Melatonin Dependent Modulation in microRNAs Relative Expression Pattern and Levels of cfDNA in Mature Oocytes of Unexplained Infertile Patients.

Haroon Latif Khan  
Lahore Institute of Fertility and Endocrinology

Shahzad bhatti (drshahzadbhatti@yahoo.com)  
Lahore Institute of Fertility and Endocrinology  https://orcid.org/0000-0003-1937-0100

Sana abbas  
Lahore Institute of Fertility and Endocrinology

Celal Kaloglu  
Cumhuriyet Universitesi

Syeda Qurat-ul-ain  
Kinnaird College for Women

Yousaf Latif Khan  
Lahore Institute of Fertility and Endocrinology

Zahira Hassan  
Royal Free London NHS Foundation Trust

Hikmet Hakan Aydin  
Ege Universitesi

Nilgün Öztürk Turhan  
Bayindir Hastanesi Sogutozu

Nour El Houda Bousnane  
Universite Batna 2

Aysegul Yildiz  
Mugla Sitki Kocman Universitesi

Isadora Saruhashi  
Universidade Tuiuti do Parana

Research

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Abstract

Background

Intrafollicular melatonin maintains the DNA integrity of granulosa cells and protects them against apoptosis. This ubiquitous indoleamine compound serves as a potent free radical scavenger. It reduces oxidative stress and modulates DNA damage response, which improves oocyte's quality with a higher fertilization rate.

Methods:

This prospective study was designed to investigate the antioxidant property of intrafollicular melatonin and its impact on IVF outcome parameters by exploring the relative expression of five microRNAs (miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p) and levels of cfDNA by real-time PCR in unexplained infertile patients. We collected 425 follicular fluid (ff) samples containing mature oocytes from 295 patients undergoing IVF.

Results:

Patients were sub-grouped based on intrafollicular melatonin concentration (Group A; ≤ 30 pg/mL, Group B; >70 to ≤110 pg/mL, Group C; >111 to ≤ 385 pg/mL). Our results showed that patients with ≤ 30 pg/mL intrafollicular melatonin levels have a significantly higher cfDNA levels and lower relative expression of miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p compared to other subgroups (p<0.001). Similarly, they have a low fertilization rate and a reduced number of high-quality day 3 embryos. For the probability of obtaining a good quality embryo, miRNAs had AUC value of 0.89 [95% CI; 0.71; 0.93] with 87% sensitivity and 83 % specificity (p = 0.001), whereas cfDNA have AUC value 0.76 [cut-off points, 95% CI: 0.61, 0.67; 0.85] along with 82.4 % sensitivity and 66.7 % specificity.

Conclusion:

Conclusively, our study provided evidence that melatonin's antioxidant capability significantly impacts cfDNA concentration and miRNAs relative expression profile in the follicular microenvironment for optimal oocyte development and embryo quality. Therefore, it may be a potent non-invasive diagnostic tool to select high-quality day 3 embryos with such promise.

Background

Melatonin (N-acetyl-5-methoxytryptamine) is a potent immunomodulatory synthetic product of the pineal gland that manages the circadian rhythm, sleep-wake cycle, blood pressure, and monitors mammalian reproductive activities. Moreover, this ubiquitous indoleamine compound serves as a potent free radical scavenger. Melatonin, in part, functions through the activation of two high-affinity G-protein-coupled
receptors. However, it was initially uncovered as a pleiotropic regulatory molecule that has shown considerable antioxidant/antinitrosant activity in various cell types and biological fluids, including ff [1]. Intrafollicular melatonin concentration is almost 3-fold higher than that in blood plasma, which directly protects oocyte from oxidative stress within the ovarian follicle [2]. Human follicular fluid (ff) may be considered a "biological window" that reflects allosteric metabolic regulation. It comprises various hormones, polysaccharides, reactive oxygen species (ROS), and antioxidant defense systems [3]. Paradoxically, ROS act as powerful signaling molecules that initiate the process of various growth-related responses and are primarily involved in cellular metabolism. However, the acquisition of ROS has harmful effects on the follicular microenvironment. Surprisingly, it favors the process of ovulation, thus might function as a double-edged-sword in the cellular processes [4]. Under moderate concentrations, ROS are responsible for mediating inter-and intracellular signaling cascades provided protection against apoptosis while massive production of ROS result in oxidative stress. Apoptosis is mainly due to the upregulation of oxidant species and the antioxidant defense system's limited efficacy. However, within the follicles, a delicate equilibrium exists between the pro-oxidant and antioxidant defense system, maintained by antioxidants' prominence. Non-enzymatic antioxidants, such as vitamins, minerals, superoxide dismutase (SOD), Glutathione (GSH), ascorbic acid, and melatonin, are required necessarily during ovulation [5].

Oxidative stress is an incessant cause of DNA damage and epigenetic modifications in developing oocytes. The molecular mechanism of biological processes underlying cellular response to broad-spectrum actions of melatonin is not restricted to its interaction with intracellular proteins but also has functional effects on non-protein-coding RNA species [6]. Owing to their wide plethora of functional activities and dynamic stability, miRNAs have a promising function as diagnostic and prognostic biomarkers [7]. Notably, miRNAs expression profile has been recognized as a novel mechanism of intercellular communication, thereby acting similarly to hormones and a potential source of non-invasive biomarkers in the oocyte microenvironment to predict embryo quality [8].

Oxidative or nitro-oxidative stress leads to DNA damage when the antioxidant capability is insufficient, thereby playing a crucial role in intrinsic/extrinsic pathways of apoptosis by the integrated release of various pro-apoptotic factors. The amount of cfDNA in ff significantly related to the oocyte's quality and reflects the extent of cell damage by apoptosis [9]. In contrast, intrafollicular melatonin maintains the DNA integrity of granulosa cells, protects them against apoptosis, and stimulates estradiol and progesterone production that improves oocyte's quality with a higher fertilization rate [10]. Considering the possible effect of melatonin on different miRNA expression levels, which affects oocyte maturation quality, it should be of primary importance to focus on this relationship. Currently, limited studies are focused on the interactions of melatonin concentration and miRNAs relative expression profile in the ff samples, which might serve as a potent non-invasive tool for predicting oocyte development capability in infertile patients.

Therefore, this study was designed to investigate the melatonin-dependent modulation in five miRNAs (miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p) relative expression patterns constitutively
in mature oocytes' ff microenvironment to predict embryo quality. Previous studies demonstrating their involvement in oocyte maturation and embryo quality [11, 12]. Additionally, we sought to explore whether melatonin's antioxidative property affects the concentration of apoptotic cfDNA in ff samples from which mature oocytes were obtained.

