Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Toxic Effects of Benzo[b]fluoranthene on Developmental Competence of Ovine Oocytes During In Vitro Culture

Atieh Hajarizadeh

atihajari@gmail.com

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Marziyeh Mehrabadi

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Iman Rad

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Nastaran Ansari Noghlebari

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Niloofar Mohammad Ebrahim

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Elaheh Esmaeili

Stem Cell Technology Research Center (STRC), Iran university of medical science (IUMS)

Article

Keywords: B[b]F, sheep oocyte, oocyte maturation, preimplantation development

Posted Date: July 22nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10212101/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Benzo[b]fluoranthene (B[b]F) as one of the common Polycyclic aromatic hydrocarbons (PAHs) with ≤ 2.5 μm (PM 2.5) can be found in various environmental sources polluted air, water, and soil. Previous reports have revealed that B[b]F may reproductive toxicity in the female reproductive system through of oxidative stress, excessive autophagy, and apoptosis. In this study, we sought to investigate the impact of B[b]F on meiotic and cytoplasmic maturation, the production of internal reactive oxygen species (ROS), blastocyst formation, and the expression levels of mRNA associated with apoptotic (*BAX* and *Caspase3*), anti-apoptotic (*BCL2*), Growth Differentiation Factor 9 (*GDF9*), and Bone Morphogenetic Protein 15 (*BMP15*) genes in ovine oocytes. In this study, in vitro maturation (IVM) of oocytes was conducted using varying concentrations of B[b]F (0, 1, 2, 5, and 10 μM). After a 24-hour period of IVM, assessments were made regarding meiotic and cytoplasmic maturation, as well as the levels of intracellular reactive ROS. The approach mentioned facilitates the assessment of the impact that varying concentrations of B[b]F have on the maturation processes and oxidative stress levels in oocytes. Embryonic cleavage and blastocyst formation following *in vitro* fertilization were also assessed in each group. Alterations of *BAX*, *Caspase3*, BCL2, *GDF9*, and *BMP15* were analysed following *IVM*. In the presence of 5 μM B[b]F, the number of oocytes reaching the metaphase II stage decreased and the oocytes presented with lower intracellular levels of GSH ($P < 0.05$). Furthermore, intracellular ROS production in matured oocytes reached the highest-level following exposure to 2, 5, and 10 μM of AFB1 ($P < 0.05$). Rates of cleavage and blastocyst formation observed in the presence of B[b]F were lower than those recorded in the Control group ($P < 0.05$). Apoptosis-related gene analysis in AFB1 treated groups (5 μM) revealed a higher expression of the *BAX* and *Caspase3* genes, and a lower expression of the *BCL2* gene as compared with the Control group ($P < 0.05$). Additionally, our data showed that expression patterns of *GDF9* and *BMP15* decreased in the 5 μM group when compared to the Control group ($P < 0.05$). We showed that exposure of oocytes to B[b]F leads to a reduced nuclear and cytoplasmic maturation that may ultimately impair the embryonic development in the sheep. Furthermore, alterations in GDF9 and BMP15 and initiation of apoptosis through excessive ROS generation could be a prime molecular mechanism responsible for the disruption of oocyte developmental competence in the presence of B[b]F in the ovine model.

1. Introduction

Environmental pollution components are related to many diseases including infertility. One of the most important members is Polycyclic aromatic hydrocarbons (PAHs) which play an important role in air pollution is formed by incomplete organic materials combustion and diesel exhaust that is well known as a carcinogen, mutagen, and teratogen. Some PAH compounds that are identified as probable human health effects are Phenanthrene(PHE), Benzo[a] pyrene(BaP), Benzo[b] fluoranthene, naphthalene, and Benzo[k] fluoranthene(Geier et al. 2018).

PAHs metabolism can occur via cytochrome p450(Abdel-Shafy and Mansour 2016), they can convert to active metabolic such as epoxides, diol-epoxides, and o-quinones through phase I activation. This

generation of active metabolites is banished due to phase II and then can bind to DNA(Branco et al. 2021).

Benzo[b]fluoranthene (B[b]F) is a type of PHA that is found in food, water, solids, and the atmosphere[1]. The ovary is a major target of B[b]F, therefore excessive exposure to B[b]F may cause reproductive toxicity in the female reproductive system(Guo et al. 2020). Following exposure, B[b]F is absorbed by ingestion, inhalation, and dermal contact and then transported by the blood circulation to another organ(Sousa et al. 2022). B[b]F such as other PAH components is converted to epoxide, an electrophilic form that produces reactive oxygen species (ROS), leading to oxidative stress(Branco et al. 2021). Oxidative stress can reduce oocyte maturation, fertilization, and embryonic development via changes in cellular progression. Excessive ROS can also induce DNA definition, mitochondrial dysfunction, apoptosis and reduce ATP production(Hajarizadeh et al. 2022; Kala et al. 2017). It has been reported exposure to B[b]F caused DNA damage and apoptotic incidence in mice oocytes(Guo et al. 2020).

Fertilization and the early stage of embryonic development depend on the in vitro progression of an oocyte from germinal vesicle (GV) to metaphase meiosis II (MII) and cytoplasmic maturation(Sirait et al. 2021).

The coordination of Meiotic and cytoplasmic maturation that occurs during oogenesis to MII is regulated by Multiple factors including different pathways, growth factors membranes, and epigenetic molecules(Han et al. 2016). Oocyte maturation relies on maintaining the normal distribution and function of organelles in oocytes. This incurs maintaining normal physiological activity and correct distribution of mitochondria and other organelles in the cytoplasm(Pan et al. 2021).

Expression of Growth differentiation factor 9 (*GDF9*) and the Bone morphogenetic protein 15 (*BMP15*) are found in oocyte coordinate the genes involved in the cumulus cell expansion during oocyte maturation(Paulini and Melo 2011). *GDF9* belongs to the superfamily of transforming growth factor-β (*TGF-β*), which are recognized as significant regulators of cell proliferation in ovine oocytes, and the biological impact of *GDF9* is conveyed through the involvement of the BMPs family(Monte et al. 2021). *BMP15* has been detected in the oocytes of humans, bovines, and ovine species. which has a vital role in granulosa cells, inducing their proliferation.*BMP15* expression reaches its highest level during the expansion of cumulus cells, after oocyte maturation(Paulini and Melo 2011).

In the present study, we evaluated the hypothesis that the developmental competence of ovine oocytes is influenced by B[b]F. The aims of the present study were: (1) to evaluate the concentration of B[b]F to study the B[b]F on oocyte nuclear maturation by evaluating the nuclear status, intracellular glutathione (GSH) content, and intracellular ROS level after in vitro maturation (IVM); (2) to investigate the effect of B[b]F on oocyte developmental potential and quality of derived blastocysts; and (3) to analyze the effect of B[b]F on expression *GDF9*, *BMP15* and profile of genes involved in apoptosis (*Bax*, *Caspase3*, and *Bcl2*,) in mature ovine oocytes.

2. Materials and methods

All chemicals and media used in this study were purchased from the Sigma–Aldrich Corporation (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA). Ovaries were collected from adult ewes at a local slaughterhouse and transported to the laboratory within 2–3 h in saline solution at a temperature of approximately 35–37°C.

