

## ORIGINAL ARTICLE

# Evaluating the Functional Status of Spermatozoa in Smokers by Using Hypo-Osmotic Swelling Test

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## Abstract

**Background:** Smoking damages sperm, making them less likely to fertilize eggs and making the embryos they do manage to create less likely to survive, as smoking degrades sperm protein needed for fertility and embryo survival. There exists a strong negative relationship between smoking and fertility due to the fact that genetic material in the sperm can be damaged by the chemicals in cigarette smoke. The available data do not conclusively demonstrate that smoking decreases male fertility. However, with much debate for its impact on various semen parameters, it is regarded as an infertility risk factor. Apart from genetic damage, we intended to study the membrane integrity of spermatozoa, a main factor in fertilization. Therefore, this study has hunted to assess the relationship between smoking and male infertility by evaluating the semen parameters and the membrane integrity of the spermatozoa by hypo-osmotic swelling test among different subgroups of smoker, as per smoking index and non-smoker infertile males.

**Methods:** Membrane integrity of spermatozoa is analyzed by hypo-osmotic swelling test, the simplest and least expensive assay to assess the functional integrity of sperm-membrane among 204 infertile smokers who were subgrouped depending on the strength of smoking and 30 controls.

**Results:** Infertile smokers showed decreased membrane integrity among asthenospermia, teratozoospermia, asthenoteratozoospermia, and oligospermic compared to infertile non-smokers with a significant variation ( $P < 0.05$ ) among the different subgroups and also with control group.

**Conclusion:** Hypo-osmotic swelling test is an important tool for assessing the extent of membrane damage caused by smoking resulting in severity of infertility.

**Keywords:** Cigarette Smoking; Hypo-Osmotic Swelling Test; Infertility.

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## INTRODUCTION

Hazardous effects of cigarette smoking affect the male reproductive system as well as the respiratory and cardiovascular systems. Smoking reduces sperm production, motility, normal forms and fertilising capacity through increasing seminal oxidative stress and DNA damage to sperms (1). Thus, semen analysis is an important diagnostic tool to assess fertility status of the male. Conventional parameters used for evaluation of semen have narrow application since they only help to assess the structural integrity of the cell (2) rather than permeability and integrity of sperm membrane, which plays a leading role in fertilization. Each sperm consists of multiple sub-cellular compartments with different functions, all of which must be intact for successful fertilization (3). The most important mechanisms of fertilization, such as capacitation, acrosome reaction, and binding of spermatozoa to the egg surface are believed to be dependent on the functional integrity of the sperm membrane. Sperm function tests such as hypo-osmotic swelling (HOS) test (4) and zona-free hamster egg

penetration assay (5), have been projected for measuring male fertilization potential. However, the HOS test, modified for human use by Jeyendran et al, stands out as the simplest and least expensive assay to assess the functional integrity of sperm membrane (6). This test is also useful for the detection of damage during cryopreservation, toxic effects of drugs and chemicals, and to assess the quality of sperm (7). Several studies have shown that smoking leads to a decline in semen parameters such as sperm concentration, viability, forward motility, and morphology (1). Compounds of cigarette smoke like polycyclic aromatic hydrocarbons and smoking metabolites may act as chemotactic stimuli and thus induce an inflammatory response, recruitment of leukocyte and subsequent generation of Reactive Oxygen Species (ROS) (8). In males, it has been suggested that cigarette smoking negatively affects every system involved in the reproductive process. Spermatozoa from smokers have reduced fertilising capacity, and embryos display lower implantation rates (9, 10).

The available biological, experimental and epidemiological data indicate that up to 13% of infertility may be attributed to cigarette smoking, and sperm function tests are also of poorer

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quality in smokers than in non-smokers. To clinch the diagnosis of infertility due to loss of membrane integrity in smokers, the goal of this study is to evaluate a simple and potential parameter to test the altered capacity of spermatozoa for fertilization.

