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Analysis of quality of frozen thawed bull sperms treated with bisphenol A

K BRINDHA✉[1], M ARTHI[2] and M PARTHIBAN[3]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 051 India

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Bisphenol A (BPA) is one of the largely produced endocrine disruptor chemical (EDC) used in the production of several consumer products including lining of canned foods and personal care products. BPA exhibits both estrogenic and anti-androgenic effects and impairs the normal functioning of homeostasis, and developmental process leading to systemic, neurological, immune and reproductive disorders in human and animals (Belcher *et al.* 2019). Although the major route of exposure to BPA is ingestion of contaminated feed or water, exposure could happen through absorption via skin or inhalation also. With respect to male fertility, BPA interferes with sperm functions through receptors on cell membrane. Thus, BPA-exposed sperms are known to exhibit decreased motility, abnormal acrosome reaction, altered mitochondrial activities, decreased fertilisation and embryo development (Chioccarelli *et al.* 2020). In mouse models, BPA exposure was shown to affect sperm quality in terms of decreased sperm count and semen volume, impaired motility, malformed sperms, oxidative stress and apoptotic induction. These adverse effects vary with species, dosage and exposure period (Siracusa *et al.* 2018). Also, it is documented that BPA induces oxidative stress in sperms by accelerating the accumulation of reactive oxygen species in the rat testis through morphological alterations and induction of apoptosis (Migliaccio *et al.* 2019). However, there is no sufficient data on the effect of BPA on sperm from higher animal species. The present study is conducted to investigate the detrimental effect of BPA on bull sperms as a higher animal model under *in vitro* conditions by exposing frozen semen to different concentrations of BPA for different time periods.

Sperms obtained from frozen bull semen straws were treated with BPA (Sigma, USA). The BPA doses were fixed taking into consideration those employing a variety of species, from lab animals to human as reviewed by Kortenkamp *et al.* (2022). Accordingly, four treatment groups were studied: Group 1 – sperms treated with 10 µM BPA, Group 2 – sperms treated with 25 µM BPA, Group 3 – sperms treated with 50 µM BPA, Group 4 – sperms treated with 100 µM BPA and Control group – sperms with no exposure to BPA. Sperms from each of the four treatment groups (1-4) were analyzed at three time points namely 1 h, 2 h and 3 h post BPA treatment. Short term exposure of sperms to BPA was chosen so as to suit the duration of time that sperms are exposed during collection, processing and preservation, simulating the exposure duration that would occur in a semen station or an andrology lab. Swim-up technique was adopted to retrieve sperms from frozen semen straws as described by Shamsuddin *et al.* (1993) except that incubation was carried out for 1 h, 2 h and 3 h in the presence of BPA. The acrosomal integrity of sperms was assessed following the Giemsa staining protocol of Watson (1975). From every treatment and sub group at different time points, minimum of 300 sperms were counted and number of sperms with intact acrosome was expressed as percentage. The viability of sperms was assessed by differential eosin-nigrosin staining method of Bakst and Long (2014). About 300 sperms per treatment group were observed under oil immersion and number of unstained sperms was expressed as percentage. The functional integrity of bull sperm membrane was determined by hypo-osmotic swelling test (HOST) following the procedure of Correa and Zavos (1994). A total of 100 sperms were evaluated per field with three different fields per treatment and the number of osmotolerant sperms was expressed as percentage.

For successful fertilization to occur, the transfer of paternal genome to the egg is mediated by acrosome reaction. Thus the intactness of sperm acrosome is a major factor influencing fertilization. Our study aimed at investigating whether *in vitro* exposure of sperms to BPA could induce changes in acrosomal integrity of frozen thawed sperms in a dose-dependent and time-dependent manner. Upon visual observation of BPA treated Giemsa stained sperms, acrosomal integrity was scored as intact, altered or completely lost (Fig. 1A). Loss of acrosomal integrity could be due to the fact that BPA interferes with the acrosomal reaction by damaging the acrosomal cap leading to altered mitochondrial activities and decreased fertilization as documented by Rahman *et al.* (2015).

