



Effect of L-ascorbic acid supplementation at different gaseous environments on *in vitro* development of preimplantation sheep embryos to the blastocyst stage

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Abstract

Ascorbate is an important water-soluble antioxidant. Oxidative stress during *in vitro* gamete and embryo culture leads to defective development of gametes and embryos. In this context, the present study aimed to evaluate the effects of L-ascorbic acid supplementation to oocyte maturation media and embryo culture media and subsequent culture at different gaseous environments on the overall yield of ovine embryos. Rates of cleavage, morula and blastocyst, blastocyst total cell count and comet assay were taken as indicators of developmental competence of embryos. In experiments 1 and 2, L-ascorbic acid at concentrations of 0, 50, 100, 200, 400 and 500 μM was supplemented to oocyte maturation medium and cultured in an environment of 5% or 20% oxygen. L-ascorbic acid supplementation to oocyte maturation medium did not have any significant effect on yield of mature oocytes or embryos. In experiments 3 and 4, L-ascorbic acid at the aforesaid concentrations was supplemented to embryo culture media followed by culture in 5% or 20% oxygen environment. It was observed that 50 μM L-ascorbic acid in embryo culture medium at 5% oxygen levels significantly increased the rates of morulae ($P < 0.001$), blastocysts ($P < 0.01$) and blastocyst total cell number ($P < 0.05$) when compared to control. 100 μM L-ascorbic acid in embryo culture medium at 20% oxygen levels significantly increased rates of cleavage ($P < 0.01$), morulae ($P < 0.05$), blastocyst ($P < 0.01$) and blastocyst total cell number ($P < 0.01$) compared to control. Supplementation of embryo culture media with 400 μM and 500 μM L-ascorbic acid significantly retarded the rates of embryo formation and development. We conclude that optimal L-ascorbic acid supplementation may enhance the *in vitro* developmental competence of ovine embryos by protecting them from oxidative damage.

Keywords: embryo culture, *in vitro* fertilization, ovine, oxidative stress, vitamin C.

Introduction

In vitro fertilization (IVF) and other assisted reproductive technologies (ART) have been experimented in farm animals to enable production of

viable and high-yielding offspring in large numbers. Oxidative stress is a major factor in IVF that affects the overall yield of viable embryos (Guerin *et al.*, 2001). Free radicals generated by endogenous processes such as normal cellular metabolism and exogenous factors such as chemicals added to culture media, hyperoxia, exposure to light etc., cause oxidative damage to gametes and embryos. The innate antioxidant defense systems in embryos are insufficient to protect the embryos from high levels of oxidative stress during *in vitro* culture. Damages caused by pro-oxidants and reactive oxygen species (ROS) to gametes and embryos during IVF have been documented in bovine (Silva *et al.*, 2007), porcine (Kitagawa *et al.*, 2004) and murine (Nasr-Esfahani and Johnson, 1991) gametes and embryos.

Therefore, controlling the levels of ROS by supplementing antioxidants to gamete and embryo culture media is essential for the proper formation and interaction of gametes, fertilization and embryo development *in vitro* (Miesel *et al.*, 1993; Sanocka and Kurpisz, 2004). Several compounds including antioxidant enzymes, proteins, vitamins, thiol compounds and metal chelators have been supplemented to culture media as antioxidants to counter the oxidative stress. Ascorbate is a potent antioxidant that is known to protect human sperm against endogenous oxidative DNA damage (Fraga *et al.*, 1991). It is the major antioxidant in the seminal plasma of fertile men (Lewis *et al.*, 1995). Ascorbic acid has been associated with fertility in primates and may have evolutionary significance (Millar, 1992). Ascorbate is known to scavenge a wide range of ROS (Sies *et al.*, 1992). Supplementation of ascorbate to culture media has been reported to improve blastocyst production in mice (Eppig *et al.*, 2000). Thus ascorbate plays a key role in vertebrate gamete and embryo development both *in vivo* and *in vitro*. For embryo culture *in vitro*, a 5-10% oxygen environment was reported to favor the most while a 20% oxygen environment was reported to favor the least (Bernardi *et al.*, 1996; Leoni *et al.*, 2007). Therefore, we intended to determine the antioxidant potential of L-ascorbic acid under conditions of low and high oxygen levels in *in vitro* sheep oocyte and embryo culture.