2.1. Oocyte collection and *in vitro* maturation

Cumulus oocyte complexes (COCs) were aspirated from follicles with 2–6 mm diameters and selected COCs with more than two layers of cumulus were washed three times in HEPES-buffered tissue culture medium-199 (HTCM199) containing 10% fetal bovine serum (FBS). After washing, COCs were transferred into micro drops containing TCM-199 supplemented with 10% FBS, 1µg/mL 17β-estradiol, 10 IU/mL PMSG, 10 IU/mL hCG, 100 IU/mL penicillin, 100 µg/mL streptomycin and incubated in maximum humidity with 5% CO2 at 38.5°C for 24 hours(Mohammadi-Sangcheshmeh et al. 2012; Hajarizadeh et al. 2022).

2.2. Nuclear chromatin evaluation

After 24 h following IVM, oocytes were released from the cumulus mass by treating them with 0.1% hyaluronidase (100 IU/ml). Then, oocytes are stained with 2.5 mg/ml Hoechst 33258 in 3:1 (v/v) glycerol/PBS. The meiotic stages of the oocytes are evaluated under an epifluorescence microscope (Nikon, TE-2000 Japan) and each oocyte is classified as GV, germinal vesicle breakdown (GVBD), metaphase I (MI), or MII(Hajarizadeh et al. 2022).

2.3. Measurement of intracellular GSH content

After IVM, a group of COCs is denuded and incubated in Tyrodes medium plus 5 mg/ml polyvinyl alcohol containing 10 µM Cell Tracker blue for 30 min. The oocytes are subsequently washed in PBS, placed into 10 µl droplets, observed under an epifluorescence microscope with UV filters, and then all fluorescent images are recorded as graphic files. Image J software is used for oocyte fluorescence intensities analysis(Ataei-Nazari et al. 2023).

2.4. In vitro fertilization

After IVM, COCs were washed two times in HEPES-buffered synthetic oviductal fluid (HSOF) and 10 COCs were placed in 44-µl drops overlaid with equilibrated mineral oil. The fertilization medium was SOF supplemented with 4IU/ml heparin, PHE (20µM penicillamine,10 µMhypotaurine,1µM epinephrine), and 2% (v/v) estrous sheep serum. Frozen sperm in straws (Nahadehaye Dami Jahed, Tehran, Iran) were thawed and used for in vitro fertilization. To separate the viable sperm for in vitro fertilization, a swim-up method was applied and high-quality sperm was added to COCs at a concentration of 2 × 106 spermatozoa/ml in fertilization drops. Oocytes and sperm were incubated together for approximately 18 h under mineral oil at 39°C, 5% CO2, 5% O2, and 90% N2 in the air with maximum humidity(Rasekhi et al. 2020)

2.5. In vitro culture

Following either activation or in vitro fertilization, presumable zygotes were after 20 h freed from excess sperm and cumulus cells. After three times washing in CR1aa medium, presumable zygotes were cultured

in CR1aa medium supplemented with 10% FBS for 8days at 38.5°C in a humidified incubator with 5% O2, 5% CO2, and 90% N2. The cleavage rate and blastocyst rate were recorded on days 3 and 8 of culture, respectively. The day of activation/fertilization was considered to be day 0(Hajarizadeh et al. 2022).

2.6. RNA extraction, reverse transcription, and real-time PCR

Fifty mature oocytes in each experimental group were separated from the cumulus cells and pooled in three biological replicates for total RNA extraction(Mishra et al. 2017; Hajarizadeh et al. 2022). For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total cellular RNA was extracted using TRI-reagent. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase and random hexamer primers, according to the manufacturer's instructions (Fermentas; St. Leon-Rot, Germany). PCR amplification was performed using a standard procedure with Taq DNA polymerase with denaturation at 94°C for 15s, annealing at 55–60°C for 30s according to the melting temperature of each primer, and extending at 72°C for 45 s. The number of cycles varied between 30 and 40, depending on the abundance of a particular *mRNA*. Real-time PCR reactions of 25 µl were conducted in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia), by adding 12.5 µl2 × SYBR Premix ExTaq, 0.4µM of final concentration for each primer, 2 µl template, and distilled water to reach the volume of 25 µl. Real-time PCR was performed in two steps with the following thermal setting: 3 min at 95°C for initial enzyme activation, followed by 40 amplification cycles (each 5 s at 95°C, and 20 s at 60°C with fluorescence detection) and a final step of melting curve analysis. All samples were analyzed in duplicate, and the average value of the duplicate was used for quantification. The data were normalized to β-actin and 2-ΔΔCt methodology was used for relative quantification(Fang et al. 2016; Suocheng et al. 2017). Expression of β-actin transcript was used as an internal housekeeping gene(Desmarchais et al. 2020; Ataei-Nazari et al. 2023).

2.7. Experimental design

B[b]F was dissolved in DMSO and then diluted with the maturation medium to final concentrations of 0 (Control), 1,2, 5, and 10 µM (Lu et al. 2018; Liu et al. 2015) with DMSO concentration < 1% in the culture medium(Branco et al. 2021; Guo et al. 2020).

2.7.1. Experiment 1. Effect of B[b]F in maturation medium on oocyte meiotic maturation and GSH level

After IVM, COCs of each group were denuded and oocytes were evaluated for the stage of nuclear maturation and intracellular level of GSH. This experiment was performed in five replicates. A total of 110, 110, 106, 108, and 110 oocytes were analyzed for the Control, 1, 2, 5, and 10 µM groups, respectively.

2.7.2. Experiment 2. Effect of B[b]F in maturation medium on ROS level of matured oocytes

To evaluate the effect of B[b]F on ROS level after IVM, cumulus cells were denuded and then assayed as described above. This experiment was repeated five times with roughly 20 to 25 oocytes per replicate per

group. A total of 112, 110, 111, 112, and 111 oocytes were evaluated for the Control, 10, 50, and 100 μM groups, respectively.

2.7.3. Experiment 3. Effect of B[b]F in maturation medium on embryo development after fertilization in vitro

To investigate the effects of B[b]F on embryonic development following IVM, the oocytes were fertilized, and the presumptive zygotes were cultured *in vitro*. Rates of cleavage and blastocyst formation were recorded and compared between the B[b]F-treated and the Control group.

2.7.4. Experiment 4. Effect of B[b]F in maturation medium on the expression of candidate genes

Matured oocytes derived either from the Control, 5 μM (as minimum concentration with the detrimental effect on meiotic maturation obtained from the finding of *Experiment 1*) groups were evaluated for the expression of *GDF9*, *BMP15*, pro-apoptotic (*BAX* and *Caspase3*) and anti-apoptotic (*BCL2*) genes. Three biological replicates, each containing 50 matured oocytes were used for RNA extraction, reverse transcription, and real-time PCR.

