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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Use of platelet activating factor for inducing bull sperm capacitation *in vitro*: A novel approach

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Abstract

Several chemicals viz., hyamine, Ca^{+2} ionophore, high ionic strength (HIS) media, glycosaminoglycans (GAGs), lysophosphatidyl choline (LPC), lipids, chondroitin sulphate, heparin sulphate, caffiene, oviductal fluid, follicular fluid etc. have been reported to induce *in vitro* capacitation of spermatozoa. Off late, a new potent vasoactive phospholipid named Platelet Activating Factor (PAF) has been reported to have multifold involvement in a wide spectrum of reproductive activities in both the sexes. Though the exact role of PAF on sperm function is not very clear yet it has been observed that it plays a significant role in sperm motility, acrosome reaction and the fertilization process. A special attribute of this source of capacitation is that it requires very little time for acrosome reaction and the motility of spermatozoa is maintained at a reasonable level. The results of work done in our laboratory indicated that PAF treatment of spermatozoa caused a significant increase in acrosome reaction, which is an indirect yardstick of capacitation and the motility decrement was much within the permissible limits. Despite increment, all the sperm abnormalities were pretty much within the permissible limits. The stress indicator enzymes viz. seminal GOT and GPT were not affected with PAF treatment of spermatozoa and the proteolytic enzyme-hyaluronidase had a significant increase. Based on these precepts, PAF can be recommended as an ideal substance to induce capacitation and improve fertilizing ability of spermatozoa for *in vitro* fertilization (IVF).

Keywords: Buffalo; cattle; *in vitro* capacitation; platelet activating factor; sperm

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Introduction

Successful fertilization, irrespective of the site (*in vivo* or *in vitro*) requires maturation and capacitation of spermatozoa. The maturation takes place in two phases of pre- and post-ejaculation, the former occurs in epididymus where human intervention is beyond reach whereas the latter phenomenon takes place in female reproductive tract (when fertilization is *in vivo*) and can be manipulated if the fertilization system is *in vitro*. Capacitation also involves some events like agglutination of spermatozoa at an early stage, vigorous whiplash movements (hyper activation) and finally, disruption of acrosomal cap which involves fusion of outer acrosomal membrane with sperm plasma membrane, called as the acrosome reaction. It is generally presumed that capacitation is immediately followed by the acrosome reaction. Several chemicals viz., hyamine, Ca^{+2} ionophore, high ionic strength (HIS) media, glycosaminoglycans (GAGs), lysophosphatidyl choline (LPC), lipids, chondroitin sulphate, heparin sulphate, caffiene, oviductal fluid, follicular fluid etc. have been reported to induce *in vitro* capacitation of spermatozoa. Off late, a new potent vasoactive phospholipid named Platelet Activating Factor (PAF) has been reported to have multifold involvement in a wide spectrum of reproductive activities in both the sexes. Though the

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exact role of PAF on sperm function is not very clear yet it has been observed that it plays a significant role in sperm motility, acrosome reaction and the fertilization process. A special attribute of this source of capacitation is that it requires very little time for acrosome reaction and the motility of spermatozoa is maintained at a reasonable level.

Role of PAF in male reproductive system

It has been suggested that endogenous PAF plays an essential physiological functions especially in the reproductive system. It was suggested that PAF was present in seminal vesicles and other male reproductive organs of guinea pigs and rats viz. testis, epididymis, vas deferens and prostate (Muguruma et al., 1993). It has also been shown that PAF concentrations in seminal vesicles are influenced by androgens (Muguruma et al., 1993). The sperm content of PAF in investigated species is reported to relate positively to the fertility status of the male (Roudebush and Purnell, 2000).

