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Advancement In Oocyte Collection and Preservation

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ABSTRACT

Vitrification has become a prevalent and effective technique for cryopreservation in the field of reproductive biology. Recent findings indicate that this method is not only reliable but also serves as a highly efficient means for storing human oocytes at low temperatures. The latest techniques, when paired with the right selection of cryoprotectants, tools, and methods, along with optimized conditions, can achieve nearly 100% morphological survival rates. By standardizing this technique and addressing biosafety concerns while preserving its advantages, vitrification could be integrated into the routine practices of human embryo laboratories. This could provide a viable therapeutic option in various biological and social situations, as well as in addressing common logistical challenges currently encountered. Ovulation induction (OI) is a cornerstone of infertility treatment, particularly for anovulatory women, accounting for a significant proportion of female infertility cases. This review studies the body's processes that cause ovulation via the hypothalamic-pituitary-ovarian axis and provides information on common medical drugs used for ovulation induction. Both traditional agents such as Clomiphene Citrate and Letrozole, gonadotropins and hCG are discussed together with newer treatments that include kisspeptin analogs and SERMs. Laser-assisted hatching improved IVF. At Dr. Shobha's Fertility Center, Muktsar Sahib Punjab conducted the study from July 2024, to December 2024. The study included 80 ICSI-treated infertile couples. Women aged 20-30 and 30-40 were tested utilizing laser exposure. A noncontact RI-SATURN V laser was employed for zona pellucida manipulation. All participants had hormone tests, transvaginal ultrasounds, and hysteroscopy or laparoscopy done during treatment cycle. Fixed and extended ovarian hyperstimulation treatments were used. IVF success rates vary by age, with 30-40 having 83%, 20-30 (84%). The laser exposure hatching had the highest success rate (92%), and the lowest success rate (48%). A significant positive correlation was established between laser exposure and clinical pregnancy outcomes. The study reveals age, especially 30-40, may impair IVF success. Additionally, a laser exposure hatching increases IVF success rates. The recommended laser exposure period for all age groups is 4.5 µs, with younger women recommended at 2 µs and older women at 8 µs. Laser-assisted hatching methods, specifically diode laser exposure periods, were evaluated for in vitro fertilization success. The study evaluated how age influences IVF couples' clinical pregnancy outcomes. The findings optimize laser parameters for assisted reproductive technology.

Keywords: *Laser-assisted hatching, In vitro fertilization, Success rates, embryo implantation.*

1. INTRODUCTION

Vitrification is a highly accelerated manual cooling method carried out at subzero temperatures, resulting in a solidified substance with a glass-like quality, devoid of ice crystals. This technique inhibits the production of ice crystals during cooling via facilitating oocyte dehydration and utilizing high concentration from cryoprotectants, which significantly enhances the viscosity of the solutions involved (Chang et al., 2022). Since oocyte viability and clinical pregnancy rates have significantly improved over the last decade, this technique and procedure have been widely used for a variety of applications. This method was initially primarily used in oocyte donation programs, which solved the issue of donor-recipient synchronization and enabled an effective distribution of oocytes among various recipients. However, its application would also be advantageous in fertility preservation programs that are medical and non-medical (De Munck et al, 2018).

Potential effects of vitrification and warming on oocytes:

It is still unclear whether vitrification has any detrimental effects on oocyte quality, including temperature change/chilling effect, osmotic stress, cryoprotectant toxicity, and/or phase transitions, causing damage to oocyte spindle, DNA and ultrastructural changes, even though it can cryopreserve oocytes without ice crystal formation (Lai, D., 2014).

Effects of CPAs' exposure and osmotic shocks:

Rapid variations in the external osmotic pressure that the oocytes undergo during vitrification and thawing are referred to as osmotic shocks (Lai, D., 2014).

Even while CPAs are necessary for a proper vitrification process, their exposure may present several difficulties.

Toxicity:

Oocytes may be injured by prolonged exposure or high cryoprotectant concentrations. Alteration on oocyte include oxidative stress, membrane damage, and altered cellular function can result from CPAs' ability to penetrate the cell membrane and interfere with cellular functions. The concentration of cryoprotectants and the duration of exposure are the main factors that affect toxicity (Best, B. P., 2015).

Osmotic Imbalance:

As the cryoprotectants are added and the oocytes are quickly cooled or heated throughout the vitrification process, osmotic alterations occur. Cell swelling or shrinking may result from this osmotic imbalance. The oocyte may sustain irreparable damage in the event of warming if cryoprotectants are not sufficiently eliminated and the osmotic balance is not restored (Best, B. P., 2015).

Membrane Integrity:

Oocytes are susceptible to membrane disruption throughout the CPA exposure phase. Membrane instability may result from cryoprotectants' disruption of the lipid bilayer as they enter the oocyte. This may lead to the loss of cellular activities, such as the capacity to divide normally after fertilization, and the leakage of intracellular contents (Best, B. P., 2015).

Vitrification is a cryopreservation technique that enables cells and their surrounding extracellular environment to transition into a glass-like state without the formation of ice, differing from the slow-freezing method. For vitrification, it is essential for there to be a reduction in water content and for the cytoplasm to become highly viscous, facilitating the achievement of this glass-like

Aneuploidy:

Both the sperm and the egg are expected to provide the embryo exactly one copy of each chromosome during conception. Aneuploidy is a disorder, which is far more common occurs in eggs than in sperm or the majority of somatic cells, occurs when human eggs have an incorrect number of chromosomes. Inversely, one of the main causes of infertility, miscarriages, and congenital disorders is aneuploidy in eggs. Aneuploidy results when the oocyte, the egg's progenitor cell, undergoes abnormal meiosis during development. Chromosomes frequently segregate improperly in human oocytes. Age-related infertility is the result of increased chromosome segregation mistakes in women starting in their mid-thirties, which raise the levels of aneuploidy in eggs from older mothers (Charalambous *et al*, 2023).

Ultrastructural changes:

Transmission electron microscopy can be used to identify ultrastructural alterations in human oocytes in the metaphase II (MII) stage following vitrification or warming. Increased vacuoles, cortical granules (CGs), and smooth endoplasmic reticulum of mitochondria accumulate with vesicle complexes were the primary ultrastructural alterations. Vacuoles had the same ultrastructural features regardless of their size: they were rounded irregularly, looked empty, and had a membrane enclosing them. Golgi and/or SER membrane swelling and coalescence can result in vacuoles. The fusion of damaged CGs or changed and swollen mitochondria could be involved in the genesis of vacuoles in vitrified oocytes, accompanied by a significant reduction in electron-dense granules. Moreover, peripheral vacuoles also could be formed by aggregations of endocytic vesicles in the oocyte, or elevations of the oolemma and/or aggregations of endocytic vesicles in the cortex of the oocyte, in the oocyte surface as seen in oocytes treated only with the CPAs (Chang et al., 2022).

