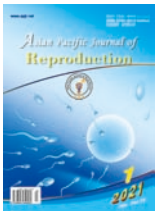

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305-0500.306436

Supplementation of *L*-ascorbic acid improves the *in vitro* development of buffalo (*Bubalus bubalis*) embryos and alters the expression of apoptosis-related genes

Mayank Roshan✉, Diksha Dua, Ankur Sharma, Manish Tiwari, Manoj Kumar Singh, Suresh Kumar Singla, Prabhat Palta, Radhay Sham Manik, Manmohan Singh Chauhan

Embryo Biotechnology Lab, Animal Biotechnology Centre, ICAR–National Dairy Research Institute, Karnal, India

ABSTRACT

Objective: To study the effect of *L*-ascorbic acid supplementation on the *in vitro* development of buffalo embryos and evaluate the relative mRNA abundance of some pro-apoptotic, anti-apoptotic, and embryonic development-related genes.

Methods: In experiment 1, we evaluated the effect of the addition of 0 (control), 50, and 100 μM *L*-ascorbic acid to the *in vitro* maturation medium on the developmental competence in terms of blastocyst rate and relative mRNA abundance of some pro-apoptotic (*BAX*, *BID*), anti-apoptotic (*BCL-XL*, *MCL1*), and embryonic development (*GDF9*, *BMP15*) related genes. Based on the results, we chose 50 μM as the suitable dose of *L*-ascorbic acid for the subsequent experiments. We further evaluated the blastocyst rates following the addition of 50 μM *L*-ascorbic acid to the *in vitro* culture medium (experiment 2), and *in vitro* maturation and *in vitro* culture media (experiment 3). In all three experiments, the maturation and culture media devoid of *L*-ascorbic acid served as the control group.

Results: The blastocyst rate after adding 50 μM *L*-ascorbic acid to the *in vitro* maturation medium was significantly higher than the control group ($P<0.05$), whereas 100 μM *L*-ascorbic acid exhibited a negative effect on the blastocyst rate. The blastocyst rates for embryos cultured in 50 μM *L*-ascorbic acid in the *in vitro* culture medium alone and both *in vitro* maturation and *in vitro* culture media were significantly higher than their corresponding control groups ($P<0.05$). The relative mRNA abundance of *BAX* significantly decreased in blastocysts produced after the addition of 50 μM *L*-ascorbic acid as compared with the control group ($P<0.05$), whereas, for *MCL1*, it significantly decreased in blastocysts produced after the addition of 100 μM *L*-ascorbic acid ($P<0.05$).

Conclusions: The supplementation of 50 μM *L*-ascorbic acid to *in vitro* maturation and *in vitro* culture media supports *in vitro* embryonic development in buffaloes by improving developmental competence and altering the expression of apoptosis-related genes.

KEYWORDS: Buffalo; Blastocyst; *In vitro* embryo production; *L*-ascorbic acid; Oocyte

1. Introduction

Owing to its immense contribution to milk, meat, draught power, and employment generation, buffalo plays a vital role in the rural economy of the Indian subcontinent. However, it has been considered as a sluggish breeder due to its poor reproductive performance as denoted by the delayed arrival of puberty, low conception rate, seasonality, anestrous, and prolonged calving intervals[1]. Among the several assisted reproductive technologies adopted to improve the reproductive efficiency and faster multiplication of elite germplasm in buffaloes, *in vitro* embryo production through *in vitro* fertilization (IVF) holds a prominent place. Despite the successful implementation of *in vitro* embryo production in buffaloes, the blastocyst rate has remained very poor at only <20%[2,3] as compared with that of 35%–48% in cattle[4,5]. The low blastocyst rate for buffalo *in vitro* embryo production is principally due to suboptimal culture conditions and requires substantial improvements.

The oxidative stress-mediated by the generation of reactive oxygen species (ROS) during the embryo metabolism and culture environment causes various types of embryo damages, including DNA, RNA, and protein damages, lipid peroxidation, mitochondrial dysfunction, and apoptosis[6]. Mammalian embryos at the early stage of development are susceptible to oxidative damage[6], and they produce elevated levels of ROS when cultured *in vitro*[7]. The

✉To whom correspondance may be addressed. E-mail: mayank.ndri02@gmail.com

How to cite this article: Roshan M, Dua D, Sharma A, Tiwari M, Singh MK, Singla SK, et al. Supplementation of *L*-ascorbic acid improves the *in vitro* development of buffalo (*Bubalus bubalis*) embryos and alters the expression of apoptosis-related genes. *Asian Pac J Reprod* 2021; 10(1): 36-42.

