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Research

Effect of Transport Condition on the Structural Integrity of Ovarian Tissue and the Development of Sheep Embryos *In-Vitro*

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Abstract: The oocyte quality decreases during ovarian tissue transport to the laboratories of in vitro embryo production. To provide additional information on how the conditions of transporting sheep ovaries impact the ovarian tissue and oocytes' ability to develop into blastocyst stages, we have studied new transport media Ankara University Zootekni (AUZ1, AUZ2) supplemented with antioxidants (melatonin, Vit E, and Vit A), buffer solution, and energy substrates, and compared them with the traditional transport media: Phosphate-Buffered Saline (PBS), and Charles Rosenkrans 1 (CR1), Normal Saline (NS) at different temperatures (-6 to 30 °C). We also studied and compared how well different transport media preserve the ovarian tissue's structural integrity while transporting sheep ovaries at 4°C. Our findings indicated that various temperatures and transport media play critical roles in embryo development. The embryo development rates showed that when sheep ovaries are transported in AUZ1, they produce oocytes with a higher embryo development rate than other transport media at any temperature. In addition, histology examination revealed that the transport of sheep ovarian tissue in any medium at a temperature of 4 °C did not negatively impact the viability and histomorphology of the primordial, primary, and secondary follicles. In contrast to other transport media, the AUZ1 medium maintained the normal morphology of antral follicles, Graafian follicles, and the cumulus oophorus of sheep ovarian tissue. In conclusion, adding melatonin, buffer solution, and energy substrates to the transportation medium of ovarian tissues has a beneficial and positive role in maintaining ovarian tissue and increasing the rates of embryonic development.

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1. Introduction

The improvement of the efficiency of the in vitro embryo production (IVEP) protocols in sheep requires the use of ovaries from slaughtered animals because a significant number of oocyte samples must be obtained (García-Álvarez et al., 2011). Oocytes obtained from slaughtered animals have lower developmental potential than those retrieved from living animals (Jiao et al., 2016). Oocytes can be

harvested from live goats and sheep by ovum pick-up (OPU) methods (Wieczorek et al., 2014). However, this procedure needs technical competence and comes at a hefty price (Galli et al., 2014).

Since the quality of cumulus-oocyte complexes (COC) influences the developmental potential of embryos following in vitro fertilization (IVF), the maintenance of oocyte integrity from the time an animal is slaughtered and during the ovary transport from the slaughterhouse to the laboratory is crucial (Martín-Maestro et al., 2020). Medium composition, temperature, and storage period are all recognized to play a role in organ preservation (Combelles et al., 2009). Low oocyte in vitro maturation ratio and developmental competence could be caused by improper transportation and conservation circumstances (Wang and Sun, 2006). Often, slaughterhouses are located far from research centers requiring extended periods of preservation of the ovaries collected which may threaten the oocyte viability (Tellado et al., 2014).

The female reproductive organs (ovaries) have one of the highest blood flow rates per unit of tissue of any adult mammalian organ, as well as a fast metabolic rate (Reynolds et al., 2002). During the transportation to the laboratory, blood flow would be blocked, lowering oxygen delivery to the ovaries, and causing them to become ischemic (Wongsrikeao et al., 2005). Ischemia affects the viability of follicles in the ovaries, with oxygen-free radicals being pivotal contributors to ovary damage during preservation (Cruz et al., 2014). Low antioxidant enzymes and reactive oxygen species (ROS) generated in the follicular milieu due to ischemia may activate programmed cell death during transportation of the ovaries.

