

EFFECTS OF SODIUM FLUORIDE ON THE APOPTOSIS AND THE DEVELOPMENT OF PREIMPLANTATION BOVINE EMBRYOS

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ABSTRACT: Oocyte quality, which can determine embryonic viability, is easily perturbed. Thus, the factors affecting normal oocyte maturation require further study. The fluoride ion (F) is an endocrine system-disrupting chemical that elicits a variety of deleterious reproductive effects. F has previously been found to disrupt meiosis during gestation. However, its embryonic effects in mammals are not well documented. Here, bovine oocytes were matured *in vitro* either with or without sodium fluoride (NaF) treatment. Our study found that increased NaF concentration also increased the generation of reactive oxygen species (ROS) and disturbed the oxidant-antioxidant balance. The resulting embryos exhibited decreased embryonic development rates. Moreover, the NaF-induced apoptosis increased the levels of caspase-3 and caspase-9 mRNA in addition to the levels of the apoptotic protein Bax. Treatment with NaF also decreased the gene expression associated with embryo development (Oct4, Nanog, Sox2, and Cdx2) in blastocysts. NaF exposure during oocyte maturation *in vitro* can, therefore, decrease oocyte quality, embryo quality, and the developmental potential in a dose-dependent fashion. In summary, our findings suggest that NaF exposure causes oxidative damage during bovine embryonic development and leads to apoptosis.

Keywords: Apoptosis; Bovine embryos; Oocytes; Oxidative stress; Sodium fluoride.

INTRODUCTION

The fluoride ion (F) exists naturally in varying amounts in soil, water, and food. It has been widely used as an additive in toothpaste, mouthwash, and drinking water to prevent dental caries. However, the range of benefits is limited, and an excessive F intake over a prolonged period may pose an increased risk to public health.¹ There is increasing evidence of a close link between elevated environmental F levels and decreasing fertility rates.^{2,3} Studies have verified toxic effects of NaF on the male reproductive system.⁴⁻⁹ These effects include damage to testicular structures and a decrease in number of spermatozoa capable of oocyte fertilization which are related to disorders in the oxidative system.^{10,11} Further reports found alterations in reproductive hormones, fertility, histological structures, and the developmental outcomes in individuals exposed to relatively high concentrations of F.^{12,13}

Oxidative stress (OS), a cellular condition caused by the accumulation of reactive oxygen species (ROS), is inducible by F and is thought to contribute significantly to defective embryo development in mice.^{14,15} Several defense mechanisms are present in both embryos and their environments to remove excess

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ROS. As a non-enzymatic antioxidant, glutathione (GSH) is able to react with ROS and maintain the ROS at appropriate levels. In addition, enzymatic defense systems, including glutathione peroxidase (GSH-Px) and catalase (CAT), also suppress oxidative injury efficiently, and weaken the damage caused by ROS.¹⁶ However, if the level of ROS goes beyond the capacity of the antioxidative defense systems, OS will occur.

To the best of our knowledge, there is no direct evidence linking the toxic effects of F to the preimplantation bovine embryo. In this study, we show an embryo-toxicity effect of NaF on the bovine embryo for the first time. Furthermore, impairment of embryo viability was associated with increased ROS levels and decreased activities of antioxidants.

MATERIALS AND METHODS

Ethics statement: All the chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Disposable, sterile plasticware were purchased from Nunclon (Roskilde, Denmark). All the procedures in this experiment were approved by the Animal Care and Use Committee of Gansu Agricultural University (Lanzhou, China) and performed in accordance with animal welfare and ethics.

Oocyte collection, sodium fluoride (NaF) treatment, and in vitro maturation (IVM): Oocyte collection and *in vitro* maturation (IVM) were performed as described previously.¹⁷ Briefly, bovine ovaries were transported from the slaughterhouse to the laboratory within 2 hr after the animal was killed in a thermos bottle with sterile saline at 20–25°C. A 12-gauge needle attached to a 10 mL syringe was used to aspirate cumulus-oocyte complexes (COCs) from antral follicles with a diameter of between 2 and 8 mm. COCs were recovered and washed in PBS containing 5% (v/v) FBS. Only oocytes surrounded by a minimum of 3 cumulus cell layers and with uniform cytoplasm were selected, washed in PBS containing 5% (v/v) FBS, and cultured for 20 hr in bicarbonate-buffered tissue culture medium 199 (TCM-199, Gibco) containing 10% (v/v) FBS, 1 mg/mL 17 β -estradiol, and 0.075 IU/mL Human Menopausal Gonadotropin (HMG) in 95% humidified air with 5% CO₂ at 38.5°C. Oocytes were exposed to 0 (control), 20, 40, 80, and 160 μ M NaF/L for 12 hr, respectively. We used 0, 20, 40, and 80 NaF/L for further studies as no blastocysts were obtained in the 160 μ M NaF/L group.

Determination of ROS products: To measure ROS levels, after exposing germinal vesicle (GV) oocytes to NaF for 12 hr, each group was incubated in SOF supplemented with dichlorodihydrofluorescein diacetate (DCFH-DA; 2 mM, Molecular Probes) for 30 min at 37°C as previously described.¹⁸ The intracellular ROS level in oocytes was analyzed using a commercial ROS assay kit (Beyotime Institute of Biotechnology). In brief, 20–40 oocytes from each group of the MII stage were mixed with 100 μ L of schizolysis solution supplied by the kit for lysis on ice for 1 hr. The supernatants were then collected by centrifuging at 11 000 \times g for 10 min at 4°C for the following tests. All the operations were carried out on

ice. Finally, the test tubes containing 100 μL of supernatants and 100 μL of test solutions were placed at room temperature for 20 min. A clear supernatant was removed from each group and stored at -70°C until use. The optical density was determined using a microplate reader (Bio-TEK, USA) set at 560 nm.

Antioxidant capability assay: After exposure to NaF, a total of 30–50 MII oocytes from each group were lysed and vortexed for 5 min, then frozen at -80°C and thawed at 37°C three times. The mixture was centrifuged at 10,000 g for 10 min at 4°C and put on ice. Then, according to the manufacturer's instructions to determine the level of total antioxidant capacity (T-AOC) and glutathione (GSH) and the activities of GSH-Px and CAT (Nanjing Jiancheng Biochemistry Reagent Co; Nanjing, Jiangsu, China).

In vitro fertilization (IVF) and embryo culture: IVF was carried out in accordance with the methods of previous study.¹⁷ Two-pronuclear embryos were selected for further culture at 38.5°C in 5.5% CO_2 in air for 7 days.

