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Molecular and ultrastructure studies on cumulus ovarian cells in cases of unexplained infertility undergoing intracytoplasmic sperm injection (ICSI) treatment

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ABSTRACT

Unexplained infertility cases pose a puzzling concern of *in vitro* fertilization (IVF) protocols. The related mechanisms and signalling pathways of the microenvironment surrounding the cumulus-oocyte complex remain unclear. Herein, the current study is conducted to elucidate the causes of these cases. Patients undergoing unexplained infertility during intracytoplasmic sperm injection (ICSI) and donor females with proven fertility were included in this study. Cumulus cells (CCs) were aspirated and isolated, and the corresponding follicular fluid (FF) was collected. A significant increase was found in malondialdehyde (MDA), caspase-3, and tumor necrosis factor-α (TNF-α) concentrations in CCs of all infertile patients compared to the controls, but no significant changes were noticed in calcium ion concentrations. A marked DNA fragmentation pattern was observed, along with a significant increase in the calculated DNA length, in the patients' cumulus cells. Additionally, a significant decrease in reduced glutathione (GSH) levels was detected in the follicular fluid (FF) of all infertile patients compared to the controls. A significant increase in the concnetration of phosphatase and tensin homolog (PTEN). Zinc ion concentration was significantly increased in patients. Ultrastructurally, distinct alterations in the nuclei, loss of microvilli, hypertrophied Golgi bodies, abnormal mitochondria, large autophagic vacuoles, and increased smooth endoplasmic reticulum were evident in CCs of patients. Under molecular and biochemical levels, the intricacy of componentry of microenvironment, like caspase-3, TNF-α, PTEN and DNA damage, MDA, GSH, may likely play a crucial role in creating defective conditions for unexplained infertility and may be linked to oocytes' failure to complete maturation and subsequent competency. These were also evident by ultrastructural alterations of CCs. Understanding this complexity could be key to unlocking solutions for those facing challenges in conception. we suggest that PTEN in the FF represents a valuable diagnostic biomarker for unexplained infertility. Further investigation under a large-scale cohort is required to prove the validity of these findings and to explore possible implementation in unexpected infertility.

Keywords: Unexplained infertility; *In vitro* fertilization; Follicular fluid; Cumulus cells; PTEN; Electron microscope; Tumur necrosis factor

1. Introduction

Infertility is the inability to achieve pregnancy within twelve consecutive menstrual cycles of regular and unprotected sexual intercourse. Males and/or females may be affected by infertility as a couple, but unexplained factors may even be implicated (Practice Committee of

the American Society for Reproductive Medicine, 2020). Despite extensive fertility screening, testing and standard medical examination being normal, the patient experiences unexplained infertility without discernible or rational reason or reproductive system abnormalities (Penzias et al., 2020). According to the evidence-based treatment about 30% of infertile couples are diagnosed with unexplained infertility, showing regular ovarian physiology, and normal fallopian tubes, uterus, cervix and pelvis (Guideline Group on Unexplained Infertility., 2023). The ovarian follicles are composed of oocytes, granulosa cells (GCs) and theca cells. According to their structure and function, granulosa cells are composed of mural granulosa cells (MGCS) and cumulus cells (CCs). MGCS line the follicular wall, supplying steroidogenesis, cholesterol, lactate, and pyruvate biosynthesis essential to the oocyte development via gap junctions (Turathum et al., 2021). The tightly junctional CCs with the extracellular matrix create the cumulus oophorus complexes surrounding the oocyte, leading differentiation and expansion of CCs. In addition, this intricate complex regulates gene expression and protein production that is crucial for oocyte growth, development, maturation, and quality (Martinez et al., 2023).

Therefore, the ovary performs hormone production and the second for controlling the follicular growth, recruitment, and release of a mature oocyte. The development and enhancement of the GCs to secrete steroid hormones is consistent with changes in pituitary secretion (Holesh et al., 2021). During nuclear maturation, mature growing oocytes resume and progress to complete meiosis II (MII) and become competent for fertilization. (Aizawa et al., 2025). Multiple studies reported that CCs can contribute to successful oocyte maturation, fertilization, and early embryogenesis through protecting the oocyte against apoptosis induced by oxidative stress in *in vitro* fertilization (IVF) (Yaka et al., 2022; Govahi et al., 2024). In conventional assisted reproductive techniques (ART), oxidative stress is implicated among the negative factors affecting unexplained female infertility, such as reactive oxygen species (ROS) levels in follicular fluid (FF), ovaries and 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress in GCs (Mauchart et al., 2023) can affect oocyte competence, fertilization, implantation, and embryonic development (Chen et al., 2023). Additionally, oocyte quality is influenced by oxidative stress, which causes DNA damage and impairs oocyte fertilization (Yan et al., 2022). Due to its active biological role, FF influences many regulators of folliculogenesis relevant to the competence of oocyte development and follicle maturation (Nejabati et al., 2023). Fluctuations in the FF milieu may exert an impact on egg competence, prenatal, and postnatal development (Da Broi et al., 2018).

Under pathological conditions such as endometriosis and polycystic ovary syndrome (PCOS), the altered follicular microenvironment may adversely affect oocyte quality, fertility, and preimplantation embryo development, producing poor outcomes in IVF (Moreira et al., 2023; Pan et al., 2024).

Developmental oocyte competence depends on the transported ions and macromolecules through the signaling junction between CCs and oocytes (Russell et al., 2016). As a key messenger in various signaling pathways, calcium (Ca^{2+}) is vital for the quality and functionality of oocytes. It regulates resumption of meiosis, microtubule assembly, and prevents exit from MII through intramembranous Ca^{2+} channels and transporters (Meng et al., 2021). Increased Ca^{2+} levels in CCs increase cAMP concentrations and trigger the MAPK pathway, leading to the expression of EGF-like proteins for oocyte development (Pei et al., 2023). Zinc is another ion that enhances oocyte quality and embryo development by boosting antioxidant activities (Vickram et al., 2021; Yao et al., 2023). A significant relationship exists between the level of FF zinc, the number and quality of oocytes retrieved from the ovaries of infertile PCOS patients in IVF therapy, and the number of high-quality embryos (Janati et al., 2021). Zinc is also crucial for completing meiosis during puberty, which helps to prevent meiosis arrest before puberty (Kumar et al., 2018). Zinc influx during meiosis I (MI) is also required to transition from MI to MII, independently. Additionally, it reduces endoplasmic reticulum

stress in oocytes, promoting normal oocyte development and embryogenesis (Jeon et al., 2014). Blocking zinc influx results in meiotic failure at telophase (Mendoza et al., 2022). Ovarian function and fertility depend on cumulus cell apoptosis. CCs DNA fragmentation can affect the meiotic ability of oocytes (Pereira et al., 2019). In mammalian oocytes, cell cycle delays and arrest, as well as impaired oocyte quality, are linked to DNA fragmentation and ultimately lead to infertility and miscarriage (Park et al., 2021).

The relationship between cumulus cell apoptosis and fertility and oocyte quality has been investigated, focusing on the proximity of CCs to the oocyte. High apoptotic rates of GCs may cause follicular development issues and directly affect egg quality, consequently limiting the capacity of oocyte development (Kawano et al., 2025). Furthermore, low levels of DNA may cause oocytes without pronuclei and fail to complete maturation or fertilization (Rodrigues et al., 2024). Tumor necrosis factor-α (TNF-α) regulates follicular development to the preovulatory stage in human oocytes, theca and GCs, corpus luteum, FF, and during the menstrual cycle (Salmassi et al., 2019; Silva et al., 2022). Liang et al. (2022) found that TNF-α levels increased with follicle expansion and were higher in the FF of PCOS patients compared to healthy women.

There is a relationship between the number of oocytes, CCs, and FF content, and apoptosis. The increase in malondialdehyde (MDA) and reduced glutathione (GSH) may increase the activity of the caspase-3 pathway, leading to oxidative stress that may affect the number of oocytes and quality (Wang et al., 2023). On the other hand, the encoding phosphatase and tensin homolog (*PTEN*) is one of the most important genes in cell growth, regulating the cell cycle and specifically inhibiting oocyte activation. Also, it functions as a mediator of apoptosis and is important for both follicular expansion and oocyte maturation because it regulates apoptosis (Yao et al., 2022). Therefore, good-quality oocytes and successful IVF treatment may be promoted by the inhibition of *PTEN* expression. PTEN levels are connected to lower pregnancy rates, and any changes in its expression and activity have been linked to changes in the female reproductive system (Maidarti et al., 2020). PTEN has also been shown to inhibit the PI3K/AKT pathway, a key regulator of cell survival and proliferation (Chesnokov et al., 2024). Niu et al. (2023) found that increased *PTEN* expression encouraged GCs apoptosis. To the best of our knowledge, it is the first study to investigate the PTEN in human FF from an unexplained infertile patient. Consequently, we hypothesize that cumulus cell apoptosis and elevated PTEN in the FF may contribute to the potential mechanisms underlying unexplained infertility. To better understand the causes of unexplained infertility in women undergoing IVF treatment, we will employ advanced molecular analyses of FF and CCs, exploring the apoptotic implications of the findings. Additionally, we aim to enhance our understanding of the mechanisms underlying unexplained infertility both *in vivo* and during IVF procedures. We will also focus on identifying the ultrastructure of CCs associated with unexplained infertility.

