

The in vitro effect of benzo[a]pyrene on human sperm hyperactivation and acrosome reaction

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Objective: To evaluate the in vitro effect of benzo[a]pyrene on sperm hyperactivation and acrosome status in normozoospermic semen samples of nonsmokers analyzed by computer-assisted semen analysis (CASA).

Design: Experimental in vitro study.

Setting: Andrology laboratory.

Patient(s): Thirteen proven fertile, normozoospermic, and nonsmoking men.

Intervention(s): Spermatozoa were washed free of seminal plasma and were treated with different concentrations of benzo[a]pyrene and compared with controls treated with medium alone. The benzo[a]pyrene concentrations were: 100, 50, 25, and 12.5 $\mu\text{g/mL}$.

Main Outcome Measure(s): Effect of varying concentrations of benzo[a]pyrene on sperm hyperactivation and acrosomal reaction.

Result(s): A statistically significant increase in sperm hyperactivation was observed in presence of benzo[a]pyrene at concentrations of $\geq 50 \mu\text{g/mL}$. The result of the acrosome halo test showed that concentrations of benzo[a]pyrene $\geq 25 \mu\text{g/mL}$ statistically significantly decreased the percentage of halo formation, indicating an inappropriate (false) acrosome reaction.

Conclusion(s): Benzo[a]pyrene statistically significantly affected sperm functional competence as evidenced by increased hyperactivation as well as premature acrosomal reaction. (Fertil Steril® 2010;94:595–8. ©2010 by American Society for Reproductive Medicine.)

Key Words: Benzo[a]pyrene, computer assisted semen analysis, hyperactivation, in vitro, tobacco, acrosome

The effects of tobacco smoking on reproductive function have been widely studied (1–3). It has been suggested that certain components in cigarette smoke interact directly or indirectly with the male or female gametes, affecting their function (4). Cigarette smoking has been found to be significantly related to decreased sperm motility, decreased sperm density, and decreased normal morphology (5, 6). Tobacco combustion yields about 4000 compounds; the major components known to affect semen parameters and sperm function are lead, cadmium, and nicotine. The polycyclic aromatic hydrocarbon benzo[a]pyrene is one of the most harmful components of tobacco, and this component has been a study candidate in the field of reproductive medicine for its effect on testicular steroidogenesis (7), apoptosis (8), and sperm DNA damage (9, 10). These studies on benzo[a]pyrene were performed on its mutagenic and carcinogenic properties.

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However, few studies have ascertained whether this polycyclic aromatic hydrocarbon has an effect on sperm motion characteristics and acrosomal properties. It is worth mentioning here that epidemiologic surveys on smoking have revealed that the seminograms of smokers differ considerably from those of nonsmokers, and that the parameters affected include sperm motility and morphology (5). Because benzo[a]pyrene is an active component of tobacco smoke, its role cannot be discounted. Our investigation is a part of a broad-spectrum study on the effect of tobacco smoke components on the parameters of sperm as ascertained by computer-assisted semen analysis (CASA). One of the important parameters of the CASA-based study is the hyperactivation module whereby one can analyze the percentage of hyperactivated spermatozoa. Mammalian sperm hyperactivation is calcium dependent and is typified by a change in normal motility parameters after successful capacitation. We used CASA to evaluate the in vitro effect of benzo[a]pyrene on sperm hyperactivation and acrosome status in normozoospermic semen samples of nonsmokers.

MATERIALS AND METHODS

Patients

After approval by the University of Calcutta ethics committee, a questionnaire was used to screen prospective donors. Only healthy, proven fertile, nonsmoking men were selected.

