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SUPEROXIDE DISMUTASE MIMICS (TEMPOL) IMPROVES IN VITRO OOCYTES MATURATION IN EGYPTIAN BUFFALOES

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SUMMARY

The purpose of this study was to examine how Tempol, an exogenous antioxidant, affected the in vitro maturation of Egyptian buffalo oocytes at the molecular level. Cumulus-oocyte complexes (COCs) were recovered from the ovaries of slaughtered animals. Cumulus-oocyte complexes were matured in a maturation medium supplemented with different concentrations of Tempol (0, 0.5, 1, and 2 μM)2-24 h and then nuclear maturation and cumulus cells expansion rates were evaluated. Profiled the expression of two candidate genes regulating metabolic activity and antioxidant status were analyzed in the matured oocytes using Real-Time PCR. Tempol increased the rate of buffalo COC expansion. The nuclear maturation rate was significantly improved in the 0.5 μM group compared to the 1 and 2 μM groups and the control group. A significant decline in the transcriptional abundance of CPT2 was observed with the increase in the Tempol concentration in the maturation medium. While, the expression level of the antioxidant-related gene showed a lower level of expression of different genes used in comparison with all experimental groups. In conclusion, the 0.5 μM concentration of Tempol may improve oocyte maturation and molecular competence.

Keywords: Tempol, in vitro maturation, Buffalo, oocytes

INTRODUCTION

Buffaloes (*Bubalus bubalis*) have occupied an advanced site in the agricultural economy, especially with significant concern to the animal livestock sector in Egypt. This is because of their significant contribution to milk and meat production. Egyptian buffaloes, however, have been characterized by low fertility performance which is related to the low number of ovarian follicles, silent ovulation, and the unordinary follicular dynamics, (Barkawi *et al*., 2008). These fertility-related problems could be ameliorated through the application of assisted reproductive technologies (ART).*In vitro* oocyte maturation, fertilization, and subsequent embryo production (IVP) are intensively used in order to improve oocyte quality and embryo yield in this species (Marin *et al*., 2019). Although this technique has led to a steady increase in the quality of produced embryos in different mammalian species, still *in vitro* produced embryos are different from their *in vivo* counterparts. Under *in vitro* environment, several stressors could be generated which in turn play a crucial role in accumulating products of biochemical returns that happen during the processes of respiration and photosynthesis in organelles such as mitochondria and free radicals (Hernández-García *et al*., 2010).It was acknowledged that culture media composition and supplements can contribute to ROS production in *in vitro* embryo production systems (Martín-Romero *et al*., 2008). A certain amount of ROS production is necessary for gene expression, cell signaling, and redox homeostasis. While, the excessive accumulation of ROS could disrupt the normal cellular antioxidant defense mechanism resulting in oxidative stress (Torres-Osorio *et al*., 2018).

Oxidative stress is a significant biological danger that has a severe impact on the quality of oocytes, as well as their future maturity and embryonic developmental competence. Under normal physiological conditions, the cellular antioxidant system, such as an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O−2) radical into ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), is critical for maintaining redox balance in the cell. Hence *in vitro* conditions could compromise redox homeostasis, different In vitro culture systems typically use compounds that inhibit oxidation to improve oocyte maturation. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) is a chemical compound that play a role in hydrogen peroxide metabolism and is functionally similar to SOD enzyme (Saker *et al*., 2019). The benefit of Tempol as exogenous antioxidant is to overcome the lack of media from serum or other macromolecules that serve as reactive oxygen species scavengers. Moreover, could be act as a molecule that fights damage by free radicals and unstable molecules that can harm cellular structures (Santiani *et al*., 2014).Several studies were concerned with the effectiveness of using Tempol antioxidant for improving semen quality and post-thawing viability (Santiani *et al*., 2014 and Saker *et al*., 2019),

however till now, there may be no available implementation for oocytes. As a result, the current study looked at the impact of a product that catalyzes the dissociation of radicals as an external antioxidant on Egyptian buffalo oocyte maturation rate *in vitro* and at the molecular level.

