RESEARCH ARTICLE

Effect of α-Tocopherol on Viability, Lipid Peroxidation and Oxidative Stress of Cryopreserved Ovine Preantral Follicle

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Abstract

Vitrification of preantral follicles is a promising technique to preserve female fertility. The aim of the present study was to evaluate the effect of supplementation of α-tocopherol in the vitrification solution on the viability, lipid peroxidation and mRNA expression of superoxide dismutases (SOD1 and SOD2) in vitrified cultured ovine preantral follicles at day-6 and day-12. Preantral follicles (200-300 µm) were isolated from the ovine ovaries by the mechanical method and were distributed separately to the vitrification medium supplemented with 10 mM and 20 mM of α-tocopherol. After a week, the follicles were thawed and analyzed for follicular viability by trypan blue dye exclusion method and subjected to in vitro culture (IVC) for 6 and 12 days. Our results revealed that the significant increase ($p<0.05$) of viability in 20 mM α-tocopherol supplemented vitrified group when compared to the vitrified without α-tocopherol group. On day-6 of IVC, malondialdehyde (MDA) concentration was significantly($p<0.05$) higher in vitrified group without α-tocopherol in comparison to vitrified supplemented with 20 mM of α-tocopherol and control fresh groups. However, no significant difference in MDA content was found among the groups at day-12. The mRNA expression level of SOD1 at day-6 was significantly ($p<0.05$) higher in vitrified with 20 mM of α-tocopherol and control fresh groups compared to the vitrified without α-tocopherol and with10 mM α-tocopherol groups. The expression pattern of SOD2 was significantly ($p<0.05$) higher in control fresh group compared to the other groups at day-6 and day-12 of IVC. We conclude that lowering the vitrification-induced lipid peroxidation of preantral follicles by α-tocopherol at 20 mM concentration may be mediated by increasing SOD expression during IVC.

Keywords: Tocopherol, Gene, Ovary, preanatral, Sheep, Vitrification.

Ind J Vet Sci and Biotech (2022): 10.48165/ijvsbt.18.5.05

Introduction

Vitrification of preantral follicles is considered a feasible option for fertility preservation because germ cells contained in preantral follicles are less vulnerable to cryogenic damage (Hovatta, 2005). High cooling rate of vitrification suppresses the intra and extra cellular ice formation and also cell damage (Sadat *et al*., 2017).The detrimental effects of vitrification are it causes cryo and osmotic injuries and produces reactive oxygen species (ROS) and oxidative stress. In the cellular membrane, ROS oxidises polyunsaturated fatty acids (PUFAs), produces reactive aldehydes, and increases the production of MDA (Yamauchi et al., 2008). Therefore, MDA concentration can be regarded as marker of lipid peroxidation level (Ahmed *et al*., 2011). Vitrification can also inhibit total superoxide dismutase (SOD) activity, which eliminates ROS, by directly damaging the mitochondria (Salehnia *et al*., 2013). Since vitrification increase the oxidative stress and decrease the cellular enzymatic antioxidant SOD activity, the supplementation of antioxidants may contribute for the improvement of vitrification of preantral follicles. It has been reported that antioxidants like α-tocopherol reduce the generation of free radicals and protect the cell membrane from lipid peroxidation and avoid irreversible damages and cellular death (Jimenez *et al*., 2016). Considering the advantage of an antioxidant, we hypothesized that the addition of α-tocopherol in the vitrification solution may increase the viability, decrease ROS and lipid peroxidation via altering the gene expression of antioxidant enzymes. Therefore, the present study aimed to evaluate the supplementation of α- tocopherol on the viability of vitrified preantral follicle, lipid peroxidation, and SOD1 and SOD2 gene expression after *in vitro* culture.

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How to cite this article: Kaushik, K., Pulukuri, J., Tej, N.K., Krishna, K., Gupta, P. S., Nandi, S., & Mondal, S. (2022). Effect of α-Tocopherol on Viability, Lipid Peroxidation and Oxidative Stress of Cryopreserved Ovine Preantral Follicle. Ind J Vet Sci and Biotech. 18(5), 24-28.

Source of support: DBT (DBT/2016/NIANP/672), Ministry of Science & Technology, Government of India.