Materials and Methods

Ethical approval for IVF study

The study protocol was approved by the institutional Ethical Committee of the University of Medicine, Alexandria University, Alexandria, Egypt, IRB No: 00012098/2024 - FWA No: 00018699/2024 and complied with the good practice guidelines. All female participants were informed about the study and gave a written informed consent, which was signed and returned to the center.

Baseline patient characteristics and selection

A total of 100 oocytes from 20 infertile human female patients and 100 healthy oocytes from 20 human donors. The infertile female patients who underwent diagnostic evaluation with unexplained infertility and who had regular menstrual cycles visited the REPRO FERTILITY CENTER, Alexandria, Egypt, for IVF treatment. Female patients aged 25 - 33 years (median 29 years) had a history of at least one year duration with regular unprotected intercourse and without a child. They consented to confess to the case study throughout April and September 2023. Informed consent was obtained from all participants. All women who

took part in this study met the following criteria:

Inclusion criteria:

(i) Good ovarian reserve, defined based on follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH) values, (ii) physiologic range for estradiol and luteinizing hormone (LH) hormones, (iii) normal endocrine function, (iv) body mass index (BMI) < 30 kg/m^2, (v) age < 35 years and they had a history of at least 1-year duration with regular unprotected intercourse without a child. a complete medical history without past or current exposure to radiation, hazardous chemicals, or drug abuse, and any reproductive history complications, and these investigations were conducted by gynaecological investigators.

Exclusion criteria:

(i) Absence of uterine disease or congenital anomalies (i.e., fibroid, uterine polyp, uterine septum, etc.), and PCOS (ii) smokers (iii) complete medical history without past or current exposure to radiation, hazardous chemicals, or drug abuse, and any reproductive history complications, and these investigations were conducted by gynecological investigators. On the other hand, 100 oocytes from 20 healthy and proven fertile women aged 25-33 years had a history of spontaneous pregnancy and underwent intracytoplasmic sperm injection (ICSI) for gender selection. No history of infertility or reproductive disorders affected their fertility during the elapsed 12 months. Also, they have a complete medical history of the absence of current or past exposure to radiation or hazardous chemical substances, or drug abuse. They had normal physical and gynecological examinations. They were selected, enrolled, and consented as normal control responders.

Sample size calculation

The required sample size was calculated using G*Power version 3.1.9.2 and PASS 2020 software (NCSS, LLC, Kaysville, Utah, USA). Based on a recent study published by The Guideline Group on Unexplained Infertility (2023), approximately 30% of infertile couples are diagnosed with unexplained infertility. Assuming an effect size of 30% (ranging from 15% to 50%), a two-tailed α of 0.05, and a statistical power of 80%, the minimum total sample size was estimated to be 40 participants (20 per group) for detecting significant differences using the Chi-square test. Due to the rarity of eligible cases within the study period, 40 patients were ultimately enrolled, achieving an estimated post hoc power of 85% based on the collected data.

Ovarian triggering protocol

For ovarian stimulation, patient and control women underwent the same protocol, applying the guidelines on ovarian stimulation for IVF and the prevention of ovarian hyperstimulation syndrome. Triggering was achieved with a single injection of human menopausal gonadotropin (Menogon; Ferring, Kiel, Germany) or recombinant FSH (Serono or Organon, Munich, Germany) following the gonadotropin-releasing hormone (GnRH) antagonist protocol (Fig. 1). To initiate ovarian stimulation, a dose of 225-300 IU of recombinant FSH was given starting from day 2 of the menstrual cycle. Following a multiple-dose protocol, once follicles reached 14 mm in diameter and/or estradiol levels reached 400 pg/mL, a dose of 0.25 mg of GnRH antagonist was administered. Using GnRH antagonist protocols is to enhance clinical pregnancy rates. When two or more follicles reached a diameter of ≥17 mm, oocyte retrieval was conducted under sedation 36 hours after the administration of recombinant human chorionic gonadotropin (HCG) (Ovitrelle®,Merck, Madrid, Spain).

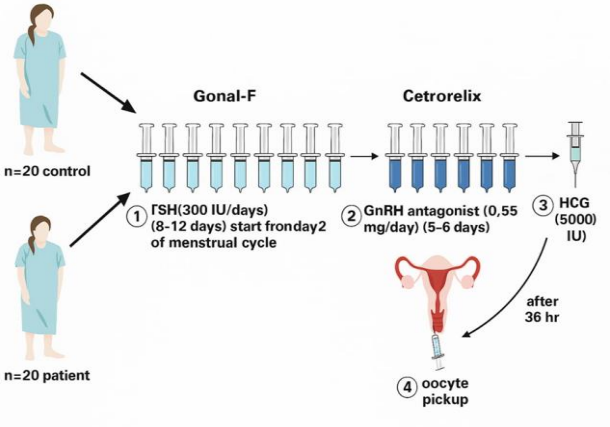

Fig.1. Schematic representation of controlled ovarian stimulation protocol used for both control (n = 20) and patient (n = 20) groups.
1. Follicle-Stimulating Hormone (FSH) at a dose of 300 IU/day was administered starting from Day 2 of the menstrual cycle for a duration of 8–12 days using Gonal-F. 2. Starting midway through the stimulation, GnRH

antagonist (Cetrorelix) was administered at 0.55 mg/day for 5–6 days to prevent premature LH surge. 3. When follicles reached appropriate maturity, Human Chorionic Gonadotropin (HCG) was administered at 5000 IU to trigger ovulation. 4. Oocyte pickup was performed 36 hours after HCG injection.

Collection of FF and isolation of CCs

Isolation of CCs

By Transvaginal ultrasound, several follicles (diameter 7–15 mm) in the same cycle were targeted for aspiration. Using an 18-G needle. After Ovum pick up (OPU) by transvaginal ultrasound–guided needle aspiration. Cumulus oocyte complexes (COCs) were collected. They were washed in several dishes containing Global® Total w/w/HEPES (LifeGlobal, Cooper Surgical, USA) to remove residual mural GCs, blood cells, and debris. Complete removal of CCs was achieved through enzymatic and mechanical denudation, as described by Caponnetto et al. (2022). Briefly, oocytes from each patient and control group were treated with GM501 Hyaluronidase (80 IU/mL; Gynemed GmbH and Co. KG, Germany) and gently denuded using a stripper pipette (EZ-Tip, RI-CooperSurgical, USA). under a stereomicroscope, CCs surrounding the oocyte are loose, have clear cytoplasm, and are cloud-shaped (Fig. 2) (Raad and Bazzi, 2020). The morphological examination of oocytes was performed under the RI Integra 3™ Micromanipulator on a Nikon microscope Ti2-U connected through RI ViewerTM Software.

Collection of FF

From each antral follicle (5 - 6 follicles/patient and controls), aspirated samples of FF were collected from the antrum, following the methodology of Caponnetto et al. (2021) (Fig. 2). The FF were then filtered using a 40 µm cell strainer (Fisherbrand, Fisher Scientific, Madrid, Spain).

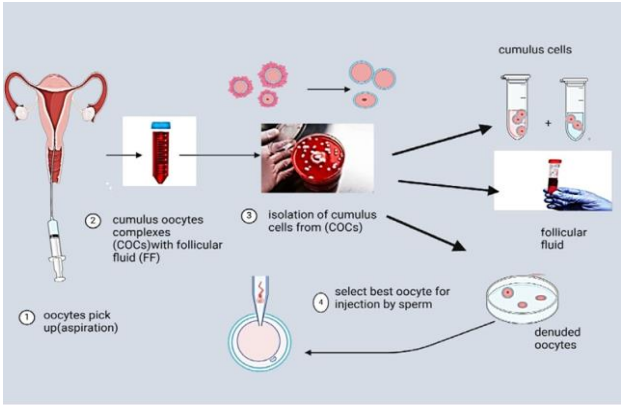

Fig. 2. Cumulus cells isolation and follicular fluid collection. 1. Oocyte pickup (aspiration) is performed from ovarian follicles ,2. Cumulus-oocyte complexes (COCs) are collected with follicular fluid (FF),3. Cumulus cells are isolated from COCs for analysis,4. Denuded oocytes are selected for intracytoplasmic sperm injection (ICSI).

