 5–6 days. Blastocysts were morphologically classified in Top Quality (TQ), Good Quality (GQ), Average Quality (AQ), Poor Quality (PQ), artificially collapsed and individually vitrified using Vit Kit – Freeze (Fujifilm, Irvine Scientific). When indicated, TE biopsy for PGT was done just before vitrification.

Endometrial preparation

Patients were treated with estradiol (Progynova; Bayer, Milano, Italy 2 mg × 3 daily) starting on day 2 of their menstrual cycle. Approximately 15 days after the commencement of estradiol treatment, patients were scanned by transvaginal ultrasound to determine the thickness of the endometrium. If the endometrium did not reach a satisfactory thickness, the dosage of estradiol could be adjusted or supplemented with transdermal Estradiol (Climara; Bayer, Milano, Italy). Once the thickness of the endometrium reached 8 mm, patients were instructed to commence vaginal progesterone (Amelgen 400 mg × 2 daily) for 4–5 full days of progesterone supplementation until FER.

Blastocyst warming and re-expansion evaluation

For warming procedure, Vit Kit – Thaw (Fujifilm, Irvine Scientific) was used. Survived blastocysts (< 50% of necrotic

Figure 1 Study groups according to the blastocyst re-expansion. Fully Re-Expanding blastocyst (FRE, 1), Quickly Re-Expanding blastocyst (QRE, 2), Slowly Re-Expanding (SRE, 3), Not Re-Expanding (NRE, 4).



cells) were incubated in G-TL™ continuous culture medium (Vitrolife, Sweden) under 37°C, 5% CO₂, 6% O₂ until FER (4 hours later). Two hours after warming, blastocyst re-expansion was evaluated as follow: Fully Re-Expanding (FRE, Group 1); Quickly Re-Expanding (QRE, ≥ 50% of the original blastocyst volume, Group 2); Slowly Re-Expanding (SRE, < 50% of the original blastocyst volume, Group 3); and Not Re-Expanding (NRE, collapsed, Group 4) (Figure 1). Before FER a second blastocyst re-expansion check was made in comparison to the previous one. A photographic record was kept of blastocyst immediately post-warming, 2 hours post-warming and before FER.

FER and implantation assessment

Equilibrated EmbryoGlue® transfer medium (Vitrolife, Sweden) was used. The embryos were injected as gently as possible while withdrawing the catheter very slightly, approximately 0.5 cm. The catheter was slowly withdrawn completely from the uterus, making sure to keep the plunger depressed and taking care to confirm that the fluid is not trailing the catheter back as visualized by ultrasound. In the occurrence of pregnancy, detected by s-hCG measurement 11 days after FER, the hormonal treatment continued until the 8th week of pregnancy. Embryo implantation was assessed by ultrasound evaluation of the gestational sac with fetal heartbeat.

Statistical analysis

Descriptive statistics have been reported for the female characteristics and implants overall and according to the clinical pregnancy outcome. The data have been synthesized by taking into account both the absolute and relative frequencies for categorical factors, as well as the interquartile ranges for the continuous variable. The focus has been on clinical preg-

nancy as a result. To address the quasi-separability problem that emerges in this outcome, a Logistic Regression model with Fifth bias reduction was computed ^[14]. The Univariable and Multivariable models present the Odds Ratio (OR) alongside 95% confidence intervals and corresponding P-values. The correlation between repeated implants within the same subject was managed using a robust cluster standard errors approach through 1000 iterations of bootstrap resampling. The computations have been performed with R 3.4.2 ^[15] and logistic ^[16] packages.

Results

One hundred and sixty-four patients submitted to 205 single FER were enrolled. A total of 205 blastocysts were included in the study, 89 euploid (89/205, 43.4%) and 116 untested (116/205, 56.6%). After 2 hours post-warming, 67 blastocysts out of 205 (32.7%) were classified as FRE (Group 1); 73 blastocysts out of 205 (35.6%) were classified as QRE (Group 2); 40 blastocysts out of 205 (19.5%) were classified as SRE (Group 3); 25 blastocysts out of 205 (12.2%) were classified as NRE (Group 4). After 4 hours post-warming, 147 blastocysts out of 205 (71.7%) were classified as FRE (Group 1); 25 blastocysts out of 205 (12.2%) were classified as QRE (Group 2); 16 blastocysts out of 205 (7.8%) were classified as SRE (Group 3); 17 blastocysts out of 205 (8.3%) were classified as NRE (Group 4). Both the univariable and multivariable models indicate that a decreased degree of blastocyst re-expansion and a lower quality grade of blastocyst significantly and independently lowered the likelihood of clinical pregnancy, regardless of the warming

timing. These results are consistent across both the Univariable and Multivariable model outputs (Table 1) and are supported by the predictive probability plots for blastocyst re-expansion and quality (Figures 2, 3). Furthermore, the findings were corroborated by conducting separate models for expansion at 2 hours and 4 hours post-warming (Table 2).

