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Glyphosate decreases bovine oocyte quality by inducing oxidative stress and apoptosis

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Research Article

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Summary

Glyphosate is a universal herbicide with genital toxicity, but the effect of glyphosate on oocytes has not been reported. This study aimed to evaluate the effect of glyphosate (0, 10, 20, 50 and 100 mM) on bovine oocyte *in vitro* maturation. We showed that 50 mM glyphosate adversely affects the development of bovine oocytes. Exposure of oocytes to 50 mM glyphosate caused an abnormal reduction in oxidative (redox) levels compared with that in the control group, with a significantly higher reactive oxide species level ($P < 0.05$) and significantly lower glutathione (GSH) expression ($P < 0.05$). Additionally, the mRNA levels of antioxidant genes (*SOD1*, *SOD2*, *SIRT2*, *SIRT3*) and the mitochondrial membrane potential (MMP) were significantly reduced ($P < 0.05$). Furthermore, treatment with 50 mM glyphosate-induced apoptosis, and the mRNA levels of the apoptotic genes *Caspase-3* and *Caspase-4* were significantly higher than those in the control group ($P < 0.05$); however, the mRNA level of *BAX* was significantly higher than that in the control group ($P < 0.01$). Additionally, the mRNA levels of the anti-apoptotic genes *Survivin* and *BCL-XL* were significantly lower than those in the control group ($P < 0.05$), and oocyte quality was adversely affected. Together, our results confirmed that glyphosate impairs the quality of oocytes by promoting abnormal oocyte redox levels and apoptosis.

Introduction

Agriculture is the main industry in human society, and the use of modern pesticides has markedly increased crop yields and has created large economic value regarding agricultural production (Helander *et al*., 2019). However, environmental and health problems resulting from the large-scale use of pesticides have increasingly affected people in recent years, and numerous conditions, such as tumours, cancer, neurodegenerative lesions, endocrine disorders, and autoimmune diseases, have been linked to the proximity to accumulative pesticides (Martínez *et al*., 2020; Meftaul *et al*., 2020). Therefore, the problem of pesticides has affected human lives.

Glyphosate (*N*-(phosphonate methyl)glycine) is currently the most highly produced herbicide (Beckie *et al*., 2020; Khan *et al*., 2020). Because glyphosate is a broad-spectrum agent with high efficiency, low toxicity, a low cost and targeted specific effects, it has been widely used and considered a highly reliable herbicide (Fuchs *et al*., 2021). However, in recent years, glyphosate has been reported to adversely affect the health of organisms (Caiati *et al*., 2020). Regarding its application, glyphosate is mainly diluted and sprayed. During the spraying process, some of the herbicide is absorbed by plant tissue, and these types of grass and crop straw constitute most farm feed sources for ruminants, such as cows, sheep, and deer. Glyphosate solution can also enter the groundwater environment through rainwater, affecting the drinking water of animals, with consequent toxic effects (Gillezeau *et al*., 2019).

Glyphosate shows nerve, blood, liver, kidney, endocrine, and reproductive toxicity. Glyphosate and its metabolites promote apoptosis and autophagy pathways, hindering neuronal development (Martínez *et al*., 2020). After exposure to drinking water with 1000 mg/l of glyphosate for 72 h, mice showed abnormal and pulverized cell tumours and disorders in organs such as the kidney and lymphatic organs (Wang L *et al*., 2019). *In vitro* rainbow trout hepatocyte lines previously exposed to glyphosate exhibited DNA damage, indicating the effects of glyphosate on the hepatocyte system (Weeks Santos *et al*., 2019). In a study examining exposure to glyphosate during pregnancy via drinking water, a decline in testicular function in F1 was observed, the number of sperm in the epididymis was reduced, and adjustments within the hypothalamus–pituitary–testicular axis were disrupted (Teleken *et al*., 2020). In a study of glyphosate exposure in female mice, the mice exhibited long-term deformation of the mammary gland after contact with glyphosate (Zanardi *et al*., 2020).

In mammals, oocyte maturation is essential for female reproduction. Oocytes with maternal genetic material contribute most of the cytoplasm and cell membrane to develop subsequent

embryos. *In vivo*, oocyte maturation is inhibited during the germinal vesicle (GV) stage. After luteinizing hormone (LH) stimulation, maturation of the oocytes begins (Pan and Li, 2019; Arroyo *et al*., 2020). Discharge of the oocyte is then assessed by measuring the release of the first polar body in the middle of the second meiotic division (Pan and Li, 2019; Arroyo *et al*., 2020). However, oocytes are often the basis for infertility (Duan and Sun, 2019). Synchronous maturation of the nucleus and cytoplasm of the oocyte is crucial for fertilization and embryo development (Nie *et al*., 2020). At the same time, oxidative stress affects the maturation and development of oocytes.