The PAF binds to the surface receptor and activates a phospholipase, which in turn converts diacylglycerol (DAG) to inositol triphosphate (IP_3), and thus increases the intracellular Ca^{2+} concentration, either by release from intracellular stores or by an extracellular Ca^{2+} influx via Ca^{2+} channels (Reinhardt et al., 1999). This intracellular Ca^{2+} increase may thus be responsible for the induction of sperm capacitation and acrosome reaction. Moreover, changes in the distribution of PAF receptors on spermatozoa surfaces may play a key role in modulating sperm function.

Effect of PAF on sperm maturation

In spite of significant progress made over the last few decades in reproduction, there are some conditions in male infertility that still lack specific treatment, e.g. meiotic defects, sperm maturational arrest and defects in oocyte activation. For years, sperm separation methods have been performed to treat spermatozoa *in vitro* in order to improve and maintain their functional capacity for successful fertilization. Many substances including serum, follicular fluid or other chemically defined pharmacological substances like PAF have been proposed to stimulate sperm functions.

In several species, it has been demonstrated that PAF can influence sperm function by affecting the maturation process, as defects in maturation can lead to infertility (Yang et al., 2003). The presence of many vital enzymes involved in the de-novo synthesis of PAF has found in the rat epididymal spermatozoa (Muguruma and Johnston, 1997). Interestingly, it was concluded by Yang and his colleagues that epididymal PAF concentrations were decreased during the sperm maturation processes and that this may be due to utilization of PAF by maturing spermatozoa (Yang et al., 2003). Their results also suggested that immature spermatozoa have higher concentrations of PAF compared with mature spermatozoa.

Effect of PAF on sperm cryopreservation

Cryopreservation of spermatozoa from infertile males has been complicated by the high loss of post-thaw motility (Hellstrom et al., 1991), hence intra-cellular sperm injection (ICSI) that need only one viable spermatozoa per oocyte has become a valuable alternative after cryopreservation and storage of poor semen samples (Kahraman et al., 1996). Loss of sperm function and impaired fertilizing ability due to the damaged sperm plasma membrane after cryopreservation and thawing is irreversible. This can be partly explained by a reduced percentage of spermatozoa with normal intact acrosomes and diminished acrosine activity. In that direction, PAF has been shown to enhance post-thaw motility of cryopreserved spermatozoa in human (Wang et al., 1993). An improvement in the recovery of motile spermatozoa from unwashed and washed post-thaw samples was observed when sperm samples were cryopreserved in the presence of PAF. Moreover, an investigation by Briton-Jones et al. (2001) on the motion characteristics of poor quality cryopreserved human sperm showed the increase in number of motile sperm on incubating the oligospermic cryopreserved spermatozoa with PAF, which enabled the easier identification of viable spermatozoa for ICSI in samples with severe asthenozoospermia. Based on these results, we can deduce that PAF could be a good cryoprotectant, as incubation with PAF increased the number of motile spermatozoa in both poor and good quality cryopreserved sperms. Furthermore, it could be suggested that the success of less intrusive assisted reproductive techniques, such as IUI and traditional IVF, may be enhanced by PAF-treated cryopreserved sperm.

Effect of PAF on sperm motility

Ability of sperm to move progressively towards an egg is called as the sperm motility, which imply the quality, a crucial factor for successful pregnancies. Two types of physiological motility has been displayed in most of the mammalian spermatozoa viz. the activated motility, as seen in freshly ejaculated sperm, and the hyper-activated motility, as is seen in most spermatozoa recovered from the site of fertilization (Florman et al., 1992). The sperm motility was reported to be a problematic issue when the cattle were crossbred (Geetha et al., 2011) and the rejection rate was as high as 50 %. However, the sperm motility and forward progression appear to be the most important factors in determining sperm function and thus, the fertilization potential (Roudebush and Purnell, 2000).