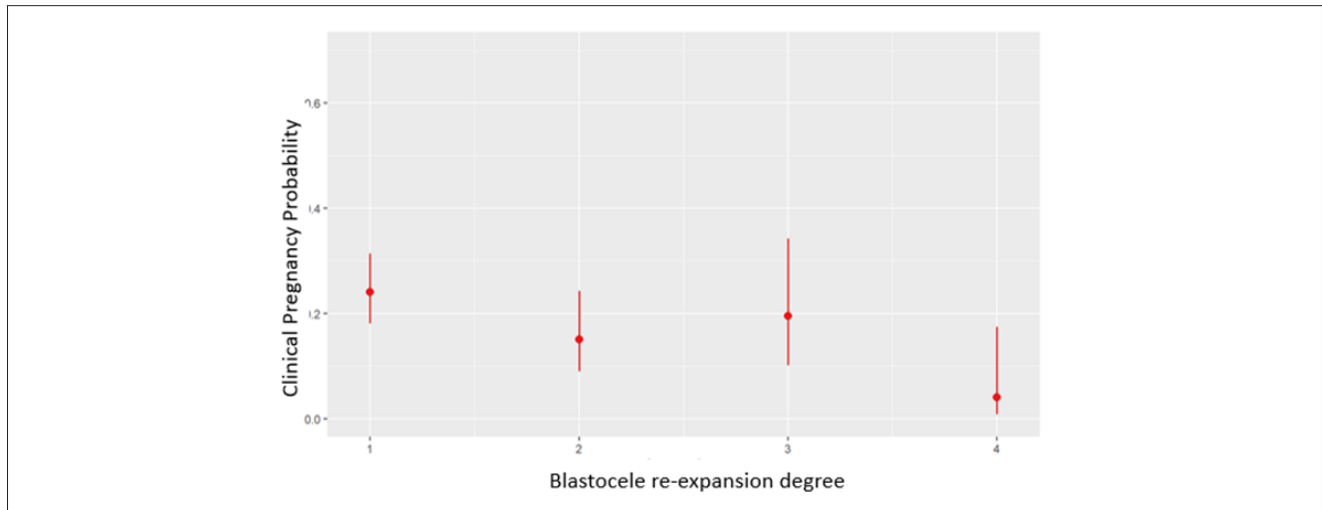
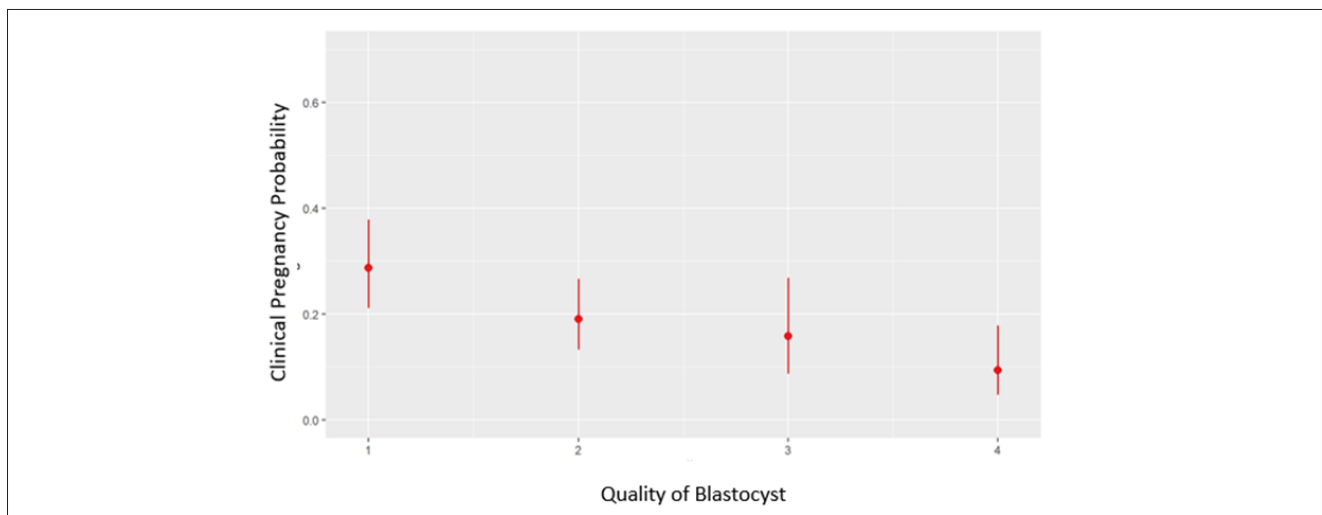
Discussion

We conducted a retrospective study on 205 vitrified-warmed blastocysts to evaluate the impact of post-warming blastocyst re-expansion on implantation potential. In fact, blastocysts are usually collapsed after warming so a post-warming culture could provide the chance to assess embryo viability. Our results showed a statistically significant higher implantation rate of full re-expanded blastocysts respect to not re-expanding ones 4 hours post-warming. This is in agreement with published data indicating that the post-warming re-expansion degree is the best predictor of clinical outcome in FER. Some investigators reported 39% increased odds of live birth for each 10% increase in degree of blastocyst post-warming re-expansion ^[4]. Du et al. showed a significant correlation between the post-warming blastocyst re-expansion and live birth rate after FER ^[17].

Similarly, Desai et al. showed a 4-fold increase in live birth rate in FER with expanded blastocyst ^[9]. More recently Coello et al. reported a 44.6% implantation rate of re-expanded blastocysts respect to 6.5% of not re-expanded blastocysts ^[7]. The correlation between re-expansion and implantation potential could be explained by the reconstitution of TE cell-to-

Table 1 Fifth bias reduced logistic regression model. Odds Ratio (OR) and 95% confidence intervals have been reported with the p-value for the univariable and multivariable models.

	UNIVARIABLE				MULTIVARIABLE			
	OR	LOWER BOUND	UPPER BOUND	P-VALUE	OR	LOWER BOUND	UPPER BOUND	P-VALUE
Age	0.999	0.923	1.082	0.985	1.006	0.9486	1.067	0.8373
Days from defrosting to implantation	0.998	0.996	1.001	0.228	–	–	–	–
Degree of expansion at freezing	0.899	0.560	1.444	0.661	1.076	0.748	1.548	0.695
PGT	0.566	0.287	1.117	0.101	–	–	–	–
Artificial collapsing	1.750	0.844	3.630	0.133	–	–	–	–
Blastocoele re-expansion degree								
2	0.611	0.322	1.161	0.133	0.694	0.380	1.266	0.233
3	0.677	0.318	1.443	0.312	0.936	0.443	1.976	0.863
4	0.100	0.015	0.661	0.017	0.112	0.018	0.699	0.019
Blastocoele re-expansion degree								
2	0.573	0.260	1.259	0.166	0.580	0.326	1.030	0.063
3	0.431	0.147	1.260	0.124	0.461	0.212	1.000	0.050
4	0.275	0.089	0.849	0.025	0.254	0.109	0.589	0.001

Figure 2 Multivariable model predicted probability of clinical pregnancy plot for blastocoele re-expansion degree.**Figure 3** Multivariable model predicted probability of clinical pregnancy plot for quality of blastocyst.**Table 2** Fifth bias reduced logistic regression model for 2 hours and 4 hours post warming time. The Odds Ratio (OR) and 95% Confidence Intervals (CI) have been reported with the p-value for the multivariable models.

