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PROTECTIVE EFFECTS OF VITAMIN E AGAINST BISPHENOL-INDUCED TOXICITY IN PORCINE SPERMATOZOA *IN VITRO*

Nguyen Lam Khanh Duy[1], Cao Thi Tai Nguyen[2], Nguyen Hoang Gia Han[3], Tran Thi Thanh Khuong[1,*]

[1]Stem cell laboratory, Institute of Biotechnology and Food, Can Tho University, Vietnam
[2]Faculty of Basic Sciences, Can Tho University of Medicine and Pharmacy, Can Tho University, Vietnam
[3]Student of course 44, major in Biotechnology, Can Tho University, Vietnam

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ABTRACT

Bisphenol A (BPA) is a toxic compound that negatively impacts reproductive function, particularly affecting male gametes. Vitamin E, a potent antioxidant, is known for its ability to neutralize reactive oxygen species. This study aimed to further elucidate the toxic effects of BPA and the protective effects of vitamin E on sperm quality through in vitro experiments using porcine sperm. In the first experiment, sperm samples were exposed to BPA concentrations of 0 µM, 1 µM, 10 µM, and 100 µM for 1, 2, and 4 hours. In the second experiment, sperm samples were treated with 0 µM or 100 µM BPA, or 100 µM BPA combined with 200 µM vitamin E for 1 hour. Results showed a time- and dose-dependent decline in sperm quality with increasing BPA exposure. Notably, the 100 µM BPA concentration caused the most severe sperm inactivation at all time points. However, the co-treatment of 100 µM BPA with 200 µM vitamin E significantly mitigated BPA's toxic effects. After 1 hour of co-treatment, porcine sperm exhibited improved overall motility, progressive motility, viability, antioxidant capacity, and reduced DNA fragmentation rates, with values of 87.39%, 92.42%, 74.83%, and 11.61%, respectively. These findings demonstrate both the detrimental effects of BPA and the protective role of vitamin E in maintaining sperm quality.

Keywords: Bisphenol A, DNA fragmentation, porcine sperm, reproduction, toxicity, vitamin E.

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[*]*Corresponding author email*: tttkhuong@ctu.edu.vn

INTRODUCTION

Bisphenol A (BPA) is a synthetic chemical extensively used in the production of polycarbonate plastics and epoxy resins, commonly found in everyday items such as food containers, water bottles, and medical devices (Juan-García et al., 2015). Its widespread use has resulted in environmental prevalence, leading to significant human exposure through ingestion, inhalation, and dermal contact (Vandenberg et al., 2013). BPA is recognized as an endocrine disruptor, mimicking estrogen and interfering with hormonal systems, which has been linked to various reproductive health issues, particularly in males (Fenichel et al., 2013). Many researches indicated that BPA exposure significantly impairs sperm quality, reducing motility, viability, and overall sperm count, while also increasing oxidative stress and DNA fragmentation (Meeker et al., 2010; Meeker et al., 2011; Lassen et al., 2014). Animal studies in rodents and pigs demonstrate that chronic BPA exposure can lead to decreased fertility, raising similar concerns for human reproductive health (Salian et al., 2009; Liu et al., 2021; Torres-Badia et al., 2023). Consequently, BPA's impact on male reproductive function has prompted investigations into protective interventions.

Reactive oxygen species (ROS) are highly reactive molecules that can both regulate normal sperm function and cause damage when present in excess (Dinesh et al., 2012). While ROS are involved in essential processes such as sperm capacitation and acrosome reaction, elevated ROS levels lead to oxidative stress, resulting in sperm membrane, protein, and DNA damage (Lamirande et al., 1998). This oxidative damage is detrimental, leading to impaired motility, decreased viability, and increased DNA fragmentation, ultimately compromising fertilization potential and embryonic development (Morielli & O'Flaherty, 2015). BPA exposure is strongly associated with heightened oxidative stress in both animal models and in vitro studies, as it promotes excessive ROS production, overwhelming the sperm's antioxidant defenses (Rahman et al., 2019). This cascade of oxidative damage underscores the importance of interventions to counteract BPA's harmful effects.