CCs, storage and experimental protocol

The retrieved CCs were centrifuged at 300 ×*g* for 5 minutes, and the supernatant was discarded. The resulting CC pellets were immediately frozen at −80°C until further use. For subsequent analyses, the samples were divided into two aliquots. The first aliquot (500 µL) was placed in Eppendorf tubes (Denville Scientific Inc., Holliston, MA, USA) containing 4F1G in phosphate-buffered saline (PBS; pH 7.2) and stored at 4°C for 3 hours before electron microscopy. The second aliquot (500 µL) was homogenized in 0.5 mL PBS using a TT-13K Mini Handheld Homogenizer, followed by centrifugation at 6000 rpm for 15 minutes at 4°C. The resulting pellets of homogenized CCs were further divided into two other aliquots: one stored at −20°C for up to two weeks for determination of MDA, caspase-3, and TNF-α, and calcium ion; and the other aliquot for DNA fragmentation analysis.

FF purification and storage

Only FF samples that showed no visible blood contamination were used for further individual analysis. To remove residual follicular cells and blood traces, the FF samples were centrifuged at 1000 ×*g* for 20 minutes at 4°C. The supernatant from each FF sample (patient and control) was aliquoted and preserved at –20°C for a maximum of two weeks before analysis.

Estimated parameters of CCs and FF

Biomarkers of oxidants and antioxidants

The *GSH level* (Ellman, 1959) in the FF and MDA levels (Tappel and Zalkin, 1959) in the CCs were *determined.* The Biuret method, as described by Gornall et al. (1949), was used to determine the total protein concentration. *Briefly, a* reagent containing 6 mM copper sulfate, 6 mM potassium iodide, 750 mM sodium hydroxide, and 21 mM sodium-potassium tartrate, with 6 g/dL bovine serum albumin (Sigma) was used as a standard. *Exactly,* 200 µL of the Biuret reagent and 10 µL of each supernatant sample (FF and cumulus cell samples), standard, and distilled water (blank) were mixed and incubated at 25°C for 5 minutes. The absorbance of the sample and the standard was measured at 540 nm against the blank, and total protein concentration was expressed in g/dL.

Apoptotic markers

The ELISA method (Abcam Company, Abcam, Cambridge, UK) was used to estimate the concentration of: 1. The human PTEN, by Simple Step ELISA® Kit (ab206979), in the FF. The detection range (not specified in the provided source) and sensitivity of the ELISA kit for PTEN is calculated, the minimal detectable dose (MDD) is 39.9 pg/mL.

2. The human cleaved caspase-3 (Asp175), by Simple Step ELISA® Kit (ab220655), in the CCs. The detection range (0.313 – 20 ng/mL) and sensitivity of the ELISA kit for caspase-3 are calculated, the MDD is < 0.188 ng/mL.

3. The human TNF-α, by Simple Step ELISA® Kit (ab181421), in the CCs. The detection range (15.63 – 1000 pg/mL) and sensitivity of the ELISA kit for TNF-α are calculated, the MDD is 4.32 pg/mL. A volume of supernatant containing 100 µg of protein was added from each sample or standard to individual wells of an ELISA plate and brought to a final volume of 100 µL with carbonate buffer. The plate was covered with adhesive plastic and incubated for 2 hours at room temperature, followed by overnight incubation at 4°C. After this incubation period, 200 µL of 5% skimmed milk was added per well, the plate was covered with adhesive plastic and incubated for 1 hour at 37°C. Subsequently, the plate was washed three times with 200 µL of PBS.

Next, 100 µL of diluted capture antibodies (prepared in coating buffer) were added to each well. The plate was covered with adhesive plastic, incubated for 2 hours at room temperature, and washed three times with PBS. After that, 100 µL of diluted detector antibody (prepared in coating buffer) was added to each well. Finally, 100 µL of tetramethylbenzidine (TMB) substrate solution was added per well and incubated for 30 minutes at 37°C in the dark. Then, 50 µL of stop solution (5% sulfuric acid) was added. The concentration of PTEN, caspase-3 and TNF-α was calculated by reading the optical density absorbance at 450 nm using a microplate reader and referring to the standard curve shown in Fig. (3a,b,c), respectively. Caspase-3 and PTEN results were expressed in ng/mL, while TNF-α results were expressed in pg/mL.

Estimation of zinc and calcium ions

Samples from each supernatant of the FFs as well as of the CCs of each patient and the controls were digested with the concentrated HNO_3 (1.0 N). Exactly, 1.0 mL of concentrated HNO_3 was mixed with 1.0 mL of each supernatant. The mixture was gently shaken to suspend particulate matter and allowed to react for 100 minutes in a microwave, leaving the mixture to cool at room temperature, then centrifuged at 4525×*g* for 10 minutes. Exactly, 1.0 mL of the supernatant from each test tube was brought to a final volume of 15 mL with deionized water in a Falcon tube and stored at 4°C until analysis for zinc and calcium ion concentrations. Zinc levels were determined using a colorimetric assay based on the chelation of zinc present in the sample by zincon (2-carboxy-2'-hydroxy-5-sulphoformazyl-benzene) in the reagent at alkaline pH. The formation of this complex was measured at a wavelength of 610 nm (Hayakawa, 1961). Calcium levels were determined using a colorimetric assay based on the reaction of calcium present in the CCs with a chromogen reagent composed of methyl thymol blue (0.2 mmol/L), hydroxy 8-quinoline (140 mmol/L), and hydrochloric acid, along with ethanolamine (6 mol/L) at an alkaline pH, a blue color the intensity of which is in proportion to the calcium concentration. The formation of this complex was measured at a

wavelength of 585 nm (Gindler and King, 1972).

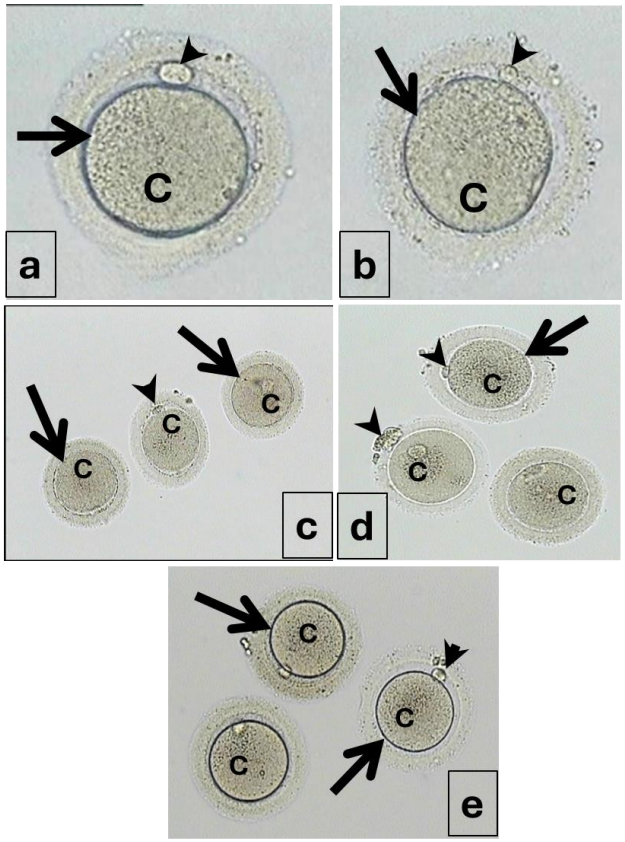

Fig. 3. Various types of oocyte morphology of controls and unexplained infertile patients. a and b: Control oocytes showing clear cytoplasm (C), an intact single polar body (arrowhead), and uniform zona pellucida (arrow). c: No polar body. d: dark granular cytoplasm (C), fragmented or absence of polar body (arrowhead). e: very thick zona pellucida (arrow), central dark cytoplasm (C), absence of polar body (arrowhead).

Molecular analysis of DNA fragmentation in the CCs

The integrity of cumulus cell DNA was assessed using a DNA fragmentation technique. This technique was employed as a molecular marker to evaluate genotoxicity between control and patient samples. The total DNA extraction from isolated CCs was carried out using the Nucleospin Tissue Kit® (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations, as previously described (Desquiret-Dumas et al., 2017).

Exactly, 15 µg of DNA per lane was loaded onto an agarose gel (1.5%, weight/volume), stained with ethidium bromide (10 µg/mL), and separated by electrophoresis (75 V, 150 mA). DNA bands were visualized under ultraviolet light (Consort, Turnhout, Belgium). The Gel Doc-It gel Documentation System (UVP, Cambridge, UK) was used for data analysis with Total Lab Analysis software (version 1.0.1, www.totallab.com).