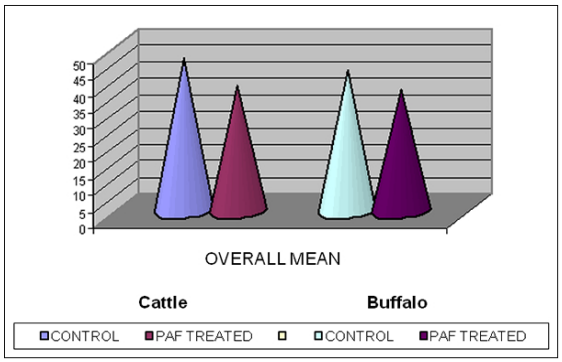

Fig. 1: Effect of PAF on sperm Motility

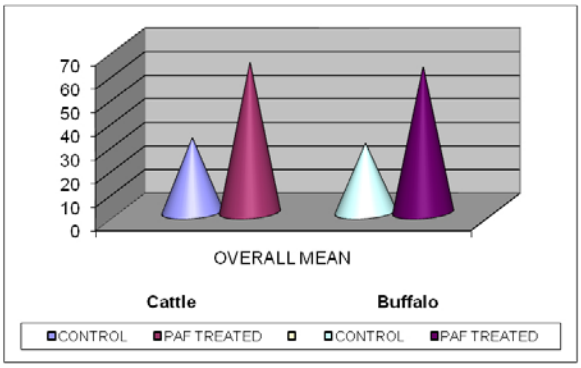

Fig. 2: Effect of PAF on sperm Acrosome Reaction

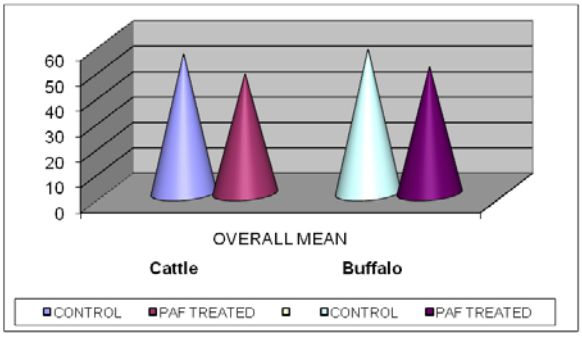

Fig. 3: Effect of PAF on sperm Live Count

One of the potential regulators of sperm motility is PAF. The concentration of PAF in human sperm was originally found to be inversely related to sperm quality (Angle et al., 1991), which was based on the comparison between PAF content of normal and abnormal specimens and they did not process the samples to look at PAF content in different subpopulations (i.e. motile vs. non-motile) of the ejaculates. Also, significant decrease in motility in buffalo bull sperm motility in buffaloes (Kumar and Sharma 2008), which was higher than those reported by Aravindakshan and Sharma (1996) using the same dose of PAF and time of incubation. This overall reduction in motility was much lesser than that caused by the treatment of heparin (13.75%), caffeine (15.67%), and calcium chloride (8.14%) (Ramesha, 1991; Kumar, 1992; Kumar et al., 1994; Kumar and Sharma, 2001). In bulls, PAF treatment has found to be ineffective in enhancing the sperm motility (Bosch et al., 2009). However, significantly higher level of PAF was reported in non-motile sperms than motile sperms (Roudebush and Purnell, 2000) which may perhaps be due to the inability of non-motile sperms to utilize their endogenous PAF because of poor and / or ineffective receptor activities (Roudebush and Purnell, 2001).

