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Boric acid supplementation promotes the development of *in vitro*-produced mouse embryos by related pluripotent and antioxidant genes

Ali Cihan Taskin[1], Ahmet Kocabay[2], Seref Gul[3], Gizem Nur Sahin[4], Sercin Karahuseyinoglu[4], I. Halil Kavakli[5] and Ibrahim Sogut[6,*]

[1]Department of Laboratory Animal Science, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey; [2]Animal Research Facility, Center for Translational Medicine (KUTTAM), Koç University, Istanbul, Turkey; [3]Life Sciences and Biotechnology Institute, Department Of Biotechnology, Bezmialem Vakıf University, Istanbul, Turkey; [4]Translational Medicine Research Center and School of Medicine, Koç University, Istanbul, Turkey; [5]Department of Chemical and Biological Engineering, Koç University, Istanbul, Turkey and [6]Department of Biochemistry, Faculty of Medicine, Demiroglu Bilim University, Istanbul, Turkey

Research Article

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Corresponding author:
Ali Cihan Taskin;
Email: ataskin@istanbul.edu.tr

*Contributed equally.

Summary

Boric acid (BA) is an important mineral for plants, animals and humans that assists metabolic function and has both positive and negative effects on biological systems. The present study aimed to investigate the effects of different concentrations of BA added to the culture media, the quality and *in vitro* development potential of mouse embryos. Superovulated C57Bl6/6j female mice were sacrificed ~18 hours after human chorionic gonadotropin (hCG) injection. Single-cell-stage embryos were collected from the oviduct, divided into experiment groups and cultured in embryo medium with supplemented BA+ in 5% CO_2 at 37 °C until 96 hours at the blastocyst stage. The blastocyst development rates of 0, 1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA were 51.52%, 73.47%, 77.36% and 81.13%, respectively. The *in vitro* development rates were significantly higher in the 1.62×10^{-3} ($p < 0.05$) and 1.62×10^{-4} µM BA groups than in the control group ($p < 0.001$). These results indicated that low BA doses influenced embryo development by positively affecting *in vitro* development rates, embryo cell numbers, biochemical parameters and development at the molecular level by pluripotent and antioxidant genes. Therefore, BA seems to play an important role on *in vitro* embryo development.

Introduction

Reproductive biotechnology studies focus on such topics as the collection of greater numbers of embryos, the long-term storage of embryos (cryopreservation), embryo culture, the genetic diagnosis of embryos and embryo transfer (Taşkın *et al*., 2020). The development of embryos *in vitro* is affected by, among other factors, the composition of the culture medium (nutritional minerals, protein sources, etc.), atmospheric conditions (CO_2 and O_2 proportions), ambient temperature, the osmotic pressure of the culture medium, the volume of culture drops and embryo manipulation (Quinn and Harlow, 1978). Embryos are exposed to severe oxidative stress under *in vitro* culture conditions, and the resulting reactive oxygen species can damage *in vitro* embryo development. The specific conditions of an embryo culture can affect the blastocyst quality and cell counts (Umaoka *et al.,* 1992; Smith, 2001). Antioxidants act as free radical scavengers, protecting cells or reducing the damage caused by free radicals. The addition of antioxidants to the embryo culture medium has been shown to improve *in vitro* embryo development (Zarbakhsh, 2021). While some free radical scavenging antioxidants in follicular fluids can protect oocytes against oxidative stress under *in vivo* conditions (Wang *et al.,* 2002), this antioxidant environment is weaker under *in vitro* culture conditions, exposing oocytes or embryos to severe oxidative stress (Nagina *et al.,* 2016). The most effective approach to overcoming this problem involves the supplementation of the culture medium with antioxidant agents.