Ultrastructural examination

Isolated aspirated CCs obtained from all participants (infertile patients and fertile controls) were immediately fixed in 4% formalin and 1% glutaraldehyde (4F1G, fixative mixture, 15- 30 minutes) and rinsed in phosphate buffer (0.1 M, pH 7.4) at 4°C for 24 hours. Washing in cacodylate buffer for about 2 minutes. Post-fixation was performed using 1% buffered osmium tetroxide (OsO_4) at 4°C for 2 hours. Thereafter, the samples were washed in phosphate buffer for 30 minutes and dehydrated through ascending ethanol concentrations at 4°C for about 14-16 minutes. The specimens were then treated with propylene oxide and embedded in an Epon Araldite resin mixture (1:1) (Bozzola and Russell, 1998). Ultrathin sections (60 nm thick) were cut with a glass knife on an LKB ultra-microtome, mounted on 200 mesh naked copper grids, and double-stained with uranyl acetate and lead citrate for 30 minutes. Finally, the samples were examined and photographed using a JEM-1400 Plus Transmission Electron Microscope (TEM) (Faculty of Science, Alexandria University, Alexandria, Egypt).

Statistical analysis

Data were analyzed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p<0.05$. Results are presented as mean ± standard deviation (SD). The Shapiro–Wilk test assessed normality ($p>0.05$ indicating normal distribution). Levene's test assessed variance homogeneity ($p>0.05$, indicating equal variances). An unpaired t-test was used to compare group means.

3. Results

In the current study, all clinical investigations conducted on couples undergoing IVF treatment were normal, and male variables were excluded. According to WHO guidelines, unexplained female infertility was explored to rule out ovarian causes by examining the hormonal

profile (LH, FSH, AMH, and prolactin hormones) and performing a uterine ultrasound examination. LH levels ranged from 3 to 13 IU/L, FSH levels from 3.5 to 21.5 IU/L, AMH values from 0.7 to 2.27 ng/mL, and prolactin levels from 4 to 23 ng/mL. All these parameters were evaluated for the participating group and were within normal limits.

Clinical data of patients and oocyte retrieval

Table. 1 and Fig. 3 depict the recorded clinical baseline patient characteristics and morphology of aspirated oocytes from unexpected infertile females and normal fertile participants. The mean number of aspirated oocytes in infertile females showed no significant difference compared to the control fertile women. In all infertile patients, insignificant morphological abnormalities were recorded in the oocytes compared to the control females. The control oocytes showed a thick zona pellucida, fragmented polar body, central darkness, and granular cytoplasm. In other oocytes, a polar body is absent or is fragmented.

CCs and FF-linked findings in unexpected infertility

Comprehensively, the CCs displayed a significant increase in the means of MDA concentrations in all infertile patients compared to the control fertile ones (Fig. 4). MDA increased in patients. The results revealed significant increases in the level of human caspase-3 in the CCs of all female patients compared to the control fertile donors (Fig. 4). By comparison with the control donors, TNF-α in human CCs was significantly increased, as shown in Figure "4". No significant differences in the level of calcium ions in the CCs of all infertile patients compared to the controls (Fig. 4). It is essential to evaluate DNA integrity in the CCs of women with unexplained infertility, as it is associated with oocyte competence. The results revealed significant DNA fragmentation in the CCs of all infertile patients compared to those of the control fertile donors (Fig. 5a,b). Figure 5c illustrates the various fragmentation patterns observed. This analysis indicates that assessing DNA integrity serves as an effective marker for identifying and addressing cases of unexplained infertility. The results revealed that the level of GSH in the FFs of all patients showed statistically significant decreases compared to the control value (Fig. 6). Only the concentrations of zinc increased significantly in the aspirated FF, compared to the control (Fig. 6). PTEN is a tumor suppressor gene that functions as a mediator of apoptosis. Statistical analysis revealed that the level of PTEN in the FF of all unexplained infertile patients increased significantly compared to the control value (Fig. 6). tumor suppressor gene that functions as a mediator of apoptosis. The statistical analysis revealed that the level of PTEN in the FF of all unexplained infertile patients increased significantly compared to the control value (Fig. 6).

Table. 1. Baseline of clinical records of unexpected infertile patients and normal donor women.

	Donor women	**Infertile patient**
Total number of females	20	20
Mean age of human females (years)	29.02±2.54	28.69±3.65 #
Total number of oocytes	100	100
Number of cycles of oocyte retrieval	1	1
Body mass index(kg/m^2)	24.94± 2.15	26.24± 1.95 #
Period of infertility for patients/year	0	2.4 ± 0.02 #
Morphology of oocytes	1. Uniform zona pellucida. 2. Homogeneous and translucent cytoplasm. 3. Single and intact-sized polar body.	1. Thick zone pellucida. 2. Fragmented polar body. 3. Central darkness and granular cytoplasm
Rate of fertilization after ISCI (%)	85-100	55-75
LH (IU/L)	7.14 ± 2.0	6.83 ± 2.41#

FSH (IU/L)	9.54 ± 3.28	10.06 ± 4.22#
AMH (ng/mL)	1.63 ± 0.37	1.52 ± 0.54#
Prolactin (ng/mL)	12.52 ± 2.83	14.34 ± 2.32#

Data are presented as mean ± standard deviation (SD). The sample size for the patient and control groups is n=40. #: Indicates statistically insignificant differences compared with control (P<0.0001).

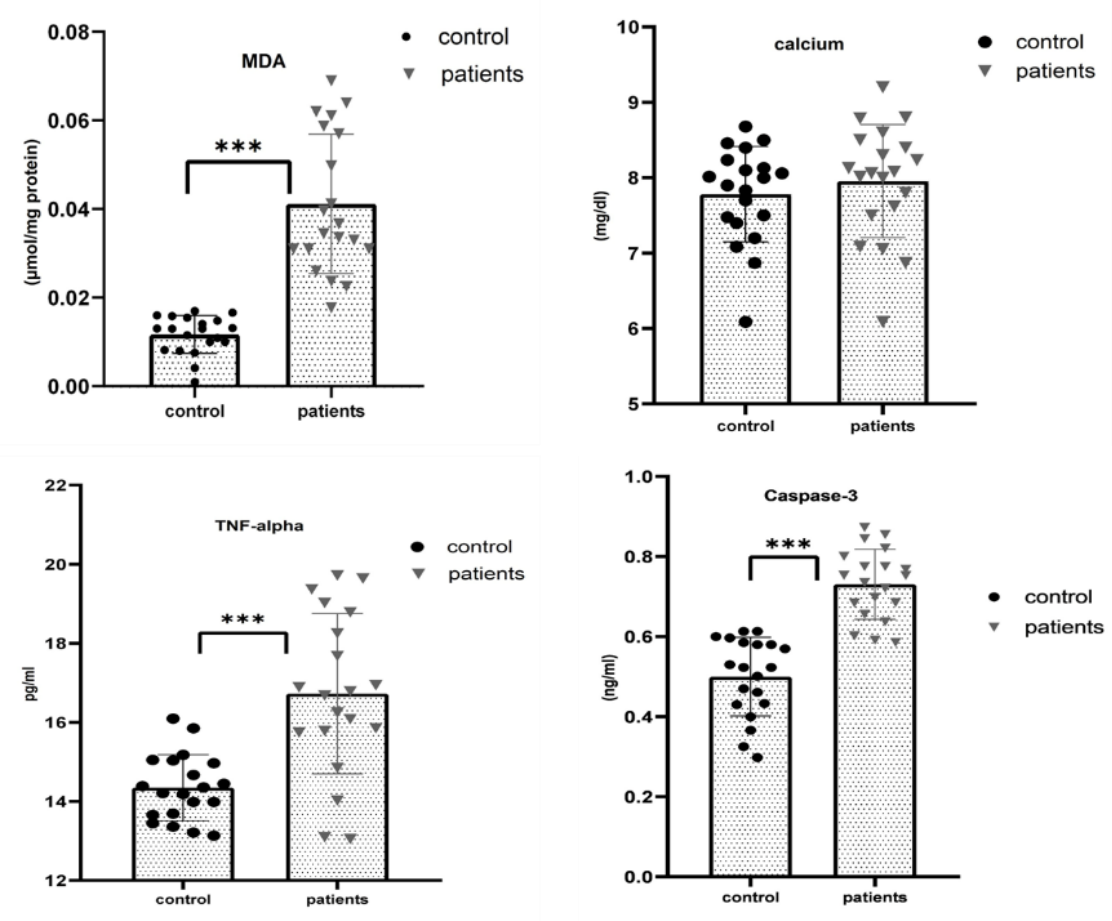

Fig. 4. Malondialdehyde levels, calcium concentration, activities of tumor necrosis factor, and caspase-3 in human cumulus cells of unexplained infertile patients and the normal fertile donor. Data are presented as mean ± standard deviation (SD). The sample size for the patient and control groups is n=40. ***Indicate statistically significant differences compared with control (P<0.0001).