PREDICTORS	2 H POST-WARMING			4 H POST-WARMING		
	OR	CI	P-VALUE	OR	CI	P-VALUE
Age	1.01	0.93 – 1.09	0.897	1.01	0.93 – 1.09	0.894
Quality of blastocyst						
GQ vs TQ	0.59	0.26 – 1.31	0.195	0.59	0.27 – 1.29	0.19
AQ vs TQ	0.47	0.16 – 1.36	0.157	0.47	0.16 – 1.36	0.157
PQ vs TQ	0.25	0.08 – 0.80	0.013	0.29	0.09 – 0.90	0.026
Degree of expansion at freezing	1.07	0.65 – 1.76	0.802	1.06	0.64 – 1.74	0.832
Blastocyst re-expansion						
QRE vs FRE	0.57	0.26 – 1.25	0.164	0.93	0.34 – 2.55	0.885
SRE vs FRE	0.97	0.39 – 2.42	0.944	0.67	0.17 – 2.72	0.573
NRE vs FRE	0.16	0.03 – 0.89	0.013	0.1	0.01 – 1.52	0.02

cell bonds, the reactivation of mitochondria, which influences the activity of the ATP-dependent sodium/potassium pump, the reconstitution of aquaporin channels on the membrane of these cells, water influx, and the formation of the blastocoel^[11]. Thus, re-expansion may reflect the viability of the blastocyst allowing to express its implantation potential. Although the implantation potential of blastocysts is significantly influenced by their post-warming re-expansion, our data do not show any statistically significant correlation between timing of re-expansion and implantation, as demonstrated by other authors as well^[5]. This result suggests that the implantation potential of blastocysts is not influenced by the speed of their re-expansion, but rather by whether it is completed within 4 hours post-warming^[7]. Probably, the timing of re-expansion is not linked to the viability of the blastocyst but rather to any damage it may have suffered during the vitrification-warming processes.

In other words, if the blastocyst has been damaged, its re-expansion may be slower as it will use its energy to repair the damage. On the contrary, if the blastocyst has not been damaged, its re-expansion may be faster. However, if re-expansion is completed within 4 hours post-warming, blastocysts can express their implantation potential. Regarding the pre-vitrification blastocysts morphology, several authors have suggested that the quality of the TE and ICM can be an indicator of post-warming implantation potential, as it is for fresh cycles^[4-8]. Our results are in line with those previously published, indicating that blastocysts characterized by excellent quality of ICM and of TE have a significantly higher probability of implantation compared to those with poor quality of ICM and TE. Our findings seem to suggest that the complete re-expansion of blastocysts 4 hours post-warming is a predictive factor for blastocyst implantation, regardless of their euploidy. In our sample, 89 blastocysts were euploid (43.4%) and 116 were untested (56.3%). Although the sample size of our study did not allow for a sub-analysis comparing PGT-tested blastocysts with untested ones, this result seems to be in line with the data from Allen et al., which show that blastocyst re-expansion is correlated with a higher live birth rate both in euploid and untested blastocysts^[13].

This result would suggest that euploid blastocysts not fully re-expanded post-warming, may exhibit reduced implantation potential. Once again, it can be hypothesized that complete re-expansion of blastocysts after warming is crucial to allow them to express their full implantation potential. To confirm this hypothesis, increasing the number of blastocysts would be necessary to compare the post-warming re-expansion of euploid blastocysts with those with unknown chromosomal complement.

Finally, our analysis shown that most blastocysts that do not re-expand after 2 hours post-warming also do not re-expand after 4 hours post-warming (17/25, 68.0%) showing a limited implantation potential (3 biochemical pregnancies were obtained from these blastocysts). The rate of failed re-expansion observed in our data (8.2%) is consistent with what reported by other authors^[7]. This data suggests that the total absence of re-expansion after 4 hours post-warming is indicative of a significantly reduced chances of success^[18]. This observation has a direct impact on clinical practice since, in these

cases, and if other blastocysts are available, the observation of re-expansion after 2 hours post-warming could be a useful tool to evaluate the possibility of thawing a second blastocyst, balancing this choice with the risk of a twin pregnancy. In conclusion, the results of this study confirm that the quality of the pre-vitrification ICM and TE, and the complete re-expansion of blastocysts within 4 hours post-warming are significantly predictive of embryo implantation in FER. As for the timing of re-expansion, this variable does not seem to influence embryo implantation.

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Intracytoplasmic injection of testicular sperm: blastulation rate in patients with obstructive azoospermia, non-obstructive azoospermia, and cryptozoospermia

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ABSTRACT

Purpose: The influence of sperm origin on embryo development remains a crucial issue in assisted reproduction. This study evaluates blastocyst development in patients with obstructive azoospermia (OA), non-obstructive azoospermia (NOA), and cryptozoospermia undergoing testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). **Methods:** A retrospective analysis was performed on 874 TESE-ICSI cycles: 368 OA, 253 NOA, and 253 cryptozoospermia. Data were stratified by female age (<35 and ≥35 years). Oocytes were injected via ICSI and cultured to the blastocyst stage. Embryo development was evaluated using the Istanbul Consensus criteria. ANOVA and Tukey's tests assessed differences among groups. **Results:** Blastocyst formation rates were significantly higher in OA patients across both age groups. The OA group had a superior oocyte-to-blastocyst conversion ratio and better-quality embryos. The NOA and cryptozoospermic groups showed delayed development and fewer hatching blastocysts. Female age impacted ovarian reserve but did not significantly influence blastulation outcomes within each diagnostic group. **Conclusions:** The study underscores the role of sperm quality, linked to the underlying male diagnosis, in influencing embryonic development. OA cases showed the most favorable outcomes following TESE-ICSI, likely due to preserved spermatogenesis despite obstruction. Conversely, testicular sperm from cryptozoospermic patients were less competent, possibly due to subtle defects not addressed by surgical retrieval. These findings highlight the importance of male factor considerations in ART success and suggest further investigation into optimizing lab protocols for patients with severely compromised spermatogenesis

KEYWORDS

ICSI, azoospermia, blastocyst, sperm quality, obstructive azoospermia (OA), non-obstructive azoospermia (NOA).

Introduction

This study investigates the influence of the origin of testicular sperm on embryonic development in patients with obstructive azoospermia (OA), non-obstructive azoospermia (NOA), and cryptozoospermia undergoing intracytoplasmic sperm injection (ICSI) with testicular sperm extraction (TESE) ^[1,2]. Previous meta-analyses have shown that sperm retrieval site and sperm integrity—particularly DNA fragmentation—can significantly impact fertilization and embryo developmental competence ^[3,4]. A total of 874 cycles were analyzed, stratified by diagnosis and the female partner's age (<35 and ≥35 years). Blastulation rates, blastocyst expansion grades, and other embryonic parameters were evaluated, revealing significant differences associated with the severity of the spermatogenic defect ^[3,5].