2.8. Statistical analyses

All statistical analyses to compare the Control and experimental groups (0, 1, 2, 5, and 100 μM B[b]F) were subjected to ANOVA using the SAS 9.2 software and the GLM procedure. Tukey's multiple comparison test was applied to compare the means of the experimental groups. The average values for each biological replicate were used for statistical analyses and an orthogonal comparison was used to test the increasing level of B[b]F with the level of significance of $P < 0.05$ (GSH level, and ROS level). The logistic regression analysis was performed to determine the association between the dependent variable (oocytes nuclear maturation, cleavage, and blastocyst rates) and independent variables (experimental treatments) using the generalized linear model function (GLM) with binomial family and logit link in the R (3/1/0) statistical software. The strength of the association was estimated by an odds ratio (OR) measure. The relative gene expression level of different genes was analysed by one-way analysis of variance (ANOVA). The results are expressed as the mean ± standard error of the mean (SEM) of at least three independent replicates. A probability of $P < 0.05$ was considered to be significant.

3. Results

3.1. Experiment 1. Effects of B[b]F in the maturation media on the oocyte meiotic competence and GSH level

The percentage of oocytes that reached the MII stage decreased in the 5 and 10 μM group ($P < 0.05$). There was no difference ($P \geq 0.05$) between the control group and oocytes in the 1 and 2 μM group. Accordingly, the odds ratio of MII oocytes decreased with an increasing B[b]F concentration in the maturation medium (Table 2).

Table 1
Primer sequences used for the gene expression assay in ovine oocytes treated with B[b]F.

Gene name	Accession number	Primer sequence	Annealing temperature (°C)	Product size (bp)
GDF9	NM_001142888.2	F: GCGCCAGATGACAGAGC R: CTATGGAAAGGTTCCTGC	60	174
BMP15	NM_001114767.2	F: AAGGCCTCTCAGAGGCTC R: GAGGTGGGAATGAGTTAGG	60	130
BAX	XM_027978593.3	F: TGGAGATGAATTGGACAGTA R: GCCTTGAGCACCAGTT	60	174
Caspase3	XM_060406949.1	F: AACAGTGAGGTAGGAGGAA R: TTCTTGAAGCAGTAATCTAT	60	136
BCL2	XM_012103831.5	F: CAGGAGAAATCAAACA R: GTGTGTGGAGAGCGTCA	60	171
β-actin	NM_001009784.3	F: CGTGACATCAAGGAGAA R: AGGAAGGAAGGCTGGA	60	173
F: Forward				
R: Reverse				

Table 2
Effect of BbF in maturation medium on meiotic status of ovine oocytes after maturation *in vitro*.

Groups	No. Oocyte	Degenerate n (%)	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	MII P value	MII OR
Control	110	2 (1.8)	5 (4.5)	8 (7.3)	7 (6.4) [b]	88 (80) [a]	-	-
1 μM	110	2 (1.8)	5 (4.6)	8 (7.3)	14 (12.9) [b]	80 (73.4) [a]	0.7	0.66
2 μM	106	1 (1)	7 (7.1)	7(7.1)	11(11.1) [b]	73 (73.7) [ab]	0.3	0.55
5 μM	108	5 (5.2)	13(13.4)	15 (15.5)	11 (11.3) [b]	53 (54.6) [bc]	< 0.0001	0.24
10 μM	110	7 (6.9)	11 (10.9)	14 (13.9)	24 (23.8) [a]	45 (44.5) [c]	< 0.0001	0.17
Five replicates were carried out.								
[a, b, c] Values with different superscripts in each column are significantly different (*P 0.05*).								
OR: odds ratio (OR is described as comparison of each treated group, with the control).								

A significant demotion of the GSH content was observed in the 5/10μM treated oocytes when compared to the control group (Fig. 1).

3.2. Experiment 2. The impact of B[b]F in the maturation media on the oocyte ROS level

The intracellular ROS level in *in vitro* matured oocytes treated with different B[b]F concentrations is shown in Fig. 2. Our data revealed that in the 10 μM group level of ROS was highest and the lowest level of ROS was observed in the 1 μM group (Fig. 2). ROS levels in oocytes exposed to 2, 5, and 10 μM groups were significantly higher in comparison to the control or the 1 μM group.

3.3. Experiment 3. The impact of B[b]F effects on the developmental competence of oocytes

Our results show that different concentrations of B[b]F had significant effects on the cleavage rate with an increase in the AFB1 concentration to 10 μM in the maturation medium. According to Table 3, different concentrations of B[b]F reduced ($P < 0.05$) the number of fertilized oocytes that reached the blastocyst stage when compared to the control group. As the concentration of B[b]F in the maturation medium increased to 1,2, 5, and 10 μM, the odds ratio of the blastocysts decreased (Table 3).

Table 3
The effect of B[b]F on the embryonic development of ovine oocytes following in vitro fertilization.

Groups	No. Oocyte	Cleaved n (%)	P value	OR	Blastocyst n (%)	P value	OR
Control	95	88 (92.6) [a]	-	-	33 (34.7) [a]	-	-
1 µM	95	81 (85.2) [ab]	0.5	0.46	28 (29.4) [ab]	0.9	0.78
2 µM	93	55(59.1) [bc]	< 0.0001	0.11	16 (17.2) [bc]	0.05	0.39
5 µM	95	42 (55.7) [cd]	< 0.0001	01	11 (11.5) [cd]	0.002	0.24
10µM	94	30 (31.9) [d]	< 0.0001	0.03	4 (4.25) [d]	0.0001	0.8
Five replicates were carried out.							
a, b, c, d Values with different superscripts in each column are significantly different *(P□0.05).*							
OR: odds ratio (OR is described as a comparison of each treated group with the Control).							

3.4. Experiment 5. Impact of B[b]F on the Expression of Apoptosis-Related Genes and GDF9/BMP15 in MII Stage Oocytes

Our findings indicate that the expression levels of apoptotic genes (Fig. 3), specifically *BAX* and *Caspase3*, were significantly elevated in the treated group (5 µM as minimum concentration with the detrimental effect on meiotic maturation obtained from the finding of *Experiment 1*) compared to the control group ($P < 0.05$).

Conversely, the expression of the anti-apoptotic gene *BCL2* was markedly diminished in the treated group. Additionally, the expression of the candidate genes *GDF9* and *BMP15* during oocyte maturation was significantly lower in the treated group relative to the control group (Fig. 4).

4. Discussion

In contemporary times, the topic of Environmental pollution has garnered significant attention owing to its pervasive qualities and detrimental impacts. Studies have shown that B[b]F as one of the common PAHs with ≤ 2.5 µm (PM 2.5) can be found in various environmental sources polluted air, water, and soil which can lead to carcinogenesis, teratogenesis, and mutagenesis(Ambroz et al. 2016). B[b]F has been related to fertility issues via the ovary is the principal organ of the B[b]F target(Guo et al. 2020). Here we investigated the cytotoxicity of B[b]F involved in ovine oocyte maturation and its competence to reach the blastocyst stage. We have shown that exposure to different concentrations of B[b]F caused an interruption of early embryonic development due to an arrest in the oocyte maturation by excessive ROS levels, leading to alterations in *GDF9* and *BMP15* and apoptotic-related *mRNA* abundances.

The progression of oocyte meiosis is a crucial factor in determining the quality of oocytes. The process of oocyte maturation is intricate and involves arrest at the MII stage until fertilization. Throughout meiosis, oocytes exhibit sensitivity to external harmful substances, leading to impaired nuclear and cytoplasmic maturation, consequently reducing the potential for fertilization and embryonic development(Guo et al. 2020).

