ORIGINAL ARTICLE

EFFECT OF WORKSTRESS AND SMOKING TOWARDS SPERM QUALITY AMONG INFERTILE MALE

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ABSTRACT

Male infertility is a relatively common condition affecting approximately 1 in 20 of the male population. DNA fragmentation is an important factor in the etiology of male infertility. Men with high DNA fragmentation levels have significantly lower odds of conceiving, naturally or through procedures such as intrauterine insemination and IVF. The most common contributing factor of male infertility is smoking. Studies have shown that smoking intensity is positively associated with job demands and stress. Therefore, we believe that work stress increases the nicotine-dependent thus causing lower male fertility rate. As proper protamine to histone ratio is essential to produce viable sperm, smoking is strongly suspected to reduce sperm viability through histone-to-protamine transition abnormalities. These abnormalities, results in sperm with high DNA damage when exposed to excessive free radical. This present study was undertaken to evaluate the relationship of work stress, smoking and sperm quality. A total of 210 infertile patients attending Medical Assisted Contraceptive Clinic (MAC), UKMMC were selected for the study. Smoking status and stress level of patients were collected after obtaining relevant consent. Histone-to-protamine ratio was acquired using Aniline Blue staining and Chromomycin A3 staining respectively. Sperm DNA fragmentation was estimated using Comet Assay. Result revealed that smokers tend to be more stressful (r = .446, p <. 001). The result showed a significantly increased level of histone (r = .385, p <. 001) and incomplete protamination (r = .492, p <. 001) in smokers. The imbalance of histone-to-protamine ratio lead to increase of DNA damage. All the data were analyzed using SPSS version 20.0. Result revealed that patients who smoke are more stressful at work. Higher proportion of abnormal sperm histone to protamine ratio were found among smokers suggesting that cigarette smoking may inversely affect male fertility.
Keywords: *male, stress, smoking, sperm quality*

INTRODUCTION

In recent decade, infertility affects 10% - 15% of couples worldwide and it is recognized as a public health problem[1]. From the afore mentioned prevalence, male infertility accounts for half of the problem[2]. Drilling deeper into the subject, numerous studies have reported that cigarette smoking plays a major role in sperm quality and consequently male infertility.[3,4,5].

Cigarette smoking is well known to be a leading source of preventable morbidity and morbidity[6]. There are many reason one can pick up the habit of smoking[7]. One of which is job stress. Stress present physiological and psychological challenges to the body and mind and can be a result of real or perceived challenge to homeostasis[8]. In response to stress, individuals try to maintain homeostasis through a number of mechanism, one potential mechanism is self-medication by smoking[9]. Many smoking studies have been carried out for the past three decades among adolescents who belongs in the working age group population[10].

Job-related stress might affect smoking behavior because smoking may relieve stress and stress can make individuals more focused[11]. Some studies have showed that heavy smoking is associated with high job demands[12] low job control[13] or peer influence at work[14]. This evidence suggest that job stress could causally related to smoking.

Smoking can be detrimental to human sperm due to high concentration of free radicals in the smoke and can potentially induce the production of cellular reactive oxygen species in the human body[15]. Due to high concentration of polysaturated fatty acids on human sperm plasma membrane, spermatozoa are especially prone to oxidative damage caused by smoking[16]. The exact pathophysiology underlying cigarette smoking and sperm deterioting is still unclear[17]. However, it has been reported that cigarette smoking may affect sperm DNA integrity[18].

Normally, sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins[19]. Chromatin's condensed and insoluble nature features, allows it to protect genetic integrity and facilitate transport of the paternal genome through the male and female reproductive tracts[20]. For a spermatozoon to be fertile, chromatin must be capable of undergoing decondensation at an appropriate time in the fertilization process[21]. Infertile men manifest various nuclear alterations, including an abnormal chromatin structure, chromosomes with microdeletions, aneuploidies and DNA strand breaks[22].

