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The impact of various antioxidant supplementation on ram's sperm quality, fertilization, and early embryo development, in vitro

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Abstract

The in vitro embryo production (IVEP) is very stressful for gametes. Gametes are subjected during in vitro manipulation to many different types of stress; oxidative stress is the most prominent one, which will cause damage or alter the genetic material of the sperm and reduce the quality of the oocytes, and has a crucial impact on the possibility of developing embryos even after implantation. This study aimed to determine the influence of antioxidants on the achievement of In vitro culture (IVC) and sperm's ability to adhere to and penetrate further into In vitro maturated oocytes. For this purpose, we have incubated ram sperm using four different treatments in terms of antioxidants: melatonin, cysteamine, vitamin C, and vitamin E. They were incubated by the standard methods of maturation and capacitation of sperm. The oocytes were fertilized by spermatozoa that had been capacitated with two groups of fertilization media, the first group containing melatonin and the second group containing cysteamine. Compared with other groups, sperms treated with melatonin demonstrated hyperactivity, and the fertilization rate was significantly increased. As for the IVF medium containing melatonin, it was superior to cysteamine in embryo development rates. In conclusion, melatonin could be a promising tool for improving sperm competence for fertilizing oocytes and embryo development in sheep.

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Introduction

In the last decade, in vitro fertilization and sperm capacitation techniques have significantly advanced. However, only a tiny percentage of Assisted reproductive technology (ART)- produced embryos develop fully (1). Scientists attempting to discover the basic requirements for successful embryo development have a hurdle when using in vitro embryo manufacturing strategies (2). To fertilize the oocytes, sperm must undergo a series of physiological and biochemical alterations in vitro (3). Only capacitated sperm can bind to the oocyte's zona pellucida (ZP) and undergoing the acrosome reaction (4). Oxidative stress is one of the key factors contributing to a decrease in sperm quality during the maturation and capacitation of the sperm (5). Oocytes can also be exposed to high levels of free radicals during in vitro fertilization (IVF) due to the surrounding dead spermatozoa; thereby, oocytes and sperm are highly vulnerable to reactive oxygen species (ROS) attacks, affecting their quality and viability (6). ROS are free radicals that are continuously produced by oxidative metabolic reactions and increase in vitro conditions, causing aging of both the oocyte (7) and sperm (8), as well as perhaps impairing fertilization and embryo development. Oocytes, sperms, and embryos are shielded from oxidative stress in their natural environment by free radical scavengers found in oviductal and follicular fluids. Oocytes and embryos are not provided with this level of protection when in vitro production (9). As a result, developing a culture medium capable of effectively reducing ROS in vitro embryo production (IVEP) protocols is of tremendous importance (10).

There is a growing interest in melatonin and cysteamine as antioxidants for IVEP (11). The antioxidant power of melatonin is 5-15 times that of glutamine. Glutamine is an

amino acid that plays a crucial role in cell metabolism and biosynthesis (12). Furthermore, glutamine helps cells reduce oxidative stress (13). Melatonin is a chemical messenger that quickly crosses through cellular membranes (14). Melatonin acts as an antioxidant by directly destroying free radicals, indirectly increasing antioxidant enzymes, and inhibiting peroxidation enzymes like nitric oxide synthetase (15). Melatonin supplementation has been shown to promote bovine and porcine oocytes' maturation rate and increase their blastocyst of IVF embryos (16). These findings are linked to melatonin's ability to reduce ROS and its adverse effects on oocytes, such as aging, apoptosis, and its capacity to bind to specific DNA sequences and interact with cytosolic molecules to regulate antioxidant genes (17). During sperm capacitation, melatonin may have a chemotactic effect that helps guide sperm to fertilization. Melatonin has also improved sperm motility and sperm agglutination (18). Melatonin presumably performs these functions in the sperm via binding to the MT2 and MT1 receptors that belong to the G-protein-coupled receptor (GPCR) superfamily; spermatozoa contain these receptors (19).