Impact to oocyte spindle:

During meiosis, the chromosomes are arranged by the microtubule-based oocyte spindle, which makes sure they are properly aligned and segregated. Chromosome mis segregation, aneuploidy (an aberrant number of chromosomes), and poor fertilization or embryo development can result from spindle structural disruptions (Bennabi *et al*, 2016). The second meiosis requires timely chromosome attachment to ensure efficient chromosome segregation, and the disappearance of cohesion is necessary for the medial gonads and successful sperm. Sister chromatid cohesion is of great importance since it guarantees that sister chromatids are correctly aligned on the spindle during the MII stage. Finally, the bipolar linkage of spindle microtubules to sister chromatids is a critical step in producing tension at the centromere. Upon entry of a spermatozoon and

initiation of the second meiosis, a lack of correct alignment and tension of the sister chromatids may result in aneuploid embryos. Among other things, the spindle is sensitive to low temperatures. Cryopreservation exposes the oocyte spindle to low temperatures, which may cause ice crystals to form and harm the spindle. Whether the spindle structure has been damaged due to the phase shift and cold effects of vitrification and warming in the absence of formation of ice crystals is unknown. Vitrification and MII oocyte spindle configuration dynamics.

Parthenogenetic activation:

The process of intentionally triggering an oocyte (egg cell) to start developing without sperm fertilization is known as parthenogenetic activation. Similar to what occurs after fertilization, this can be accomplished via a variety of chemical, physical, or electrical techniques that cause the egg to start dividing. Research on parthenogenetic activation in human oocytes has significance for developmental biology, stem cell research, and fertility (Kharche and Birade, 2013). The occurrence of oocyte activation during cryopreservation poses a potential risk. The fusion of sperm with mammalian oocytes leads to an elevation in the intracellular concentration of calcium ions (Ca2+). Ultimately, fertilization triggers a widespread increase in Ca2+ levels throughout the oocyte. This surge in calcium is primarily due to the release from the internal cell reticulum, starting at the site where the sperm enters and propagating in a wave-like manner; however, it can also originate from larval reserves, especially from the stores within the endoplasmic reticulum. Additionally, exocytosis may be stimulated by an increase in Ca2+, which can arise from external sources or a combination of both. Furthermore, commonly used cryoprotectants in vitrification, such as DMSO and EG, have the potential to significantly elevate intracellular calcium levels. The duration of the amplitude can be 50% longer than the typical Ca2+ spike, with the initial surge beginning at fertilization and persisting for approximately this extended duration. This influx of calcium is believed to contribute to the zona hardening that occurs due to exposure to these cryoprotectants (Chang et al., 2022).

Deoxyribonucleic acid (DNA) integrity:

Vitrification has a high survival rate but insufficient mechanisms to protect oocytes from sublethal damage. Even though most successful vitrification protocols for oocytes use relatively high amounts of cryoprotectants (CPAs), a few of these substances have shown concerns for related side effects and could induce DNA damage. Germ cell DNA damage may elicit genotoxic responses, and when unresolved, produce chromosomal abnormalities that will be transmitted to offspring. Importantly, CPAs are genotoxic agents also in the absence of cryopreservation. The genotoxic effects of three of the most commonly used vitrification CPAs, including ethylene glycol (EG), propanediol (PrOH), and dimethyl sulfoxide (DMSO), have been evaluated in both somatic cells and oocytes (Chang et al., 2022).

Epigenetic effects:

The term "epigenetics" refers to alterations in cellular phenotype or gene expression that are not caused by changes to the DNA sequence. Numerous physiological and environmental factors, including age, stress, and—most importantly—reproductive technologies like cryopreservation, can have an impact on these changes. Epigenetic control is essential for early development, fertilization, and cellular differentiation in human oocytes. Important epigenetic markers that control gene expression during oocyte maturation and fertilization include histone changes, DNA methylation, and non-coding RNA (Mazzio and Soliman, 2012).

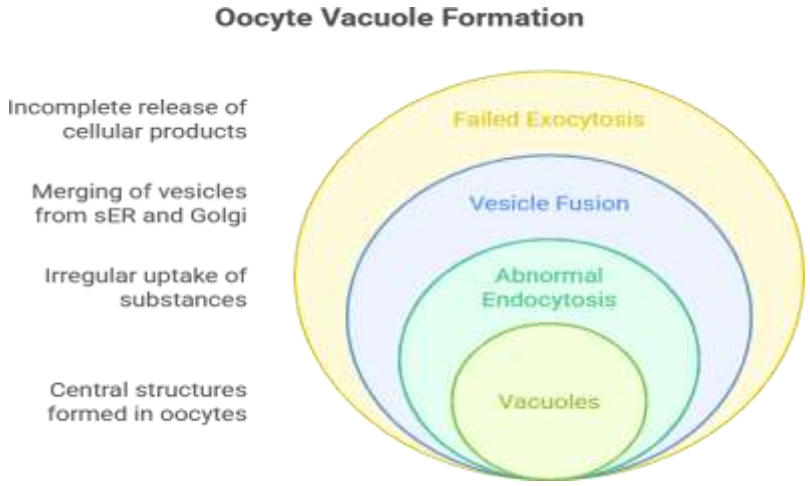
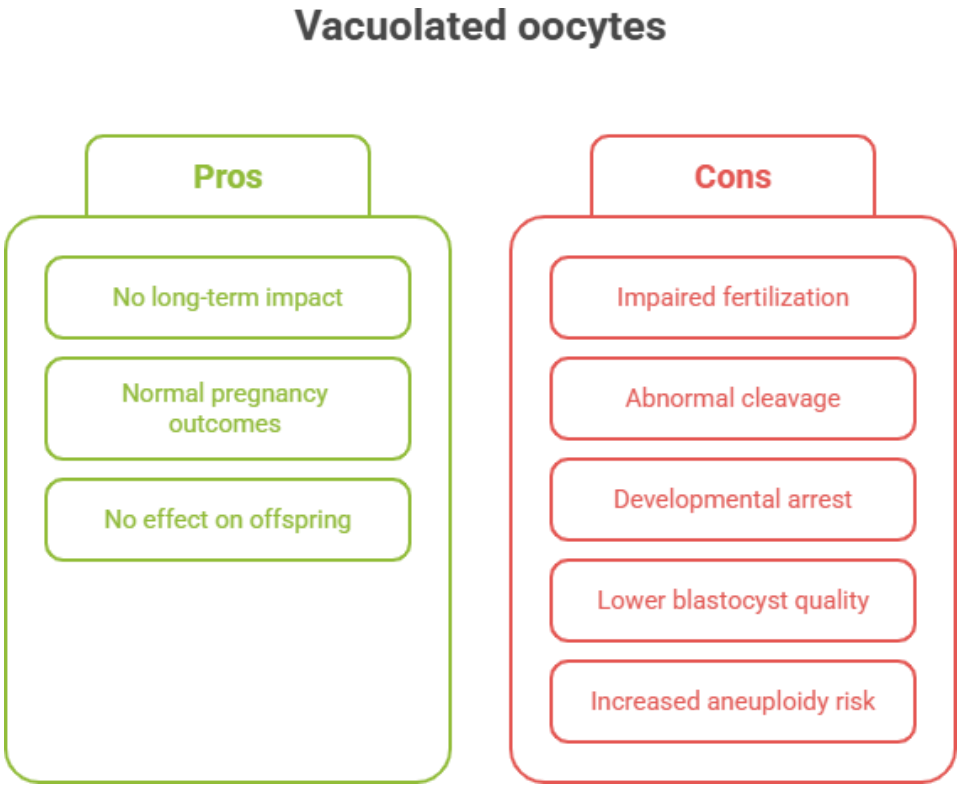