Several antioxidants have been used to reduce the harmful effects of ROS in the follicular milieu during ovary transportation (Sánchez-Ajofrín et al., 2020; Soto-Heras and Paramio, 2020). The protective properties of melatonin as a potent direct scavenger of free radicals have been well-documented (Tian et al., 2014). These therapeutic effects have been related to its ability to decrease pro-apoptotic and increase anti-apoptotic gene expression, as well as its ability to neutralize ROS. Under hypoxic conditions, glycolysis is the most common anaerobic pathway for ATP synthesis, resulting in lower pH and higher lactate levels (Guibert et al., 2011). Hypoxic and acidic environments adversely affect the net electric charge, cell membrane permeability, and chromosomal instability (Schomack and Gillies, 2003). As a result, the absence of buffer systems in the ovarian tissue transportation medium could significantly influence the oocyte's eventual developmental ability (Bohlooli et al., 2015). The removal of organs from the blood supply exposes them to induced hypothermia which protects them from damage (Guibert et al., 2011). At low temperatures, the metabolic activities of cells are reduced or entirely stopped, and the metabolic rate is bisected for every 10 °C reduction in temperature. As a result, the remaining metabolic rate at 4 °C is around 10 % of normal condition (Cantu and Zaas, 2011). A study has found that transporting ovaries at 38 °C for several hours reduces the blastocyst rate in several animals (Naoi et al., 2007).

With this background, this study hypothesized that the use of unconventional transport medium may have beneficial effects by maintaining the follicular quality of ovaries. Therefore, the first part of this study was designed to assess the impacts of five different ovaries transport media: Phosphate-Buffered Saline (PBS), Charles Rosenkrans 1 (CR1), Normal Saline (NS), Ankara University Zootekni 1 (AUZ1), and Ankara University Zootekni 2 (AUZ2) at four different temperatures (-6 to -4, 2 to 4, 8 to 12, and 24 to 30 °C), on the embryo development rate. The second part of this study was designed to compare the efficiency of NS, PBS, CR1, AUZ1, and AUZ2 transport media in maintaining the structural integrity of sheep ovarian tissue during transport at 4 °C.

2. Material and Methods

In this study, IVEP was performed at the Reproductive Biology and Animal Physiology Laboratory, Ankara University, Turkey. Histological analysis of the ovarian tissue was performed at the Department of Histology and Embryology, Faculty of Veterinary Medicine, Ankara University. Unless mentioned otherwise, all chemicals used in this study were purchased from Sigma-Aldrich Chemical Company. Materials procured from other companies are described in the relevant sections.

2.1. *In vitro* embryo production

Post-mortem ovaries (n = 825) were collected from adult sheep of various Turkish breeds from a local slaughterhouse from January 2022 to April 2022; the range of temperature on the days of the