Caspase-3 and caspase-9 activity detection: Caspase-3 and 9 activities in MII oocytes were determined with a caspase assay kit (Beyotime, China), which detects the production of the chromophore p-nitroanilide after its cleavage from the peptide substrate DEVD-p-nitroanilide and LEHD-p-nitroanilide.

Quantitative real-time PCR: A single day 7 blastocyst was used per sample, and 5 to 8 embryos were used for each group. The total RNA of the embryos was isolated using the Cells-to-Signal™ Kit (Ambion Co., USA) according to the manufacturer's protocol. The RT reaction was achieved using the M-MLV RT included in the Cells-to-Signal Kit. The mRNA levels were quantified using SYBR Premix ExTaq™ II (Takara, Japan) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Carlsbad, CA, USA). Samples were denatured at 95°C for 1 min and then subjected to 40 cycles of amplification (95°C , 5 sec; 60°C , 30 sec). Each data point was the average of duplicate assays performed on three independently obtained samples and transcript levels were calculated relative to the transcription of the housekeeping gene Histone 2a (H_2A) in every sample. Fold changes for each gene were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. All the primers used in this study are shown in Table 1.

Apoptosis assay: Embryo cell apoptosis was tested according to the instructions. Briefly, blastocysts were washed 3 times (5 min each) in PBS (Beyotime, China) containing 0.2% PVA, and then fixed in Immunol Staining Fix Solution (Beyotime, China) for 30 min, and then embryos were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After that they were equilibrated for 8 min after being washed 3 times. Samples in treatment group were incubated with rTdT incubation buffer (45 μL equilibration buffer, 5 μL Nucleotide Mix, and 1 μL rTdT Enzyme) in the dark for 1 hr at 37°C . The tailing reaction was terminated in $2 \times$ standard saline citrate for 15 min. The DNA was stained with DAPI (Beyotime, China). Finally, the samples were placed on slides and analyzed with a Nikon eclipse Ti-S microscope. The experiments were carried out 3 times.

Immunoblotting: Antibodies against H₂A, Bax, and Bcl-2 were purchased from Santa Cruz (Santa Cruz, CA, USA). Western blot analysis was performed as described previously¹⁵ using the protocols provided by the primary antibody suppliers.

Table 1. Primers for qRT-PCR

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
H ₂ A	GTCTTGGAGTACCTGACCGC	AGTCTTCTTCGGGAGCAACA
BAX	TCTCCCCGAGAGGTCTTTTT	TGATGGTCCTGATCAACTCG
BCL-2	GGTATTGGTGAGTCGGATCG	CAAGACGACCCGAGGAGAA
CASPASE-3	GTTTCATCCAGGCTCTTTG	TCTATTGCTACCTTTTCG
CASPASE-9	CGCCACCATCTTCTCCCTG	CCAACGTCTCCTTCTCCTCC
OCT4	CCACCCTGCAGCAAATTAGC	CCACACTCGGACCACGTCTT
NANOG	CGTGTCTTGC AAACGTCAT	CTGTCTCTCCTTCCCTCCTC
SOX2	GGTTGACATCGTTGGTAATTT	CACAGTAATTTTCATGTTGGTTTCA
CDX2	GCAAAGGAAAGGAAATCAACAA	GGGCTCTGGGACGCTTCT

Genes	Accession	Production size
H ₂ A	NM_001098097.2	201 bp
BAX	NM_173894.1	151 bp
BCL-2	NM_001166486.1	201 bp
CASPASE-3	NM_001077840.1	96 bp
CASPASE-9	NM_001205504.1	99 bp
OCT4	NM_174580.3	68 bp
NANOG	NM_001025344.1	66 bp
SOX2	NM_001105463.2	88 bp
CDX2	NM_001206299.1	120 bp

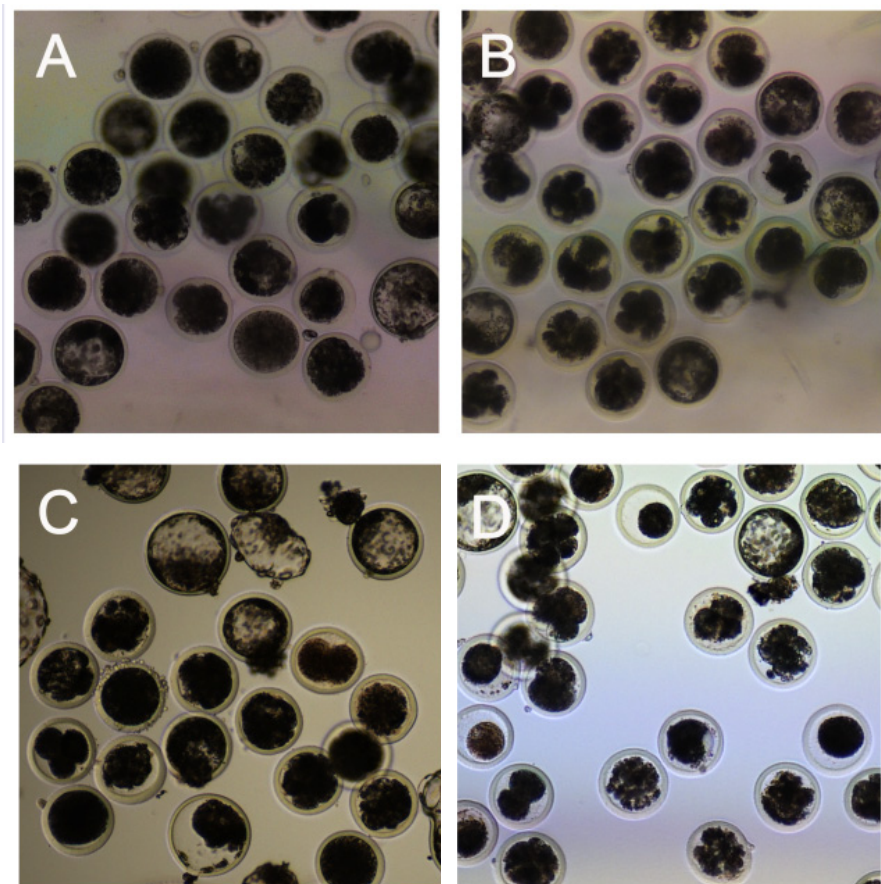
Immunofluorescence staining of embryos: Embryos were washed 3 times (5 min each) in PBS containing 0.2% PVA, and fixed in Immunol Staining Fix Solution (Beyotime, P0098, Jiangsu, China) for 1 hr. All steps were performed at room temperature unless otherwise stated. Embryos were permeabilized with 0.2% Triton X-100 in PBS for 30 min. After 3 washes, they were blocked in the Immunol Staining Blocking Solution (Beyotime, P0102) for 12 hr at 4°C and then incubated with the first antibodies for 12 hr at 4°C. Anti-CDX2 mouse monoclonal antibody (BioGenex, Inc., San Ramon, CA) was diluted 1:200 using Immunol Staining Primary Antibody Dilution Solution (Beyotime, P0103). After 3 washes,