Methods

Participant's Selection:

Subjects

This prospective study included 425 individual ff samples related to mature oocytes from 295 women (mean age; 33.87 ± 1.98 years) with unexplained infertility. The subjects were registered in the tertiary-care hospital's assisted reproductive center between January 2017 and December 2018. The participants were sub-grouped based on intrafollicular melatonin concentration (Group A; ≤ 30 pg/mL, Group B; >70 to ≤ 110 pg/mL), Group C; >111 to ≤ 385 pg/mL). The study was approved by the Institutional Review Board (IRB). All patients provided written consent to participate.

Inclusion Criteria

Unexplained infertile women with less than four previous attempts and have normal physical and mental health, basal FSH level ≤8.85 IU/mL, and ovaries appeared in normal shape and sizes, were the part of this study. Moreover, male partners have normal semen parameters.

Exclusion Criteria

We excluded patients with metabolic disorders, communicable diseases, body mass index (BMI) ≥35 kg/m², hyperandrogenemia, polycystic ovary syndrome, hyperprolactinemia, and reported any pelvic surgery. Moreover, we excluded females with known chromosomal translocation, endometriosis, diminished ovarian reserve, and autoimmune diseases.

Assessment of Clinical Parameters

BMI was calculated based on height and weight. While baseline hormones such as follicular stimulating hormone (FSH), luteinizing hormone (LH), 17β-estradiol (E₂), thyroid-stimulating hormone (TSH) and anti-mullerian duct hormone (AMH) were assessed on 2nd day of the menstrual cycle through electrochemiluminescence immunoassay, according to the manufacturer's instructions (Elecsys® Roche Diagnostics, Indianapolis, USA). The antral follicle count (AFC) was assessed using transvaginal ultrasonography (TVS) on the 2nd or 3rd day of the menstrual cycle.
**Therapeutic Regimen**

To minimize the possible confounding bias by varied controlled ovarian stimulation (COS) procedures, we only include patients in which ovarian stimulation was done through long GnRH agonist (decapeptyl®: Ferring, USA, ATCO pharma) administered in the middle of the luteal phase of the previous cycle. Ovarian stimulation with rFSH (Follitropin β, Purigon®: Organon Schering-plough, Oss, France) was evaluated by TVS and by quantifying serum 17β-estradiol level. The dosage of rFSH was adjusted according to ovarian response, BMI, bFSH levels, and AFC. A single dose of human chorionic gonadotrophin (6500-10,000 IU: Merk Serono, Lyon, Spain) was injected when more than two follicles reached a mean diameter of 18 mm or more by TVS inspection.

**Follicular fluid collection and estimation of melatonin and E₂ concentration**

Oocytes were retrieved by TVS guided puncture after 36 hours of hCG treatment. Clear follicular fluid without blood contents was aspirated independently from the two or three individual follicles. FF samples of mature MII-oocytes were centrifuged separately at 1500 × g for 20 minutes at 4 °C by preventing high-intensity bright light exposure. The supernatant was filtered through a 0.85 µL filter and stored immediately as aliquots of 500 µL × 2 at -80 °C. Each mature oocyte, its related embryo, and ff sample were handled separately in the IVF laboratory. Subsequently, to avoid potential confounding bias by dissimilar follicles of different maturation statuses, as they might contain a varied miRNA and cfDNA profile, we only include those follicles with greater than 18mm diameter. Finally, mature oocytes were subject to intracytoplasmic sperm injection (ICSI) procedure. Melatonin and E₂ concentrations were evaluated by diluting ff samples 1:100 through radioimmunoassay kits (MP® diagnostics, Santa Ana, California, USA). Intra-assay variations for melatonin and E₂ was <10%.

**Assessment of embryo quality**

Fertilization check was done 18-24 h after ICSI, and the embryo quality was determined through manual grading, using standard criteria based on cytoplasmic appearance, the extent of fragmentation, number, and regularity in the symmetry of blastomeres [13].

**RNA extraction from follicular fluid and relative expression analysis by RT-qPCR**

500 µL frozen aliquots were thawed on ice, and cell debris was removed by centrifugation for 20 min at 3000 ×g at 4 °C. RNA was extracted from individual follicles using the Silica-based membrane purification technique (miRNAeasy kit, Qiagen, USA) following the given instructions except that we diluted the sample 3:1 ratio with XBP buffer to optimize its use with the ff. Total RNA was dissolved in 30
µL of RNAs free water, and its concentration was measured through Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Expression-based digital gel electrophoresis (Bio-Rad, Hercules, CA, USA) was also used to confirm the total RNA concentration. MicroRNA profiling and data normalization were achieved as narrated by (Mestdagh et al 2009). Complementary DNA (cDNA) was generated using the TaqMan MicroRNA reverse transcription kit (Life Technologies, USA) in combination with RNA specific stem-loop Megaplex primers (Applied biosystems). A total of 15 µL reaction mixture contains 5 µL of a sample (10 ng miRNA), 0.5 µL of dNTP (100mM), 1.5 µL RT buffer (10X), 1 µL /50 IU of multiScribe RT enzyme, 0.19 µL of RNase inhibitor, 3 µL of stem-loop RT primers and 4.16 µL of nuclease-free water. Reverse transcription was performed in pulsating RT reaction: 40 cycles of 16 °C for 2 min, 42 °C for 60 seconds, and 50 °C for 60 seconds. Inactivation of reverse transcriptase was done at 85 °C for 5 min and hold step at 4 °C. Amplification was done with the following conditions: enzyme inactivation at 95 °C for 10 min and 40 cycles of two thermal amplification steps of 95 °C for 15 seconds 60 seconds for 1 min and a hold step at 4 °C. Q-PCR was duplicated for each sample using a CFX-96® touch RT-PCR detection system (Bio-Rad, Life Sciences, USA). We used Allele ID software® to design primers and probe. For quantification of follicular fluid miRNAs expression levels, PCR reaction was performed in a total volume of 20 µL, having 3 µL of cDNA, 10 µL of TaqMan Universal PCR MasterMix (Applied Biosystems), 0.8 µL of each primer and 5.4 µL double distilled water. Amplification was carried out in a 96-well plate, and thermocycling conditions were 10 minutes at 95 °C for enzyme activation, followed by 45 cycles of 95 °C for 20 seconds, 60 °C for 60 seconds. miRNA expression levels were normalized against the expression of MiR-16, which was used as an internal control because of its constant expression in ff samples. The relative expression of the five miRNAs such as miR-320a, miR766-3p, miR-132-3p, miR-16-5p, and miR-663b was calculated using equation $2^{-\Delta Ct}$, while $\Delta Ct = Ct$ target miRNA – $Ct$ miR-16. To calculate the fold change (FC), we estimated the relative expression levels between high quality and impaired quality embryo on day 3 using $2^{\Delta \Delta Ct}$ formula [11].