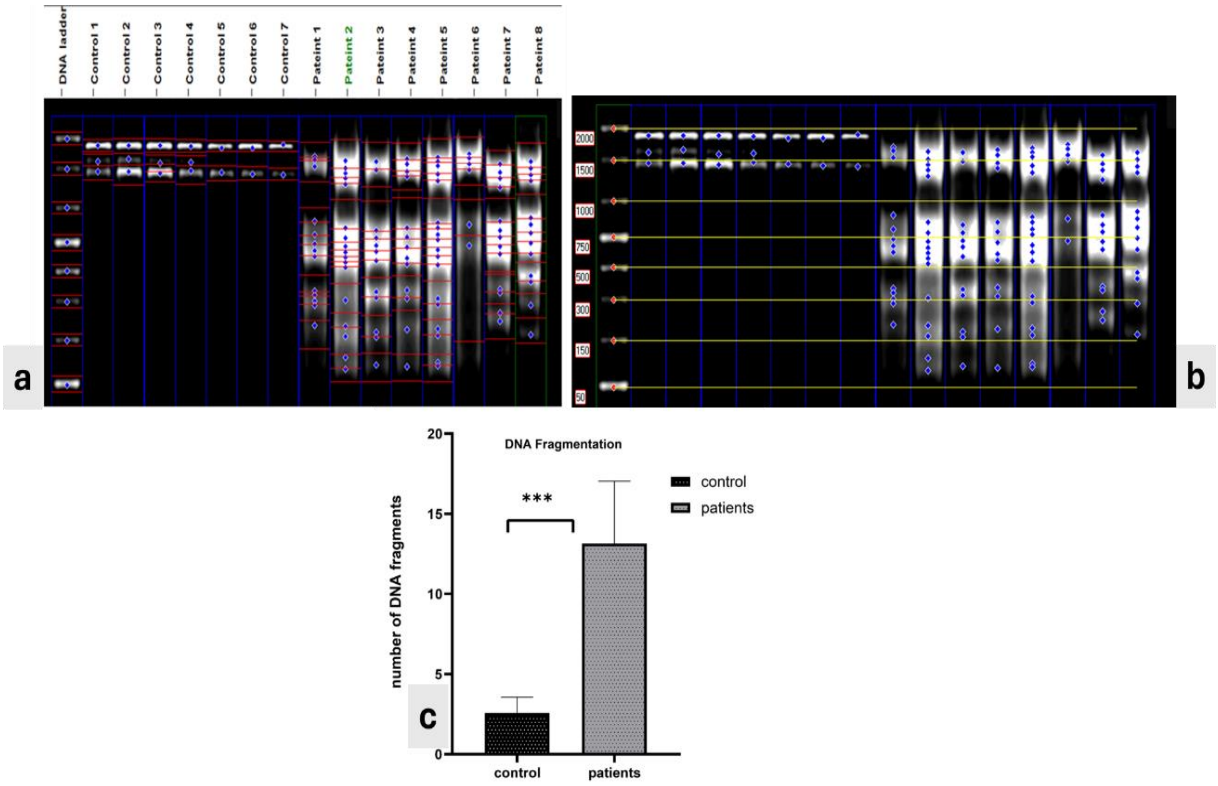

Fig. 5. Genomic DNA fragmentation pattern and calculated DNA length in the cumulus cells of unexplained infertile patients and the normal fertile donors. Data are presented as mean ± standard deviation (SD). The sample size for the patient and control groups is n=15. ***Indicate statistically significant differences compared with control (P<0.0001).

CCs and FF-linked findings in unexpected infertility

Comprehensively, the CCs displayed a significant increase in the means of MDA concentrations in all infertile patients compared to the control fertile ones (Fig. 4). MDA increased in patients. The results revealed significant increases in the level of human caspase-3 in the CCs of all female patients compared to the control fertile donors (Fig. 4). By comparison with the control donors, TNF-α in human CCs was significantly increased, as shown in Fig. 4. No significant differences in the level of calcium ions in the CCs of all infertile patients compared to the controls (Fig. 4). It is essential to evaluate DNA integrity in the CCs of women with unexplained infertility, as it is associated with oocyte competence. The results revealed significant DNA fragmentation in the CCs of all infertile patients compared to those of the control fertile donors (Fig. 5a,b). Figure 5c illustrates the various fragmentation patterns observed. This analysis indicates that assessing DNA integrity serves as an effective marker for identifying and addressing cases of unexplained infertility.

The results revealed that the level of GSH in the FFs of all patients showed statistically significant decreases compared to the control value (Fig. 6). Only the concentrations of zinc increased significantly in the aspirated FF, compared to the control (Fig. 6). PTEN is a tumor suppressor gene that functions as a mediator of apoptosis. Statistical analysis revealed that the level of PTEN in the FF of all unexplained infertile patients increased significantly compared to the control value (Fig. 6).

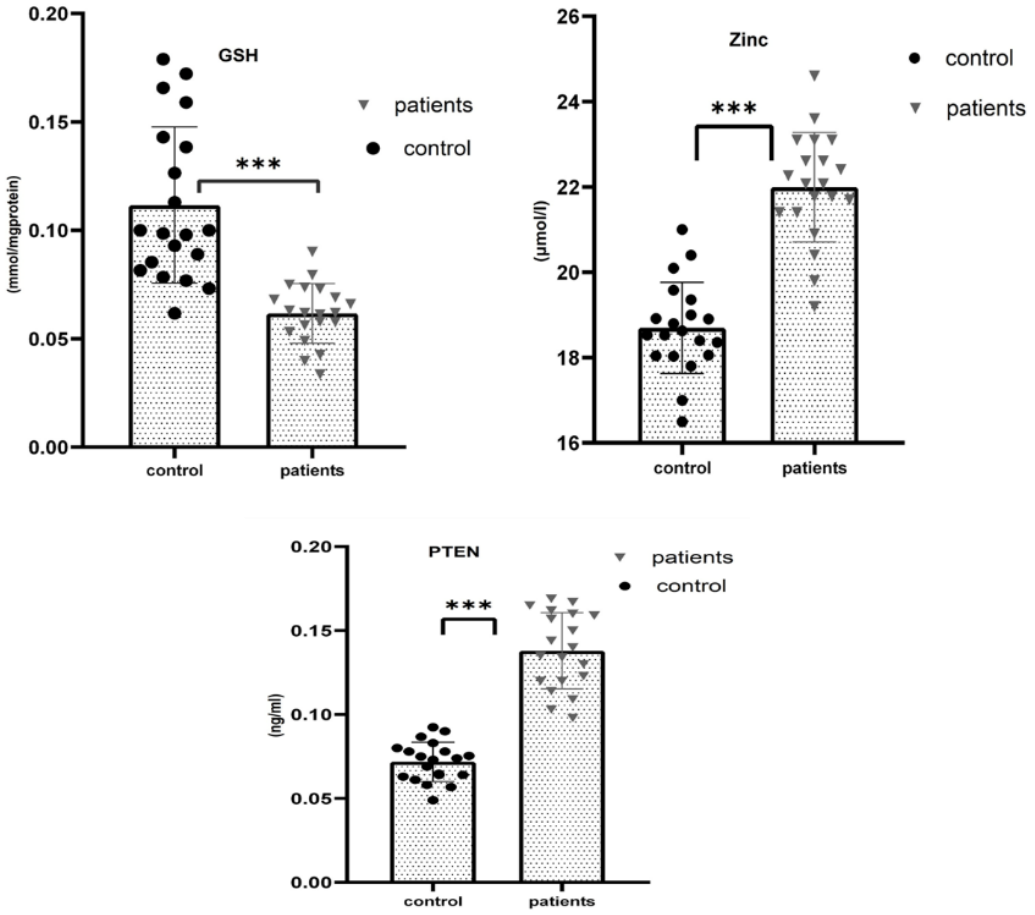

Fig. 6. Activities of reduced glutathione (GSH), zinc concentration, encoding phosphatase and tensin homolog (PTEN) concentration in human follicular fluids of five unexplained infertile patients and the normal fertile ones. Data are presented as mean standard deviation (SD). The sample size for the patient and control groups is n=40.***Indicate statistically significant differences compared with control (P<0.0001).

