

## The in-vitro effects of nicotine, cotinine and leptin on sperm parameters analyzed by CASA system

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### Abstract:

**Background:** Cotinine (COT) is a major degradation product of nicotine (NIC). The participation of leptin in female reproduction is well-established, but any role in male reproductive function is at the best tenuous.

**Objective:** The aim of this study was to evaluate the in-vitro effects of nicotine, cotinine and leptin on sperm parameters in normal semen of non-smokers fertile men.

**Materials and Methods:** Ten healthy nonsmokers aged 25-40 years old were divided into 7 groups, Their semens were divided into 7 aliquots. (A) was layered with basal solution 70 ng/ml NIC, (B) 35 µg/ml NIC, (C) 300 ng/ml COT, (D) 200 µg/ml COT, (E) 30 ng/ml leptin, (F) 300 ng/ml leptin respectively and (G) was layered with mHTF. After migration, the samples were examined at time 0, +1, +2, +4, +8, and + 24 h of incubation.

**Results:** These findings were obtained: sperm count:  $75.66 \pm 66.25 \times 10^6$  /ml, forward motility:  $75.55 \pm 14.80\%$ , progress:  $33.66 \pm 13.01$ , VSL:  $51.58 \pm 6.99$  µm/s, VCL:  $103.33 \pm 14.52$  µm/s, ALH:  $4.33 \pm 0.77$  µm, BCF:  $25.60 \pm 2.97$  HZ, STR:  $79.33 \pm 8.04$  %, LIN:  $52.55 \pm 10.52$  %, ELO:  $74.22 \pm 12.76$  % and ARE:  $3.04 \pm 1.50$  u/sq. The parameters were similar before 8 hr and were being decreased after that.

**Conclusion:** According to the results. nicotin and cotinin have negative effects on the sperm parameters but despite the positive effect of leptin, there is no correlation between leptin concentration in semen and its physical characteristics.

**Key words:** Nicotine, Cotinine, Leptin, Sperm parameters.

### Introduction

Review of literature indicates that cigarette smoking is associated with modest reduction in semen quality including sperm concentration, motility and morphology (1), semen volume and acidity (2); and could cause specific lesions in the development of spermatozoon, and it might be either directly or indirectly toxic to

spermatogenesis (3). Smokers have a significantly greater percentage of abnormal forms in sperm morphology than non-smokers (4). Many studies have assessed the association between cigarette smoking and sperm density, motility and morphology with mixed result (5, 6). Besides a few articles, it can be believed that cigarette smoke contains more than 3000 different chemical compounds, and some of these chemicals are genotoxic by entering the blood circulation of the testes and can have a direct cytotoxic effect on spermatozoa by damaging DNA (7).

The smoke can be divided into a gaseous phase and a phase made up of particles; the main

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component of the particle phase is nicotine (8). As much as 1 mg of nicotine can be absorbed by smoking a single cigarette (9), and about 80 - 90 % of it is metabolized by the organism (10). Cotinine is a major degradation product of nicotine (11). Because of its specificity and detectable concentration in human body fluids, such as serum, urine, follicular fluid (11) and seminal plasma (12), it is more stable than nicotine, with a longer half-life of ~ 20 h (nicotine is 2 h) (13). Also experimental studies in male rats exposed to smoking have indicated that serum levels of nicotine and cotinine were increased which adversely affected spermatogenesis, epididymal sperm content, motility and fertilizing potential (14). Nicotine is reported to have toxic effects on gonadal functions by making nicotine-induced biochemical changes in testes (15). The nicotine causes thickening of the tunica properia, increases in the collagen fibers under the irregular basal lamina and degeneration of junctional specialization between the Sertoli cells; and the germ cells may be degenerated and spermatids retained excess cytoplasm (16).

Besides, nicotine may cause reduction in the epididymal sperm count, and decrease in the surface epithelial cell height of epididymis and seminiferous ducts (17). The effect of nicotine on fertility is much greater in male than in female and some earlier studies have shown that female rodents have a greater tolerance to nicotine than their male counterparts (18). The cotinine concentration in blood and seminal plasma were of similar magnitude with high correlation; therefore an active transfer may be suggested between the compartments from the arteriae testicularis via the Sertoli cells in to the seminiferous tubules and the seminal plasma (19).