Evidence suggest that any level of severity damage to the organization of sperm nuclei genetic material will negatively correlated with the fertility potential of sperm, irrespective of either in vivo or in vitro fertilization[23]. The formation of mature spermatozoa is a unique process involving a series of meiotic and mitotic changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins, and the final addition of protamine leading to a highly packaged chromatin[24]. Sperm DNA is organized in a specific manner that keeps the chromatin in the nucleus compact and stable[25].

Environmental stress such as smoking can disturb the biochemical events that occur during spermatogenesis, and this can ultimately lead to an abnormal chromatin structure that leave the sperm DNA more vulnerable to damage thus causing impairment in fertility[26]. Alternatively, it could be the result of free radical-induced damage[27] or a consequence of apoptosis[28]. The exact mechanisms by which chromatin abnormalities or DNA damage arise in human spermatozoa is not precisely understood, but three main theories have been proposed, namely defective sperm chromatin packaging, apoptosis and oxidative stress[29].

Therefore, this study was determined to find the correlation between stress, smoking and sperm quality in men. So far, studies on the effect of smoking towards the sperm compaction protein are limited. Highly likely due to difficulty obtaining sample from patients resulting from religious and psychological issues.

METHODOLOGY

Ethic Statement

Ethical approval was obtained from UKMMC Scientific Research and Ethical Committee with Grant Code No: NN-2014-100.

Study Participants

Participants were male patients aged between 25-45 years old who attended Medically Assisted Contraceptive Clinic (MAC) of Universiti Kebangsaan Malaysia Medical Centre (UKMMC) for fertility procedures. A total no of 210 participants were involved in this study. The inclusion criteria of this study was male with sperm count more than 15 million (normospermia) and sperm count less than 15 million (oligospermia). However, patients with no sperm count (azoospermia) is was excluded from the study. Patient's smoking status and demographic data were collected during the interview.

Stress Measures

Union of Shops, Distribution and Allied Worker (USDAW) Questionnaire Participant's job stress were assessed using the Union of Shops, Distribution and Allied Worker (USDAW) questionnaire. USDAW questionnaire is an instrument used to evaluate individual's job stress level. The questionnaire consisted of four components physical, environment, socio-economy and behavior at work. It takes about 15 minutes to answer all the questions. The stress level was determined by calculating the average score of participants. Scale from 33 to 83 was considered less stressful, while a scale from 84 to 165 was considered as stressful.

Sample Collection

All participants were given a sterile container for semen collection. Semen collection was done in accordance with WHO (2010) guideline[25].

Semen Analysis

A total of 10 µl of sperm sample was pipetted and placed on MaklerR Counting Chamber. Sperm were counted under a light microscope using X200 magnification. Sperm count of more than 15 million is considered normospermia (normal) while sperm count less than 15 million is considered as oligospermia.

Aniline Blue Staining

The Aniline Blue stain is able to discriminates between lysine-rich histones and arginine/cysteine-rich protamines. This technique provides a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the other hand, protamine rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by the aniline blue stain.

Slides were prepared by smearing 5 µl of washed semen sample. The slides were air-dried and fixed for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS). The smear was dried and stained for 5 minutes in 5% aqueous aniline blue solution (pH 3.5). Sperm heads containing immature nuclear chromatin were stained blue, and those with mature nuclei do not take up the stain. The percentage of spermatozoa stained with aniline blue was determined by counting 200 spermatozoa per slide under bright field microscopy.

Chromomycin A_3 Staining

Chromomycin A_3 is a guanine–cytosine-specific fluorochrome that reveals chromatin that is poorly packaged in human spermatozoa through indirect visualization of protamine-deficient DNA. Chromomycin A_3 and protamines compete for the same binding sites in the DNA. Therefore, high CMA_3 fluorescence is a strong indicator of the low protamination state of spermatozoa.