Article history: Received: 24 March 2020; Revision: 19 September 2020; Accepted: 28 October 2020; Available online: 15 January 2021

cells have adopted specific complex antioxidant defence mechanisms to retrench the damages caused by the oxidative free radicals, which rely upon certain enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and also small molecule scavengers like vitamins A, C, E, taurine, hypotaurine, and cysteamine. The shielding effect of the enzymatic protectants is mainly limited to intracellular protection, whereas small molecule scavengers act as a sink to the spare electrons and play a critical role in the extracellular milieu. In this regard, vitamin C (*L*-ascorbic acid) is such a crucial antioxidant that improves the *in vitro* embryonic development by counteracting the apoptosis induced by ROS[8-11]. Its significance for *in vitro* embryo production in buffaloes in terms of improved developmental competence has been reported in previous studies[9,11]. Despite the antioxidant role of *L*-ascorbic acid and its beneficial effect on embryonic development, the selection of its optimum dose is crucial, as, at a high dose, it might act as a pro-oxidant and significantly hamper embryonic development[12,13].

Since the ROS manifest their deleterious effects on embryonic development mainly by inducing apoptosis, the evaluation of the effect of *L*-ascorbic acid on developmental competence along with relative mRNA abundance of some crucial genes related to apoptosis and embryonic development would provide a better understanding regarding its antioxidant effect on the *in vitro* embryo production in buffaloes. However, limited information is available regarding of *L*-ascorbic acid's effect on the relative expression of apoptosis and development-related genes in the *in vitro* produced buffalo embryos. With this backdrop, the present study aims to evaluate the effect of *L*-ascorbic acid on the developmental competence and relative mRNA abundance of some crucial apoptosis and development-related genes and to assess its effect on the developmental competence after supplementation to the *in vitro* maturation and *in vitro* culture media individually and also to both *in vitro* maturation and *in vitro* culture media together.

2. Materials and methods

2.1. Experimental design

L-ascorbic acid supplementation during maturation and development had been reported to improve the blastocyst rate during *in vitro* embryonic production in the water buffaloes. To gain a better understanding of *L*-ascorbic acid's antioxidant effect on developmental competence, we evaluated its effect on blastocyst rate after addition to the maturation- and culture media both individually and in combination with each other. In experiment 1, we supplemented the *in vitro* maturation medium with 0 (control), 50, and 100 μM doses of *L*-ascorbic acid[11,14] and determined the blastocyst rate. These blastocysts were further used to evaluate the relative mRNA abundance of pro-apoptotic (*BAX*, *BID*), anti-apoptotic (*BCL-XL*, *MCL1*), and embryonic development (*GDF9*, *BMP15*) related genes. Our results showed that 50 μM dose of *L*-ascorbic acid supported the embryonic development better than 100 μM and therefore we selected 50 μM dose of *L*-ascorbic acid for subsequent experiments. We then evaluated the effect of 50 μM *L*-ascorbic acid on blastocyst rate after its addition to *in vitro* culture medium alone (experiment 2) and *in vitro* maturation and *in vitro* culture media together (experiment 3). For these experiments, the respective media devoid of *L*-ascorbic acid supplementation served as the control group.

2.2. Chemicals

All the reagents and media were procured from Sigma Chemical (St Louis, MO, USA) unless otherwise specified. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA), and plastic wares were purchased from Nunc (Rosklide, Denmark).

2.3. Oocyte collection and in vitro maturation

Buffalo ovaries were collected from Ghazipur slaughterhouse, Delhi, and washed three times with 0.9% saline containing 500 μg/mL streptomycin and 400 IU/mL penicillin and transported to the laboratory within 6 h of collection. Follicular oocytes (2-8 mm in diameter) were aspirated using an 18 G needle attached with a 10-mL syringe in the aspiration medium [tissue culture medium-199 (TCM-199) + 2 mM *L*-glutamine + 0.3% bovine serum albumin (BSA) + 50 μg/mL gentamicin sulfate] and compact cumulus-oocyte complexes having >3 layers of cumulus cells with homogenous granular ooplasm were searched. Subsequently, the oocytes were washed 4-5 times in washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 2 mM *L*-glutamine + 50 μg/mL gentamicin sulphate) and then washed thrice with *in vitro* maturation medium (washing medium supplemented with 10% buffalo follicular fluid + 5 μg/mL pFSH + 1 μg/mL β-estradiol). Groups of cumulus-oocyte complexes (about 15-20) were transferred to 100 μL droplets of the *in vitro* maturation medium covered with sterile mineral oil and then incubated at 38.5 ℃ for 24 h with proposed treatments in a 5% CO_2 incubator.

For experiment 1, the number of oocytes used for groups 0, 50, and 100 μM doses of *L*-ascorbic acid were 403, 447, and 420, respectively. In experiment 2, the number of oocytes used for groups 0- and 50 μM *L*-ascorbic acid were 330 and 329, respectively. In experiment 3, we used 383 and 399 oocytes for groups 0- and 50 μM *L*-ascorbic acid, respectively.