MATERIALS AND METHODS

This study has been conducted in Cairo University Research Park (CURP), Faculty of Agriculture - Cairo University.

Ethics Statement:

The Institutional Animal Care and Use Committee, (IACUC, Vet. CU. IACUC), has approved the animal use protocol (AUP) used in this experiment. (Vet CU code: 03162023656).

Chemicals:

All reagents and media supplements used in this study were of embryo-culture grade and were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise.

Experimental design:

The present study was designed to assess the impact of Tempol antioxidant addition to buffalo oocytes maturation medium with different concentrations (0.5, 1, and 2 µM) on: (1) *in vitro* oocytes maturation rate including cytoplasmic maturation (expansion rate) and nuclear maturation (oocytes reached Metaphase II stage); and (2) the transcriptional abundance of *CPT2* and *NEF2L2* genes in matured oocytes.

Ovaries collection and oocytes aspiration:

Buffalo ovaries were collected from local abattoirs in Giza Governorate during the winter months (November to January). Within two hours post-slaughtering, ovaries were transported to the laboratory in a thermos containing Dulbecco's phosphate buffer saline (DPBS) at a temperature ranging from 34 to 36°C. At the laboratory, ovaries were trimmed from extraneous tissues and washed three times with fresh warm DPBS followed by rinsing in 70% alcohol for 30 seconds to avoid any source of contamination. Then after, ovaries were kept in warm DPBS in a water bath till oocytes collection.

Cumulus–oocytes complexes collection and maturation:

Cumulus–oocytes complexes (COCs) were aspirated from antral follicles (2–8 mm diameter) with an 18-gauge needle. Good quality COCs, based on their morphological appearance, with granulated ooplasm and surrounded by at least four layers of compacted cumulus cells were washed in TCM-199 HEPES medium supplemented with 2% FBS, 0.3 mg/ml glutamine, 50 µg/ml gentamycin according to Faheem *et al*. (2014). Then COCs were assigned randomly to four groups of maturation medium-TCM (TCM-199 with HEPES supplemented with 10% FBS, 5 µg/ml of FSH, 1 µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22 µg/ml Na-pyruvate, 50 µg/ml gentamycin); (1) TCM with 0.5 µM of Tempol, (2) TCM with 1 µM of Tempol, (3) TCM with 2 µM of Tempol and (4) TCM without Tempol (control group). After 22-24 h of maturation in an incubator under 5% CO_2 in air in a humidified atmosphere at 38.5°C, the expansion rate of the oocytes from all groups of the study was recorded and then oocytes were subjected to denudation (mechanical denudation using micropipette) for further nuclear assessment and genetic analysis.

Nuclear maturation assessment:

Denuded oocytes were fixed in a petri dish containing fixation solution (methanol: glacial acetic acid, 3:1). After 24 h of fixation period, oocytes were placed on a glass slide and then covered immediately with a glass slip and stained with 1% aceto-orcein for 5 min before examining under an inverted microscope.

Separation of RNA and creation of cDNA:

With the Trizol reagent kit, total Ribonucleic acid was obtained from three biological replicates of each experimental group of oocytes, following the manufacturer instructions (Life Technologies, Inc., Carlsbad, CA, USA) according to Pavani *et al*. (2015). Nano Drop was used to quantify total RNA level and clarity at a 260/280 nm ratio. RNA samples from each treatment group were reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription Kit that included random primers and MultiScribe™ Reverse messenger after the RNA level of all samples was adjusted.