For CMA_3 staining, semen smears were first fixed in methanol–glacial acetic acid 3 : 1 at 4°C for 20 minutes and were then allowed to air-dry at room temperature for 20 minutes. The slides were treated for 20 minutes with 100 µl CMA_3 solution 0.25 mg/ml CMA_3 in McIlvain's Ph 7.0 buffer and 10 mmol/l $MgCl_2$. The slides were then rinsed in buffer and mounted with 1 : 1 v/v PBS–glycerol. The slides were then kept at 4°C overnight after which evaluation of fluorescence was performed using a fluorescent microscope. A total of 200 spermatozoa were randomly evaluated on each slide. Evaluation of CMA_3 staining was done by distinguishing spermatozoa that stain bright yellow (CMA_3 positive) from those that stain a dull yellow (CMA_3 negative).

Comet Assay

The comet assay, also known as single-cell gel electrophoresis is used for the analysis of DNA damage in an individual cell. Neutral electrophoresis buffer conditions were used to show that the migration of double-stranded DNA loops from a damaged cell in the form of a tail unwinding from the relaxed supercoiled nucleus was proportional to the extent of damage inflicted on the cell. This finding took on the appearance of a comet with a tail when viewed using the fluorescent microscope and DNA stains. The damage is quantified by measuring the displacement between the genetic material of the nucleus "comet head" and the resulting tail. The tail lengths are used as an index for the damage. Also, the "tail moment," which is the product of the tail length and intensity (fraction of total DNA in the tails), has been used as a measuring parameter. The tail moment can be more precisely defined as being equivalent to the torsional moment of the tail.

Sperm cells were cast into miniature agarose gels on microscope slides lysed in situ to remove DNA-associated proteins and to allow the compacted DNA in the sperm to relax. The lysis buffer (Tris 10 mmol/l, 0.5 mol/l EDTA, and 2.5 mol/l NaCl, pH 10) contains 1% Triton X-100, 40 mmol/l dithiothreitol, and 100 µg/ml proteinase K). Microgels were then electrophoresed (20 minutes at 25 V/ 0.01 A) in neutral buffer (Tris 10 mmol/l containing 0.08 mol/l boric acid and 0.5 mol/l EDTA, pH 8.2), during which the damaged DNA migrates from the nucleus towards the anode. The DNA was visualized by staining the slides with fluorescent DNA binding dye SYBR Green I. Comet measurements were then performed using fluorescent microscopy. All of the images were analyzed using the Comet Assay Software Project (CASP).

RESULTS

Table 1: ***Demographic Data of Participant***

Demographic variable	Frequency	Percentage
Age		
20 – 30 years	60	28.6
31-40 years	119	56.7
41-50 years	28	13.3
51- 60 years	3	1.4
Race		
Malay	159	75.7
Chinese	38	18.1
Indian	12	5.7
Others	1	0.5
Education		
No formal education	1	0.5
Primary School	9	4.2
Lower Secondary School	6	2.9
Upper Secondary School	43	20.5
Vocational School	21	10.0
University/ College	130	61.9
Working Status		
Goverment sector	60	28.6
Private sector	125	59.5
Own job	25	11.9
Retired	0	0
Unemployed	0	0
Smoking Status		
Smoker	107	51.0
Non smoker	103	49.0

Table 1 shows that a total population of 210 participants aged within 20 to 60 from Universiti Kebangsaan Malaysia Medical Centre (UKMMC) were recruited in this study. Most of the participant in this study were within 31 to 40 years old (56.7%). A total of 75.7% of the population of this study were Malays followed by Chinese (18.1%), Indian (5.7%) and others (0.5%). Besides that, nearly half of the participants received tertiary education (61.9%) and working at private sectors (59.5%). 51% of the population of the study were smokers and 49% were nonsmokers.

Table 2: ***Correlation between job stress and intensity of cigarette smoking***

	Number of cigarette smoked (r)
Job stress	(r = .446, p < .001)

(Notes : * p < 0.05, ** p < 0.01)

Table 2 shows there is significant positive correlation between job stress and number of cigarette smoked. This shows that number of cigarette smoked is influenced by job stress.

