Effect of Alpha-Tocopherol Supplementation in TCM199 Medium on In Vitro Maturation and Cleavage of Buffalo Oocytes

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ABSTRACT

An experiment was conducted to assess the effect of supplementing α-tocopherol at two different concentrations to the in vitro maturation (IVM) medium (TCM 199 culture medium) on in vitro maturation and cleavage of buffalo oocytes. Ovaries were collected from local slaughter house. Oocytes were collected by aspiration method and were matured in IVM medium (T0, control), IVM medium supplemented with α-tocopherol @ 10 µg/ml (T1) and IVM medium supplemented with α-tocopherol @ 20 µg/ml (T2). The mean cytoplasmic maturation rate was 82.39 ± 0.81, 88.81 ± 1.08, 81.67 ± 1.82 % and nuclear maturation rate was 62.50 ± 8.33, 87.50 ± 5.59, 66.66 ± 7.68 % in T0, T1 and T2 groups, respectively. The fertilized oocytes reaching 2-cell and 4-cell stages of cleavage for T0, T1 and T2 groups were 14.92 ± 1.52, 32.39 ± 1.01 and 16.39 ± 1.25 %, respectively. Significantly (p < 0.05) higher level of cytoplasmic maturation rate, nuclear maturation rate and cleavage rate was observed in IVM medium supplemented with α-tocopherol @10 µg/ml than other two groups.

Key words: α-tocopherol, Buffalo oocytes, Cleavage, In vitro maturation, In vitro fertilization.

Ind J of Vet Sci and Biotech (2020): 10.21887/ijvsbt.15.4.14

INTRODUCTION

Inherent reproductive problems limit the productivity of buffalo. Assisted reproductive technologies such as artificial insemination (AI), superovulation, in vitro fertilization (IVF), and embryo transfer (ET) have been introduced to overcome these problems, to increase the number of offspring from selected females, and to reduce the generation intervals in buffalo. Laboratory production of embryos (IVF technology) provides an excellent and inexpensive source of embryos for carrying on basic research in developmental physiology, farm animal breeding, and for commercial application of the emerging bio-techniques like cloning and transgenesis (Suresh et al., 2009). In vitro culture results in higher level of oxygen than in vivo environment, leading to an increased level of reactive oxygen species (ROS) that cause lipid peroxidation of cellular membranes. Oxidative stress has recently appeared as one of the most important factors contributing to low oocyte quality in vitro. Oxidative oocyte injury is detrimental to fertility potential (Guérin et al., 2001) and early embryo development (Kitagawa et al., 2004). Alpha-tocopherol (an active form of Vitamin-E) is the predominant lipid soluble antioxidant which is found to have powerful function as an antioxidant in vitro to protect the cells from ROS (Hamideh et al., 2015). Hence the present experiment was designed with an aim to assess the effect of supplementing α-tocopherol at two different concentrations to the in vitro maturation medium on maturation and subsequently on in vitro fertilization (IVF) and cleavage of buffalo oocytes.

MATERIALS AND METHODS

Buffalo ovaries were collected from organised abattoir, M/s. Frigerio Conserva Allana Limited, Zaheerabad located near Bidar, Karnataka. Immediately after slaughter, the ovaries were collected and placed into thermos (27°C to 30°C) in normal saline (0.9% NaCl) containing gentamicin (50 µg/ml) and transported to the laboratory within two hours post

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The ovaries were processed and cumulus oocyte complexes (COCs) were recovered from matured follicles into oocyte collection media (OCM) as per standard aspiration method described by Suresh and Maurya (2000).

**In Vitro Maturation and Cleavage Study**

The COCs were evaluated and graded according to cumulus morphology under a stereomicroscope (Motic, Germany). Only excellent (>5 layers of cumulus cells and evenly granulated cytoplasm) and good (>3 layers of cumulus cells and evenly granulated cytoplasm) COCs were isolated and used for the experiment. The separated COCs were washed three times in the in vitro maturation (IVM) medium (TCM199 culture medium containing 10% FCS (Fetal calf serum), 5% sterile BFF (Buffalo follicular fluid), 0.3% BSA, 50 IU/ml Gonadotrophin, 10 IU/ml of hCG and 50 µg/ml gentamicin). After washing, 20-25 COCs were cultured in 50 µl droplets of IVM medium (control group, T0) in 35 mm sterile petridish. The droplet was covered with warm non-toxic mineral oil and cultured at 38°C, 5% CO2, 90-95% RH for 24 hrs in CO2 incubator (Nuaire, USA). For treatments, the IVM medium was supplemented with α-tocopherol (Sigma-Aldrich, USA) @ 10 µg/ml (T1) and 20 µg/ml (T2). The experiment was repeated in 6 replicates for all the groups.