Quantitative real-time PCR (qRT-PCR):

Primer3 software (http://primer3.wi.mit.edu//) was used to construct gene-specific primers based on sequences provided in the GenBank database, as indicated in Table 1. (www.ncbi.nlm.nih.gov). In a 20ul final volume, 10ul of Power SYBR Green PCR Master Mix (Thermofisher Scientific, California, USA), 0.2l primers, 7.6l nuclease-free water, and 2.0l cDNA were used. The following thermal cycling conditions were used for PCR reactions: 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C, followed by a final 1 minute at 60°C. A Step One Plus TM Real-Time PCR apparatus was used for the qRT-PCR (Applied Biosystems, California, USA).Results were quantitatively analyzed using delta Ct method and were provided as the comparative regulation of genes under investigation. (CPT2 and NFE2L2) to the calibrator after normalization of the target transcript to reference gene (GAPDH) as shown in (Table 1).

Table 1. Oligonucleotide primers used for quantitative real-time polymerase chain reactions (qPCR)

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)
CPT2	NM_001045889	F: 5'-CCGAGTATAATGACCAGCTC-3' R: 5'-GCGTATGAATCTCTTGAAGG-3'	152
NFE2L2	NM_001011678	F: 5'-TAAAACAGCAGTGGCTACCT-3' R :5'-GAGACATTCCCGTTTGTAGA-3'	159
GAPDH	NM_001034034.2	F: 5'- AGGTCGGAGTGAACGGATTC -3' R: 5'- GGAAGATGGTGATGGCCTTT -3'	219

Data analysis:

Oocyte maturation rates were analyzed using one-way analysis of variance (ANOVA) and expressed as mean± standard error of the mean (SEM). Comparisons were significantly different if $P<0.05$. All analyses were performed using the IBM SPSS Statistics 22 program (SPSS Inc., Chicago, Illinois, USA). Gene expressions were analyzed using SAS GLM procedure (SAS 2004). Duncan's multiple range test was used to detect differences among the means.

RESULTS

For oocytes maturation rate**,** as shown in Figure 1, the expansion rate of buffalo COCs was increased in the 0.5 µM group (88.0±4.0%) as compared to the control (75.7±3.0%), and the other two groups (1 and 2µM; 79.9±4.1 and 68.1±4.2%, respectively)but with no significant differences. Among the greater and lower concentrations of Tempol, there were significant differences (P≤0.05). The metaphase II oocytes (Figure 2) were higher in 0.5 µM (84.4 %) in comparison to 1 and 2 µM (68.9 and 76.7 %, respectively) and the control group (75.8 %).