Table 3: ***Correlation between intensity of cigarette smoking and number of immature histone protein in sperm DNA***

Variable	Number of Immature Histone Protein in sperm DNA (r)
Intensity of cigarette smoking	(r = .385, p < .001)

(Notes : * p < 0.05, ** p < 0.01)

Table 3 shows there is significant positive correlation the number of cigarette smoked and number of immature histone protein in sperm DNA. This shows that number of immature histone protein in sperm DNA is influenced by the intensity of cigarette smoked.

Table 4: Correlation between intensity of cigarette smoking and number of incomplete protamine protein in sperm DNA

Variable	Number of Incomplete Protamine Protein in sperm DNA (r)
Intensity of cigarette smoking	(r = .492, p < .001)

(Notes : * p < 0.05, ** p < 0.01)

Table 4 shows there is significant positive correlation the number of cigarette smoked and number of incomplete protamine protein in sperm DNA. This shows that number of incomplete protamine protein in sperm DNA is influenced by the intensity of cigarette smoked.

Table 5: ***Correlation between number of immature histone protein and sperm DNA damage***

Variable	Sperm DNA damage (r)
Number of immature histone protein	(r = .697, p < .001)

(Notes : * p < 0.05, ** p < 0.01)

Table 5 shows there is significant positive correlation the number of immature histone protein and DNA damage. Results shows that the increase of the number of immature histone protein increases the sperm DNA damage.

Table 6: ***Correlation between number of incomplete histone protein and sperm DNA damage***

Variable	Sperm DNA damage (r)
Number of incomplete protamine protein	(r = .775, p < .001)

(Notes : * p < 0.05, ** p < 0.01)

Table 6 shows there is significant positive correlation the number of incomplete protamine protein and DNA damage. Results shows that the increase of the number of incomplete protamine protein increases the sperm DNA damage.

DISCUSSION

The main purpose of this study was to determine the correlation between job stress, smoking and the quality of sperm among men. The result shows the intensity of cigarette smoking is correlated with job stress. It is hypothesized that unfavorable work environments and adverse psychosocial work conditions may play important roles in increasing smoking intake[30]. This finding is in line with the study conducted by Ayyagari[9] that shows, job related stress might affect smoking behavior because smoking may relieve stress. Then stress can make individuals more alert and focused.

Alternatively, individuals may both self-select into stressful jobs and choose to smoke based on unobserved factors[31].

In addition, findings had also showed that smoking is associated with a significant increase in histone-to-protamine ratio in human sperm. Most DNA in mature human sperm is bound to protamines, as somatic histones are replaced during spermiogenesis[32]. However, biochemical analyses of human sperm proteins indicate retention of some histones resulting in a nuclear protein composition that is about 90% protamine and 10% histone[33]. The protamine-bound DNA is coiled into tightly compacted toroids that contain about 50 kb of DNA[34]. All of these levels of compaction and organization help to protect sperm chromatin during transport through the male and female reproductive tract and also ensures the paternal genome is delivered in a form that allows developing embryo to accurately express genetic information[35]. The histone-to-protamine ratio abnormalities might be affected by the smoking related reactive oxygen species[36]. A study by Hammadeh[37] also demonstrated that oxidative stress induced by cigarette smoking may have a significant inverse effect on the protamination process. It is suggested that cigarette smoking may impair the mRNA expression of protamine, which may partially contribute to the deficient replacement of histone by protamines in sperm[38].

This study also demonstrated abnormal histone-to-protamine ratio increase DNA damage in sperm. It is known that protamine-insufficient sperms have increased DNA damage in the sperm cells[39]. This could be due to the consequence of abnormalities in the correct nucleohistone to nucleoprotamine transition during spermiogenesis, resulting in abnormal protamination in the mature sperm cell and thus making the DNA more vulnerable to oxidative stress, nucleases, or mutagens[40]. This finding is in agreement with Yu[41] who also observed increase DNA damage in men with abnormal histone-to-protamine transition.