After 24 hr of maturation period, the cytoplasmic maturation was assessed (Kobayashi et al., 1994) under inverted microscope as per the degree of cumulus expansion and graded as degree 0 (no cumulus cell expansion), degree 1 (cumulus cells were non-homogenously spread and clustered cells were still observed) and degree 2 (cumulus cells were homogenously spread and clustered cells were no longer present). The cytoplasmic maturation rate (CMR) was calculated as:

\[
\text{Cytoplasmic maturation rate (CMR)} = \frac{\text{Total number of degree 1 and 2 matured oocytes}}{\text{Number of oocytes utilised for maturation}} \times 100
\]

The nuclear maturation was assessed by nuclear staining under phase contrast microscope as per method of Wael et al. (2013) and they are graded as germinal vesicle (GV) stage, germinal vesicle breakdown (GVBD) stage, M1 and M2 stage. Nuclear maturation rate was calculated as:

\[
\text{Nuclear maturation rate (CMR)} = \frac{\text{Number of oocytes in GVBD, M-I and M-II stage}}{\text{Total number of matured oocytes stained}} \times 100
\]

To prepare the sperms for the IVF, semen straw was thawed in warm water (35-37°C) for 1 min, emptied in to 5 ml of Tyrode’s Albumin Lactate Pyruvate (TALP) medium supplemented with 3 mg/ml BSA, 10 µg/ml heparin, 0.25 mM pyruvate (Sperm TALP medium) in a centrifuge tube and centrifuged at 800-1000 rpm for 5 min. The supernatant was removed and process was repeated again by adding 5 ml sperm TALP. Sperm pellet was dissolved in 1 ml of TALP medium supplemented with 6 mg/ml fatty acid free BSA, 10 µg/ml heparin, 0.25 mM pyruvate (Fertilization TALP medium), kept in CO2 incubator for 30 min before inseminating the matured oocytes. The sperm concentration was adjusted to 4-5x10⁶ cell/ml.

Following IVM, only degree 1 and 2 cumulus expanded oocytes were considered as matured and utilized for in vitro fertilization. They were washed several times with fertilization TALP medium to remove expanded cumulus cells. Denuded oocytes were transferred to 60 µl droplet of fertilization TALP medium in 35 mm sterile petridish and were inseminated with 40 µl of processed spermatozoa. The droplet was covered with sterile mineral oil and kept in CO2 incubator at 38°C, 5% CO2, 90-95% RH for 18 hr. After 18 hr of sperm oocyte co-incubation, oocytes were washed several times with modified synthetic oviductal fluid (mSOF) to remove the attached and dead spermatozoa from the oocytes. Washed oocytes were cultured in mSOF supplemented with 0.8% BSA, essential and nonessential amino acids. Cleavage rate was observed after 48 hr of culture, and it was calculated as:

\[
\text{Cleavage rate (%) =} \frac{\text{Number of cleaved oocytes (2-4 cell stage embryos) at 48 hr}}{\text{Number of oocytes utilised for maturation in vitro fertilization}} \times 100
\]

The data was subjected to statistical analysis using General Linear Model (GLM) procedures of SAS 9.3 to assess the significant difference at 5% level between the groups for cytoplasmic maturation rate, nuclear maturation rate and cleavage rate.

**Results and Discussion**

The mean cytoplasmic and nuclear maturation rate of buffalo oocytes cultured in IVM medium supplemented with two different concentrations of α-tocopherol are presented in Table 1 and 2, respectively. In the present study, the oocytes cultured in IVM medium supplemented with α-tocopherol @ 10 µg/ml showed significantly (p < 0.05) higher level of cytoplasmic and nuclear maturation rate assessed (Figs. 1 and 2) than the control group (T0) and α-tocopherol 20 µg/ml (T2) group. The beneficial effect of addition of tocopherol to the maturation media in improving cytoplasmic and nuclear maturation of oocytes has also been documented earlier by Cavalcante et al. (2009) in dogs, and Miclea et al. (2009) and Ileana et al. (2010) in pigs. Dalvit et al. (2005) reported that during in vitro maturation, α-tocopherol content naturally present in the membranes of COCs diminished by 50% in bovine, indicating the partial loss of antioxidant activity during the period of in vitro culture. Therefore, the favourable effect of additional supplementation of α-tocopherol in the
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The number of fertilized oocytes that reached 2 cells stage (Fig. 3) and 4 cells stage (Fig. 4) of cleavage and the