Transmission Electron Microscopy inspection

Control fertile

Under a stereomicroscope, the CCs showing apparent morphological deformity were further inspected using Transmission Electron Microscopy. TEM inspection displayed round or oval-shaped CCs, with a continuous cytoplasmic membrane and a thin, short extension of microvilli. Their nuclei were slightly eccentric, with evident nucleoli and heterochromatin patches in the center and periphery. The cytoplasm was visible as tiny, with prominent, rounded, and ovoid mitochondria (mt), rough endoplasmic reticulum, electron-dense lipid droplets, and a few lysosomal particles (Fig. 7a, b).

3.3. b. Unexplained infertile patients

Ultrastructural examination of an unexplained infertile woman, exhibiting cumulus cell abnormalities, showed marked disarray in structure. The structure alterations in the nuclei featured a crescent-shaped appearance, associated with changes in the topological constituents of the nuclear envelope (Fig. 7c). Most microvilli of CCs were distinctly lost, indicating a deficiency of cell gap junctions (Fig. 7c-f). The cytoplasm appeared to confine several big lipid droplets. A hypertrophied Golgi body, a tiny appearance

of mt, and a large smooth endoplasmic reticulum also appeared in the cytoplasm (Fig. 7d). Wrinkling of the nuclear membrane was also evident. Besides, a necrotic area and a big myelin figure (autophagosome) were detected among the cytoplasmic abnormalities (Fig. 7f).

Under the electron microscope, the nuclei in deformed CCs were distinguished by corrugation or blebbing of the nuclear membrane (Fig. 8b). Vast intercellular gaps were identified during TEM examination, indicating a significant discontinuity in the membrane and loss of connection. Herein, although the tiny-sized and dark aggregates of mt were detected in the CCs, in other cumulus samples, they were vacuolized, and their cristae were not visible (Fig. 8b, c). In the CCs, most cytoplasmic inclusions contain hypertrophied Golgi bodies (Figure 8b), a large autophagic vacuole, and numerous cytoplasmic protrusions compared with control CCs (Fig. 8e). In addition, many large lysosomal particles and large vacuoles were prominent (Fig. 6e, f). Taken together, these morphological features suggest that the affected CCs were a pathological case.

Other CCs collected from unexplained infertile females undergoing IVF protocol showed marked alterations in their nuclei. Irregular outlines and folding were the most common nuclear envelope deformities. Small-sized mt were prominent, solitary, or associated with other abnormalities, and were typically evident in their disintegration and cristae loss. Besides numerous smooth endoplasmic reticulum and hypertrophied Golgi bodies, many small- and large-sized vacuoles were recognized (Fig. 8b, d). TEM examinations exhibited a vacuolar structure that was evident as a massive assembly of elongated tubular-type smooth endoplasmic reticulum (sER; Fig. 8a).

Other characteristic alterations were observed in the CCs of the patient, where most of their nuclei revealed the appearance of an infoldings nuclear envelope with a given suture that may divide the nucleus into two parts (Fig. 8c,d). In the cytoplasm of these cells, most of the mt were thin, elongated, and fused in appearance (Fig. 8c). In other cells, they were swollen and had lost their cristae. Furthermore, many dark lysosomal particles were observed (Fig. 8d).

It was revealed that the electron micrograph examination of the CCs exhibited fragmented chromatin karyolitic nuclei in some of them. Other nuclei showed pyknotic features (Fig. 8e). Long projections of the cell membrane, giving blebbing, were another characteristic alteration observed in the cytoplasm of the CCs (Fig. 8e, f), alongside the appearance of apoptotic bodies (Fig. 8e). Along with the fewer mt appearances, many large lipid droplets, hypertrophied Golgi bodies, and the disappearance of the rough endoplasmic reticulum were observed (Fig. 8e,f). Different sizes of vacuoles were scattered in the cytoplasm, indicating impairment of the functional potency of the CCs (Fig. 8a,b,d).

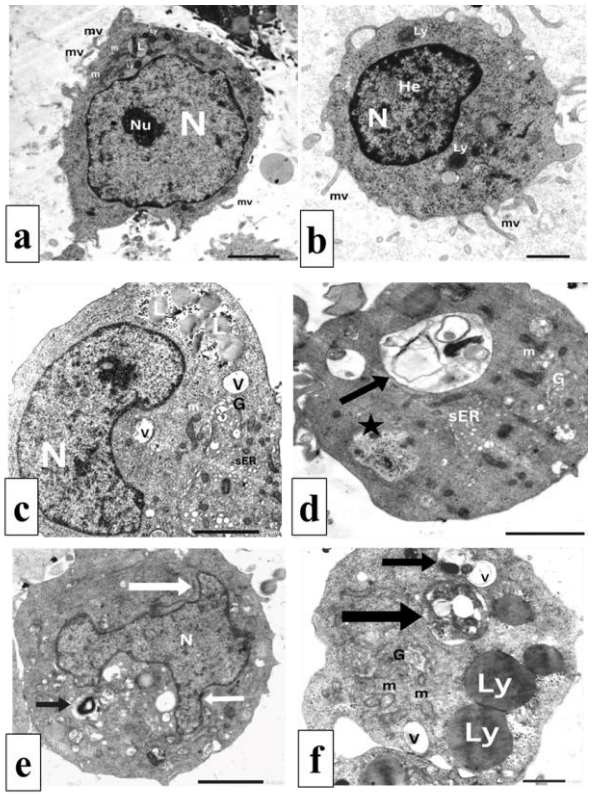

Fig. 7. Electron micrographs of the cumulus cells of control (a and b) showing the rounded and oval-shaped nuclei (N) with a nucleolus (Nu). mitochondria (m), lysosomes (Ly), thin short microvilli (mv), and lipid droplets (L). c – e: Patient showing a crescent-shaped nucleus (N), autophagosome (black arrow), small-sized mitochondria (m), hypertrophied Golgi area (G), vacuoles (V), smooth endoplasmic reticulum (sER), necrotic tissue in the cytoplasm (asterisks), wrinkling of the nuclear membrane (white arrow), autophagosome (black arrow), hypertrophied Golgi (G), small-sized mitochondria (m), vacuoles (V), and large-sized lysosomes (Ly). a: ×3000; b: ×3000; c: ×4000; d: ×4000; e: ×5000; f: ×4000.

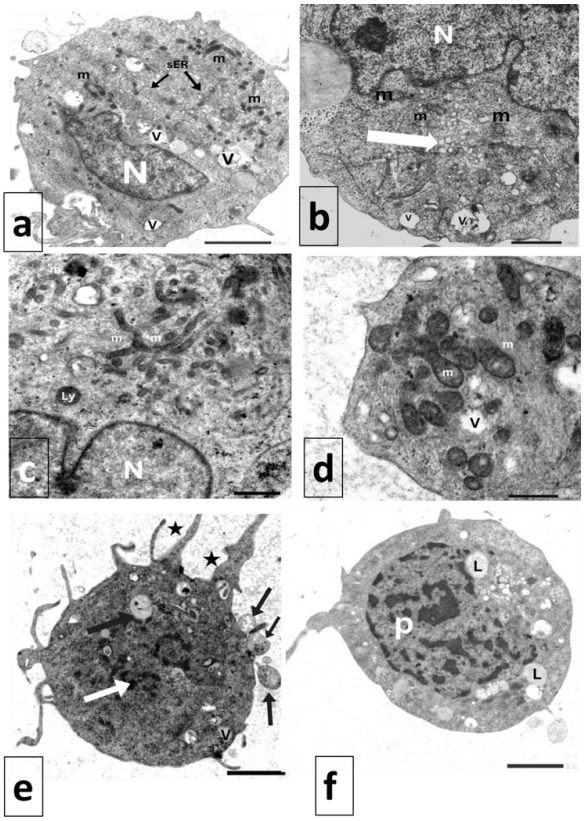

Fig. 8. Electron micrographs showing some misconfigurations of the cumulus cell of patients evident as a corrugation and blebbing outline of the nuclear membranes, pyknotic nucleus (P); karyolitic nucleus (small white arrow), vacuoles (V), mitochondrial swelling (m), hypertrophied smooth endoplasmic reticulum (sER), lysosomes (Ly), apoptotic bodies (3 black arrows), hypertrophied Golgi area (big white arrow); lipid droplets (L); autophagosome (black arrow); necrotic tissue (asterisks). a: ×4000; b: ×6000; c: ×5000; d: ×12000; e: ×3000; f: ×3000.