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Materials and Methods

Unless otherwise stated, all chemicals used in this experiment were purchased from Sigma-Aldrich Chemicals Pvt Ltd., Bangalore, India.

Oocyte collection

Sheep ovaries were obtained from a local abattoir and transported to the laboratory suspended in 0.9% saline supplemented with 50 µg/ml gentamycin in insulated containers within 1 h of slaughter. Upon arrival, the ovaries were washed repeatedly in normal saline, trimmed free of extraneous tissue and rinsed in normal saline. The cumulus-oocyte complexes (COCs) were isolated from follicles by the slicing method (Pawshe *et al.*, 1994) and subsequently washed three times in Tyrode's lactate – N – [2-hydroxyethyl] piperazine – N' – [2-ethanesulphonic acid] (TL-HEPES) medium. The COCs were assessed morphologically and only those that had a compact non-atretic cumulus oophorus – corona radiata and a homogenous ooplasm were selected for *in vitro* maturation.

In vitro maturation

The COCs were washed three times – first in TL-HEPES medium and subsequently in maturation medium composed of TCM 199 (Invitrogen Corporation, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco Laboratories, Grand Island, USA), 5.5 mg/ml sodium pyruvate, 25 µg/ml gentamycin sulphate, 5.0 µg/ml LH (ovine LH; Sigma, L5269), 0.5 µg/ml FSH (porcine FSH; Sigma, F8001) and 1 µg/ml Estradiol (E2). Twenty COCs were placed in 100 µl droplets of maturation medium, covered with sterile mineral oil and matured for 24 h at 39°C at 5% CO₂ in air (which is approximately 20% O₂) or at 5% CO₂, 5% O₂ and 90% N₂, based on the design of the experiment.

In vitro fertilization

Sheep testis obtained from the local abattoir was transported to the laboratory suspended in 0.9% saline supplemented with 50 µg/ml gentamycin in an insulated container within 1 h of slaughter. Sheep testis was washed in saline and semen was extracted by a procedure partly similar to that of Wani *et al.*, (2000); the difference being swim-up procedure used as sperm separation technique by these authors. The testis was trimmed free of covering tissues and the tail of the epididymis, presumed to contain mature sperm, was cut using a sterile blade. The sperm-rich fluid that oozed out was directly laid on Bovine Serum Albumin-free Brackett and Oliphant (BSA free BO) medium (Brackett and Oliphant, 1975) (containing 10 mM caffeine sodium benzoate and 10 µg/ml Heparin) in a Petri dish. Sperm

selection was carried out in a Percoll (Pharmacia, Uppsala) density gradient (45/90%) placed in CO₂ incubator at 39°C for 2 h. Approximately 2 - 3 ml of BSA free BO medium containing the semen sample was layered over the pre-incubated gradient solution in sterile centrifuge tubes, and centrifuged at 1400 rpm for 10 min at room temperature. The supernatant was discarded and the sperm sediment was rewashed three times by centrifugation at 1400 rpm for 10 min in BSA-free BO medium described above. The final pellet was resuspended in 1 ml of BSA-free BO medium diluted with 1 ml BO medium containing 20 mg/ml BSA supplemented with 10 µg/ml heparin. A final sperm concentration of approximately 1-2 x 10⁶/ml BO medium was used for fertilization. Mature sheep COCs were then washed in BO medium and distributed at a rate of 20/100 µl drop of fertilization medium under mineral oil. Capacitated spermatozoa (2 µl) were added to these fertilization drops and incubated for 18 h at 39°C at 5% CO₂, 5% O₂ and 90% N₂.