However, the mechanism of the toxicity of cotinine on sperm morphology is not clear, but there is a minor effect of smoking on male factor subfertility, which is probably due to compounds in cigarette smoke other than nicotine (cotinine) (19). On the other hand, a small but statistically significant correlation was found between cotinine concentration in seminal plasma and the percentage of abnormal sperm morphology (19). Thus there is an idea that the associations between cotinine concentration in seminal plasma and semen parameters have been inconclusive (20). Leptin is a 167-amino acid (21), 16-kD protein (22) and pleiotropic hormone that produced primarily by white adipose tissue (23,24) and also maybe the testes contribute to its secretion.

Therefore, the source of leptin can be also the seminal vesicle or the prostate tissue (23).

Normal leptin secretion is necessary for normal reproductive function (24). Although the participation of leptin in female reproduction is well-established (25), but any role in male reproductive function is at the best tenuous. Its role in male gonadal function is less evident than in females and participation of it in seminal production or sperm capacity has not been completely assessed (23). Generally its contribution to the proper functioning of the male reproductive system has been less clear (26). It has been shown that the testes are direct targets for leptin actions (26). Besides, leptin plays a role in other target reproductive organs (27). Leptin may play an important role in the regulation of the fertility in underweight males (28) and probably plays a role in spermatozoid capacity and also have an effect on testosterone secretion (23).

In recent years, it was strongly suggested that leptin is able to act at different levels of the hypothalamic pituitary-testicular axis (26) and influence the mechanisms involved in motility development of spermatozoa (21). Therefore future application of leptin may be directed to the treatment of infertility (29). The aim of this study was to evaluate the in-vitro effects of nicotine, cotinine and leptin on sperm kinetic parameters in normal semen sample of non-smokers fertile men.

## Materials and methods

In this study, ten healthy nonsmokers aged 25-40 years old were selected. They were not normally exposed to passive smoking and not treated medically in 3 months ago. The semen specimens were obtained via masturbation after 3-4 days of ejaculatory continence. Standard semen analyses (WHO, laboratory manual, Third edition, 1992) and analysis of sperm parameters by computer aided sperm analysis (CASA system - Hamilton - Thorn IVOS system - Micron UK Ltd., UK) were carried out.

The following variables were taken into consideration: volume of ejaculate (ml), sperm concentration ( $\times 10^6/\text{ml}$ ), forward motility (%), average path velocity (VAP,  $\mu\text{m/s}$ ), straight-line velocity (VSL,  $\mu\text{m/s}$ ), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ), beat cross-frequency (BCF, Hz), percentage of straightness (STR, VSL/VAP, %), percentage of linearity (LIN=VSL/VCL, %), percentage of elongation and head area (U/sq.).

The subjects with the following seminal characteristics were included in this work: volume  $\geq 3.0$  ml, sperm concentration/ml  $\geq 50 \times 10^6$  and forward motility  $\geq 50\%$ . The basal solution for nicotine {sigma, (-) - nicotine, ([-] -1-methyl-2 - [3-pyridyl] - pyrrolidine), 98 - 100 % purity, N-3876}, cotinine {sigma, (-) - cotinine, ([s] - 1-Methyl-5- [3-pyridyl] - 2 pyrrolidinone), purity approx. 98 %, N-5923} and leptin {sigma, (OB) Human; recombinant. Expressed in E.Coli, purity =97%, No, L4146} was modified human tubal fluid (m. HTF, PH, 7.5 osmolarity  $284 \pm 10$ ) that used as a buffer in the preparation of nicotine, cotinine and leptin at various concentrations. Then these solutions were prepared: A; nicotine base 70 ng/ml B; nicotine base 35  $\mu\text{g/ml}$ , C; cotinine base 300 ng/ml, D; cotinine base 200  $\mu\text{g/ml}$  (30) , E; leptin base 30 ng/ml and F; leptin base 300 ng/ml (31) The PH of solution A, B, C, D, E and F was then confirmed as being the same as the basal solutions (PH=7.5).