Antioxidants are critical for maintaining reproductive health, particularly in mitigating the detrimental effects of ROS and preventing oxidative stress. They play a vital role in preserving sperm quality by scavenging free radicals, stabilizing cell membranes, and protecting spermatozoa from oxidative injury (Santiago et al., 2021). Among antioxidants, vitamin E (α-tocopherol) stands out as a powerful agent for safeguarding male reproductive function. As a lipid-soluble compound, vitamin E effectively protects the lipid-rich membranes of sperm from peroxidation and oxidative degradation (Kurmi et al., 2018). Numerous studies across various species, including rodents, pigs, and humans, have demonstrated vitamin E's protective effects on male gametes, improving sperm motility, viability, and membrane integrity while reducing DNA fragmentation and apoptosis (Rahman et al., 2019; Doostabadi et al., 2021; Marin-Guzman et al., 1997; Sabetian et al., 2021). Due to its effectiveness, vitamin E is frequently explored as a therapeutic agent for enhancing sperm quality, particularly under oxidative stress conditions such as BPA exposure.

Despite the extensive research on BPA's toxic effects on male reproductive health, significant knowledge gaps remain regarding the protective role of vitamin E against BPA-induced toxicity, especially in porcine sperm. The porcine model is particularly advantageous for studying reproductive toxicity due to its physiological and reproductive similarities to humans, including comparable sperm structure and function. The porcine reproductive system is sensitive to environmental toxins like BPA, leading to observable effects on sperm quality and fertility. Additionally, studies involving porcine sperm provide critical insights into the mechanisms of toxicity and the efficacy of protective interventions, making them relevant not only for agricultural contexts but

also for broader implications for human health. Thus, findings from porcine sperm studies can enhance our understanding of BPA toxicity and antioxidant protection, informing potential strategies to safeguard fertility in both livestock and humans.

MATERIALS AND METHODS

Chemicals

The chemicals used in the study included Absolute ethanol (Fisher, USA, 64-17-5), ABTS (Sigma, USA, 30931-67-0), Bisphenol A (shyuanye, China, 80-05-7), Dithiothreitol (AK Scientific, USA, 3483-12-3), LMP Agarose (Thermo Fisher Scientific, USA, 39346-81-1), Nigrosin (Himedia, India, 8005-03-6), NMP Agarose (Thermo Fisher Scientific, USA, 9012-36-6), Vitamin E (Biosharp, China, 10191-41-0).

Animals

Porcine (Duroc, 8 months old) semen samples were sourced from a reputable breeding farm located in Hung Loi Ward, Ninh Kieu District, Can Tho City. The breeding farm was selected to ensure the procurement of semen from healthy pigs, free from infectious diseases and reproductive disorders, thus maintaining a consistent and reliable source for the experiments.

Experiment design

The collected porcine sperm samples were diluted in LVCN medium at a 1:1 ratio. Following dilution, the samples were stored at 5 ^{o}C and transported to the laboratory for further analysis.

In experiment 1, the diluted sperm samples were treated with Bisphenol A (BPA) at concentrations of 0 µM (control), 1 µM, 10 µM, and 100 µM. The samples underwent incubation for three durations: 1 hour, 2 hours, and 4 hours. Subsequent assessments were conducted to evaluate sperm quality.

In experiment 2, the sperm samples were divided into three treatment groups: 0 µM BPA (control), 100 µM BPA, and 100 µM BPA combined with 200 µM vitamin E. All samples were incubated for 1 hour. The quality of the treated sperm was assessed based on four parameters: motility, viability rate, DNA fragmentation, and antioxidant capacity.