Protocol and Sample Preparation

Samples were collected according to World Health Organization protocols (11). The men were instructed to abstain from ejaculation for 2 to 3 days before producing the semen. Each man produced a semen sample by masturbation into a sterile, wide-mouthed, plastic specimen container, and 13 normozoospermic samples were selected from these donors for use in the study. Each sample was allowed to liquefy at 37°C for 20 minutes before the computer-assisted semen analysis (Sperm Class Analyzer, version 3.2.0; Microptic S.L., Barcelona, Spain; Olympus CX41 microscope; Olympus Corporation, Tokyo, Japan). After liquefaction, spermatozoa were washed free of seminal plasma, mixed with media, and a swim-up was performed. Retrieved cells were treated with medium alone or with the different concentrations of benzo[a]pyrene. The CASA was performed at 1 hour after incubation to determine the percentage hyperactivated spermatozoa. Simultaneously, the acrosomal status was monitored by evaluating the level of halo formation by means of the gelatin-based acrosome halo test.

Media and Doses

We used minimal essential media (MEM, GIBCO BRL/Invitrogen, Carlsbad, CA) with HEPES buffer and human serum albumin (HSA) added for this study. Benzo[a]pyrene concentrations were selected by taking into account studies previously described elsewhere (12) and also were based on sperm-survival studies in our laboratory. The benzo[a]pyrene concentrations were 100, 50, 25, and 12.5 µg/mL.

Hyperactivation Assessment by Sperm Class Analyzer

The SCA Motility Module software generates objective seminograms quickly and accurately with a high repeatability factor. The analysis of individual paths and the acquisition of kinetic parameters of sperm motility are extremely reliable and accurate. One of the important features of SCA is that it can display the percentage of hyperactivated spermatozoa in a given sample directly; if needed, the detailed motility parameters of a particular hyperactivated spermatozoon can be analyzed. Thus, there is no need to recalculate hyperactivation from other motility parameters. We took advantage of this facility and recorded the percentage of hyperactivated spermatozoa in samples treated with or without different concentrations of benzo[a]pyrene. The parameters for hyperactivated motility were curvilinear velocity (VCL) $\pm 150 \mu\text{m}/\text{second}$; linearity (LIN) $\leq 50\%$, and maximum amplitude of lateral head displacement (ALH_{max}) $\pm 7.0 \mu\text{m}$.

Assessing the Acrosomal Status of the Benzo[a]Pyrene-treated Sperm

To further investigate whether the hyperactivation status attained by sperm with benzo[a]pyrene treatment indeed induces a false acrosomal reaction, the acrosomal status of the benzo[a]pyrene-treated sperm was monitored by evaluat-

ing the level of halo formation in the gelatin-based acrosome halo test.

Spermatozoa from normal fertile, normozoospermic, non-smoking men that had been washed free of seminal plasma were treated with medium alone (control) and with different concentrations of benzo[a]pyrene. After 1 hour of sample incubation, the acrosome halo test was performed.

Slide test for acrosome intactness For the slide test for acrosome intactness (13), semen is smeared on a gelatin-coated plate. The digestion of gelatin and the formation of a halo around the sperm head depend on the availability and release of acrosomal proteases. The percentage of halo formation provides a good indication of acrosome activity and demonstrates whether an acrosome reaction has already occurred.

Preparation of gelatin slides First, a solution of 5% gelatin in distilled water is prepared at 50°C, then 40 µL of the gelatin solution (warm) is spread on precleaned microscope slides. The gelatin-coated slides are kept horizontal for 24 hours at 4°C after which they are fixed in 0.05% glutaraldehyde in phosphate-buffered saline (PBS) for 2 minutes. The fixed gelatin slides are washed twice in distilled water.

Acrosome halo test procedure Samples were diluted 1:20 with PBS-D-glucose and equilibrated. We gently smeared 20 µL of the diluted sample on the gelatin slide and incubated the slide for 5 to 10 minutes at room temperature to remove excess moisture. The slide was then incubated for 2 hours at 37°C in a Petri dish and covered with wet filter paper. The slides were observed under a phase contrast microscope (Olympus) at $\times 400$ magnification. The spermatozoa with halos were expressed as a percentage of total cells.