Materials and methods

Patients were retrospectively divided into three groups:

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obstructive azoospermia (OA, $n=368$), non-obstructive azoospermia (NOA, $n=253$), and cryptozoospermia ($n=253$). Each group was further stratified by the female partner's age (<35 or ≥35 years). Semen and testicular samples were processed according to WHO 2010 guidelines. All NOA patients underwent genetic screening, including karyotype and Y-chromosome microdeletion testing (AZF regions); cases with genetic abnormalities were excluded ^[5].

Cryptozoospermia was diagnosed by microscopic examination of the pellet following centrifugation (3000g for 15 minutes). TESE was indicated in cryptozoospermic patients when post-processing sperm concentration was <0.1 million/mL and

no motile sperm were retrieved from the ejaculate [3,5]. Ovarian reserve was assessed using Anti-Müllerian Hormone (AMH) levels and antral follicle count (AFC). Female infertility factors were recorded and stratified by age group, including tubal factor, PCOS, endometriosis, idiopathic, and advanced maternal age. Testicular sperm were used fresh in approximately 80% of

cases; the remaining 20%, predominantly in NOA and cryptozoospermic patients, used cryopreserved samples [6]. Fertilization was performed by ICSI, and embryonic development was monitored up to the blastocyst stage. Embryos were assessed using the Istanbul Consensus criteria. Statistical analysis was performed using ANOVA and Tukey's test [3].

Results

Table 1 General parameters of patients stratified by diagnosis and age group.

GROUP	CYCLES (n)	FEMALE AGE (MEAN ± SD)
OA (<35 years)	175	31.48 ± 3.13
OA (≥35 years)	193	38.78 ± 2.32
NOA (<35 years)	132	30.92 ± 3.45
NOA (≥35 years)	121	39.11 ± 2.29
Crypto (<35)	120	30.78 ± 3.18
Crypto (≥35)	133	38.92 ± 2.41

Table 2 Blastocyst data for OA, NOA, and cryptozoospermia groups.

GROUP	BLASTULATION RATE (%)	HATCHING BLASTOCYSTS (%)
OA (<35 years)	67.3	42.1
OA (≥35 years)	64.8	40.9
NOA (<35 years)	59.2	36.7
NOA (≥35 years)	56.3	35.1
Crypto (<35)	49.8	30.2
Crypto (≥35)	47.6	28.9

Table 3 Female infertility factors by group and age (<35 and ≥35).

DIAGNOSIS/AGE	TUBAL (%)	PCOS (%)	ENDOMETRIOSIS (%)	IDIOPATHIC (%)	AMA (%)
OA <35	12.5	18.3	14.2	41.0	14.0
OA ≥35	13.5	12.7	16.5	28.3	29.0
NOA <35	10.2	20.5	11.9	45.0	12.4
NOA ≥35	11.3	13.1	18.1	32.6	24.9
Crypto <35	9.8	19.4	13.6	44.2	13.0
Crypto ≥35	10.7	11.9	17.0	30.1	30.3

Table 4 Ovarian reserve (AMH, AFC) and mean number of MII oocytes inseminated.

GROUP	AMH (NG/mL, MEAN ± SD)	AFC (MEAN ± SD)	MII OOCYTES INSEMINATED (MEAN ± SD)
OA	3.1 ± 1.2	14.8 ± 3.9	8.7 ± 2.6
NOA	2.9 ± 1.3	13.7 ± 4.2	8.2 ± 2.4
Crypto	2.7 ± 1.4	12.9 ± 3.8	7.9 ± 2.7

Table 5 Fresh vs cryopreserved testicular sperm use by group.

GROUP	FRESH SPERM (%)	CRYOPRESERVED SPERM (%)
OA	91.6	8.4
NOA	76.2	23.8
Crypto	73.5	26.5

Table 6 Grading of blastocysts obtained on days 5 and 6.

GROUP	DAY 5 BLASTOCYSTS (N)	DAY 6 BLASTOCYSTS (N)	EXPANDED BLASTOCYSTS (%)	HATCHING BLASTOCYSTS (%)
OA (<35 years)	95	80	85.3	42.1
OA (≥35 years)	88	76	84.5	40.9
NOA (<35)	85	69	74.1	36.7
NOA (≥35)	79	62	73.6	35.1
Crypto (<35)	64	55	72.5	30.2
Crypto (≥35)	61	50	71.2	28.9

Discussion

This study underscores the substantial impact of sperm origin on blastocyst development. OA patients demonstrated the most favorable outcomes in terms of blastulation rate, embryo expansion, and hatching. These results are consistent with previous evidence indicating that testicular sperm from OA patients exhibit lower DNA fragmentation and better developmental potential than sperm from NOA or cryptozoospermic men^[1,3,5].

In contrast, NOA and cryptozoospermic groups showed poorer outcomes, likely reflecting underlying testicular damage and spermatogenic dysfunction^[3,4]. Although female age affected ovarian reserve markers (AMH, AFC), its impact on blastulation was minimal when controlling for sperm origin.

The use of cryopreserved testicular sperm, while sometimes necessary, may reduce embryo quality^[6]. Future improvements in sperm selection techniques (e.g., IMSI, MACS), enhanced embryo culture systems (e.g., co-cultures, antioxidant-enriched media), and personalized ICSI protocols may help optimize outcomes for patients with compromised spermatogenesis^[4,6]. A deeper understanding of sperm–oocyte interactions and female resilience may further enhance ART success rates^[4,6].

Conclusions

Sperm origin plays a pivotal role in embryonic development following ICSI. Patients with OA benefit the most from TESE-ICSI, achieving higher blastulation and embryo quality^[1,3]. Conversely, NOA and cryptozoospermic men exhib-

it reduced blastocyst formation, likely due to intrinsic sperm nuclear defects^[4,5].

These findings support targeted laboratory and clinical strategies to optimize ART outcomes, particularly in cases with severely compromised spermatogenesis.