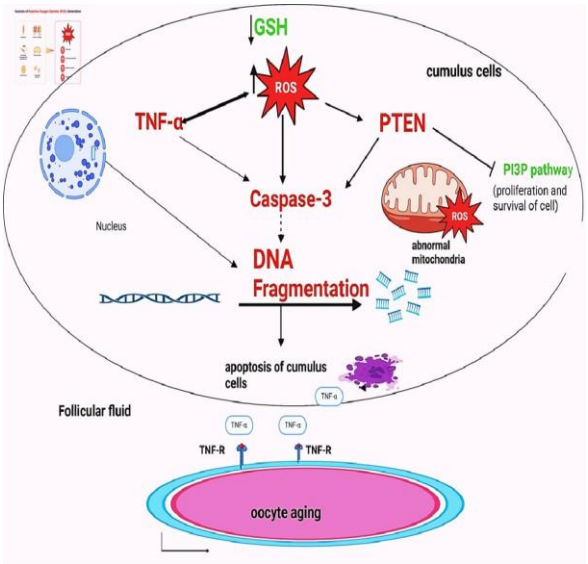

Fig. 9. Schematic illustration of the molecular mechanism by which oxidative stress contributes to oocyte aging in unexplained infertility patients.

4. DISCUSSION

Herein, the current study aims to elucidate the causes of unexplained infertility in infertile females undergoing the IVF protocol by conducting advanced analysis of the FF and CCs. Until now, no primary symptoms, precise factors, or related complications have emerged that can elucidate the mechanisms of unexplained infertility. This study observed no significant oocyte morphological abnormalities in all unexplained infertile patients' samples, compared to the control fertile donors. From the current results, the morphological screening of oocytes with the surrounding milieu, CCs, and the FF of unexplained infertile women can predict high-quality oocytes with normal developmental potential, without disorders in their physiological or structural makeup (Da Broi et al., 2018; Gabrys et al., 2022). However, under IVF, this displayed a significant failure in the rate of zygote development with altered CCs and/or FF, suggesting that CCs and/or FF were implicated in zygote quality and growth.

As one of the most important biomarkers of oxidative stress, MDA exhibits a significantly higher concentration in the CCs of unexplained infertile patients (Karabulut et al., 2020). Zaha et al. (2023) reported that oxidative stress can damage cells in some ways, including mt dysfunction, protein oxidation, DNA damage, and LPO. The elevation in ROS was linked with a decline in fertilization ability, embryo quality, and oocyte implantation capacity (Al-Saleh et al., 2021). The current findings showed a significant decrease in antioxidant GSH. The GSH is essential for cellular antioxidant defenses, where it directly scavenges free radicals and stops the oxidation of -SH groups in proteins. Indirectly, it is a required cofactor for the antioxidant enzymes glutathione peroxidase and glutathione S-transferase, which detoxify lipid peroxides and H_2O_2 **(**Averill-Bates, 2023).

Furthermore, the current study clarified the effects of minerals (the amounts of calcium and zinc ions) on the CCs' activity, and their connection to oocyte quality is also crucial. The signal communication between CCs and oocytes is facilitated by the transport of certain ions and small molecules between the two cell types (Chen et al., 2024). The current results demonstrated significant increases in zinc concentration in the FF of unexplained infertile patients, compared to the control. This declared the relation between zinc levels in the FF and the quality of oocytes.

Zinc has strong antioxidant properties, regulating intracellular ROS, reducing oxidative stress, protecting and maintaining DNA integrity, shielding mt from oxidative stress, and preventing apoptosis (Uh et al., 2019; He et al., 2020). According to Costa et al. (2023), a moderate intake of zinc is necessary to increase the antioxidant capacity of ovaries. Kageyama et al. (2022) talked about the crucial role of zinc signaling in mammalian reproduction, and Liao et al. (2023) clarified how the increased availability of zinc facilitates GCs proliferation, follicle development, oocyte maturation, fertilization, and embryo development. Liu et al. (2024) added that zinc deficiency diminishes significantly the number of mature ovarian follicles. This is directly linked to decreased anti-Müllerian hormone levels and disturbances in gene expression that regulate hormone secretion. Furthermore, zinc deficiency adversely affects mt efficiency, leading to the production of ovarian oxidative stress, which not only inhibits essential autophagy processes and exacerbates ovarian apoptosis and breakdown of the impaired germinal vesicle and compromises oocyte quality. Addressing zinc deficiency is therefore crucial for maintaining ovarian fitness and enhancing embryonic competency. Fluctuations of intracellular zinc concentrations may suggest a role for zinc in the control of the cell cycle, controlling cell growth in hyperplasia, neoplasia, and diseases associated with aberrant differentiation. Zinc ion concentrations in humans fluctuate due to various factors, including acute infections, inflammation, stress, hypoalbuminemia, nutritional deficiencies, malabsorption syndromes, chronic diseases, cell cycle regulation, and environmental conditions (Li et al., 2009). Additionally, in comparison to the control fertile donors, the current results showed no discernible differences in the calcium contents in the CCs of any unexplained infertile patients. So, this indicates that calcium detection did not affect the occurrence of

infertility cases since it did not reflect the opposite effect of elevated oxidative stress parameters like MDA. Numerous signal pathways use calcium as a second messenger, and variations in intracellular Ca^{2+} concentration play a significant role in oocyte maturation, activation, fertilization, GCs and CCs function regulation (Rodrigues et al., 2024).

According to the current study, the infertile patients' CCs revealed much higher caspase-3 levels than the control fertile donors, indicating apoptosis in these cells. Caspase-3 is one of the most well-known crucial downstream apoptotic initiators, which may activate caspase-activated DNase, an active endonuclease that fragments DNA by cleaving it during apoptosis (Nakamura and Takada, 2021). This finding was supported by the proposal of Guo et al. (2023), who concluded that apoptosis is caused by the concentration of Caspase-3 in CCs. The increased caspase-3 in the CCs of unexplained infertile women reflect necrotic and apoptotic cumulus cell damage (Stringer et al., 2023). The apoptosis of CCs in unexplained infertility conditions may create adverse follicular microenvironments and weaken oocyte development. Relatively, this may release cell-free DNA associated with unexplained infertility, resulting in the degeneration of oocyte quality, declining frequency of fertilization and ultimately embryogenesis (Ge et al., 2025).

Fan et al. (2019) described that the increased levels of cell-fragmented DNA in the CCs may be attributed to the contribution of various aspects of ovarian apoptosis. These mechanisms of apoptotic sequences may be, in part, among the reasons which elucidate the unexplained infertility in IVF undergoing ICSI.

TNF-α is one of the main signaling mechanisms of apoptosis. CCs induce oocyte apoptosis by release of cytokines, Fas ligand, TNF-α, and the interleukins (ILs) IL1A and IL1B (Wen et al., 2023; Bao et al., 2024). The present results revealed statistically significant increases in TNF-α in CCs of all infertile patients. By releasing soluble Fas ligand, a type II transmembrane protein that is a member of the tumor necrosis factor family, or TNF and the apoptotic CCs accelerated oocyte ageing (Kong et al., 2018). Gong et al. (2025) suggested that CCs contribute to the premature ageing of oocytes by triggering the TNF-α signaling pathway. It was reported that TNF-α secreted by COCS causes follicular rupture during ovulation by stimulating collagenase production (Zaniker et al., 2023). It has been documented that TNF-α influences the PTEN expression **(**Yang et al., 2018). It plays a critical role in fostering the development of primary ovarian follicles. PTEN mediates apoptosis in CCs (Chang et al., 2015; Carbognin et al., 2019). As compared to the control, the currently acquired results showed that the FF of people with unexplained infertility had higher levels of *PTEN*, which may indicate that it triggers apoptosis**.**

PTEN plays a crucial role in the cell cycle and has dual phosphatase activity, making it essential for various cellular processes, including differentiation, cell growth, and survival (Brandmaier et al., 2017; Namlı Kalem et al., 2023).

Its primary substrate, phosphatidylinositol-3,4,5-trisphosphate, acts as an important second messenger in cellular signaling pathways (Adhikari et al., 2012; Quan et al., 2017). Consistent with the present results, Wu et al. (2023) declared that the activation and over-expression of caspases-9 and caspases-3 demonstrate that *PTEN* over-expression triggers mt-based intrinsic apoptotic pathways. Overexpression of *PTEN* causes elevated apoptosis in GCS, resulting in decreased pregnancy rates, while downregulation of PTEN might enhance cell growth and survival (Wu et al., 2020; Yao et al., 2021). Hence, failed IVF outcomes might be related to the high *PTEN* transcript levels in GCs, which might function as a mediator of apoptosis. Furthermore, Hu et al. (2024) reported that *PTEN* is a legitimate target of miR-29-3p, and PTEN appeared to foster autophagy and apoptosis in chicken GCs. An increase is detected in *PTEN* expressions in GCs and FF in PCOS, and PTEN knockdown could strengthen proliferation as well as weaken the apoptotic activity of GCs (Zho et al., 2022). It was found that elevated *PTEN* expression may encourage GCs death and inhibit the PI3K pathway, which is essential for controlling cell migration, proliferation, survival, and