In vitro embryo culture

Upon completion of the incubation period, the oocytes were washed to remove the cumulus cells by repeated pipetting through a small-bore pipette. They were then cultured in modified synthetic oviductal fluid medium (Tervit *et al.*, 1972) containing 2% (v/v) BME essential amino acids and 1% (v/v) MEM non-essential amino acids, 3 mg/ml BSA, 0.6 mM sodium pyruvate, 10 µg/ml gentamycin at a rate of 20 embryos/100 µl droplet for 8 days at 39°C under 5% CO₂ in air (which is approximately 20% O₂) or under 5% CO₂, 5% O₂ and 90% N₂ based on the design of the experiment. The medium was changed once every 48 h to replenish the nutrients.

Blastocyst cell number analysis

Blastocyst cell number analysis was done to assess the morphological quality of embryos. Expanded day 8 blastocysts from each treatment group were fixed and stained as per the method described by Mermillod *et al.* (1993). These expanded blastocysts were then individually transferred onto glass microscopic slides and dried at room temperature. They were then fixed with 70% ethanol for 24 h. The fixed blastocysts were then stained with 10 µg/ml bisbenzamide (Hoechst 33342) and 2.3% sodium citrate. The slides were observed under an epifluorescence microscope, fitted with an excitation filter (330 to 380 nm) and barrier filter (420 nm). The total numbers of nuclei in each blastocyst were counted.

Comet assay

DNA damage in individual embryos cultured for 3 days under 5% O₂ or 20% O₂, in the



presence/absence of vitamin C supplementation was assessed by comet assay (Takahashi *et al.*, 2000). About 10 to 20 embryos were washed twice in a mixture of phosphate buffered saline (PBS) and polyvinylpyrrolidone (4 mg/ml). Then embryos from each experimental group were transferred to a 200 μ l drop of 1% low-melting temperature agarose (Genei, Bangalore) in PBS at 39°C; and the agarose drop was placed on a 35 mm plastic Petri dish. Using a stereo-dissection microscope to visualize the embryos, the embryos were gently mixed with the 1% low-melting temperature agarose and then captured in a total volume of about 10 μ l using a mouth-operated glass pipette. The embryos were then quickly transferred onto a glass microscopic slide pre-coated with 1% high melting temperature agarose (Genei, Bangalore). Then the slides were placed for 5 min on ice to solidify the agarose. The embryos were then lysed by incubating the slides for 3 h at ambient temperature in lysing buffer composed of 10 mM Tris, pH 10, containing 100 mM sodium EDTA, 2.5 mM NaCl, 10 μ g/ml proteinase K, 1% sodium sarcosinate and 1% Triton X-100. Then the slides were removed from the lysing solution and placed on a horizontal gel electrophoresis unit. The unit was filled with fresh electrophoresis buffer (1 mM sodium EDTA, 300 mM NaOH) to a level 0.25 cm above the slides, followed by equilibration of the slides in electrophoresis buffer for 20 min. Electrophoresis was then carried out at 25 V for 20 min. The slides were then neutralized by immersing in 0.4 M Tris-HCl (pH 7.5) for 5 min at ambient temperature. Staining of DNA was carried out by adding a 20 μ l drop of acridine orange (5 μ g/ml) to the slide for 2 min followed by 1 min of washing in sterile distilled water. Stained DNA was observed under fluorescence microscope. Quantification of DNA damage was done by measuring the length of the streak of DNA comet tail. The length was calculated by comparing with a photograph of a micrometer of the same magnification as that of the embryos.

Experimental design

Vitamin C (L-ascorbic acid sodium; Sigma-Aldrich Chemicals Pvt Ltd., Bangalore, India) concentrations were standardized. In experiment 1, varying concentrations of L-ascorbic acid (0, 50, 100, 200, 400 and 500 μ m) were added to oocyte maturation medium followed by culture in 5% O₂ environment, while in experiment 2, the aforesaid concentrations of L-ascorbic acid were added to oocyte maturation medium followed by culture in 20% O₂ environment. Experiment 3 was carried out in 5% O₂ environment wherein varying concentrations of L-ascorbic acid (0, 50, 100, 200, 300 and 400 μ m) were added to embryo

culture medium and in experiment 4, the aforesaid concentrations of L-ascorbic acid were added to embryo culture medium followed by culture in 20% O₂ environment.