**Extraction and assessment of follicular fluid cell-free DNA (cfDNA)**

CfDNA was quantified, as previously described [14]. For cfDNA extraction, each ff sample was diluted with an equal volume of buffer solution (Tween-20, Tris-50 mmol/l, EDTA-1mmol) and incubated with proteinase K (Qiagen) at 55°C for at least 30 min, followed by inactivation at 98 °C for 10 min. After denaturation, each ff sample was centrifuged at 3000 rpm for 15 min and then immediately stored at -80 °C until quantification. The cfDNA concentration in each follicle with mature oocyte was estimated relative to the corresponding amplification of β-globin and GAPDH measured by the real-time PCR-SYBR green detection method previously described [15].

**Antioxidant status and oxidative stress markers measurements in follicular fluid samples**
The frozen (-80 °C) ff samples were thawed and evaluated for oxidative status. Average values of triplicate measurements were carried out from each ff sample to avoid inter-assay variations. ROS levels were measured by chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) as a probe [16]. The total antioxidant capacity (TAC) was assessed using the colorimetric assay based on the manufacturer's instructions (BioVision, Inc, CA, USA). Lipid peroxidation was evaluated by calculating the concentration of Thiobarbituric acid reactive substances (TBARS) [17] while 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured using a kit based on the manufacturer's instructions (BioVision, Inc, CA, USA). Both TBARS and 8-OHdG values were expressed as µM/L and ng/mL, respectively.

**Pathway analysis:**

We performed in-silico analysis to predict targets of miRNAs using web-based bioinformatics tool DIANA miRPath-v3 available on [http://snf-515788.vm.okeanos.grnet.gr](http://snf-515788.vm.okeanos.grnet.gr). Pathways were identified in both the regression analysis and fold-change. The results were demonstrated as a heat map. The more intense red color directed an increased probability that a specific miRNA targets a unique pathway supplemented with target genes.

**Statistical Analysis:**

Baseline characteristics are presented as means ± SD, number percentage [n%], and median with 95% population limits as applicable. We used Kruskal-Wallis/two-tailed test to explore the outcome differences in parameter levels between patients with low (≤30 pg/ml), intermediate (>70 to ≤110 pg/ml), and high intrafollicular melatonin concentration (>111 to ≤385 pg/ml). Based on the evaluation of the normality of the distribution by Kolmogorov-Smirnov test and Shapiro-Wilk test, we used the [Mann-Whitney](http://snf-515788.vm.okeanos.grnet.gr) test to determine the Pairwise comparison between different groups. χ²-test was used to address the categorical variables. Spearman rank test was used to determine the correlation between intrafollicular melatonin levels and other parameters. Receiving operating characteristics (ROC) curves were used to calculate AUC with a 95% confidence interval (CI). The sensitivity and specificity for optimal cut-off were calculated using XLSTAT 2020 software. SPSS (version 27; SPSS Inc., Chicago, IL. USA) was used for further statistical analysis. P<0.05 was considered statistically significant.

**Results**

**Baseline clinical characteristics of participants**

The demographic parameters of participants are listed in Supplementary Table 1. Patients were divided into three groups based on melatonin concentration in their follicular fluid samples: Group A; ≤ 30 pg/mL, Group B; >70 to ≤110 pg/mL), and Group C; >111 to ≤ 385 pg/mL. Of the 425 oocytes with associated ff samples, 55 oocytes did not fertilize nor reach the 1PN stage. From the results of our study, it was evident that increased concentration of cfDNA is found in ff samples of group A (median, 95% Cl:
2.01 (1.66; 3.42) compared to groups B (median, 95% CI: 1.03 (0.41; 1.98) and C (median, 95% CI: 0.86 (0.11; 1.03; p<0.001). Subsequently, ROS, TBARS, and 8-OHdG were significantly higher in group A than that found in group B and C patients (p <0.001). Similarly, we observed that patients exhibiting decreased intrafollicular melatonin concentration (≤ 30 pg/mL) have a significantly lower total antioxidant capacity [median, 95% CI: 238 (198; 305; Kruskal-Wallis test, p<0.001] (Table 1). However, we could not find any significant association among subgroups of patients regarding age, BMI, and baseline endocrine parameters such as FSH, AMH, LH, and TSH levels. Additionally, we also screened for five selected microRNAs and detected miR-663b in 266/295 samples, miR-320a (284/295 samples), miR-766-3p (279/295 samples), miR-132-3p (268/295) and miR-16-5p in 273 out of 295 samples.
Table 1

Relationship between different concentrations of melatonin and intrafollicular parameters