### Semen preparation

All semen samples were collected in separate sterile containers and allowed to liquefy for 20 min at 37°C temperature. Then the kinetic parameters were evaluated by using computer-aided sperm analyzing system (CASA system). Each sample was then allowed to swim up in basal solution modified human tubal fluid for A, B, C, D, E, F and G; without medicine (exact used pure m. HTF) for control group in the following experimental protocol.

### Experiment

The semen sample was divided into seven aliquots of 0.3 ml and then layering swim-up by using of m.HTF. The first aliquot (A) was layered with basal solution 70 ng / ml nicotine, 2nd (B) 35  $\mu\text{g}$  / ml nicotine, 3rd (C) 300 ng / ml cotinine, 4<sup>th</sup> (D) 200  $\mu\text{g/ml}$  cotinine, 5<sup>th</sup> (E) 30ng/ml leptin, 6<sup>th</sup> (F) 300 ng/ml leptin respectively and 7<sup>th</sup> (G) aliquot was layered with m.HTF without any medicine. The whole of tubes, labeled with the corresponding letters. For providing the best spermatozoa, the total samples were allowed to migrate for 30 minutes at 37° C, in 5 % CO2 incubator.

After migration, the seven solutions were collected and continued incubation in 37° C for maximum 24 h. So, the samples were examined with computer aided sperm analysis (CASA) at time O(the end of migration), +1, +2, +4, +8, and + 24 h of incubation in order to determine the effect of nicotine, cotinine and leptin on sperm kinetic

parameters at low and high levels of concentration. As the control, we used spermatozoa migrated in basal m. HTF solution (G).

### Statistical analysis

The statistical analysis were used as following methods; one-way analysis of variance was used for evaluating the association of the level of medicines (Nicotine, cotinine and leptin) and sperm kinetic parameters. The comparisons of grouped means was performed using the grouped student's t-test.

### Results

The 10 seminal samples from selected subjects had the following mean values: volume  $\geq 3.0$  ml, sperm concentration  $\geq 50 \times 10^6$  /ml and forward motility  $\geq 50\%$ . The mean  $\pm$  SD of the sperm parameters of the 10 samples after swimming up in the basal solution (m. HTF) at time 0 were as follows; sperm concentration  $75.66 \pm 66.25 \times 10^6$  /ml, forward motility  $75.55 \pm 14.80\%$ , progress  $33.66 \pm 13.01$ , VSL  $51.58 \pm 6.99$   $\mu\text{m/s}$ , VCL  $103.33 \pm 14.52$   $\mu\text{m/s}$ , ALH  $4.33 \pm 0.77$   $\mu\text{m}$ , BCF  $25.60 \pm 2.97$  HZ, STR  $79.33 \pm 8.04\%$ , LIN  $52.55 \pm 10.52\%$ , ELO  $74.22 \pm 12.76\%$  and ARE  $3.04 \pm 1.50$  u/sq. The results at +1, +2, +4, +8 and + 24 h were used as the control for the subsequent experiment.

The analysis of the results showed that kinetic parameters at +1, +2 and +4 h were similar, but there was a statistically significant decrease in forward motility, progress and VSL starting at +8 h; whereas for other parameters significant decrease was found only after 24 h. The significance also varied according to the variables considered from  $p < 0.05$  to  $p < 0.001$ .

### Experiment «Incubation at low and high dosage of Nicotine, Cotinine and Leptin

The analysis of the results for A, C and E demonstrated as follows:

For sample A, there was no significant difference at +1, +2 and +4 h but a statistically significant decrease in parameters started at +8 h. The significance varied according to the variables considered from  $p < 0.05$  to  $p < 0.001$ .

For samples C and E there were no statistically significant difference at +1, +2 ,+4 and +8 h , but there was a statistically significant decrease at +24 h .

The analysis of the results for B, D and F demonstrated as follows: For sample B variables

started statistically significant decreasing at +1 h ( $p < 0.05$ ) whereas for samples D and F a statistically significant decrease was found only at +24 h of incubation ( $p < 0.05$ ). The analysis of variance for repeated measures performed on the seven groups (A, B, C, D, E, F, G) showed a highly significant statistical difference between the mean values of each considered variable caused by the influence of treatment and time. This significance was due only to data for treatment B.

The results of A, C and E versus G, referring to the various times of incubation are reported in figure 1. It can be seen that at time 0 the data were very similar and at +1, +2, +4, +8 and +24 h, they did not show a statistically significant modification for the variables considered ( $p > 0.05$ ).