Thus the experimental design can be summarized as follows.

Experiment 1: Oocyte maturation with L-ascorbic acid at 5% O₂ environment and embryo culture without L-ascorbic acid at 5% O₂ environment;

Experiment 2: Oocyte maturation with L-ascorbic acid at 20% O₂ environment and embryo culture without L-ascorbic acid at 5% O₂ environment;

Experiment 3: Oocyte maturation without L-ascorbic acid at 5% O₂ environment and embryo culture with L-ascorbic acid at 5% O₂ environment;

Experiment 4: Oocyte maturation without L-ascorbic acid at 5% O₂ environment and embryo culture with L-ascorbic acid at 20% O₂ environment.

Statistical analyses

Statistical Package for Social Sciences (SPSS 11.0, Chicago, USA) software was used for statistical analysis. In each experimental group, oocytes were randomly distributed. The percentages of oocytes that were fertilized and embryos that had developed to morula and blastocyst were subjected to arcsine transformation before analysis. The total cell count data (of blastocyst) were directly subjected to analysis. All data were subjected to one-way ANOVA followed by Tukey's test to determine differences among experimental groups. Differences $P < 0.05$ were considered statistically significant.

Results

Experiment 1

The results of experiment 1 are shown in Table 1. Addition of L-ascorbic acid to *in vitro* oocyte maturation medium and subsequent culture under 5% O₂ environment did not result in significant increases in the percentage of cleavages, morula, blastocyst or total cell count when compared to control.

Experiment 2

Data from experiment 2 are shown in Table 2. No significant change was observed upon supplementation of L-ascorbic acid to *in vitro* oocyte maturation medium and subsequent culture under 20% O₂ environment with respect to rates of cleavage, embryos that developed to morula and blastocyst or blastocyst total cell number when compared with control.



Table 1. Effect of L-ascorbic acid supplementation to oocyte maturation medium on development of preimplantation sheep embryos cultured in 5% oxygen environment. Experiment 1.

L-ascorbic acid conc. (μM)	No. of oocytes inseminated	Cleavage (%)	Morula (%)	Blastocyst (%)	No. of blastocysts evaluated	Total cell number
0	372	64.7 \pm 1.8	33.2 \pm 2.2	10.4 \pm 1.5	17	88.3 \pm 3.2
50	390	64.6 \pm 1.5	32.8 \pm 1.7	10.1 \pm 0.9	17	88.5 \pm 3.8
100	366	65.6 \pm 1.6	33.8 \pm 2.2	11.0 \pm 1.1	19	89.7 \pm 4.4
200	375	65.9 \pm 1.2	34.9 \pm 2.0	11.5 \pm 1.3	18	95.8 \pm 4.8
400	381	63.3 \pm 1.6	32.8 \pm 1.8	9.7 \pm 1.4	18	91.8 \pm 3.6
500	362	62.8 \pm 2.4	32.2 \pm 2.1	9.4 \pm 1.2	19	87.2 \pm 3.8

Values are listed as mean \pm SEM. No significant difference was observed between control and supplemented groups or among any of the supplemented groups.

Table 2. Effect of L-ascorbic acid supplementation to oocyte maturation medium on development of preimplantation sheep embryos cultured in 20% oxygen environment. Experiment 2.

L-ascorbic acid conc. (μM)	No. of oocytes inseminated	Cleavage (%)	Morula (%)	Blastocyst (%)	No. of blastocysts evaluated	Total cell number
0	385	57.7 \pm 1.0	25.1 \pm 0.8	8.2 \pm 0.9	16	86.7 \pm 3.1
50	365	58.2 \pm 1.3	26.2 \pm 1.1	8.9 \pm 1.0	20	87.0 \pm 3.0
100	372	60.3 \pm 1.3	27.2 \pm 1.7	10.0 \pm 1.7	18	92.2 \pm 2.6
200	377	59.4 \pm 1.4	26.5 \pm 1.8	9.7 \pm 0.7	17	90.3 \pm 3.9
400	396	60.7 \pm 1.3	27.7 \pm 2.1	10.4 \pm 1.4	19	91.7 \pm 3.9
500	393	58.6 \pm 1.0	26.7 \pm 1.5	9.3 \pm 1.0	18	89.8 \pm 3.0

Values are listed as mean \pm SEM. No significant difference was observed between control and supplemented groups or among any of the supplemented groups.