Boron (B) is an essential trace element for the metabolism of plants, animals and humans. Boric acid (BA) is a Lewis acid that plays an important role in the regulation of many enzymes, cell proliferation and development, and energy metabolism. Generally, B exists in the environment in an oxidized state, which is BA. After being taken into the body, it quickly enters the bloodstream and is excreted without accumulation. BA contains approximately 17.5% B (Nielsen, 2000; Nielsen, 2008; Sogut *et al*., 2015). It is not an antioxidant but rather plays a role in controlling the antioxidation system. In addition to being used for its antioxidant effect, and as

an anti-inflammatory and anti-cancer agent, BA has also been reported to positively affect embryonic and bone development, the immune system, and psychomotor and cognitive functions (Nielsen, 2000; Henderson *et al.,* 2009; Sogut *et al.*, 2015; Hazman *et al.*, 2018; Cikler-Dulger and Sogut, 2020). Recent studies have focused on the potential beneficial effects of BA in rat Sertoli and mouse Leydig cells and Angora Buck, Ram Sperm, and fetal embryo development (Tirpan and Tekin, 2015; Ince *et al.*, 2016; Lu *et al.*, 2020; Yalcin and Abudayyak, 2020). The B concentration in serum is 0.22 micrograms ml^{-1} (Laurent-Pettersson *et al.,* 1992).

This investigation aimed to monitor embryo development and optimize beneficial therapeutic supplemental BA to *in vitro* mouse embryo cultures. To this end, *in vitro* development rates, embryo development quality, total oxidative stress, and antioxidant levels and the developmental pathways of embryos derived through different supplemental BA preparations of the embryo culture medium were investigated.

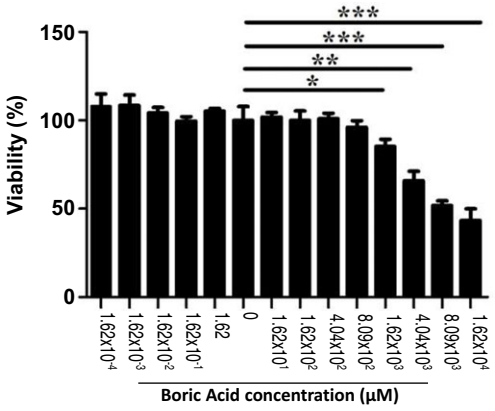

Figure 1. *In vitro* toxicity and determination of the beneficial BA dose by MTT testing: cell viability of 13 different BA doses 48 hours after exposure were evaluated, revealing that 4 doses (1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA) BA negatively affected (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). 1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA).

Materials and method

BA supplementation

The purity of the BA (Merck catalogue number:100165) was <= 100, and the molecule weight was 61.83. The stock solution was 1.62×10^{-2} µM BA. Experimental solutions were prepared by adding 0, 10 and 1 ml of stock for a final volume of 100 ml media, thus creating from 1.62×10^{-3} to 1.62×10^{-4} uM BA embryo culture. The culture media and final supplemented media were not analysed for BA. The experimental BA concentrations had BA values that were additional to the unknown concentrations of the culture media.

In Vitro *dose selection by MTT testing*

Before starting the animal studies, the therapeutic dose was first determined *in vitro* to determine the beneficial dose of BA (using the 3R model of replacement, reduction and refinement). U2-OS (human osteosarcoma) cells were used for the cytotoxicity assay, this being the cell line most suitable for reproductive studies due to its pluripotency-like stem cells. Accordingly, 4000 cells/well were seeded into clear 96-well plates and grown for 48 hours, after which the cells were treated with BA in 13 different supplemental BA preparations (from 1.62×10^{-4} to 1.62×10^{4}) in which the final solvent (DMSO) concentration was adjusted to 0.5% (v/v) (Figure 1). The cells were incubated for another 48 hours, after which cell viability was determined using tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) salt. MTT is the substrate of the mitochondrial enzymes and converts to insoluble purple colour formazan. After 48 hours, the MTT:DMEM mixture was replaced with the medium and incubated for four hours. The intensity of the purple colouring was measured to determine cell viability (Doruk *et al.*, 2020). Cells with MTT reagent medium were replaced with DMSO:EtOH (50:50) mixture to dissolve the formazan salt. Finally, the absorbance of the wells was measured at 570 nm using a spectrophotometer. As a positive control, the cells were treated with a final 5% DMSO concentration (known as toxic to cells). The experiment was repeated twice in triplicate.

Animal experimentation and ethical approval

C57Bl/6j mouse strains were used in this study. The animals were kept under a 12:12 light–dark cycle in a room with 55 ± 10% humidity at a temperature of 22 ± 2 °C in the Animal Research Laboratory of the Koç University Translational Medical Center (KUTTAM), Istanbul, Turkey, and provided with pellets (Special Diets Services; UK), bedding (TAPVEI®, Estonia), and filtered potable water *ad libitum*. All experiments with mice were approved by the Koç University Animal Experiments Local Ethics Committee (Approval No: 2021-05).