2.4. Production of IVF embryos

The spermatozoa used for IVF were processed as described previously[15] with minor modifications. Briefly, frozen-thawed buffalo semen was washed twice with washing Bracket and Oliphant (BO) medium containing 10 μg/mL heparin, 1.942 mg/mL caffeine sodium benzoate, and 137.0 μg/mL sodium pyruvate. The pellet was resuspended in approximately 0.5 mL of the capacitation and fertilization BO medium (washing BO medium + 10 mg/mL fatty acid-free BSA). The *in vitro*-matured oocytes were then washed

twice with washing BO medium and transferred to 50 μL droplets of the capacitation and fertilization BO medium in 15-20 oocytes/ droplet groups. A 50 μL volume of spermatozoa resuspended previously in capacitation and fertilization BO medium was added to each droplet containing the oocytes, overlaid with mineral oil, and incubated at 38.5 ℃ for 18 h in a humidified 5% CO_2 incubator for IVF.

Following sperm-oocyte incubation, the cumulus cells were then washed-off presumptive zygotes. The presumptive zygotes were washed thrice with modified Charles Rosenkrans medium with amino acids (mCR2aa) supplemented with 0.8% BSA and cultured in the same medium for 48 h post-insemination. Subsequently, the embryos were transferred to the *in vitro* culture medium (mCR2aa + 10% FBS + 0.8% BSA) and subsequently cultured in 100 μL droplets for up to 9 days post-insemination at 38.5 ℃ in a humidified 5% CO_2 incubator. The medium was replaced with 50% of fresh *in vitro* culture medium at 48 h intervals, and cleavage rate was recorded on day 2 post-insemination, followed by 4-cell, 8- to 16- cell, morula, blastocyst, and hatched blastocyst on days 3, 4, 5, 8 and 9 of culture, respectively (Figure 1). The blastocyst rate index was the percentage of total blastocysts formed to the number of total embryos cleaved for the respective group.

2.5. Quantitative real-time polymerase chain reaction (qPCR) analysis

qPCR was performed as described previously[16]. Briefly, total RNA was isolated from embryos (blastocysts n=5 to 6 each) using RNAqueous- Micro Kit (Ambion Inc. The RNA Company, Austin, TX, USA) as per the manufacturer's instructions. The concentration and purity of RNA were determined by Nanoquant (Teccan, Salzburg, Austria). Following DNase treatment, the cDNA was prepared using Superscript Ⅲ, first-strand cDNA synthesis kit (Invitrogen) and stored at -80 ℃ until use for qPCR.

The relative mRNA abundance was determined on a C.F.X. 96 I Cycler (Bio-Rad, Hercules, CA, USA) using 10 μL reaction volume incorporating 5 μL of SYBR Green Master Mix (Maxima SYBR Green Mastermix; Fermentas; Fisher Scientific, Pittsburgh, PA, USA), 0.2 μL of 10 μM of each primer and cDNA. The following thermal cycling conditions were used: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles at 95 ℃ for 15 s, primer specific annealing temperature (given in Table 1) for 15 s, 72 ℃ for 15 s, and a final extension step at 72 ℃ for 5 min. The melting cycle started from 65 ℃ up to 95 ℃ with a 0.5 ℃/s transition rate. The relative gene expression was determined by using the method as described previously[17]. Gene expression data were normalized against GAPDH expression and were analyzed by using CFX Manager (Bio-Rad). The calibrator in each study consisted of cDNA from the corresponding control group. Relative mRNA abundance was expressed as n-fold mRNA expression relative to the calibrator. The specificity of the PCR products was confirmed by the melting curve analysis, whereas the appropriateness of product size was validated by 2% agarose gel electrophoresis. In all experiments, three trials were executed, each in duplicate.

2.6. Statistical analysis

SPSS 17.0 (IBM, USA) software was used for data analysis. The means between different *L*-ascorbic acid groups supplemented to *in vitro* maturation medium were compared by one-way analysis of variance followed by Fisher's least significant difference test. In contrast, the Student's t-test was used to compare the mean difference between the control and treatment groups for *L*-ascorbic acid supplementation to *in vitro* culture media or *in vitro* maturation and *in vitro* culture media. Each experiment was repeated five times using 70 to 90 oocytes in each group per replicate. The mean and standard deviation were calculated by using these replicate values, and the ct values obtained from real-time PCR were arcsine transformed before analysis. The differences were considered statistically significant at a 5% level of significance (P<0.05).

2.7. Ethics statement

Buffalo ovaries were collected from a government-approved abattoir (Ghazipur slaughterhouse, Delhi) and handled as per the guidelines by the Institute Animal Ethics Committee. Since the biological samples were collected *via* a noninvasive method, a separate ethical approval number was not received.