the embryos were treated with secondary antibodies of Alexa Fluor 555-labeled Goat Anti-Mouse IgG (Beyotime, A0459). Finally, the DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1005) for 3 min, the samples were mounted on glass slides with a drop of Antifade Mounting Medium (Beyotime, P0126) and they were then analyzed using a Nikon eclipse Ti-S microscope equipped with a 198 Nikon DS-Ri1 digital camera (Nikon, Tokyo, Japan). The experiments were carried out 3 times. In each experiment, 10 to 15 embryos per group were processed.

Statistical analysis: Outcomes were tested by one-way ANOVA and LSD tests using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$. Data were presented as mean \pm SEM.

RESULTS

As shown in Figures 1A-1D and Table 2, we found that NaF decreased the developmental potential of oocyte, as indicated by the maturation rate, or 2-cell rate, and the blastocyst rate, in a dose-dependent manner.



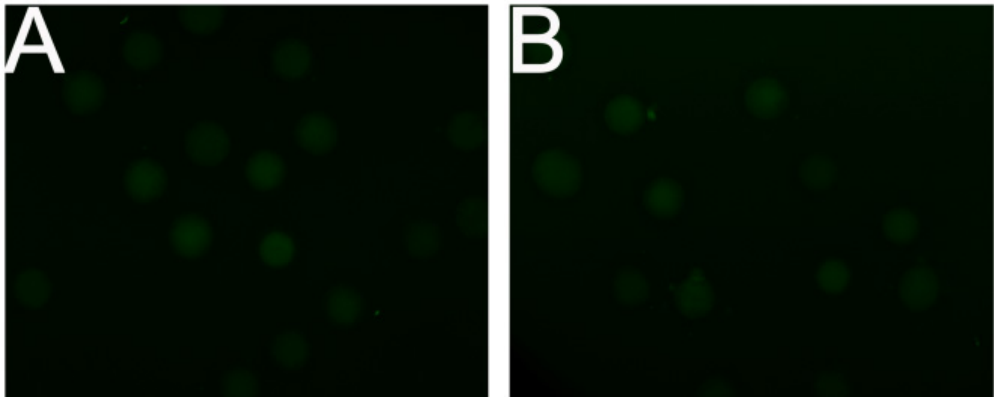
Figures 1A-1D. Representative photographs of day 7 bovine blastocysts developed from each group. A: control, B: 20 μ M NaF/L, C: 40 μ M NaF/L, and D: 80 μ M NaF/L.

Table 2. Development potential of oocyte after treatment with NaF

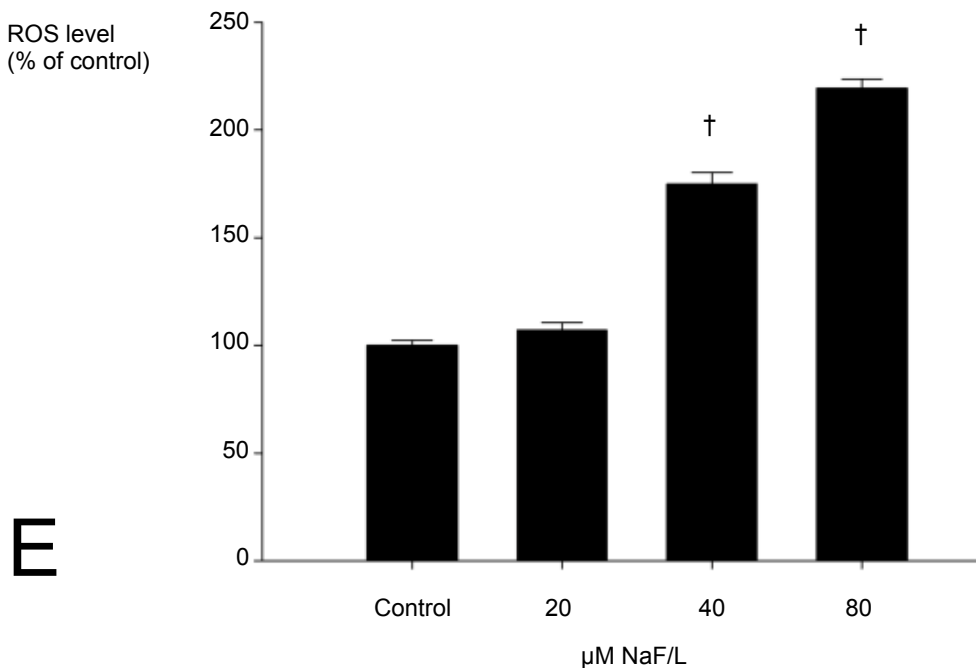
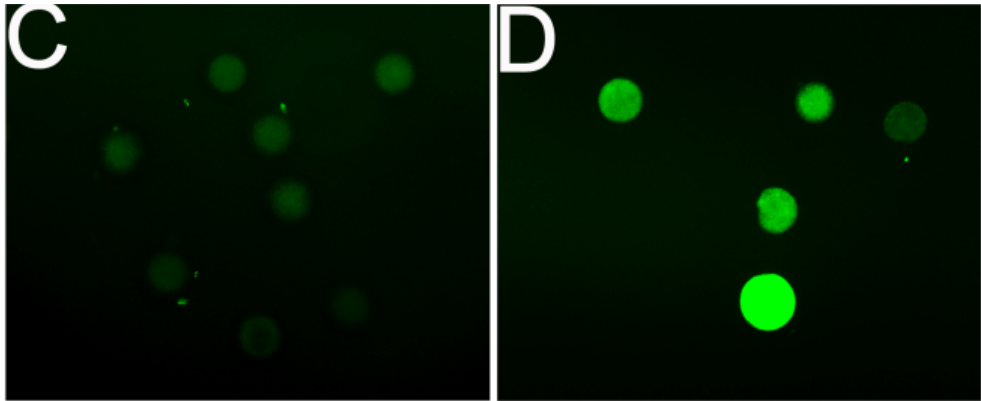
Treatment (μM NaF/L)	Number	Maturation rate No. (mean[%] \pm SEM)	≥ 2 -Cells No. (mean[%] \pm SEM)	Blastocyst No. (mean[%] \pm SEM)
0 (control)	214	179 (82.27 \pm 1.65)	147 (81.01 \pm 1.87)	73 (40.81 \pm 1.18)
20	186	151 (81.15 \pm 1.92)	122 (79.09 \pm 1.83)	59 (38.39 \pm 0.64)
40	194	137 (70.27 \pm 1.91)*	93 (68.80 \pm 0.87)*	38 (29.55 \pm 1.31) [†]
80	248	140 (55.86 \pm 2.33) [†]	52 (36.73 \pm 3.13) [†]	20 (13.84 \pm 2.11) [†]
160	188	49 (25.38 \pm 0.70)	0	0

Compared to the control group: * $p < 0.05$, [†] $p < 0.01$. The data are shown as mean (%) \pm SEM.