Based on the abovementioned knowledge, this study aimed to assess cow oocyte maturation, reactive oxygen species (ROS) levels, GSH levels, gene expression, and mitochondrial function.

The effects of glyphosate accumulation on the quality of cow oocytes and reproductive developmental capacity of mammalian oocytes were explored.

Materials and methods

Drug

All the drugs were obtained from Sigma but without special instructions.

Collection and transport of test materials

The cattle ovaries used in this study were obtained from a slaughterhouse in China (Yanji). The ovarian specimens were placed in a thermos (temperature 23–30°C) and transported to the laboratory within 3 h. The surrounding tissues were excised in sterile saline (0.9% sodium chloride, 75 μg/ml of penicillin G, and 50 μg/ml of streptomycin). To address the formation of sulfate, the specimens were washed until the solution became clear, and the entire process was performed at 25°C.

Acid of oocytes and cultures *in vitro*

Fresh ovaries were cleaned once or twice with sterile saline (25°C), and the ovarian surface was disinfected with 75% alcohol. After the water absorbing paper was saturated, the specimen was washed once with physiological saline and then air dried. An 18-g needle was used to aspirate the cumulus–oocyte complexes (COCs) from follicles 2–8 mm in diameter, and the COCs were precipitated in another tube for 10 min. The supernatant was removed, and 2–3 ml of *in vitro* maturation (IVM) culture solution (90% TCM-199, 10% FBS, 25 μg/ml of gentamycin, 0.2 mM pyruvate, 1 μg/ml of FSH, 0.57 mM cysteine, 1 μg/ml of oestradiol) was added to the precipitate. The COCs were added to a disposable culture dish containing IVM solution and observed under a microscope. The COCs were washed three times using IVM culture solution. To evaluate whether glyphosate had dose-dependent effects on the *in vitro* culture, different concentrations (0, 10, 20, 50, 50 or 100 mM) of glyphosate were added, and the COCs were incubated at 38.5°C for 24 h. After determining the glyphosate culture concentration, glyphosate was added to the IVM solution and cultured oocytes according to the above method.

In vitro *fertilization and* in vitro *development*

Oocytes that were matured and cultured *in vitro* for 24 h were selected, and the outer cumulus cells of COCs were removed by gentle blowing with 1% hyaluronidase (HY). Next, the oocytes were cleaned 3–5 times with *in vitro* semen (IVF, Japan), and then the oocytes were placed into 100 IVF droplets and then in an incubator for fertilization. The semen were removed from the liquid nitrogen, left to stand at room temperature for 20 s, and then thawed in a water bath at 38°C for 20 s. The thawed sperm were placed in a 1.5-ml sperm product (BO) centrifuge. After the completion of sperm capacitation, the bottom fraction of the semen was injected into 100 IVF droplets, mixed with the oocytes, covered with mineral oil, cultured in a 38°C, 5% CO_2 incubator, and cultured *in vitro* to complete IVF. After oocyte fertilization *in vitro*, the oocytes were blown with 1% HY to remove all cumulus cells and obtain bare oocytes. Afterwards, the naked oocytes were washed five times with 0.4% bovine serum albumin (BSA)-CRI (IVC) culture media at 10 μl of IVC droplets per wash and covered with mineral oil. Next, the cells were cultured at 38.5°C in a 5% CO_2 incubator until the 8-cell stage (72 h), and then statistical analyses of the ovulation and 8-cell rates were performed. The cultivation in 10% FBS-CRI continued to the seventh experimental dose, and the test was repeated at least three times.

Determination of meiosis progression in oocytes

Fresh bovine oocytes were cultured in IVM for 22 h. Ruptured oocytes were eliminated with 0.1% HY, and cumulus cells were removed to obtain denuded oocytes. The cells were washed three or four times with 0.1% BSA-PBS, and denuded oocytes were incubated with Hoechst 33342 stain at 37°C for 20 min. Next, BSA-PBS was used to wash the residual stain, and a fluorescence microscope was used to photograph and count the number of discharged polar bodies. As shown in Figure 1, the nuclei of the stained oocytes were evaluated under a fluorescence microscope.

Immunofluorescence staining

The intracellular ROS content in oocytes was detected using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, China). The cumulus cells of COCs were removed, and the naked oocytes were incubated in 10 μM DCFH-DA in the dark at 37°C for 30 min. After washing with 0.1% BSA-TC M199 three times, quantitatively labelled oocytes were observed using a fluorescence microscope (Nikon, JP) with a 460-nm UV filter. Finally, Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used to analyze the fluorescence intensity of the oocytes (green fluorescence, UV filter, 490 nm). Each experiment was repeated five times.