## METHODS

After the institutional ethical clearance (IHEC- UOM No 34/Ph.D/2009-10) and informed written consent, membrane integrity of spermatozoa was analyzed by hypo-osmotic swelling test, a simple and inexpensive assay, to assess the functional integrity of sperm membrane among 204 infertile smokers who were sub-grouped depending on the strength of smoking and 30 fertile non-smokers as controls. The semen samples were collected from different IVF centers and hospitals, Mysore, Karnataka, India. Genetic register was used to collect the information regarding lifestyle and reproductive history. The subjects were classified into smokers and non-smokers based on their smoking history. The smokers were grouped into 3 groups depending on smoking index: those who had smoked less than 10 cigarettes per day (< 3,625 cigarettes/year), 10 to 20 cigarettes per day (7, 305-3, 652/year), and more than 20 cigarettes per day (>7, 305/year). Semen was collected in sterile jars by masturbation process after 3 days of abstinence. Microscopic semen analysis was performed and subjects were classified into different infertile conditions (WHO) (11).

**Exclusion criteria:** Azoospermia (ejaculation of semen without sperms), retrograde ejaculation and aspermia (ejaculation failure) condition, subjects with previous reproductive tract infection and common primary ciliary dyskinesias like chronics in pulmonary disease manifested as chronic sinusitis, otitis media, nasal polyposis, and bronchiectasis were excluded. In addition to Kartagener (infertility and situs inversus) nephronophthisis, Bardet-Biedl syndrome, Alstrom syndrome, and Meckel-Gruber syndrome were also excluded.

### Hypo-osmotic swelling test (HOS)

Hypo-osmotic swelling test was carried out by the

modified protocol described by Jeyenderan et al (4). The semen samples were diluted with equal volumes of aqueous fructose and sodium citrate solution. The solution was then incubated at 37°C for 30 min. 10µl of the incubated mixture was placed in a clean glass slide and approximately 200 spermatozoa were scanned under microscope using a 40X objective. Sperms with coiled tail indicate positive reaction to the HOS test and uncoiled tail indicate as the negative response. The obtained data were documented and statistically analyzed using Prism software (version 3). ANOVA test was carried to find out the significant difference between and within the groups.

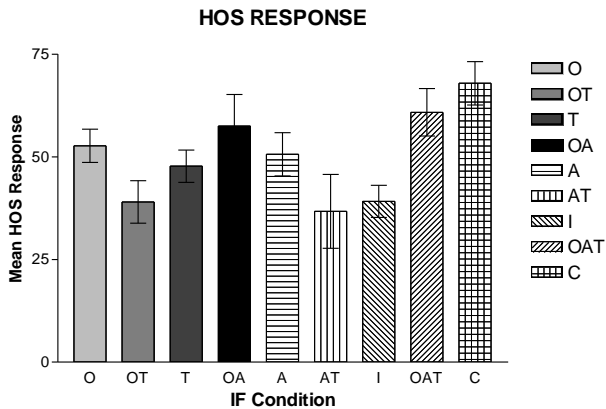
## RESULTS

Semen samples of both infertile and control groups were analyzed, based on the sperm characteristics. Infertile groups were categorized in to 10 different subgroups, which is depicted in Table 1. Response to the HOS test by the spermatozoa's was illustrated in the Figure 1 where oligoteratozoospermic (OT), Teratozoospermic(T), asthenoteratozoospermic(AT) and Idiopathic (I) conditions showed less than 50% of HOS response compared to other conditions, including control. Analysis of variance also revealed the significance of mean difference ( $p < 0.05$ ) existing between different infertile groups and the control group. Based on the smoking and non-smoking habits, further study and control groups were classified into infertile smoker (S IF), infertile nonsmokers (NS IF), control smokers (SC) and control nonsmokers (NS C). The HOS response of these groups were recorded and illustrated in Figure 2. Decrease in the HOS response is observed in smokers of both cases as well as in control groups compared to the nonsmokers. Among infertile smoking group, HOS response was significantly decreased compared to nonsmokers.

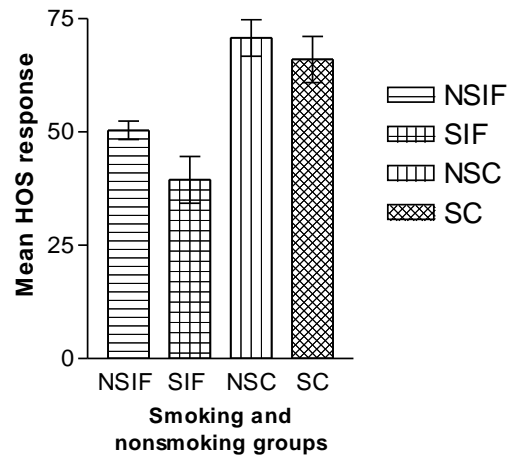
Of 204 infertile subjects, 53 were smokers. Based on the smoking index, further smoking infertile groups were analyzed and categorized into 3 groups, which is depicted in Table 2. Out of 10 different infertile subgroups, 4 groups were associated with the smoking habit. The teratozoospermic