Thiol substances like cysteamine or mercaptoethanol can promote embryonic growth by stimulating glutathione synthesis and lowering H2O2 levels. Cysteamine (NH2-CH2-CH2-SH, β-mercaptoethylamine) is a chemical that affects animal endocrine and metabolic function. By causing intracellular cysteine buildup, this substance exerts antioxidant and anti-apoptotic properties (20).

For sheep IVPE media, many researchers have utilized many antioxidants like cysteamine, Vit E, Vit A, and Vit C to detect their impact on IVEP (21). The addition of cysteamine to the maturation medium improved nuclear maturation and the fertilization rate of sheep oocytes (21). Many studies in buffalo have discovered that cysteamine supplementation improves nuclear maturation rates by increasing GSH synthesis and improving male pronucleus formation (22). Vit C has also been demonstrated to play a role in buffalo oocytes' in vitro maturation and developmental competence; this effect was enhanced with the added cysteamine (23). The addition of antioxidants to bovine sperms inhibits the overproduction of reactive oxygen species, enhancing the antioxidant ability of sperm, improving in vitro fertilization (IVF), and embryo development outcomes (24).

To our knowledge, no literature exists that shows the impact of melatonin and cysteamine as antioxidants on sperm capacitation, but a few show the effect of melatonin and cysteamine on the IVM of sheep. As a result, an experiment was implemented to assess the impact of (1) Cysteamine and Melatonin to sperm capacitation medium and IVF medium on IVF and embryonic development, and (2) Using traditional antioxidants in the sperm capacitation medium, such as vitamin C and E, and comparing their effects on IVF and embryonic development with melatonin and cysteamine.

Materials and methods

Ethical Statement

Scientific Ethical Committee on Animal Experimentation at universities and research institutions in Turkey should supervise scientific studies on live vertebrate animals. Since our study was performed using only slaughtered animal material collected from the slaughterhouse, there was no need to apply it to the Scientific Ethical Committee on Animal Experimentation.

Recovery of oocytes and in vitro maturation

Post-mortem ovaries were collected from adult sheep (various Turkish breeds) from a local slaughterhouse during the winter season and transported to the laboratory at four °C in the transport medium Normal Saline (NS). Ewe ovaries were manipulated 1 to 3 h after the post-mortem. Ovaries were sliced with a scalpel to obtain the cumulus-oocyte complexes (COCs), were then washed in Oocyte Collection Medium (OCM) TCM-199 with HEPES (N-2-Hidroxyethylpiperazine-N-2-ethane sulfonic acid) supplemented with sodium bicarbonate (S5761, Sigma) 0.005 mol/L, 1 g/mL Heparin (H3149, Sigma), 0.5 mg/ml glutamine (A8185, Sigma), and 10 mg/mL gentamycin (GEN-10B, CAPRICORN). Oocytes were graded according to the character of the cumulus cells during the wash (25); grade A COC oocytes were surrounded by five or more layers of compact cumulus cells. Oocytes surrounded by two to four layers of cumulus cells were classed as grade B, which was less compact. On the other hand, grade C oocytes were either half-naked or enclosed by less than two cumulus cell layers. Were chose oocytes that had two or more layers of compact cumulus cells and homogeneous cytoplasm. (Figure 1). Then oocytes were transferred in groups of 30–50 into a 50 µl micro drop of IVM medium\ HEPES-buffered TCM-199 medium (Gibco, Life Technologies, Milan, Italy). They were also supplemented with 0.36 mmol/L Na Pyruvate (P4562, Sigma), 1 IU/mL follicle-stimulating hormone (FSH) and 1 IU/mL luteinizing hormone (LH) (Plusnet; Bio98, Milan, Italy), 10 mg/mL of gentamycin (GEN-10B, CAPRICORN), 750 µM of Glutamax (35050-061, Life Technologies), and 10% Fetal Bovine Serum (FBS) (FBS-16A, CAPRICORN) covered with mineral oil (M8410, Sigma), for 24 hrs at 38.5 °C in a 90% relative humidity with 5% CO_2.