To assess the MMP, a JC-1 (Beyotime, China, AUM) assay kit was used. This process was performed according to the instructions given in the kit. The oocytes were incubated in a working solution containing 10 μM JC-1 in the dark at 38.5°C for 30 min. After washing with JC-1 buffer solution, the oocytes were observed in the same scan of each sample under the microscope. JC-1 aggregates (red fluorescence) were detected using the tetramethylrhodamine (TRITC) channel, indicating high membrane potential, while the JC-1 monomer (green fluorescence) was detected using the FITC channel, indicating a low membrane potential. The ratio of aggregates (red fluorescence) to monomers (green fluorescence) was calculated to quantify changes in MMP.

The colliculus cells of the COCs were removed to obtain denuded oocytes, and then the naked oocytes were washed with 1% BSA-PBS two or three times. The denuded oocytes were placed in 10 μL of the cell-tracking blue-fluorescent dye 4-chloromethyl-6,8-difluoro-7-hydroxy-coumarin at a concentration of 10 μmol/l. The oocytes were incubated at 37°C for 30 min in the dark and then

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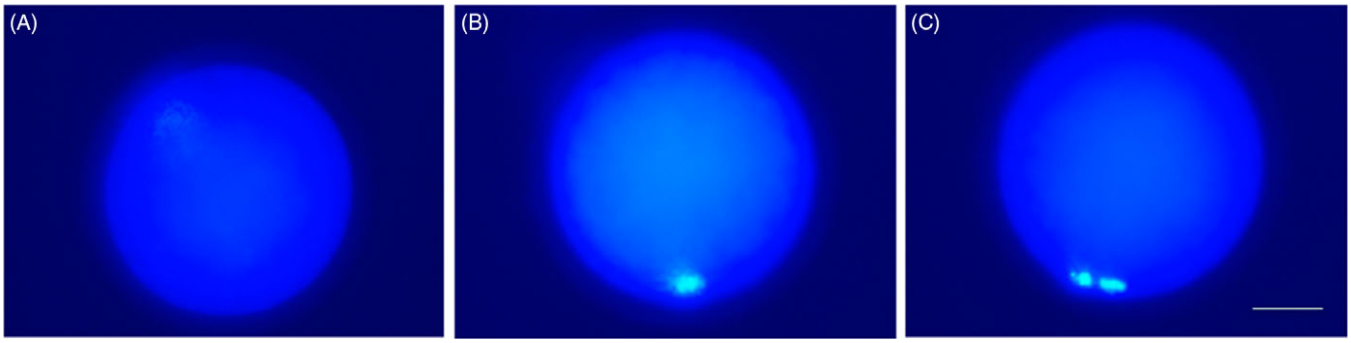

Figure 1. Schematic diagram of meiosis of bovine oocyte. (A) Oocytes with diffusion or slight concentrated chromosomes are classified as the GV phase or GVBD phase. (B) Oocytes with clumped or strongly condensed chromatin form individual divalent (prometaphase) or metaphase plates, but irregular networks without polar bodies are classified as being in the MI phase. (C) Oocytes with polar bodies or two bright chromatin spots are classified as being in the MII stage. Blue, chromosome. Scale, 40 μm.

washed with 1% BSA-PBS. They were observed and photographed under a fluorescence microscope using blue fluorescence.

RT-PCR

Each group of 30 oocytes cultured for 24 h was removed from the cumulus and washed with 1% BSA-PBS, and the samples were added to 50 μl of lysis buffer (5 mmol/ml of dithiothreitol, 20 U/mol of RNase inhibitor, 1% NP-40) according to the product's instructions. The samples were fast frozen in liquid nitrogen and stored at −80°C for later use. mRNA was extracted using the Dynabeads mRNA Direct Kit according to the specifications. Next, a Dynabeads mRNA Direct Kit was used to synthesize cDNA by reverse transcription of the improved mRNA. Real-time fluorescence quantitative PCR was then performed using a Kapa SYBR® FAST kit, and qPCR was performed using CFX Connect Optics Module PCR. The 20 μl PCR system comprised the following: 2 μl of cDNA, primer (comprising 1 μl each of the upstream primer and downstream primers), 10 μl of SYBR GREEN, and 7 μl of DDH_2O. The PCR procedure was as follows: predenaturation at 95°C for 3 min, 95°C for 3 s, 60°C for 30 s, and 72°C for 20 s. Forty target genes were identified, such as *SOD1*, *SOD2*, *SIRT1*, *SIRT2*, *SIRT3*, *Caspase-4*, *Caspase-3*, *Survivin* and *BCL-XL*. The reference gene was *Actin*. The primer sequences used to amplify each gene are shown in Table 1, and quantitative RNA data were statistically analyzed using the $2^{-\Delta\Delta Ct}$ method.