Assessment of sperm concentration

Following the loading of 9µL of the sample, the counting chamber was given four minutes to acclimate to ambient temperature. A minimum of 200 intact spermatozoa (with entire heads and tails) were counted in each counting chamber under a 40X magnification microscope. Spermatozoa with heads positioned on the dividing line above and to the left of a square were included in the count, whilst those on the line separating two squares were tallied once to prevent double-counting. The WHO criteria were followed in order to calculate the sperm count (WHO, 2010).

Assessment of sperm motility

Two wet mounts, each with a depth of about 20 µM, were made on a counting chamber for each sample. Spermatozoa motility was assessed by classifying them into three groups: immotility, non-immotility, and progressive motility. A random counting area was chosen, eliminating areas with only motile spermatozoa present, to guarantee an objective evaluation. In every field, an initial assessment was carried out without waiting for spermatozoa to enter the assessment region. In each wet mount, spermatozoa from a minimum of five fields were counted. Two distinct wet mounts underwent two counts, and the outcomes of the two mounts were compared. The average was determined if the rate of sample variation was within an acceptable range (Fumuso et al., 2018).

Assessment of sperm viability

Sperm viability was assessed using the Eosin-Nigrosin method. A volume of 50 µL of the semen sample was mixed with 50 µL of Eosin-Nigrosin solution and allowed to incubate for 30 seconds. Subsequently, the mixture was placed on a glass slide and air-dried. Under a microscope, 100 spermatozoa were examined and categorized. Viability spermatozoa were identified by their white appearance or partial red or dark pink staining

in the neck region, while the remaining head portion remained unstained. In contrast, dead spermatozoa exhibited a reddish or dark pink coloration in the head region. The rate of viability spermatozoa was calculated based on the observed counts (Agha-Rahimi et al., 2014).

Assessment of sperm antioxidant

The ABTS+ decolorization method was used to determine the free radical scavenging activity using the approach of Nenadis et al. (2004). The ABTS+ solution gradually lost its blue color corresponding to the presence of antioxidants. Pipetting 10 µL of the sperm sample into 990 µL of the ABTS+ free radical solution was used to determine the test and incubating in the dark for 6 minutes at room temperature (28–30 ^{o}C). The spectral absorbance (Abs) was measured at 734 nm; the lower the Abs value, the stronger the antioxidant capacity.

Assessment of sperm ability to DNA fragmentation

The sperm Chromatin Dispersion (SCD) method was used for evaluating the fragmentation of sperm DNA (Absalan et al., 2012). This technique allows assessment of DNA fragmentation based on the characteristics of the halo formed by the removed sperm nuclear proteins after acid denaturation. Sperm nuclei with severely damaged DNA form very small or no halo, while sperm with less damaged DNA form large halos.

Statistical analysis

Data analysis was performed using Excel (2016) and the R.4.3.1 program. The results are presented as mean ± standard error (SE). A linear mixed model ANOVA was employed to analyze the data, followed by mean comparisons between treatments using the Turkey method in the R.4.3.1 program. Statistical significance was set at $p < 0.05$, indicating a high level of confidence in the obtained results. The independent variables of the study were BPA concentrations. The dependent variables of the study were sperm quality parameters such as motility, viability, antioxidant and DNA fragmentation.

RESULTS

Fresh sperm quality

The quality parameters of fresh semen are summarized in Table 1.

Figure 1. Assessment of porcine sperm viability using Eosin-nigrosine staining. Live sperm (unstained, white arrow); dead sperm (stained, black arrow). Scale bar = 50µm

Table 1. The quality of fresh semen (mean ± SEM, N = 3)

Criteria	Sample 1	Sample 2	Sample 3
Color	Milky white	Milky white	Milky white
pH	7.33 ± 0.03	7.41 ± 0.02	7.29 ± 0.04
Concentration ($\times 10^6$ cells/mL)	268 ± 14	273 ± 1	257 ± 14
Viability (%)	99 ± 0.34	97 ± 1.48	97 ± 0.70
Overall motility (%)	96 ± 0.40	94 ± 0.21	94 ± 0.55

All samples exhibited normal ranges for concentration, viability, and motility, indicating that the semen was of high quality and suitable for assessing the effects of chemical treatments on sperm function. Figure 1 showed the results of sperm vitality assessment, Figure 2 showed the results of sperm DNA fragmentation assessment.