In contrast to the above findings, PAF positively affects sperm motility, a positive correlation of PAF content in human sperm (motile population) with seminal parameters and pregnancy rates has been reported (Purnell and Roudebush, 2001; Shilo and Roudebush, 2013). Also, synthetic PAF treatment was reported to increases motility of fresh or frozen–thawed human sperm (Hellstrom et al., 1991; Ricker et al., 1989). In addition, a brief exposure of spermatozoa to exogenous PAF was found to significantly enhance the sperm motility and hyperactivation (Roudebush et al., 2001). The brief exposure of spermatozoa to PAF will significantly improve motion parameters (eg, track speed and lateral head amplitude). Lateral head amplitude is an excellent gauge of hyperactivation, a key indicator of capacitation. This was buttressed by the report that motility in rhesus monkey spermatozoa was significantly greater in high-PAF ($\geq$2 picomoles/10^6 cells) group (31.0 ± 7.6) than low-PAF (<2 picomoles/10^6 cells) group (6.8 ± 2.1) and forward progression was significantly greater in high-PAF ($\geq$2 picomoles/10^6 cells) group (3.0 ± 1.0) than low-PAF (<2 picomoles/10^6 cells) group (0.7 ± 0.3) (Roudebush et al., 2002).

PAF has shown to affect the sperms motility in either way. However, the decrease in motility upon PAF treatment is less detrimental to spermatozoa as compared to other agents commonly used to induce the capacitation. However, several studies have indeed demonstrated increased motility in spermatozoa upon exposure to PAF, which may lead to enhanced fertilization rates and subsequently higher pregnancy rates (Sengoku et al., 1992; Jarvi et al., 1993; Wild and Roudebush, 2001). Since PAF positively affects sperm motility, it can also be used to increase the chances of success in intrauterine insemination (IUI) as recently demonstrated (Wild and Roudebush, 2001; Roudebush et al., 2004; Grigoriou et al., 2005). Addition of PAF to the human spermatozoa has been reviewed to increases sperm motility, sperm penetration of cervical mucus and to enhance the results of sperm penetration assays. Also, exogenous PAF addition has been found to

augment the in-vitro fertilization (IVF) rates in murine and rabbit spermatozoa. Though, PAF is found to improve fertilization rate it has not been found to affect the embryo development detrimentally either in-vitro or in-vivo (Naz and Minhas, 1995). Therefore, PAF is substantial in enhancing the animal production and it can be recommended to use in *in-vitro* fertilization researches.

Effect of PAF on sperm capacitation and acrosome reaction

After the ejaculation the sperm cells go through several vital physiological changes during their time in the female genital tract before they, at the end, are able to penetrate the oocyte plasma membrane. The first change in this cascade is capacitation, a penultimate biochemical step in the maturation of mammalian spermatozoa and is required to render them competent to fertilize an oocyte. Sperm cells accomplish this during the ascension through the female genital tract (in contact with its secretions). It has to do with a physiological maturation process of the sperm cell membranes, which is seen as the precondition for the next step to follow, namely the acrosome reaction. Capacitation step is dispensable for non-mammals and is preceded by acrosome reaction. Sperm capacitation and hyperactivation, so that spermatozoa can penetrate oocytes, are required for the successful fertilization either within the female reproductive tract or under suitable in-vitro conditions.

Sperm capacitation is believed primarily to involve membrane modifications, including changes in lipid composition, surface properties, fluidity, permeability to calcium and lowered concentration of cholesterol in membranes (Davis, 1981). Capacitation involves the destabilization of the acrosomal sperm head membrane allowing greater adhesion of sperm and oocyte. This change is facilitated by the removal of steroids (e.g. cholesterol) and non-covalently bound epididymal/ seminal glycoproteins. The destabilization of acrosomal membrane results an increased permeability to Ca^{2+}. An influx of Ca^{2+} produces increased intracellular cAMP levels and thus, an increase in motility i.e. hyperactivation, a visible consequence of capacitation. This increase in the intracellular calcium levels may be influenced by PAF and hence plays a decisive role in sperm capacitation.

The PAF-acetylhydrolase in the seminal plasma is believed to decapacitate the sperms (Letendre et al., 1992) and its removal during the capacitation process thought to promote PAF synthesis, which in turn would allow the increase in spermatozoa motility and eventually the acrosome reaction and fertilization (Ricker et al., 1989; Hellstrom et al., 1991). This was also supported by the report of Krausz et al. (1994) and Sengoku et al. (1992) who have found the calcium dependent increase in sperm capacitation and acrosome reaction.