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Confluency-dependent gene expression in trophoblast cell lines: implications for appropriate physiologic and pathologic models set up

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ABSTRACT

Purpose: This study aimed to determine how culture confluency and intrinsic cell line differences influence the expression of angiogenic mediators and immune markers in commonly used trophoblast models. **Methods:** JEG-3 and JAR cells were cultured at sub-confluent (60%) and fully confluent (100%) states. Immunofluorescence was performed to visualize the actin cytoskeleton and nuclei. Gene expression of EPHA1, FLT4, ANGPT4, and HLA-G was assessed by qPCR using GAPDH as reference. Expression levels were calculated with the $2^{-\Delta\Delta C_t}$ method. Statistical analysis was performed using the unpaired t test to compare cell lines and confluency states. **Results:** A notable distinction between the two models was observed, where JEG-3 cells exhibited higher levels of FLT4 and ANGPT4 compared with JAR. A subsequent analysis of each cell line revealed additional modulations associated with cell density, with JEG-3 demonstrating a heightened susceptibility to culture confluency. In the case of fully confluent JEG-3 cells, there was a significant increase in HLA-G expression, as well as a substantial rise in the expression of FLT4 and ANGPT4. In contrast, EPHA1 remained constant across the various conditions, suggesting that its expression is not influenced by changes in cell density. **Conclusions:** These findings demonstrate that both cell density and model choice critically shape marker expression in trophoblast cell lines. Careful reporting and control of confluency are essential for reproducibility and for selecting the most appropriate in vitro model depending on the biological question.

KEYWORDS

Trophoblasts, JEG-3, JAR, cell density, vascular remodeling.

Introduction

A wide range of *in vitro* trophoblast models has been established to investigate placental biology and its pivotal role at the maternal–fetal interface. Among these, choriocarcinoma-derived cell lines such as JEG-3 and JAR are frequently employed due to their ease of culture, reproducibility, and retention of several trophoblast characteristics, including hormone secretion and barrier-related functions^[1]. Although these immortalized lines are widely used, they differ substantially in physiological properties, raising important questions about their interchangeability in experimental settings^[2–6].

Both JEG-3 and JAR have been applied to studies on endocrine activity and on nutrient and glucose transport across trophoblast monolayers^[7]. They are also used to model barrier integrity, tight junction dynamics, and nanoparticle or drug transfer^[8]. These applications make them indispensable for investigating placental mechanisms when primary tissue is unavailable due to ethical and practical limitations.

More complex and physiologically representative platforms have emerged in recent years, including primary first-trimester trophoblast cultures⁹, trophoblast stem cell lines^[10], and 3D organoids^[11]. These models better recapitulate cellular diver-

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sity and architecture, providing new opportunities to interrogate trophoblast differentiation and maternal–fetal interactions. Despite their advantages, access to early gestation tissue, high costs, and technical complexity limit their widespread use, and many laboratories continue to rely on immortalized cell lines such as JEG-3 and JAR for practical, reproducible assays^[10].

Importantly, functional outcomes in trophoblast cell lines may depend not only on the cell line chosen but also on culture variables such as seeding density and confluence, as well described in other cell types^[13–17]. A similar impact of cell density on cell behaviour can be speculated for trophoblast cell models, as suggested by a comparative work on placental cell lines which showed that seeding density and model choice markedly alter barrier function, hormone release and transport properties of BeWo, JAR and JEG-3 cell lines^[8].

Trophoblast differentiation and invasion are tightly regulated by the interaction with maternal uterine cells, including decidual stromal cells, immune populations and endothelial cells [18,19]. Paracrine signals deriving from these neighboring cells, as well as extracellular matrix composition and biomechanical features of maternal decidua, influence trophoblast cell growth and density [20-22]. In particular, several molecules are pivotal mediators of trophoblast-endothelial crosstalk. A peculiar mediator of placentation is HLA-G, a non-classical MHC class I molecule which is exclusively expressed by invasive extravillous trophoblasts and is central for the establishment of maternal-fetal immune tolerance [23] and for an efficient vascular remodeling [24]. In addition, higher levels of HLA-G are known to correlate with increased migration/invasion by trophoblast cells [25].

Among the molecular mediators of trophoblast-endothelial cell crosstalk, EPHA1, CXCL12, FLT4 and ANGPT4, expressed by trophoblasts, and their interacting partners EFNA1, EFNA5, VEGFC and TEK, expressed by ECs, have been proposed to play an essential role [26]. FLT4 (VEGFR-3) contributes to lymphatic and blood vascular remodeling, playing a critical role in uterine vascular adaptation during pregnancy. In trophoblasts, FLT4 signaling intersects with mechanical cues such as shear stress, thereby linking trophoblast invasion to the hemodynamic remodeling of maternal vessels [27]. ANGPT4 and related angiopoietins are angiogenic modulators [28,29] which in the placenta fine-tune endothelial/trophoblast communication, balancing vessel permeability and structural integrity [30]. Nonetheless, the specific role of ANGPT4 in trophoblast physiology is still poorly characterized. Finally, the tyrosine kinase receptor EPHA1 regulates cell adhesion, repulsion, and directional migration at sites of cell-cell contact. Although poorly studied, evidence from placental tissues indicates that EPHA1 signaling influences trophoblast-endothelial interactions, potentially guiding extravillous trophoblast through the decidual vasculature [31].

In our study, we explored the impact of cell density on the expression of these molecules and found that their level changes according with cell confluency. For the first time, we quantified the expression of EPHA1, FLT4, ANGPT4 in JEG-3 and JAR cell lines, with the aim to provide practical guidance for model selection and to highlight the importance of reporting and controlling confluence as an experimental variable in trophoblast research.

Methods

Cell Culture and Harvesting

JAR cells were maintained in RPMI 1640 with GlutaMa-xTM-I (61870-036, Thermofisher Scientific) and JEG-3 cells in EMEM completed basic (820100c, Cell Lines Services). All culture media were supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were cultivated in a humidified atmosphere at 37°C and 5% CO2. The cell lines were seeded in 6-well plates at either 3.5×10⁵ or 5.0×10⁵ cells per well and maintained in culture until they reached the desired confluence (60% or 100%). For JAR cells, the culture plates were pre-coated with 2% gelatin.

RNA extraction, quantification and reverse transcription

Cells were lysed with Buffer RL (Norgen) and total RNA extracted with a dedicated kit (NR17200, Norgen Biotek), according to the manufacturer’s instructions.

RNA was quantified using the NanoDrop instrument and stored at -80°C until use. Reverse transcription of RNA was made using Takara Kit (RR036A, Takara) following the manufacturer’s instructions.

Real-Time PCR (qPCR)

cDNA was analyzed by quantitative PCR using specific primers for EPHA1, FLT4, ANGPT4, and HLA-G, as listed in Table 1. Reactions were performed according to the supplier’s recommendations in Hard-Shell® 96-Well PCR Plates (Bio-Rad). Amplification was carried out on a CFX Opus 96 thermocycler (Real-Time PCR System, Biorad) where the following thermal protocol was performed: 95°C, 30 sec/ 95°C, 10 sec + 60°C, 30 sec for 40 cycles. A melting curve analysis was performed at the end of each run: 65°C - 95°C, 0.5 sec, increase every 5 sec. Expression levels of target genes were obtained through the 2-ΔΔCt method; GAPDH was used as reference gene.