Experiment 3

The effects of L-ascorbic acid in embryo culture medium at 5% oxygen level are shown in Table 3. Rates of cleavage were significantly less in 400 μM supplemented group ($P < 0.05$) and 500 μM supplemented group ($P < 0.001$) than control. The 50 μM supplemented group showed significantly higher rates of morulae ($P < 0.001$), blastocyst ($P < 0.01$) and

blastocyst total cell number ($P < 0.05$) than control. The percentage of morulae was significantly less in 400 μM ($P < 0.01$) and 500 μM ($P < 0.001$) supplemented groups than control. The blastocyst percentage was significantly less in 200 μM ($P < 0.01$), 400 μM ($P < 0.001$) and 500 μM ($P < 0.001$) supplemented groups when compared to control. The 400 μM ($P < 0.05$) and 500 μM ($P < 0.01$) supplemented groups showed significantly less blastocyst total cell count than control.

Table 3. Effect of L-ascorbic acid supplementation to embryo culture medium on development of preimplantation sheep embryos cultured in 5% oxygen environment. Experiment 3.

L-ascorbic acid conc. (μM)	No. of oocytes inseminated	Cleavage (%)	Morula (%)	Blastocyst (%)	No. of blastocysts evaluated	Total cell number
0	363	65.0 \pm 1.0 ^{ab}	30.0 \pm 0.8 ^{bc}	13.0 \pm 2.0 ^b	19	92.0 \pm 3.5 ^b
50	388	69.7 \pm 1.2 ^a	40.8 \pm 1.5 ^a	19.5 \pm 0.6 ^a	18	108.0 \pm 3.4 ^a
100	376	67.3 \pm 1.4 ^{ab}	34.0 \pm 1.5 ^b	15.0 \pm 1.2 ^{ab}	19	94.8 \pm 2.6 ^{ab}
200	368	63.8 \pm 2.2 ^{bc}	23.6 \pm 2.1 ^{cd}	6.9 \pm 0.9 ^c	15	83.5 \pm 4.6 ^{bc}
400	391	58.1 \pm 0.7 ^{cd}	21.0 \pm 1.2 ^d	3.2 \pm 0.6 ^{cd}	10	76.5 \pm 2.4 ^c
500	378	56.1 \pm 1.2 ^d	17.3 \pm 1.9 ^d	2.1 \pm 0.2 ^d	08	72.5 \pm 0.8 ^c

Footnote: Values are listed as mean \pm SEM. Means in the same column with different superscripts were significantly different ($P < 0.05$).

The extent of DNA damage in individual embryos on day 3 of culture at 5% oxygen levels, in absence or presence respectively of L-ascorbic acid supplementation is illustrated in Fig. 1. Embryonic DNA that migrated in the

gel was visualized as a comet tail-like streak under the fluorescence microscope. Supplementation of 50 μM L-ascorbic acid resulted in a significant reduction ($P < 0.05$) in comet tail length when compared with that of control.