Our findings indicate that exposure to B[b]F results in a notable decrease in the number of oocytes progressing to the MII stage, especially in the 5 and 10 μM groups. This aligns with earlier research conducted on porcine oocytes(Miao et al. 2018) and other studies regarding the toxicity of PAHs. Previous investigations have demonstrated that PAHs like BaP and PHE can impact the cytoskeletal structure in mouse oocytes(Wang et al. 2023; Wang et al. 2024). The findings indicated that exposure to PAHs resulted in the disruption of GVBD and the extrusion of polar bodies, implying that PAHs and components had a substantial impact on oocyte maturation. The spindle apparatus is an essential organelle during oocyte meiosis, and abnormalities in spindle formation often lead to the failure of meiotic progression(Wang et al. 2024; Wang et al. 2023). However, there were no significant differences observed between the Control group and the oocytes in the 1and 2 μM group.

GSH, a tripeptide, is the most prevalent nonprotein thiol found within cells, typically existing at concentrations in the millimolar range. GSH plays a variety of crucial roles inside the cell, contributing not only to antioxidant defense mechanisms but also to various metabolic processes(Lim and Luderer 2018). In addition, GSH plays a crucial role in the cytoplasmic maturation of oocytes. This molecule protects mammalian cells from excessive ROS and oxidative stress and is essential for the formation of the male pronucleus during fertilization, which is critical for facilitating proper early embryonic development before implantation(Hajarizadeh et al. 2022; Lim and Luderer 2018). The research indicates that there was a significant decrease in GSH levels in oocytes when exposed to concentrations of 1, 2, 5 and 100 μM of B[b]F This observation is consistent with previous research indicating that the levels of GSH in mature oocytes PAHs were significantly reduced(Lim and Luderer 2018). The developmental competence of oocytes is significantly influenced by GSH levels and the maintenance of cellular redox balance. findings indicate that GSH is essential for mitigating the ovotoxic effects of PAHs, which are critical for preserving the redox environment within the ovary and oocytes.(Malott 2022; Wang et al. 2024)

ROS are oxygen-derived free radicals generated during metabolic activities in all organisms. These molecules function as signaling agents within follicular fluid, playing a crucial role in the regulation of oocyte maturation, meiotic arrest, and the resumption of meiosis(Kala, Shaikh, and Nivsarkar 2017). Reactive ROS play a crucial role in facilitating the resumption of meiosis by activating the maturation-promoting factor and promoting the extrusion of the first polar body(Barros et al. 2019).

In this investigation, consistent with earlier research conducted on mouse [24] and pig [23] oocytes, exposure to B[b]F a membrane of PAHs family resulted in elevated ROS levels in ovine oocytes at concentrations of 2, 5, and 10 μM. These findings align with prior studies indicating that PAHs enhance ROS production during the maturation of oocytes(Wang et al. 2024; Wang et al. 2023; Miao et al. 2018).

The overproduction of ROS can adversely affect cellular function, resulting in oxidative stress. This condition may cause enzyme inactivation, lipid peroxidation, depletion of ATP, and disturbances in mitochondrial function. The oxidative damage incurred can trigger the release of cytochrome C and other pro-apoptotic factors from the mitochondria, ultimately culminating in cellular apoptosis(Schoevers et al. 2016; Zhou et al. 2019).

We propose that the elevated levels of ROS lead to a significant reduction in *BCL2* expression in the groups treated with 5 μM. Furthermore, the expression levels of *BAX* and *Caspase3* were found to be increased in the oocytes treated with 5 μM, aligning with prior findings that associate exposure to B[b]F and other PAHs with an increase in apoptotic genes and a decrease in anti-apoptotic genes in mouse oocytes (Guo et al. 2020; Wang et al. 2023) and mouse Leydig cells(Shi et al. 2023).

GDF9 and *BMP15* are suggested to serve as valuable non-invasive indicators for assessing the developmental potential of oocytes. Within the ovary, these factors are crucial in the processes of folliculogenesis, ovulation (Sirait et al. 2021) and are crucial in the process of follicle maturation by influencing granulosa cell activity (Kathirvel, Soundian, and Kumanan 2013). In addition to their primary functions, *GDF9* and *BMP15* are also critically involved in steroidogenesis, the inhibition of luteinization, and the differentiation of cumulus and granulosa cells. Notably, in most species, the expression of these genes is restricted to the oocyte. Evidence from studies in both humans and sheep indicates that *BMP15* and *GDF9* are secreted by the oocyte during follicular development(Paulini and Melo 2011). This suggests that these two growth factors may interact and operate synergistically on target cells, thereby acting as a cohesive functional complex(de Castro, Cruz, and Leal 2015). This research demonstrated that the expression levels of *GDF9* and *BMP15 mRNA*s during oocyte maturation were notably reduced in ovine oocytes subjected to B[b]F, in comparison to the Control group. This finding is consistent with previous research indicating that a decrease in *GDF9* levels is influenced by B[b]F(Mourikes and Flaws 2021). According to this premise, exposure to varying concentrations of B[b]F (2, 5, and 10 μM) disrupted the development of oocytes, preventing them from progressing to the blastocyst stage in comparison to the Control group. This observation is corroborated by the occurrence of incomplete cleavage, which is attributed to deficiencies in the meiotic process. This observation is corroborated by the association of elevated levels of *GDF9* and *BMP15* with improved quality and enhanced developmental competence of oocytes, which in turn may contribute to the higher blastocyst rates observed in sheep(Singh 2020). Furthermore, this aligns with prior findings indicating that PAHs adversely affect preimplantation embryo development and subsequent embryo quality by impairing oocyte maturation. This impairment occurs through the induction of ROS, oxidative stress, DNA damage, and apoptosis. This result is consistent with existing literature that links exposure to B[b]F(Guo et al. 2020)and other PAHs (Miao et al. 2018; Wang et al. 2023; Wang et al. 2024)with a reduction in mitochondrial membrane potential within oocyte. Additionally, elevated levels of ROS during oocyte maturation impair mitochondrial function and spindle assembly, resulting in diminished oocyte quality and nuclear maturation, which ultimately impacts blastocyst development(Hajarizadeh et al. 2022). While this study provides valuable insights into the reproductive toxicity of B[b]F, several limitations should be acknowledged. First, this research was conducted entirely under in vitro culture conditions, which may not fully replicate the complex

physiological microenvironment of the ovary and the protective maternal systems present in vivo. Second, although we identified alterations in key gene transcripts (such as Caspase3, BAX, BCL2, GDF9, and BMP15), these changes were evaluated solely at the mRNA level; further protein expression analyses (e.g., Western blotting or immunofluorescence) are required to confirm these findings at the translational level. Lastly, while our results demonstrate a strong association between B[b]F exposure, oxidative stress, and impaired development, the precise downstream molecular signaling pathways and receptor-mediated mechanisms warrant further investigation.