Data processing and analysis

SPSS 19.0 (Inc., Chicago, IL, USA) software was used for statistical analysis, and all the test results were expressed as means ± standard deviation (SD). A highly significant difference between the two groups was indicated by $^{**}P < 0.01$, and a significant difference was indicated by $^{*}P < 0.05$. An independent sample *t*-test was used for comparisons between the two groups. GraphPad Prism 6.01 software was used to generate the graphics. Fluorescence intensity analysis was performed using ImageJ software. The test was repeated at least three times.

Results

Glyphosate causes meiotic arrest of bovine oocytes

To investigate the effect of glyphosate on bovine oocyte maturation *in vitro*, meiosis in oocytes was examined. After the oocytes were cultured *in vitro* for 24 h, meiosis was quantified after oocyte staining (Table 2). Oocytes exposed to 50 mM and 100 mM glyphosate were more likely to stay in the GV/germinal vesical breakdown (GVBD) phase. The percentage of oocytes reaching the MII stage in the 50 mM and 100 mM groups was significantly lower than that in the control group ($P < 0.05$). Therefore, 50 mM glyphosate treatment was used in subsequent experiments. Exposure to glyphosate inhibited the maturation of bovine oocytes.

Glyphosate affects oocyte development

To investigate the effect of glyphosate on oocyte maturation and development *in vitro*, oocytes were exposed to 50 mM glyphosate for *in vitro* culture. Both the cleavage and blastocyst rates in the glyphosate treatment group were significantly lower than those in the control group (Table 3) ($P < 0.05$). Therefore, glyphosate reduced the developmental capacity of oocytes.

Glyphosate causes oxidative stress

To investigate the effects of glyphosate on oxidative stress during IVM of bovine oocytes (Figure 2), we examined the levels of ROS and GSH in the cytoplasm of bovine oocytes. The ROS fluorescence intensity in the 50 mM glyphosate-treated group was significantly higher than that in the control group ($P < 0.05$), indicating that the glyphosate-treated group enhanced ROS production. The immunofluorescence intensity of GSH in the 50 mM glyphosate-treated group was significantly lower than that in the control group ($P < 0.05$), indicating that glyphosate can reduce GSH production.

Glyphosate causes abnormal mitochondrial function

MMP is an important indicator of the maturation and quality of oocytes. To determine the effect of glyphosate on MMP, the MMP of treated oocytes was detected by immunofluorescence staining (Figure 3). MMP in the 50 mM glyphosate treatment group was significantly lower than that in the control group ($P < 0.05$). In conclusion, glyphosate interferes with the mitochondrial function of bovine oocytes.

Effects of glyphosate on antioxidant gene expression in bovine oocytes

RT-PCR was used to measure the mRNA expression levels of oxidative stress-related genes (*SOD1*, *SOD2*, *SOD3*, *SIRT1*, *SIRT2* and *SIRT3*) (Figure 4). Compared with the control group, 50 mM glyphosate treatment downregulated the *SOD1*, *SOD2*, *SIRT2* and *SIRT3* transcription levels in oocytes ($P < 0.05$). This finding also provides further evidence that glyphosate disrupts the oocyte

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Table 1. Primer sequences expressed in oocytes were analyzed by RT-PCR

Gene	Primer sequences (5′–3′)	Fragment length (bp)	Annealing temperature (°C)
Actin	F:GCACCACTGGCATTGTCATG; R:CCATCTCCTGCTCGAAGTCC	160	60
SOD1	F:ATCCACTTCGAGGCAAAGGG; R:TGTCACATTGCCCAGGTCTC	249	60
SOD2	F:CCCTAACGGTGGTGGAGAAC; R:GCGTCCCTGCTCCTTATTGA	269	60
SOD3	F:CCAAGGTGACGGAGATCTGG; R:CAAACTGGTGCACGTGGATG	250	60
SIRT1	F:TGGCCAGCTAGACTTGCAAA; R:AACTTGGACTCTGGCACGTT	173	60
SIRT2	F:ATAGACACCCTGGAGCGAGT; R:CATGCAGGAGAAGAAACGCG	505	60
SIRT3	F:CAGCATCCTCCAGCAGTACA; R:GAGCTTTGAGTCAGGGATGC	238	60
Caspase-4	F:TTTCTGGCCTTTTGGATGAC; R:AGCTGCAGATCCCACTGACT	248	60
Caspase-3	F:TGGTGCTGAGGATGACATGG; R:GAGCCTGTGAGCGTGCTTTT	163	60
Survivin	F:GCCAGATGACGACCCCATAG; R:GGCACAGCGGACTTTCTTTG	199	60
BCL-XL	F:AGGCAGGCGATGAGTTTGAA; R:AGAAAGAGGGCCAAATGCGA	159	60
BAX	F: AGAAGGATGATCGCAGCTGTG; R: AGTCCAATGTCCAGCCCATG	234	60

Note: annealing temperature is 60°C. F: forward primer. R: reverse primer.