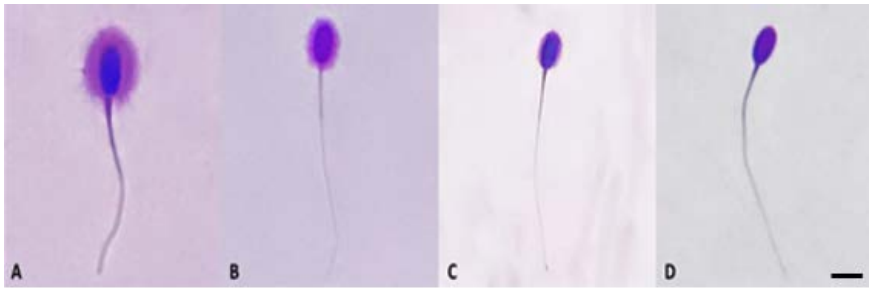

Figure 2. Sperm sample tested for DNA fragmentation. (A) Sperm with large halo, (B) Sperm with medium halo, (C) Sperm with small halo, (D) Sperm without halo. Scale bar = 50 µm

Porcine sperm quality after BPA treatment

The overall motility of porcine sperm treated with BPA is shown in Figure 3. Data showed that BPA concentration and time affected the overall motility rate of porcine sperm. The overall motility rate of porcine sperm decreased with increasing treatment time and increasing BPA concentration. At all three time levels (1 h, 2 h and 4 h), BPA concentration of 100 µM reduced sperm motility to the lowest level (41.65%, 22.65%, 3.98%). The difference was statistically significant when compared with the remaining concentrations ($p < 0.05$).

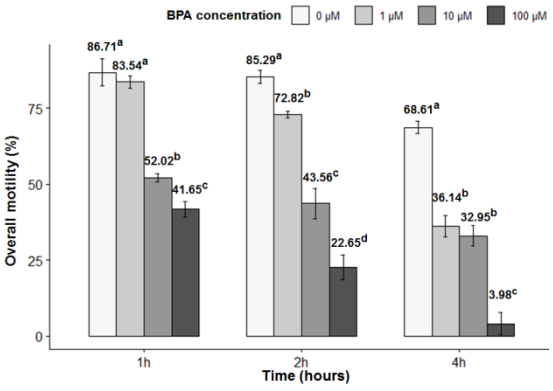

Figure 3. The overall motility of porcine sperm treated with BPA. a,b,c,d Values in each time point with different superscripts are statistically significantly different; $p < 0.05$

Figure 4 depicts the viability of BPA-treated porcine sperm. The viability rate of porcine sperm was found to be influenced by both time and BPA concentration. As treatment duration and BPA concentration increased, so did the viability rate of porcine sperm. BPA at a concentration of 100 µM decreased sperm viability to the lowest level (75.64%, 59.91%, 50.90%) at all three time points (1 h, 2 h and 4 h). When compared to the remaining concentrations, the difference was statistically significant ($p < 0.05$).