collection was (-4–30 °C), and the days of ovaries collection was 28. The ovaries were randomly distributed into 100 ml tubes containing 60 ml of NS as the control medium (154 mM NaCl (S5886)), PBS (137 mM NaCl, 2.70 mM KCl (P5405), 8 mM Na_2HPO_4 (S5136), and 2 mM KH_2PO_4 (P5655)), CR1 (135 mM NaCl, and 10 mM KCl), AUZ1 (108 mM NaCl, 7 mM KCl, 3.6 mM $NaHCO_3$ (S5761), 16.9 mM L-Glutamine (G8540), 520 mM TRIS (252859), 1.11 mM Glucose (G7021), 1.5 µl/ml Na Pyruvate (P4562), and 1 µl/ml Melatonin (M5250)), or AUZ2 (51 mM NaCl, 2.7 mM KH_2PO_4 (P5655), 1.15 mM Sucrose (S0389), 1 µl/ml Vit E, and 1 µl/ml Vit A) at -6 to -4, 2 to 4, 8 to 12, or 24 to 30 °C. The temperature was continuously monitored with thermometers during transportation. Then the ovaries were transported to the laboratory in an ice chest 1 to 2 h post-mortem. The cumulus-oocyte complexes (COC) were collected by aspiration from visible follicles with a 3–5 mm diameter, and oocytes with two or more compact cumulus cell layers and homogeneous cytoplasm were chosen. Then, the oocytes were placed in groups of 40–60 into 500 µl of IVM medium based on TCM-199 supplemented with: 0.36 mmol/L Na Pyruvate (P4562), 5 µl/ml gentamycin (GEN-10B), 750 µM Glutamax (35050-06), 1 IU/ml FSH (Life Technologies),1 IU/ml LH (Bio98), and 1% FBS (16A) in a 4-well dish (Nunc) for 24 h at 38.5 °C in a humidified atmosphere with 5% CO_2. At the end of the IVM period, the oocytes were evaluated under an inverted microscope (LEICA DM IL LED; Wetzlar, Germany), and only those that had expanded cumulus cells were selected for in vitro fertilization (IVF) (Nikiforov et al., 2020). The oocytes were washed twice in BO (IVF Bioscience, 71001) medium 40-60 oocytes were placed in 500 µl of BO medium supplemented with 5 µl/ml gentamycin, 1.25 mM sodium pyruvate, 5 mg/ml BSA (A3311), 1 µl/ml heparin (P4562), 40 µl/ml D- penicillamine, Hypotaurine and epinephrine (B2794), 2 mM caffeine, and 10% FBS. For IVF, sperm cells were examined for quality and assessed according to individual/collective motility (Majeed et al., 2019) and viability. The COC was fertilized with sperm concentrations of 7 x 106/ml and co-incubated at 38.5 °C in 5% CO_2 and 90% humidity. After IVF, the oocytes were evaluated as fertilized when showing a 2nd PB or sperm heads in the cytoplasm (Arat et al., 2016). After 18 h of the IVF, cumulus cells were partially removed using 1 µl hyaluronidase (H3884) followed by pipetting. The resulting zygotes were cultured in 500 µl of SOF medium supplemented with 4 mg/ml BSA, 10 µl/ml glutamax, 10 µl/ml sodium citrate, 10 µl/ml Non-Essential AA (M7145), 20 µl/ml Essential AA (B6766), 10 µl/ml Myo-inositol (P4562), 0.5 µl/ml Na pyruvate, and 5 µl/ml gentamycin (GEN-10B), and incubated at 38.5 °C, 5% CO_2, and 90% humidity. After 48 h of IVF, the cleavage, morula, and blastocyst rates were evaluated at 24 h intervals using an inverted microscope (LEICA DM IL LED; Wetzlar, Germany) (Ferraz et al., 2018).

2.2. Histomorphology of ovaries

After slaughtering the animals, the ewes' abdomen was opened, the ovaries removed, collected (n = 120), and randomly assigned to the different transport media (PBS, CR1, NS, AUZ1, and AUZ2) at 4 °C. The samples were transported to the laboratory and fixed for 24 hours in a container with 10% neutral buffered formalin. The samples were rinsed in distilled water after fixing for 24 hours and dehydrated in a graded alcohol series (70%, 80%, 95%, 100% ×3), then were cleared in xylene (×3), embedded in paraffin, and sectioned into 5 µm thick slices. For each block, 8 serial sections were collected per slide with 100 µm (20 sections) discarded between slides. The slides were stained with Masson's trichrome (hematoxylin, acid fuchsin, and aniline blue) (MT, Sigma Aldrich) to determine the morphological integrity of ovarian tissue. They were examined under a light microscope (Leica DM2500, LEICA Microsystems GmbH, Wetzlar, Germany) and photographed with a digital microscope camera (Leica DFC450, Leica Microsystems GmbH, Wetzlar, Germany). Diameter and density measurements were not included in this assessment. The follicles were classified according to the method developed by Chaves et al. (2008) and Youm et al. (2014). The following morphological standards were used to assess the follicles: basement membrane integrity, granulosa cells (GC), oocyte, and cell density. The follicles were then categorized as normal (spherical oocyte, homogenous cytoplasm, well-organized GC) or degenerative (deformed and disordered GC, poor cell density). Sections selected for analysis were distributed throughout the tissue and all sections were assessed blindly.

2.3. Statistical analysis

The data were expressed as the mean (±S.E.M). Data analysis was done using two-way ANOVA in the SPSS version 23.0 statistical software. Duncan's Multiple Range test was carried out to compare the mean values, and p-values < 0.05 were considered significant.