Conflict of interest: None

Submitted: 15/07/2022 **Accepted:** 19/09/2022 **Published:** 10/11/2022

Materials and Methods

All chemicals were purchased from Sigma (Sigma chemical Co., St. Louis, MO, USA). Sheep ovaries (n=116) were procured

from local abattoir (Bengaluru, India) and immediately placed in 0.9% normal saline supplemented with 50 mg/mL streptomycin and 100 IU/mL penicillin and transported to the laboratory within 2 h.

Isolation and Selection of Preantral Follicles

Procedure of Gupta *et al*. (2022) was followed for Isolation of preantral follicle (200-300 um). After isolation, preantral follicles with a visible oocyte, surrounded by at least two granulosa cell layers, an intact basement membrane and with no antral cavity were selected for this study (Ferreira *et al.* , 2018).

Vitrification and Thawing Procedures

The vitrification of preantral follicles was performed using the protocol as described by Gupta *et al*. (2022). A total of 480 preantral follicles were selected for the study and distributed into four groups: (1) Fresh preantral follicles (n =121), (2) Vitrified preantral follicles (n=118) without any supplementation of α-tocopherol, (3) Vitrified follicles (n=122) with 10 mM of alpha tocopherol supplementation and (4) Vitrified follicles (n=119) with 20 mM of α-tocopherol supplementation. Preantral follicles were exposed to two vitrification solutions (VS),vitrification solution1 (VS 1) consisted of base medium (BM) (TCM-199 supplemented with 50 µg/mL gentamicin, 25 mM HEPES) with 10 % fetal bovine serum, 10 % ethylene glycol (EG), 10 % dimethyl sulfoxide (DMSO) and 0.3 M sucrose) and VS2 had a similar composition of VS1 but with higher concentration of cryoprotectants (25 % EG and 25 % DMSO). Follicles were initially exposed to VS1 for 4 min followed by VS2 with or without α-tocopherol for 45 s. The α-tocopherol concentrations (10 mM and 20 mM) used in this study was based on our preliminary study with α -tocopherol supplementation in ovine preantral follicles (unpublished data). Follicles were transferred to 1.8 mL cryotube containing 200 µL of the VS2 and immediately stored in the cryogenic cylinder (-196^{o}C) for 7 days. For warming, cryotube containing follicles were removed from liquid nitrogen, air-warmed for 45 s and then immersed into water bath at 37 °C until the vitrification solution was completely liquefied. Then the follicles from different groups (fresh follicles or with VS exposed) were sequentially exposed to thawing solutions for 5 min in each solution. The thawing solution consisting of base medium (BM) supplemented with decreasing concentrations of sucrose (0.3M, 0.15 M ,0.075M and 0 M).

Assessment of Follicular Viability by Trypan Blue Exclusion Method

The viability of follicles was assessed by trypan blue staining technique (Gupta *et al*., 2002). Evaluation of trypan blue staining was carried out on a stereozoom microscope (Euromex Stereo blue, Holland) and classified as nonviable or viable if they were positively or negatively stained, respectively, with trypan blue.

In Vitro Culture of Secondary Follicles

The *in vitro* culture of preantral follicles was adapted from an earlier study (Gupta *et al*., 2022). The preantral follicles was incubated in a CO_2 incubator at 38°C, 5% CO_2, and 95% RH for 6 or 12 days. The culture medium was partially replaced on alternate days and the preantral follicles were harvested on day-6 as well as on day - 12. Day-6 and day-12 media were used for estimation of MDA concentration and the follicles were used for RNA isolation.

Assessment of MDA Level

Lipid peroxidation was evaluated using MDA level as an index of lipid peroxidation based on method adapted from Ohkawa *et al*. (1979). The levels of lipid peroxidation were presented as µmol MDA/mL and calibration was performed using 1,1,3,3-tetramethoxypropane as a standard.