**Table 1.** Incidence of different infertile conditions with count and motility

Condition	n=204 (%)	Number	Motility (a + b)
		Mean ± SD	Mean ± SD
Oligozoospermia	23 (11.27)	11.45 ± 1.1	48.24 ± 4.0
Oligoteratozoospermia	11 (5.39)	9.645 ± 1.5	46.67 ± 5.1
Teratozoospermia	25 (12.25)	51.46 ± 4.0	56.92 ± 3.9
Oligoasthenozoospermia	13 (6.37)	8.379 ± 1.4	20.15 ± 7.6
Asthenozoospermia	16 (7.9)	45.06 ± 3.0	21.94 ± 5.2
Asthenoteratozoospermia	8 (3.9)	47.76 ± 7.5	27.43 ± 8.9
Idiopathic condition	23 (11.27)	69.18 ± 6.3	65.75 ± 3.9
Oligoasthenoteratozoospermia	16 (7.8)	6.233 ± 1.2	18.33 ± 5.7
Azoospermia	56 (27.4)	-	-
Aspermia	13 (6.37)	-	-
Control	30	87.2 ± 5.32	62.5 ± 2.5



**Figure 1.** Response of spermatozoa in different infertile Condition for HOS test. (One-way Anova analysis  $P < 0.05$ ,  $F = 4.017$ )  
 O=Oligozoospermia, OT= Oligoteratozoospermia, T=Teratozoospermia, OA=Oligoasthenozoospermia, A=Asthenozoospermia, AT=Asthenoteratozoospermia, I=Idiopathic, OAT=Oligoasthenoteratozoospermia, C=Control.



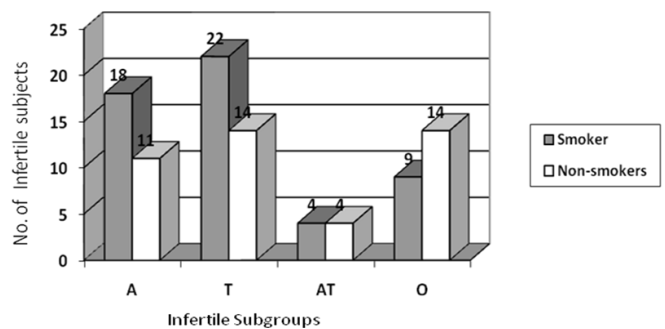
**Figure 2.** Comparison of HOS test response of spermatozans in smoker and non smoker infertile group with smoker and non smoker control group. (NS IF =Non smoker Infertile, NS C= Non smoker Control, S IF = Smoker Infertile, S C= Smoker Control).

individuals (n=22) associated with the smoking, followed by asthenozoospermia (n=18), oligospermic (n=9) and asthenoteratozoospermic (n=4). Figure 3 illustrates the association of infertile subgroups with respect to smoking and nonsmoking habit, out of 36 Teratozoospermic condition [T+ (O+T)] 22(61%) of them were associated with smoking habit, followed by Asthenospermic condition [A+ (O+A)]. Of 29 Asthenospermic subject 18(62%) of them were smokers who showed positive association with smoking. Furthermore, out of 8 Asthenoteratozoospermia, 4 (50%) subjects and 9 out of 23 (39%) oligospermic subjects showed smoking habits.

## DISCUSSION

In males, it has been suggested that cigarette smoking negatively affects every system involved in the reproductive process. Spermatozoa from smokers have reduced fertilising capacity, and embryos display lower implantation rates (9, 10) resulting in altered fertilisation and implantation. Diminished fertilising capacity at the chromosomal level, with a significantly higher ratio of single-stranded/double-stranded breakage of DNA in spermatozoa, was also found in smokers (12). Analysis of sperm DNA fragmentation after capacitation detected a detrimental effect produced by tobacco, altering the sperm swim-up selection process in smokers (13). A negative association of smoking and DNA fragmentation in the spermatozoa of healthy light and heavy smokers compared with non-smokers was also evident, especially with respect to disturbance of plasma membrane phospholipids asymmetry (14, 15).