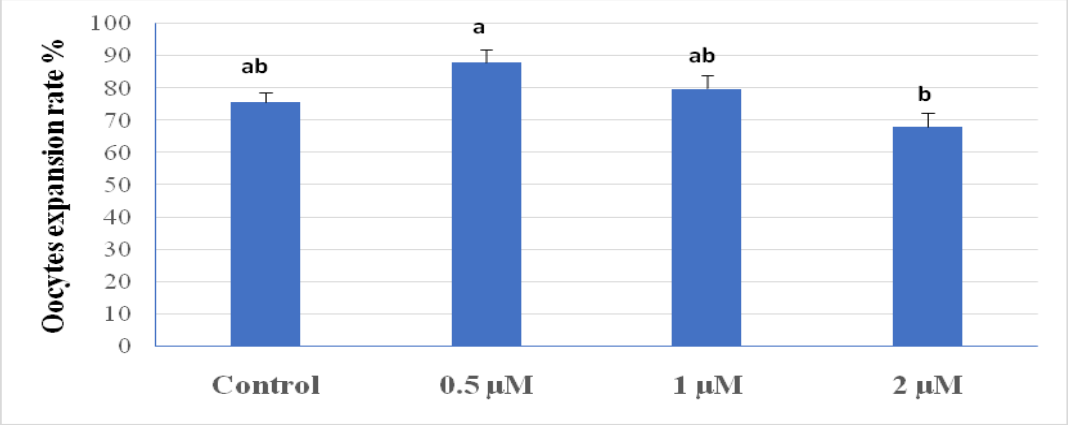
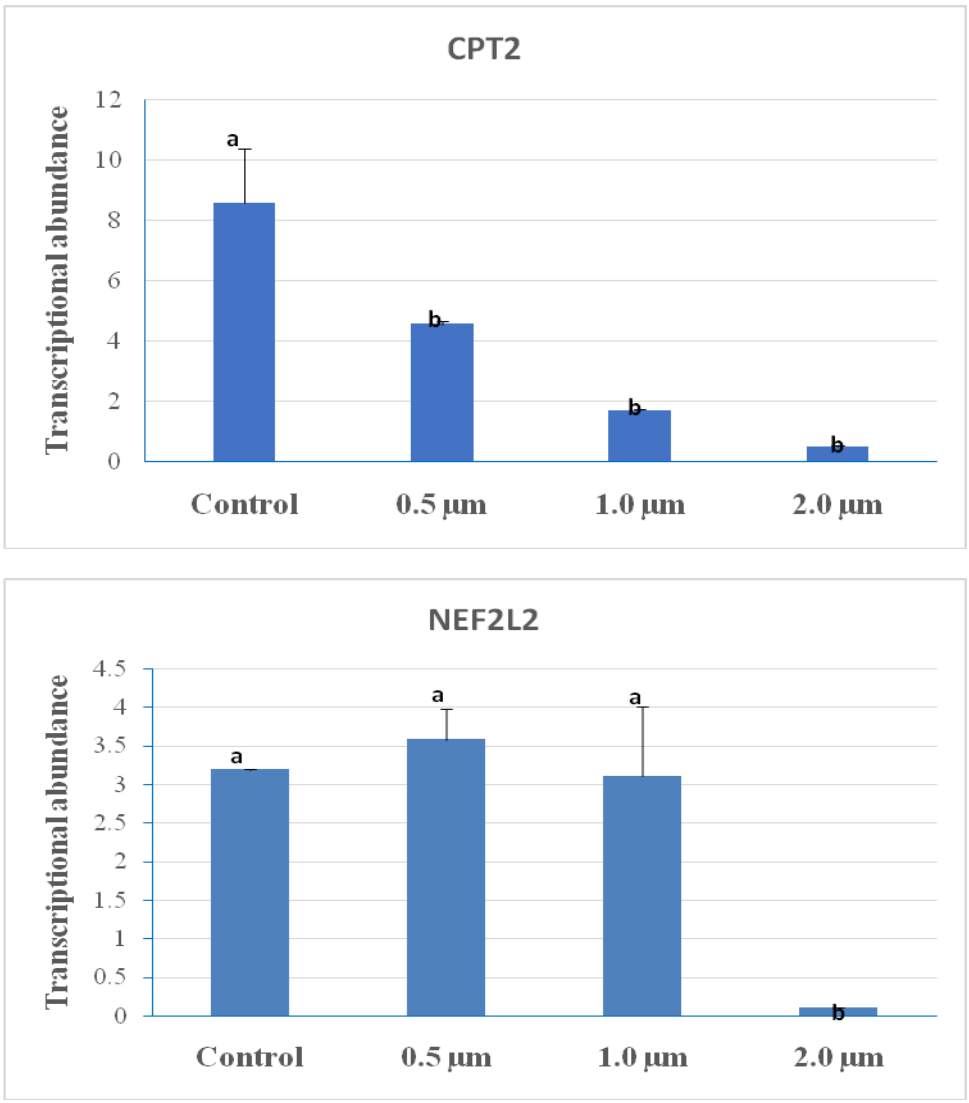


Figure 1. Buffalo oocytes expansion rate after 24h of *in vitro* maturation.

Figure 2. Microscopic image represents morphology of oocyte DNA in meiotic division. a. Metaphase Ib. Metaphase II. Arrow indicates the location of the first polar body.

The Transcriptional level of CPT2 revealed a substantial reduction trend in the molecular analyses, as did the growth of Tempol level in the maturation medium, which was also considerably lower than the control group. While the levels of transcription of the NEF2L2 gene were similar in the control group and

the oocytes developed with 0.5 and 1 µM of Tempol, The lowest (P<0.05)level of NEF2L2 expression was found with 2 µM of Tempol (Figure 3).



a,b (P< 0.05)

Figure 3. Transcriptional abundance of CPT2 and NEF2L2 genes.