**Table 1:** Effect of α-tocopherol supplementation in IVM medium on cytoplasmic maturation rate (CMR %) of buffalo oocytes

| Groups   | No. of replicates | Culturable oocytes | Degree of COC expansion (%) | No. of oocytes matured (degree 1 and 2) | CMR (%) |
|----------|-------------------|--------------------|-------------------------------|----------------------------------------|---------|
|          |                   |                    | 0                            | 1                                      | 2       |
| Control  | 6                 | 142                | 17.43 ± 0.81 (25)             | 30.07 ± 1.30 (43)                      | 52.45 ± 1.58 (74) | 117    | 62.50 ± 8.31 |
| T1       | 6                 | 143                | 10.95 ± 1.07 (16)             | 32.14 ± 2.7 (46)                       | 56.91 ± 3.64 (81) | 127    | 87.50 ± 5.59 |
| T2       | 6                 | 131                | 18.00 ± 1.81 (24)             | 29.44 ± 2.12 (38)                      | 52.57 ± 2.54 (69) | 107    | 81.67 ± 1.82 |

Mean values with different superscripts differ significantly (p ≤ 0.05). The values in parenthesis are number of oocytes showing different stages of nuclear maturation.

**Table 2:** Effect of α-tocopherol supplementation in IVM medium on nuclear maturation rate (NMR, %) of buffalo oocytes

| Groups   | No. of replicates | Culturable oocytes | Degree of COC expansion (%) | No. of oocytes matured (degree 1 and 2) | NMR (%) |
|----------|-------------------|--------------------|-------------------------------|----------------------------------------|---------|
|          |                   |                    | 0                            | 1                                      | 2       |
| Control  | 6                 | 142                | 37.50 ± 10.70 (9)             | 12.50 ± 5.59 (3)                       | 20.83 ± 4.16 (5) | 29.17 ± 7.68 (7) | 117    | 62.50 ± 8.31 |
| T1       | 6                 | 143                | 12.50 ± 5.59 (3)             | 20.83 ± 4.16 (5)                       | 25.00 ± 0.00 (6) | 41.67 ± 5.27 (10) | 127    | 87.50 ± 5.59 |
| T2       | 6                 | 131                | 33.33 ± 10.5 (8)             | 8.33 ± 5.27 (2)                       | 20.83 ± 7.68 (5) | 37.50 ± 5.59 (9) | 107    | 81.67 ± 1.82 |

Mean values with different superscripts differ significantly (p ≤ 0.05). The values in parenthesis are number of oocytes showing different stages of nuclear maturation.

IVM medium on oocyte maturation rate can be attributed to the fact that α-tocopherol protects the polyunsaturated fatty acids in membranes against free radicals. It is probable that the lipid soluble α-tocopherol could be better distributed throughout the lipid rich environment of the oocyte and thus contributes to maintaining cell viability. Other possible functions of α-tocopherol is, it prevents the DNA fragmentation of cumulus cells and therefore, maintaining the GSH synthesis in COCs (Tao et al., 2004). In contrast to our findings, Natarajan et al. (2010) and Hamideh et al. (2015) have concluded that supplementation of α-tocopherol in maturation medium had no beneficial effect on ovine oocyte maturation.

The number of fertilized oocytes that reached 2 cells stage (Fig. 3) and 4 cells stage (Fig. 4) of cleavage and the
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67

b

4

32.39 ± 1.01

No. of replicates

10

9

O

16.39 ± 1.25

embryo

6

6

14.92 ± 1.52

Fig. 4: Four-cell stage embryos

cleavage rates are presented in Table 3. The cleavage rate was significantly (p < 0.05) higher in the oocytes matured in IVM medium supplemented with α-tocopherol @ 10 µg/ml as compared to T0 and T2 groups. These findings are in agreement with the reports of Olson and Seidel (2000) in bovine, Natarajan et al. (2010) in ovine, and Kitagawa et al. (2004) and Jeong et al. (2006) in porcine, who have documented positive effect of α-tocopherol supplementation on early development of embryos. Feugang et al. (2004) reported that vitamin E could protect bovine embryos exposed to oxidative stress generated through an increase in ROS production or a decrease in antioxidant protection. Kitagawa et al. (2004) stated that vitamin E added to culture medium was shown to decrease the H₂O₂ content and increase the developmental ability to the blastocyst stage and the cell number in porcine IVF embryos.

Table 3: Effect of α-tocopherol supplementation in IVM medium on cleavage rate (%) of in vitro fertilized buffalo oocytes

| Groups | No. of replicates | Number of matured oocytes fertilized | Number of fertilized oocytes 2 cell stage | Number of fertilized oocytes 4 cell stage | Cleavage rate (%) |
|--------|-------------------|-------------------------------------|------------------------------------------|------------------------------------------|-------------------|
| Control | 6                 | 67                                  | 10                                       | 0                                        | 14.92 ± 1.52² (10) |
| T1      | 6                 | 71                                  | 19                                       | 4                                        | 32.39 ± 1.01b (23) |
| T2      | 6                 | 61                                  | 9                                        | 1                                        | 16.39 ± 1.25a (10) |

Mean values with different superscripts differ significantly (p ≤ 0.05). The values in parenthesis are number of fertilized oocytes showing cleavage.

of α-tocopherol @ 10 µg per ml to the IVM medium is suitable approach to manage oxidative stress to improve in vitro maturation of buffalo oocytes and to achieve higher cleavage rate upon in vitro fertilization.