| Characteristics                          | Group A (Intrafollicular Melatonin Concentration ≤30 pg/ml) | Group B (Intrafollicular Melatonin Concentration >70 to ≤110 pg/ml) | Group C (Intrafollicular Melatonin Concentration >111 to ≤385 pg/ml) | Kruskal-Wallis test (P-value*) | P-value** |
|-----------------------------------------|-------------------------------------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------|-------------------------------|-----------|
| **Number of women [n (%)]**             | 97 (32.8)                                                   | 145 (49.1)                                                          | 53 (17.9)                                                           | -                             | -         |
| **Age (Years) [mean ± SD]**             | 33.85 ± 2.11                                                | 32.10 ± 3.03                                                        | 30.14 ± 4.41                                                        | 0.332                         | -         |
| **BMI (kg/m²) [mean ± SD] [median (95% population limit)]** | 28.52 ± 0.99                                                | 26.29 ± 1.77                                                        | 25.89 ± 2.25                                                        | 0.455                         | -         |
| **Intrafollicular parameters**          |                                                             |                                                                     |                                                                     |                               |           |
| Intrafollicular E2 level (ng/ml) [mean ± SD] [median (95% population limit)] | 411.2 ± 113.69                                              | 598.15 ± 258.74                                                    | 745.36 ± 288.98                                                    | <0.001                        | <0.001<0.002 ab, 0.002 c |
|                                         | 389.3 (295.7; 524.2)                                        | 566.1 (488.6; 830.1)                                               | 698.3 (497; 915.3)                                                 |                               |           |
| Intrafollicular cfDNA (ng/µl) [mean ± SD] [median (95% population limit)] | 2.97 ± 1.36                                                 | 0.97 ± 0.85                                                         | 0.81 ± 0.52                                                        | <0.001                        | <0.001<0.001 a, b |
|                                         | 2.01 (1.66; 3.42)                                           | 1.03 (0.41; 1.98)                                                  | 0.86 (0.11; 1.03)                                                  |                               |           |
| **Intrafollicular antioxidant status and oxidative Stress markers [median (95% population limit)]** |                                                             |                                                                     |                                                                     |                               |           |
| ROS (cpm)                               | 38.98 (25.8; 77.9)                                          | 8.98 (10.98; 15.09)                                                | 6.89 (3.09; 10.99)                                                 | <0.001                        | <0.001<0.001 a, b |
| TAC (µM/L)                              | 238 (198; 305)                                              | 965 (698; 1067)                                                    | 1275 (883; 1765)                                                   | <0.001                        | <0.001<0.001 a, b, 0.003 c |
| TBARS (µM/L)                            | 1.98 (1.45; 2.87)                                           | 0.95 (0.60; 1.02)                                                  | 0.65 (0.45; 0.87)                                                  | <0.001                        | <0.001<0.001 a, b |
| 8-OHdG (ng/mL)                          | 1.68 (0.89; 2.12)                                           | 0.97 (0.76; 1.09)                                                  | 0.71 (0.43; 0.91)                                                  | <0.001                        | <0.001<0.001 a, b |

Nd [n (%)]
| miRNA  | Group A/C | Group A/B | Group C/B | Group C/B | Group C/B |
|--------|-----------|-----------|-----------|-----------|-----------|
| miR-663b | 74 (76.2) | 134 (92.4) | 47 (88.6) | -         | -         |
| miR-320a | 83 (85.5) | 141 (97.2) | 51 (96.2) | -         | -         |
| miR-766-3p | 69 (71.1) | 129 (88.9) | 49 (92.4) | -         | -         |
| miR-132-3p | 62 (63.9) | 135 (93.1) | 50 (94.3) | -         | -         |
| miR-16-5p   | 52 (53.6) | 127 (87.5) | 48 (90.5) | -         | -         |

Note: a Group A/C, b Group A/B, c Group C/B; # $\chi^2$-test. d Number of samples in which each mRNA was detected out of a total number of samples analyzed. E2, 17β-estradiol; ROS, reactive oxygen species; TAC, total antioxidant capacity; LPO, lipid peroxidation; TBARS, Thiobarbituric acid reactive substances; 8-OHdG, 8-hydroxy-2-deoxyguanosine. Intra-follicular data for patients are also subjected to statistical differences, $P^*$ = Kruskal-Wallis test, $P^{**}$ = pairwise comparisons between sub-groups, and p<0.05 considered statistically significant.

**Intrafollicular melatonin concentration can predict IVF outcome parameters**

The characterization of IVF outcome parameters based on a pairwise comparison test between the groups is shown in Table 2. Patients with $\leq$ 30 pg/mL intrafollicular melatonin levels have significantly decreased IVF outcome parameters, particularly they have a low fertilization rate, a reduced number of mature oocytes, and high-quality day 3 embryos compared to other subgroups. Our results showed that IVF outcome parameters were better in group B and achieved a maximum in group C (Kruskal-Wallis test, p<0.001) with the same rising tendency as the intrafollicular melatonin levels between the groups (Kruskal-Wallis test, p<0.001). Correlation of intrafollicular melatonin concentration on IVF outcome parameters was given in supplementary Table 2, which shows that melatonin levels have a positive correlation with the number of MII- oocytes ($r_s = 0.712$; p<0.001), normal fertilized oocytes ($r_s = 0.731$; p<0.002), early cleaved zygotes ($r_s = 0.697$; p<0.001), blastomeres (6-8 cells) with regular symmetry ($r_s = 0.641$; p<0.001), high-quality day 3 embryos ($r_s = 0.745$; p<0.003) and have a negative correlation with fragmentation rate ($r_s = -0.812$; <0.001).
### Table 2
Association between intrafollicular melatonin levels and IVF outcome parameters.

| Characteristics                                                                 | Group A (Intrafollicular Melatonin Concentration ≤30 pg/ml) | Group B (Intrafollicular Melatonin Concentration >70 to ≤110 pg/ml) | Group C (Intrafollicular Melatonin Concentration >111 to ≤385 pg/ml) | Kruskal-Wallis test (P-value*) | P-value** |
|---------------------------------------------------------------------------------|-------------------------------------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------|--------------------------|----------|
| No. of Follicles with >18mm size [mean ± SD] [median (95% population limit)]   | 15.91 ± 2.43                                                | 16.18 ± 2.12                                                        | 17.31 ± 1.08                                                        | 0.165                   | -        |
|                                                                                  | 13.2 (12.11 ± 17.12)                                        | 13.5 (8.9; 16.21)                                                   | 14.1 (9; 17)                                                        |                          |          |
| No. of Retrieved oocytes [mean ± SD] [median (95% population limit)]            | 13.34 ± 3.88                                                | 14.09 ± 2.90                                                        | 15.4 ± 2.09                                                         | 0.543                   | -        |
|                                                                                  | 11.6 (2; 14)                                                | 12 (9; 15)                                                          | 14 (11; 16)                                                        |                          |          |
| No. of MII-oocytes [mean ± SD] [median (95% population limit)]                  | 4.41 ± 0.94                                                 | 10.12 ± 1.11                                                        | 13.31 ± 2.11                                                        | <0.001                  | <0.001ab |
|                                                                                  | 4.11 (1-4.5)                                               | 9.83 (6.89; 11.11)                                                  | 12.12 (10.21; 15.12)                                               |                          |          |
| No. of Fertilized oocytes [mean ± SD] [median (95% population limit)]           | 3.99 ± 0.95                                                 | 9.98 ± 2.12                                                         | 12.98 ± 2.01                                                        | <0.001                  | <0.001a,0.003b |
|                                                                                  | 3.21 (1; 3.5)                                              | 8.11 (6.98; 10.87)                                                  | 11.91 (9.07; 14.41)                                                |                          |          |
| No. of Normal Fertilized oocytes [mean ± SD] [median (95% population limit)]    | 3.16 ± 0.34                                                 | 9.65 ± 1.98                                                         | 12.39 ± 1.88                                                        | <0.001                  | <0.001abc |
|                                                                                  | 2.98 (1; 3)                                                | 8.69 (6.32; 9.21)                                                   | 11.01 (9.10; 14.13)                                                |                          |          |
| No. of Early cleaved zygotes [mean ± SD] [median (95% population limit)]        | 2.10 ± 0.54                                                 | 8.91 ± 1.09                                                         | 11.59 ± 1.87                                                        | <0.001                  | <0.001abc |
|                                                                                  | 1.89 (1; 2.8)                                              | 8.21 (7.09; 9.01)                                                   | 10.61 (8.14; 12.58)                                                |                          |          |
| No. of blastomeres (6-8 cells) with regular symmetry at day 3 [mean ± SD] [median (95% population limit)] | 2.01 ± 0.12                                                | 7.26 ± 1.01                                                         | 10.84 ± 1.88                                                        | <0.001                  | <0.001abc |
|                                                                                  | 1.62 (1; 2)                                                | 7.15 (6.09; 8.81)                                                   | 10.2 (9.18; 11.86)                                                  |                          |          |
| No. of High-Quality day 3 embryos [mean ± SD] [median]                           | 1.06 ± 0.76                                                | 6.32 ± 1.43                                                         | 9.65 ± 1.92                                                         | <0.001                  | <0.001a, 0.004b |
|                                                                                  | 1 (0; 2)                                                   | 6.21 (5.31; 6.81)                                                   | 7.94 (6.98; 9.87)                                                   |                          |          |
Intrafollicular cfDNA content and IVF outcome parameters among idiopathic patients