Fusion of sperms with the oocyte plasma membrane and then penetration of egg is pivotal for fertilization. Fusing to the egg usually causes little problem, whereas penetrating through the egg's hard shell can present more of a problem to the sperm. Therefore, sperms undergo a process known as acrosome reaction, a reaction that occurs in acrosome of sperms as it approaches the egg. Acrosome reaction (AR) of mammalian spermatozoa is the second process that is essential for fertilization and is a calcium-dependent exocytotic event in the sperm head (Yanagimachi, 1981). This crucial reaction is reported to be actuated *in-vitro* by substances such as progesterone, follicular fluids, calcium ionophore A23187, PAF and others.

PAF has increased the acrosome reaction rate in human spermatozoa incubated with PAF (Angle et al., 1991; Lee et al., 1997). This increase in acrosome reaction and capacitation as well are calcium dependent processes (Sengoku et al., 1992; Krausz et al., 1994). PAF appears to initiate the formation of IP3 and DAG and increasing the intracellular calcium levels once fastened to its receptors on the cell membrane. This elevated calcium signals causes the depolymerization of inter-membrane actin network and activation of phospholipases, leading to an acrosome reaction (Benoff, 1998). Significant increase in the acrosome reaction was reported in buffaloes (Kumar and Sharma, 2008). They also reported the positive associations of group-wise mean AR for treated spermatozoa with fertility status of bulls, which was also observed by Kumar and Sharma (2001) in crossbred cattle. Similar association was observed in increase in AR due to PAF treatment and fertility status of bulls. The overall increase in AR was much higher than those by Kumar (1992) using calcium chloride and heparin supplemented with lysophosphatidyl choline, respectively, and Aravindakshan and Sharma (1996) using same dose (200 mM) of PAF and time of incubation (15 min). Hence, PAF seems to be advantageous in inducing AR in spermatozoa as compared to other agents.

Several studies showed that PAF can induce sperm capacitation (Huo and Yang, 2000) and/or the acrosome reaction AR (Hellstrom et al., 1991; Aravindakshan and Sharma, 1995) and these effects explain, at least partially, the observed beneficial effect of PAF on *in vitro* fertilization (Parks and Hough, 1990). Contrastingly, PAF treatment has found ineffective in enhancing the proportion of either capacitated sperm or acrosome reacted sperms in bulls. PAF inability to promote capacitation and acrosome reactions contrasts with previous studies in which PAF supplementation in the culture media enhanced the capacitation and AR of

bull (Parks and Hough, 1990) and buffalo (Aravindakshan and Sharma, 1995, 1996) spermatozoa. This discrepancy in terms of AR response has been accounted to the differences in PAF formulations. Bosch et al. (2009) has used a synthetic hexadecyl (C16) PAF whereas, Parks and Hough (1990) and Aravindakshan and Sharma (1995, 1996) have studied a natural purified PAF (a mixture of hexadecyl and octadecyl PAF isoforms)

In many species, PAF has been implicated to promote sperm capacitation, acrosomal reaction and prolong sperm viability (Sengoku et al., 1992; Jarvi et al., 1993; Wild and Roudebush, 2001; Kumar and Sharma, 2005). It was noted that PAF improved sper m capacitation and acrosomal reaction without loss of motility (Wild and Roudebush, 2001; Kumar and Sharma, 2005). Moreover, the supplementation of semen with PAF at physiological concentrations may enhance plasma lemma integrity, which is one of the factors facilitating the fertilization process (Odeh et al., 2003). Kordan et al. (2009) and Kordan et al. (2010) observed the effect of PAF on boar and canine spermatozoa also. Ming et al. (2012) studied the effect of PAF in vitro fertilizing ability of spermatozoa fro giant panda.