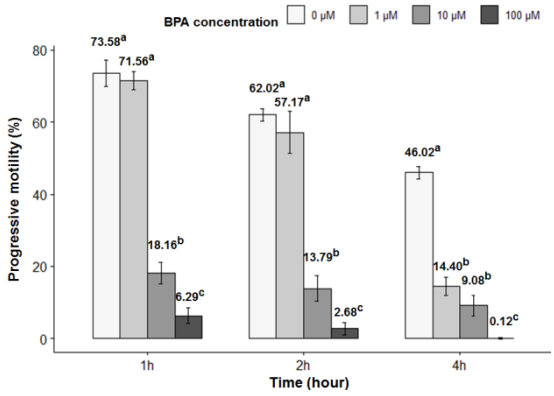

Figure 4. The viability of porcine sperm treated with BPA. a, b, c, d) Values in each time point with different superscripts are statistically significantly different; $p < 0.05$

Figure 5 shows the antioxidant capacity of porcine sperm treated with BPA. Data

revealed that BPA concentration and time influenced the antioxidant capacity rate of porcine sperm. The antioxidant rate of porcine sperm decreased as the treatment time and BPA concentration increased. At all three time intervals (1h, 2h and 4h), BPA concentrations of 100 µM lowered sperm antioxidant capacity to its lowest (23.99%, 17.34%, 7.98%). The difference was statistically significant when compared to the other concentrations ($p < 0.05$).

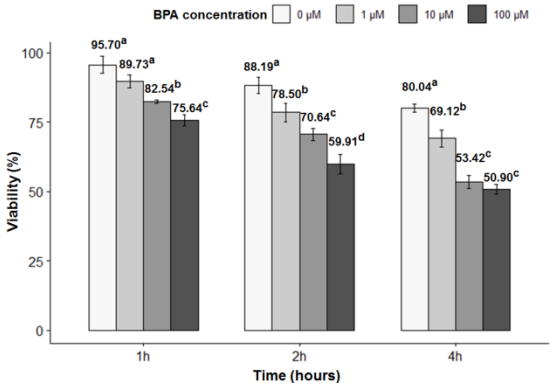

Figure 5. The antioxidant capacity of porcine sperm treated with BPA. a, b, c, d) Values in each time point with different superscripts are statistically significantly different; $p < 0.05$

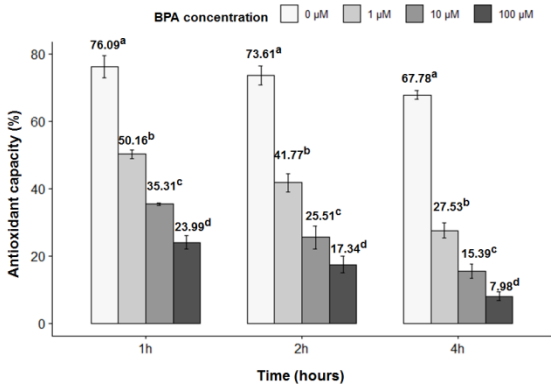

Figure 6. The DNA fragmentation of porcine sperm treated with BPA. a, b, c, d) Values in each time point with different superscripts are statistically significantly different; $p < 0.05$

The results of the evaluation of the DNA fragmentation rate of BPA-treated porcine spermatozoa were recorded and presented in Figure 6. The effects of BPA concentration and time on the DNA fragmentation rate of porcine spermatozoa were recorded. The DNA fragmentation rate of porcine spermatozoa increased as the treatment time and BPA concentration increased. The highest DNA fragmentation rate (28.67%, 54.84%, 75.60%) was recorded in the treatment of 100 µM BPA concentration at all time points (1 h, 2 h, and 4 h). There was a statistically significant difference when compared with other concentrations ($p < 0.05$).

Porcine sperm quality after BPA and vitamin E treatment

The effects of treating porcine sperm with 100 µM Bisphenol A (BPA) in combination with 200 µM vitamin E were illustrated in Figures 7 and 8. The results indicate that the addition of vitamin E effectively mitigated the detrimental effects of BPA on sperm overall motility and viability (Fig. 7). After 1 hour of co-treatment with BPA and vitamin E, the overall motility and viability of porcine sperm were recorded at 87.39% and 92.42%, respectively. Importantly, the differences in sperm overall motility and viability between the samples treated with 100 µM BPA combined with 200 µM vitamin E and the control samples (0 µM BPA) were not statistically significant ($p > 0.05$), suggesting that vitamin E provided significant protection against BPA-induced toxicity.