Table 2. Effects of glyphosate on the *in vitro* maturation of bovine oocytes

Groups	Total no. of presumptive oocytes in culture	GV/GVBD (%)	MI (%)	MII (%)
Control	122	21 (17.16 ± 1.232)	37 (30.33 ± 1.902)	64 (52.5 ± 2.640)
10 mM	121	22 (18.17 ± 0.379)	36 (29.74 ± 1.352)	63 (52.10 ± 1.516)
20 mM	144	23 (17.04 ± 1.347)	44 (32.55 ± 2.704)	68 (50.40 ± 2.306)
50 mM	132	29 (22.01 ± 1.572)*	52 (39.44 ± 1.092)*	51 (38.56 ± 2.503)*
100 mM	122	53 (43.61 ± 4.549)**	30 (24.52 ± 5.805)*	39 (31.87 ± 2.532)*

N: Number of oocytes allocated to each group. All the experiments shown represent three replicates, and all the data are presented as means ± SD of three independent experiments. Control group compared with other groups.
*Indicates that the expression level was significantly different ($P < 0.05$). A highly significant difference between the two groups is indicated by ** ($P < 0.01$).

Table 3. Effects of glyphosate on bovine early embryo development

			Blastocyst yield	
Groups	Total no. presumptive zygotes in culture	Cleavage rate, *n* (%)	Day 7, *n* (%)	Day 8, *n* (%)
Control	137	102 (74.45 ± 5.33)	29 (21.12 ± 2.68)	33 (24.07 ± 1.78)
50 mM	139	80 (57.62 ± 4.52)*	25 (17.89 ± 1.07)*	27 (19.40 ± 1.76)*

n: The number of oocytes allocated to each group. All the experiments shown represent three replicates, and all the data are presented as means and SD of three independent experiments.
*Indicates that the expression level was significantly different ($P < 0.05$).

reduction–oxidative (redox) system, causing excessive intracellular accumulation of ROS.

Effects of glyphosate on apoptosis-related gene expression in bovine oocytes

Based on the oxidative stress of bovine oocytes induced by glyphosate, the mRNA expression levels of apoptosis-related genes (*Caspase-3*, *Caspase-4*, *BAX*, *Survivin* and *BCL-XL*) were further determined. Compared with those in the control group, the mRNA expression levels of the apoptotic genes *Caspase-3* and *Caspase-4* in oocytes exposed to 50 mM glyphosate were significantly increased (Figure 5; $P < 0.05$). Additionally, the mRNA expression level of the apoptosis gene *BAX* was significantly higher than that in the control group ($P < 0.01$), and the mRNA expression levels of the anti-apoptotic genes *Survivin* and *BCL-XL* in exposed oocytes were significantly lower than those in the control group ($P < 0.05$). These results suggest that glyphosate negatively affects bovine oocyte quality.

Discussion

Because of various unreasonable uses of glyphosate and its accumulation in organisms, the environment, animals and plants are affected by the toxicity of glyphosate to differing degrees. The