metabolism (Xu et al., 2025). Recently, Yao et al. (2025) added that overexpression of *PTEN* is not beneficial to normal follicle development, resulting in a poor ovarian reserve. Compared to the control fertile donors, the current data demonstrated a significant increase in DNA fragmentation in the CCs of all unexplained infertile patients. We noticed that the reduction in retrieved oocytes correlated with higher levels of fragmented DNA in aspirated CCs and collected FF in unexplained infertile patients. Debbarh et al. (2023) concluded that oocyte competence and embryo quality were associated with the integrity of follicular DNA. The higher levels of cell-fragmented DNA, a greater decline in oocyte maturation and a greater decline in embryo quality. Pereira et al. (2019) and Baratas et al. (2022) found that the oocyte's developmental competence was inversely correlated with the CCs' DNA damage. According to reports by Buyuk et al. (2011), the high levels of fragmented DNA may be linked to the downregulation of tropomyosin-related kinase. This kinase is a crucial mediator of cell survival, which maintains the viability of CCs. Consequently, its downregulation may enhance the increase of follicular apoptosis in women experiencing unexplained infertility. Winship et al. (2018) explained that the accumulation of DNA damage causes chromosomal fragmentation, which in turn impacts meiosis, spindle assembly, and mt distribution in the oocyte, all of which have an impact on embryo development. Furthermore, according to the current findings, the electron micrographs of CCs from unexplained infertile patients displayed several unique anomalies in the cytoplasm and nucleus. The ultrastructural features of apoptosis can be demonstrated by chromatin condensation, DNA fragmentation, nuclear envelope collapse, plasma membrane blebbing, cell disassembly, and the development of apoptotic bodies (Erkaya et al., 2021). One of the main causes of the age-related reduction in oocyte quality is thought to be mt dysregulation (May-Panloup et al., 2016; Kasapoğlu and Seli, 2020). The mt damage alterations in the CCs may be considered an additive reason that induces unexplained infertility. Ra et al. (2023), and Mauchart et al. (2023) reported that oxidative stress can damage cells in some ways, including mt dysfunction, protein oxidation and DNA damage. The oocyte's most abundant organelles, mt, supply enough energy to support fertilization and preserve development (Kirillova et al., 2021)**.** They participate in crucial oocyte meiotic processes such as cell maturation, chromosomal segregation, and spindle assembly. High-quality oocytes exhibit an optimized mt structure and number, enabling the production of a sufficient quantity of ATP (Jiang and Shen, 2022). Overgeneration of ROS affects mt DNA expression. This exerts the efficiency of mt production of ATP, which increases the intensity of apoptotic signaling in the follicular microenvironment, causing a decline in oocyte quality and subsequently hindering embryonic development (Sasaki et al., 2019; Wang et al., 2021)**.** According to Cecchino et al. (2018)**,** oocyte developmental potential is somewhat influenced by the GCS's ATP synthesis, mt shape, and mtDNA expression. ATP is crucial for the maturation of meiotic oocytes MII, fertilization, and the early stages of embryonic development (cleavage). Different studies have indicated that high levels of circulating fragmented DNA (cell and/or mt) correlate with the production of embryos of poor quality (Huang and Zhou, 2021; Kari et al., 2022; Zhang et al., 2025)**.** Hence, depletion in the mt ATP generation usually correlates with the decline in oocyte developmental competence, while elevated oxidative stress is closely linked to lower oocyte quality. Accordingly, this evidence is considered an additive crucial parameter implicated in unexplained infertility. In the current results, the mt vacuolization, swelling and cristae damage are related to rising apoptosis in all unexplained infertile women patients may be due to the failure of ATP generation (Van Blerkom, 2011; Lee et al., 2014). The mt function and the mt DNA content in CGCS were tightly correlated with CGCS in oocytes, indicating that mt CGC could functionally act as markers of oocyte quality (May-Panloup et al., 2016; Zhang and Wu, 2023). Based on the present findings, mt dysfunction can be linked to ROS accumulation, enhancement of apoptotic pathways, mtDNA mutations, calcium ion fluctuation, and lowering in ATP synthesis, ultimately hindering follicular growth, oocyte meiosis and quality. Many

investigations have interpreted that the impaired quality of the oocyte may be due to a reduction in the levels of cAMP and inhibition in the biosynthesis of steroid hormones, creating a condition of ovarian hypoestrogenism (Yu et al., 2021). Taking into consideration, nuclear-free DNA and mt free-DNA are released products of follicular apoptosis. Gabrys et al. (2024) explained that it may be due to changes in the extracellular vesicles of FF. The FF-derived extracellular vesicles reflect the status of the microenvironment milieu, affecting various molecular and phenotypic alterations in both cumulus and GCs (Battistelli and Falcieri, (2020)**.** These vesicles play a substantial role in delivering molecular signals and transporting genetic elements, such as mRNA and miRNA, between follicular cells in addition to cumulus expansion. Additionally, the gene expression of the Wnt signaling pathway is linked to signal transduction during embryogenesis (Spate et al., 2014). Many expressed components of the Wnt signaling pathway occurred in the cumulus oophorus complex, oocytes, ovaries, and cleavage stage embryos. These expressed elements are essential for demarcating the direction of the meiotic spindle (Harwood et al., 2008). Consequently, any deterioration in this mechanism may explain how the byproducts of extracellular vesicles from FF can disrupt COCs during IVF, potentially leading to unexplained infertility.

Another possibility of unexplained infertility in the current study may be consistent with the explanation of Jenabi et al. (2022), who reported that it may be due to an elevation of the chemokine CXCL8 protein and its mRNA levels in CCs and FF, causing ovarian inflammation by hyperactivity of immune system cells and leukocytes. Pro-inflammatory cytokines are crucial in the maturation process of the ovarian follicle, in addition to the process of embryo implantation. Immune imbalances harm the prognosis of the effectiveness of IVF and possibly on natural fertilization. According to some studies, the role of IL-12 in FF seems to be of particular interest; its higher concentration is correlated with effective fertilization of an ovum and the development of an embryo. Additionally, studies suggest that IL-18, both in blood serum and FF, positively correlate with the number of oocytes (Han et al., 2023). Furthermore, the electron micrographs revealed the appearance of vacuolized Golgi bodies, hypertrophied in sER and fragmented rough endoplasmic reticulum (rER) in the cytoplasm of CCs. This was in addition to many large electron-lucent lipid droplets. This may explain why the breakdown of Golgi proteins could disrupt lipid trafficking, leading to lipid accumulation. The Golgi apparatus is an important membrane-bound organelle involved in many physiological events, such as packaging of materials for secretion, trafficking, processing and sorting new membranes and secretory proteins and lipid products received from the endoplasmic reticulum (Gimenez-Escamilla et al., 2025).

Conclusion

Based on present findings and evidence, it is suggested that DNA damage and the molecular markers of apoptosis, such as caspase-3, TNF-α, and PTEN in CCs and FF, are the predictive primary causes of unexplained infertility. This was supported by elevated levels of oxidative stress markers, MDA, and GSH. Moreover, the multifactorial microenvironment surrounding oocytes may also account for the most conducive factor behind unexplained infertility in women undergoing IVF. In this context, we can assume that unexplained infertility may be caused either by the excessive accumulation of unspecified or unknown signals in the multiparametric microenvironment, which disturbs the consequences of oocyte competency and embryonic development, or by a certain deficit of normal, transferred signals within the microenvironment. We can also suggest that, even ignoring the environmental micro-junction, the molecules secreted by CCs can exert an adverse effect on both oocytes and target cells. Furthermore, the disruption of extracellular vesicle-derived FF or even the internalization of extracellular vesicle-derived FF into the cytoplasm of GCs and/or oocytes may be implicated as inducible factors in human oocyte maturation and developmental competence in IVF and ICSI. Interestingly, we noticed that the functional roles of PTEN related to oocyte growth, maturation, and subsequent embryonic developmental potential. Future work needs to

be further explored and studied could involve combining PTEN as a valuable potential biomarker for evaluating and improving oocyte quality

Author Contributions:

Azza Attia: Supervision, Conceptualization, Data curation, Investigation, Methodology, Supervision, Visualization, Writing - original draft; Ahmed Abdelkareem: Supervision, Conceptualization, Investigation, Visualization, Writing - original draft; Ahmed Galal: Supervision, Conceptualization, Investigation, Visualization, Methodology, Writing - original draft; Ahmed Etman: Supervision, Conceptualization, Investigation, Visualization, Methodology, Writing - original draft; Reda ElMazoudy: Writing - original draft, Writing - review & editing, Validation, Data curation, Interpretation of data for the work, Final approval of the version to be published; Nada Fawzy: Reasearcher, Data curation, Formal analysis, Methodology, Resources, analysis of data for the work.

Conflict of interest.

All the authors declare that they have no conflict of interest.

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