Superovulation and embryo culture

First, an intraperitoneal (IP) injection of 10 IU of pregnant mare serum gonadotropin (Sigma G4877-PMSG) was administered to the female mice at between 12:00 and 13:00, followed 48 hours later by 10 IU of human chorionic gonadotropin (Organon-hCG), administered intraperitoneally between 12:00 and 13:00. The mice were then mated with males of the same strain. The next day, at 08:00, vaginal plug control was performed. The superovulated female mice were sacrificed, and the embryos were collected through the rupture of the oviduct ampullae. After washing three times in 80 IU/mL hyaluronidase (Sigma H-3506) + 4 mg/mL bovine serum albumin (BSA) (SigmaA-3311) + Human Tubal Fluid + HEPES-buffered (HTF, global total w/ HEPES) medium, the embryos were washed again in Hepes buffer medium supplemented with 4 mg/mL BSA.

At least 2 h before embryo collection, the culture media was kept in high humidity incubator at 37 °C in 5% CO_2 for air gassing. The collected embryos were kept in culture (LifeGlobal Media, LGGG-020) medium (4 mg/ml BSA Fraction V. A3311) for 60 minutes in an incubator at 37 °C with 5% CO_2 for quality assessment. Selected, good-quality embryos were then cultured in a Life Global medium (4 mg/ml BSA) supplemented with 0 (control), 1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA in an incubator at 37 °C with 5% CO_2 (Taskin *et al.*, 2019).

Differential labelling of inner cell mass (ICM) and trophectoderm (TE) nuclei

The blastocysts were kept in 100 µg/ml propidium iodide (PI) + HTF Hepes buffer medium + 1% TritonX100 (Sigma CAS No. 9002-93-1) solution for 10–12 seconds, then transferred to a 100 µg/ml 100% ethanol (EMPROVE) +25 µg/ml Hoechst 33258 (H1398, Molecular Probes, Inc.) solution and incubated overnight

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Table 1. Quantitative real-time polymerase chain reaction primers list

Gene	Forward primer	Reverse primer
NANOG	GCGGACTGTGTGTTCTCTCAGGC	TTCCAGATCCGTTCACCAGATAG
CDX-2	GCAGTCCCTAGGAAGCCAAGTGA	CTCTCGGAGAGCCCGAGTGTG
SOD1	TGCGTGCTGAAGGGCGAC	GTCCTGACAACACAACCTGGTTC
SOD2	GGAGCAAGGTCGCTTACAGA	GTGCTCCCACACGTCAATC
GPx1	TGTTTGAGAAGTGCGAAG	GTGTTGGCAAGGCATTCC
GPx4	TAAGAACGGCTGCGTGGT	GTAGGGGCACACACTTGTAGG

at 4 °C. A 5-µl glycerol drop was placed on the slide to fix the blastocysts, which were transferred into the drop and covered with a coverslip. In the prepared blastocyst cell staining samples, the cell counts of trophectoderm (TE) and inner cell mass (ICM) were calculated under an inverted microscope with red and blue fluorescent attachments (Taskin *et al.*, 2019; Mallol *et al.*, 2013).

Determination of oxidant and antioxidant levels from mouse embryos

Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) were detected as described previously (Söğüt *et al.*, 2021) using commercial assay kits (Rel Assay Diagnostics, Gaziantep, Turkey) in phosphate-buffered saline (PBS). Briefly, antioxidants convert the 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical from a dark blue-green hue to a colourless reduced ABTS form. The sample's TAS level (mmol Trolox eq/L) is correlated with the change in absorbance at 660 nm. The ferrous ioneo-dianisidine complex is oxidized to the ferric ion by oxidants present in the sample. In an acidic media, the ferric ion and xylenol orange form a colourful complex. The sample TOS levels (µmol H2O2 eq/L) was correlated with the change in absorbance at 530 nm. The OSI levels (arbitrary unit) in the sample were detected as the ratio of the TOS level to the TAS level.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RNA isolation was performed using a Quick-RNA® Kit (Macharey-Nagel) following the manufacturer's instructions. RNA measurement was performed using Nanodrop (ThermoScientific) with a spectrophotometric reading at 260 nm. A 250 ng cDNA preparation was obtained through the reverse transcription of RNA using M-MLV Reverse Transcriptase. The relative mRNA expression levels of glutathione peroxidase 1 (GPX1), glutathione peroxidase 4 (GPX4), CDX2, superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), CDX2 and NANOG genes were determined using a Light Cycler® 480 SYBR Green I Master (Taskin *et al.*, 2019) (Table 1).