To determine whether NaF induced oxidative stress through upregulation of ROS levels, the ROS production was measured using DCFH fluorescent reaction and colorimetry, respectively. As shown in Figures 2A-2E, after treatment with 40 or 80 μM NaF/L, the ROS levels increased in the oocytes compared with the controls without exposure to NaF.



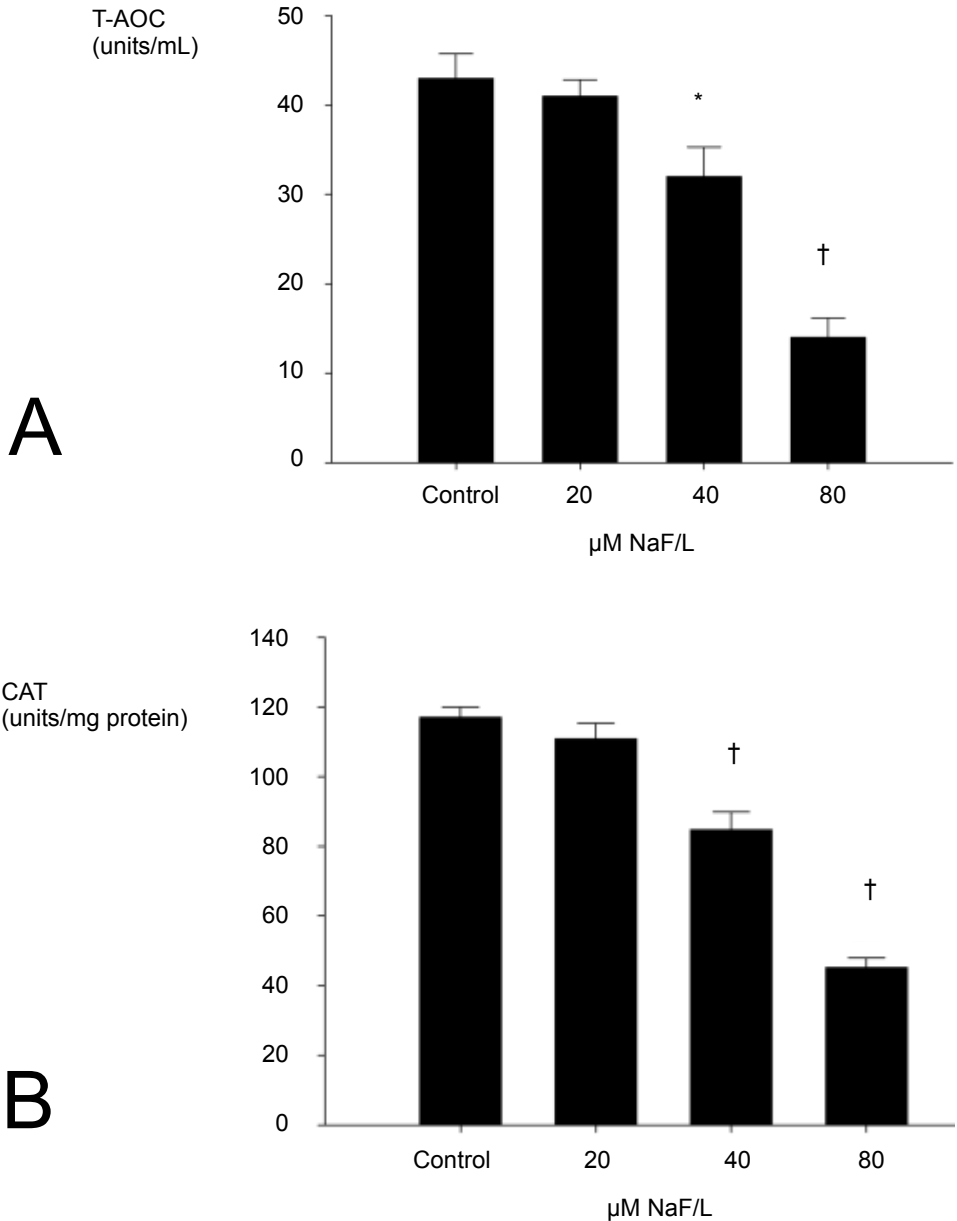
Figures 2A and 2B. NaF increases intracellular ROS level in bovine MII oocytes. Representative images of ROS levels in germinal vesicle oocytes treated with A: 0 μM NaF/L, and B: 20 μM NaF/L.