DISCUSSION

The current study was designed to investigate heimpact of Tempol antioxidant on buffalo oocyte maturation competence. Three different concentrations of Tempol (0.5, 1 and 2 µM) were added to the maturation medium then after, the oocytes expansion rate and nuclear status as well as the gene expression after maturation, the expression of two target genes that regulate cellular metabolic activity (CPT2) and antioxidant status (NFE2L2) was tested. Tempol was used in these concentrations due to its impact showed in the previous studies. For instance, Iman *et al*., 2022 came to the conclusion that Mito TEMPO, at a concentration of 1.00 M, improved the quality and potential for fertilization of bovine oocytes. Yousefian *et al.*, 2021 demonstrated through investigations that one M Mito TEMPO has a favorable impact on the ability of the bovine oocyte to grow as well as the standard of the produced blastocyst. Also, Jae-Hoon *et al.*, 2021 found that lowering mitochondrial superoxide during vitrification, combined with Mito-TEMPO, improved survivability in vitrified cow embryos. Our oocyte maturation rate results revealed a significant impact of adding Tempol in a concentration of 0.5 and1 µM over 2 µM and in addition over the control group. This, in turn, reveals that *in vitro* condition adversely affects the oocyte maturation competence and at the same time it could be used as evidence for oxidative stress shock during oocyte maturation. This is could be attributed to the fact that Tempolis a SOD mimic antioxidant (Santiani *et al*., 2014).SOD performs the initial enzymatic step in protecting cells from harmful oxygen radicals, producing hydrogen peroxide (H_2O_2) as a by-product, which is then removed by catalase or glutathione peroxidase (Guérin *et al*., 2001). Tempol supplementation was

observed to reduce ROS generation and lipid peroxidation during the semen chilling process. This improves the motility and survivability of ram sperm cells after they have been thawed. In ram spermatozoa, Tempol has been shown to be effective in eliminating intracellular ROS and preventing DNA fragmentation (Mata-Campuzano *et al.*, 2012). The results of the current genetic analysis matched the maturation rate results. Tempol improved the *in vitro* culture conditions for oocytes by minimizing the production of free radicals in the extracellular environment, which lowers cell damage, hence can affect oocyte competence and maturity. Carnitine palmitoyltransferases (CPT1 and CPT2) are functionally critical for free fatty acid transport into mitochondria where mitochondrial fatty acid oxidation takes place in order to produce ATP (Bonnefont *et al*., 2004). The severe down regulation of CPT2 as stated from our results revealed that lipid metabolism was impaired by the addition of Tempol antioxidant. This is could be a supportive mechanism of Tempol to reduce the metabolic pathway which results in, excessive generation of endogenous ROS since buffalo oocytes are characterized by high lipid content. This finding was confirmed by increasing the reduction of CPT2 expression in a Tempol concentration-dependent manner.

The involvement of NFE2L2 as a major antioxidant gene involved in the defense system against oxidative stress is governed by the quantity of reactive oxygen species (ROS) (Khadrawy *et al*., 2019). The NRF2/KEAP1 system is a cellular self-defense mechanism that activated in response to oxidative stress. The antioxidant response factor signaling pathway NRF2 (NF-E2-related factor 2) is a growth factor that works with KEAP1 protein (Kelch-like ECH-associated protein 1) as a system to activate a battery of antioxidant genes in response to oxidative stress, with the final outcome of reducing ROS levels (Jaiswal, 2004; Zhang *et al*., 2006 and Wu *et al*., 2012). The NFE2L2 upregulation with Tempol addition (0.5 and 1 µM), as noted in the present result, might be related to the coinciding excessive level of ROS (Alemu *et al*., 2018) as generated under *in vitro* culture conditions. This may be due to the fact that ROS as NRF2 inducing agent results in the dissociation of NRF2 from its inhibitor KEAP1 and subsequently induces the expression of downstream target genes of NRF2 and leading to the overproduction of cytoprotective genes (Liu *et al*., 2010).The protective mechanism of NFE2L2 could be achieved through intrinsic upregulation pattern under high ROS induction as observed with the addition of 0.5 and 1 µM of Tempol which exhibit benefits on oocytes maturation outcomes. While this mechanism was severely disrupted when 2 µM of Tempol was used.