Figure 2 shows the comparison between B, D and F versus G over the various times of incubation. For sample B at time 0 the data are similar, but a statistically significant difference between mean values of variables progress, VSL, ALH, BCF, STR, LIN, ELO and ARE seen after +1 h or more of incubation. These variables decreased with the time of incubation. For the variable «percentage of forward motility» a significant decrease was only found after +8 h of incubation ( $p < 0.05$ ).

For samples D and F there were no statistically significant changes from +1 to +24 h of incubation ( $p > 0.05$ ); except the variable progress which showed statistically significant declining from +8 h with treatment D ( $p < 0.05$ ) and at +24 h with treatment F ( $p < 0.05$ ).

The same significant differences were also found between treatments A, C, E and treatments B, D and F. No difference was found between A versus C or E, and B versus D or F respectively.

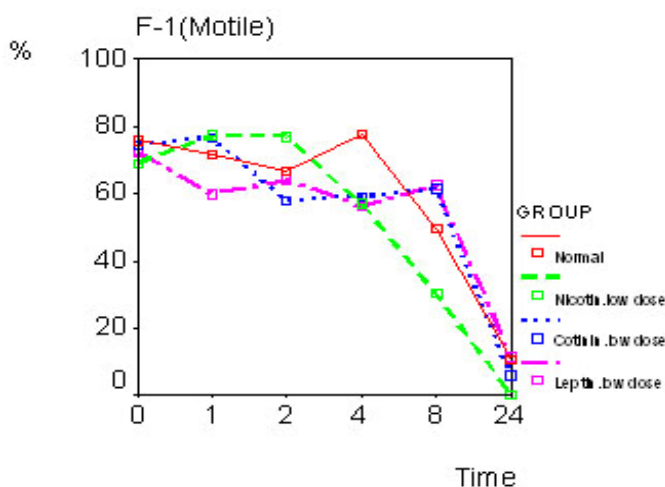
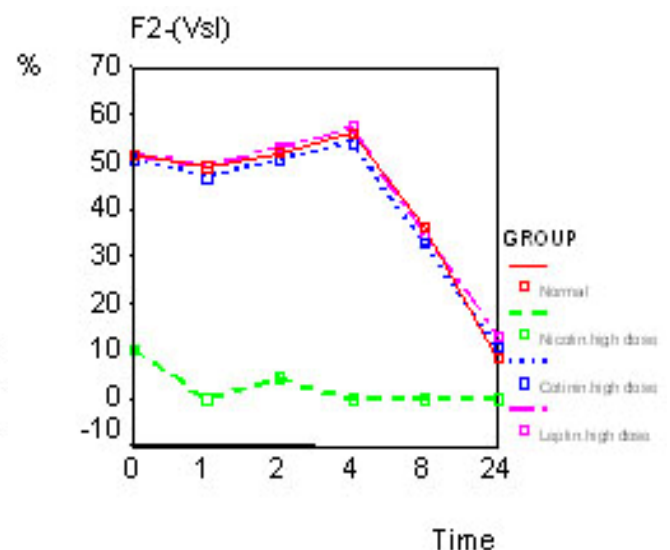
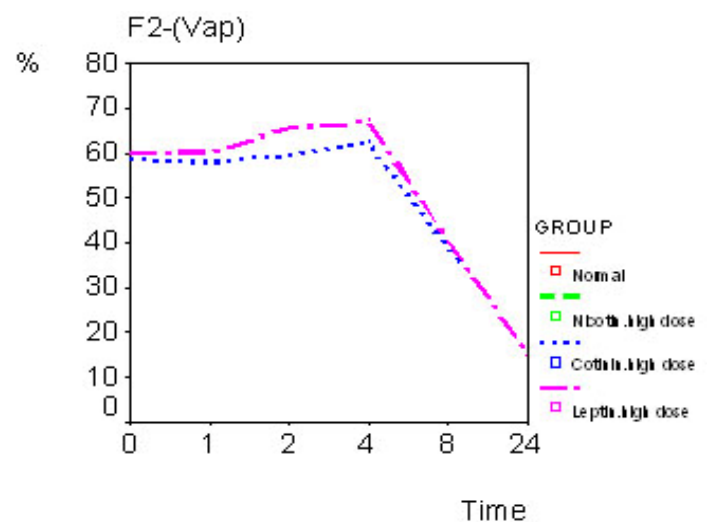
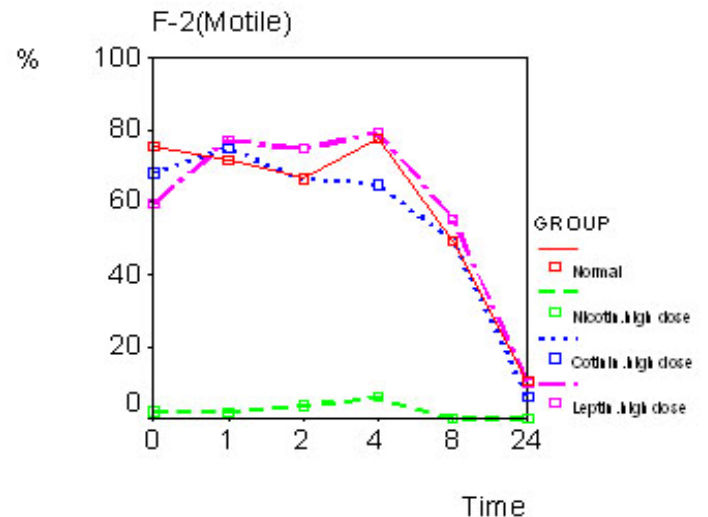
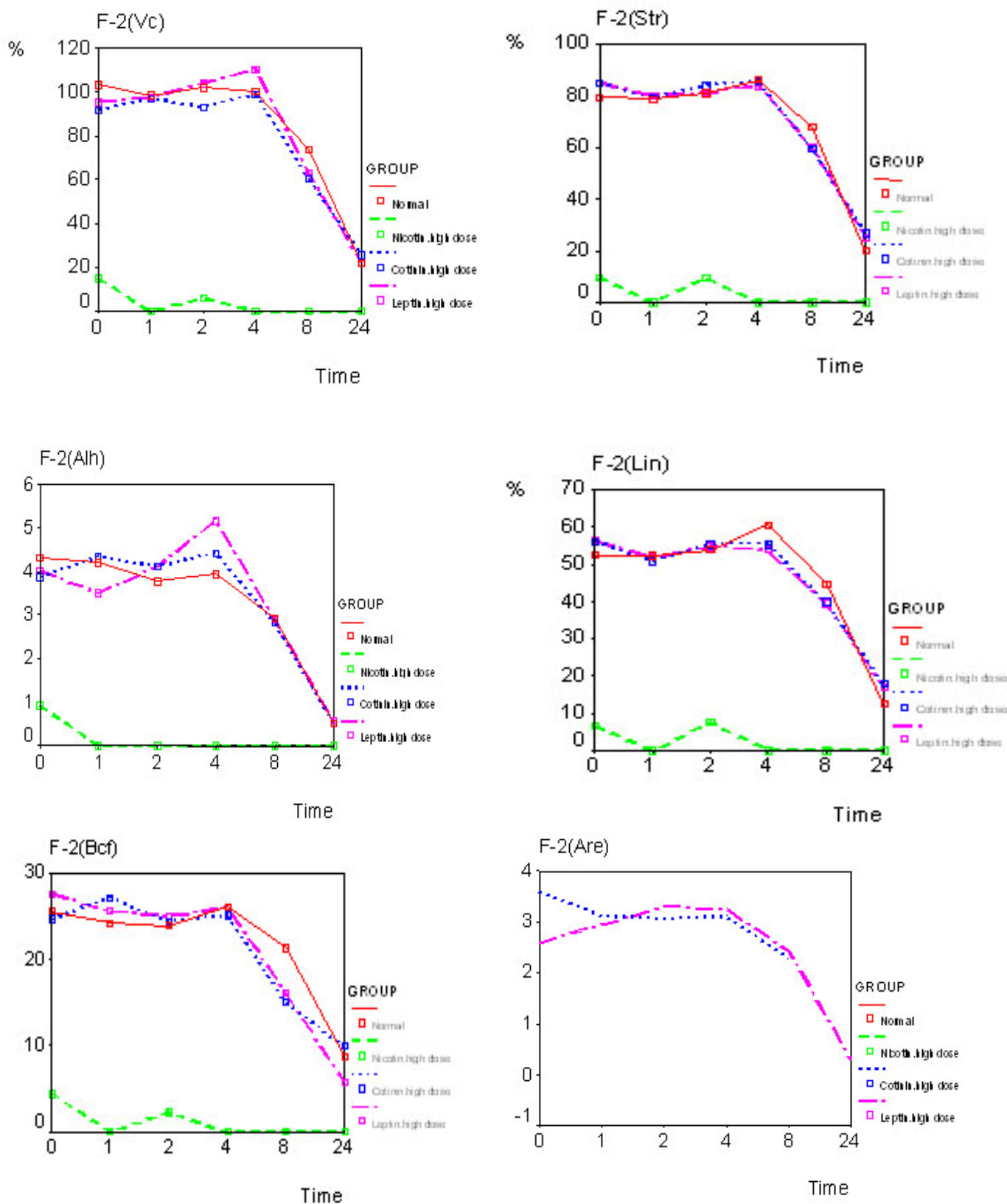