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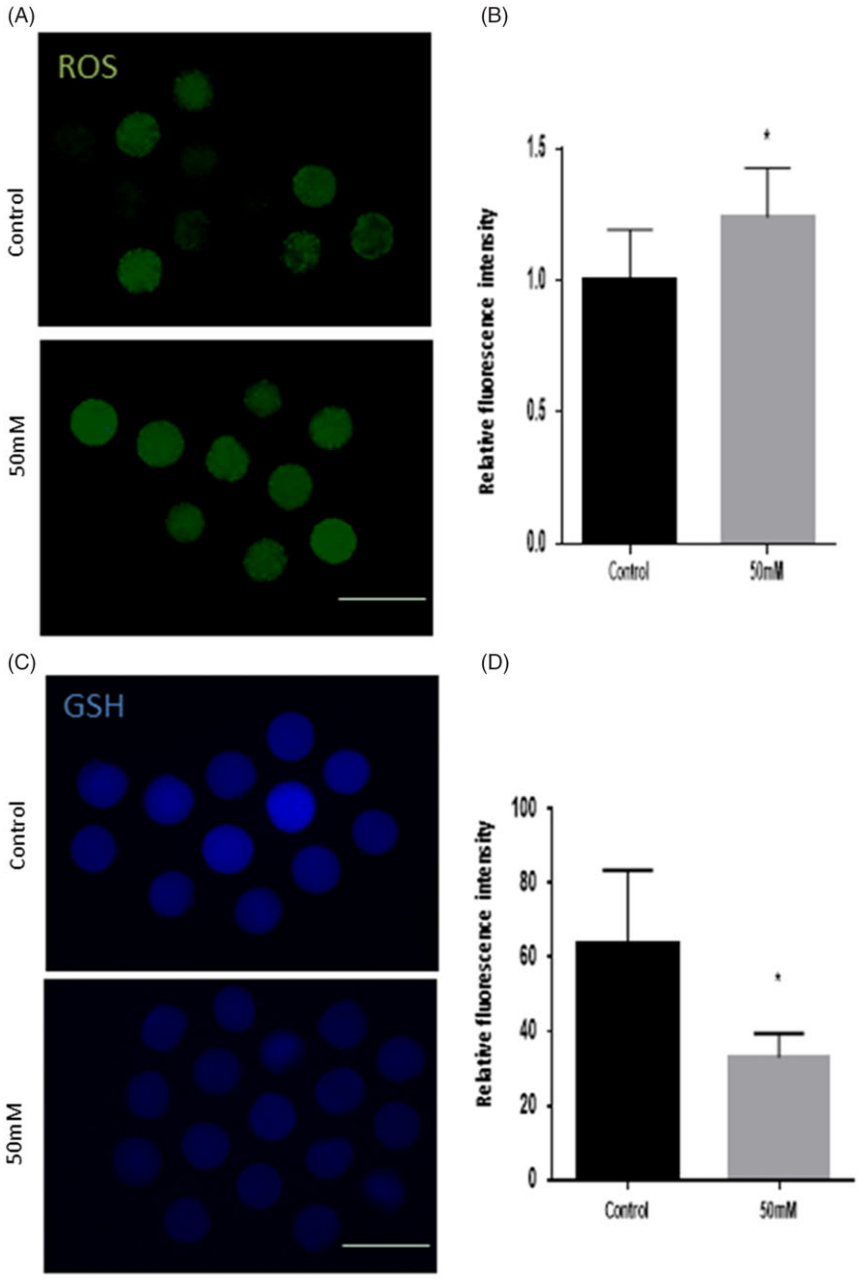

Figure 2. Effects of glyphosate on oxidative stress levels in oocytes. (A) Representative images of ROS staining of oocytes in the control group and 50 mM glyphosate treatment group. (B) Specific data analysis ROS fluorescence intensity. The number of oocytes allocated to each group (50 mM group: 122, control group: 118). (C) Representative images of GSH staining of oocytes in the control group and 50 mM glyphosate treatment group. (D) Data analysis of GSH fluorescence intensity. The number of oocytes allocated to each group (50 mM group: 101, control group: 108). All experiments shown are representations of three replicates, and all data are presented as the mean and SD of three independent experiments. *Indicates that the expression level was significantly different ($P < 0.05$). Scale bars, 200 μm.

average daily intake of glyphosate in dairy cows is 122.7 μg/kg of body weight/day, and 10 ng/ml of glyphosate disturbs the secretory functions of the ovaries and uterus, leading to the deregulation of uterine contractions and impairment of fertilization or difficulties in the maintenance of gestation (Schnabel *et al.*, 2020; Wrobel, 2018). Additionally, subcutaneous injection of glyphosate (2 mg/kg of body weight/day) can lead to abnormal uterine development of female sheep larvae (Alarcón *et al.*, 2020). Some studies have shown that exposure of human peripheral white blood cells to glyphosate at 500 μM and 750 μM significantly increases DNA-damage parameters (Nagy *et al.*, 2019). In recent years, many studies have shown that glyphosate toxicity damages the health of various organisms (Van Bruggen *et al.*, 2018). However, few studies have examined the toxic effects of glyphosate on oocytes. Therefore, in this study, the effects of glyphosate on bovine oocytes were investigated by adding glyphosate to the IVM medium of bovine oocytes. First, we hypothesized that glyphosate adversely affects oocyte maturation.

Oocyte maturation includes nuclear maturation and cytoplasmic maturation, and the expulsion of the first polar body represents nuclear maturation. In our study, Hoechst 33342 stain was used to label chromatin to evaluate the process of meiosis, revealing that the first polar body excretion rate of oocytes decreased significantly after glyphosate exposure. Our results are consistent with those of other studies involving animal models (Maskey *et al.*, 2019; Zhang *et al.*, 2019; Cao *et al.*, 2021). Additionally, glyphosate exposure in IVM reduced the cleavage and blastocyst rates of embryos, suggesting that glyphosate affects oocyte maturation and development.