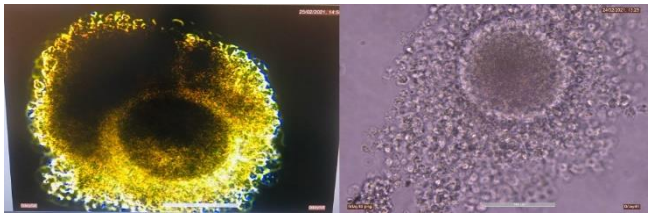

Figure 1: Oocytes surrounded by several layers of cumulus cells × 40.

Sperm collection and preparation

Fresh testes-epididymides were recovered in the scrotal sacs of mature rams slaughtered at a local abattoir. Individually packed testes were placed on ice and brought to the laboratory in an ice chest for processing. The epidermis tail is rinsed with a protective Phosphate Buffered Saline (PBS) (P-5493, Sigma) to eliminate blood clots and debris upon arrival at the laboratory. After that, the surface of the epididymis tail was removed via a sharp scalpel, and the epididymal contents were aspirated using a sterile syringe of 5 ml. For sperm maturity, cauda epididymides contents were collected and disseminated in HEPES-TL medium (26) with four different treatments; The first treatment was the culture medium (CAP+C) containing 50 µM Vit C (A4403, Sigma). In the second treatment, the contents of the epididymis were added to the (CAP+E), which contains 50 µM Vit E (Sigma). In the third treatment, the epididymis contents were added to the culture medium (CAP+M), containing 0.5 µM Melatonin (M5250, Sigma). Finally, in the fourth treatment, epididymis contents were added to the culture medium, which contained 100 µM cysteamine (M9768, Sigma). The concentrations of antioxidants applicable in the study according to (27,28).
Ram sperm were cultured for roughly 4 hrs at 37 °C in a humidified atmosphere with 5% CO_2 in the air. Samples were examined for sperm quality and assessed according to individual and collective movement of spermatozoa. Samples with less than 60% individual motion were rejected (29).

In vitro fertilization

Twenty-four hours post IVM, COCs were observed under a LEICA inverted microscope (LEICA DM IL LED; Wetzlar, Germany), and only the COCs that presented an expansion of cumulus cells or oocytes with the first polar body were selected for IVF (30) (Figure 2 A and B). Oocytes were collected and mechanically denuded cumulus cells. Oocytes were randomly divided into two groups, group 1 IVF medium (BO) (31) supplemented with 100 µM cysteamine (IVF+Cys). In contrast, group 2 supplemented 0.5 µM Melatonin (IVF+M). The oocytes were then subjected to in vitro fertilization by four treatments for the sperm capacitation media, each treatment separately at a temperature of 38.5 °C, 5% CO_2, and 90% relative humidity for 24 hrs. Oocytes were evaluated as fertilized oocytes with a second polar body or oocytes with sperm heads in the cytoplasm 24 hrs after insemination (29).

In vitro embryo culture

Zygotes were cleaned three times and put into well plates containing 500 µL droplets of Synthetic Oviductal Fluid (SOF) medium (26) supplemented with bovine serum albumin (BSA) (3 mg/mL) (A6003, Sigma) under mineral oil (M8410, Sigma). Embryos were incubated for eight days at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 air with 90% relative humidity. At 48 hrs after IVF, the division rate was assessed, and the blastocyst formation rate was measured under a LEICA inverted microscope (LEICA DM IL LED; Wetzlar, Germany) at 24 hrs intervals (Figure 3).

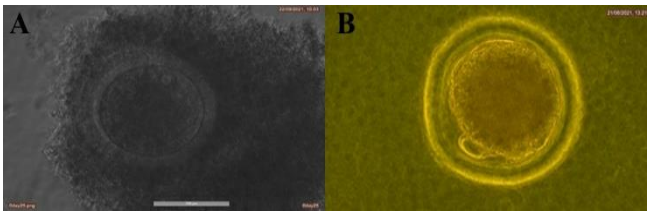

Figure 2: (A) Matured oocyte with an expansion of cumulus cells × 40. (B) Matured oocyte with the first polar body × 40.