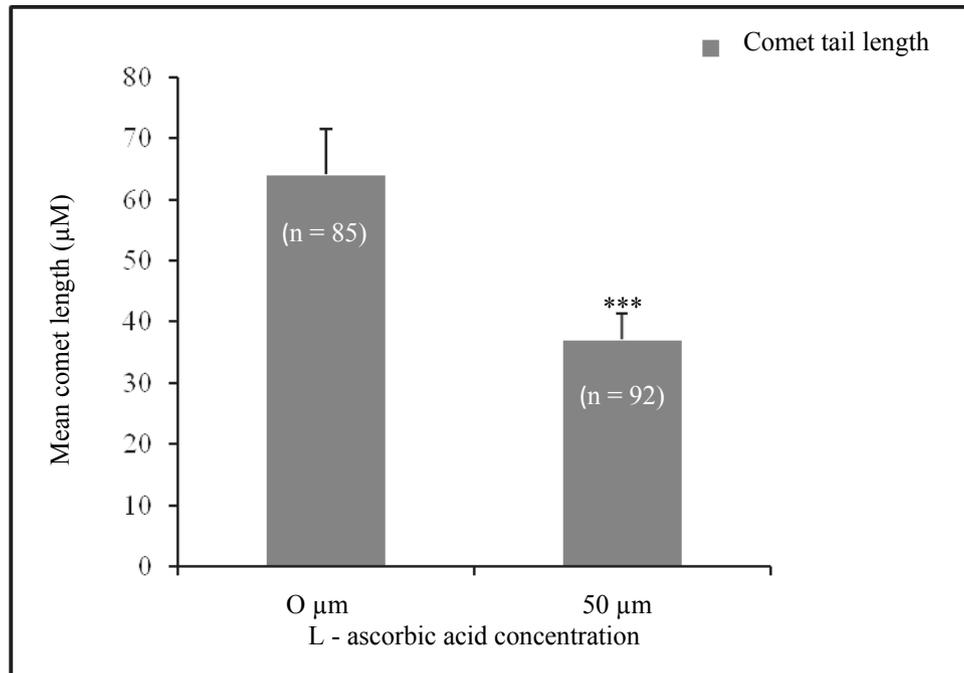


Figure 1. Effect of L-ascorbic acid supplementation to embryo culture medium at 5% oxygen levels on DNA damage in individual sheep embryos determined via comet assay. Values are expressed as mean \pm SEM. n = number of embryos used for comet assay. ***Significant difference ($P < 0.05$) was observed in 50 μM supplemented group when compared to control group.

Experiment 4

The results of experiment 4 are shown in Table 4. The rates of cleavage ($P < 0.01$), morula ($P < 0.05$), blastocyst formation ($P < 0.01$) and blastocyst total cell number ($P < 0.01$) were significantly higher in 100 μM supplemented group than control. Supplementation of 400 μM L-ascorbic acid significantly lowered the

rates of cleavage ($P < 0.001$), morula ($P < 0.01$), blastocyst formation ($P < 0.001$) and blastocyst total cell number ($P < 0.01$) compared to control. The 500 μM supplemented group also showed significantly lower rates of cleavage ($P < 0.001$), morulae ($P < 0.001$), blastocyst formation ($P < 0.001$) and blastocyst total cell number ($P < 0.001$) than control.

Table 4. Effect of L-ascorbic acid supplementation to embryo culture medium on development of preimplantation sheep embryos cultured in 20% oxygen environment. Experiment 4.

L-ascorbic acid conc. (μM)	No. of oocytes inseminated	Cleavage (%)	Morula (%)	Blastocyst (%)	No. of blastocysts evaluated	Total cell number
0	388	57.9 ± 0.6^b	25.2 ± 1.7^b	9.0 ± 1.3^b	15	93.3 ± 2.9^b
50	374	60.3 ± 0.8^{ab}	27.6 ± 1.6^{ab}	11.2 ± 1.2^{ab}	20	101.5 ± 2.1^{ab}
100	390	63.6 ± 1.2^a	32.3 ± 1.6^a	14.3 ± 0.6^a	17	110.3 ± 2.8^a
200	366	56.6 ± 1.5^b	23.8 ± 1.4^b	7.5 ± 1.1^b	15	89.8 ± 4.4^b
400	393	51.0 ± 0.9^c	16.1 ± 0.8^c	3.2 ± 0.4^c	09	75.3 ± 1.9^c
500	371	49.0 ± 0.6^c	15.9 ± 1.5^c	2.1 ± 0.3^c	07	69.8 ± 1.7^c

Values are listed as mean \pm SEM. Means in the same column with different superscripts were significantly different ($P < 0.05$).