When oocytes are exposed to harmful stimulation during *in vitro* culture, the resulting imbalances in the intracellular oxidant–antioxidant system lead to excessive ROS production in

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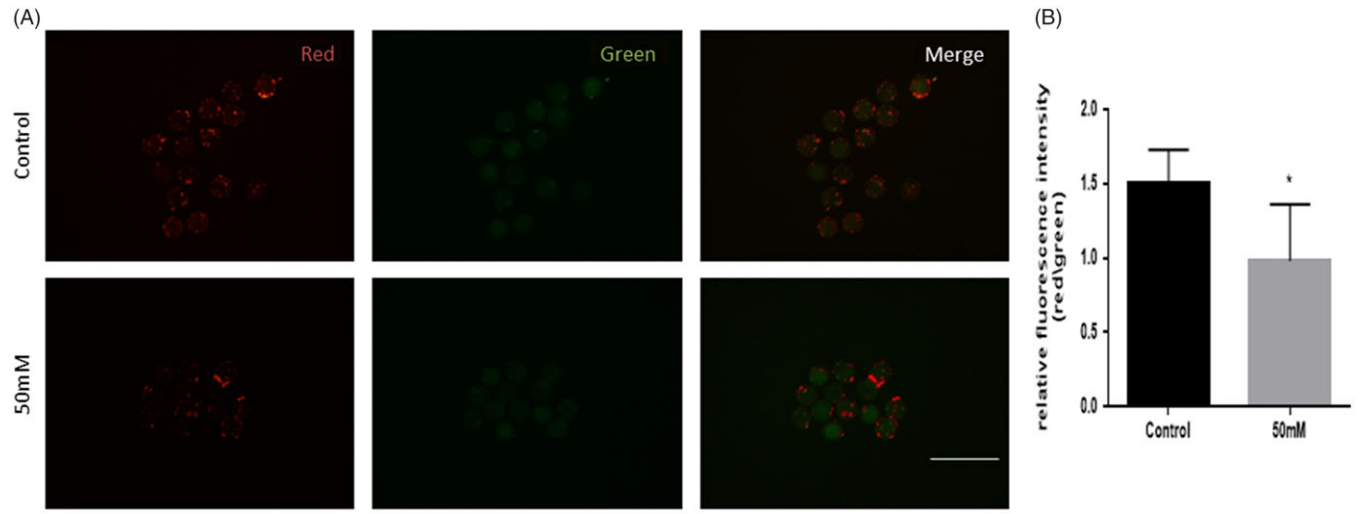

Figure 3. Glyphosate affects mitochondrial function in oocytes. (A) Representative image of JC-1 staining of oocytes in the control group and 50 mM glyphosate treatment group. Mitochondria with high MMP were stained red, while those with low MMP were stained green. (B) Quantitative analysis of the MMP of oocytes in the two groups. The number of oocytes allocated to each group (50 mM group: 101, control group: 108). All experiments were repeated three times, and all data are presented as the mean and SD of three independent experiments. *Indicates that the expression level was significantly different ($P < 0.05$). Scale bar, 200 μm.

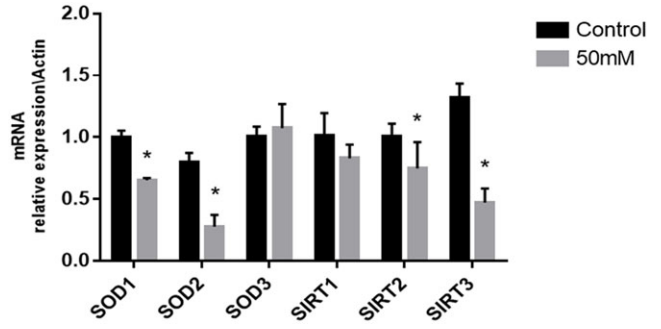

Figure 4. Effects of glyphosate on the mRNA expression of antioxidant-related genes in bovine oocytes. qRT-PCR analysis of mRNA expression of the antioxidant-related genes *SOD1*, *SOD2*, *SOD3*, *SIRT1*, *SIRT2*, and *SIRT3*. All experiments were repeated three times, and all data are presented as the SD of three independent experiments. *Indicates that the expression level was significantly different ($P < 0.05$).

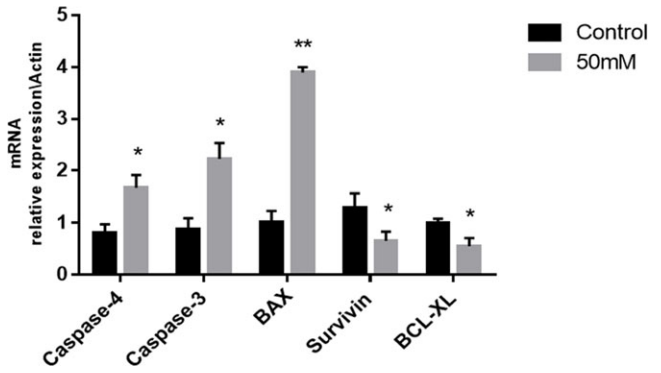

Figure 5. Effects of glyphosate on the mRNA expression of apoptosis-related genes in bovine oocytes. mRNA expression of the apoptosis-related genes Caspase-3 and Caspase-4 and BAX and the anti-apoptotic genes Survivin and BCL-XL were analyzed by qRT-PCR. All experiments are three repetitions, and all data are presented as the mean and SD of three independent experiments. *Indicates that the expression level was significantly different ($P < 0.05$). **Indicates that the expression level was significantly different ($P < 0.01$).