Table 1. Primers used for gene expression study.

Gene	Primer sequence	Size (bp)	Annealing temperature (℃)	Accession No.
BMP15	F- CATCCCTTACGGTATATGCTG R- GTTTGGTCTCAGAGGAAAGTC	179	56	DQ463368.1
GDF9	F- CCCTAAATCCAACAGAAGCC R- GTTCCACAACAGTAACACGA	148	60	NM_174681.2
BCL-XL	F- TTGTGGCCTTTTTCTCCTTC R- GATCCAAGGCTCTAGGTGGT	128	60	ENSBTAT00000008572
MCL1	F- TCGGAAACTGGACATCAAAA R- CCACAAAGGCACCAAAAGAA	128	58	ENSBTAT00000020159
BID	F- CTGTCGGAGGAGGACAGGAG R- GTGGTCGGCTATCTTTTTGG	135	60	NM_001075446.1
BAX	F- CCTTTTGCTTCAGGGTTTCA R- CGCTTCAGACACTCGCTCA	123	60	NM_001191220.1
GAPDH	F-TCAAGAAGGTGGTGAAGCAG R-CCCAGCATCGAAGGTAGAAG	122	57	GU324291.1

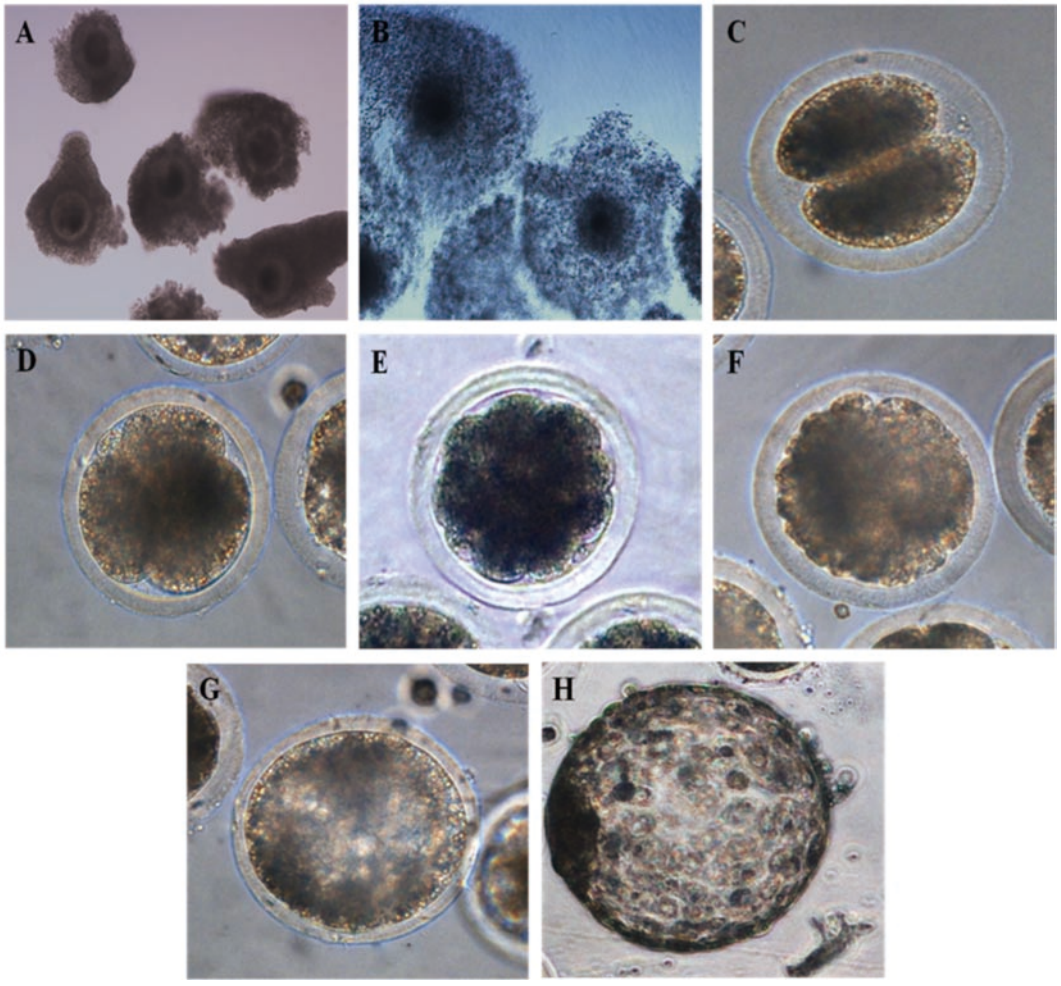

Figure 1. *In vitro* produced buffalo embryos at different stages of development: (A) Immature oocytes of usable quality (grade A & B); (B) *In vitro* matured oocytes; (C) 2-cell; (D) 4-cell; (E) 8-16 cell; (F) Morula; (G) Expanded blastocyst; and (H) Hatched blastocyst. Magnification: 100×.