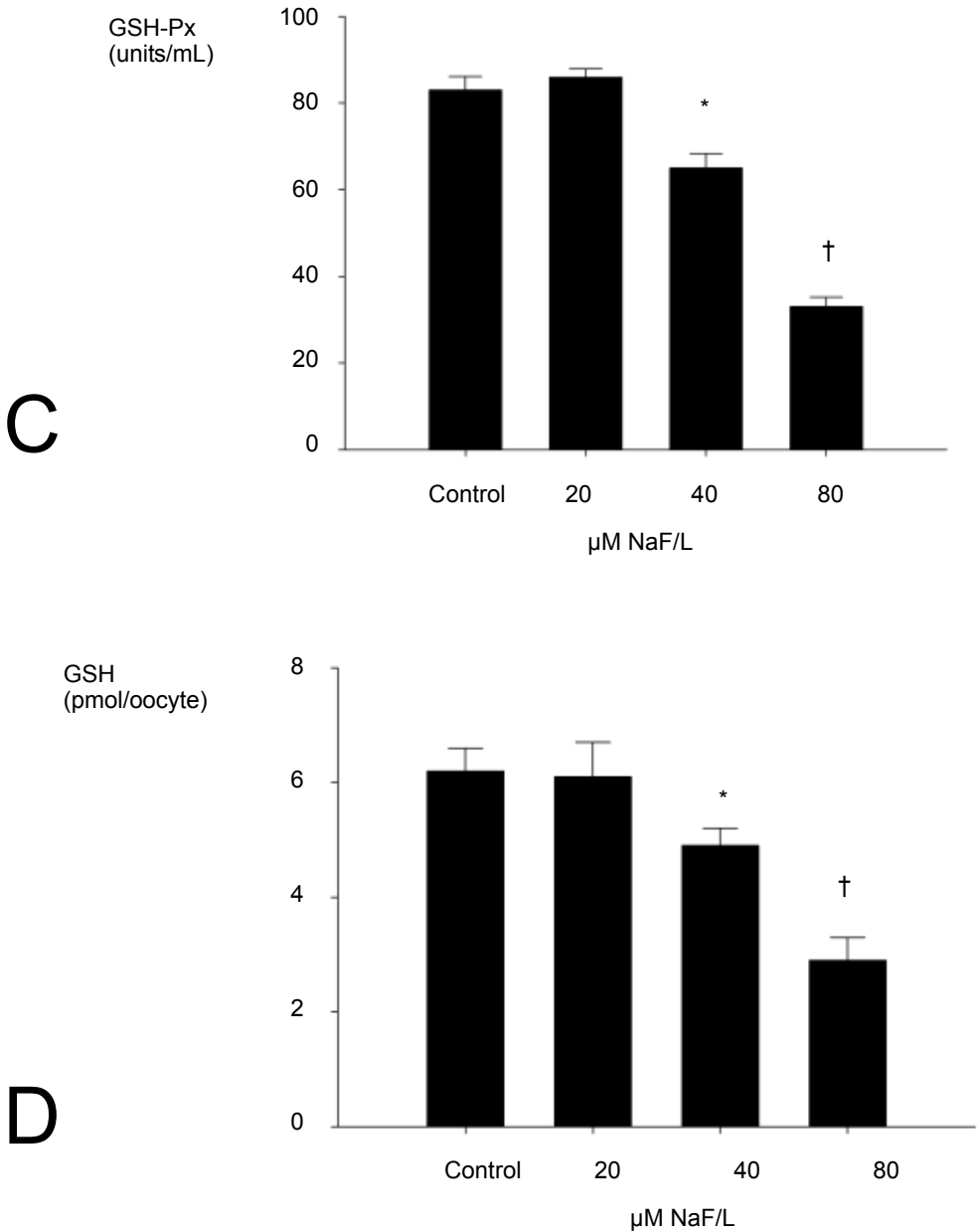
Figures 2C, 2D, and 2E. NaF increases intracellular ROS level in bovine MII oocytes. Representative images of ROS levels in germinal vesicle oocytes treated with C: 40 μM NaF/L, and D: 80 μM NaF/L. E: Intracellular levels of ROS (% of control) measured by the spectrophotometer in MII oocytes from the control and NaF-treated groups. Compared to the control group: [†] $p < 0.01$. Data are presented as mean \pm SEM from at least three independent experiments.

Oxidative stress indicators including total antioxidant capacity (T-AOC), catalase (CAT), GSH-Px, and GSH activities were further analyzed. As shown in Figures 3A-3D, NaF treatment significantly decreased the levels of T-AOC and

GSH, as well as the activities of GSH-Px and CAT. These data demonstrated that NaF could induce oxidative stress in oocytes.



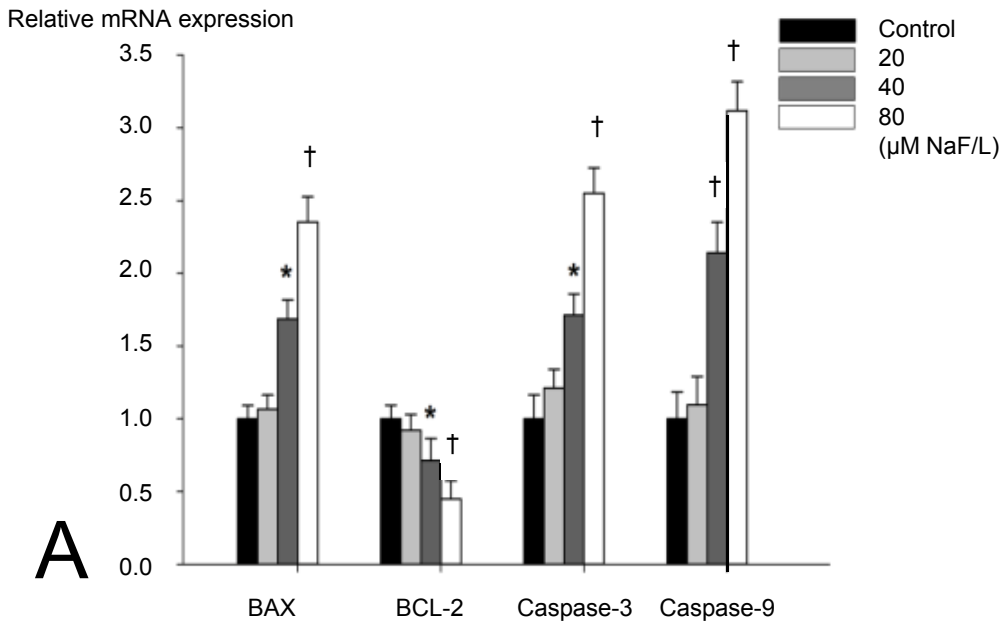
Figures 3A and 3B. NaF-induced oxidative injury in bovine MII oocytes by sparing antioxidative enzymes. A: T-AOC (units/mL), and B: CAT activity (units/mg protein). Compared to the control group: * $p < 0.05$, † $p < 0.01$. Data are presented as mean \pm SEM from at least three independent experiments.



Figures 3C and 3D. NaF-induced oxidative injury in bovine MII oocytes by sparing antioxidative enzymes. C: GSH-Px activity (units/mL), and D: GSH levels (pmol/oocyte). Compared to the control group: * $p < 0.05$, † $p < 0.01$. Data are presented as mean \pm SEM from at least three independent experiments.