ACKNOWLEDGEMENTS

Authors are thankful to M/s Frigerio Conserva Allana Limited, Zaheerabad, Telangana for providing buffalo ovaries for the experiment.

REFERENCES

Cavalcante, L.F., Artur, E.F.S., Berenice, D.A.R. and José Luiz, R. (2009). In vitro nuclear maturation of canine oocytes in the presence of α-tocopherol. Master’s dissertation, Federal University of Rio Grande do Sul; Porto Alegre, RS, Brazil.

Dalvit, G., Llanes, S.P., Descalzo, A., Insani, M., Beconi, M. and Cetica, P. (2005). Effect of alpha-tocopherol and ascorbic acid on bovine oocyte in vitro maturation. Reprod. Dom. Anim., 40(2): 93-97.

Feugang, J.M., De Roover, R., Moens, A., Leonard, S., Dessy, F. and Donnay, I. (2004). Addition of beta-mercaptoethanol or trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. Theriogenology, 61: 71-90.

Guerin, P.E.L., Mouatassim, S. and Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod., 7: 175-189.

Hamideh, A., Saeed, Z., Hamid, K., Mahmoud, A.R. and Mortezaz, D.J. (2015). In vitro maturation of ovine oocyte in a modified granulosa cells co-culture system and alpha-tocopherol supplementation: effects on nuclear maturation and cleavage. J. Anim. Sci. Tech., 57: 1-6.

Ileana, M., Andrea, H., Iulian, R., Marius, Z. and Vasile, M. (2010). Exposure to α-tocopherol, lutein or ascorbic acid improve cumulus expansion, viability and maturation of swine oocytes. Anim. Sci. Biotechnol., 43(1): 289-293.

Jeong, Y.W., Park, S.W., Hossein, M.S., Kim, S., Kim, J.H., Lee, S.H., Kang, S.K., Lee, S.K. and Hwang, W.S. (2006). Antiapoptotic and embryotrophic effects of α-tocopherol and L-ascorbic acid on porcine embryos derived from in vitro fertilization and somatic cell nuclear transfer. Theriogenology, 66: 2104-2112.

Kitagawa, Y., Suzuki, K., Yoneda, A. and Watanabe, T. (2004). Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology, 62(7): 1186-1197.

CONCLUSION

The success of in vitro fertilization and in vitro embryo production depends on the proper cytoplasmic and nuclear maturation of oocytes. Improved cytoplasmic and nuclear maturation leads to an increase in the number of oocytes that are able to undergo fertilization and hence more embryos will be available for embryo transfer. It can be concluded from the experiment that the supplementation of α-tocopherol at 10 µg per ml to the IVM medium is suitable approach to manage oxidative stress to improve in vitro maturation of buffalo oocytes and to achieve higher cleavage rate upon in vitro fertilization.
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Kobayashi, K., Yanagamachi, S. and Hoshi, H. (1994). Influence of epidermic growth factor and transforming factor-alpha on in vitro maturation of cumulus enclosed bovine oocytes in defined medium. J. Reprod. Fertil., 100(2): 439-446.

Miclea, I., Andrea H., Marius, Z., Iulian, R. and Vasile, M. (2009). The effect of several α-tocopherol concentrations on swine oocyte maturation and embryo culture. Bulletin UASVM Anim. Sci. Biotech., 66(2): 385-392.

Natarajan, R., Shankar, M.B. and Munuswamy, D. (2010). Effect of alpha-tocopherol supplementation on in vitro maturation of sheep oocytes and in vitro development of preimplantation sheep embryos to the blastocyst stage. J. Assist. Reprod. Genet., 27(8): 483-490.

Olson, S.E. and Seidel, G.E.J.R. (2000). Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. Biol. Reprod., 62: 248-252.

Suresh, K. and Maurya, S.N. (2000). Evaluation of oocyte retrieval methods from bubaline ovaries. Indian J. Anim. Res., 34(2): 130-132.

Suresh, K.P., Nandi, S. and Mondal, S. (2009). Factors affecting laboratory production of buffalo embryos: A meta analysis. Theriogenology, 72: 978-985.

Tao, Y., Zhou, B., Xia, G., Wang, F., Wg, Z. and Fu, M. (2004). Exposure to L-ascorbic acid or α-tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and prevents cumulus cells from fragmentation. Reprod. Dom. Anim., 39(1): 52-57.

Wael, A., Khalil, Waleed, F.A., Marei and Muhammad, K. (2013). Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. Theriogenology, 80: 161-168.