Statistical Analysis

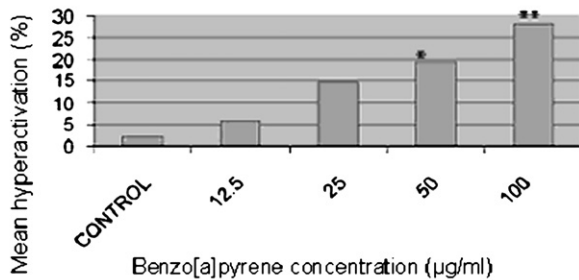
The statistical tools used were GraphPad Prism 4 (GraphPad Software, La Jolla, CA) and Microsoft Excel for Office XP (Microsoft, Redmond, WA). All data except for those obtained after stimulation with 12.5 µg/mL of benzo[a]pyrene were normally distributed, so we performed the nonparametric analysis of variance (ANOVA; Kruskal-Wallis test) with Dunn's multiple comparisons post hoc test. For the acrosome status data, Student's *t*-test was performed.

RESULTS

The mean of the percentage of hyperactivation in response to the different concentrations of benzo[a]pyrene was statistically significantly different; the *H* value of the Kruskal-Wallis test for the reported data was highly statistically significant at $P=.0052$ (Fig. 1). Dunn's multiple comparisons post hoc test showed that spermatozoa stimulated with concentrations of 50 µg/mL ($19.47 \pm 12.87\%$, $P<.05$) and 100 µg/mL ($28.40 \pm 22.42\%$, $P<.001$) of benzo[a]pyrene, respectively, were statistically significantly different from those of the control ($2.12 \pm 0.66\%$). Figure 2A depicts the typical motion characteristics of a normal sperm cell as

FIGURE 1

Graphical representation of the effect of different concentrations of benzo[a]pyrene on human sperm hyperactivation.



p Value for Kruskal-Wallis Test = 0.0052, considered very significant.

* $p < 0.05$ vs. control

** $p < 0.01$ vs. control

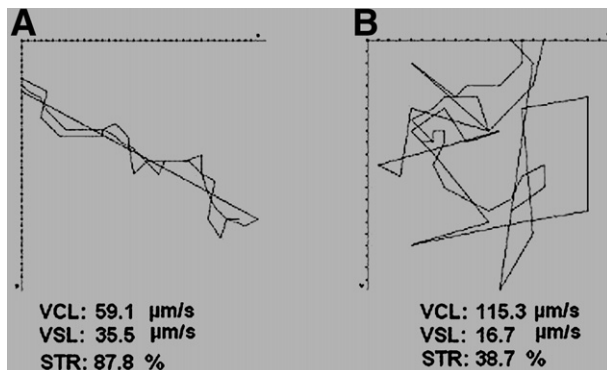
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observed by CASA; the tracks in Figure 2B show a typical hyperactivated sperm cell.

Figure 3 is a visual representation of typical halo formation of an acrosome-intact (see Fig. 3A) and acrosome-reacted (see Fig. 3B) spermatozoa as observed by phase contrast microscopy. The results of the acrosome halo test (Fig. 4) showed that concentrations of 25 µg/mL ($48 \pm 0.095\%$, $P < .05$), 50 µg/mL ($32 \pm 0.117\%$, $P < .05$), and 100 µg/mL ($14 \pm 0.093\%$, $P < .05$) of benzo[a]pyrene statistically signif-

FIGURE 2

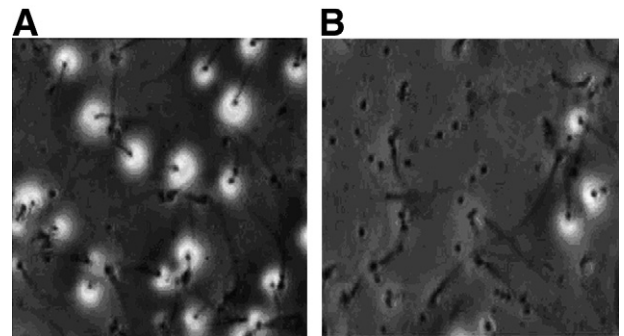
Graphical comparison of motion characteristics of a (A) normal spermatozoon and (B) hyperactivated spermatozoon (after benzo[a]pyrene treatment) as captured from the Sperm Class Analyser (CASA) along with their respective velocity parameters.



