REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 18 (2017)
http://www.veterinaria.org
Article Received: Revised: Accepted:

Effect of Nicotine on Ejaculated Cattle Bull Spermatozoa Acrosome Reaction and Motility—An *in vitro* Study

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Abstract
Nicotine, the addictive component of tobacco, is known to impair male reproductive function in rodents and humans. This *in vitro* study investigates the impact of nicotine on the motility and acrosome reaction of ejaculated cattle bull spermatozoa. Recognising the growing concern over environmental and occupational exposures to nicotine, this research aimed to elucidate its potential reproductive toxicity in livestock. Semen samples were incubated with 0.5 mM and 1.0 mM concentrations of nicotine, and sperm motility and acrosomal integrity were assessed over time. Results revealed a dose- and time-dependent decline in both total and progressive sperm motility, with significant reductions observed after 90 and 120 minutes of exposure.
Furthermore, nicotine exposure led to premature acrosome reaction and structural sperm damage, including head decapitation, rendering spermatozoa non-fertilisable. These findings suggest that nicotine disrupts sperm calcium homeostasis and bioenergetics, likely through competitive inhibition of Ca^{2+}-ATPase activity and the induction of oxidative stress. The study underscores the reproductive hazards posed by nicotine exposure in cattle, with implications for herd fertility and livestock productivity. Future studies should explore in vivo effects, protective interventions, and broader environmental implications.

Keywords: nicotine, bull spermatozoa, motility, acrosome reaction, reproductive toxicology

Introduction

Recent interest in the effects of smoking on male reproduction has come about for two reasons. First, smoking is probably the most prevalent voluntary action that modifies susceptibility to a diverse variety of diseases in a generally deleterious fashion (US Department of Health and Human Services, 2004). Second, the growing realisation that a small but increasing number of environmental and occupational exposures can impair male reproduction. Reproductive problems such as foetal loss have traditionally been associated with women, and reproductive research, including that dealing with tobacco, has centred on women (CDCP(US), 2010). The growing realisation of a paternal component of reproductive impairment suggests that studying men is also appropriate (Stillman *et al*, 1986). Chronic exposure to nicotine, the primary psychoactive component of tobacco, adversely affects male reproductive function across species. Nicotine is rapidly absorbed through mucous membranes and reaches seminal plasma within minutes, achieving concentrations up to 1–3 μM in chronic smokers (Mattison, 1982). In humans and rodents, epidemiological and experimental studies have reported decreased sperm count, motility, and increased morphological defects in smokers compared to non-smokers (Evans *et al*., 1981; Rodriguez, 1982). Nicotine's interaction with nicotinic acetylcholine receptors (nAChRs) on sperm membranes modulates ion fluxes, particularly calcium, critical for motility activation and the acrosome reaction (Nelson, 1978; Stewart & Forrester, 1978). There are also ample experimental data to support the acute and chronic adverse effects of smoking (especially nicotine exposure) on the hypothalamic-pituitary-testicular axis, showing effects on LH, testosterone, adrenal corticotropic hormone (ACTH), growth hormone, thyroid stimulating hormone (TSH), and prolactin concentrations (Wilkins *et al*, 1982; Funabashi *et al*, 2005; Mendelson *et al*, 2005 and Friedman & Friedman,2012). . Whether such impairment in spermatogenesis translates into clinical impairment of fertility remains unclear.
In cattle bulls, fertility is paramount for livestock productivity; however, the impact of environmental toxins, such as nicotine, remains underexplored. Bulls may be exposed to nicotine via second-hand smoke in indoor housing, contaminated feed, or proximity to tobacco-processing facilities. Disruptions in sperm function result in decreased conception rates, economic losses, and welfare concerns. According to Peterson & Freund (1976), pharmacological agents, including tranquillisers, anticholinergics, antihistamines, local anaesthetics, and alpha-and beta-adrenergic blockers, inhibit spermatozoan motility and metabolism. It has been reported that spermatozoa of mammals, such as rams (Stewart & Forrester, 1978), bulls (Sekine, 1951), rabbits (Bishop *et al*., 1976), and humans (Sastry *et al*., 1981), have cholinergic systems comprising acetylcholine, acetylcholinesterase, and choline acetyltransferase. Nelson (1978, 1985) and Stewart & Forrester (1978) observed that spermatozoa contain nicotinic-type acetylcholine receptors. Intracellular calcium binding sites have been demonstrated histochemically in thin sections of bull spermatozoa (Nelson, 1985). Premature AR renders sperm incapable of fertilisation (Yanagimachi & Usui, 1974). Calcium homeostasis in spermatozoa relies on membrane-bound ATPases and ion channels; nicotine-induced oxidative stress further impairs these pathways (Guraya, 1987).
Mammalian spermatozoa undergo the acrosome reaction in the female genital tract as an absolute prerequisite (Bedford, 1983). Because capacitation of guinea pig spermatozoa in vitro does not occur in calcium-deficient medium, but

