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# Effects of cotinine on sperm motility, membrane function, and fertilizing capacity in vitro

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**Abstract** We evaluated the effect of cotinine on sperm fertilizing capacity in vitro. Human spermatozoa were washed and re-suspended in medium containing albumin and various concentrations of cotinine (0, 100, 200, 400, or 800 ng/ml). After an 8-h incubation period, sperm motility, hypoosmotic swelling test (HOST) outcome, and the percentage of hyperactivated spermatozoa were assayed. Aliquots of spermatozoa were then processed for the zona-free hamster oocyte sperm penetration assay (SPA) or hamster ooplasmic injections. Spermatozoa exposed to concentrations of cotinine equal to 400 or 800 ng/ml demonstrated significantly smaller outcomes for all of the above with the exception of after hamster ooplasmic injections, where high cotinine concentrations did not affect sperm viability or sperm capacity to undergo decondensation and activate hamster oocytes. It appears that cotinine concentrations of 400 or 800 ng/ml exert a detrimental effect on sperm motility, membrane function, and the ability to undergo capacitation. In addition, the current findings suggest that smokers with a high seminal plasma cotinine concentration who participate in assisted reproduction programs may be treated with intracytoplasmic sperm injections (ICSI) rather than conventional in vitro fertilization (IVF) trials.

**Key words** Smoking · Cotinine · Sperm · Fertilization · Oocyte activation

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## Introduction

Infertility is both a private and a social problem. Male infertility is responsible for at least 40–50% of all infertile couples. Toxic agents and environmental factors have been implicated in the etiology of male infertility. Several reports suggest that smoking may contribute to the impaired reproductive potential of a subpopulation of males [5–8, 13, 16]. Most studies evaluating the effect of smoking on the capacity of men to reproduce use one of two methods. The most direct method is to appreciate and compare the man's ability to achieve pregnancy in relation to the smoking habits or to the serum concentrations of nicotine and its metabolites. However, such studies cannot control the maternal reproductive potential. Even if a woman is considered fertile, having undergone the most contemporary examinations to evaluate fertility potential, one cannot rule out the possibility that the infertility of a couple is due to an undetermined female factor. The other method is to evaluate the effect of smoking on the standard parameters of semen analysis. This approach has three inherent limitations: (a) the standard parameters of semen analysis cannot predict the outcome of in vitro fertilization (IVF) [15, 24]; (b) there are no satisfactory correlation coefficients between standard sperm parameters and the percentage of fertilized oocytes in a number of assisted reproduction programs [15]; and (c) male smokers often consume alcohol, occasionally use drugs, and their private lives often make them susceptible to prostatic infections [2, 16]. Therefore, when the effect of smoking on sperm parameters is assessed using semen samples collected from smokers, the results may be influenced by other factors (i.e., alcohol, drug use, or leukocytospermia) [2, 16]. For these reasons, the current investigation on the influence of smoking on sperm fertilizing capacity focuses on the effects of smoking substances/metabolites on sperm function in vitro.

Cotinine is the major metabolite of nicotine, which is the major psychoactive substance found in cigarette