The significant impact of intrafollicular melatonin concentration on cfDNA content of ff is given in Table 3, which shows that the higher level of intrafollicular melatonin significantly reduces the cfDNA content between groups. Among the analyzed idiopathic patients, no statistically significant differences have existed for the association of cfDNA concentration, and the number of oocytes retrieved, normally fertilized oocytes and early cleaved zygotes. However, when we compared the IVF outcome parameters such as number of MII- oocytes, blastomeres with a regular symmetry, high-quality day 3 embryos and fragmentation rate, we observed that cfDNA content was significantly higher in group A than group B and C (p<0.001) (Table 3). Studying the correlation between cfDNA levels and IVF outcome parameters shows that the highest cfDNA levels are related to the lowest IVF outcome parameters and decreased melatonin concentration (Supplementary Table 3 and Fig.1S).
Table 3
Association between cfDNA concentration in follicular uid of mature follicles and IVF laboratory parameters.

| Characteristics | Intrafollicular cfDNA concentration (ng/µl) in Group A | Intrafollicular cfDNA concentration (ng/µl) in Group B | Intrafollicular cfDNA concentration (ng/µl) in Group C | Kruskal-Wallis test \( (P\text{-value}^*) \) | \( P\text{-value}^{**} \) |
|-----------------|------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|------------------------------------------|------------------|
| Retrieved oocytes (mean ± SD) [(95% population limit)] | 2.91 ± 0.78 [1.5; 2.9] | 2.53 ± 0.43 [1.02; 2.78] | 2.21 ± 0.13 [1.01; 2.5] | 0.421 | - |
| MII- oocytes (mean ± SD) [(95% population limit)] | 2.00 ± 0.86 [0.98; 2.18] | 1.64 ± 0.97 [0.78; 1.93] | 1.53 ± 0.32 [0.98; 1.82] | <0.001 | <0.001\(^a\) |
| Fertilized oocytes (mean ± SD) [(95% population limit)] | 1.86 ± 0.98 [1.21; 1.98] | 1.51 ± 0.65 [0.98; 1.80] | 1.43 ± 0.14 [0.91; 1.73] | 0.214 | - |
| Normal fertilized oocytes (mean ± SD) [(95% population limit)] | 1.81 ± 0.71 [1.14; 1.86] | 1.48 ± 0.88 [0.92; 1.91] | 1.40 ± 0.77 [0.81; 1.69] | 0.143 | - |
| Early cleaved zygote (mean ± SD) [(95% population limit)] | 1.78 ± 0.65 [1.08; 1.81] | 1.32 ± 0.54 [0.87; 1.53] | 1.01 ± 0.62 [0.93; 1.32] | 0.145 | - |
| Blastomeres (6-8 cells) with regular symmetry at day 3 (mean ± SD) [(95% population limit)] | 1.98 ± 0.54 [1.02; 2.31] | 0.98 ± 0.76 [0.19; 1.09] | 0.51 ± 0.98 [0.16; 0.99] | <0.001 | <0.001\(^a\), 0.002\(^b\) |
| High-Quality day 3 embryos (mean ± SD) [median (95% population limit)] | 1.59 ± 0.97 [1.04; 1.94] | 0.88 ± 0.12 [0.15; 1.13] | 0.43 ± 0.31 [0.49; 0.99] | <0.001 | <0.001\(^a\), 0.01\(^b\) |
| Embryos suitable for transfer (mean ± SD) [(95% population limit)] | 1.81 ± 0.56 [1.12; 1.99] | 1.46 ± 0.49 [0.56; 1.01] | 0.91 ± 0.64 [0.71; 0.88] | 0.214 | - |
| Fragmentation rate (≤25%) | 1.91 ± 0.76 [1.03; 1.96] | 0.92 ± 0.97 [0.17; 0.88] | 0.49 ± 0.29 [0.17; 0.53] | <0.001 | <0.001\(^a\), 0.003\(^b\) |

**Note:** \(^a\) Group A/C, \(^b\) Group A/B, \(^c\) Group C/B; Group A (Intra-follicular Melatonin Concentration ≤30 pg/ml), Group B (Intra-follicular Melatonin Concentration >70 to ≤110 pg/ml), Group C (Intra-follicular
Melatonin Concentration >111 to ≤385 pg/ml). Intra-follicular data for patients are also subjected to statistical differences, P* = Kruskal-Wallis test, P** = pairwise comparisons between sub-groups, and p<0.05 considered statistically significant.