Figure 1: Formation of Oocyte Vacuole

The clinical significance of vacuolated oocytes is underscored by their association with impaired fertilization, abnormal embryonic cleavage, and a higher likelihood of developmental arrest. Large or multiple vacuoles can distort the oocyte's

internal architecture, sometimes displacing the metaphase II (MII) spindle, which is essential for accurate chromosome segregation. Such displacement increases the risk of aneuploidy and embryonic arrest[1]. Embryos derived from vacuolated oocytes frequently demonstrate delayed cleavage, abnormal pronuclear morphology, and increased rates of developmental arrest before reaching the blastocyst stage. Vacuolization in embryos on days 3 and 4 of in vitro development is associated with reduced blastocyst formation rates and lower quality blastocysts[1][2][3]. However, some studies suggest that if blastocysts from vacuole-positive embryos are transferred, their pregnancy and neonatal outcomes may not be significantly affected, indicating that vacuole-associated variation in preimplantation embryos may have no long-term impacts on embryo development or the health of offspring[1]

(Figure 2).



Despite the clear association between vacuolization and reduced developmental competence, the precise impact of vacuolated oocytes on long-term reproductive outcomes remains an area of active investigation. Some studies have reported that while vacuolization impairs the competence of blastocyst formation and blastocyst quality, pregnancy and neonatal outcomes following the transfer of blastocysts derived from vacuole-positive embryos are not significantly different from those of vacuole-negative embryos[1]. This suggests that the negative effects of vacuolization may be limited to preimplantation development, and that embryos able to reach the blastocyst stage may have compensated for earlier cytoplasmic defects.

Classification and Morphological Features of Cytoplasmic Vacuoles in Human Oocytes

The clinical impact of cytoplasmic vacuoles in human oocytes is largely influenced by their size, number, and localization (Figure). These morphological characteristics offer critical insight into the developmental competence of the oocyte and its potential for successful fertilization and embryo formation.

Size of Vacuoles- Vacuoles within the ooplasm are typically classified by diameter into small (<5 µm), medium (5–10 µm), and large (>10 µm) categories. Macro-vacuoles, often defined as those exceeding 14 µm or, in some cases, 25 µm, are considered particularly detrimental to oocyte quality. Ebner et al. reported that oocytes harboring a single vacuole up to 14 µm exhibited a fertilization rate of 51.6%, whereas oocytes with vacuoles exceeding 14 µm rarely achieved fertilization [4]. Larger vacuoles, especially those surpassing 25 µm in diameter, can occupy a significant portion of the cytoplasm, distort intracellular architecture and disrupting key events such as sperm-oocyte fusion, resumption of meiosis, and early embryonic cleavage [5,6]. These macro-vacuoles often arise during the transition from metaphase I (MI) to metaphase II (MII), coinciding with the extrusion of the first polar body [4].

Number of Vacuoles- The number of vacuoles observed per oocyte also holds prognostic value. Data suggest that approximately 66% of vacuolated oocytes present with a single vacuole, 21.3% with two, and 12.7% with multiple vacuoles

[4]. The presence of multiple vacuoles—commonly defined as more than three—is associated with a further reduction in fertilization potential. Comparative analyses show that fertilization rates decline from 51.6% in oocytes with a single vacuole to 43.8% in those with multiple vacuoles [4,6]. In addition to reduced fertilization, multiple vacuoles increase the likelihood of developmental arrest and impaired blastocyst formation, likely due to cumulative cytoplasmic disorganization [6,7].

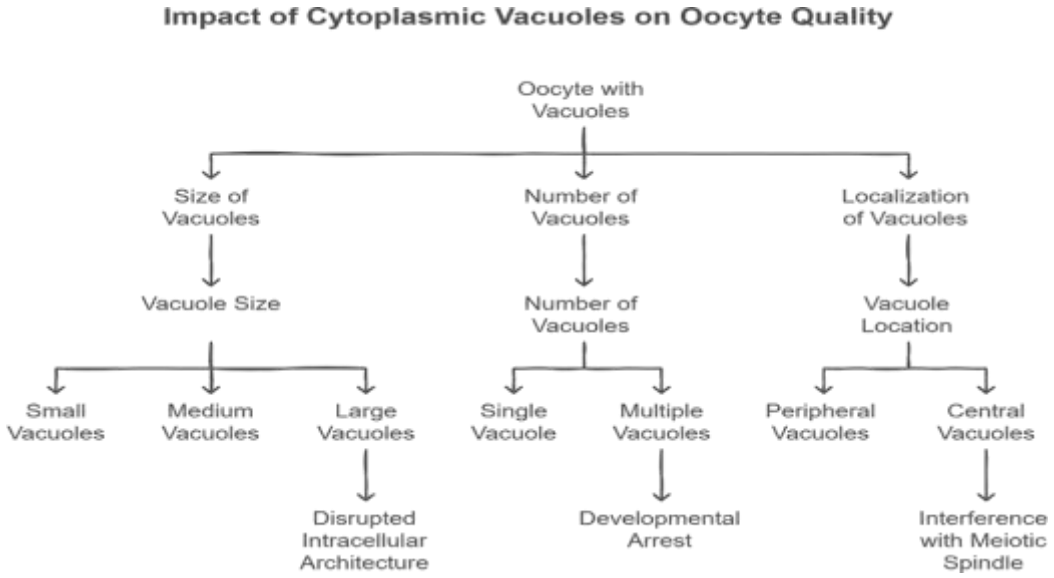


Figure -3

Localization of Vacuoles

The spatial distribution of vacuoles within the oocyte cytoplasm also modulates their developmental impact. Centrally located vacuoles are generally regarded as more disruptive than those at the periphery. Central vacuoles can potentially interfere with the meiotic spindle apparatus or displace critical organelles, thereby compromising spindle function and chromosome segregation [8]. While some studies have not observed direct spindle displacement by central vacuoles, their proximity to the metaphase plate raises concerns about subtle mechanical or biochemical interference [8,9]. In contrast, peripheral vacuoles are thought to have a lesser effect on meiotic dynamics, possibly due to their distance from critical cytoskeletal structures.

Morphological Characteristics

Under light microscopy, vacuolated oocytes are recognized by the presence of one or more sharply demarcated, round, translucent inclusions within the cytoplasm. These vacuoles may be either central or peripheral and frequently coexist with other cytoplasmic abnormalities such as refractile bodies, increased granularity, or abnormal polar body morphology [4,6]. Severe vacuolization may result in cytoplasmic asymmetry, oocyte distortion, or a darkened, uneven cytoplasmic texture—features commonly linked to poor oocyte quality and compromised developmental potential [6,7].