Immunofluorescence

Cells were seeded on 12-mm glass coverslips (CB00120 RA120MNZ0, EpreDia) previously degreased with 100% ethanol. Once reached 60% and 100% confluency, cells were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 in PBS for 2 min at RT.

Coverslips were blocked with 2% BSA in PBS for 1h at RT, followed by incubation for 1 hour at RT with with AlexaFluorTMPlus-647 Phalloidin (A30107, Thermofisher Scientific) in 0,2% BSA + PBS and Hoechst (33342, Cell Signaling). Coverslips were mounted in SuperfrostTM Plus Adhesion Microscope Slides (J1800AMNZ, EpreDia) using Antifade Mounting Medium (H-1700, Vectashield). Images were acquired with Confocal Microscope (Zeiss Lsm 900), using the ZEN Microscopy Software and analyzed with ImageJ.

Table 1 List and sequence of primers used for expression analyses.

PRIMER	DAY 5 BLASTOCYSTS (N)
GAPDH_Fw	5'- TCGGAGTCAACGGATTGGT
GAPDH_Rv	5'- TTCCCGTTCTCAGCCTTGAC
EPHA1_Fw	5'- ACCGATGTGGATCGAGAGGA
EPHA1_Rv	5'- TGACAGTGTCCACCATCAGC
FLT4_Fw	5'- CCTTGCCCGGGACATCTA
FLT4_Rv	5'- TTGTCGAAGATGCTTTCAGGG
ANGPT4_Fw	5'- GAATCACTGGCCAACCCACT
ANGPT4_Rv	5'- TGATGGCCCTCTCTAGCTTC
HLA-G_Fw	5'- CACGCACAGACTGACAGAATG
HLA-G_Rv	5'- GCCATCGTAGGCATACTGTTCA

Results

JAR and JEG-3 cells express different levels of ANGPT4 and FLT4. In order to explore potential differences in trophoblast behavior and morphology relevant to trophoblast–endothelial interactions, we examined sub-confluent (60%) and fully confluent (100%) JAR and JEG-3 cell cultures (Figure 1).

The expression level of EPHA1, FLT4, ANGPT4 and HLA-G were subsequently analyzed and key differences emerged between the two cell models (Figure 2A). In particular, FLT4 expression showed a lower level in JAR cells, compared with JEG-3, in a similar manner at both 60% and 100% cell confluency (Figure 2B). Noteworthy, the expression of ANGPT4 changed along with the culture conditions: when seeding the cells at 60% confluency, no differences emerged between the two cell lines, whereas at 100% cell density JAR cells showed a significantly lower expression level, compared with JEG-3 (Figure 2C), reinforcing the cell line-specific regulation of these angiogenesis-related gene. Conversely, EPHA1 expression in the two cell lines was found comparable in both experimental conditions (Figure 2D).

As already known⁶, HLA-G expression was completely absent in JAR cells, whereas it was expressed in JEG-3, supporting the use of JEG-3 as a model for HLA-G positive trophoblast subtypes (Figure 2E).

The expression levels of FLT4, ANGPT4 and HLA-G is differentially influenced by cell density in trophoblast models

To assess the impact of cell confluency on features key for

the crosstalk with endothelial cells, we evaluated the effect of different cell density on the expression of EPHA1, FLT4, ANGPT4, and HLA-G in each trophoblast cell line. We assessed that JEG-3 and JAR cells differently responded to cell density, as illustrated in Figure 3.

In JEG-3, EPHA1 expression remained largely unchanged between the two conditions, suggesting that this receptor is relatively insensitive to variations in confluency (Figure 3A). In contrast, both FLT4 and ANGPT4 displayed a pronounced induction when cells reached full confluency. The increase in ANGPT4 was particularly striking, with expression levels rising more than fivefold compared to sub-confluent cultures. Also, HLA-G exhibited an upregulation at 100% of confluency in JEG-3, further underlining the crucial effect of cell density on the immune properties of trophoblasts models.

In JAR cell line, a different pattern emerged, which suggests a limited effect of cell density on their phenotype (Figure 3B). EPHA1 again showed no significant changes between 60% and 100% confluency. However, the induction of FLT4 and ANGPT4 at full confluency was less relevant compared to JEG-3, with only moderate increases observed.

Discussion

Our findings reveal that trophoblast biology *in vitro* cannot be disentangled from the conditions under which cells are cultured. Both the intrinsic properties of trophoblast cell lines and

Figure 1 Morphological features of JAR and JEG-3 trophoblast cells at different confluency levels, stained with Phalloidin (red) to visualize actin filaments and Hoechst (blue) to label nuclei. Representative images are shown at low and high magnification. Scale bar: 100µm.