Statistical assessment of results

IBM SPSS Statistics for Windows (Version 24.0. Armonk, NY: IBM Corp.) was used for the statistical assessment of the results with sufficient repetitions. The results were evaluated with a one-way ANOVA and Bonferroni *post hoc* test. A *p*-value of <0.05 was considered statistically significant. All the experiments were repeated three times.

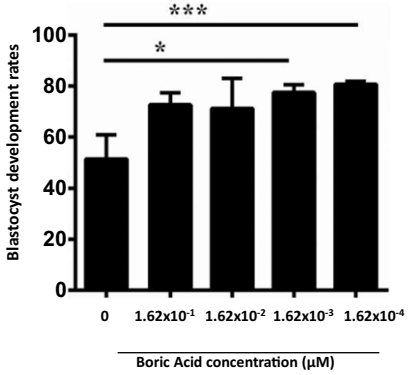

Figure 2. Effect of supplemental BA on *in vitro* development rates of mouse embryo: effect of BA on blastocyst mouse embryo development rates (*$p < 0.05$ and ***$p < 0.001$). 1.62×10^{-3}, and 1.62×10^{-4} µM BA doses beneficial mouse embryo development.

Results

Determination of the BA dose by In Vitro *cytotoxicity*

The cytotoxic effects of the 13 different BA doses were evaluated after 48 hours of exposure, revealing that 1.62×10^3, 4.04×10^3, 8.09×10^3 and 1.62×10^4 µM BA significant negatively affected ($p < 0.05$) both cell viability and cell count, while 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA were nontoxic (Figure 1).

Embryo culture

There was a statistically significant difference between the control group and the 1.62×10^{-3} ($p < 0.05$) and 1.62×10^{-4} ($p < 0.001$) µM BA groups (Figure 2). The difference between the control group and the 1.62×10^{-1} and 1.62×10^{-2} µM BA groups was insignificant ($p > 0.05$) (Figure 2).

Results of cell counting by differential staining

The mean cell and inner cell counts differed significantly between, 1.62×10^{-3} ($p < 0.05$) and 1.62×10^{-2} ($p < 0.01$) µM BA groups and the control group. There was a significant difference in the mean TE cell count between the 1.62×10^{-2} and 1.62×10^{-4} µM BA groups and the control group ($p < 0.05$) (Figure 3).

Oxidant and antioxidant levels

A comparison of TOS levels revealed a statistical difference between the control group and the 1.62×10^{-4} µM groups

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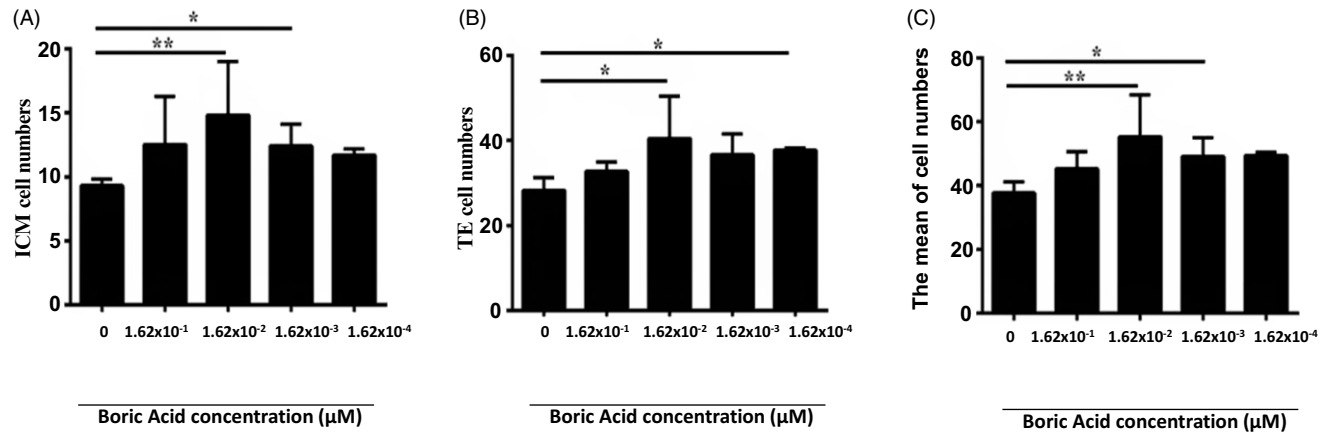