Thus there was dual opinion among these factors which led us to prove the importance of HOS test and demonstrate that the membrane integrity is an important marker in fertilisation. HOS test is based on the semi-permeability of the intact cell membrane, which causes spermatozoa to swell under hypo-osmotic conditions when an influx of water



**Figure 3.** Representation of smokers and nonsmokers in different associated infertile conditions  
 A= Asthenospermic condition [A+ (O+A)], T=Teratozoospermic condition [T+ (O+T)]  
 AT=Asthenoteratozoospermia condition, O=Oligospermic condition

results in an expansion of cell volume (16-18). It has recently been hypothesized that a defect in the functional integrity of the sperm membrane, which is detectable by the HOS test, may reduce fertility potential by causing implantation disorders rather than fertilization problems (18).

In the present study, decreases in HOS scoring were observed in different sub groups of infertile conditions compared to the control group. Teratozoospermic and associated teratozoospermic condition [T+ (O+T), (A+T)] showed decreased HOS scoring compared to control. Teratozoospermic semen samples were characterized by a higher content of morphologically abnormal and immature spermatozoa. Furthermore, this may increase ROS production. This acts adversely on the membrane and thereby causing membrane damage which decreases HOS Scoring (19, 20). What appears to be happening is that smoking-damaged sperm lose much of their ability to fight off destructive oxygen molecules such as free radicals in the

**Table 2.** Conditions associated with infertile smokers with respect to smoking index (Infertile cases).

Condition	> 20 cigarettes/day	10-20 cigarettes/day	<10 cigarettes/day	Total
Asthenozoospermia (A)+(O+A)	8	6	4	18
Teratozoospermia (T)+(O+T)	7	10	5	22
Asthenoteratozoospermia (A+T)	-	3	1	4
Oligozoospermia	6	2	1	9

seminal fluid. Interestingly, in addition to making sperm cells more sensitive to oxidative stress, smoking itself increases the concentration of free radicals in the seminal fluid (21).

Nicotine has a significant influence on sperm morphology and sperm count (22). Change in sperm morphology is the contributing factor in altered membrane integrity. Smokers have been shown to have seminal cotinine and trans-3-hydroxycotinine levels similar to the serum, while seminal nicotine was significantly increased compared to the serum. Elevated seminal cadmium in smokers has been observed if more than 20 cigarettes/day were smoked, with a significant negative correlation between seminal cadmium in blood with smoking and sperm density (23, 24). Seminal cadmium in normozoospermics was shown to be higher in smokers compared with non-smokers, being correlated with number of cigarettes consumed/day (15). Also, lead levels in seminal plasma have been shown to be higher in infertile smokers compared to fertile men and infertile non-smokers (25) who showed that benzo(a)pyrenediol epoxide-DNA adducts in sperm cells are increased by smoking. The formation of adducts in spermatozoa is a potential source of variation in membrane integrity. The sperm ultrastructure changes have been noted in smokers in the form of the number and arrangement of axonemal microtubules (26, 27). Yeung et al observed and suggested that the percentage of coiled sperm had correlated with heavy smoking and electron microscopy revealed coiling, which was similar to findings in the present study where the HOS test revealed its potentiality (28).

The general population is acutely aware of the role of smoking in lung and heart diseases, while the adverse effects of smoking on male reproductive health is less known. During the past two decades, the whole perception of smoking as having an insignificant effect on male fertility has changed cigarette smoking probably tilts the delicate balance of ROS and antioxidant levels (34). Increased ROS have been shown to be detrimental to the DNA of spermatozoa, thus producing a negative effect on the viability and morphology of spermatozoa. Gaur et al., demonstrated that teratozoospermic condition would be produced in heavy and moderate smokers (33). Other conditions like oligospermic were also observed in the infertile smokers group. The above conditions may be due to other etiologies, which should be considered besides smoking with other habits that need additional in depth exploration.

## CONCLUSION

HOS test play a vital role in evaluating sperm functional status. In the present study it is evident that sperm membrane integrity is affected in different subgroups of infertile male, and cigarette smoking still causes declines in the membrane integrity status of spermatozoa. The defective membrane in the spermatozoa is detected through HOS test, which may be helpful in improving success rate in fertilization and HOS a simple effective mode in detection of morphologically defective sperms in terms of membrane integrity.

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