**Effect of melatonin concentration on intrafollicular oxidative balance and day 3 embryo quality**

The association of intrafollicular melatonin levels and oxidative stress markers is shown in Supplementary Table 4. The comparison between different subgroups shows significant differences. Besides the expected differences regarding oxidative status, patients with lower melatonin concentration (≤30 pg/ml) have higher levels of oxidative stress markers and exhibited a higher number of impaired quality day 3 embryos than patients with moderate to high levels of melatonin. Of note, TAC levels have markedly increased in superior quality day 3 embryos [median (95% CI; 1164.3 (877.45; 1467.75; p<0.001] developed from oocytes containing higher levels of intrafollicular melatonin (>111 pg/ml). Likewise, they distinctively exhibit a positive correlation (R² = 0.870; p<0.001) (Fig. 2S).

| Table 4 | Expression of miRNAs between the three groups. |
|---------|---------------------------------------------|
| **Group A (Intra-follicular Melatonin Concentration ≤30 pg/ml)** | **Group B (Intra-follicular Melatonin Concentration >70 to ≤110 pg/ml)** | **Group C (Intra-follicular Melatonin Concentration >111 to ≤385 pg/ml)** |
| Raw Ct | ΔCt | RQ | Raw Ct | ΔCt | RQ | Raw Ct | ΔCt | RQ |
| miR-663b | 31.986 | 12.765 | 0.231 | 25.976 | 6.143 | 13.987 | 23.927 | 4.345 | 49.432 |
| miR-320a | 32.098 | 12.987 | 0.227 | 24.987 | 4.823 | 38.945 | 22.987 | 3.545 | 88.766 |
| miR-766-3p | 33.987 | 13.098 | 0.113 | 25.156 | 5.980 | 10.765 | 24.183 | 4.839 | 38.098 |
| miR-132-3p | 33.997 | 13.154 | 0.143 | 24.432 | 4.909 | 42.981 | 23.546 | 4.198 | 44.456 |
| miR-16-5p | 29.688 | 10.213 | 0.9754 | 26.098 | 7.654 | 5.443 | 25.872 | 5.587 | 24.098 |

**Note:** Internal reference miR 16. ΔCt = Raw Ct target miRNA – Raw Ct miR-16. Inclusion criteria: high expression -level miRNAs with (Raw Ct <30 and ΔCt (mRNA) < 10). Low expression -level miRNAs with (Raw Ct >30 and ΔCt (mRNA) >10).
Melatonin mediated miRNAs expression profile in follicular fluid samples is associated with IVF outcome parameters

Under the influence of melatonin, miRNAs have shown different expression patterns that vary from group A to C with the same trend as the intrafollicular melatonin levels differ among the groups (see Fig. 1). As shown in Table 4, the relative expression levels of miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p were significantly upregulated in group B and C (Raw Ct < 30) compared to group A (Raw Ct > 30). In group A the relative expression levels of miR-766-3p and miR-132-3p were significantly reduced (p<0.002) in comparison to group B and C. While the relative expression of miR-766-3p and miR-132-3p in ff of groups B and C were slightly upregulated but did not reach the significant threshold (see Fig. 1). To examine the possible correlation between miRNAs in ff and day 3 embryo quality, we compared cases of high-quality embryos to impaired-quality embryos. The correlation analysis results were summed up in Supplementary Table 5, which shows a significant positive correlation between miRNAs’ relative expression and day 3 embryo quality. In all studied subjects, when we compared the fold change ($2^{-\Delta\Delta\text{Ct}}$) of miRNAs in ff samples between high quality and impaired quality embryos on day 3, we observed that miR-663b (FC = 1.97, p = 0.02), miR-320a (FC = 2.01, p = 0.01), miR-766-3p (FC = 2.52, p = 0.03), miR-132-3p (FC = 2.41, p = 0.04) and miR-16-5p (FC = 1.89, p = 0.05) exhibited significantly different expression levels respectively (see Table S5).

Predictive model for high-quality embryo selection

A predictive model for high-quality embryos is depicted in Fig. 2. Accordingly, the largest areas under the ROC were 0.89 [95% CI; 0.83; 0.96] (p = 0.001) at cut-off values of ≥ 0.79 along with 75% sensitivity and 92.5% specificity for miR-320a and 0.88 (0.82; 0.94), with 95% sensitivity and 72.5% specificity (p = 0.003, at cut-off value ≥ 0.59) for miR-132-3p (see Fig. 2A). These results showed that miR-320a predicts IVF outcome parameters better than miR-132-3p (see Table 5). Likewise, combination of all miRNAs did not improve the AUC of miR-320a (0.89) but enhance the sensitivity to 87% and slightly decrease the specificity to 83% which may offer a potent non-invasive diagnostic tool in the selection of high-quality day 3 embryos (see Table 5). ROC analysis for oxidative stress markers showed that TAC have the highest AUC value 0.85 [95% CL; 0.77; 0.93] with cut-off value of 0.89 and showed 90.2 % sensitivity and 80.3 % specificity (p = 0.001). On the other hand ROS have lowest AUC value 0.65 [95% CI; 0.54; 0.76] at cut-off value of 1.88 exhibited 63.3 % sensitivity and 70 % specificity (p = 0.021) (see Fig. 2B). Furthermore, the AUC value for the combination of all evaluated stress markers was 0.78 with sensitivity of 83.2 % and specificity of 78.3% (p = 0.001) as given in (see Table 5). For melatonin AUC value was 0.85 [cut-off points, 95% CI: 0.80, 0.78; 0.92] with sensitivity 90.7 % and specificity 72.6 % whereas cfDNA have AUC value 0.76 [cut-off points, 95% CI: 0.61, 0.67; 0.85] along with 82.4 % sensitivity and 66.7 % specificity (see Fig. 2C).
Table 5
Predictive values of sensitivity and specificity estimates for the probability of obtaining the best quality embryo develops from mature oocytes.