smoke [11, 16]. Significantly larger concentrations of cotinine have been found in smokers [mean ± standard deviation (SD); serum 303 ± 128 ng/ml; seminal plasma 279 ± 155 ng/ml] than in non-smokers (undetectable cotinine in both serum and seminal plasma) [11, 16]. Heavy smokers have serum cotinine levels above 400 ng/ml, whereas in average smokers it is $<400$ ng/ml [16]. In the latter study [16], giving up smoking altered the serum cotinine concentration from 211 ± 63 ng/ml to undetectable levels and significantly improved hypoosmotic swelling test (HOST) outcome, sperm acrosin profiles, and the zona-free hamster oocyte sperm penetration assay (SPA) result. Significantly negative correlations between peripheral serum or seminal plasma cotinine levels and semen parameters or sperm functional assays have been reported [11, 16]. High cotinine concentrations in seminal plasma or serum are linked to diminished sperm motility, diminished sperm membrane function, and a diminished ability of sperm to undergo capacitation and acrosomal reaction [11, 16]. However, because of the above-mentioned limitations of in vivo studies, the probability that the significant correlations between serum or seminal plasma cotinine and impaired sperm fertilizing capacity are due to other compounds induced by/correlated with cotinine (i.e., cotinine metabolites, products generated by chemical reactions in which cotinine participates, or substances/drugs that are consumed by a subpopulation of smokers) cannot be excluded. Thus, it appears that undertaking an in vitro study evaluating the effects of smokers' seminal plasma cotinine concentrations on sperm fertilizing capacity represents an attractive alternative approach. In the current study, we evaluated the effects of small and large cotinine concentrations on the quantitative and qualitative motility of sperm, sperm viability, sperm membrane function, and sperm ability to undergo capacitation, acrosomal reaction and fuse with the ooplasm. Furthermore, the influence of various cotinine concentrations (similar to those present in the smokers' seminal plasma) on sperm capacity to activate oocytes after ooplasmic injections was examined. Such a study evaluates whether the effects of smoking on sperm parameters are due to cotinine or whether they should be attributed to other compounds included in cigarette smoke or consumed by smokers.

## Materials and methods

### Participants

Twenty-six semen samples were collected from 26 fertile healthy donors (non-smokers; one sample from each man) known to have a sperm concentration $>50 \times 10^6$/ml after 3 days of sexual abstinence. These participants were known to be negative for alcohol consumption, drug usage, semen infections, and leukocytospermia.

### Semen sample processing

The standard parameters of semen analysis were evaluated as previously described by the World Health Organization (WHO) [26]. Semen volume, sperm concentration, quantitative sperm motility (percentage of motile spermatozoa), and percentage of morphologically normal spermatozoa were assessed. In addition, sperm viability was evaluated using trypan blue stain [10, 16]. Qualitative motility (motility grade) was evaluated using an arbitrary grading system on a scale of 0–4 [16].

Semen samples were divided into 0.4–0.5-ml aliquots. One milliliter of human tubal fluid (HTF; Irvine Scientific, La Jola, Calif., USA) was gently layered onto each aliquot. The tubes were inclined at 45°. A 60-min incubation at 37 °C allowed motile spermatozoa to swim up in the HTF medium. The supernatants (0.7 ml) from all of the tubes used were pooled and centrifuged at 400 *g* for 20 min. The final sperm pellet was re-suspended in 3 ml HTF medium supplemented with 35 mg/ml human serum albumin (HSA; Sigma, St. Louis, Mo., USA) (modified HTF medium). A small volume (0.2 ml) was processed for sperm quantitative and qualitative motility assay. Five aliquots of 0.5 ml were also prepared (aliquots A, B, C, D, and E) from each final sperm sample post-swim up.

Aliquots A–E were mixed with 0.5 ml modified HTF medium or modified HTF medium supplemented with cotinine (Sigma) so that the final concentration of cotinine was equal to 0, 100, 200, 400, or 800 ng/ml for aliquots A–E, respectively. All aliquots were incubated at 37 °C under 5% carbon dioxide for 8 h. At the end of the incubation period, a volume of 0.2 ml was removed and processed for assessing sperm viability, quantitative sperm motility, and qualitative sperm motility. Additional volumes of 0.1, 0.2, and 0.05 ml were processed for the HOST, the assay of hyperactivated spermatozoa, and ooplasmic injections of spermatozoa into hamster oocytes, respectively. The remaining volume of each aliquot (0.45 ml) was centrifuged at 300 *g* for 15 min; the sperm pellet was then re-suspended in a volume of modified HTF medium to prepare final sperm samples of concentration equal to $10 \times 10^6$ spermatozoa/ml [17]. Two sperm drops of 0.1 ml were prepared from aliquots A–E (each drop contained $10^6$ spermatozoa). Ten zona-free hamster oocytes were transferred to each sperm drop and co-incubated at 37 °C under 5% carbon dioxide for 3 h as previously described [17]. The oocytes were then rinsed in HTF medium and observed using a confocal scanning laser microscope computer-assisted system [18–22]. The presence of a swollen sperm head or male pronucleus with a visible tail in the ooplasm was considered to be indicative of positive penetration. The penetration rate was defined as previously described [17]: (number of positive oocytes/total number of oocytes examined) × 100. The average penetration rate from the two sperm drops of each aliquot was calculated.