5. Conclusion

In conclusion, the findings of this study suggest that in vitro exposure to B[b]F may impair meiotic maturation and compromise subsequent embryonic development in ovine oocytes. These toxic effects are potentially mediated by an altered redox balance, characterized by increased ROS accumulation and a concomitant reduction in intracellular GSH levels, which is associated with the initiation of apoptotic pathways. This hypothesis is supported by the observed transcriptional upregulation of the pro-apoptotic genes Caspase3 and BAX, alongside the downregulation of the anti-apoptotic gene BCL2. Furthermore, the decreased expression of GDF9 and BMP15 transcripts during oocyte maturation suggests a potential mechanism through which B[b]F exposure might negatively affect early preimplantation embryonic development.

Declarations

Data Viability:

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Funding:

This research received no external funding.

Author Contribution

Atieh Hajarizadeh: Conceptualization, Validation, Investigation, Supervision. Marzieh Mehrabadi: Methodology. Iman Rad: Supervision, Writing editing. Nastaran Ansari Noghlebari: Methodology, Writing Orginal Draft. Niloofar MohammadEbrahim: Methodology, Soft Ware. Elaheh Esmaeili: Data curation, Supervision.

Acknowledgement

We would like to thank the members of Stem Cell Technology Research Centre for their helpful discussion. This work has been supported by the Stem Cell Technology Research Centre- University of IRAN.

Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Abdel-Shafy, Hussein I, and Mona SM Mansour. 2016. 'A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation', *Egyptian journal of petroleum*, 25: 107-23.
2. Ambroz, Antonin, Veronika Vlkova, Pavel Rossner Jr, Andrea Rossnerova, Vlasta Svecova, Alena Milcova, Jana Pulkrabova, Jana Hajslova, Milos Veleminsky Jr, and Ivo Solansky. 2016. 'Impact of air pollution on oxidative DNA damage and lipid peroxidation in mothers and their newborns', *International journal of hygiene and environmental health*, 219: 545-56.
3. Ataei-Nazari, Sara, Amin Rahimi, Mohammad Reza Bakhtiarizadeh, Pooya Jahandideh-Golroodbari, Ali Assadi-Alamouti, Atieh Hajarizadeh, Hanieh Haji-Rahimi, Banafsheh Mansouri-Bahrani, Ali Afshar-Bahrabad, and Irfan Ozturk. 2023. 'Alpha-linolenic acid alleviates the detrimental effects of lipopolysaccharide during in vitro ovine oocyte development', *Theriogenology*, 212: 64-72.
4. Barros, Fabiana Dakkach de Almeida, Paulo Roberto Adona, Samuel Guemra, and Bruno Cesar Michelette Damião. 2019. 'Oxidative homeostasis in oocyte competence for in vitro embryo development', *Animal Science Journal*, 90: 1343-49.
5. Branco, Vasco, Beatriz Matos, Carolina Mourato, Mário Diniz, Cristina Carvalho, and Marta Martins. 2021. 'Synthesis of glutathione as a central aspect of PAH toxicity in liver cells: A comparison between phenanthrene, Benzo [b] Fluoranthene and their mixtures', *Ecotoxicology and Environmental Safety*, 208: 111637.
6. de Castro, Fernanda Cavallari, Maria Helena Coelho Cruz, and Claudia Lima Verde Leal. 2015. 'Role of growth differentiation factor 9 and bone morphogenetic protein 15 in ovarian function and their importance in mammalian female fertility—a review', *Asian-Australasian journal of animal sciences*, 29: 1065.
7. Desmarchais, Alice, Ophélie Téteau, Pascal Papillier, Manon Jaubert, Xavier Druart, Aurélien Binet, Virginie Maillard, and Sebastien Elis. 2020. 'Bisphenol S impaired in vitro ovine early developmental oocyte competence', *International journal of molecular sciences*, 21: 1238.
8. Fang, Yi, Xiaosheng Zhang, Jinlong Zhang, Rongzhen Zhong, and Daowei Zhou. 2016. 'Global DNA methylation and related mRNA profiles in sheep oocytes and early embryos derived from pre-pubertal and adult donors', *Animal reproduction science*, 164: 144-51.

9. Geier, Mitra C, Anna C Chlebowski, Lisa Truong, Staci L Massey Simonich, Kim A Anderson, and Robert L Tanguay. 2018. 'Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons', *Archives of toxicology*, 92: 571-86.
10. Guo, Jing, Jiayu Huang, Liqun Zhang, Chong Li, Yinhua Qin, Weiwei Liu, Jingyu Li, and Guoning Huang. 2020. 'Benzo [b] fluoranthene impairs mouse oocyte maturation via inducing the apoptosis', *Frontiers in Pharmacology*, 11: 1226.
11. Hajarizadeh, Atieh, Akram Eidi, Ehsan Arefian, Eva Tvrda, and Abdollah Mohammadi-Sangcheshmeh. 2022. 'Aflatoxin B1 impairs in vitro early developmental competence of ovine oocytes', *Theriogenology*, 183: 53-60.
12. Han, Jun, Qiao-Chu Wang, Cheng-Cheng Zhu, Jun Liu, Yu Zhang, Xiang-Shun Cui, Nam-Hyung Kim, and Shao-Chen Sun. 2016. 'Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation', *Toxicology and applied pharmacology*, 300: 70-76.
13. Kala, Manika, Muhammad Vaseem Shaikh, and Manish Nivsarkar. 2017. 'Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation', *Reproductive medicine and biology*, 16: 28-35.
14. Kala, Manika, Muhammad Vaseem Shaikh, Manish Nivsarkar, and Manish Nivsarkar. 2017. 'Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation', *Reproductive medicine and biology*, 16: 28-35.
15. Kathirvel, Muralidharan, Eswari Soundian, and Vijayarani Kumanan. 2013. 'Differential expression dynamics of Growth differentiation factor9 (GDF9) and Bone morphogenetic factor15 (BMP15) mRNA transcripts during in vitro maturation of buffalo (Bubalus bubalis) cumulus–oocyte complexes', *Springerplus*, 2: 1-6.
16. Lim, Jinhwan, and Ulrike Luderer. 2018. 'Glutathione deficiency sensitizes cultured embryonic mouse ovaries to benzo [a] pyrene-induced germ cell apoptosis', *Toxicology and applied pharmacology*, 352: 38-45.
17. Liu, Jun, Qiao-Chu Wang, Jun Han, Bo Xiong, and Shao-Chen Sun. 2015. 'Aflatoxin B1 is toxic to porcine oocyte maturation', *Mutagenesis*, 30: 527-35.
18. Lu, Yujie, Yue Zhang, Jia-Qian Liu, Peng Zou, Lu Jia, Yong-Teng Su, Yu-Rong Sun, and Shao-Chen Sun. 2018. 'Comparison of the toxic effects of different mycotoxins on porcine and mouse oocyte meiosis', *PeerJ*, 6: e5111.
19. Malott, Kelli F. 2022. *The Roles of Oxidative Stress, Mitochondria, and Lipid Droplets in Glutathione Deficiency and Benzo (a) Pyrene-Induced Germ Cell Death and Reduced Oocyte Developmental Competence* (University of California, Irvine).
20. Miao, Yilong, Changyin Zhou, Qingyun Bai, Zhaokang Cui, Xiayan ShiYang, Yajuan Lu, Mianqun Zhang, Xiaoxin Dai, and Bo Xiong. 2018. 'The protective role of melatonin in porcine oocyte meiotic failure caused by the exposure to benzo (a) pyrene', *Human reproduction*, 33: 116-27.