Effect of PAF on live sperm count

The PAF has reported to significantly drop the live count (LC) in buffalo bulls (Kumar and Sharma, 2008). Additionally, they also reported the group-wise increasing trend of LC with respect to fertility status of bulls which clearly explained the higher LC for highly fertile bulls compared to low fertility bulls, which was also substantiated by Kumar and Sharma (2001) in crossbred cattle. The overall LC in controls was higher than those reported by Kumar et al. (1994) while lower than those by Nath et al. (1991), Kataria and Tuli (1992) and Pramanik (1996).

Effect of PAF on sperm abnormalities

Kumar and Sharma (2008) have reported the significant increase in head abnormalities (HD-ABN) in buffalo spermatozoa due to PAF treatment, ranging from 0.25 to 0.60%. This increased HD-ABN in control and treated spermatozoa was lesser than those reported in buffalo by Pramanik (1996) whereas comparable to crossbred cattle (Kumar and Sharma, 2001). The increase in HD-ABN followed a positive pattern with respect to fertility status of the bulls hence it can be concluded that high fertility bull spermatozoa are more susceptible to undergo abnormity with respect to sperm head.

PAF has found not to follow any specific trend in causing mid piece abnormalities (MP-ABN) however the net change in the same ranged from -15 to 0.30% and the effect of PAF on MP-ABN was non-significant (Kumar and Sharma, 2008). The overall MP-ABN for controls, treated spermatozoa, (net change) averaged 0.93±0.07, 1.00±0.05, (0.07%) respectively, which was lesser than those reported in buffalo by Pramanik (1996) where as comparable to crossbred cattle (Kumar and Sharma, 2001).

Following PAF treatment, tail abnormalities (TL-ABN) inclined to increase significantly in buffalo spermatozoa and the increase is ranged from 0.50 to 1.20% (Kumar and Sharma, 2008). These values were comparable to crossbred cattle (Kumar and Sharma, 2001).

After the PAF treatment, the frequency of total abnormalities (TTL-ABN) in the spermatozoa has increased significantly in buffalo sperms owing to an increase in head and tail abnormalities and the increase is ranged from 0.80 to 1.90% (Kumar and Sharma, 2008). The overall mean TTL-ABN for controls and treated spermatozoa was found lesser than those reported by Nath et al. (1991), Kataria and Tuli (1992) and Pramanik (1996) and these values were comparable to those of crossbred cattle (Kumar and Sharma, 2001). The significant increase in the TTL-ABN had no pragmatical importance as the increased values were much below the limits defined by Blom (1948) and Mickelson and Memon (1993) who have revealed that fertility was impaired only if the abnormal forms exceeded 15%. Hence, it can be hypothesized that PAF has practically no detrimental effect on the morphological well being of spermatozoa.

The PAF has been found to be a wonder molecule of reproductive biology and has been corroborated to improve spermatozoal characteristics. However, the same PAF has found to significantly increase the HD-ABN, TL-ABN, TTL-ABN while decrease in motility and LC though no effect was observed on MP-ABN. Since, the magnitude of increase in AR was considerably high, decrease in motility and LC were quite low and the increase in abnormalities were too low to cause any impairment in fertility, PAF can be recommended as an ideal source to improve the reproductive performance under *in vitro* system. One of the advantages of using PAF is that it is a natural substance and therefore considered as non-toxic.

We also resorted to identify the potentiality of PAF as an agent for inducing *in vitro* capacitation. To accomplish this, we evaluated the effect of PAF treatment of spermatozoa with untreated spermatozoa (controls) for a variety of parameters in crossbred (*Bos taurus x Bos indicus*) cattle and murrah buffalo bulls which could be categorized into three (low, moderate and high) fertility groups. The parameter evaluated were progressive motility, acrosome reaction, live count, abnormalities, hyaluronidase, GOT and GPT enzyme activities.