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FIGURE 3

Microscope image capture of (A) acrosome intact (able to produce halos) and (B) acrosome reacted (mostly with no halo formation) spermatozoa after benzo[a]pyrene treatment.



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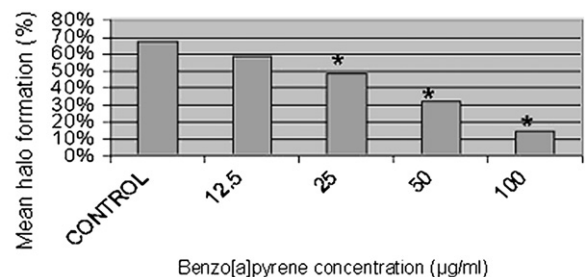
icantly decreased the percentage of mean halo formation when compared with the control ($67 \pm 0.087\%$).

DISCUSSION

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon, a potent carcinogen and mutagen. Apart from its other environmental sources such as automobile exhausts and coal tar, it is an active component of tobacco smoke. A person who smokes a pack of 20 tobacco cigarettes per day is expected to inhale anything from 0.067 µg to 0.568 µg of benzo[a]pyrene per day (14). Previous studies of nicotine, another important component of tobacco smoke, have yielded contradictory results with respect to its effect on sperm motility (6, 15, 16). Although benzo[a]pyrene has been studied with respect to sperm DNA adducts (9, 10) and apoptosis

FIGURE 4

Graph showing effect of benzo[a]pyrene on the degree of halo formation in the acrosome reaction test.



* $p < 0.05$ vs. control

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(8), few studies have evaluated its possible effect on sperm motion characteristics. A study of adult male F-344 rats showed that subchronic exposure to inhaled benzo[a]pyrene contributed to reduced testicular and epididymal function (17). Because the motion characteristics, hyperactivation status, and acrosomal reaction of spermatozoa indicate functional status and fertilizing potential, it is worthwhile to note whether benzo[a]pyrene has any effect on these parameters.

In a previous study by our group (unpublished data), we noted that heavy smokers (more than 20 tobacco cigarettes per day) showed an increased percentage of hyperactivated spermatozoa compared with nonsmokers. This finding prompted us to specifically focus on the effect in vitro of benzo[a]pyrene on sperm hyperactivation. Sperm hyperactivated motility is characterized by high flagellar bend amplitude and asymmetrical beating, which are detected by computer-assisted sperm motility analysis as increased curvilinear velocity (18) and lateral head movement (see Fig. 2). Normally, hyperactivation is required for sperm penetration of the oocyte zona pellucida during fertilization and is induced by an increase in flagellar Ca^{2+} influx. Our study has proved that escalating hypermotility of spermatozoa occurs with increasing concentrations of benzo[a]pyrene (an active component of tobacco) as a result of premature capacitation. For further substantiation, the capacitation status of the benzo[a]pyrene-treated sperm was monitored by evaluating the level of halo formation in the gelatin-based acrosome halo test, which showed that concentrations of benzo[a]pyrene $\geq 25 \mu\text{g/mL}$ can statistically significantly decrease the percentage of halo formation (see Figs. 3 and 4). Thus, the hyperactivation status attained due to benzo[a]pyrene treatment induces false acrosomal reactions in the spermatozoa.

Previous studies have shown that polycyclic aromatic hydrocarbons can alter intracellular Ca^{2+} homeostasis (19). The Ca^{2+} elevation is coupled with increased tyrosine phosphorylation, and it has also been shown that benzo[a]pyrene and especially its metabolites can increase intracellular Ca^{2+} with an increase in tyrosine phosphorylation activity (20). It was shown that a P450-induced BaP metabolite is responsible for Ca^{2+} elevation in human B cells. It is possible that such a mechanism is also applicable to sperm cells exposed to benzo[a]pyrene. Thus, the increase in the flagellar Ca^{2+} , which is noted during sperm hyperactivation, may be induced by the action of benzo[a]pyrene which ultimately leads to a false acrosomal reaction. Thus, these hyperactivated sperms exhaust their acrosomal enzymes and can never attain fertilizing potential.

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