21. Mishra, Ashish, Ippala Janardhan Reddy, Paluru Subramanyam Parameswara Gupta, and Sukanta Mondal. 2017. 'Expression of apoptotic and antioxidant enzyme genes in sheep oocytes and in vitro produced embryos', *Animal biotechnology*, 28: 18-25.
22. Mohammadi-Sangcheshmeh, Abdollah, Masoud Soleimani, Hamid Deldar, Mohammad Salehi, Sara Soudi, Seyed Mahmoud Hashemi, Karl Schellander, and Michael Hoelker. 2012. 'Prediction of oocyte developmental competence in ovine using glucose-6-phosphate dehydrogenase (G6PDH) activity determined at retrieval time', *Journal of assisted reproduction and genetics*, 29: 153-58.
23. Monte, APO, MES Bezerra, VG Menezes, BB Gouveia, RS Barberino, TLBG Lins, VRP Barros, JMS Santos, NJ Donfack, and MHT Matos. 2021. 'Involvement of phosphorylated Akt and FOXO3a in the effects of growth and differentiation Factor-9 (GDF-9) on inhibition of follicular apoptosis and induction of granulosa cell proliferation after in vitro culture of sheep ovarian tissue', *Reproductive Sciences*, 28: 2174-85.
24. Mourikes, Vasiliki E, and Jodi A Flaws. 2021. 'Reproductive toxicology: effects of chemical mixtures on the ovary', *Reproduction*, 162: F91-F100.
25. Pan, Meng-Hao, Yu-Ke Wu, Bi-Yun Liao, Hui Zhang, Chan Li, Jun-Li Wang, Lin-Lin Hu, and Baohua Ma. 2021. 'Bisphenol A exposure disrupts organelle distribution and functions during mouse oocyte maturation', *Frontiers in Cell and Developmental Biology*, 9: 661155.
26. Paulini, Fernanda, and Eduardo O Melo. 2011. 'The role of oocyte-secreted factors GDF9 and BMP15 in follicular development and oogenesis', *Reproduction in domestic animals*, 46: 354-61.
27. Rasekhi, Mahsa, Abdollah Mohammadi-Sangcheshmeh, Morteza Daliri, Mohammadreza Bakhtiarizadeh, Vahid Shariati, Maryam Rahimi, Atieh Hajarizadeh, Sara Ataei Nazari, Pablo J Ross, and Eva Tvrdá. 2020. 'Transcriptional profile of ovine oocytes matured under lipopolysaccharide treatment in vitro', *Theriogenology*, 157: 70-78.
28. Schoevers, Eric J, Regiane R Santos, Johanna Fink-Gremmels, and Bernard AJ Roelen. 2016. 'Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development', *Reproductive Toxicology*, 65: 159-69.
29. Shi, Chao-feng, Fei Han, Xiao Jiang, Zhonghao Zhang, Yingqing Li, Jiankang Wang, Shengqi Sun, Jin-yi Liu, and Jia Cao. 2023. 'Benzo [b] fluoranthene induces male reproductive toxicity and apoptosis via Akt-Mdm2-p53 signaling axis in mouse Leydig cells: integrating computational toxicology and experimental approaches', *Food and Chemical Toxicology*, 179: 113941.
30. Singh, Aanchal. 2020. 'Effects of BMP15 and GDF9 on Ovine Oocytes in an In Vitro Maturation System', Open Access Te Herenga Waka-Victoria University of Wellington.
31. Sirait, Batara, Budi Wiweko, Ahmad Aulia Jusuf, Dein Iftitah, and R Muharam. 2021. 'Oocyte competence biomarkers associated with oocyte maturation: a review', *Frontiers in cell and developmental biology*, 9: 710292.
32. Sousa, Gabriel, Joana Teixeira, Cristina Delerue-Matos, Bruno Sarmento, Simone Morais, Xianyu Wang, Francisca Rodrigues, and Marta Oliveira. 2022. 'Exposure to PAHs during firefighting activities: A review on skin levels, in vitro/in vivo bioavailability, and health risks', *International Journal of Environmental Research and Public Health*, 19: 12677.

33. Suocheng, Wei, Gong Zhuandi, Sheng Li, Liang Haoqin, Lai Luju, and Deng Yingying. 2017. 'Maturation rates of oocytes and levels of FSHR, LHR and GnRHR of COCs response to FSH concentrations in IVM media for sheep', *Journal of Applied Biomedicine*, 15: 180-86.
34. Wang, Peng-Xia, Si-Le Wu, Jia-Qian Ju, Le Jiao, Yuan-Jing Zou, Kun-Huan Zhang, Shao-Chen Sun, Lin-Lin Hu, and Xi-Bang Zheng. 2024. 'Benzo [a] pyrene exposure disrupts the organelle distribution and function of mouse oocytes', *Ecotoxicology and Environmental Safety*, 281: 116630.
35. Wang, Yi, Si-Hong Li, Shu-Jie Yang, Xiao-Qing Li, Lu Liu, Xiang Ma, Dong Niu, and Xing Duan. 2023. 'Exposure to phenanthrene affects oocyte meiosis by inducing mitochondrial dysfunction and endoplasmic reticulum stress', *Cell Proliferation*, 56: e13335.
36. Zhou, Wenjun, Ying-Jie Niu, Zheng-Wen Nie, Yong-Han Kim, Kyung-Tae Shin, Jing Guo, and Xiang-Shun Cui. 2019. 'Fipronil induces apoptosis and cell cycle arrest in porcine oocytes during in vitro maturation', *Apoptosis*, 24: 718-29.