oocytes. This large amount of ROS can reduce the *in vitro* maturation efficiency of oocytes and lead to cell damage (Wang Y *et al.*, 2019, 2020; Barros *et al.*, 2019). To examine this, we measured the ROS levels in oocytes exposed to glyphosate, and the ROS levels were elevated. This finding indicates that, under the influence of glyphosate, bovine oocytes produce excessive ROS, reducing the developmental potential of oocytes. High GSH expression in the oxidation–antioxidant system in oocytes indicates a strong ability to eliminate ROS, and GSH levels are a critical indicator of oocyte activity (Barros *et al.*, 2019). GSH production can also protect mitochondria and prevent mitochondrial oxidative damage, protecting oocytes from the toxic damage caused by oxidative stress (Zhou *et al.*, 2019). Therefore, we measured glutathione levels in oocytes exposed to glyphosate, which reduced glutathione production, suggesting that glyphosate reduces the ability of oocytes to scavenge free radicals.

Mitochondria transform energy through phosphorylation pathways and are the primary organelles responsible for the energy supply of the oocyte. The quality, structure, location and activity of mitochondria play an essential role in oocyte maturation (Al-Zubaidi *et al.*, 2019). MMP, an indicator of mitochondrial activity, plays an indispensable role in ATP production and redox balance maintenance in oocytes (Sasaki *et al.*, 2019). The MMP of oocytes exposed to glyphosate decreased, suggesting that glyphosate reduces mitochondrial activity. This study is consistent with Jingwen Zhang *et al.*'s study on melatonin-mediated regulation of the effects of glyphosate on mouse oocytes (Zhang *et al.*, 2021).

To further understand the effect of glyphosate on the antioxidant system of bovine oocytes, the mRNA expression levels of the antioxidant genes *SOD1*, *SOD2* and *SOD3* were measured by RT-PCR. These genes are protective factors against antioxidant stress in oocytes (Mukherjee *et al.*, 2011; Malvezzi *et al.*, 2018; Nie *et al.*, 2018), and their expression levels represent the antioxidant levels of oocytes. Compared with those in the control group, the expression levels of the *SOD1* and *SOD2* genes in the glyphosate-treated group were significantly decreased. This finding also suggests that glyphosate inhibits oocyte maturation and development. At the same time, the levels of members of another recognized

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antioxidant group family – namely, *SIRT1*, *SIRT2* and *SIRT3* – were measured (Yang *et al.*, 2018; Xu *et al.*, 2019; Iljas *et al.*, 2020). Compared with those in the control group, the mRNA expression levels of *SIRT2* and *SIRT3* genes in the glyphosate-treated group were significantly decreased. These results further demonstrate the adverse effects of glyphosate on bovine oocyte maturation.

Based on the expression of oxidative stress-related factors induced by glyphosate exposure of oocytes, we assessed apoptosis-related genes, among which the pro-apoptotic gene *BAX* and anti-apoptotic gene *BCL-XL* play crucial roles in regulating apoptosis (Somfai *et al.*, 2020). In the mitochondria-mediated apoptosis pathway, abnormal signal transduction in oocytes activates *BAX*, inhibiting the expression of *BCL-XL*, and mitochondria secrete cytochrome *c*, which activates *Caspase-3* and promotes apoptosis (Chen *et al.*, 2019; Escobar *et al.*, 2019; Gao *et al.*, 2019). *Caspase-4* primarily affects ER stress-induced apoptosis (Tatsuta *et al.*, 2013). The *Survivin* gene inhibits cell apoptosis mainly by inhibiting chromosome damage (Chen *et al.*, 2018). Glyphosate treatment upregulated the transcription levels of proapoptosis-related genes (*Caspase-3*, *Caspase-4* and *BAX*) and downregulated the transcription levels of antiapoptosis-related genes (*Survivin* and *BCL-XL*). Therefore, we suggest that exposure of oocytes to glyphosate can disrupt the oocyte oxidation–antioxidant system and induce the production of a large amounts of ROS, activating the early apoptotic pathway.

In summary, glyphosate exposure inhibited the development of bovine oocytes, increased oxidative stress and decreased free radical scavenging in the oocytes, decreased the MMP of the oocytes, and induced apoptosis of oocytes. Our results provide a partial basis for the reproductive toxicity of glyphosate and improvements in the embryo culture environment.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199422000181

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