Clinical Implications

The classification of vacuoles by size, number, and localization provides valuable information for embryologists in assessing oocyte quality during ART procedures. Oocytes with large, multiple, and centrally located vacuoles exhibit significantly lower fertilization rates, a higher risk of early embryonic arrest, and reduced likelihood of reaching the blastocyst stage [4,5,7,8]. Persistent vacuolization observed across multiple ART cycles in the same individual may suggest an underlying intrinsic or genetic abnormality, potentially related to cytoskeletal or vesicular trafficking dysfunction [6,10]. In such cases, alternative therapeutic strategies, including oocyte donation or modified stimulation protocols, may be warranted.

Ultrastructure and Origin of Vacuoles

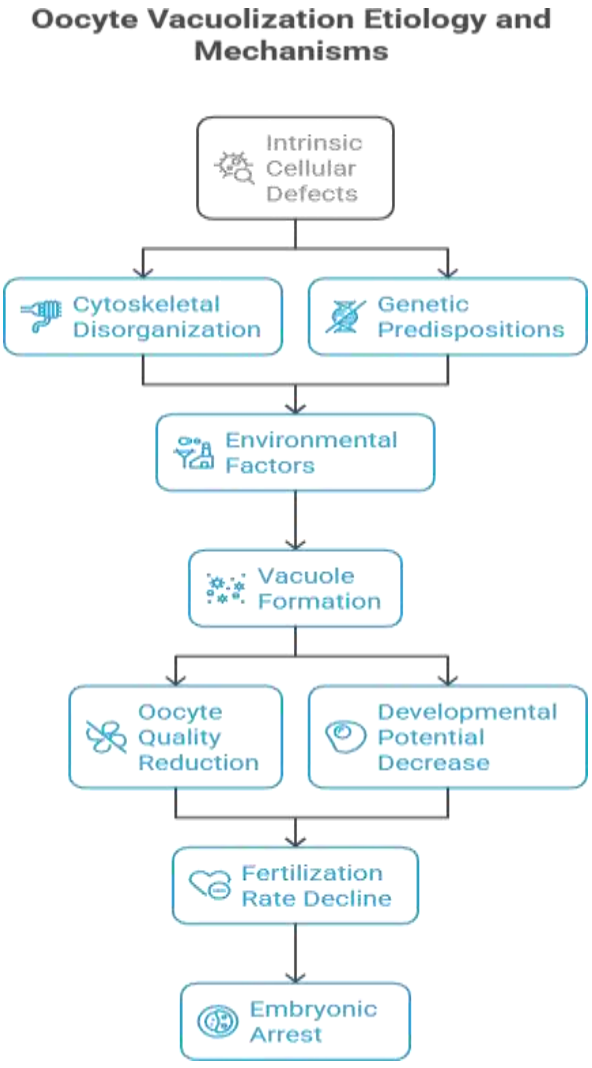
Ultrastructural studies using transmission electron microscopy (TEM) and advanced confocal imaging have significantly advanced our understanding of the origin and nature of vacuoles in human oocytes. These studies reveal that vacuoles are not a uniform entity but may arise from several distinct intracellular processes and organelle alterations.

One of the most commonly identified origins of vacuoles is the aggregation of smooth endoplasmic reticulum (sER). TEM analyses have shown that sER can cluster to form membrane-bound inclusions within the ooplasm, sometimes referred to as sER clusters (sERCs). These clusters are particularly significant because they can disrupt calcium signaling and organelle distribution, both of which are critical for oocyte maturation and subsequent fertilization. Large sER aggregates have been

observed in poor-quality oocytes and are linked to diminished fertilization potential and adverse developmental outcomes [1][11][12]. In addition to sER aggregations, vacuoles may also originate from lysosomes or autophagosomes. These organelles are involved in cellular degradation and recycling processes, and their abnormal accumulation or fusion can create vacuole-like structures within the cytoplasm. While less commonly described in human oocytes than sERCs, lysosomal and autophagic vacuoles have been documented as part of the spectrum of cytoplasmic dysmorphisms, particularly in oocytes exposed to stress or toxic insults [1][11].

The etiology and mechanisms of oocyte vacuolization

The etiologic and mechanisms of oocyte vacuolization are highly complex, reflecting a convergence of intrinsic cellular defects, cytoskeletal disorganization, genetic predispositions, and environmental or procedural factors encountered during ovarian stimulation and ART. Expanding on the current understanding, recent research and ultrastructural analyses have illuminated several key pathways and processes involved in vacuole formation and their implications for oocyte quality and developmental potential



The cytoskeleton, especially the actin network, plays a pivotal role in maintaining ooplasm integrity and the proper distribution of organelles during oocyte maturation. Recent ultrastructural and experimental studies have shown that disruption of the actin cytoskeleton-not microtubules- leads to excessive clustering of organelles and the formation of vacuole-like inclusions [1]. Inhibition of actin polymerization in oocytes, either pharmacologically or due to intrinsic defects, results in an ultrastructural pattern remarkably similar to that seen in dysmorphic eggs retrieved during IVF cycles. This suggests that actin is a key regulator of organelle distribution, and its malfunction is central to the genesis of vacuolization and other common cytoplasmic aberrations[1].

Vacuole formation often occurs during the transition from metaphase I (MI) to metaphase II (MII), particularly around the time of polar body extrusion. The process of nuclear and cytoplasmic maturation is highly coordinated, involving germinal

vesicle breakdown, chromosome segregation, asymmetric meiotic division, and the expulsion of the first polar body. Disruption of these processes-whether due to cytoskeletal instability, abnormal vesicle trafficking, or hormonal imbalance-can lead to the rapid appearance of vacuoles within the ooplasm[2][11]. For example, insufficient endogenous luteinizing hormone (LH) secretion at the time of ovulation trigger has been linked to defective luteal function and asynchronous maturation of the cytoplasm and nucleus, predisposing oocytes to vacuolization[2].

Cytoplasmic abnormalities that occur during oocyte maturation, especially those that interfere with the meiotic spindle and the intricate cytoskeletal structure, can adversely affect the oocyte's ability to undergo normal fertilization and embryo development. Disruption of the actin cytoskeleton, rather than microtubules, has been shown to induce excessive clustering of organelles and the formation of vacuole-like structures in experimental models. Pharmacological agents that destabilize the action network can induce vacuolization, highlighting the importance of cytoskeletal integrity in maintaining ooplasm organization[11]. Furthermore, the actin-microtubule interplay is essential for spindle assembly, chromosome segregation, and asymmetric division. Disruption of this crosstalk through genetic mutations or environmental insults can exacerbate cytoplasmic disorganization and vacuolization[1][11]. Biochemical and molecular changes further contribute to the susceptibility of oocytes to vacuolization. With aging or prolonged in vitro culture, oocytes experience a decline in intracellular glutathione (GSH), increased lipid peroxidation, elevated reactive oxygen species (ROS), and decreased ATP content-all markers of oxidative stress and metabolic compromise[14]. These changes impair the oocyte's ability to regulate intracellular calcium, which is crucial for fertilization and subsequent embryonic development[14][15]. Moreover, the expression of anti-apoptotic proteins such as BCL2 decreases in aged oocytes, increasing susceptibility to degeneration and cytoplasmic anomalies, including vacuoles[14].