Figure 3. Effect of BA on mouse embryo cell count by differential staining; (A) effect of BA on inner cell mass (ICM) mean numbers of mouse blastocysts; (B) effect of BA on trophectoderm cell (TE) mean numbers of mouse blastocysts; (C) effect of BA on total cell mean numbers of mouse blastocysts.

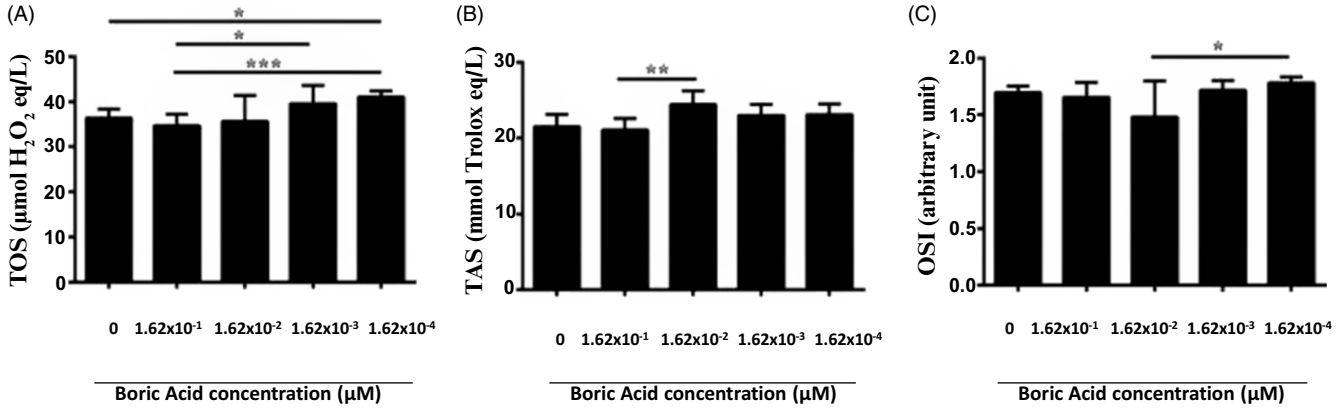

Figure 4. Oxidant and antioxidant effects of different doses of BA on mouse embryo. (A) total oxidant status (TOS, µmol H_2O_2 eq/L), (B) total antioxidant status (TAS, mmol Trolox eq/L) and (C) oxidative stress index (OSI, arbitrary unit) (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

(Figure 4A). A comparison of TAS levels showed no significant difference between the control group and the 1.62×10^{-2} µM BA group ($p > 0.05$) (Figure 4B). The comparison of OSI levels did not reveal any statistical difference between the control group and the 1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} µM BA groups ($p > 0.05$) (Figure 4C).

Quantitative real-time PCR

We performed qPCR analyses of the control and BA-treated blastocysts to evaluate whether the observed changes in the cell counts of TE and ICM within the embryo were reflected in the expression of the respective genes in these two structures. The BA-treated embryos were seen to significantly upregulate the expression of CDX2, a TE cell marker (Figure 5A), and NANOG, an ICM marker (Figure 5D) ($p < 0.05$). The oxidative stress marker genes GPX1, SOD1 and SOD2 (Figure 5B,E,F) differed significantly between the BA-treated groups and the control group ($p < 0.05$). Furthermore, the level of GPX4 – a marker gene identifying the negative effect of oxidative stress – was significantly lower in the BA-treated groups than in the control groups ($p < 0.05$).