3. Results

3.1. Effect of L-ascorbic acid supplementation on the in vitro embryonic development

The supplementation of *in vitro* maturation medium with 50 μM *L*-ascorbic acid significantly increased blastocyst rate compared with the control group (P<0.05), although the increase in other developmental stages was nonsignificant. However, the supplementation of 100 μM *L*-ascorbic acid to the *in vitro* maturation medium showed a non-significant decrease at all the developmental stages compared with the control group (Table 2).

The supplementation of *in vitro* culture medium with 50 μM *L*-ascorbic acid significantly improved the developmental competence for all the development stages except the 2-cell stage (P<0.05) (Table 3). The addition of 50 μM *L*-ascorbic acid to both *in vitro* maturation and *in vitro* culture media significantly improved developmental competence for all the stages of embryonic development except the 2-cell stage (P<0.05) (Table 4).

3.2. Effect of supplementation of L-ascorbic acid on the relative mRNA abundance of apoptosis and embryonic development-related genes

For pro-apoptotic genes, the relative mRNA abundance of *BAX* decreased significantly upon supplementation of 50 μM *L*-ascorbic acid to the *in vitro* maturation medium as compared with the control group (P<0.05) (Figure 2). The relative mRNA abundance for *BID* did not significantly decrease after the addition of 50 μM *L*-ascorbic acid. There was a nonsignificant increase in relative mRNA abundance of *BAX* and *BID* after supplementation of 100 μM L-ascorbic acid.

For anti-apoptotic genes, the relative mRNA abundance of *MCL1* decreased significantly after the addition of 100 μM L-ascorbic acid as compared with the control group (P<0.05), whereas 50 μM *L*-ascorbic acid showed a non-significant increase in the relative mRNA abundance of both *MCL1* and *BCL-XL*. The relative mRNA abundance of embryonic development-related genes *GDF9* and *BMP15* was not significantly affected after supplementing *L*-ascorbic acid to the *in vitro* maturation medium.

Table 2. Effect of *L*-ascorbic acid supplementation to *in vitro* maturation medium on the developmental competence of buffalo embryos.

Group	Oocytes cultured (*n*)	Percentage development [*n*(%)]				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	403	253(62.78±6.07)[a]	184(72.73±3.96)[a]	135(53.36±1.58)[a]	62(24.51±2.41)[a]	27(10.67±0.55)[a]
50 μM *L*-ascorbic acid	447	298(66.67±6.35)[a]	235(78.86±6.20)[a]	176(59.06±3.70)[a]	97(32.55±2.97)[a]	50(16.78±2.83)[b]
100 μM *L*-ascorbic acid	420	227(54.04±4.93)[a]	141(62.11±5.81)[a]	75(33.04±5.10)[a]	36(15.86±0.84)[a]	14(6.17±0.84)[a]

Given values are percentages (mean±SD) as well as absolute values (*n*) from the total number of respective oocytes taken (*n*). a, b: Percentages within a column having different superscripts differ significantly ($P<0.05$). [1]2-cell%: number of embryos cleaved/number of embryos cultured. [2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

Table 3. Effect of supplementation of 50 μM *L*-ascorbic acid to the *in vitro* culture medium on the developmental competence of buffalo embryos.

Group	Oocytes cultured (*n*)	Percentage development [*n*(%)]				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	330	190(57.58±3.11)[a]	125(65.79±4.03)[a]	89(46.84±2.50)[a]	44(23.16±2.16)[a]	18(9.47±1.73)[a]
50 μM *L*–ascorbic acid	329	228(69.30±6.48)[a]	183(80.26±4.11)[b]	139(60.96±4.11)[b]	78(34.21±2.08)[b]	46(20.18±1.73)[b]

Given values are percentages (mean±SD) as well as absolute values (*n*) from the total number of respective oocytes taken (*n*). a, b: Percentages within a column having different superscripts differ significantly ($P<0.05$). [1]2-cell%: number of embryos cleaved/number of embryos cultured. [2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

Table 4. Effect of supplementation of 50 μM *L*-ascorbic acid to both *in vitro* maturation and *in vitro* culture media on the developmental competence of buffalo embryos.