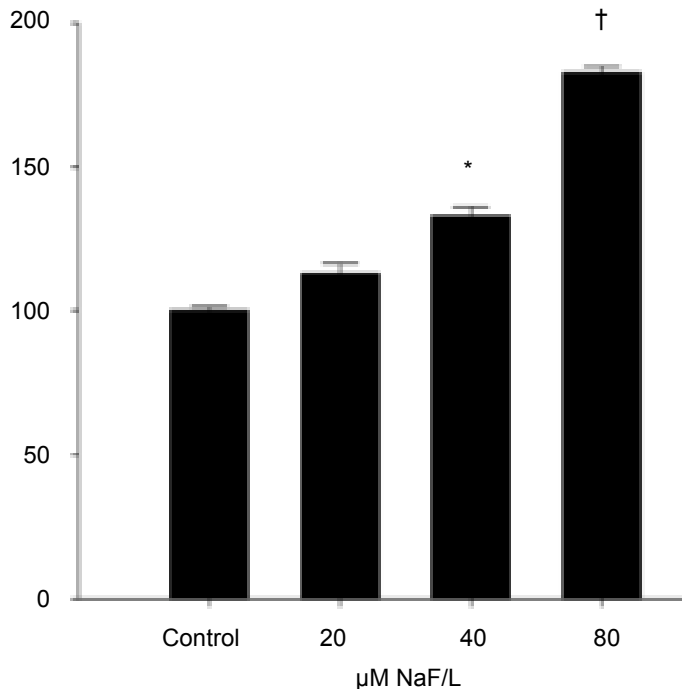
As shown in Figures 4A-4D, NaF exposure increased levels of the apoptotic protein Bax in addition to activities of caspase-3 and caspase-9, and reduced anti-

apoptotic protein Bcl-2 in oocytes. Moreover, increased apoptosis rates were further analyzed in blastocysts developed from the NaF-treated oocytes.



A

Caspase 3 activity
(% of control)

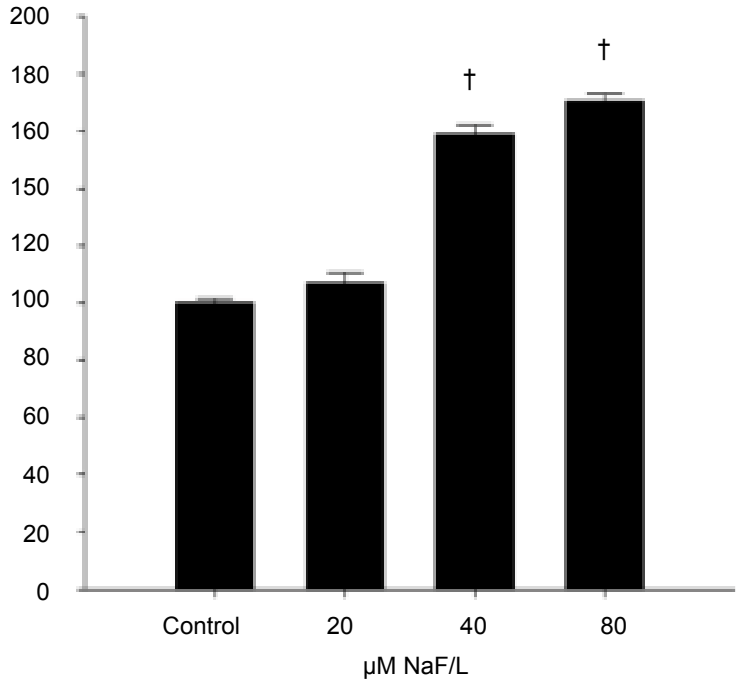


B

Figures 4A and 4B. Effects of NaF on the apoptosis of MII oocytes. A: relative mRNA levels of the apoptosis genes (Bax, Bcl-2, caspase-3, and caspase-9) and B: the activity of caspase-3. Compared to the control group: * $p < 0.05$, † $p < 0.01$. Data are presented as mean \pm SEM from at least three independent experiments.

Caspase 9 activity
(% of control)