### Hypoosmotic swelling test (HOST)

To perform the HOST, the sample of recovered spermatozoa was mixed with 1.0 ml of hypoosmotic solution [7] and incubated at 37 °C for 1 h. Post-incubation, the spermatozoa were examined for typical swelling abnormalities as described by Jeyendran et al. [7].

### Sperm hyperactivation assay

Sperm motility was analyzed using a motility analyzer (Hamilton Thorn, Boston, Mass., USA) [28]: curvilinear velocity (VCL; total distance traveled divided by the total time the cell was tracked), linearity (the ratio of the straight line distance to the actual track distance × 10), and the amplitude of lateral head displacement (ALH; maximal deviation of the sperm head from the mean trajectory) were determined [1, 28]. The criteria for hyperactivation were VCL ≥ 100 μm/s, linearity ≤ 6.5, and ALH ≥ 7.5 μm. The percentage of hyperactivated spermatozoa was recorded.

### Sperm injections into hamster oocytes

Droplets of sperm samples were prepared as we previously described [18, 21]. Spermatozoa were aspirated via micropipettes of

diameter 6–7 μm and injected into zona intact metaphase II hamster oocytes [22]. Ten oocytes were injected with spermatozoa from each aliquot of each participant. Micropipettes were prepared at our Reproductive Physiology and IVF Center using a pipette puller (P-97 IVF; Sutter, Tokyo, Japan) and a micro-forge (MF-90, Narishige, Tokyo, Japan). A Piezo-micromanipulator Unit (PMAS-CT 140, Nihon Ham, Tokyo, Japan) was used for ooplasmic injections because it is difficult to penetrate the highly elastic hamster oocyte vitelline membrane using conventional micromanipulator systems. Post-injection oocytes were transferred to hamster embryo culture medium (HECM)-3 medium [10] and incubated at 37 °C under 5% carbon dioxide for 2 h. The percentage of activated oocytes (resumption of meiosis) [9, 19, 21, 22] was recorded. Ten additional zona intact hamster oocytes were injected with a small amount of medium (0.7–0.9 pl) equal to that injected into the hamster oocytes during spermatozoal injections. The latter ten oocytes were cultured in HECM-3 medium at 37 °C under 5% carbon dioxide for 2 h.

### Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) ($P < 0.05$) followed by the multiple range Duncan's test ($P < 0.05$). A $P$ value of $<0.05$ was considered to be statistically significant. Values were expressed as mean ± SD.

## Results

### Semen samples

All 26 semen samples had a semen volume >2.4 ml (3.1 ± 0.3), sperm concentration $>73 \times 10^6$/ml (98 ± $12 \times 10^6$/ml), quantitative sperm motility >52% (72 ± 9), motility grade >2.3 (3.0 ± 0.3), percentage of morphologically normal spermatozoa >39% (63 ± 10), and percentage of alive spermatozoa >91% (95 ± 2).

### Post-swim up sperm parameters

All post-swim up sperm samples (prior to their incubation with cotinine) had a quantitative sperm motility >71% (85 ± 6%) and qualitative sperm motility >2.8 (3.3 ± 0.2).

### Effects of cotinine on sperm motility

Data on the effects of cotinine on sperm motility are given in Table 1. There were no significant differences in quantitative and qualitative sperm motility among aliquots A, B, and C. However, aliquots D and E demonstrated significantly smaller quantitative and qualitative sperm motility than aliquots A, B, and C.