Impact of Vacuolization on Fertilization Outcomes

Oocyte vacuolization exerts a significant and multifaceted impact on fertilization outcomes, affecting both the likelihood of successful fertilization and the quality of subsequent embryonic development. The degree of this impact is closely related to the size, number, and persistence of vacuoles within the oocyte cytoplasm, as well as the underlying mechanisms that lead to their formation.

Reduced Fertilization Rates and Embryonic Arrest

Clinical studies consistently report that vacuolated oocytes are associated with lower fertilization rates compared to morphologically normal oocytes. In a detailed analysis by Ebner et al., vacuoles were present in 3.9% of collected oocytes, with 66% containing a single vacuole, 21.3% double, and 12.7% multiple vacuoles[11]. Fertilization rates were significantly reduced in vacuolated oocytes: oocytes with single vacuoles up to 14 µm had a fertilization rate of 51.6%, while those with multiple vacuoles had a rate of 43.8%. Critically, fertilization was rarely observed in oocytes with vacuoles exceeding 14 µm in diameter[11]. This finding is echoed by other studies, which also note that the presence of large or multiple vacuoles is a predictor of poor fertilization potential and early embryonic arrest.

Mechanistic Insights: Spindle Disruption and Ooplasm Integrity

The mechanisms by which vacuolization impairs fertilization are rooted in its effects on the oocyte's cytoskeletal and organelle organization. Macro-vacuoles (>25 µm) can theoretically displace the metaphase II (MII) spindle, which is crucial for accurate chromosome segregation during fertilization. However, advanced imaging (e.g., polscope) has shown that not all large vacuoles physically displace the spindle; yet, their presence is logically expected to interfere with the fine architecture of the oocyte, including spindle positioning, sperm-oocyte signaling, sperm binding, and meiotic resumption[11]. Even when spindle displacement is not observed, vacuoles may still disrupt the cytoplasmic environment essential for fertilization and early development.

Fertilization Failure and Oocyte Activation Defects

Fertilization failure in vacuolated oocytes may also be linked to impaired oocyte activation. Normally, fertilization triggers a series of calcium oscillations that release the oocyte from metaphase II arrest and initiate embryogenesis. However, vacuolization may interfere with the proper transmission of activation signals or disrupt the machinery required for the M-G1 transition in MII oocytes[16]. In some cases, even after ICSI, oocytes with vacuoles fail to activate and fertilize, suggesting that cytoplasmic anomalies can override the benefits of micromanipulation. Assisted oocyte activation (AOA) using chemical agents has been explored as a potential remedy, but human oocytes often do not respond robustly to universal activators, and the efficacy of AOA in vacuolated oocytes remains uncertain[16][17].

Genetic and Biological Basis for Persistent Fertilization Failure

Persistent and homogeneous vacuolization across multiple cycles in a single patient suggests a possible genetic etiology, potentially involving defects in vesicle trafficking or cytoskeletal regulation[11][18]. Such intrinsic abnormalities can lead to recurrent fertilization failure and poor embryo quality, regardless of sperm quality or ART technique. This is supported by cases where macro-vacuolization is present in nearly all oocytes from a patient, regardless of stimulation protocol, indicating a stable biological cause[11].

Clinical Observations and Prognosis

While some vacuolated oocytes can be fertilized and may even cleave, the resulting embryos are typically of poor quality and have a low prognosis for successful implantation and pregnancy. In the rare cases where fertilization and cleavage occur, the embryos often arrest early, likely due to cytoplasmic defects that prevent proper embryonic genome activation or disrupt critical developmental pathways[11][2]. The overall prognosis for cycles dominated by vacuolated oocytes is poor, and such cases may require alternative strategies, including the consideration of oocyte donation or advanced micromanipulation techniques.

Cell Fate During Compaction and Self-Correction

A key observation is the fate of vacuole-containing blastomeres during compaction. If these abnormal cells are excluded from the compacting embryo, the resulting blastocyst has a low rate of chromosomal mosaicism, supporting the hypothesis that exclusion of abnormal blastomeres is a self-correction mechanism[2]. Conversely, if vacuole-positive blastomeres are incorporated into the compacting embryo, the resulting blastocyst exhibits a significantly higher mosaicism rate, which may impact further development and implantation potential[2]. This phenomenon is supported by studies observing that excluded cells during compaction have a high aneuploidy rate, suggesting that embryos can eliminate abnormal or aneuploid cells to preserve developmental potential[2].

Mechanistic Insights and Oocyte Quality

The developmental impairment associated with vacuolization is thought to arise from several mechanisms:

- ***Cytoskeletal Disruption:*** Large vacuoles can distort the oocyte's internal architecture, interfering with spindle positioning, chromosome segregation, and organelle distribution, all of which are critical for normal cleavage and compaction[1][11].
- ***Impaired Ooplasmic Signaling:*** Vacuoles may disrupt calcium signaling and metabolic pathways necessary for embryonic genome activation and blastocyst formation [3].
- ***Genetic and Biological Factors:*** Persistent vacuolization across cycles in some patients suggests a genetic etiology, affecting vesicle trafficking or cytoskeletal regulation, which may predispose to recurrent developmental arrest [1][11].

Oocytes recovered from hyperstimulated ovaries often display multiple morphological anomalies, including vacuoles, refractile bodies, and cytoplasmic granularity, which are suspected to compromise their fertilization and developmental potential[1]. The development of competence is a gradual process, shaped by follicular growth as well as the events of meiotic resumption and cytoplasmic maturation.

Clinical and Neonatal Outcomes

Despite the well-documented adverse effects of vacuolization on early embryonic development, such as reduced blastocyst formation and lower rates of high-quality blastocysts, recent large-scale studies have shown that, once the blastocyst stage is achieved and abnormal (vacuole-containing) cells are excluded during compaction, the prognosis for vacuole-positive embryos is comparable to that of vacuole-negative embryos [2][3]. Specifically, there is no significant difference in key clinical outcomes, including euploidy rates, implantation, ongoing pregnancy, and live birth rates, between embryos with and without vacuoles, provided the blastocyst is well-formed and abnormal cells have been eliminated during compaction [2][3].

Clinical Implications and Management Strategies

For oocyte and embryonic vacuolization, it is clear that this morphological anomaly presents nuanced challenges for both clinicians and embryologists in assisted reproductive technology (ART).

Clinical Implications

1. **Fertilization and Embryo Development**

Vacuolization is consistently linked to lower fertilization rates, impaired embryonic development, and increased risk of early embryonic arrest. Large or multiple vacuoles, particularly those exceeding 25 µm, can distort the oocyte's cytoskeletal structure, disrupting processes such as sperm-oocyte signaling, sperm binding, and meiotic resumption[11][21][22]. This can result in failed fertilization in conventional IVF and significantly reduced fertilization rates even with ICSI. Embryos derived from vacuolated oocytes frequently arrest at the 2-cell stage, rarely progressing to the blastocyst stage[11][21][2].

2. **Blastocyst Formation and Quality**

Multiple studies and meta-analyses confirm that vacuolization in oocytes or embryos is associated with reduced blastocyst formation rates and lower proportions of high-quality blastocysts[21][2]. The negative effect is more pronounced with larger

or more numerous vacuoles. Embryos with vacuolization observed on days 3 and 4 of culture have lower rates of both blastocyst formation and high-quality blastocyst development compared to vacuole-negative embryos[2].