|                              | AUC [(95% population limit)] | S.E.M | *P-Value | **Cut-off point | Sensitivity | Specificity |
|------------------------------|------------------------------|-------|----------|-----------------|-------------|-------------|
| miR-663b                     | 0.87 (0.80; 0.94)            | 0.036 | 0.002    | ≥ 0.64          | 88.3        | 80          |
| miR-320a                     | 0.89 (0.83; 0.96)            | 0.032 | 0.001    | ≥ 0.79          | 75.0        | 92.5        |
| miR-766-3p                   | 0.86 (0.79; 0.94)            | 0.037 | 0.001    | ≥ 0.57          | 93.3        | 72.5        |
| miR-132-3p                   | 0.88 (0.82; 0.94)            | 0.032 | 0.003    | ≥ 0.59          | 95          | 72.5        |
| miR-16-5p                    | 0.85 (0.78; 0.93)            | 0.039 | 0.001    | ≥ 0.68          | 88.8        | 80.5        |
| Combination of ff miR-663,   | 0.89 (0.71; 0.93)            | 0.034 | 0.001    | -               | 87          | 83          |
| 320a, 766-3p, 132-3p and 16-5p |                              |       |          |                 |             |             |
| ROS                          | 0.65 (0.54; 0.76)            | 0.055 | 0.021    | 1.88            | 63.3        | 70          |
| TAC                          | 0.85 (0.77; 0.93)            | 0.049 | 0.001    | 0.89            | 90.2        | 80.3        |
| LPO                          | 0.83 (0.75; 0.91)            | 0.042 | 0.001    | 0.78            | 91.7        | 72.5        |
| TBARS                        | 0.69 (0.58; 0.80)            | 0.052 | 0.003    | 0.71            | 80          | 40          |
| 8-OHdG                       | 0.75 (0.65; 0.85)            | 0.058 | 0.001    | 0.75            | 93.3        | 60.1        |
| Combination of ff evaluated oxidative stress markers | 0.78 (0.69; 0.85) | 0.045 | 0.001    | -               | 83.2        | 78.36       |
| cfDNA                        | 0.76 (0.67; 0.85)            | 0.047 | 0.001    | 0.61            | 82.4        | 66.7        |
| Melatonin                    | 0.85 (0.78; 0.92)            | 0.037 | 0.001    | 0.80            | 90.7        | 72.6        |

Note: *The null hypothesis was true area = 0.5, after the adjustment of a number of attempts and the number of embryos. P-values in bold letters considered statistically significant p<0.05.

** Estimated cut points that maximize sensitivity and specificity for observed range predictors.
Multitargeting activity of melatonin upregulated miRNAs

MicroRNAs are the genetic switches that fine-tune essential cellular responses and are required to streamline the signal transductions in several cell types. They are depicted as multivalent with single miRNA able to target numerous genes, thus regulating structural and functional molecules’ expression within a pathway. In the present study, we identified several pathways that involve at least one of the studied miRNAs, including cytoskeletal organization, post-translational protein modification, cell cycle, oocyte meiosis, p53 signaling pathway, TGF-beta signaling pathway, and estrogen signaling pathway (see Fig. 3 and 3S).

Discussion

The intrafollicular microenvironment is a highly complex and critical indicator of an individual oocyte's developmental capability to be fertilized and mature into a good quality embryo. Primarily melatonin's positive impact on the intrafollicular microenvironment has been reported through melatonin-mediated gene expression [18]. This is the first attempt in unexplained infertile patients to establish a direct association between melatonin's antioxidant property and its impact on oocyte quality, fertilization rates, and embryo quality by exploring the relative expression patterns of five miRNAs and levels of cfDNA.

Our results established that patients with higher melatonin concentration were associated with elevated 17β-estradiol (E$_2$) levels in their ff samples. E$_2$ is a key player in the final steps of oocyte's nuclear and cytoplasmic maturation [19]. The non-human primate study demonstrated that it can improve oocytes’ developmental competence during in vitro maturation (IVM) [20]. Our study showed that patients with lower intrafollicular melatonin concentration exhibited a considerable oxidative imbalance in their ff (e.g., lower TAC levels and higher ROS, 8-OHdG, and TBARS levels), which jeopardizes the quality of oocytes and thus, hampers the oocyte's maturation along with limiting IVF outcome parameters. These findings follow much earlier research reporting that an imbalance between lipid peroxidation marker (TBARS) and antioxidant system plays a significant role in the pathogenesis of unexplained infertility [21]. Our study's outcome agrees with Jana et al, which revealed a direct relationship between low TAC levels and poor embryo quality and a sharp decline in fertilization rate [22]. Additionally, recent studies validated that decreased melatonin concentration is responsible for reduced TAC levels in the ff samples [23, 24]. These studies suggest that elevated follicular lipid peroxidation and lower TAC levels have a negative impact on IVF outcomes.

Over the past decade, advances in scientific knowledge have established that depreciation in intrafollicular melatonin assets resulted in excessive ROS production, which is responsible for single or double-strand DNA breaks, thus introducing mutations in nuclear DNA and reduces in mitochondrial function [25]. Together with this reference, our study tends to confirm that the concentration of ROS
raised high in those patients who had lower levels of melatonin (≤30 pg/mL) than those who had moderate to higher concentrations in their ff samples. ROS production might be a result of suboptimal intrafollicular microenvironment or impaired metabolism of the developing oocyte. Increasing evidence highlights that melatonin is responsible for the upregulation of specific miRNAs, which control antioxidative enzymes' expression, more likely by acting as a potent free radical scavenger [26]. Similarly, another group reported that melatonin significantly upregulates genes' expression and the formation of proteins responsible for synthesizing antioxidant enzymes. Therefore, compromised antioxidant capacity leads to oxidative stress and flare-up the process of apoptosis [27].

One of the significant obstacles for IVF is oxidative damage to nuclear DNA that can be estimated by assessing intrafollicular cfDNA concentration and levels of 8-OHdG. The increased concentrations of cfDNA and 8-OHdG levels also indicate the embryo's inappropriate quality and often have a low success rate of pregnancy [9, 28]. In the same context, our results showed that intrafollicular cfDNA concentration and 8-OHdG levels were significantly raised in good quality embryos of those patients who have decreased intrafollicular melatonin concentration (≤30 pg/mL). Alternatively, those patients who exhibited a higher concentration of melatonin (>111 pg/mL) in their ff samples have decreased intrafollicular cfDNA concentration and levels of 8-OHdG, which is in agreement with previous observations [29, 30]. Furthermore, in the present study, higher intrafollicular melatonin resulted in an enrichment of high-quality day 3 embryos leading to an increase in the availability of embryos suitable for transfer, ultimately resulting in an augmented future pregnancy rate per embryo transfer [31].