3. Results

The AUZ1 transport medium at 2 - 4 ºC exhibited 72.5% ($p<0.05$) fertilization rate and embryo development with a significant superiority over the other treatments (Table 1). The AUZI medium also showed a significant superiority (60.83%, $p<0.05$) in IVF rate at 8 - 12 ºC when compared with the other transport media. At -6 - -4ºC, the fertilization rates showed no significant differences ($p> 0.05$) among transport media. Similarly, there were no significant differences in IVF rates ($p>0.05$) among the employed transport media at 24 - 30 ºC.

Table1. Impacts of different temperatures and transport media on the IVF rate and embryo development

Temperature	Media	No. of oocytes	No. of fertilized oocytes	IVF rate (%)	Cleavage rate (%)	Morula rate (%)	Blastocysts rate (%)
-6 - -4 ºC	NS	120	48	40±1.63 ^{a}	39.58±1.24 ^{a}	14.58±0.37 ^{ab}	3.77±0.24 ^{ab}
	PBS	120	40	35.33±1.78 ^{a}	37.5±0.54 ^{a}	12.5±0.60 ^{ab}	2.17±0.20 ^{c}
	CR1	120	39	32.5±1.01 ^{a}	28.20±0.66 ^{a}	5.12±0.44 ^{b}	-
	AUZ1	120	59	49.16±1.59 ^{a}	42.37±1.58 ^{a}	16.94±0.67 ^{ab}	6.77±0.20 ^{a}
	AUZ2	120	40	33.72±2.09 ^{a}	25±0.89 ^{a}	2.5±0.24 ^{b}	-
2-4 ºC	NS	120	60	50±1.78 ^{b}	58.33±0.22 ^{b}	30±0.50 ^{b}	10±0.37 ^{b}
	PBS	120	48	40±2.27 ^{b}	58.33±1.63 ^{b}	18.75±0.80 ^{bc}	6.25±0.24 ^{bc}
	CR1	120	51	42.5±1.77 ^{b}	39.21±1.18 ^{b}	5.88±0.40 ^{c}	1.9±0.20 ^{c}
	AUZ1	120	87	72.5±3.66 ^{a}	67.81±2.41 ^{a}	35.9±1.01 ^{a}	16.09±0.37 ^{a}
	AUZ2	120	54	45±1.39 ^{b}	40.74±1.28 ^{b}	7.40±0.37 ^{c}	1.85±0.20 ^{c}
8-12 ºC	NS	120	53	44.16±1.24 ^{b}	41.50±0.92 ^{b}	22.64±1.16 ^{ab}	5.66±0.40 ^{ab}
	PBS	120	44	36.66±1.39 ^{b}	25±0.73 ^{b}	15.90±0.50 ^{b}	2.27±0.20 ^{b}
	CR1	120	43	35.83±1.43 ^{b}	25.58±0.66 ^{b}	4.65±0.40 ^{b}	-
	AUZ1	120	73	60.83±2.11 ^{a}	50.68±1.07 ^{a}	26.02±0.37 ^{a}	10.95±0.67 ^{a}
	AUZ2	120	41	34.16±2.72 ^{b}	41.46±0.92 ^{b}	7.31±0.24 ^{b}	-
24-30 ºC	NS	120	50	41.66±1.70 ^{a}	40±0.70 ^{b}	22±0.66 ^{b}	4±0.24 ^{b}
	PBS	120	40	33.33±0.83 ^{a}	42.5±1.20 ^{b}	15±0.24 ^{bc}	2.5±0.20 ^{b}
	CR1	120	41	34.16±0.80 ^{a}	29.26±0.74 ^{b}	4.87±0.24 ^{c}	2.43±0.20 ^{b}
	AUZ1	120	62	51.66±1.69 ^{a}	54.83±0.73 ^{a}	29.03±0.50 ^{a}	11.29±0.24 ^{a}
	AUZ2	120	42	35±1.74 ^{a}	30.95±1.12 ^{b}	4.76±0.24 ^{c}	2.38±0.20 ^{b}

Data from 7 replicates; a, b Different superscripts indicate significant differences between transport media at the same temperature ($p< 0.05$). The total number of oocytes used was 2400; 120 were divided into each treatment.