RNA Isolation and Quantitative Real-Time PCR Analysis (qPCR)

For RNA isolation, follicles (n=10) from each group and 6 replicates were collected on day- 6 and day-12. Total RNA was isolated by Trizol™ (Invitrogen, São Paulo, SP, Brazil) method. Concentration and purity of the RNA was determined with Nanodrop spectrophotometer at 260 and 280 nm wavelengths. Total RNA (100 ng from each sample) was then transcribed into cDNA immediately using iScript cDNA synthesis kit (BIORAD iScript TM cDNA synthesis kit) following the manufacturer's instructions. Quantitative PCR was performed using 10 µL reactions mixture containing 5.0 µL of iTaq™ Universal 2x SYBR Green Supermix (Biorad), 0.5 µL (0.5 pmol each) primer mix (sense and antisense primers), 1.5 µL of cDNA and 2.5 µL of nuclease free water. Ribosomal protein S18 (RPS18) was used as the internal reference (Gupta *et al., 2022), and* the order-specific primer SOD1 and SOD2 were designed by a NCBI website and Primer 3 software. The sequences of primer, product length and GenBank accession numbers are mentioned in Table 1.

Statistical Analysis

The effect of supplementation of α-tocopherol in vitrification solution on viability, lipid peroxidation and mRNA expression of SOD1 and SOD2 were analyzed by one way ANOVA and post ANOVA test of Tukey Multiple comparison test using Graph Pad Prism' San Diego, USA. The results obtained on various parameters were presented as mean ± SEM and a value of $p<0.05$ was considered statically significant. All Percentage values of viability were subjected to Arcsine transformation before the statistical analysis.

Results and Discussion

Preantral Follicle Viability after the Thawing

The viability of preantral follicles was significantly higher (95%, $p<0.05$) in the vitrification with 20mM of α-tocopherol

Table 1: Sequence of primers for real-time quantitative polymerase chain reaction (qPCR)

S.No	Genes	Primer Sequence (5"→3")	Accession no.	Product Size (bp)
1	RPS 18S	CCTGAGAAGTTCCAGCACATCT	NM_001285639.1	229
		AGTCTGGGATCTTGTATTGGCG		
2	SOD1	GGTTCCACGTCCATCAGTTT	NM_001145185.1	159
		CAATGGCAACACCATTTTTG		
3	SOD2	GTCAGGCCCGATTATCTGAA	NM_001280703.1	196
		AAGCCACGCTCAGAAACACT		

Table 2: Effect of supplementation of α-tocopherol in the vitrification solution on the viability (%) of vitrified preantral follicles

Treatment	% Viability
Control fresh	88.4±4.9^{ab}
Vitrified –Control	86.0± 1.80 ^{a}
Vitrified with 10 mM Tocopherol	94.90±2.4^{ab}
Vitrified with 20 mM Tocopherol	95.20±2.4^{b}

Means different superscripts differ significantly ($p < 0.05$) within the column

than vitrified follicles without α-tocopherol (86%). There were no significant differences in the viability rate among the control fresh, vitrified follicle group without α-tocopherol, and vitrified follicle group with 10 mM α-tocopherol (Table 2). The significant increase in viability with 20 mM of α-tocopherol group might be due to its role as a primary free radical scavenger in biological cell membranes. The present finding was in contrast with the other report where supplementation of 10 mM of α-tocopherol in vitrification solution increased the rate of viability in bovine preantral follicles (Jimenez *et al*., 2016).

Lipid Peroxidation

On day-6 culture MDA concentration of the preantral follicles was significantly ($p<0.05$) higher in vitrified group, as compared to vitrified with 20 mM of α-tocopherol and control fresh groups. In contrast, no significant differences in MDA content were found among control fresh and vitrified groups (with and without supplementation of α-tocopherol) in day 12 culture of vitrified preantral follicles (Table 3).

In the present study we found the lipid peroxidation occurred in the vitrified group without supplementation of α-tocopherol. This adverse effect may be attributed due to oxidative stress triggered by vitrification. The vitrification results in significant damage to the cellular structure, such as plasma membrane and mitochondria, thereby leads to oxidative stress (Liu *et al*., 2003). Oxidative stress compromises the developmental competence of ovarian follicle and oocyte quality (Zavareh *et al*., 2009; Talebi *et al*., 2012; Hatami *et al*., 2014) by damaging the cell membrane integrity. Such damage consequently increases the process of lipid peroxidation, a unique mode of oxidative injury and is the main manifestation of oxidative stress (Lee *et al*., 2012).

Table 3: MDA content of preantral follicles at Day-6 and Day-12 of IVC of ovine preantral follicles.