Group	Oocytes cultured (*n*)	Percentage development [*n*(%)]				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	383	210(54.83±4.36)[a]	139(66.19±6.22)[a]	98(46.67±7.99)[a]	54(25.71±3.63)[a]	24(11.43±3.19)[a]
50 μM *L*-ascorbic acid	399	266(66.67±4.97)[a]	217(81.58±5.08)[b]	166(62.41±2.77)[b]	103(38.72±3.91)[b]	76(28.57±0.84)[b]

Given values are percentages (mean±SD) as well as absolute values (*n*) from the total number of respective oocytes taken (*n*). a, b: Percentages within a column having different superscripts differ significantly ($P<0.05$).[1]2-cell%: number of embryos cleaved/number of embryos cultured. [2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

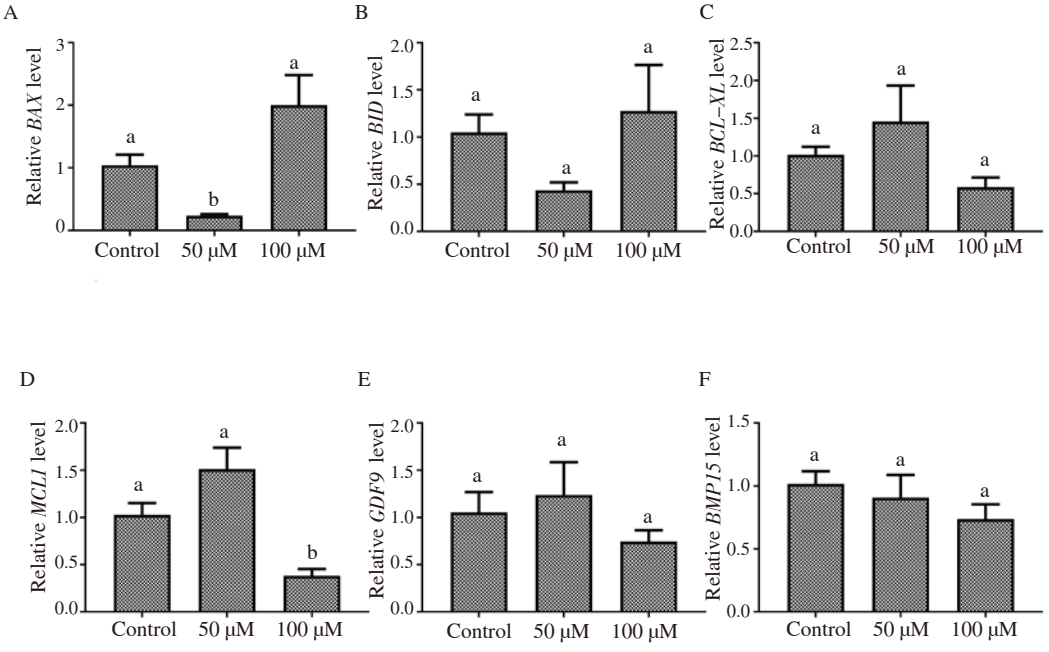

Figure 2. Effect of supplementation of *in vitro* maturation medium with 50- and 100 μM *L*-ascorbic acid on the relative mRNA abundance of some pro-apoptotic (*BAX*, *BID*), anti-apoptotic (*BCL–XL*, *MCL1*), and embryonic development (*GDF9*, *BMP15*) related genes in the *in vitro* produced buffalo blastocysts. Bars with different superscripts differ significantly ($P<0.05$). 50 μM: 50 μM *L*-ascorbic acid group; 10 μM: 10 μM *L*-ascorbic acid group.

4. Discussion

During the past several years, researchers have emphasized the inclusion of antioxidants in the culture medium to alleviate oxidative stress and its detrimental effects on oocytes and embryos during *in vitro* embryo production. *L*-ascorbic acid is a crucial antioxidant that significantly improves embryonic development in terms of increased blastocyst rate and total cell number and reduced apoptotic index[8–11]. In the present study, the *L*-ascorbic acid concentrations were chosen based on the earlier studies in buffalo[11] and sheep[14]. Our results showed that the supplementation of 50 μM *L*-ascorbic acid to the *in vitro* maturation medium exhibited a profound increase in the blastocyst rate compared with the control group, whereas its 100 μM dose exhibited an adverse effect on the blastocyst rate. These results agree with a previous study in buffalo[11] wherein the supplementation of 50 μM *L*-ascorbic acid showed better cleavage and blastocyst rate than 25- and 100 μM doses. It has been previously reported that supplementation of *L*-ascorbic acid to the *in vitro* maturation medium decreases the ROS level and elevates the mature oocytes' glutathione level, culminates in superior ooplasmic maturation and better embryonic development[10]. Our results support the notion from the earlier studies[10,11] that a narrow window exists for concentration dependency of *L*-ascorbic acid for its antioxidant effect on embryonic development. Moreover, supplementing a high *L*-ascorbic acid dose might act as a pro-oxidant and significantly retard embryonic development[12,13].