3. Cryopreservation Outcomes

Vacuolization also negatively impacts the cryosurvival of oocytes and embryos. Oocytes with vacuoles or smooth endoplasmic reticulum (sER) disks are not recommended for cryopreservation, as their post-thaw survival and developmental competence are compromised[21][23]. The Istanbul Consensus highlights that giant oocytes and those with cytoplasmic vacuoles should be carefully evaluated before inclusion in vitrification protocols[21][23].

4. Genetic and Biological Considerations

Persistent, homogeneous vacuolization across cycles in a single patient may indicate a genetic predisposition, possibly originating during fetal oogenesis and affecting vesicle trafficking or cytoskeletal regulation[11][21]. This can lead to repeated poor outcomes and may necessitate alternative reproductive strategies, such as oocyte donation[21].

5. Clinical Decision-Making

Despite the negative associations, current consensus guidelines (e.g., Istanbul Consensus 2024) state that oocytes with vacuoles are not automatically excluded from clinical use, especially in ICSI cycles[21]. However, care should be taken to avoid injecting sperm directly into a vacuole, as this may further compromise developmental potential[21].

Management Strategies

1. Morphological Assessment and Selection

- ***Oocyte Screening:***

Careful morphological assessment is a cornerstone of managing vacuolated oocytes. Embryologists routinely screen retrieved oocytes under high magnification, identifying those with large or multiple vacuoles. Oocytes presenting with macro-vacuoles (typically

>14–25 μm) or more than three vacuoles are flagged due to their strong association with poor fertilization rates and frequent embryonic arrest [11][13]. In cases of repeated fertilization failure or recurrent early embryo arrest, the use of such oocytes may be reconsidered, and patients may be counselled about the potential impact on clinical outcomes [11][13][2].

- ***Embryo Selection:***

Similarly, embryos are evaluated for vacuolization during early cleavage and compaction stages. Embryos with significant vacuolization—especially those where vacuole- containing blastomeres are incorporated during compaction—are at higher risk for chromosomal mosaicism and developmental arrest [13][3][24]. Such embryos may be deprioritized for transfer, with preference given to embryos lacking significant vacuoles or those where abnormal cells have been excluded during compaction, as this self-correction can improve developmental potential [3][24].

Laboratory and Procedural Techniques

- ***ICSI Technique:***

During ICSI, it is critical to avoid injecting sperm directly into a vacuole, as this may further compromise oocyte viability and developmental competence. Targeting the injection away from vacuoles helps minimize additional cytoplasmic disruption and increases the likelihood of normal fertilization and embryo development [13].

- ***Experimental Micromanipulation:***

There is growing interest in micromanipulation techniques, such as puncturing and draining large vacuoles using a fine ICSI or biopsy needle. While these interventions are theoretically promising potentially alleviating cytoplasmic distortion caused by large vacuoles, they are not yet standard clinical practice and may require special regulatory approval. There is also concern that draining vacuoles could reduce the oocyte's cytoplasmic volume, potentially impairing viability or releasing toxic substances [11]. Thus, such approaches are experimental and should be considered with caution.

Culture Optimizing Conditions:

Maintaining optimal laboratory conditions is essential for minimizing the formation and impact of vacuoles. This includes strict control of pH, temperature, and osmolality throughout all stages of oocyte handling and embryo culture. Minimizing light exposure during manipulation and culture is also recommended, as excessive light can induce cellular stress. These measures help preserve oocyte and embryo integrity, reducing the risk of vacuole formation and other cytoplasmic anomalies [13][25]. Additionally, gentle handling during denudation, insemination, and culture transfers further reduces the likelihood of iatrogenic vacuolization.

Cryopreservation Policies

Cryopreservation policies in assisted reproductive technology (ART) have evolved to prioritize the selection of oocytes and embryos with optimal morphological integrity for vitrification, as this directly impacts post-thaw survival and developmental competence. The latest expert consensus and laboratory guidelines recommend excluding oocytes with giant vacuoles or smooth endoplasmic reticulum (sER) disks from vitrification protocols. These cytoplasmic abnormalities are markers of compromised oocyte quality and are associated with reduced fertilization rates, impaired embryo development, and poorer clinical outcomes after thawing [23][27][29].

Patient Counseling Counselling and Alternative Strategies

- ***Genetic Counselling***

For patients whose cycles are repeatedly dominated by vacuolated oocytes—especially those with large, homogeneous macro-vacuoles—genetic counselling is an important step. Persistent vacuolization is increasingly recognized as a marker of underlying biological or genetic abnormalities, potentially originating during fatal oogenesis and affecting cytoskeletal organization or vesicle trafficking within the oocyte. Genetic counselling provides an opportunity to discuss the possibility of heritable defects that may be contributing to the abnormal oocyte phenotype. During counselling, patients can receive information about the nature of their oocyte abnormalities, the likelihood of recurrence in future cycles, and the potential for these issues to be passed on to offspring. Additionally, a detailed family and reproductive history may be taken, and in select cases, referral for genetic testing or further evaluation by a reproductive geneticist may be considered, especially if there is a history of unexplained infertility or repeated ART failure [11].

- ***Oocyte Donation***

When repeated ART cycles yield predominantly vacuolated oocytes and result in poor fertilization rates, frequent embryonic arrest, or failed pregnancies, oocyte donation becomes a practical and evidence-based alternative [11][13]. Oocyte donation offers the best chance for pregnancy in these challenging cases, as it bypasses the intrinsic cytoplasmic and genetic defects present in the patient's own oocytes. The literature documents cases where, despite optimal stimulation protocols and laboratory techniques, patients with persistent macro-vacuolization experience universal fertilization failure or early embryonic arrest, making further attempts with autologous oocytes unlikely to succeed [11][13]. Counselling should address the emotional, ethical, and practical aspects of oocyte donation, including the high success rates, the process of selecting a donor, and the implications for family building. For many couples, this strategy provides a realistic path to parenthood when conventional ART options are exhausted.

Research and Future Directions

- ***Further Investigation:***

Ongoing research is needed to clarify the molecular mechanisms underlying vacuolization and to develop targeted interventions. The clinical utility of micromanipulation for vacuole removal and advanced culture systems (e.g., microfluidics) are promising areas for future study[11][21].

- ***Consensus and Standardization:***

Updated guidelines (such as the Istanbul Consensus 2024) continue to refine best practices for the assessment, selection, and management of vacuolated oocytes and embryos, promoting standardization across ART laboratories [21].

Genetic Counselling and Prognosis

Genetic counselling and prognosis for patients exhibiting oocyte or embryonic vacuolization require a nuanced, evidence-based approach, as these morphological anomalies may reflect underlying genetic, biological, and clinical factors that influence reproductive outcomes.