Figures

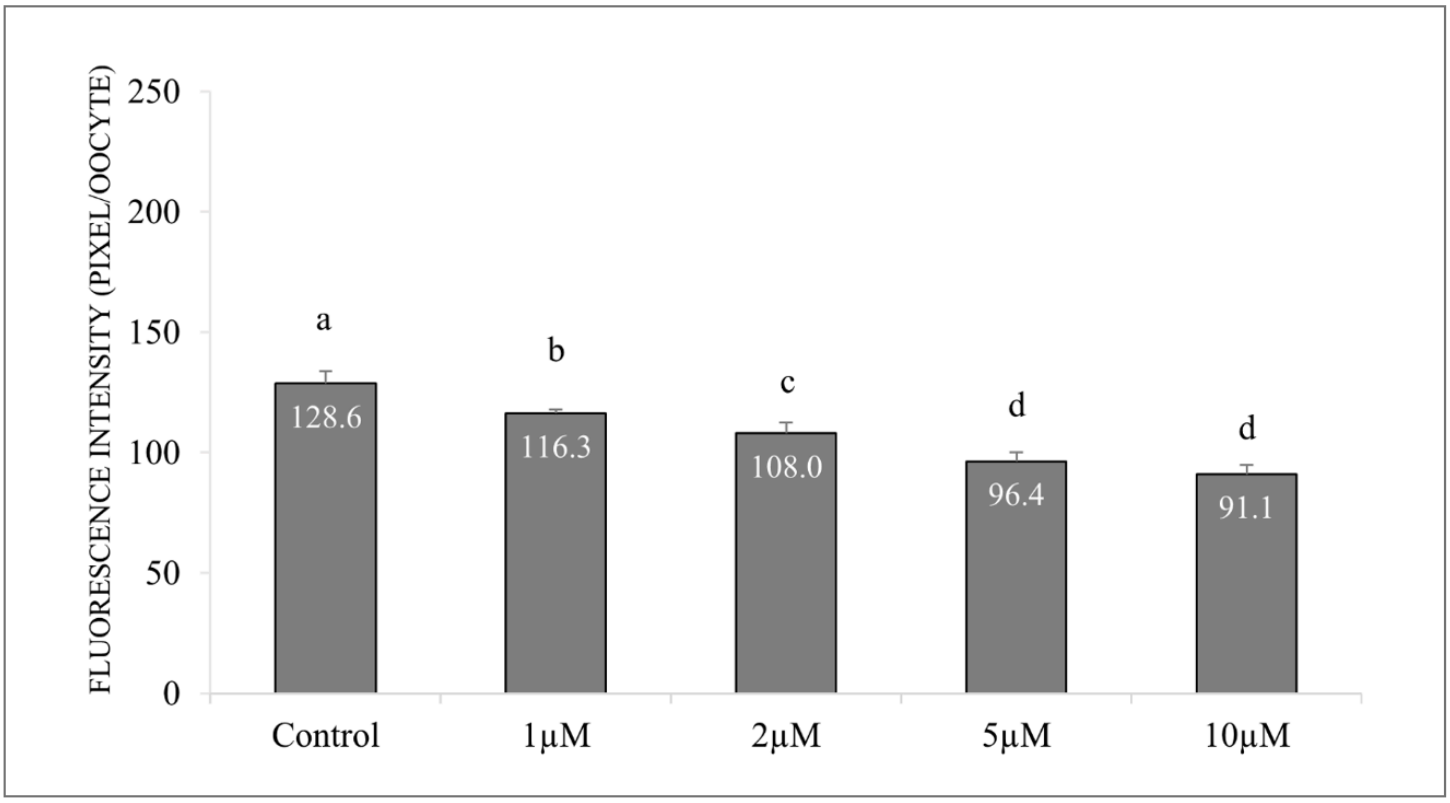


Figure 1

GSH level of ovine oocytes treated with different concentrations of B[b]F during*in vitro* maturation. Bars with uncommon superscripts are different (*P<0.001*).

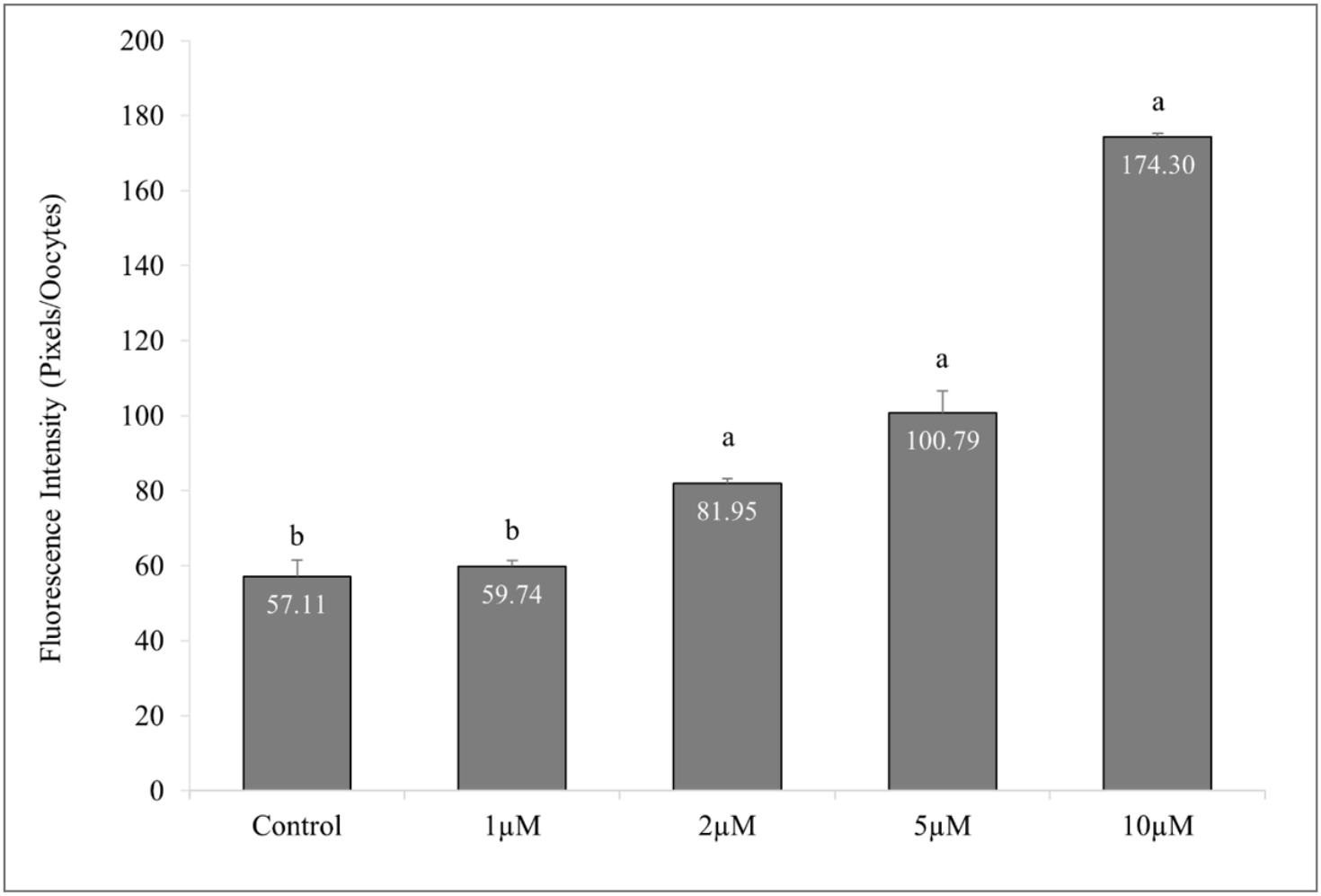


Figure 2

The effect of B[b]F on the intracellular ROS levels in ovine oocytes. Bars with different superscript letters are different (*P<0.001*).