Present address: [1]Madras Veterinary College, Chennai, Tamil Nadu. ✉Corresponding author email: narayananbrindha@gmail.com

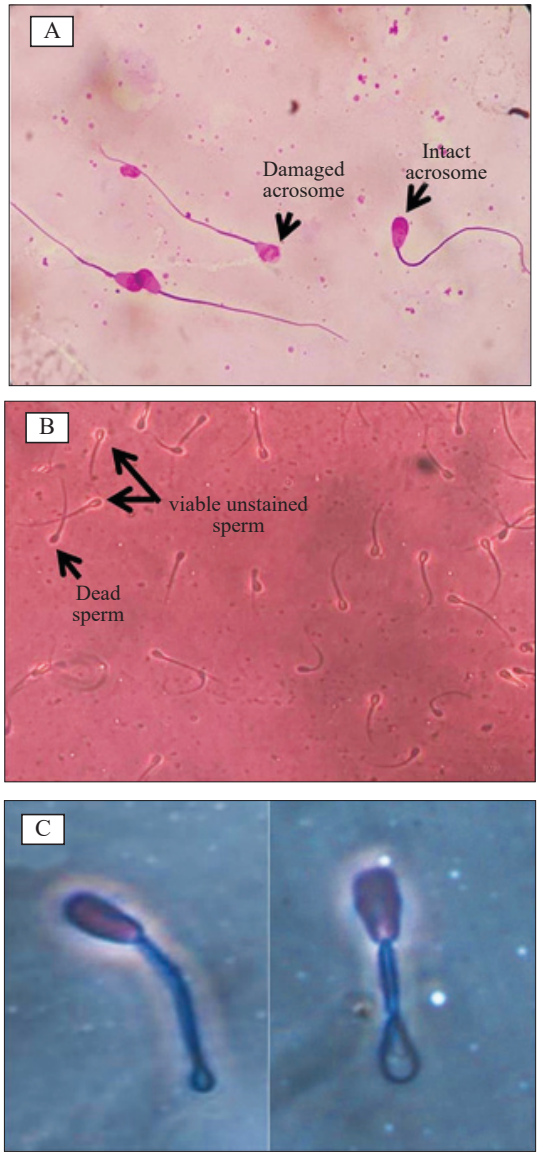

Fig. 1. Sperm quality assessment. A. Giemsa staining for demonstration of acrosomal integrity of sperms; B. Differential staining for demonstration of sperm viability; C. Demonstration of osmotolerance of sperms by hypo osmotic swelling test.

It could be seen from Fig. 2A, that there is a significant difference in the acrosomal integrity between control and treatment groups at any given time point ($p \leq 0.05$). Higher the dose of BPA, greater was the loss of acrosomal integrity. Similar dose-dependent reduction in acrosomal integrity up to 8% and 16% was documented by Wisniewski *et al.* (2015), when rats were exposed post natally to 5 mg and 25 mg of BPA/kg feed. *In vitro* exposure studies in rats by Rahman *et al.* (2015) proved that BPA at 100 µM dose was able to bring about precocious acrosome reaction leading to 30% increase in the number of acrosome reacted sperms. Comparison of the acrosome integrity of BPA treated sperms reveal that there is a significant difference in the number of acrosome reacted sperms at different time points. The loss of acrosomal integrity was maximum at 3 h time point in all the treatment groups analyzed. This could be due to the fact that *in vitro* administration of BPA could cause acceleration of sperm capacitation-associated protein tyrosine phosphorylation by activation of protein kinase A in a time-dependent manner, as previously reported by Wan *et al.* (2016) in rat model.

Sperm viability determined by differential staining technique is presented in Fig. 1B, live sperms remain unstained, whereas dead stained pink. It could be observed that, the mean percentage of viable sperms after exposure to BPA at different concentrations significantly decreased with increasing concentrations of BPA exposure *in vitro* (Fig. 2B). The loss of viability could be attributed to BPA induced imbalance in the production of reactive oxygen species creating an oxidative stress and induction of apoptosis mediated cell death (Migliaccio *et al.* 2019). Also, longer exposure time was found to significantly decrease the viability rate of BPA treated sperms. Interestingly, at 100 µM exposure, the loss of viability of BPA treated sperms was 50% at 1 h which drastically dropped to 25% at the end of 3 h when compared to that of control. The above findings corroborates with the observations of Lukacova *et al.* (2015) with regard to the analysis of viability of BPA treated bovine sperms by MTT cytotoxicity assay. The fact that sperm viability is amenable to BPA exposure even at low doses is worth mentioning. Kocabas *et al.* (2020) recorded a notable reduction of 48%, 42%, 35% and 30% of viable sperms of wild spirlin (*Alburnoides bipunctatus)* when exposed to 0.5, 1.25, 2.5 and 5 µg/l of BPA. It could be observed that even at the lowest concentration of 10 µM BPA, there was a reduction of almost 20% viability at every hour interval, when compared to control. Thus the findings of the present study indicate that the viability of sperms is adversely affected with varying doses and exposure periods.