Genetic Counseling

1. Indications for Genetic Counseling

Persistent or homogeneous macro-vacuolization across multiple ART cycles in a single patient strongly suggests a possible genetic or biological etiology. Case reports have documented patients whose oocytes consistently display large vacuoles (>25 μm) in nearly all retrieved cohorts, regardless of stimulation protocol. This pattern is unlikely to be explained solely by external factors such as ovarian stimulation or laboratory technique. Instead, it points toward a stable, intrinsic defect-potentially originating during fetalfatal oogenesis-that affects cytoskeletal organization or vesicle trafficking within the oocyte[11].

2. Possible Genetic Mechanisms

The biological basis for such vacuolization may involve mutations or dysregulation in genes governing endocytosis, exocytosis, or cytoskeletal dynamics. These defects could lead to uncontrollable endocytosis or the fusion of vesicles that would normally be exocytosedexocytosis, resulting in persistent vacuole formation[11]. While direct genetic mutations have not been universally identified in all patients with vacuolization, the homogeneity and recurrence of the phenotype within individuals across cycles support a genetic underpinning.

Prognosis

1. Fertilization and Embryo Development

Persistent vacuolization, especially when large and homogeneous, is associated with very poor fertilization rates and high rates of embryonic arrest. In documented cases, IVF often results in failed fertilization, and even with ICSI, only a small proportion of oocytes fertilize, with most embryos arresting at the 2-cell stage[11]. The underlying cytoplasmic abnormality impairs key processes such as sperm-oocyte signaling, meiotic resumption, and embryonic cleavage.

2. Blastocyst Formation and Chromosomal Stability

Large cohort studies confirm that vacuolization in embryos on days 3 and 4 is associated with significantly reduced rates of blastocyst formation and high-quality blastocyst development [2]. Notably, when vacuole-positive blastomeres are incorporated into the embryo during compaction, there is a significantly higher rate of chromosomal mosaicism, which may further compromise developmental potential [2]. However, embryos that exclude vacuole-containing cells during compaction demonstrate a lower mosaicism rate, indicating a possible self-correction mechanism[2].

3. Euploidy, Implantation, and Neonatal Outcomes

Despite the negative impact on blastocyst formation and quality, large studies report no significant difference in euploidy rates, implantation, ongoing pregnancy, or live birth rates between vacuole- positive and vacuole-negative embryos, provided that a blastocyst forms and abnormal cells are excluded during compaction[2]. Neonatal outcomes-including birth weight, length, preterm birth, and birth defect rates-are also comparable, suggesting that the adverse effects of vacuolization are largely confined to preimplantation development and do not necessarily compromise long-term offspring health[2].

4. Clinical Recommendations and Patient Counseling

- ***Embryo Selection:*** When vacuole-positive embryos are the only option, clinicians may consider transferring blastocysts that have excluded vacuole-containing cells during compaction, as these have outcomes similar to vacuole-negative embryos[2].
- ***Realistic Expectations:*** Patients with persistent, severe vacuolization should be counselled regarding the high likelihood of poor fertilization and embryo development outcomes, and the potential need to consider alternative options such as oocyte donation[11].
- ***Role of Hormonal and Environmental Factors:*** While genetic factors are important, clinicians should also assess and optimize hormonal triggers (e.g., LH levels) and laboratory conditions, as these can modulate the incidence and severity of vacuolization[2].

Implications of DOR on IVF/ICSI Outcomes

Diminished ovarian reserve has significant implications for IVF and ICSI outcomes. Women with DOR generally yield fewer oocytes during controlled ovarian stimulation, which translates into a lower probability of obtaining good-quality embryos and ultimately reduced live birth rates. Suqin Zhu et al. (2024) demonstrated that even in women aged ≤35 years, those with DOR had significantly lower rates of blastocyst formation (59.8% vs. 64.1%), embryo implantation (29.8% vs. 33.3%), and live birth (40.6% vs. 45.7%) compared to women with normal ovarian reserve [7].

Moreover, while oocyte quantity is evidently reduced, studies suggest that embryo quality may also be affected due to impaired cytoplasmic and mitochondrial function, although not uniformly across all age groups. Younger women with DOR may still retain acceptable oocyte quality, underlining the importance of age-reserve interaction in prognosis [15]. In ART cycles, DOR is also associated with higher gonadotropin requirements, greater cycle cancellation rates, and fewer embryos available for cryopreservation. These challenges necessitate individualized stimulation strategies, such as dual stimulation, growth hormone adjuvants, or androgen priming, to optimize outcomes in this population.

Fertilization Rate in Women with DOR

Definition of Fertilization Rate

Fertilization rate (FR) refers to the proportion of oocytes that achieve successful fertilization—typically identified by the presence of two pronuclei (2PN)—out of the total number of mature oocytes retrieved and inseminated [16]. This

embryological parameter is critical for assessing oocyte competence and IVF/ICSI efficiency, as it directly influences the number of embryos available for transfer or cryopreservation.

Mechanisms Behind Impaired Fertilization in DOR

Women with diminished ovarian reserve (DOR) often present not only with a reduced quantity of oocytes but also with compromised oocyte quality [17]. Impairments in cytoplasmic maturation, mitochondrial activity, and epigenetic regulation can hinder proper sperm interaction and fertilization. In addition, age- related alterations such as zona pellucida hardening, cytoplasmic vacuolization, and reduced spindle integrity have been reported, all of which may negatively influence fertilization potential. Studies have observed that oocytes with thick or granular zona and dense, vacuolated ooplasm tend to demonstrate poor fertilization outcomes and lead to lower-quality embryos.

IVF vs. ICSI: Which is Better in DOR Cases?

The relative efficacy of conventional IVF versus intracytoplasmic sperm injection (ICSI) in women with DOR has been extensively studied. In the absence of male factor infertility, ICSI has not consistently shown superiority. Several studies have demonstrated that fertilization rates, embryo quality, implantation rates, and live birth outcomes are comparable between IVF and ICSI in poor responders [18]. For example, in women aged 35–42 years with ≤5 retrieved oocytes, fertilization rates were similar between IVF (73%) and ICSI (76%) groups, with no significant difference in pregnancy rates. Although ICSI may reduce the risk of total fertilization failure in selected cases, its routine use in non-male factor DOR cases is not universally supported by current evidence.

Multiple retrospective and prospective cohort studies have evaluated fertilization outcomes in women with DOR [19,20]. These studies reveal that the overall fertilization rate in DOR patients is generally lower than that in women with normal ovarian reserve. However, when adjusted for age and oocyte quality, the difference narrows. One study emphasized the predictive role of oocyte morphology during ICSI—reporting that oocytes with abnormal cytoplasm or thick zona were associated with significantly reduced fertilization and blastocyst development [21]. Another large registry analysis showed that ICSI provided only a marginal improvement (approximately 2%) in fertilization rate in non-male factor DOR cases but did not translate into better live birth rates [22].

Influence of Stimulation Protocols and Gonadotropin Dose

Controlled ovarian stimulation protocols can significantly influence both the number and quality of retrieved oocytes, thereby affecting fertilization outcomes. In DOR patients, standard high-dose gonadotropin regimens may not always be effective and could lead to suboptimal follicular recruitment or overmaturation. Some evidence suggests that individualized low-dose or mild stimulation protocols—especially when guided by baseline AMH and AFC values—may result in a more physiologically favourable follicular environment and improve fertilization potential. Adjuvant strategies such as androgen priming, growth hormone supplementation, and dual stimulation cycles have also shown promise in enhancing oocyte yield and quality in poor responders.