Discussion

In the first study reported in the literature, BA was added to two-cell mouse embryo cultures until the blastocyst stage. The results showed that additions of 6 µM and 1 mM BA did not affect embryo development, while 2 to 10 mM B had a negative effect on embryo development (Lanoue *et al.*, 1998). In our study, we used BA from the one-cell stage up to the blastocyst stage, and lower doses correlated with higher significant embryo development. Rowe and Eckhert (1999) showed that B is essential for fertilization and promotes blastocyst development of zebrafish embryos. They used a low dose of B (1×10^{-1} µM). BA contains only 17.5% B and was used in five different supplemental preparations. We used BA in mouse embryos and found that low doses supported more effective mouse blastocyst development and a higher quantity of embryos by cell number. We also determined the genetic pathways affected by the blastocyst development and how the related antioxidant systems affect this, depending on the dose.

In a previous study examining the effect of B-supplemented feed on the embryonic development of mice, doses of 0.04, 2.05 and 11.8 µg B/g-diet were administered, and the negative effects on the *in vitro* development of embryos collected from those fed 0.04 µg B/g-diet were established (Lu *et al.* 2020). In the present study, to

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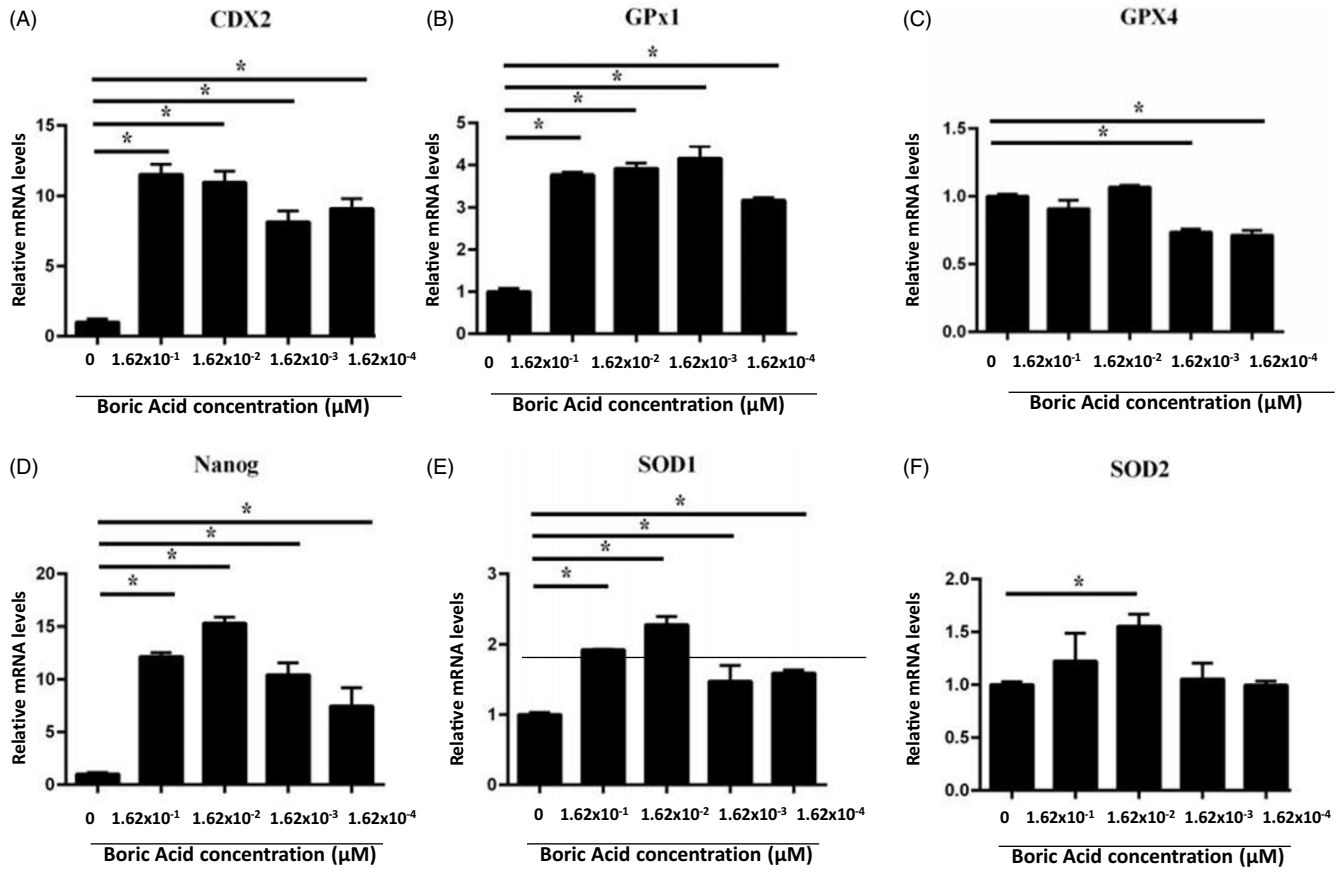