To gain better insight into *L*-ascorbic acid's antioxidant effect on embryonic development, we have further supplemented 50 μM *L*-ascorbic acid to the *in vitro* culture medium alone, and both *in vitro* maturation and *in vitro* culture media together and subsequently evaluated its effect on the developmental competence. Our results demonstrated that in both the experimental conditions, the *L*-ascorbic acid supplementation significantly improved the developmental competence at all the stages except the 2-cell stage. However, after adding *L*-ascorbic acid to both the *in vitro* maturation and *in vitro* culture medium, the blastocyst rate was considerably higher than that achieved after its addition to the *in vitro* culture medium alone. Our results demonstrated that the supplementation of 50 μM *L*-ascorbic acid to both *in vitro* maturation and *in vitro* culture media supported the embryonic development more efficiently than *in vitro* maturation or *in vitro* culture medium alone.

Since the ROS's oxidative damages induce apoptosis in cells and embryos, the evaluation of relative mRNA abundance of apoptosis-related crucial genes in response to the *L*-ascorbic acid supplementation would provide a better understanding regarding its antioxidant role in embryonic development. Among the various genes involved in apoptosis, those from the *BCL–2* gene family are the most widely studied, including pro-apoptotic cell death inducers (*BAX*, *BID*, *BAK*, *BAD*, *etc*.) and anti-apoptotic cell death suppressers (*BCL–2*, *BCL–XL*, *MCL1*, *etc*.). The protein products of these genes form complex arrays of homo- and heterodimers to regulate apoptosis[18]. The pro-apoptotic proteins form homodimers to mediate the mitochondrial pore formation and depolarization, whereas the anti-apoptotic proteins form heterodimers with the pro-apoptotic proteins to neutralize their action. Hence, the relative expression levels of these pro- and anti-apoptotic genes determine a cell's fate, whether it will survive or undergo apoptosis. Therefore, we further evaluated the effect of the addition of 50- and 100 μM *L*-ascorbic acid to the *in vitro* maturation medium on the relative mRNA abundance of some crucial pro-apoptotic (*BAX*, *BID*) and anti-apoptotic (*BCL–XL*, *MCL1*) genes at the blastocyst level.

Our results showed a significant decrease in the relative mRNA abundance of pro-apoptotic gene *BAX* after supplementation of 50 μM *L*-ascorbic acid to the *in vitro* maturation medium compared with the control group. However, for another pro-apoptotic gene, *i.e*., *BID*, it decreased non-significantly. Among the anti-apoptotic genes, the relative mRNA abundance of *MCL1* decreased significantly after the addition of 100 μM *L*-ascorbic acid. The relative mRNA abundance of both *MCL1* and *BCL–XL* showed a non-significant increase after 50 μM *L*-ascorbic acid supplementation compared with the control group. These results demonstrate that the supplementation of 50 μM *L*-ascorbic acid to the *in vitro* maturation medium has alleviated the oxidative stress in the developing embryos by downregulation of pro-apoptotic- and upregulation of anti-apoptotic genes that might be the possible reason for improved developmental competence after its use for *in vitro* embryo production in buffaloes. Our results agree with a previous study wherein *L*-ascorbic acid supplementation during *in vitro* production of porcine parthenotes reduced the oxidative stress by downregulation of *BAX* and *BCL–XL* upregulation[19].

Moreover, the elevated levels of pro-apoptotic genes and reduced anti-apoptotic genes after the addition of 100 μM dose of *L*-ascorbic acid to the *in vitro* maturation medium indicate its deleterious effect on embryonic development as evidenced by the reduced developmental competence at this dose. Additionally, we have also assessed the effect of 50- and 100 μM dose of *L*-ascorbic acid to the *in vitro* maturation medium on the relative mRNA abundance of embryonic development-related genes (*GDF9*, *BMP15*) that play a crucial role in follicular growth, oocyte maturation, and the embryo quality. However, the supplementation of *L*-ascorbic acid at both concentrations did not significantly affect these genes' relative expression.

The current study was confined to evaluate *L*-ascorbic acid's effect on the *in vitro* development of buffalo embryos and relative mRNA abundance of some crucial apoptosis-related genes only. However, the assessment of *L*-ascorbic acid's effect on ROS levels, DNA fragmentation, and cytotoxicity assays was beyond this study's scope and should be carried out in future studies.

In conclusion, the supplementation of 50 μM *L*-ascorbic acid to *in vitro* maturation and *in vitro* culture media supports *in vitro* embryonic development in buffaloes more efficiently than its addition to *in vitro* maturation or *in vitro* culture medium alone. It considerably improves developmental competence and alters the expression of apoptosis-related genes.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

The present study was funded by the National Agriculture Innovation Project Grant to Suresh Kumar Singla (C 2–1-(5)/2007) and Manmohan Singh Chauhan (C-2067 and 075).