Figure 1: The comparison between A; low dose nicotine, C; low dose cotinine and E; low dose leptin versus G; (normal) over the various times of incubation.





**Figure 2 :** The comparison between B ;high dose nicotine, D; high dose cotinine and F; high dose leptin versus G; (normal) over the various times of incubation.

## Discussion

Cigarette smoke is made up of gas and organic compounds. One of the most abundant organic particles in cigarette smoke is nicotine, which is responsible for some positive or negative effects on the various organs. Nicotine is a very toxic alkaloid (22). Inhaled Nicotine is quickly oxidized to its major metabolite cotinine (11). Cotinine has a longer half-life than NIC, which is about 20 hours versus 2 hours for NIC; for this reason COT is a better marker of the smoke absorbed (13). In addition, cigarette smoke contains a mixture of harmful components such as carbon monoxide (CO), hydrogen cyanide (HCN), ammonia, volatile hydrocarbons, Alcohol, aldehydes and ketones (30). Leptin is an adipocyte hormone important in regulating energy homeostasis and interacting with the reproductive axis at multiple sites. Leptin may act as the critical link between adipose tissue and the reproductive system, indicating whether adequate energy reserves are present for normal reproductive function (27).

In addition, leptin is expressed in several other areas, such as the hypothalamus (32), pituitary (33), syncytiotrophoblast (34) and mammary epithelium (27,35) and its receptors (ob-RS) have been identified in the hypothalamus, gonadotrope cells of the anterior pituitary (33), granulosa, theca, and interstitial cells of the ovary (36), endometrium (37) and Leydig cells (38). In reproduction, leptin is implicated in fertility regulation and acts directly on testis (39). In general leptin participates in functional regulation of the male gonadal axis (26). However, the net effect of leptin and direct inhibitory actions at the testicular level may take place in the presence of a significantly elevated leptin concentration (26). There are many debates regarding the toxic effects of cigarette smoke on human reproduction (40). In the literature several studies indicated that cigarette smoking is not associated with a reduction of fertilization rates in couples undergoing IVF (41) or with modifications of seminal parameters (42,43) such as PH, sperm concentration, motility and morphology (2,44).

Also, in-vitro, nicotine and cotinine, at the levels found in the seminal plasma of smokers, do not affect sperm motility (30), PH and sperm morphology (45). furthermore no statistically significant variation of the other kinetic parameters studied up to 24 h with average levels of NIC and COT was found; but the much higher concentrations significantly altered all the kinetic variables in relation to the time of incubation (30).

Therefore the study suggested that NIC and COT are not responsible for the harmful effects of cigarette smoke on sperm kinetic parameters (30). In contrast, some other studies have reported a reduction in fertilization rates (46), semen volume (47), concentration, motility (48), sperm density, viability, forward progression (2), linearity (20), and higher incidence of oligospermia (45) and germ cell aneuploidy (49). A worsening of sperm morphology has been shown as well as a reduced capacity of spermatozoa to undergo the acrosome reaction (50). In addition, mutagenic effects on germ cells (51) have been observed; and mutations in spermatozoa have been noted that could lead to cancer and genetic diseases (52).