### Effects of cotinine on zona-free hamster oocyte SPA

Data on the effects of cotinine on SPA outcome are given in Table 1. There were no significant differences in SPA outcome among aliquots A–C. However, aliquots D and E demonstrated significantly smaller SPA outcome than aliquots A–C.

### Effects of cotinine on sperm viability

There were no significant differences in sperm viability among aliquots A–E (Table 1).

### Effects of cotinine on sperm membrane function

There were no significant differences in the outcome of the HOST among aliquots A, B, and C. However, the HOST result was significantly smaller in aliquots D and E than in aliquots A–C (Table 2).

### Effects of cotinine on the ability of spermatozoa to undergo hyperactivation

There were no significant differences in the percentage of hyperactivated spermatozoa among aliquots A–C. In contrast, the percentage of hyperactivated spermatozoa was significantly larger in aliquots A–C than in aliquots D and E (Table 2).

### Effects of cotinine on sperm capacity to activity hamster oocytes after ooplasmic injections

There were no significant differences in the percentage of activated oocytes after sperm ooplasmic injections among aliquots A–E (Table 2). All of the activated oocytes were positive for a decondensed sperm head or a male pronucleus.

Among the ten oocytes that were injected with medium, only two were activated. The percentage of activated oocytes (20%) in the latter group of ten oocytes

**Table 1** Effects of cotinine on sperm viability, motility, and sperm penetration assay (SPA) outcome

| Aliquot | Cotinine (ng/ml) | % Motile sperm | Motility grade | Viability (%) | SPA (%) |
|---|---|---|---|---|---|
| A | 0 | 81 ± 8[a] | 3.1 ± 0.4[a] | 90 ± 3[a] | 81 ± 10[a] |
| B | 100 | 77 ± 8[a] | 2.8 ± 0.4[a] | 88 ± 4[a] | 77 ± 10[a] |
| C | 200 | 73 ± 10[a] | 2.7 ± 0.5[a] | 88 ± 5[a] | 74 ± 11[a] |
| D | 400 | 51 ± 11[b] | 1.8 ± 0.3[b] | 86 ± 6[a] | 43 ± 9[b] |
| E | 800 | 45 ± 13[b] | 1.7 ± 0.4[b] | 85 ± 7[a] | 36 ± 7[b] |

Within each column, values not sharing the same superscript, a and b, are significantly different ($P < 0.05$)

**Table 2** Effects of cotinine on hypoosmotic swelling test (HOST) result, sperm capacity for hyperactivation, and outcome of human sperm injections into hamster oocytes (% activated oocytes). *AO/IO* activated oocytes/injected oocytes

| Aliquots | Cotinine (ng/ml) | HOST | % Hyperactivated sperm | AO/IO (%) |
|---|---|---|---|---|
| A | 0 | 79 ± 7[a] | 7.3 ± 1.5[a] | 223/260 (85)[a] |
| B | 100 | 74 ± 9[a] | 6.7 ± 1.5[a] | 211/260 (81)[a] |
| C | 200 | 70 ± 10[a] | 6.0 ± 1.1[a] | 213/260 (81)[a] |
| D | 400 | 46 ± 11[b] | 3.8 ± 1.1[b] | 208/260 (80)[a] |
| E | 800 | 41 ± 14[b] | 3.7 ± 1.0[b] | 208/260 (80)[a] |

Within each column, values not sharing the same superscript, a and b, are significantly different ($P < 0.05$)

was significantly smaller than in aliquots A–E (Table 2), suggesting that the activation of oocytes in these aliquots cannot be attributed to parthenogenetic activation. The latter statement is also supported by sperm nucleus decondensation/male pronucleus formation in all activated oocytes.

## Discussion

A large percentage of men of reproductive age in the USA smoke [23]. In addition to those who voluntarily expose themselves to the risks of smoking, non-smokers are also affected by inhaling "sidestream" cigarette smoke. The Surgeon General of the United States characterized smoking as the chief single avoidable cause of death in our society and the most important public health issue of our time [25].