CONCLUSIONS

In conclusion, this study has demonstrated that smoking intensity is influenced by job stress. Smoking also affects the sperm quality but increasing the number of immature histone protein and incomplete protamine protein in sperm DNA. This causes disruption in histone-to-protamine ratio in human sperm. Considering histone and protamine plays vital role in DNA integrity, this disruption made sperm DNA more vulnerable and susceptible to damage. However, we strongly feel this condition can be improved as smoking habit and job stress are modifiable life style.

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REFERENCE

1. Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology* 2015; **13**(1): 37.

2. O'Brien, K. L. F., Varghese, A. C., & Agarwal, A. The genetic causes of male factor infertility: a review. *Fertility and sterility* 2010; **93**(1): 1-12.

3. Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. Effect of oxidative stress on male reproduction. *The world journal of men's health* 2014; **32**(1): 1-17.

4. Li, Y., Lin, H., Li, Y., & Cao, J. Association between socio-psycho-behavioral factors and male semen quality: systematic review and meta-analyses. *Fertility and sterility* 2011; **95**(1): 116-123.

5. Viloria, T., Meseguer, M., Martínez-Conejero, J. A., O'Connor, J. E., Remohí, J., Pellicer, A., & Garrido, N. Cigarette smoking affects specific sperm oxidative defenses but does not cause oxidative DNA damage in infertile men. *Fertility and sterility* 2010; **94**(2): 631-637.

6. Rostron, B. L., Chang, C. M., & Pechacek, T. F. Estimation of cigarette smoking–attributable morbidity in the United States. *JAMA internal medicine* 2014;, **174**(12): 1922-1928.

7. Jarvis, M. J. ABC of smoking cessation: Why people smoke. *BMJ: British Medical Journal* 2004; **328**(7434): 277.

8. Nash, J. M., & Thebarge, R. W. Understanding psychological stress, its biological processes, and impact on primary headache. Headache:

The Journal of Head and Face Pain 2006; **46**(9): 1377-1386.

9. Ayyagari, P., & Sindelar, J. L. The impact of job stress on smoking and quitting: evidence from the HRS. *The BE journal of economic analysis & policy* 2010; **10**(1).

10. Lim, K. H., Sumarni, M. G., Kee, C. C., Norhamimah, A., Wan Rozita, W. M., & Amal, N. M. Prevalence, smoking habit and factors related to smoking and nicotine addiction among lower secondary school male students in Kota Tinggi district, Johor, Malaysia. *Malaysian Journal of Public Health Medicine* 2010; **10**(1): 28-37.

11. Azagba, S., & Sharaf, M. F. The effect of job stress on smoking and alcohol consumption. *Health economics review* 2011; **1**(1): 15.

12. Chon, S. H., Kim, J. Y., Cho, J. J., & Ryoo, J. G. Job characteristics and occupational stress on health behavior in Korean workers. *Korean Journal of Family Medicine* 2010; **31**(6): 444-452.

13. Wong, K. Y., & Md Mizanur, R. Factors Determining Attempt-To-Quit Smoking Among Adult Current Smokers In Sarikei, Sarawak, Malaysia. *Malaysian Journal of Public Health Medicine* 2017; **17**(2): 96-102.

14. Viloria, T., Meseguer, M., Martínez-Conejero, J. A., O'Connor, J. E., Remohí, J., Pellicer, A., & Garrido, N. Cigarette smoking affects specific sperm oxidative defenses but does not cause oxidative DNA damage in infertile men. *Fertility and sterility* 2010: **94**(2): 631-637.

15. Henkel, R. R. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian journal of andrology* 2010: **13**(1): 43.

16. Aghamohammadi, A., & Zafari, M. The impact of cigarette smoking on sperm parameters: a cross-sectional study. *Age* 2011: **36**(7.76): 35-23.

17. Taha, E. A., Ez-Aldin, A. M., Sayed, S. K., Ghandour, N. M., & Mostafa, T. Effect of smoking on sperm vitality, DNA integrity, seminal oxidative stress, zinc in fertile men. *Urology* 2012; **80**(4): 822-825.