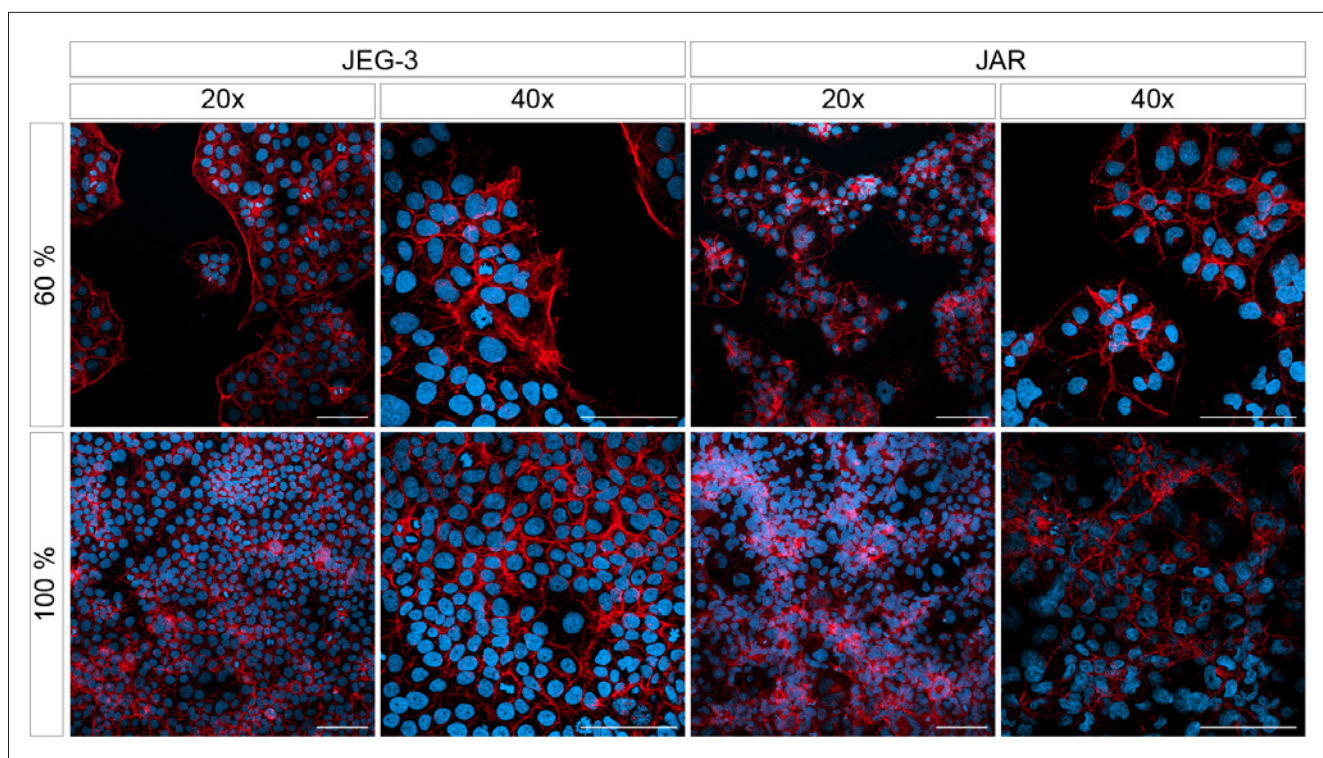
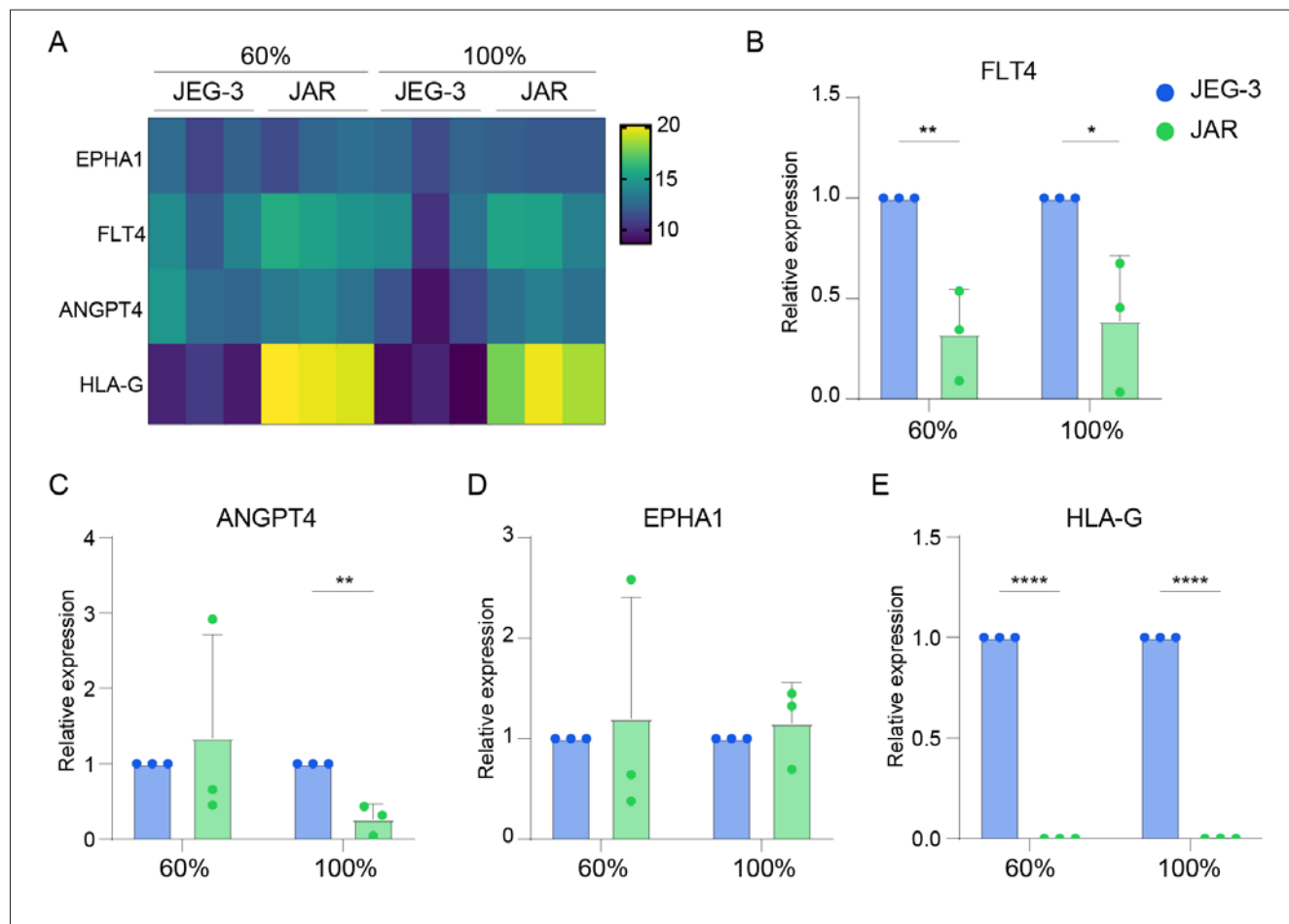


Figure 2 Comparative gene expression analysis between JEG-3 and JAR trophoblast cells. **(A)** Heatmap showing ΔCt values for selected genes involved in trophoblast–endothelial crosstalk. **(B–E)** Relative expression levels ($2^{-\Delta\Delta Ct}$) of FLT4 **(B)**, ANGPT4 **(C)**, EPHA1 **(D)**, and HLA-G **(E)** derived from the heatmap data. *, $P \leq 0.05$, **, $P \leq 0.01$, ****, $P \leq 0.0001$.



the extrinsic variable of culture density synergistically shape the expression of markers that regulate vascular remodeling and maternal–fetal immune tolerance.