The findings showed that the negative effects of BPA on sperm antioxidant capacity and DNA fragmentation were significantly reduced by the addition of vitamin E (Fig. 8). After 1 hour of co-treatment with BPA and vitamin E, the antioxidant and DNA fragmentation rate of porcine sperm were measured at 25.14% and 30.49%, respectively. Crucially, there was no statistically significant difference in sperm motility and viability between the samples treated with 100 µM BPA plus 200 µM vitamin E and the control samples (0 µM BPA), indicating that vitamin E significantly protected against the negative effect.

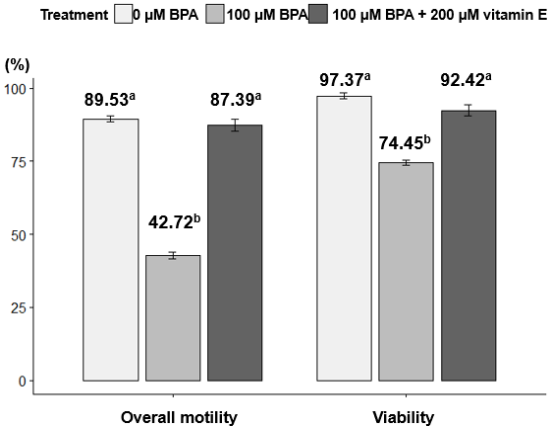

Figure 7. The overall motility and viability of porcine sperm treated with BPA and vitamin E. a, b) Values in each criteria with different superscripts are statistically significantly different; $p < 0.05$

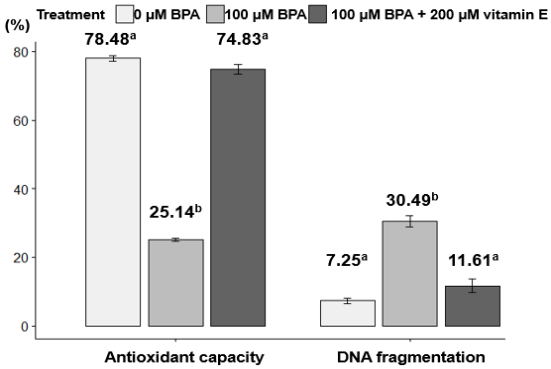

Figure 8. The antioxidant capacity and DNA fragmentation of porcine sperm treated with BPA and vitamin E. a, b) Values in each criteria with different superscripts are statistically significantly different; $p < 0.05$

DISCUSSION

This study aimed to investigate the toxic effects of Bisphenol A on porcine sperm and the protective role of vitamin E in mitigating this damage. Our results showed that BPA exposure significantly impaired sperm motility, viability, antioxidant capacity, and increased DNA fragmentation. However, the co-administration of vitamin E effectively counteracted these negative effects, supporting the hypothesis that antioxidants play a crucial role in preserving sperm function under oxidative stress.

The reduction in sperm motility and viability at BPA concentrations above 1 µM is consistent with previous findings by Rahman et al. (2019), Lukacova et al. (2015), and Yuh et al. (2013). However, Li et al. (2021) observed that even lower BPA concentrations (10^4 µM) caused significant reductions in sperm quality after short exposure times, indicating that the toxicity threshold may vary between species and experimental conditions. This variability may stem from differences in species-specific mechanisms of BPA action, variations in sperm metabolism, or differences in study methodologies. BPA's impact on porcine sperm DNA fragmentation observed in this study aligns with results from other species, such as sterlet and rats, which reported increased DNA fragmentation at BPA concentrations as low as 1.75 µg/L (Hulak et al., 2013) and 100 µg/L (Ullah et al., 2019), respectively. The higher threshold for DNA damage in porcine sperm may be attributed to species differences in sperm structure and metabolism.