Osmotic tolerance of sperms reflects the functional integrity of sperm plasma membrane. As illustrated in Fig. 1C, sperms with curled tail were considered to be HOS reacted, while sperms that lost the plasma membrane integrity were considered to be HOS unreactive. Exposure of sperms to varying concentrations of BPA *in vitro* revealed that concentrations of 25 µM and above were able to induce damage to the plasma membrane of sperms, whereas low concentrations of 10 µM did not interfere much with the osmotic tolerance of sperms (Fig. 2C). Higher concentrations of BPA concomitantly with longer exposure periods of 3 h was found to drastically reduce the osmotic tolerance of BPA treated sperms (44.83±3.09%) when compared to control (72±3.65%). The results of our study correlates with the findings of *in vivo* study of Wisniewski *et al.* (2015) wherein rats subjected to oral exposure of BPA at 5 mg per kg body weight showed 2% loss of plasma membrane integrity, accompanied by reduction in mitochondrial activity and increased levels of defective sperms. An earlier report of Alavi *et al.* (2012) support the fact that an intact plasma membrane is required for sperm functionality and that only intact

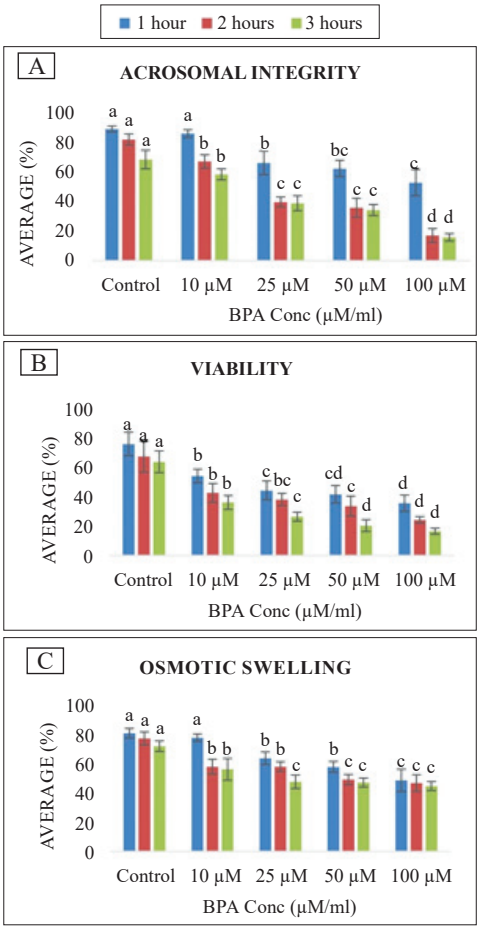

Fig. 2. Effect of cxposurc of bisphenol A on the acrosomal integrity (A), viability (B) and osmotic tolerance (C) of sperms. Significant difference ($p \leq 0.05$) between treatment groups are indicated by different superscripts (a,b,c,d).

sperms could initiate sperm motility in the presence of hypo-osmotic signals. Also, functional integrity of sperms is a prerequisite for capacitation, acrosome reaction and binding of sperms to zona pellucida of the oocyte and hence is a useful indicator of fertilizing ability of sperms. Thus it is evident from our study that *in vitro* exposure of BPA has a significant influence on the sperm membrane integrity in a dose-dependent and time-dependent manner.

SUMMARY

Bisphenol A is a pervasive endocrine disruptor that causes various detrimental health effects on animals and humans. There are several evidences to support that this persistent chemical affects male fertility by interfering with the process of functional maturation of sperms like motility, hyperactivation, capacitation and acrosome reaction in laboratory species. The present study was aimed to evaluate the effect of BPA on the quality of the frozen thawed sperms in higher animal model namely bull. Frozen thawed bull sperms were exposed to four different concentrations of BPA. Aliquots of sperms from each treatment group were examined at three time periods for assessing acrosome integrity, viability and membrane integrity. Exposure of sperms *in vitro* to BPA revealed that there is a significant influence of BPA on treatment groups when compared to control. The loss of acrosomal integrity and viability was higher even at as low a concentration of 10 µM BPA. The reduction in sperm quality was proportional to the increase in exposure period. Further *in vitro* investigation is required to elucidate the mechanism of action of BPA that alters the sperm quality.

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