Our exploratory analysis exhibited that miRNAs such as miR-320a, miR766-3p, miR-132-3p, miR-16-5p, and miR-663b were significantly decreasing their relative expression in ff samples that have a lower concentration of intrafollicular melatonin (≤30 pg/mL) and yielded a smaller number of high-quality embryos on day 3. In agreement with our study, Feng et al compared miRNAs from the ff that generate poor-quality and top-quality embryos. They found that miR-132-3p and miR-16-5p were downregulated in the ff containing mature oocytes that produce a higher number of poor-quality embryos than top quality embryos and vice-versa [12, 32]. However, the findings were statistically insignificant, and the effect of intrafollicular melatonin to regulate these miRNAs is mostly unknown. Bioinformatic analysis of studied miRNAs reveals a fundamental role in mediating genes that regulate vital aspects of multiple biological functions. They mediate cell-to-cell communication and target genes associated with follicular development, growth, and oocyte maturation, further indicating that these endogenous messengers have a critical role in oogenesis [33]. Subsequently, the molecular signature based on these miRNAs' differential levels enabled them to participate in the cell junction assembly, TGF-beta signaling, MAPK signaling, Wnt signaling PI3K-Akt pathway, Notch signaling, Estrogen signaling, and Hippo signaling pathways [34, 35].

Our study showed that miR-320 was among the highest expressed miRNA in ff samples, which have intrafollicular melatonin concentration >111 pg/mL. Similarly, Diez-frail et al observed that miR-320 was over the top-ten highest expressed miRNA in ff samples of good quality embryos [36]. Further, they reported that knockdown of miR-320a expression in mouse metaphase-II oocytes resulting in embryos
arrested at first cleavage. While very few can develop into top-quality embryos, indicating that the miR-320a has a potential role in modulating gene expression and regulating embryonic development [37]. Intriguingly, another investigation did not find miR-320 in ff [38]. This inconsistency in the results may be explained by genetic heterogeneity due to the population's different ethnic origins, resulting in varied gene expression in body fluids. Moreover, different stimulation protocols may be responsible for a varied expression of genes or miRNAs. Unsurprisingly, studies focused on the relationship between miR-320a relative expression levels in ff and embryonic development provides additional support to physiological and molecular mechanisms underlying in-vivo and in-vitro fertilization [32, 39, 40]. Evidence from another investigation has indicated that melatonin exerts its antioxidant activities by coordinating crosstalk between miRNAs and interrelated pathways [12].

Additionally, a transcriptome analysis reveals that high quality human preimplantation embryos secrete miR-320a that regulates the decidualized human endometrial stromal cells (hESCs) migration by targeting cell adhesion and cytoskeleton organization [32]. The outcome of their study specifies the promising effect of miR-320a to boost success rates in assisted reproduction. Previously, another piece of evidence indicates that miR766-3p, miR-132-3p, miR-16-5p, and miR-663b have decreased relative expression in the ff samples, yielding impaired quality embryos when compared with the top-quality embryos on day 3 [11]; this is in line with our findings. In the same frame of reference, a recent study conducted by Ragusa et al. confirmed the hypothesis that miR766-3p fine-tunes cellular responses, especially in the control of the cell cycle, play a vital role in the first phase of embryogenesis [41]. Conversely, Fu et al confirmed that miR-663b has a substantial negative correlation in ff of oocytes that produce viable blastocyst than those yielding poor-quality blastocyst [42]. However, the authors of the study did not mention the exact cause of infertility in the subjects. We speculated that it is potentially resulting from vast differences in patients’ selection, detection methodology and biological variability.

This study still holds some significant limitations, as other confounding factors were not addressed. First, the oocyte and embryo quality are associated with morphological scores and the number and composition of chromosomes; therefore, it is imperative to find the numerical chromosome euploidy by Preimplantation Genetic Diagnosis (PGD). Second, we were not able to knockdown miRNAs to assess their actual effects on embryonic development. Third, the primary goal is embryo quality, not live birth. Further studies should be planned to find a relationship between these miRNAs’ expression in ff and chromosomal anomalies.

**Conclusion**

Conclusively, our study showed that melatonin antioxidant capability significantly impacts cfDNA concentration and specific miRNAs relative expression profile in the follicular ambient microenvironment on oocyte development and embryo quality. Therefore, it may be a potent non-invasive diagnostic tool to select high-quality day 3 embryos in the future with such promise. Furthermore, information compiled herein will improve our understanding that physiology of reproduction may categorize these miRNAs as non-invasive biomarkers. These unique miRNAs signature will enhance the quality of embryo selection.
and minimize the chance of multiple gestations, thus, eventually, improve the probability of successful IVF pregnancies. More comprehensive studies should be conducted on a genome-wide scale that must confirm melatonin's effect in the follicular microenvironment to regulate miRNA expression profiles related to oocyte maturation and embryo quality.

**Abbreviations**

IVF: *in vitro* fertilization, AFC: Antral follicle count, AMH anti-Müllerian hormone, PCOS: Polycystic Ovarian Syndrome, cfDNA: Cell-free DNA, ff: Follicular fluid. PGD: preimplantation genetic diagnosis, hESCs: human endometrial stromal cells, TAC: total antioxidant capacity, ROS: reactive oxygen species, SOD: superoxide dismutase, GSH: glutathione, miRNA: microRNA, ROC: receiving operating characteristics, $E_2$: 17β-estradiol, BMI: body mass index, FSH: follicular stimulating hormone, LH: luteinizing hormone (LH), TSH: thyroid-stimulating hormone, TVS: transvaginal ultrasonography, COS: controlled ovarian stimulation, ICSI: intracytoplasmic sperm injection, TBARS: Thiobarbituric acid reactive substances, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, CI: confidence interval.

**Declarations**

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**Authors’ Contributions:**

HLK, YLK Reviewing and Editing, Software; SB Conceptualization, Methodology, Software, Data curation, Project administration, Writing-Original draft preparation. Supervision, Writing-Reviewing and Editing; SA Formal analysis, Validation, Methodology, Investigation, Writing-Reviewing and Editing; CK Writing-
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Figures
Figure 1

Comparison of miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p expression levels relative to the concentration of intrafollicular melatonin. (*) Values indicate significant differences within the expression level of each miRNAs (p<0.001).
A predictive model for high-quality embryos selection based on melatonin concentration, cfDNA levels, oxidative stress markers, and expression patterns of miRNAs in ff samples of unexplained infertile patients. (A) ROC curve analysis to estimate the discriminative significance of ff concentrations of miR-663b, miR-320a, miR-766-3p, miR-132-3p, and miR-16-5p for prediction of good quality embryos. (B) ROC curve analysis to evaluate oxidative stress markers predictive values for good quality embryos selection. (C) ROC curve analysis assesses the predictive values of melatonin concentration and cfDNA levels for good quality embryo selection.
Figure 3

MiRNAs and predictive pathways heat map. Red color indicates high expression and lower p values. Yellow color indicates intermediate expression.

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