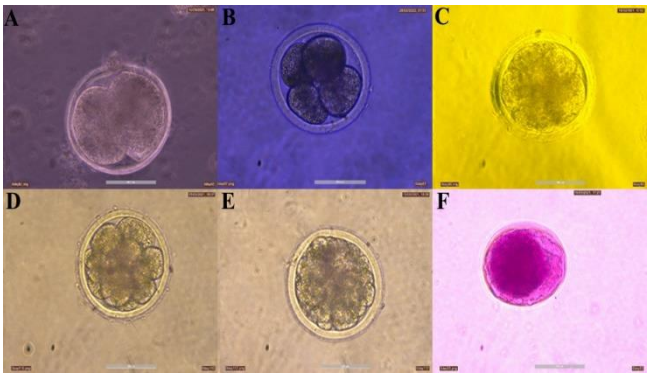

Figure 3: (A) 2- cells embryo × 40. (B) 4- cells embryo × 40. (C) 8- cells embryo × 40. (D) 10- cells embryo × 40. (E) Morula× 40. (F) Blastocyst × 40.

Statistical analysis

The experiment was replicated five times. Data were analyzed by one-way ANOVA using SPSS version 23.0 statistical software, and results are presented as the mean (± SEM). Duncan's Multiple Range Test was used to compare differences between treatments, and P-value < 0.05 was considered significant.

Results

A total of 1097 oocytes were collected, with an average ovary recovery rate of 2.54 (grade A and B oocytes). As shown in Table 1, a significant superiority was observed (60.93%, P <0.05) for (CAP+M) regarding fertilization rate. The other three groups (CAP+E, CAP+Cys, and CAP+C) have shown 53.27%, 44.52%, and 35.86% of fertilization rates, respectively. The group (CAP+C) exhibited the lowest fertilization percentage (35.86%) compared to the others in the IVF+Cys media.

Table 2 shows the cleavage and morulae rates were higher in (CAP+M) treatment (43.58% vs. 25.64%, P <0.05), respectively, compared to the other treatments. A similar result has been shown in (CAP+E) group. On the other hand, the cleavage rates were significantly decreased in each of the (CAP+E, CAP+ Cys, and CAP+C) treatments. The overall

cleavage per morulae rates was found as (34.42% and 28.84%), (11.47% and 9.61%) respectively. A significant superiority was observed in (CAP+M) treatment regarding the rate of blastocyst formation compared to (CAP+E) (7.69% vs.1.53%, P <0.05).

The results showed a significant increase in the percentage of fertilization in (CAP+M) compared to the other treatments (77.69%, P <0.05), there is no significant difference in this percentage on fertilized oocytes by (CAP+E and CAP+Cys) groups (56.75% and 54.10%), respectively. In IVF+Melatonin media, this also applies to the (CAP+C) treatment, where there are no significant differences among CAP+E and CAP+Cys, with a fertilization rate equal to 49.24% (Table 3).

Regarding the IVF+M group, the study results showed a significant increase in the percentage of cleavage and morulae (P <0.05) in (CAP+M) compared to the other treatments (72.22%) and (56.48%) respectively. There are no significant differences between (CAP+E), (CAP+ Cys), and (CAP+C) in the cleavage and morulae percentage of oocytes (59.52%, 54.43%, 43.07%), and (38.09%, 30.37, 20%), respectively. A significant superiority (P <0.05) was observed in the (CAP+M) treatment regarding the rate of blastocyst formation of (25%). Moreover, results indicate that the blastocyst formation rate was similar in (CAP+E) and (CAP+Cys), 14.28% and 11.39%, respectively (Table 4, Figure 4).

Table 1: Impacts of sperm capacitation treatments on fertilization rate (%) in IVF+Cysteamine media

Medium	Treatment	No. of oocytes	No. of fertilized oocytes	Fertilization rate (%)
IVF+ Cys	(CAP+C)	145	52	35.86±1.16 d
	(CAP+E)	122	65	53.27±2.00 b
	(CAP+M)	128	78	60.93±2.24 a
	(CAP+ Cys)	137	61	44.52±1.52 c

Mean values in the same column with different superscripts differ significantly at P<0.05.

