Sudhagar. K et al /J. Pharm. Sci. & Res. Vol.4(8), 2012, 1897 - 1900

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Correlation between Sperm Parameters and Sperm DNA Fragmentation in Fertile and Infertile Cigarette Smoking Patients.

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Abstract
The present study to investigate that the correlation between sperm parameters and sperm DNA fragmentation in fertile and infertile cigarette smoking patient. Forty men undergoing infertility evaluation were divided into four groups according their smoking ability (**A:** 2 days (n=10), **B:** 4 days (n=10), **C:** 8 days (n=10), **D:** 15 days (n=10)) sperm parameters (sperm concentration, pH, Viscosity, sperm count, motility, viability, Leukocyte concentration and comet value) . The comparisons of mean sperm count, motility viability and normal morphology among four different cigarette smoking user groups were statistical significant. Mean sperm motility viability and normal morphology were significantly different in cigarette smoking user groups within two sperm count groups. Use of cigarette smoking decrease the semen quality in men by decreasing the sperm count, motility, viability and normal morphology. The decrease in sperm parameters was dependent on the duration of 2 days exposure to cigarette smoking and independent of the initial semen quality.

Key words: Cigarette smoking, Sperm parameters, comet assay, male infertility

INTRODUCTION.

Conventional semen analysis is still one of the most popular tests for the evaluation of male fertility. However, in many circumstances, semen analysis per second is unable to predict the fertilizing capacity of ejaculated spermatozoa , because it does not assess some factors such as the integrity of sperm DNA (Mohammed ali Khalili, 2006) Assessing sperm morphology, motility and concentration is the best way to investigate males sterility. However these parameters have been proven useful in predicting the results of Assisted Reproductive Technology (Fraser, 2004) .The present techniques and principles for the analysis of human semen. However, the recent publications were listed giving further important details of semen analysis and interested readers should consult these publications (Besley *et al.*, 1980; Elisson, 1981).The disturbances in semen quality can be caused not only by such exogenous factors as chemicals and drugs. Control samples and control groups are therefore essential in most studies to the possible effects of various environmental factors especially cigarette smoking on semen quality. Current evidence suggests there may be environmental reasons for deteriorating sperm quality, including occupational exposure to various chemicals, heat, radiation and heavy metals (Lahdetie, 1995 and Thonneau *et al.*, 1998).In addition, exposure to environmental estrogens and pesticides has been linked to alternations in spermatogenesis. Life style risk factors are also significant, including cigarette smoking, alcohol consumption, chronic stress and nutritional deficiencies (De celis *et al.*, 1996).
The objectives of the present study was to assess the effects of cigarette smoking use on various parameters among patients undergoing infertility evaluation at a male infertility clinic. Our goal was to better understand the role of cigarette smoking use in male infertility and assess the need for any protective measures to prevent harmful effects of chemicals, if any on the male reproductive system.

MATERIALS AND METHODS.

The study was approved by the Institutional Review Board, and informed consent was obtained from all patients. In this observational study we examined 40 men attending an infertile clinic from January 2010 to January 2011 All the clinical disgnosis was analysed in Nandita Fertility and Research Centre, Chetpet, Chennai, Tamilnadu, India. The age of the study of population was 28.34± 7.16 years (Mean ± SE) subjects with a history of cigarette smoking in this study. In addition, patients who suffered from viral/ bacterial infection in the past 4 weeks, presented with a history of cardiac, neural or nephritic disease or had a family history of any genetic disease were also excluded.
Semen samples were collected by Masturbation in a sterile wide mouthed calibrated container after an abstinence period of 5 days. Semen analysis was performed according to World Health Organisation guidelines to evaluated eight

parameters Volume, motility, liquefaction time, pH, viscosity, sperm count, motility , viability and percentage normal morphology.(WHO, 1999).The information on cigarette smoking usage of the patients was recorded and the subjects were divided into 4 groups according to their Cigarette smoking **A:** 2 days (n=10), **B:** 4 days (n=10), **C:** 8 days (n=10), **D:** 15 days (n=10) . Sperm parameters were considered normal when sperm concentration was ≥20×106/ml, motility was ≥50% and normal sperm forms were ≥15%.The exclusion criteria were the presence of < 10 × 106 / mL total motile spermatozoa in the original (post–liquefaction) sample, azoospermia and severe oligospermia. No subjects in either group were smokers, on medication, had a history of exposure to chemotherapy or radiation, or a varicocele.