exocytotic acrosome reaction and motility activation rapidly and synchronously follow the re-addition of calcium, it was inferred that cellular alterations independent of external calcium precede the more visible calcium-dependent events in the capacitation sequence (Yanagimachi & Usui, 1974). These initial alterations were further suggested to have resulted in increased membrane permeability to calcium by the ability of various membrane-directed agents to expedite motility activation and the acrosome reaction (Yanagimachi & Usui, 1974).
The present study has been designed to consolidate the current state of knowledge regarding nicotine-induced changes in ejaculated mammalian spermatozoa, employing appropriate parameters and techniques. The principal criteria for the assessment of sperm quality are sperm motility, acrosome reaction, and morphology.

Materials and Methods
Semen Collection:
Fresh cattle bull semen was collected by interrupted intercourse through the courtesy of the Animal Breeding Complex, National Dairy Research Institute, Karnal. The semen samples, exhibiting +++ motility waves, were diluted (1:1, v/v) in a medium containing sodium citrate, citric acid, egg yolk, benzyl penicillin, and streptomycin. Chilled samples were transported in an ice bath to Chandigarh. The seminal plasma and diluting fluid were separated by centrifuging the liquefied samples at 300xg for 10 minutes. The sperm pellet was washed in 0.2 M phosphate buffered saline (PBS) and used for various experiments.

Drug preparation
Nicotine was purchased from Sigma Chemical Co., St. Louis, MO, USA. A stock solution was prepared by dissolving nicotine (molecular weight 162) in 0.2 M phosphate buffered saline (PBS) to a concentration of 10 mM. Final concentrations of 0.5 mM and 1.0 mM were used in various experiments.

Motility Test
To 1 ml of sperm sample (80 × 10^6 cells), 0.5 mM and *1.0 mM*nicotine were added. Control samples received an equal amount of 0.2 M PBS. Incubation of the samples was carried out at 37°C water bath, and at specified intervals, the number of motile and non-motile spermatozoa were counted under a low-power microscope (400x). The motility patterns were classified according to the WHO Laboratory Manual (2010). The categories used for classifying different patterns of motility have been designated as a, b, c and d and are defined as :
a - if the spermatozoon has a rapid and linear progressive motility;
b - if it has a slow or sluggish linear or non-linear movement;
c -if it has a non-progressive motility;
d - if the spermatozoon is Immotile.

Test for acrosome intactness
The acrosome intactness was studied by modifying the method of Gopalkrishnan *et al* (1992). Gelatin-coated slides were prepared by spreading 40 µL of a warm (50°C) 5% aqueous gelatin solution (5 g in 100 mL distilled water) onto clean glass slides. Slides were kept horizontal for 24 hours at 4°C. The coated slides were then immersed in 0.05% buffered glutaraldehyde solution for 2 minutes, washed twice in double-distilled water, and stored vertically at 4°C.
The collected semen samples were diluted 20 times with PBS-D-glucose solution (pH 7.8) and left to equilibrate at 37°C for 30 minutes. The sperm samples were incubated in minimum capacitation medium (pH 7.4), with or without different concentrations of nicotine, for 30 minutes. The gelatin-coated slides were brought to room temperature, and a drop (10–20 µl) of the sample was gently smeared onto them and left for 5–10 minutes at room temperature. Slides were transferred to a petri dish with moist filter paper, incubated at 37°C for 2 hours, and examined under a phase-contrast microscope (400x).