Table 2: Impacts of sperm capacitation treatments on embryonic development rate (%) in IVF+Cysteamine media

Medium	Treatment	No. of fertilized oocytes	Cleavage rate (%)	Morula rate (%)	Blastocysts rate (%)
IVF+Cys	(CAP+C)	52	28.84±0.63 b	9.61±0.54 b	-
	(CAP+E)	65	35.38±0.81 ab	16.92±1.01 ab	1.53±0.20 b
	(CAP+M)	78	43.58±0.80 a	25.64±1.00 a	7.69±0.48 a
	(CAP+Cys)	61	34.42±0.96 b	11.47±0.50 b	-

Mean values in the same column with different superscripts differ significantly at P<0.05.

Table 3: Impacts of sperm capacitation treatments on fertilization rate (%) in IVF+Melatonin media

Medium	Treatment	No. of oocytes	No. of fertilized oocytes	Fertilization rate (%)
IVF+M	(CAP+C)	132	65	49.24±1.58 bc
	(CAP+E)	148	84	56.75±2.35 bc
	(CAP+M)	139	108	77.69±2.56 a
	(CAP+ Cys)	146	79	54.10±2.26 bc

Mean values in the same column with different superscripts differ significantly at P<0.05.

Table 4: The impacts of sperm capacitation treatments on embryonic development rate (%) in IVF+Melatonin media

Medium	Treatment	No. of fertilized oocytes	Cleavage rate (%)	Morula rate (%)	Blastocysts rate (%)
IVF+M	(CAP+C)	65	43.07±1.16 b	20±1.02 b	-
	(CAP+E)	84	59.52±1.51 b	38.09±1.02 b	14.28±0.92 b
	(CAP+M)	108	72.22±2.54 a	56.48±2.08 a	25±0.89 a
	(CAP+ Cys)	79	54.43±1.69 b	30.37±1.42 b	11.39±1.09 b

Mean values in the same column with different superscripts differ significantly at P<0.05.