Single cell gel electrophoresis (comet) assay

In this study, the comet assay was performed using modification of Angelis method (1999) in order to detect both single and double stranded breaks. Pre cleaned slide (ROTH, Germany) were dipped in a solution of 1.5 %(w/v) normal melting point agarose (NMPA) in PBS, a coverslip was then placed on top, and allow solidifying at room temperature over night. The next day, coverslip was removed and 100 micro litter suspensions of spermatozoa in 1% (w/v) low melting point agarose (LMPA), at a concentration of 1×104 cell/ mL, was pipetted on the slides and covered with a coverslip. The slides were allowed to solidify at 4°C for 5 min then the coverslip was gently removed, a 1% LMPA was used to form a third layer and slides were allowed to solidify at 4°C for at last 1 h. Then the slides with coverslip were removed and placed in cold lysis buffer (2.5 M NaCL, 100 mM EDTA, 10 mM Tris, 1% Triton X- 100, 1% DMSO, and 10 mM Dithiothreitol [DDT] at a pH of 10) for 30 min at 4°C and protected from light. The slides were then incubated at 37°C in 10µg/ml of Proteinase K (Sigma) in lysis buffer for 2.5 h. Following cell lysis, all slides were washed through three changes of distilled water at 5 min intervals to remove salt and detergent from the microgels. The slides were placed in a horizontal electrophoresis tank filled with electrophoresis buffer (10 mM Tris containing 0.08 M boric acid and 0.5 M EDTA pH=8.2) and were kept for 20 min to allow the DNA to unwind. The electrophoresis buffer was adjusted at a level of ~0.25 cm above the slides surface. Electrophoresis was performed for 20 min at 25V adjusted to 300 mA by either raising or lowering the buffer level in the tank. When electrophoresis was completed, the slides were dried and flooded with three changes of neutralization buffer (0.4 mol/l Tris; pH 7.5) each for 5 min. After a neutralization step, the slides were stained with ethidium bromide (20µg/ml dissolved in distilled water) and mounted with a cover slip. Cells were visualized at 200× using a fluorescent microscope (Nikon).Each cell with fragmented DNA had the appearance of a comet with a brightly fluorescent head and a tail to one side formed by the DNA, which contained strand breaks that were drown away during electrophoresis.

Measuring comet by visual scoring

Analysis of comet cells was performed using the scoring method of Angelis (1999). According to this method, the cells divided to 5 classes from 0 (no tail) to 4 (almost all DNA in tail) according to comet appearance and each cell assigned a value of 0 to 4. At least 100 cells were randomly selected from one slide. Each cell, according to its appearance of a comet, scored between 0-4. The comet value for each slide was calculated between 0 and 400. Because two slides were prepared for each sample, the mean of comet value of two slides was calculated and considered for each case.

Statistical analysis

Data are reported as Mean±SE. The comparisons between two groups were tested by student's *t*-test using SPSS13. Correlation between two continues outcomes were evaluated using Pearson correlation coefficients. $p \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION.

The correlation was found to be between sperm concentration, pH, viscosity, count, motility, viability, leukocyte concentration, comet values were almost perfectly correlated. Semen analysis in the four cigarette smoking user groups showed a decrease in sperm count, motility, viability and normal morphology with the increase in days.(Table-1).

Table-1. Semen analysis results in four Cigarette smoking usage groups (Mean ± SE)

S.no	Parameters	A (2 days)	B (4 days)	C (8 days)	D (15 days)
1	Age	27±9.2	28±5.65	28±2.56	27±9.5
2	Sperm concentration *10 ^{6}	38.2 ±4.56*	39.4±5.67*	35.7±56.7*	30.5±58.9*
3	pH	7.5±0.34	7.6±0.67	7.7±0.45	7.6±0.45
4	Viscosity	3.25±0.67*	2.7±0.89	3.4±0.78*	2.9±0.34*
5	Sperm count x *10 ^{6} /mL	72.34±6.5*	69.45±3.4*	55.3±4.5*	45.4±7.8*
6	Motility (%)	62.6±0.65	59.78±0.67	52.7±0.45	43.5±0.76
7	Viability (%)	68.87±0.25	63.56±0.65	57.34±0.76	45.23±0.6
8	Leukocyte concentration*10 ^{6}	12.3±4.5*	10.4±7.6*	8.57±4.5*	6.98±6.5*
9	Comet value	45.6 ±7.8*	43.7±9.8*	33.6 ±7.8*	32.7±4.5*
10	WHO(morphology)	41.23±6.7	24.4±8.9	21.56±3.4	20.5±5.6