As shown in Table 1, transport media had a significant impact on cleavage, morula, and blastocyst rates ($p>0.05$). At 2-4 ºC, the AUZ1 medium achieved higher cleavage and morula rates (67.81% and 35.9%, respectively) while CR1 and AUZ2 media achieved the least rates of morula (5.12% and 2.5%) and (4.87% and 4.76%) at -6 - -4 ºC and 24 - 30 ºC, respectively. In the AUZ1transport medium, more than 16% of fertilized oocytes reached the blastocyst stage at 2 - 4 ºC, which shows significant differences ($p<0.05$) when compared with other media.

Histology analysis revealed that the primordial follicles had normal histological characteristics (Figure 1). All treatments had the structure of the primary oocyte, which is enveloped by follicular cells and marks the boundary between the follicle and the surrounding stroma. The primary follicles in PBS, CR1, NS, AUZ1, and AUZ2 transport media had normal morphology (Figure 1) that consisted of oocytes surrounded by a layer (unilaminar primary follicle) or layers of cuboidal follicular cells (multilaminar primary or preantral follicle) in addition to the basal lamina, granulosa cells, and theca interna that showed no structural changes. In secondary follicles transported with PBS, CR1, NS, AUZ1, and AUZ2 media, the follicles had oocytes that maintained normal morphology and arranged granulosa cells; there were no defects in the formation of the zona pellucida. Furthermore, ovarian tissues that were transported without a medium (NON) showed follicles with normal histological characteristics and follicle viability.

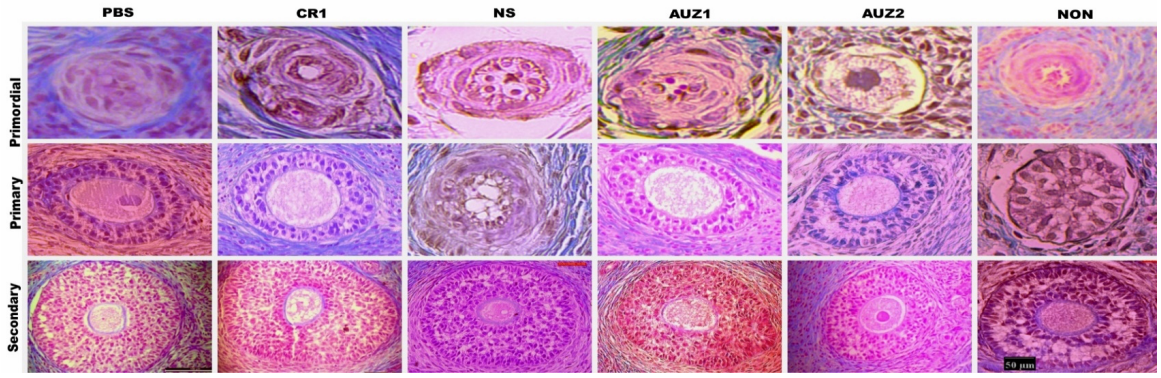

Figure 1. Histological image of primordial follicles, primary follicles, and secondary follicles of ewes' ovarian tissue transported in different media.

However, antral follicles transported in the AUZ1 medium showed normal histological profiles while those in CR1, AUZ2, or PBS exhibited disrupted granulosa cells and cumulus cells around the oocyte (Figure 2). The most significant difference was in treatments NS and NON where the oocytes were surrounded by several layers of granulosa cells separated from the antral follicles. For tissues transported in the AUZ1 medium, the Graafian follicle contained cumulus oophorus and was surrounded by theca externa and theca interna layers that maintained normal morphology. The Graafian follicles seemed to be affected by the transport conditions in the other media possibly due to the separation of the cumulus oophorus from the granulosa cells, in addition to the rupture of the corona radiata in follicles, thus exposing the oocyte to damage.