C

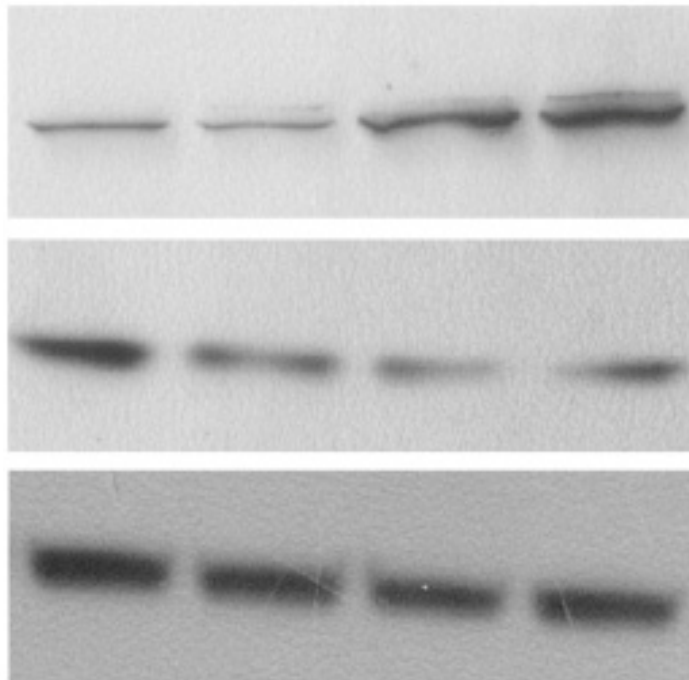


Bax

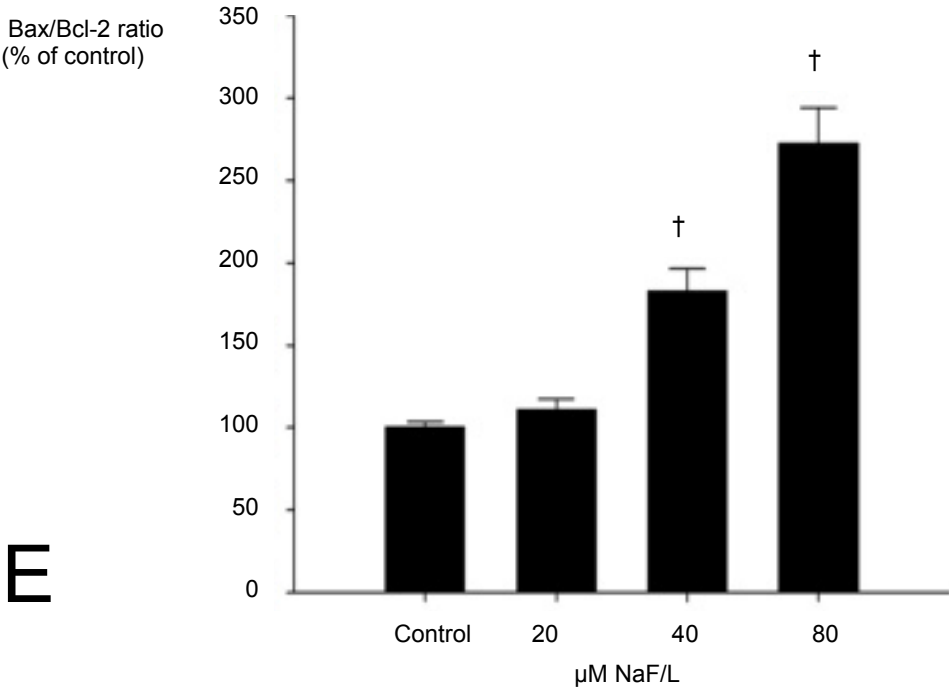
Bcl-2

D

H₂A



Figures 4C and 4D. Effects of NaF on the apoptosis of MII oocytes. C: relative mRNA levels of the apoptosis gene caspase-9 and D: representative western blots for the apoptosis genes Bax and Bcl-2t and the housekeeping gene Histone 2a (H₂A). Compared to the control group: †p<0.01. Data are presented as mean±SEM from at least three independent experiments.



Figures 4E. Effects of NaF on the apoptosis of MII oocytes. E: the quantification of Bcl-2 protein levels relative to the housekeeping gene Histone 2a (H₂A). Compared to the control group: †*p*<0.01. Data are presented as mean±SEM from at least three independent experiments.

As shown in Figure 5A-5C and Table 3, NaF exposure decreased the numbers of total, inner cell mass (ICM), and trophectoderm (TE) cells. In addition, it decreased the expression of pluripotency-related genes. Moreover, increased apoptosis rates were further analyzed in blastocysts developed from the NaF-treated oocytes.

A

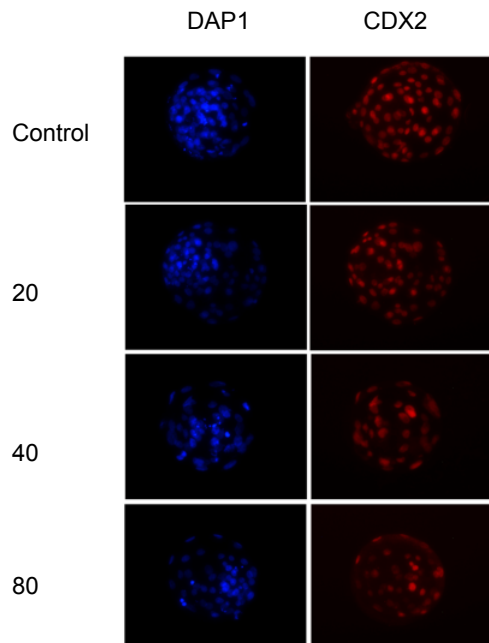
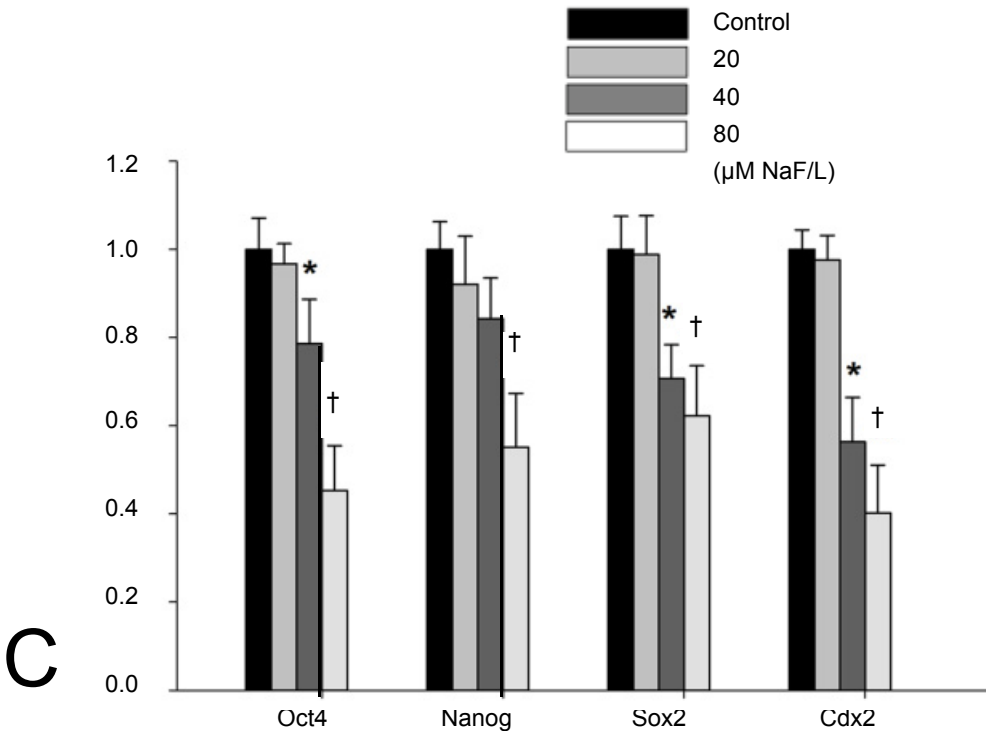
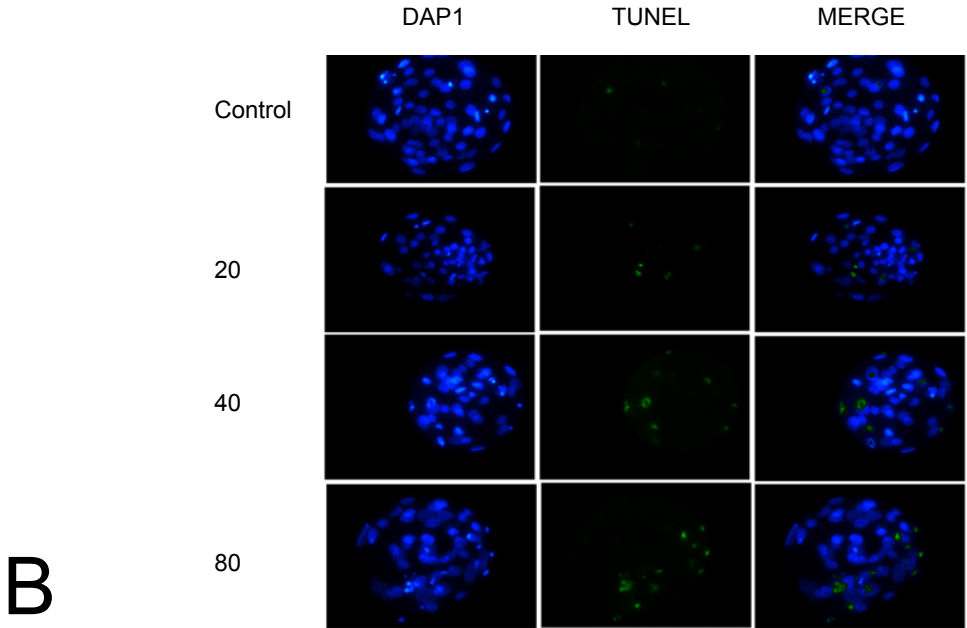