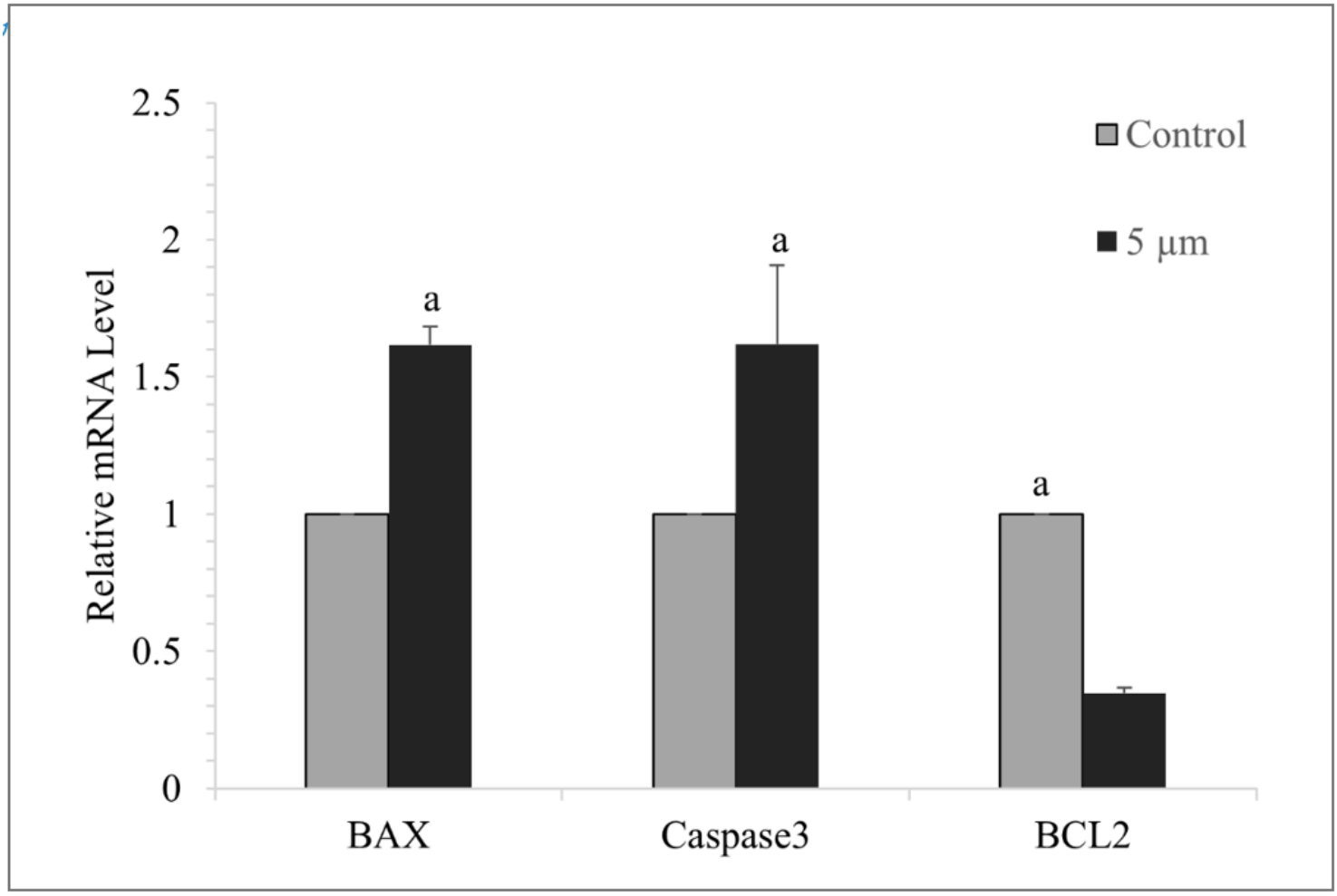


Figure 3

The effect of B[b]F on the relative abundance of *BAX*, *Caspase3* and *BCL2 mRNA*s in matured ovine oocytes. Bars with different superscript letters are different (*P<0.05*).

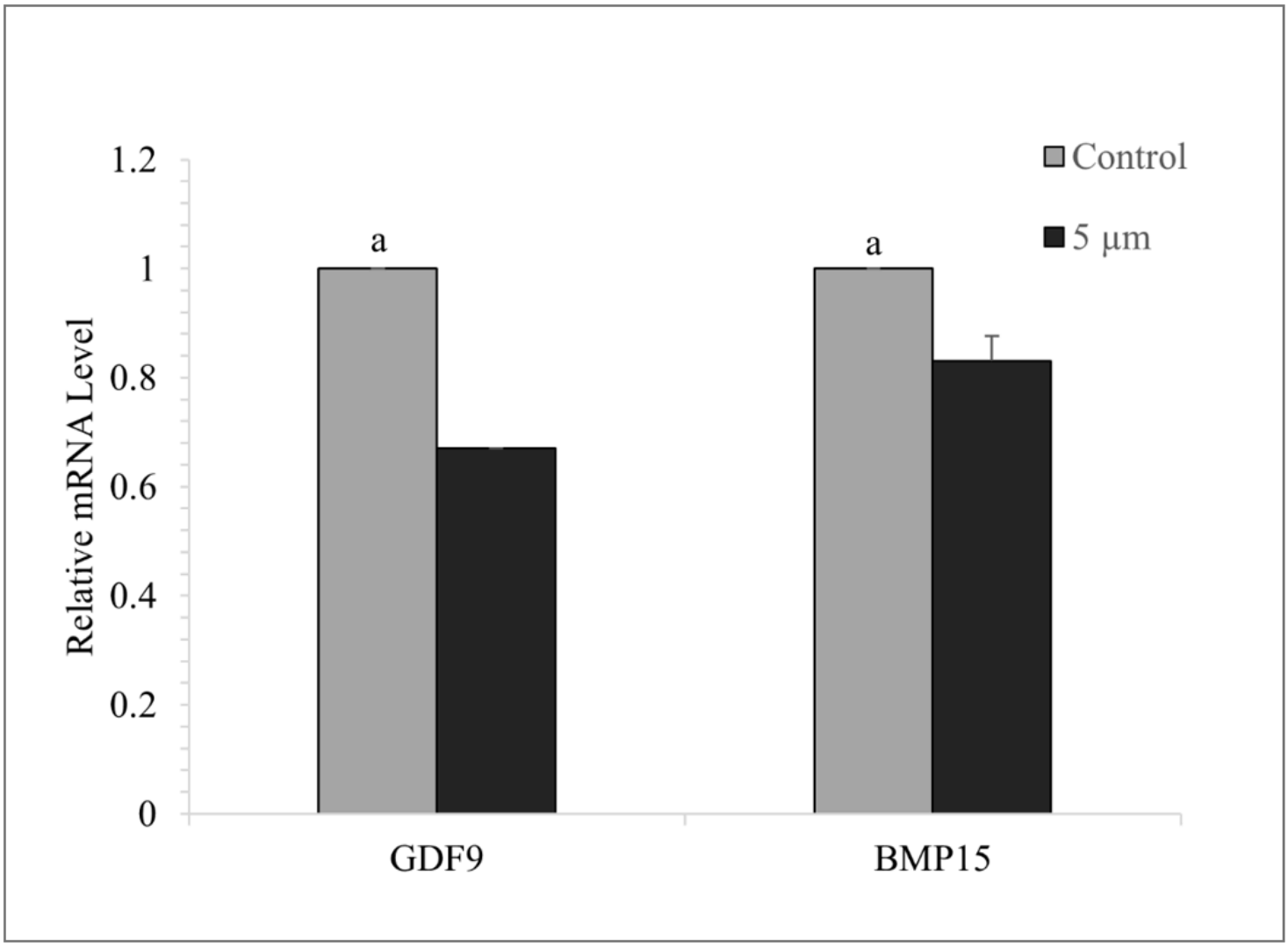


Figure 4

The effect of B[b]F on the relative abundance of *GDF9* and *BMP15 mRNAs*in matured ovine oocytes. Bars with different superscript letters are different (*P<0.05*).