We observed that the motility of controls (without PAF), PAF treated spermatozoa and net decrease for crossbred cattle and buffalo bulls averaged 46.28 ±

0.78, 38.14 ± 0.86, 8.14 % and 42.89 ± 0.85, 36.65 ± 0.85, 6.24 % respectively. On an average, the acrosome reaction of controls, treated spermatozoa and net increase for crossbred cattle and buffalo bulls were 31.23 ± 0.43, 63.22 ± 0.49, 31.99 % and 28.94 ± 0.46, 61.44 ± 0.58, 32.50 % respectively. The live count in controls, treated spermatozoa and net decrease for crossbred cattle and buffalo bulls averaged 54.78 ± 0.80, 46.85 ± 0.83, 7.93 % and 56.60 ± 0.78, 49.64 ± 0.80, 6.96 % respectively. On an average, the head abnormalities in controls, treated crossbred bull spermatozoa and net increase were 1.35 ± 0.06, 1.80 ± 0.06, 0.45 % and the corresponding values for buffalo bull spermatozoa were 1.45 ± 0.08, 1.89 ± 0.07, 0.44 % respectively. The mid piece abnormalities in controls, treated crossbred cattle spermatozoa and net change averaged 0.86 ± 0.06, 0.93 ± 0.04, 0.07 % and the corresponding values for buffalo spermatozoa were 0.93 ± 0.07, 1.00 ± 0.05, 0.07 % respectively. The average tail abnormalities in controls, treated cattle spermatozoa and net increase were 1.53 ± 0.08, 2.37 ± 0.07, 0.84 % and the corresponding values for buffalo spermatozoa were 1.45 ± 0.09, 2.13 ± 0.07, 0.68 % respectively. The total abnormalities for the same averaged 3.73 ± 0.15, 5.10 ± 0.12, 1.37 % and 3.83 ± 0.17, 5.21 ± 0.14, 1.38 % for crossbred cattle and buffalo bull spermatozoa respectively. The increase in acrosome reaction, head, tail and total abnormalities and decrease in motility and live count, due to PAF treatment was significant, whereas a non significant effect of the same was observed in mid piece abnormalities in both the species.

The GOT activity in controls, PAF treated cattle semen and net change averaged 18.99 ± 1.31, 18.77 ± 1.49, 0.22 units and the corresponding values in buffalo semen averaged 17.62 ± 1.42, 17.78 ± 1.33, 0.16 units, respectively. The corresponding values for GPT activity in cattle and buffalo semen averaged 17.18 ± 1.14, 17.73 ± 0.98, 0.55 and 13.03 ± 1.31, 17.76 ± 1.54, 0.73 units respectively. The hyaluronidase activity in controls, treated semen and net increase averaged 0.045 ± 0.003, 0.115 ± 0.004, 0.070 and 0.045 ± 0.003, 0.124 ± 0.004, 0.079 units in cattle and buffalo semen respectively. The increase in hyaluronidase activity due to PAF treatment was significant whereas non significant effect of the same was observed in GOT and GPT activities in both the species. The ANOVA revealed that with the increase in fertility status, there was a significant improvement in the acrosome reaction (in both the species) and hyaluronidase activity (in both the species).

Conclusion

To conclude, the results of our study indicated that PAF treatment of spermatozoa caused a significant increase in acrosome reaction, which is an indirect yardstick of capacitation and the motility decrement was much within the permissible limits. Despite increment, all the sperm abnormalities were pretty much within the permissible limits The stress indicator enzymes viz. seminal GOT and GPT were not affected with PAF treatment of spermatozoa and the proteolytic enzyme - hyaluronidase had a significant increase. Based on these precepts, PAF can be recommended as an ideal substance to induce capacitation and improve fertilizing ability of spermatozoa for IVF. This further substantiates the theory that PAF is intricately involved in spermatozoa function. Although limited reports have confirmed that exogenous PAF enhances pregnancy outcomes, it may prove to be an effective first-line therapeutic adjuvant in treating male factor infertility.

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