Statistical Analysis
Results were expressed as mean ± standard deviation (SD). Control and nicotine-treated data (0.5 mM or 1 mM) were compared using Student's t-test (Ipsen & Feigel, 1970).

Results
The present study aimed to investigate the effect of nicotine on sperm motility and the acrosome reaction in bull ejaculated spermatozoa. The various sperm function tests were performed on spermatozoal samples, while the biochemical assays were also conducted on the isolated plasma membrane fraction of cattle bull spermatozoa.

- **Spermatozoal motility:**

Spermatozoal samples were incubated at 37 °C, and the sperm motility was assessed at an interval of 15 min for a total of 120 min. This was referred to as the normal rate of sperm motility (control). The samples incubated with 0.5 mM and *1.0 mM*nicotine were assessed in a manner similar to the others. A drastic effect was observed on sperm motility in cattle bull ejaculated spermatozoa following incubation with different concentrations of nicotine (Fig. 1). Both the pattern and per cent motility were altered after different time intervals of incubation. The pattern of sperm motility changed from a

rapid and linear progression (category A) to slow or sluggish linear movement (category B) with 0.5 mM nicotine after 90 minutes in cattle bull spermatozoal samples. With 1.0 mM nicotine, the pattern of sperm motility changed from a rapid and linear progression (category-a) to slow or sluggish linear movement (category-b) 75 min in bull ejaculated spermatozoa. After 105 min of incubation with 1.0 mM nicotine, the pattern of motility changed from slow or sluggish movement (category-b) to a non-progressive one (category-c), which finally led to sperm inactivation or immobilisation (category-d) after 120 min in bull ejaculated spermatozoa. The percentage of motile spermatozoa decreased significantly ($p < 0.001$) at all the time points in bull spermatozoal samples treated by different concentrations of nicotine as revealed by Student's t-test (Table I).

	Percentage of motile spermatozoa		
	Nicotine (mM)		
Time (min)	**Control**	**0.5**	**1.0**
0	90.76 ± 2.47	-------	-------
15	87.94 ±1.93	86.33 ±1.06	80.52 ± 2.21***
30	86.93 ± 1.80	85.55 ±3.66	75.41 ±0.49*
45	84.55 ± 1.19	83.69 ± 0.65	72.82 ± 2.14**
60	83.92 ±0.74	80.09 ± 0.75**	65.99 ± 3.15**
75	79.18 ± 1.01	73.24 ± 0.57**	58.03 ± 2.60*
90	73.57 ± 3.83	53.11 ± 2.84**	31.16 ± 1.38*
105	69.84 ± 1.39	38.91 ± 1.72*	13.88 ± 3.42*
120	66.35 ± 1.69	21.41 ± 1.64*	0

*p<0.001, **p<0.01, ***p<0.05

Table 1: Effect of in vitro addition of different concentrations of nicotine on spermatozoal motility at different time intervals in ejaculated cattle bull spermatozoa