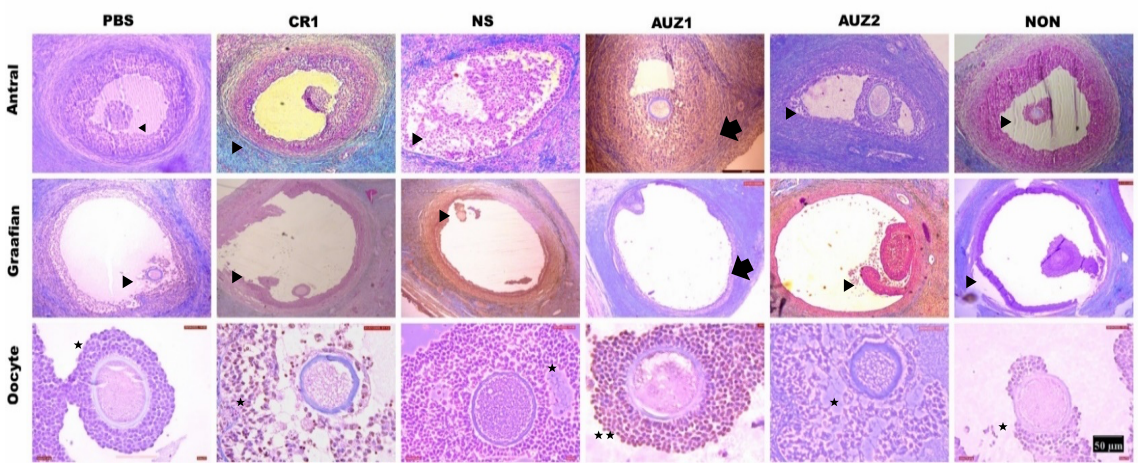

Figure 2. Histological image of antral follicles, Graafian follicles, and cumulus oophorus of ewes' ovarian tissue transported in different media. Normal follicles (arrows), degenerated follicles (arrowheads), degenerated cumulus oophorus (double asterisk), normal cumulus oophorus (asterisk).

4. Discussion

This study examined the effects of transport media on the maintenance of follicular quality after removal and during the transport of ovarian tissues, and the ability of oocytes to develop to the blastocyst stage was assessed via IVEP. To the best of our knowledge, this is the first study to verify the effects of melatonin, buffer solution, and energy substrates on the follicle quality of ewes' ovarian tissue during transportation. The main outcome of this study is that the addition of certain supplements to the AUZ1 transport medium could improve the quality of follicles and subsequent embryonic development.

The components and pH of the transport medium, as well as the period and temperature of transport, are demonstrated to affect sheep oocytes' developmental competence (Martín-Maestro et al., 2020; Sánchez-Ajofrín et al., 2020). Melatonin is an essential component in steroidogenic pathways in the follicular fluid; it is an efficient ROS scavenger that reduces oxidative stress-mediated oocyte quality deterioration and attenuates postovulatory aging oocyte abnormalities (Tamura et al., 2008; Pacchiarotti et al., 2015; Wang et al., 2017).

The data analysis showed that the addition of an antioxidant (melatonin) to the new transport medium (AUZ1) increased embryonic development rates presumably by reducing oocyte oxidative stress during transport. There are two possible mechanisms through which melatonin may have reduced oxidative stress: (i) through its direct free radical scavenging activity, and/or (ii) through the stimulation of the cellular antioxidant defense system. The transport medium (AUZ2) supplemented with Vit A and Vit E as antioxidants, had no positive effect on embryonic development rates likely because the supplements (Vit A and Vit E) had mopped up the ROS generated within the extracellular environment while melatonin scavenged the ROS produced in the COC (intracellular).

It is therefore hypothesized that transport media supplemented with biological buffers such as TRIS or HEPES are necessary for maintaining the pH levels of such media. In the present study, TRIS buffer in the AUZ1 medium also may have supported embryo development, in addition to the effects of melatonin as an antioxidant. Wongsrikeao et al. 2005 confirmed this hypothesis by suggesting that changes in pH led to a significant increase in fragmented DNA nuclei and that ZP was highly permeable to H+ ions and lacked a regulatory mechanism.