The recent growing interest on the paternal influence on fertilization and early embryonic development was translated into several studies evaluating the effects of smoking on male reproductive capacity. Stillman et al. [23] investigated the effect of smoking on male fertility. That study reviewed several considerable multifactorial risks of smoking for male fertility potential [23]. Several additional investigations have provided strong evidence of an adverse effect of smoking on the standard parameters of semen analysis and the male reproductive capacity [5, 6, 8, 12, 16]. The latter studies concluded that consumption of cigarettes is able to produce/contribute to testicular dysfunction and to alter sperm qualitative features. This thesis is also supported by a study showing detectable serum cotinine concentrations and a defect in spermatogenesis in rats exposed to cigarette smoke [27]. Alterations in the human hypothalamic pituitary testicular axis have also been described [16, 23].

The present study shows that concentrations of cotinine ≥400 ng/ml exert a detrimental effect on sperm motility and sperm membrane function. This is supported by the significantly smaller outcome of quantitative and qualitative sperm motility and HOST in aliquots D and E. Impaired sperm membrane function following the exposure of spermatozoa to cotinine levels ≥400 ng/ml may contribute to the defect in sperm ability to undergo hyperactivation under conditions known to induce sperm capacitation. The impaired sperm membrane function and the decreased sperm ability to undergo hyperactivation indicate a defect in sperm fertilizing capacity. This is because it is known that the outcome of the HOST is significantly and positively correlated with the in vitro fertilizing capacity of spermatozoa [7] and that sperm membrane function is important for sperm metabolism, motility, capacitation, and ability to fuse with the ooplasm. Furthermore, measurements of the percentage of spermatozoa showing hyperactivated motility under conditions known to induce sperm capacitation has a prognostic value for the outcome of IVF because it is thought that mammalian spermatozoa do not display hyperactivation until they are nearing completion of the capacitation process [28]. Fleming and Yanagimachi [4] showed that loss of hyperactivated motility was closely related to a decline in the ability of spermatozoa to cross the zona pellucida. Other reports have consistently suggested that hyperactivation is closely linked with the completion of acrosomal reaction and the events of fertilization [3]. Thus, the percentage of spermatozoa showing hyperactivated motility may represent a portion of the sperm population that has already undergone, or is undergoing, the capacitation process [28, 29]. Therefore, it is logical to suggest that cotinine concentrations (≥400 ng/ml) affect sperm membrane function, influence the cascade of events that constitute sperm capacitation, and impair the ability of spermatozoa to undergo hyperactivation and subsequently to fertilize oocytes. The detrimental influence of 400 or 800 ng/ml cotinine concentrations on sperm ability to undergo capacitation is additionally supported by the significantly smaller outcome of SPA in aliquots D and E because SPA evaluates sperm ability to undergo capacitation, complete acrosomal reaction, and fuse with the ooplasm [17, 29]. Considering that optimal spermatozoal motility, membrane function, ability to undergo hyperactivation, and ability to complete the capacitation process are prerequisites for the normal fertilization process, it appears that cotinine in concentrations of 400 or 800 ng/ml has a detrimental affect on the sperm fertilizing potential.

The results of the current study are compatible with a previous study demonstrating decreased sperm membrane function and fertilizing capacity in smokers' spermatozoa [16]. The detrimental effect of cotinine on sperm fertilizing capacity cannot be attributed to sperm death induced by cotinine because there were no significant differences in the percentage of living spermatozoa among aliquots A–E. The present study is the first report in the literature to show that cotinine does not affect the ability of spermatozoa to activate oocytes after ooplasmic injections. An oocyte-activating substance in human sperm (OASIS) has been considered to be responsible for the activation of human oocytes after human sperm ooplasmic injections [22]. Human OASIS has the capacity to activate hamster oocytes [22].