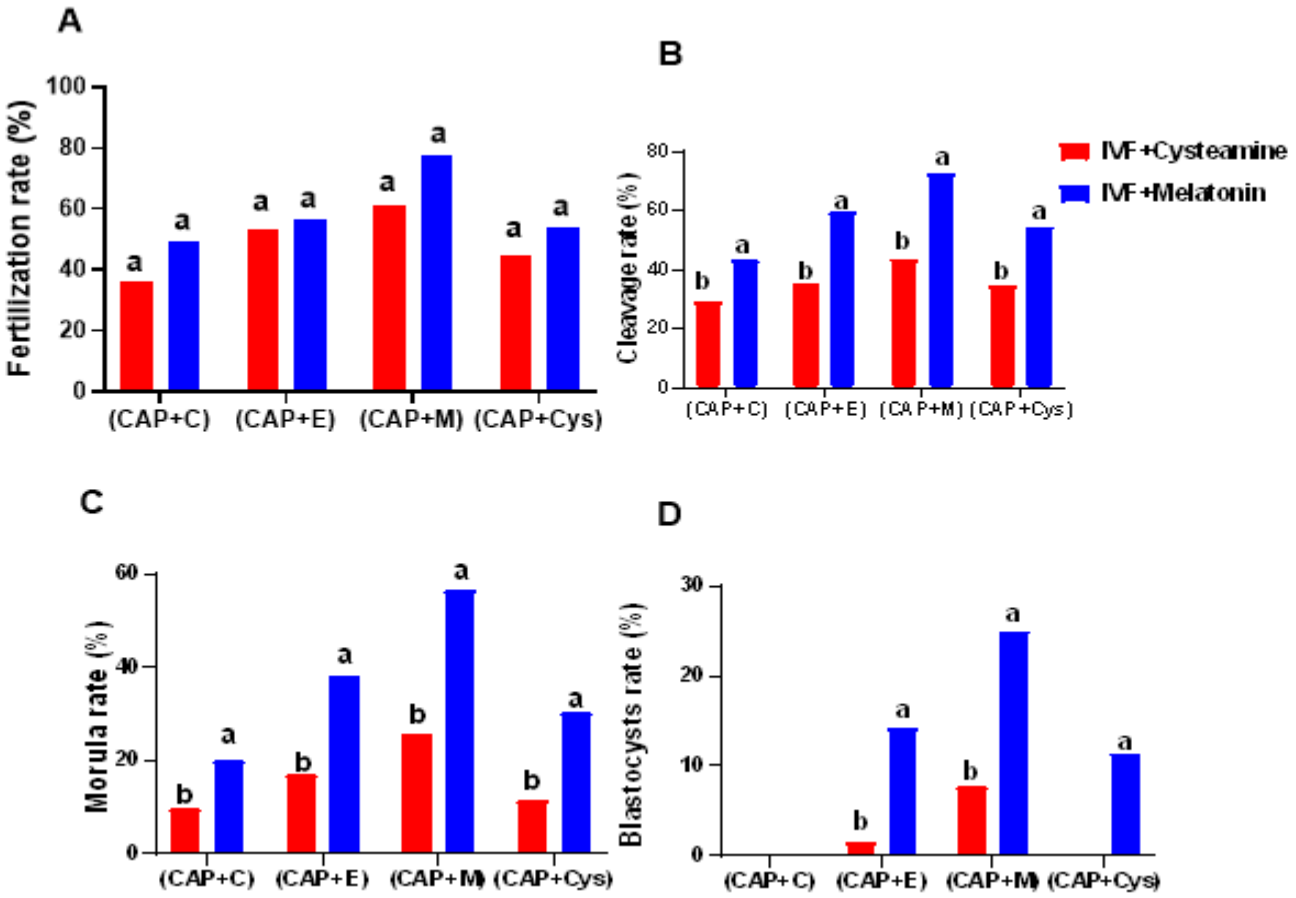

Figure 4: The relationship between sperm capacitation medium and IVF medium supplemented with antioxidants on fertilization and embryonic development rate (%). (A) Mean fertilization rate (%) of total oocytes in the different treatments. (B) Mean cleavage rate (%) of fertilized oocytes in the different treatments. (C) Mean morula rate (%) of fertilized oocytes in the different treatments. (D) Mean blastocysts rate (%) of fertilized oocytes in the different treatments. Different superscript letters (a–b) in each column represent statistical significant differences (P<0.05).

Discussion

We investigate if antioxidants would improve embryonic development by adding them to sperm capacitation media. This study involved adding melatonin, cysteamine, Vit C, and Vit E to rams' sperm capacitation and IVF media and analyzes its effect on sperm function and embryonic development rates.

The low levels of reactive oxygen species (ROS) for sperm and oocytes are required for their physiological importance of reproductive processes. The low free radical levels modulate gamete function, particularly capacitation and chemotactic acquisition by spermatozoa, the activation of oocytes by sperm, the activation of the embryonic genome, and the hatching of blastocysts from the zona pellucida (ZP), which are essential elements of the fertilization process (32).

ROS elevated levels can overwhelm the limited antioxidant defense of sperms, precipitating a state of oxidative stress (33). Oocytes and sperms, in reality, have a defensive mechanism to defend themselves from oxidative stress (21); despite this, if the formation of ROS and the availability of antioxidants are out of balance during gamete coincubation in IVEP processes, it can cause cellular damage and even early embryo mortality (34).

As a result, it has been hypothesized that adding antioxidants to sperm capacitation media and IVF media conventional potentially reduces oxidative stress, enhancing the efficiency of the IVEP process and the quality of the embryos.

Our data revealed that capacitation and IVF media with melatonin significantly increased embryonic development rates. These results are confirmed by previous studies, which elucidate that melatonin increases morula and embryonic development rates in mice (35) porcine embryo cultures (36), which are significantly helpful in porcine IVM media. Our findings align with previous research on Human IVF (37) bull spermatozoa function (38). These outcomes contrast

with a report (39) that notes that melatonin reduces blastocyst and hatching blastocyst rate in bovine.

Melatonin is a powerful antioxidant that has been shown to preserve gametes (40) and increase Ca2+ levels through increasing the IP3R and IP3 receptors on the endoplasmic reticulum, as well as cAMP (41).