The study findings also showed that the oocytes' developmental competence to the blastocyst stage was significantly decreased during the transportation of the ovaries at temperatures less than 0 °C and between 10 °C and 35 °C. However, the AUZ1 medium significantly increased the oocyte's competence to develop into the blastocyst stage compared to the other media. Although ovaries transported in a melatonin-supplemented medium showed improvement, these beneficial effects were more pronounced at low temperatures (4 °C). Previous studies had confirmed this observation that after ovaries were transported for up to 4 h at 4 °C, the IVM rate of canine oocytes was significantly higher than that of COCs transported at 38 °C (Taş et al., 2006). Furthermore, another study confirmed that ovary transport at low temperatures positively affected the oocyte maturation rates in bovines and felines (Matsushita et al., 2004; Naoi et al., 2007). However, the data on how high temperatures affected oocyte quality during transportation is inconsistent; for instance, a study had shown that transporting bovine ovaries for 4 h at 15 °C improved embryonic development and reduced programmed cell death (Wang et al., 2011) while another study opined that ovaries' transport at high temperatures was more advantageous in terms of improving IVM and embryonic development rates in equines and porcines (Love et al., 2003; Wongsrikeao et al., 2005).

The preservation of ovarian tissue is a complicated task because the tissue contains a variety of structures and cells. According to the histology analysis in this study, the quality of the primordial, primary, and secondary follicles, as well as their morphology were not affected by the transportation of the ovarian tissue in any of the transport media at a temperature of 4 °C for up to 2 h. However, the addition of melatonin to the transport medium (AUZ1) significantly maintained the antral follicle morphology. In addition, the Graafian follicles were better maintained in AUZ1 than in the other transport media. Hence, it is hypothesized that the strong antioxidant properties of melatonin may have been responsible. Zhang et al. (2020) found that follicular fluid contains more melatonin than serum. Melatonin in follicular fluid scavenges free radicals, in addition to its steroidogenic role (Adriaens et al., 2006), and diffuses readily through tissues (Reiter et al., 2009). Melatonin improves the development of oocyte-granulosa cell complexes from porcine follicles as noted by Cao et al. (2019). Moreover, the presence of melatonin receptors in oocytes indicates the potentially important roles of this hormone in

mitochondrial characteristics, cumulus expansion, and steroidogenesis. It has been reported that melatonin supplementation in sheep ovary transport medium for 24 h led to higher rates of cumulus cell expansion. Furthermore, the addition of melatonin as a supplement increases its intra-follicular levels, thereby improving fertilization rates and embryo transfer (Tamura et al., 2008). Previous studies have also reported the diverse effects of melatonin on blood vessel proliferation even though this is not involved in IVEP (Romeu et al., 2011). The anti-apoptotic properties of melatonin have also been demonstrated; for instance, melatonin has been reported to inhibit in-vivo programmed cell death in granulosa cells of follicles (Liu et al., 2022). The histological observations in this study showed that antral follicles and Graafian exhibited disruption of the granulosa cells and cumulus cells around the oocyte after ovarian tissue transport in NS, PBS, CR1, and AUZ2 transport media. Therefore, it is hypothesized that follicles deterioration could be triggered by oxidative stress which can lead to apoptosis (Figure 2), an effect that was once again reversed by melatonin in the AUZ1 medium; this melatonin effect has also been demonstrated on different tissues in other studies (Ateşşahin et al., 2006; Mohammadghasemi et al., 2012).

Conclusion

The outcome of this study showed that melatonin, buffer solution, and energy substrates supplementation in AUZ1 transport medium maintained better follicle viability in ewe ovaries and enhanced COC survival and IVEP production. Regarding ovarian tissue damage, the lack of certain components in transport media is not considered ideal for the transportation of ovaries. This study highlights the need for the development of a standard protocol that fits the needs of ovarian tissues during transportation.

Ethical Statement

Ethical approval is not required for this study because our study was performed using only animal material collected from the slaughterhouse, there was no need to apply it to the Scientific Ethical Committee on Animal Experimentation.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

The research idea was formulated by authors 1 and 3. The experiment was conducted by authors 1 and 2, with supervision from author 3. The manuscript was written by author 1

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