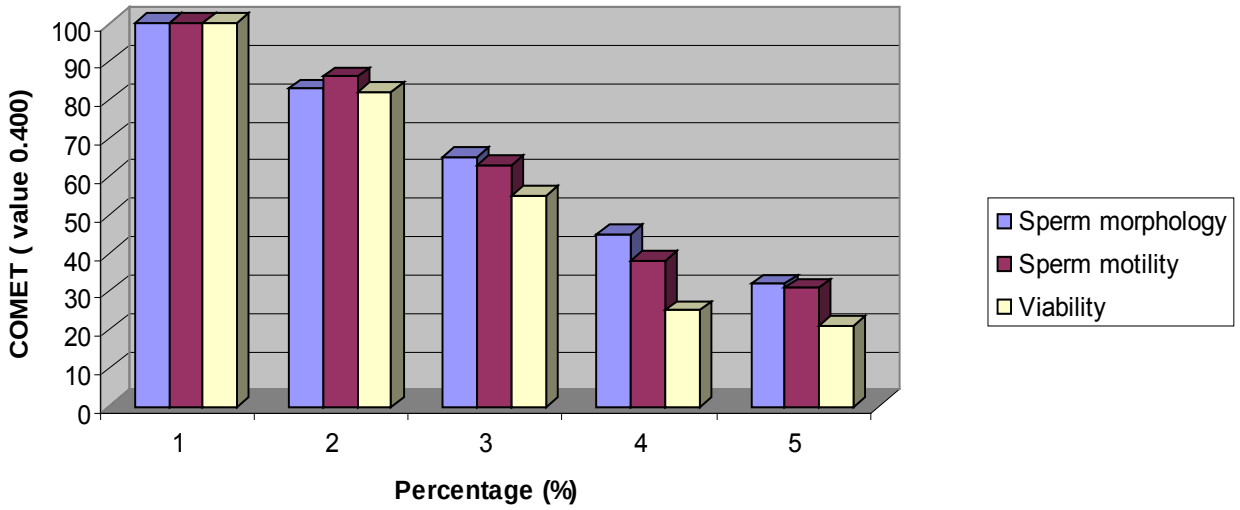
* Significant at <0.001 level.

There are no significant differences in the mean of participant's age in four groups but the mean of sperm concentration, viscosity, sperm count, leucocyte concentration, comet value were significantly higher fertile man. The mean value in the sperms of 15 days Cigarette smoking was lower (20.5±5.6 Vs 41.23±6.7) than the 2 days Cigarette smoking patients. . Use of cigarette smoking decrease the semen quality in men by decreasing the sperm count, motility, viability and normal morphology. The decrease in sperm parameters was dependent on the duration of 2 days exposure to cigarette smoking and independent of the initial semen quality (Figure -1)

The study indicate that sperm DNA damages in cigarette smoking is significantly lower (15 days) in 2days of cigarette smoking patients. Our results have provided evidence of an important relationship between some of semen parameters and sperm DNA damage. Sperm DNA damage as assessed by comet assay demonstrated a very clear negative relationship with sperm motility and morphology. Normozoospermics had a lowered percentage of sperm with DNA damage and semen quality is supported by data from other laboratories. Cigarette smoking has been associated with decreased sperm count, alternations in motility and an overall increase in the number of abnormal sperm. (Kulikauskas *et al.*, 1985). A study designed to evaluate seminal zinc levels in smokers and non-smokers found that although smokers did not have significantly lower zinc levels than non-smokers seminal Cd levels were significantly increased especially in those smoking more than one pack per day (Oldereid *et al*., 1994). Experimental evidence also suggests nicotine can alter the function of the hypothalamic-pituitary axis, affecting growth hormone, cortisol, Vasopression and oxytocin release, which then inhibits the release of luteinizing hormone (LH) and prolactin (Weisberg 1985). Cigaratte smokers were also shown to have higher levels of circulatory estradiol and decreased levels decreased levels of LH, Follicle Stimulating Hormone (FSH) and prolactin than non-smokers all of which potentially impact spermatogenesis. Smokers with low prolactin levels also demonstrated defects in sperm motility (Ochedalski *et al*., 1994).

Male fertility is a mutilfactorial disease process with a number of potential contributing causes. Considering the majority of male infertility cases are due to deficient sperm production of unknown origin, environmental factors must be evaluated. Occupational risk factors including exposure of chemicals need to be examined. Is it purely coincidence that sperm quality has diminished over the last 50 years, while ever increasing amounts of chemicals utilized by man and introduced to the environment perhaps we should consider decreased fertility in men as a physiological early warning system a" canary in the coal mine" So to speak, which is acting as a sensitive indicator of environment disruptions of cigarette smoking is utilized to decreased the sperm quality.

Figure-1. Comparative Analysis of Sperm Morphology, Motility, Viability infertile man and fertile man with comet value.



1. **Control.**
2. **A:** 2 days (n=10),
3. **B:** 4 days (n=10),
4. **C:** 8 days (n=10),
5. **D:** 15 days (n=10) .

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