CONCLUSION

Finally, the expression profiles of the *CPT2* and *NEF2L2* genes supported the *in vitro* oocyte maturation rate data. Furthermore, the Tempol, particularly 0.05 µM concentration demonstrated potential relevance for oocyte maturation competency as well as on a molecular level. However, further studies are needed to investigate the effectiveness of Tempol levels as an antioxidant on oocyte maturation, fertilization and subsequent *in vitro* embryo development and production.

REFERENCES

Alemu,T.W., H.O. Pandey, D. Salilew. Wondim, S. Gebremedhn, C. Neuhof andE. Tholen, 2018.Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress.

Barkawi, A.H., Y.M. Hafez, S.A. Ibrahim,G. Ashour, A.K. El-Asheeri and N .Ghanem, 2008. Characteristics of ovarian follicular dynamics throughout the estrous cycle of Egyptian buffaloes. Animal reproduction science,

Bonnefont, J., 2004. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Molecular Aspects of Medicine,

Faheem, M.S.,E. Baron, I. Carvalhais,A. Chaveiro,K. Pavani and F. Moreira da Silva, 2014.
The effect of vitrification of immature bovine oocytes to the subsequent in vitro development and gene expression. Zygote,1- 10 Cambridge University.

Guérin, P., S. El Mouatassim andY. Ménézo, 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. HumanReproduction Update, 7:175–89.

Hernández-García, D., C.D. Wood, S. Castro-Obregón andL. Covarrubias, 2004. Reactive oxygen species: A radical role in development? Free Radic Biol Med, 49(2):130–143.

Iman, Y., A. Zare-Shahneh, and H. Kohram, 2022. Addition of MitoTEMPO to the maturation medium improves in vitro maturation competence of bovine oocytes. Veterinary Research Forum, 13(1): 71–78.

Jae-Hoon, J., S. Yang, H. Park and D. Koo, 2021. Effects of Mito-TEMPO on the survival of vitrified bovine blastocysts in vitro. Journal of Animal Reproduction and Biotechnology, 36(4): 299-306.

Jaiswal, A.K., 2004. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radical Biology and Medicine,2004; 36(10):1199e207.

Khadrawy, O., S. Gebremedhn, D. Salilew-Wondim, M. Taqi, C. Neuhoff andE. Tholen, 2019. Endogenous and Exogenous Modulation of Nrf2 Mediated Oxidative Stress Response in Bovine Granulosa Cells: Potential Implication for Ovarian Function. International Journal of Molecular Sciences, 20:1635.

Liu, Q., H. Zhang, L. Smeester, F. Zou, M. Kesic andI. Jaspers, 2010.The NRF2- ediated oxidative stress response pathway is associated with tumor cell resistance to arsenic trioxide across the NCI-60 panel. BMC Medical Genomics, 3(37).

Marin, D.F.D., E. Baia de Souza, V. Cunha de Brito, C.V. Nascimento, A.S. Ramos, S.T.R. Filho, 2019.In vitro embryo production in buffaloes: from the laboratory to the farm. Animal Reproduction, v.16, n.2, p.260-266.

Martín-Romero, F.J., E.M. Miguel-Lasobras, J.A. Domínguez-Arroyo, E. González-Carrera andI.S. Alvarez, 2008.Contribution of culture media to oxidative stress and its effect on human oocytes. Reprod Biomed Online, 17(5):652–661.