Authors' contributions

Mayank Roshan and Diksha Dua conducted experimental studies and data analysis. Manish Tiwari and Manoj Kumar Singh contributed to the literature search and manuscript preparation. Ankur Sharma and Manmohan Singh Chauhan contributed to the concept, research design, and manuscript preparation. Suresh Kumar Singla, Prabhat Palta, and Radhay Sham Manik performed data acquisition and analysis, manuscript editing, and review. Mayank Roshan contributed to the definition of intellectual content, manuscript review, and guarantor.

References

[1] Perera BMAO. Reproduction in domestic buffalo. *Reprod Domest Anim* 2008; **43**: 200-206.

[2] Yadav A, Singh KP, Singh MK, Saini N, Palta P, Manik RS, et al. Effect of physiologically relevant heat shock on development, apoptosis and expression of some genes in buffalo (*Bubalus bubalis*) embryos produced *in vitro*. *Reprod Domest Anim* 2013; **48**(5): 858-865.

[3] Sharma A, Agrawal H, Mullani N, Sandhu A, Singh MK, Chauhan MS, et al. Supplementation of tauroursodeoxycholic acid during *in vitro* culture did not enhance *in vitro* development and quality of buffalo IVF embryos but combated endoplasmic reticulum stress. *Theriogenology* 2015; **84**(2): 200-207.

[4] Abe H, Hoshi H. Evaluation of bovine embryos produced in high performance serum-free media. *J Reprod Dev* 2003; **49**(3): 193-202.

[5] Moore K, Rodríguez-Sallaberry CJ, Kramer JM, Johnson S, Wroclawska E, Goicoa S, et al. *In vitro* production of bovine embryos in medium supplemented with a serum replacer: Effects on blastocyst development, cryotolerance and survival to term. *Theriogenology* 2007; **68**(9): 1316-1325.

[6] Burton GJ, Hempstock J, Jauniaux E. Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Reprod Biomed Online* 2003; **6**(1): 84-96.

[7] Lampiao F. Free radicals generation in an *in vitro* fertilization setting and how to minimize them. *World J Obstet Gynecol* 2012; **1**(3): 29-34.

[8] Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma RK. Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertil Steril* 2002; **78**(6): 1272-1277.

[9] Saikhun K, Faisaikarm T, Ming Z, Lu KH, Kitiyanant Y. A-tocopherol and *L*-ascorbic acid increase the *in vitro* development of *in vitro* maturation/ IVF swamp buffalo (*Bubalus bubalis*) embryos. *Animal* 2008; **2**: 1486-1490.

[10]Kere M, Siriboon C, Lo NW, Nguyen NT, Ju JC. Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. *J Reprod Dev* 2013; doi: 10.1262/jrd.2012-114.

[11]El-Naby Al-shimaa Al-HH, Mahmoud KGhM, Sosa GAM, Abouel-Roos MEA, Ahmed YF. Effect of using ascorbic acid and cysteamine supplementation on *in–vitro* development of buffalo embryos. *Asian Pac J Reprod* 2017; **6**(2): 85.

[12]Guérin P, El Mouatassim S, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001; **7**(2): 175-189.

[13]Natarajan R, Bhawani SM, Munuswamy D. Effect of *L*-ascorbic acid supplementation at different gaseous environments on *in vitro* development of preimplantation sheep embryos to the blastocyst stage. *Anim Reprod* 2018; **7**(1): 21-28.

[14]Sreenivas D, DSVGK K, Yarla NS, Thomas VM, PalniSamy A, et al. In vitro production of sheep embryos in CR1aa medium supplemented with *L*-ascorbic acid. *J Tissue Sci Eng* 2014; **5**(131): 2.

[15]Mehta P, Kaushik R, Singh KP, Sharma A, Singh MK, Chauhan MS, et al. Establishment, growth, proliferation, and gene expression of buffalo (*Bubalus bubalis*) transgenic fetal fibroblasts containing human insulin gene, and production of embryos by handmade cloning using these cells. *Cell Reprogram* 2018; **20**(2): 135-143.

[16]Sharma A, Shah SM, Saini N, Mehta P, Kumar BSB, Dua D, et al. Optimization of serum-free culture conditions for propagation of putative buffalo (*Bubalus bubalis*) spermatogonial stem cells. *Cell Reprogram* 2019; **21**(1): 1-10.

[17]Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 2001; **25**(4): 402-408.

[18]Shamas-Din A, Kale J, Leber B, Andrews DW. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harb Perspect* 2013; **5**(4): a008714.

[19]Hu J, Cheng D, Gao X, Bao J, Ma X, Wang H. Vitamin C enhances the *in vitro* development of porcine pre-implantation embryos by reducing oxidative stress. *Reprod Domest Anim* 2012; **47**(6): 873-879.