The comparison of the seminal parameters of smokers and nonsmokers indicated a statistically significant reduction of the percentage of motile spermatozoa in smokers. Also a small but statistically significant correlation was found between COT concentration in seminal plasma and the percentage of abnormal sperm morphology but not for other sperm parameters (19). Some studies showed that the semen volume, acidity, sperm density, viability and forward progression were much lower in heavy and long-term smokers than in nonsmokers ( $p < 0.01$ ) (2). Also the sperm density, viability and forward progression were negatively correlated with the amount and duration of cigarette smoking ( $p < 0.01$ ) (2). NIC at concentration of  $\geq 1$  m. M significantly decreases sperm motion characteristics after different periods of incubation, whereas 0.1 m. M concentration has the least effect (53). It was calculated that smokers had a 13%-17% reduction in sperm concentration compared with nonsmokers, but there was no clear evidence for a dose-response (54). Some investigations identified a reduction in sperm linearity among smokers, but no other smoking effects on sperm motility was detected by visual inspection or CASA (23). No deleterious effect of cigarette smoking were found on semen quality except for a non-significant trend toward decreased ejaculate volume (55). It has been found that serum leptin concentration is elevated up to 50 % in azoospermic men compared to normozoospermic fertile men. Accordingly, the mild hyperleptinemia in the azoospermic group may represent a partial leptin insensitivity that is manifested with infertility (56).

In addition, it was suggested that the concentration of leptin in seminal plasma was significantly lower in normal semen sample group than in the pathological group; and showed a significantly negative correlation with percentage

of motile spermatozoa and VCL (24). In contrast, one of the studies showed no correlation between leptin concentration in semen and physical characteristics of semen samples or physical characteristics of spermatozooids, such as concentration, motility, vitality, viability, morphology, head alterations and volume (23). However, in this study we used an in-vitro model to compare the influence of NIC, COT and LEP in low and high dosages on sperm kinetic parameters, in addition to evaluate the role of incubation time. The most interesting finding of this study is that low dose nicotine (NIC/base 70 ng/ml) and low dose cotinine (COT/base 300 ng/ml), which are the concentrations found in the seminal plasma of smokers (30,45), and low dose leptin (LEP/base 30 ng/ml) do not affect sperm kinetic parameters in-vitro. In fact, the values of percentage of forward motility, progress, VSL, VCL, ALH, BCF, STR, LIN, and ELO and ARE vary during the experiment in the same way as the control. Also, we found that high dose cotinine (COT/base 30 µg/ml) and high dose leptin (LEP/base 300 ng/ml) do not affect sperm kinetic parameters in vitro. This is in contrast with the results of Gandini et al (26) study which showed a statistically significant decrease in sperm motility with high dose cotinine. But by using high dose nicotine (NIC/base 35 µg/ml) we saw significant decrease in all sperm parameters; which is in line with the study of Gandini (26).

In fact, similar to the results in some previous studies (19,23), we showed that COT and LEP whether low dose or high dose don't have a significant effect on sperm parameters, although they decrease the parameters insignificantly. In conclusion, we showed the harmful effect of cigarette smoke on sperm kinetic parameters is not a consequence of NIC and COT. While these conditions are obviously somewhat different in vivo and the chronic contact of spermatozoa to smoke could lead in different results. These results also would suggest that other compounds (i.e. hydrocarbons, aldehydes, ketones, etc.) found in the gaseous phase of cigarette smoke are responsible for smoke - induced sperm parameters damage. These inhaled compounds are absorbed at the level of the blood circulation and after passing through the blood- testis barrier; they can act on mature spermatozoa and on their precursors, provoking the alterations of the seminal fluid in heavy smokers (57).

In addition, our results are in line with the study of Camina et al (20) who showed no correlation between leptin concentration in semen and the

physical characteristics of semen samples or physical characteristics of spermatozooids such as concentration, motility, vitality, viability, morphology, head alterations and volume. Finally this research recommends further studies on the compounds derived gaseous phase of cigarette smoke in order to understand the harmful effects of smoking on spermatozoa and also more studies about leptin effects on testicular functions and seminal parameters.

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