Therefore, the injection of human spermatozoa into hamster oocytes can serve as a test evaluating the human sperm OASIS activity/content [22]. The current study shows that although cotinine concentrations of 400 or 800 ng/ml affect sperm membrane function, they do not influence human sperm OASIS activity during ooplasmic injections. The latter finding may be of clinical importance if cotinine is not only present in the seminal plasma [27] but also on the sperm membrane of smokers. Smokers who participate in assisted reproduction programs may receive intracytoplasmic sperm injections (ICSI) as an alternative treatment to conventional IVF trials because (a) sperm membrane function and ability for capacitation may be adversely and irreversibly affected by the presence of cotinine in seminal plasma prior to sperm processing/washing for IVF, and (b) binding of cotinine to the sperm membrane may also affect sperm ability to undergo capacitation and hyperactivation. In fact, our preliminary studies have shown that the spermatozoa of smokers isolated after dilution, swim up, and centrifugation of semen samples are positive for cotinine (up to 24 ng/$200 \times 10^6$ spermatozoa; cotinine was measured using high-performance liquid chromatography (HPLC) by The Japanese Special Reference Laboratory, Matsue, Japan). Thus, if the washing of the spermatozoa of smokers during IVF treatment does not remove the cotinine bound to the spermatozoal surface, impaired spermatozoal capacitation and hyperactivation processes may be anticipated. Considering that sperm capacitation and hyperactivation are not prerequisites for the successful outcome of human ICSI techniques, and that cotinine does not affect human sperm OASIS activity during ICSI procedures, it appears that ICSI techniques represent the most optimal approach for assisted reproductive trials of smokers. The latter thesis may be supported by our recent report [27], which showed that IVF trials using the spermatozoa of rats exposed to smoke achieved diminished fertilization rates. However, in order to claim unequivocally that ICSI should be performed rather than IVF in cases of male smokers with large serum/seminal plasma cotinine concentrations ($>400$ ng/ml), fertilization and cleavage rates should be compared after assisted reproduction trials are performed using IVF techniques in half of the female partner's oocytes and ICSI procedures in the remaining oocytes.

The harmful effect (in vitro) of cotinine concentrations ≥400 ng/ml on sperm fertilizing capacity is of great clinical importance because such concentrations of cotinine are commonly found in the serum [16] and seminal plasma [11] of smokers. Thus, it may be suggested that cotinine is the main connection between smoking and impaired sperm quantitative, qualitative, and functional parameters. The latter thesis is supported by two previous studies showing significant and negative correlations between serum or seminal plasma cotinine concentrations and sperm motility, HOST result, and SPA outcome [11, 16]. The correlations between cotinine and sperm functional parameters were stronger than those between nicotine and the same sperm functional parameters [11, 16]. Additional mechanisms/factors may contribute to the detrimental effect of smoking on male reproductive potential. We have previously shown that smoking results in a Leydig and Sertoli cell secretory deficiency [16]. Impaired intratesticular/intraepididymal testosterone and androgen-binding protein profiles may cause defects in spermatogenesis and the epididymal sperm maturation process because it is known that the former are important for activating and maintaining the latter. Abnormal spermatogenesis and epididymal sperm maturation process may cause defects in the sperm cytoskeleton [16] and result in the inability of the spermatozoon to complete the series of biophysical and biochemical alterations during sperm epididymal passage, the overall result being a decrease in sperm fertilizing capacity.

The current study provides strong evidence that spermatozoal exposure to cotinine concentrations of 400 or 800 ng/ml results in sperm membrane damage, leading to an impaired sperm ability to (a) reach optimal motility, (b) undergo capacitation, and (c) achieve hyperactivation. Considering that the cotinine concentration in the semen of most heavy smokers is $>400$ ng/ml [27], it appears that cotinine may account for/contribute to the impaired sperm fertilizing capacity of smokers. ICSI techniques obviating the need for optimal sperm motility and sperm ability for capacitation and securing for OASIS-mediated oocyte activation may represent the optimal assisted reproductive technique for infertile smokers.

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