Figure 5. Relative transcript levels of CDX2, GPX1, GPX4, NANOG, SOD1 and SOD2 in control and BA-treated embryos. (A) BA has a positive effect on *in vitro* mouse blastocyst level of CDX2 (*$p < 0.05$); (B) BA has a positive effect on *in vitro* mouse blastocyst level of GPX1 (*$p < 0.05$); (C) BA has a negative effect on *in vitro* mouse blastocyst level of GPX4 (*$p < 0.05$); (D) BA has a positive effect on *in vitro* mouse blastocyst level of NANOG (*$p < 0.05$); (E) BA has a positive effect on *in vitro* mouse blastocyst level of SOD1 (*$p < 0.05$); (F) BA has a positive effect on *in vitro* mouse blastocyst level of SOD2 (*$p < 0.05$).

our knowledge, the first of its kind in the literature, low doses (1.62×10^{-1}, 1.62×10^{-2}, 1.62×10^{-3} and 1.62×10^{-4} μM) of BA were added to the culture medium to understand the effects on embryo development and its mechanism.

Ince *et al.* (2018) found that feeding rats B via a gavage tube (0.04 and 2.05 g) for 14 days improved gene expression (HEX, NANOG and OCT-3/4) in the early embryonic period after conception and improved the fetal development of the rats. Similarly, our study found increased NANOG levels were significantly associated with embryo development when compared to the control group.

Nguyen *et al.* (2009) investigated Nrf2 activation and response element by oxidative stress. The effect of BA activation of Nrf2 is a system that controls how B prevents DNA damage and antioxidant status (Yamada and Eckhert, 2019). NrF2 activation governs self-renewal and pluripotency in human embryonic cells (Jang *et al.* 2014). In the present study, it was shown that BA supports embryo development and protects DNA cells. BA has supported embryo development by the pluripotent gene levels of CDX2 and NANOG. BA has been shown to support pluripotent cell development through the NrF2 system.

In conclusion, the use of low-dose BA positively affects embryo development. The findings of the present study can be used for the development of a model for use in veterinary and clinical medicine. Embryo production is vital in farm animals (cattle, sheep, etc.). Increasing the production of healthy embryos through the use of BA can contribute to the improvement and development of livestock. Future research exploring the effects of BA-supplemented culture media on *in vitro* and *in vivo* embryo development together with studies of such processes as embryo cryopreservation, somatic cell nuclear transfer (SCNT), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) would further contribute to the literature.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199424000261

Data availability. The authors confirm that the data supporting the findings of this study are available in the article

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Author contribution. Taskin AC and Sogut I designed the study. Kavakli IH and Gul S performed the cytotoxicity experiments. Taskin AC and Kocabay A performed the animal studies and embryo culture operations. Sogut I analysed the biochemical experiments. Sahin GN and Karahuseyinoglu S performed molecular biology analyses. Taskin AC and Sogut I helped interpreted the statistical data. Taskin AC, Sogut I, Gul S and Kavakli IH wrote the paper and interpreted the data. All the authors read and approved the final manuscript.

Competing interests. The authors declare no competing interests.

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Ethics approval. All animal research protocols in this study were approved by the Koç University Institutional Ethics Committee (HADYEK, approval number 2021-05).

Consent for publication. All authors have given consent for the manuscript to be published by the corresponding authors.

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