Gaps in Literature and Future Directions

1.1. Despite considerable advances in understanding the impact of diminished ovarian reserve (DOR) on ART outcomes, several significant gaps remain. One major issue is the need for standardized measures for embryo quality. Current grading systems vary widely between clinics, and discrepancies in both morphological and genetic assessment metrics hinder the comparability of findings across studies. There is also a clear call for large, well-controlled trials that can more definitively determine the optimal stimulation protocols, insemination techniques, and adjunctive treatments for DOR patients. The majority of existing studies are retrospective or involve limited sample sizes, which reduces the power and generalizability of their conclusions. Recent technological advancements, however, offer promising avenues for future research. The exploration of artificial intelligence (AI)-based embryo assessment holds the potential to refine and standardize embryo selection by integrating morphological data with time-lapse imaging and genetic insights. AI algorithms could decrease subjectivity in embryo grading and more accurately predict developmental competence, especially within the challenging context of DOR. Furthermore, innovative fields such as oocyte rejuvenation and the development of stem cell-derived gametes are emerging, with the potential to overcome the quantitative and qualitative limitations imposed by a diminished ovarian reserve. These future approaches could revolutionize ART by providing novel interventions that enhance both oocyte quality and quantity in women with DOR.

1.2. Laser assisted Hatching Techniques

Laser-Assisted Hatching is a cutting-edge assisted reproductive technology, especially for IVF. This advanced procedure uses lasers to carefully open the zona pellucida around the embryo. This treatment helps the embryo hatch, which is crucial to IVF implantation (6).

The complexity of Laser-Assisted Hatching requires careful consideration of several parameters. To maximize precision, practitioners modify laser settings like intensity and duration. Laser kind and application method are also crucial for a precise

and controlled zona pellucida opening (7).

- **Sequential Zona Thinning:** The zona pellucida is gradually thinning out with the use of a laser in this Laser-Assisted Hatching procedure. The procedure is carried out in a structured fashion, with the laser being administered at several locations around the embryo's outer membrane. The goal of this slow thinning is to make a regulated opening so that the embryo can hatch easier later on in its development. Thanks to the laser's pinpoint accuracy, doctors may adjust the zona pellucida thickness to suit each embryo's unique anatomy (8).

- **Laser Pulsing Technique:** This method creates micro-openings in the zona pellucida by pulsing the laser. Intermittent laser use reduces heat accumulation, enabling a more regulated and measured approach. Pulsing creates precise apertures without damaging embryonic structures. Maintaining embryo integrity and optimizing hatching circumstances is especially beneficial with this strategy (9).

- **Laser-Assisted Zona Drilling:** Lasers generate small holes or perforations in the zona pellucida during zona drilling. This method allows for a more direct and concentrated intervention, allowing the embryo to hatch. Laser-assisted zona drilling precision reduces embryonic structural damage and promotes efficient and regulated hatching. When a targeted opening is beneficial for specific embryonic circumstances, Laser-Assisted Hatching treatments are more specialized (10).

1.3. Impact on Vitro Fertilization Success rates

Laser-Assisted Hatching approaches' and its effects on IVF success rates have been extensively investigated in assisted reproductive technologies. These methods try to improve embryo implantation, a critical factor in IVF success. Impact can be assessed in numerous ways:

- **Improved Embryo Implantation Rates:** Laser-Assisted Hatching helps embryos hatch and embed into the uterine lining. Laser-assisted hatching may increase implantation rates in embryos (11). These strategies aim to optimize embryo-uterus interaction by controlling zona pellucida opening, potentially improving implantation success.

- **Enhanced Pregnancy Success Rate:** A viable pregnancy is IVF's ultimate goal. Laser-assisted hatching can improve pregnancy rates when used properly. These methods may improve

implantation rates and pregnancy advancement, resulting to more clinical pregnancies in IVF couples (12).

- **Impact on Live Birth Rates:** Live birth rates are critical for assessing IVF success (13). Laser- assisted hatching may increase live birth rates by improving embryo implantation. This is important for couples considering fertility treatments because the objective is a healthy kid (14).

- **Consideration of Patient-Specific Factors:** Laser-assisted hatching may affect IVF success rates depending on patient variables. These methods are affected by maternal age, embryo quality, and infertility factors (15). To maximize benefits, laser-assisted hatching must be tailored to patient profiles.

- **Risk of Multiple Pregnancies:** Laser-assisted hatching improves success rates but has hazards. Increased implantation rates may increase the probability of multiple pregnancies, which can complicate matters for patients and doctor (16). To optimize IVF success, these risks must be carefully managed.

Conclusion:

After much research and validation of published studies on oocyte vitrification, the option of efficiently cryopreserving human oocytes has introduced great benefits to assisted reproductive technology (ART). This method is in order to help maintain women' s fertility, to postpone childbirth and to eliminate cryopreservation of embryos, which may have moral and ethical problems. It also facilitates oocyte donation programs, ensuring adequate donor recipient pairing. Although practically applied in human ART, oocyte vitrification still needs to be improved for its reliability, efficacy and safety. In order to maximize oocyte cryopreservation, the effect that vitrification has on oocyte quality must be addressed. Oocyte and embryonic vacuolization are significant indicators of reduced developmental competence in assisted reproductive technology. Their presence is linked to lower fertilization rates, impaired embryo development, and decreased blastocyst quality. However, when embryos can self-correct by excluding vacuole-containing cells, successful implantation and healthy neonatal outcomes are still possible. Careful morphological assessment, patient counselling, and individualized management strategies are essential for optimizing outcomes in cases with vacuolization. The development of ovulation induction introduced an essential component supporting female fertility treatment across multiple types of infertility. OI protocols continue to advance because of increased reproductive physiological knowledge alongside new pharmacological treatments and better monitoring tools. Medical advances in assisted reproductive technologies (ART) including in vitro fertilization (IVF) pulled together with these developments allowed the creation of patient-focused therapeutic methods. The treatment of OI has advanced substantially through clomiphene citrate evolution into letrozole therapy with both gonadotropins and

insulin treatment options enabling individualized care for women with polycystic ovary syndrome (PCOS). Clinical practitioners can now deliver optimized treatment results through combined AFC and anti-Müllerian hormone (AMH) biomarker assessment and enhanced ultrasound and hormonal assays.AI technology exhibits emerging capabilities that enhance OI protocol personalization by improving predictions about how patients will respond to multiple treatment options. Data-driven healthcare coupled with precision methods in Osteogenesis imperfecta shows potential to achieve improved clinical results through decreased costs with reduced side effects from medication. The probability of a successful clinical pregnancy is fairly impacted by age, with more seasoned ladies having a diminished possibility accomplishing clinical pregnancy. Upon multivariate examination, it was found that laser assisted hatching was multiple times bound to be connected with a preferred clinical pregnancy over non-hatching, yet laser hatching had a hardly tremendous impact in foreseeing a preferred clinical pregnancy over non-hatching and natural random hatching, and that this impact was unaffected by age.

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