Treatments	MDA concentration (μM)	
	DAY - 6	DAY - 12
Control fresh	0.38± 0.13a	1.18± 0.13
Vitrified-Control	1.43± 0.31b	1.19± 0.13
Vitrified with 10mM Tocopherol	0.72± 0.21ab	0.99 ± 0.27
Vitrified with 20mM Tocopherol	0.17± 0.05a	1.25± 0.01

Means different superscripts differ significantly ($p < 0.05$) within the column

A significant decrease in MDA level with the supplementation of α- tocopherol might be due to the fact that α- tocopherol removed the peroxyl and alcoxyl radicals, generating the poorly reactive tocopheryl radical (Olson and Seidel., 2000). α- tocopherol is a lipid soluble antioxidant and it protect cell membrane from lipid Inthe present study 20 mM alpha tocopherol decreased the level of MDA which was in accordance with an earlier report that suggest an inverse relationship exists between the antioxidant CoQ10 and MDA levels (Lee *et al*., 2012).

Gene Expression

The mRNA expression of SOD1 was significantly ($p<0.05$) higher in vitrified with 20 mM of tocopherol and control fresh groups compared to the other groups in day-6 cultured follicles. In contrast the relative abundance of SOD1 transcript was significantly higher in control fresh group compared to the other vitrified groups at day-12. The mRNA transcript of SOD2 was significantly ($p<0.05$) higher in control fresh group compared to the other groups at day-6 and day-12 cultured ovine preantral follicle (Table 4).

The low expression of SOD1 in the vitrified preantral follicles is probably due to the localization of SOD1 which is cytosolic (Zelko *et al*., 2002) and vitrification may causes some cellular damage which alters or affects the expression of cytosolic enzyme SOD1. Upregulation of SOD1 in preantral follicles exposed with 20 mM of α-tocopherol may be due to an improvement in the ability to scavenge oxygen free radicals, maintenance of the cellular morphological integrity and decrease in the lipid peroxidation level. Similar to our

Table 4: mRNA expression of SOD1 and SOD2 at Day-6 and Day-12 of IVC of ovine preantral follicles.

	SOD 1		SOD 2	
Treatments	DAY-6	DAY-12	DAY-6	DAY-12
Control fresh	1.000 ± 0.0 [a]	1.000 ± 0.0 [a]	1.000 ± 0.0 [a]	1.000 ± 0.0 [a]
Vitrified-control	0.21± 0.02[b]	0.23± 0.05 [b]	0.31± 0.01[b]	0.20± 0.08[b]
Vitrified with 10 mM	0.26± 0.02[b]	0.20± 0.03[b]	0.15± 0.06[b]	0.17± 0.06 [b]
Vitrified with 20 mM Tocopherol	0.69± 0.10[c]	0.12± 0.02[b]	0.14 ± 0.07 [b]	0.09± 0.03 [b]

Means different superscripts differ significantly ($p < 0.05$) among the Groups

finding Kashka *et al*. (2016) found that the supplementation of antioxidant CoQ10 decreased the MDA level and increased the SOD1 activity in the vitrified culture mouse preantral follicles.

In the present study, we found that SOD2 mRNA expression significantly decreased by day-6 and day-12 of cultured follicles in all vitrified groups when compared to the fresh follicles. Superoxide dismutases neutralise superoxide anions (O_2^-) and are characterized by their subcellular localization; SOD2 is located in the matrix of the mitochondria (Zelko *et al*., 2002). Thus, similar genetic expression of SOD2 in all vitrified groups (with or without supplementation of α-tocopherol) obtained in our study demonstrates no alterations in mitochondrial activity due to supplementation of α-tocopherol in vitrified ovine preantral follicles after culture.

Conclusion

We conclude that 20 mM α-tocopherol-supplemented vitrification medium increased the viability after thawing and promoted SOD1 mRNA expression level and lowered the lipid peroxidation at day-6 of IVC of vitrified follicles. Increased SOD1 mRNA expression with decrease in MDA level may be attributed to improve the ability to scavenge oxygen free radicals and decrease the lipid peroxidation in the follicles vitrified with 20 mM of α-tocopherol.

Acknowledgments

For providing workspace and support authors thank the Director, ICAR-NIANP, Adugodi, Bengaluru. The first author is thankful to DBT, Government of India for providing DBT fellowship and opportunity to do doctoral degree from Department of Biotechnology, Jain University, Bengaluru.

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