18. Ausió, J. The shades of gray of the chromatin fiber. *Bioessay*s 2015; **37**(1): 46-51.

19. Miller, D., Brinkworth, M., & Iles, D. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* 2010; **139**(2): 287-301.

20. Agarwal, A., & Said, T. M. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human reproduction Update* 2003; **9**(4): 331-345.

21. Sharma, R. K., Said, T., & Agarwal, A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian journal of andrology* 2004; **6**(2): 139-148.

22. Balhorn, R. The protamine family of sperm nuclear proteins. *Genome biology* 2007; **8**(9): 227.

23. Agarwal, A. Significance of oxidative stress and sperm chromatin damage in male infertility. *Male fertility and lipid metabolism* 2003; **13**: 157-183.

24. Aitken, R. J., De Iuliis, G. N., & McLachlan, R. I. Biological and clinical significance of DNA damage in the male germ line. *International journal of andrology* 2009; **32**(1): 46-56.

25. Sakkas, D., & Alvarez, J. G. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and sterility* 2010; **93**(4): 1027-1036.

26. Agarwal, A., & Said, T. M. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU international* 2005; **95**(4): 503-507.

27. Muratori, M., Tamburrino, L., Marchiani, S., Cambi, M., Olivito, B., Azzari, C., ... & Baldi, E.. Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. *Molecular Medicine* 2015; **21**(1): 109.

28. Yoong, F. J., See, B. L., & Baronovich, D. L. Financial literacy key to retirement planning in Malaysia. *Journal of Management and Sustainability* 2012; **2**(1): 75.

29. Tung, L. C., & Comeau, J. D. Perceived benefits and drawbacks of the retirement age policy in Malaysia: HR perspective. *International Journal of Business and Management* 2012; **7**(19): 1.

30. MOHD SIDIK, S., Rampal, L., & Kaneson, N. Prevalence of emotional disorders among medical students in a Malaysian university. *Asia Pacific Family Medicine* 2003; **2**(4): 213-217.

31. Rusli, B. N., Edimansyah, B. A., & Naing, L. Working conditions, self-perceived stress, anxiety, depression and quality of life: a structural equation modelling approach. *BMC public health* 2008; **8**(1): 48.

32. Padmaja, A. The Impact of Job Stress on Smoking and Quitting: *Evidence from the HRS*.

33. Zhang, X., Gabriel, M. S., & Zini, A. Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. *Journal of andrology* 2006; **27**(3): 414-420.

34. Ward, W. S. Function of sperm chromatin structural elements in fertilization and development. *MHR: Basic science of reproductive medicine* 2009; **16**(1): 30-36.

35. De Jonge, C. J., & Barratt, C. (Eds.). The sperm cell: production, maturation, fertilization, regeneration. *Cambridge University Press* 2006.

36. Hammadeh, M. E., Hamad, M. F., Montenarh, M., & Fischer-Hammadeh, C. Protamine contents and P1/P2 ratio in human spermatozoa from smokers and non-smokers. *Human Reproduction* 2010; **25**(11): 2708-2720.

37. Sepaniak, S., Forges, T., Gerard, H., Foliguet, B., Bene, M. C., & Monnier-Barbarino, P. The influence of cigarette smoking on human sperm quality and DNA fragmentation. *Toxicology* 2006; **223**(1): 54-60.

38. Castillo, J., Simon, L., Mateo, S., Lewis, S., & Oliva, R. Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients. *Journal of andrology* 2011; **32**(3): 324-332.

39. Aitken, R. J., Smith, T. B., Jobling, M. S., Baker, M. A., & De Iuliis, G. N. Oxidative stress and male reproductive health. *Asian journal of andrology* 2014; **16**(1): 31.

40. Yu, B., Qi, Y., Liu, D., Gao, X., Chen, H., Bai, C., & Huang, Z. Cigarette smoking is associated with abnormal histone-to-protamine transition in human sperm. *Fertility and sterility* 2014; **101**(1): 51-57.