Figure 2 demonstrates the extent of DNA damage in individual embryos on day 3 of culture at 20% oxygen levels, in absence or presence respectively of L-ascorbic acid supplementation. Under the fluorescence microscope, DNA from

individual embryos that migrated in the gel was visualized as a comet tail-like streak. Supplementation of 100 μM L-ascorbic acid resulted in a significant reduction ($P < 0.001$) in comet tail length when compared with that of control.

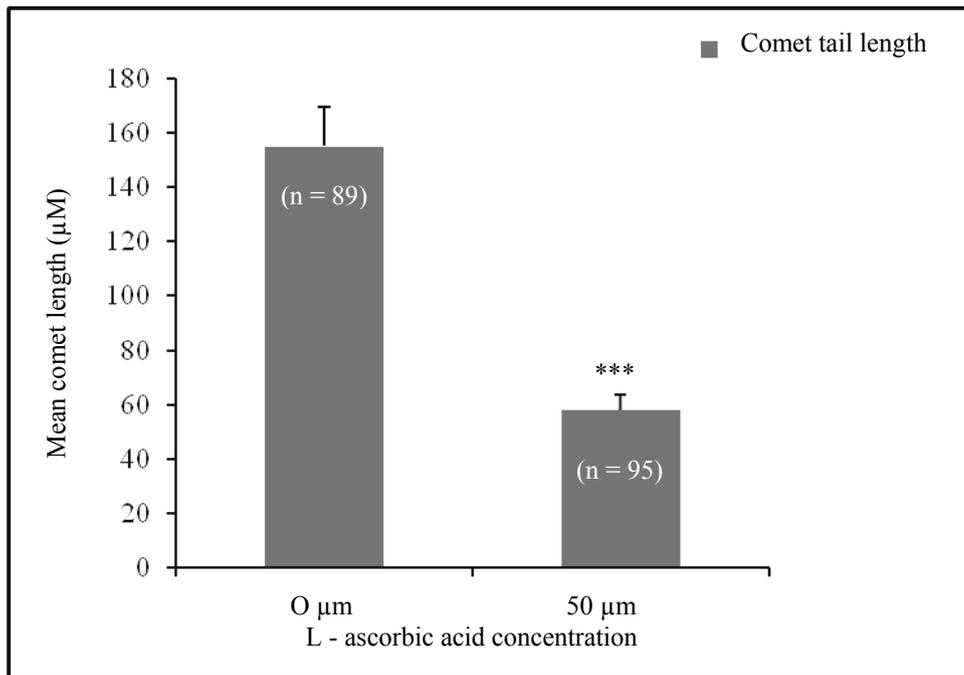


Figure 2. Effect of L-ascorbic acid supplementation to embryo culture medium at 20% oxygen levels on DNA damage in individual sheep embryos determined via comet assay. Values are expressed as mean \pm SEM. n = number of embryos used for comet assay. ***Significant difference ($P < 0.001$) was observed in 100 μm supplemented group when compared to control group.

Discussion

The present study evaluated the effect(s) of L-ascorbic acid supplementation to oocyte maturation media or embryo culture media and subsequent culture at 5% or 20% oxygen levels on *in vitro* development of ovine embryos. In this study, instead of ejaculated semen, epididymal semen was used for *in vitro* fertilization. This is because we intended to determine the maximal embryo yield from tissues of slaughtered animals rather than live ones. Use of epididymal semen for IVF has been documented by Bize *et al.* (1991) in hamsters and Wani *et al.* (2000) in sheep. However, whether sperm from fresh epididymal semen are more effective in terms of producing developmentally better competent embryos than those from fresh ejaculated semen needs to be investigated in future studies.

L-ascorbic acid supplementation to oocyte maturation medium did not significantly change the rate of oocyte maturation, embryo formation and development irrespective of the environmental oxygen concentration. This is in line with observations in bovine by Dalvit *et al.* (2005), who suggested that vitamin C in maturation medium had no effect on developmental competence of oocytes and embryos.