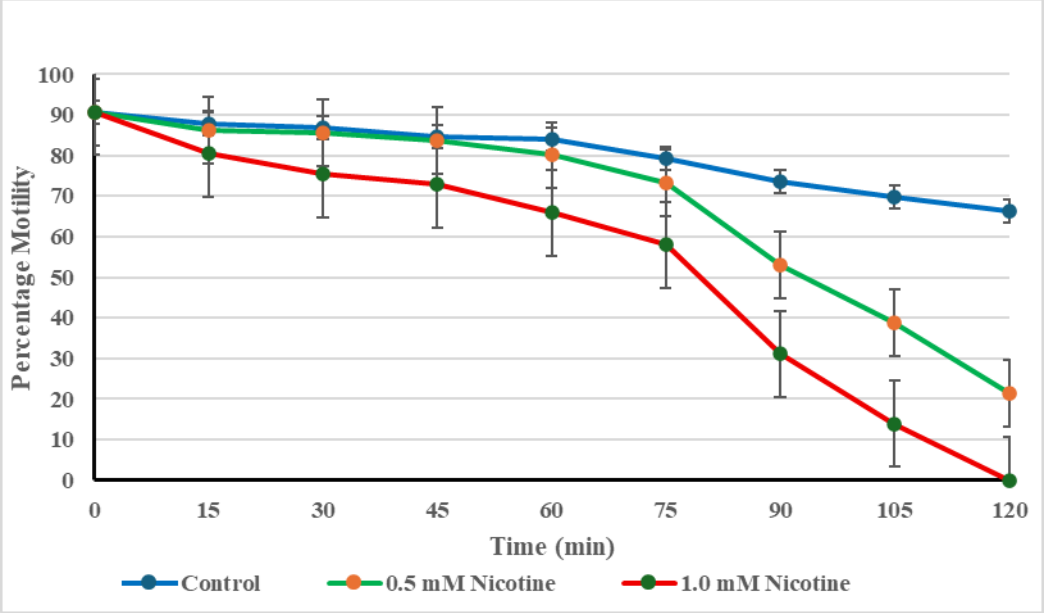
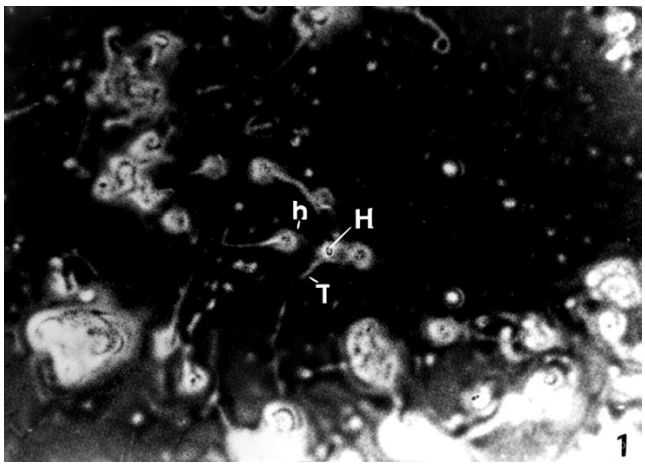


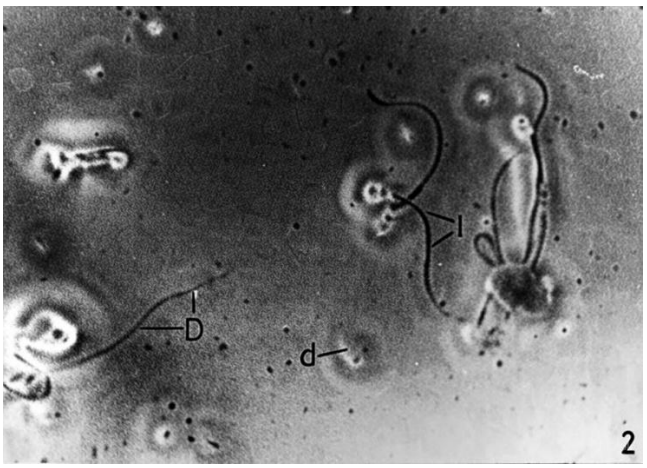
Figure 1: Effect of different concentrations of nicotine on spermatozoal motility at different time intervals in ejaculated cattle bull spermatozoa

Acrosome Reaction

The sperm samples were incubated in the minimum capacitation medium at 37 °C for 30 minutes, in the absence or presence of 0.5 mM or 1.0 mM nicotine. The number of halos formed was counted under a phase contrast microscope (400x). The control samples (Pmg l) were observed to have more than 70% sperm with intact acrosomes (i.e halos with a diameter > 120 pun). Halo formation (diameter > 120 µm) was counted under phase-contrast microscopy (400×). In control samples (Photomicrograph 1), more than 70% of the sperm had intact acrosomes. In nicotine-treated samples (Photomicrographs 2 and 3), more than 30% (0.5 mM) and 50% (1.0 mM) of sperm were decapitated, rendering them non-fertilisable. In these treated samples, more than 90% of acrosomes failed to react. Although many treated sperm retained intact acrosomes, they were non-fertilisable due to the loss of the head.