Mata-Campuzano, M., M. Alvarez-Rodriguez, M. Alvarez, L. Anel, P. de Paz andJ.J. Garde, 2012.Effect of several antioxidants on thawed ram spermatozoa submitted to 37 _C up to four hours. Reproduction Domestic Animal, 47:907–14.

Pavani, K.C., M.S. Faheem, E. Baron, I. Carvalhais and F. Moreira da Silva, 2015. Optimization of total RNA extraction from bovine oocytes and embryos for gene expression studies. Cytology and Genetics, (49) 232–239.

Sakr, O.G., A. Gad, M. Rodriguez, P.G. Rebollar and P. Millan, 2019.Superoxide dismutase mimics improves semen quality during chilled preservation of rabbit spermatozoa. Livestock Science, 221: 70–76.

Santiani, A., S. Evangelista, N. Sepúlveda, J. Risopatrón, J. Villegas and R. Sánchez, 2014. Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. Theriogenology, 82: 884–889.

Torres-Osorio, V., R. Urrego, J.J. Echeverri-Zuluaga and A. López-Herrera, 2019. Oxidative stress and antioxidant use during in vitro mammal embryo production. Revista Mexicana de CienciasPecuarias, 10(2):433-459.

Yousefian, I., A. Zare-Shahneh, A. Goodarzi, H. Baghshahi and A.A. Fouladi-Nashta, 2021. The effect of Tempo and MitoTEMPO on oocyte maturation and subsequent embryo development in bovine model. Theriogenology, 176: 182–136.

Wu, K.C., P.R. McDonald, J.J. Liu, R. Chaguturu, C.D. Klaassen, 2012. Implementation of a high-throughput screen for identifying small molecules to activate the Keap1- Nrf2-ARE pathway. PloS One, 7(10), e44686.

Zhang, D.D.,2006. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metabolism Reviews, 38(4):769e89.

التيمبول كأحد مضادات الأكسدة الخارجية يحسن نضج البويضات معمليًا في الجاموس المصري

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يهدف هذا البحث لدراسة تأثيرالتيمبول، أحد مضادات الأكسدة الخارجية، على نضج بويضات الجاموس المصري معمليًا على المستوى الجزيئي. تم تجميع البويضات من مبايض الحيوانات المذبوحة وتم إنضاجها في بيئة إنضاج تحتوي على التيمبول بتركيزاتمختلفة (صفر، ٠,٥، ١ و٢ ميكرومولار) ولمدة ٢٢-٢٤ ساعة ثم تم تقييم معدل النضج على مستوى تمدد الخلايا الحبيبية (النضج السيتوبلازمي) وكذلك نضج النواة. تم تقدير التعبير الجيني لأثنين من الجينات اتي تعبر عن النشاط التمثيلي وأكسدة الخلايا في البويضات الناضجة بإستخدام تفاعل البلمرة المتسلسل اللحظي. بالمقارنة مع مجموعة الكنترول والتركيزين الآخرين وجد ان تركيز ٠,٥ميكرومولار من التيمبول يزيد من معدل تمدد الخلايا الحبيبية لبويضات الجاموس بفروق معنوية وعلى مستوى نضج النواة كان أعلى في مجموعة ٠,٥ ميكرومولار مقارنة بمجموعات ١ و٢ميكرومولار ومجموعة الكنترول بفروق معنوية. على مستوى التعبي الجيني أظهرالجينCPT2إنخفاض معنوي مع زيادة تركيز التيمبول في بيئة الإنضاج مقارنة مع مجموعة الكنترول. بينما أظهر الجين المرتبط بمضادات الأكسدة إنخفاض معنوي في مجموعة ٢ميكرومولار مقارنة بباقي المجموعات. في الختام أظهرت النتائج أن إستخدام تركيز ٠,٥ ميكرومولار من التيمبول في بيئة الإنضاج يزيد من معدلات إنضاج بويضات الجاموس المصري على مستوى النواة.