Metabolism of molecular oxygen is a key factor in embryo development (Houghton *et al.*, 1996). Reactive Oxygen Species (ROS) are produced during aerobic metabolism. Depending on the stage of

development of embryos, there is a variation in production of ROS and susceptibility to ROS by early embryos (Morales *et al.*, 1999). This is probably due to the metabolic changes undergone by embryos during their development. In early embryos, Adenosine triphosphate (ATP) production is largely related to glycolysis with a lesser percentage being attributed to oxidative phosphorylation (Harvey *et al.*, 2002). Therefore, the predominance of anaerobic metabolism could be the factor responsible for lower levels of oxidative stress in early embryos.

L-ascorbic acid supplementation to embryo culture medium followed by culture at 5% oxygen levels did not result in significant differences in rates of cleavage among control and supplemented groups. However, supplementation of 50 μM L-ascorbic acid significantly increased the rates of morula and blastocyst formation and blastocyst total cell number when compared to control. L-ascorbic acid, at concentrations of 400 μM and 500 μM was found to significantly hinder the rates of cleavage, morula, blastocyst development and blastocyst total cell number. This could be attributed to the embryotoxic effects of vitamin C at higher concentrations. The results of comet assay indicate the protective effects of L-ascorbic acid against oxidative damage to DNA. Embryos cultured at 5% oxygen levels under 50 μM L-ascorbic acid supplementation were found to have significantly shorter comet tail length when compared to control.



ATP synthesis rates are increased at the time of blastocyst formation to support rapid protein synthesis and also to support elevated activity of membrane ion-transport systems (Leese, 1995). Increased uptake of oxygen and energy substrates such as glucose and pyruvate, together with elevated rates of ATP synthesis, indicate increased oxidative metabolism, leading to increased ROS production during embryo development (Thompson *et al.*, 1996). Hence, supplementation of antioxidants at appropriate levels to the culture media to attain optimal embryo development becomes a necessity.

Vitamin C supplementation to embryo culture medium followed by culture at 20% oxygen level resulted in significantly higher rates of cleavage, morula and blastocyst formation and blastocyst total cell count in 100 μ M supplemented group when compared to control. The protective effects of vitamin C against oxidative damage to DNA can be deciphered from the results of the comet assay, wherein 100 μ M L-ascorbic acid supplementation significantly decreased the comet tail length when compared to control.

It could be stated that the concentration of vitamin C required for optimal embryo development varies based on the environmental oxygen levels. Thus, while 50 μ M L-ascorbic acid supplementation gives best results at 5% oxygen level, 100 μ M L-ascorbic acid supplementation seems to be optimal for embryos cultured at 20% oxygen level. In other words, it can be stated that the antioxidant vitamin C concentration needed for optimal embryo development is directly proportional to the oxygen levels in the culture environment. Hence, higher the levels of oxygen in the embryo culture environment, greater the concentration of antioxidant vitamin C needed for optimal growth of embryos. Supplementation of embryo culture medium with vitamin C at concentrations of 400 μ M and 500 μ M significantly decreased the rates of cleavage, morula, blastocyst development and blastocyst total cell number than control which is again attributed to its dose-dependent embryotoxicity.

Although vitamin C has been touted to be a key water-soluble antioxidant that scavenges free radicals, reduces sulfhydryls and protects against endogenous oxidative DNA damage (Fraga *et al.*, 1991), it also exhibits pro-oxidant effects. In the presence of free transition metals in culture media, vitamin C may act as a pro-oxidant (Guerin *et al.*, 2001). The functional relationship between ascorbic acid and α -tocopherol in embryo culture *in vitro* has been well documented. Ascorbic acid is known to regenerate α -tocopherol from tocopheroxyl radicals, thereby acting in synergy with α -tocopherol (Packer *et al.*, 1979; Chow, 1991). However, simultaneous supplementation of vitamin C and vitamin E to embryo culture media has not yielded any significant benefit(s) when compared to supplementing them individually (Wang *et al.*, 2002; Jeong *et al.*, 2006; Saikhun *et al.*, 2008). Therefore, future research

may be directed towards providing insight into the molecular mechanisms by which ascorbic acid counteracts oxidative stress during *in vitro* culture of embryos.

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