One of the central insights from this study is that confluence acts as a regulator of angiogenic gene expression in trophoblast cell lines. In JEG-3, the induction of FLT4 and ANGPT4 at full density suggests that endothelial-related signaling cascades are activated as cells transit from a proliferative to a contact-inhibited state. Such density-dependent regulation has been described in other cell types, where contact inhibition, cytoskeletal tension, and junctional signaling converge to alter transcriptional programs [32]. In trophoblasts, these signals may mimic, though not perfectly,

the conditions faced *in vivo* during invasion into the compact decidual matrix. By contrast, EPHA1 remained largely insensitive to density changes, pointing to differences in how trophoblasts integrate environmental signals.

The comparison between JAR and JEG-3 provides additional details. While both lines responded to confluency, the level of induction differed, reflecting divergent regulatory architectures. Importantly, HLA-G expression was absent in JAR confirming their partial relevance as extravillous trophoblast cell model.

However, this apparent limitation can also be reframed as

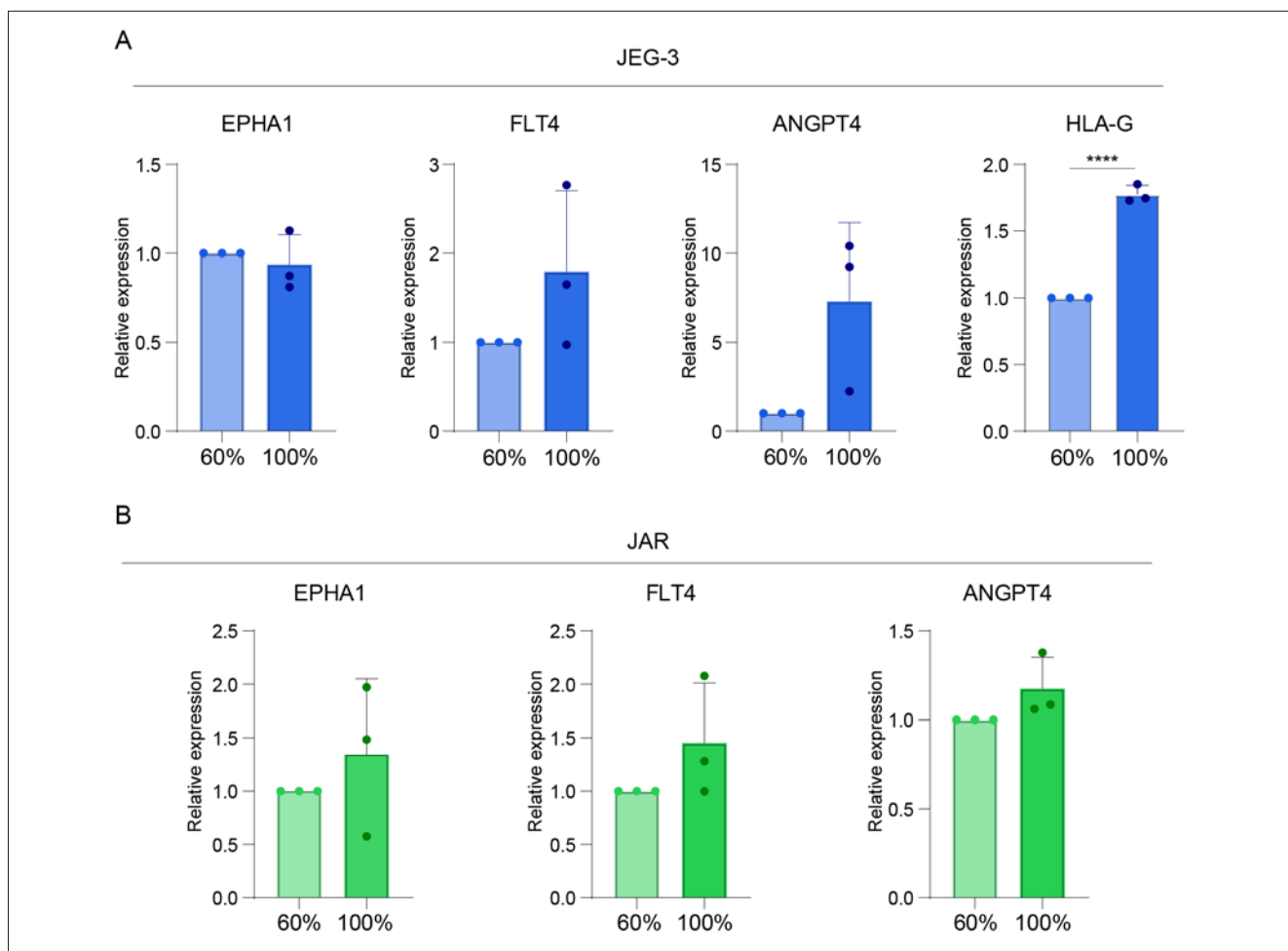
an experimental advantage offering a “blank background” for investigations focused on HLA-G–dependent phenomena.

Beyond individual markers, our results stress a broader methodological point. Although the assays employed are routine, the critical step is the precise definition and reporting of confluence states. This variable is rarely described in trophoblast literature, yet our findings indicate that it can substantially alter marker expression, posing a serious limit to experimental reproducibility. A simple but rigorous standardization, such as reporting exact seeding densities, growth times, and operational criteria for confluence, would significantly enhance the interpretability of *in vitro* trophoblast studies.

These observations also have implications for disease modeling. The balance between angiogenic signaling (e.g., via FLT4 and ANGPT4) and immune tolerance (via HLA-G) is central to successful spiral artery remodeling. Perturbations in these processes are hallmarks of pregnancy disorders such as preeclampsia and fetal growth restriction [33,34].

Our data suggest that depending on the model chosen, researchers may over- or underestimate the contribution of specific pathways. Thus, aligning the choice of model with the biological question under investigation is crucial, particularly when attempting to extrapolate mechanistic insights to pathological contexts.

Figure 3 Relative expression levels of indicated markers in trophoblast cells comparing different confluency levels. **(A)** Relative expression levels of selected markers in JEG-3 cells. **(B)** Relative expression levels of the same markers in JAR cells. ****, $P \leq 0.0001$.



Conclusions

Overall, our study underscores that cell density is not a minor technical parameter but a biologically active variable that modulates trophoblast phenotype. Combined with intrinsic differences between JEG-3 and JAR, this creates a layered experimental landscape in which marker expression is contingent on both the model and its state. A careful appreciation of these factors will not only refine *in vitro* studies but also help bridge the gap between assays and the complex biology of placenta-*in vivo*.

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