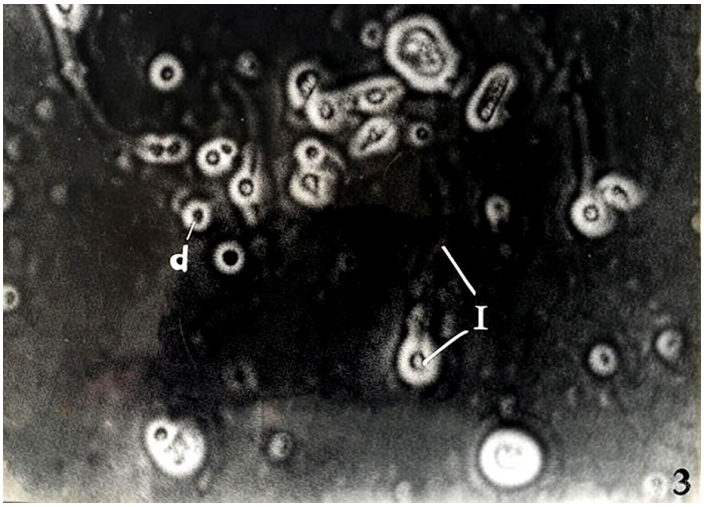
In the 0.5 and 1.0 mM nicotine-treated spermatozoal samples (Photomicrographs 2 and 3), more than 30% and 50% spermatozoa were decapitated as compared to the control, which can thus not be labelled fertilisable. The acrosomes of both the decapitated and standard forms did not undergo an acrosome reaction in over 90% of the treated samples. Although the number of unreacted acrosomes was relatively high in treated samples, the spermatozoa cannot be labelled as fertilisable, since many of them observed were decapitated.





Photomicrograph 1: Normal cattle bull spermatozoal sample showing halo formation around head(acrosome) region after gelatin test, where H=head; T=tail; h=halo

Photomicrograph 2: Cattle bull spermatozoal sample treated with 0.5 mM nicotine showing halo formation around intact and decapitated sperm after gelatin test, where = intact sperm; D decapitated or sperm without head; d=decapitated or sperm without tail



Photomicrograph 3: Cattle bull spermatozoal sample treated with 1.0 mM nicotine showing halo formation around intact and decapitated sperm after gelatin test, where I= intact sperm; D= decapitated or sperm without head; d=decapitated or sperm without tail

Conclusion

This comprehensive *in vitro* investigation demonstrates that nicotine exerts profound, dose- and time-dependent deleterious effects on the function of bull spermatozoa in cattle. By impairing both total and progressive motility and inducing premature acrosome reaction, nicotine may compromise the critical physiological processes required for fertilisation due to its effect on Ca^{2+}-ATPase activity, leading to competitive inhibition and accelerating lipid peroxidation (Kumosani *et al*., 2008). The competitive nature of Ca^{2+}-ATPase inhibition and the significant reduction in activation energy at elevated temperatures reveal mechanistic insights into how nicotine disrupts sperm bioenergetics and calcium homeostasis (Seema *et al*, 2007; Aprioku & Ugwu, 2015).

These findings have significant implications for the reproductive management of cattle bulls. Environmental or occupational nicotine exposure—through second-hand smoke, contaminated feed, or tobacco-processing byproducts—could substantially decrease bull fertility, leading to lower conception rates and economic losses in dairy and beef production systems. Preventive measures, including improved ventilation, feed quality control, and monitoring of environmental toxins, are recommended to safeguard bull reproductive performance.

Limitations of this study include its *in vitro* design and focus on a single species. Future research should validate these effects *in vivo*, examine the potential protective role of antioxidants or calcium modulators, and explore genetic polymorphisms in bulls that may confer susceptibility or resistance to nicotine toxicity. Additionally, investigating the combined effects of nicotine with other tobacco constituents will better simulate real-world exposure scenarios.
In summary, this work highlights the reproductive hazards of nicotine exposure in livestock and provides a mechanistic foundation for targeted mitigation strategies. With growing concerns over environmental contaminants in animal husbandry, integrating reproductive toxicology insights into herd management protocols is essential for maintaining cattle fertility and ensuring sustainable agricultural productivity**.**

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