Furthermore, this study is one of the first to use the ABTS method to evaluate the antioxidant capacity of boar sperm exposed to BPA. Our results align with previous studies demonstrating that BPA exposure increases intracellular reactive oxygen species, which overwhelm the sperm's natural antioxidant defenses, leading to oxidative damage (Rahman et al., 2019; Lukacova et al., 2015; Ullah et al., 2019). The observed decline in antioxidant capacity supports the hypothesis that BPA-induced oxidative stress is a major factor contributing to sperm dysfunction.

Vitamin E, a potent lipid-soluble antioxidant, significantly improved sperm motility, viability, antioxidant capacity, and DNA integrity when co-administered with BPA. These findings are consistent with Rahman et al. (2019), who demonstrated that vitamin E restored intracellular ATP levels and reduced oxidative stress in BPA-exposed sperm, enhancing spermatogenesis and early embryonic development. Although the

vitamin E concentration in our study (200 µM) was 10 times lower than that used in Rahman's study, it still effectively preserved sperm function, suggesting that vitamin E is highly efficient at mitigating oxidative stress in porcine sperm.

The mechanism by which BPA impairs sperm function likely involves mitochondrial dysfunction, as previously reported (Rahman et al., 2015). BPA-induced ROS production disrupts mitochondrial integrity, reducing ATP synthesis, which is critical for sperm motility (Gadella, 2017). Additionally, BPA enhances premature acrosome reaction, which may lead to membrane rupture, DNA fragmentation, and cell death (Engel et al., 2018). By stabilizing cell membranes and scavenging free radicals, vitamin E effectively prevents these harmful effects, as observed in this study.

The ability of vitamin E to protect sperm from BPA-induced oxidative stress has significant implications for male reproductive health. BPA is a common environmental contaminant, and human exposure to BPA is widespread. The results from this study suggest that antioxidant supplementation, particularly with vitamin E, could be a promising strategy to mitigate the adverse reproductive effects of BPA and other environmental toxins. Moreover, the results of this study, which used porcine sperm, are relevant for human reproductive health due to the physiological similarities between porcine and human sperm.

This study utilized the ABTS and SCD methods to evaluate sperm antioxidant capacity and DNA fragmentation, marking the first application of these techniques in the context of BPA exposure in porcine sperm. These methods are cost-effective and easy to implement, making them suitable for evaluating sperm quality in both livestock and humans. However, the study's limitations, including the small sample size and manual assessment of sperm parameters, may introduce variability in the results. Future research should focus on larger sample sizes and automated, objective assessments to ensure consistent and reliable outcomes.

Additionally, further investigations should explore a wider range of sperm quality parameters, such as mitochondrial activity, membrane integrity, and the specific changes in ROS and antioxidant levels. Such studies would provide a more comprehensive understanding of the mechanisms by which BPA induces oxidative stress and the protective role of antioxidants like vitamin E. Investigating the long-term effects of chronic BPA exposure and vitamin E supplementation would also be valuable in assessing their impact on fertility.

In conclusion, BPA exposure significantly impairs porcine sperm quality by inducing oxidative stress, reducing motility and viability, and increasing DNA fragmentation. Vitamin E co-administration effectively counteracts these toxic effects, highlighting its potential as a therapeutic intervention to preserve sperm function in the presence of environmental toxins. These findings contribute to our understanding of BPA's reproductive toxicity and underscore the importance of antioxidants in protecting male fertility. Further research is needed to elucidate the full extent of vitamin E's protective mechanisms and its applications in reproductive health interventions.

CONCLUSION

BPA at a concentration of 100 µM had the most detrimental impact on porcine sperm quality, significantly reducing motility, viability, and DNA integrity. However, co-treatment with 200 µM vitamin E effectively mitigated these toxic effects, preserving sperm function and antioxidant capacity. These findings suggest that vitamin E offers a protective mechanism against BPA-induced reproductive damage in porcine sperm.

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