Indeed, melatonin receptors stimulate various pathways (MEK 1/2, ERK 1/2, PI3K/Akt) (42) that are important for sperm function, particularly capacitation (38). Melatonin membrane receptors may be found in every mammalian spermatozoon (19). These explanations may be why the sperm in this study has a higher capacitation level.

In this study, Vit C and cysteamine were non-responders. This may be because the doses of Vit C and cysteamine were not meeting the antioxidant needs of the rams' sperm. These results are consistent with a study in mice where it was reported that 100 µM of Vit C could not protect the particle integrity in sperm exposed to BPA (43). Moreover, another report reported that Vit C aids in the in vitro maturation and developmental efficiency of buffalo oocytes provided that cysteamine is added to enhance this effect (23). In contrast, studies found that adding cysteamine or Vit C to the culture media improved motility in buck testicular sperm (44) boosted the rate of bovine blastocysts following IVF substantially (45).

Our results indicate that adding Vit E to the sperm capacitation medium could benefit the overall stimulation of spermatozoa activity. Results were consistent with a study on bovine, where Vit E positively affected spermatozoa protection against free radical production (46).

The melatonin-treated group resulted in higher regular fertilization and lower polyspermy rates (Table 3). It was corresponding with the report of (47), who claimed in vitro developed cumulus-oocyte-complexes fertilized with spermatozoa that had been preincubated with melatonin demonstrated increased rates of monospermic fertilization, reduced polyspermy, and improved embryo development by maintaining the amounts and localization of lovastatin and Juno's fertilization proteins (48).

Conclusion

Adding melatonin to the sperm-preparation protocol for IVF enhances sperm fertilization ability and embryo development in vitro. Selecting more suitable doses of Vit C and cysteamine should be investigated in future studies. Melatonin has a beneficial impact on embryonic development following fertilization. The melatonin + IVF group continued to present a positive trend until day 7 of culture, with a considerable improvement in blastocyst development compared to the Cys+IVF group. This study implies that spermatozoa may have a more significant impact on determining early embryos' fate than previously considered. As a result, more research on this hypothesis is required.

Competing interests

The author declares that there is no conflict of interest.

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تأثير اضافة مختلف مضادات الأكسدة على جودة الحيوانات المنوية، التخصيب والتطور المبكر للجنين، في المختبر

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الخلاصة

يعتبر إنتاج الأجنة في المختبر مجهدا جدًا للأمشاج. تخضع الامشاج أثناء معاملتها في المختبر لأنواع مختلفة من الإجهاد؛ ومن أبرزها الإجهاد التأكسدي، حيث يتسبب في تلف أو تغيير المادة الوراثية للحيوانات المنوية ويقلل من جودة البويضات، وله تأثير حاسم على إمكانية نمو الأجنة حتى بعد الزرع. هدفت هذه الدراسة إلى تحديد تأثير مضادات الأكسدة على الزرع المختبري وقابلية الحيوانات المنوية على الالتصاق والاختراق للبويضات التي تم انضاجها في المختبر. لهذا الغرض، قمنا باحتضان الحيوانات المنوية للأكباش باستخدام أربع معالجات مختلفة من مضادات الأكسدة: الميلاتونين والسيستامين وفيتامين جـ وفيتامين هـ. حضنت الحيوانات المنوية باتباع الطرق القياسية للإنضاج والمهيئة للإخصاب. تم إخصاب البويضات بواسطة الحيوانات المنوية التي تم انضاجها باستعمال مجموعتين من اوساط الإخصاب، المجموعة الأولى تضمنت الميلاتونين والمجموعة الثانية على السيستامين. بالمقارنة مع المجموعات الأخرى، أظهرت الحيوانات المنوية المعالجة بالميلاتونين فرطا بالفعالية، وزيادة معنوية في نسبة الإخصاب. أما بالنسبة لوسط التلقيح الاصطناعي الذي يحتوي على الميلاتونين، فقد كان متفوقًا على السيستامين في نسب نمو الاجنة. بالخلاصة، يمكن أن يكون الميلاتونين أداة واعدة لتحسين كفاءة الحيوانات المنوية لتخصيب البويضات وتطور الجنين في الأغنام.