Figure 5A. A: Immunostaining of CDX2. Each blastocyst in the 0, 20, 40, and 80 μM NaF-treated groups was stained with DAPI and CDX2, a marker for trophectoderm (TE). Original magnification was ×200.



Figures 5B and 5C. B: Representative images of TUNEL positive cells in mouse blastocysts after pretreatment with NaF for 12 hr during the germinal vesicle stage and C: relative expression levels of the development related genes (Oct4, Nanog, Sox2, and Cdx2. Compared to the control group: * $p < 0.05$, † $p < 0.01$. Data are presented as mean \pm SEM from at least three independent experiments.

Table 3. Characterization of day 7 bovine blastocysts

Treatment (μ M NaF/L)	Number	DAPI staining	CDX2 staining	ICM cells
0 (control)	38	111.27 \pm 5.65	76.47 \pm 5.02	35.14 \pm 3.61
20	44	105.73 \pm 8.92	71.32 \pm 3.83	33.92 \pm 2.01
40	31	87.26 \pm 7.91 [†]	59.80 \pm 0.87 [†]	27.10 \pm 2.79*
80	35	61.45 \pm 8.70 [†]	38.14 \pm 6.11 [†]	23.32 \pm 3.50 [†]

The cell numbers in the blastocysts were estimated by counting the total number of nuclei using DAPI. The number of trophectoderm (TE) nuclei was estimated using immunostaining for CDX2. The ICM cell number was assessed as the total number of nuclei minus the number of TE nuclei. The data are shown as mean \pm SEM. Compared to the control group: * p <0.05, [†] p <0.01.

DISCUSSION

In the present study, we showed that oocyte maturation, *in vitro* fertilization, and blastocyst formation were significantly impaired in the treated group compared to the control group. To our knowledge, this is the first direct evidence to show that NaF damages bovine embryo development. The result is consistent with published data in mice.¹⁹ The developmental failure could be linked to oxidative damage and apoptosis.

Accumulated evidence revealed that NaF toxicity was closely related to OS.²⁰ The disturbance in the oxidant-antioxidant balance led to potential cell injury, which is thought to play a key role in the pathogenesis of fluorosis, a chronic condition caused by excessive F intake that affects bones, teeth, ligaments, and many organ systems. Antioxidant enzyme expression is crucial for attaining homeostasis to disable redox signaling.²¹ The present investigation revealed that NaF exposure enhanced levels of ROS. During embryogenesis, cytosolic GSH-Px, an enzymatic antioxidant, is highly expressed in most cells and tissues and protects embryos from oxidative stress.²² CAT is another enzymatic antioxidant expressed in embryos.²³ Its role as an antioxidant has been demonstrated in studies with knock-out and transgenic mice.²³ Our study found that NaF treatment decreased T-AOC in addition to activities of GSH-Px and CAT, which suggests that NaF may impair bovine embryos through depletion of antioxidative enzymes. In addition to enzymatic defenses, GSH, a non-enzymatic antioxidative defense that detoxifies hydrogen peroxide via action of GSH-Px, plays a prominent role in maintaining the redox status in oocytes and embryos.²⁴ Previous studies have reported that NaF has the capability to decrease intracellular GSH levels. The

present investigation revealed that NaF exposure decreased the level of antioxidants in oocytes.

The Oct4 transcription factor is a key regulator of pluripotency that is important for maintaining ICM cell fate and pluripotency of embryonic stem (ES) cells. It has been reported that the Oct4 gene is not only expressed in ICM cells, but also in TE cells of bovine embryos.²⁵ In this study, we found that F exposure decreased expression of Oct4 in bovine blastocysts compared with the control. Sox2 is another vital regulator protein of pluripotency. Sox2 acts synergistically with Oct4 in activating Oct-Sox enhancers, which regulate the expression of Nanog, Oct4, and Sox2, themselves.²⁶ In this study, we found that F treatment downregulated the expression of Sox2 in the bovine blastocysts. Both pluripotency-related genes Oct4 and Sox2 are important for maintaining ICM cell fate. Therefore, the downregulated expression of these two genes in the NaF-treated blastocysts may be associated with the poor embryo quality.

One of the molecular hallmarks of apoptosis is the activation of caspases. Caspases are unique and closely related to the family of cysteine proteases. They play a central role in the development and regulation of cell death, which can be divided into initiator (-2, -8, -9, and -10) and effector (-3, -6, and -7) caspases.²⁷ Once activated by the signaling pathway, caspases can degrade the intracellular proteins and trigger apoptosis. Caspase-9 is the key initiator caspase for the intrinsic pathway to cell death. Upon cleavage and activation from its pro-caspase form, caspase-9 cleaves and activates caspase-3, the key effector caspase. Activation of caspase-3 leads to cytoskeletal breakdown and nuclear demise, which may provide insight into potential mechanisms involved in NaF-induced apoptosis.²⁸⁻³⁰ In the present study, NaF treatment resulted in increased activities of caspase-9 and caspase-3, which are characteristic of apoptosis. The increase in Bax levels and decrease in Bcl-2 expression further demonstrated apoptosis induced by NaF.³¹ Fluorosis-induced injury is a complex physiological and biochemical process. Because apoptosis is regulated by numerous genes, the interactions of these various factors in embryo apoptosis must be elucidated in future studies.

The findings presented here indicate that NaF exposure during *in vitro* oocyte maturation can, in a dose-dependent way, decrease embryo quality and developmental potential. Exposure of bovine oocytes *in vitro* to NaF during maturation induced poor embryonic outcomes including decreased development rates, increased apoptosis, and DNA fragmentation.

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CONFLICT OF INTEREST

The authors declare that they have no potential conflicts of interest.

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