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INFLUENCE OF MUMPS DISEASE ON PROTAMINE CONTENTS IN HUMAN SPERMATOZOA DURING A LATE CHILDHOOD

Taleb K. Odah
Email: taliboda.bio@utq.edu.iq
Department of biology, Collage of Education for Pure Sciences, University of Thi-Qar, 64001, Iraq.

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Abstract:

Protamine content is necessary for proper sperm chromatin condensation and subsequent male fertility. The exact effect of mumps disease on male fertility remains controversial. The objective of this study was to evaluate the effect of mumps disease on protamine content of sperm in mumps patients and non-patients.: Protamines 1 (P1) and 2 (P2) were quantified by gel electrophoresis in the sperm of 212 patients and 252 non-patients. Sperm DNA fragmentation was analyzed employing the terminal deoxynucleotidyl transfer ase-mediated dUTP nick-end labelling (TUNEL) assay and non-condensed chromatin was evaluated using chromomycin A3 (CMA3). Levels of oxidative stress markers were determined in seminal plasma using an enzyme linked immunosorbant assay (ELISA) and chemical reactions.: Protamine 2 concentrations were significantly lower ($P \leq 0.050$) in patients than in non-patients. In contrast P1/P2 ratios were significantly higher ($P \leq 0.010$) in patients (1.35+0.47 ng/107 sperm) than in non-patients (1.12+0.21 ng/107 sperm). The oxidative stress and mump disease markers, reactive oxygen species (ROS), family history and mobile use were significantly higher ($P \leq 0.010$) in patients than in non-patients, and correlated significantly ($P \leq 0.050$) with P1/P2 ratios. P2 showed significant negative ($P \leq 0.050$) correlations with ROS,. CMA3 and TUNEL were also significantly higher ($P \leq 0.010$) in patients (37.4+8.2 and 17.5+5.4%) than in non-patients (29.9+7.2 and 11.4+4.3%).

Keywords: protamine / sperm chromatin condensation / sperm DNA fragmentation / oxidative markers / mumps disease

1.INTRODUCTION

Human beings and other mammals express two protamines, protamine1 (P1) and protamine 2 (P2). They are the most abundant nuclear proteins present in human sperm (Aoki and Carrell, 2003; Carrell et al., 2007). They occur normally in a strictly regulated 1/1ratio (Corzett et al., 2002) and replace most of the histones, i.e. _85–90% (Li et al., 2008). Protamines are a diverse family of small and highly basic proteins (5–8 kDa) which are about half the size of a typical histone. They are positively charged due to the high content of basic amino acids, such as the arginine residues. It had been proposed that the driving forces for this arginine-rich selection could be the DNA-binding function of protamine P1 resulting in a more compact sperm nucleus and the interaction and strong activation of creatine kinase II in the oocyte by protamine (Rooney and Zhang, 1999). In addition, protamines contain a significant number of cysteine residuesthat are important in the final stage of sperm nuclear maturation as they form multiple inter- and intra-protamine disulfides cross bonds (Loir and Lanneau, 1984). The formation of these inter- and intramoleculardisulphide bonds between cysteine residues strongly stabilizes the nucleoprotamine structure in the sperm nucleus and stabilizesthe folding of different protamine domains (Vilfan et al., 2004). Therefore, both P1 and P2 are necessary for proper chromatin condensation. Animal models have shown that an insufficiency of P1 or P2causes infertility in mice and P2 deficiency causes damage of the spermDNA and embryo death (Cho et al., 2001, 2003).. The highly positive charge of protamines allows the neutralization of negatively charged DNA, resulting in a high level of compaction in the sperm nucleus. This results in sperm DNA being 6-fold more highly condensed than in mitotic chromosomes (Ward and Coffey, 1991). This highly condensed structure protects the DNA from oxidative stress and other internal or external factors (Braun, 2001) and protects the genetic material during transfer through the male and female reproductive tracts (reviewed by Oliva, 2006). A defect in spermatid protamination and disulphide bridge formation due to inadequate oxidation of thiol groups will negatively affect sperm chromatin packaging, making sperm cells more vulnerable to reactive oxygen species (ROS) induced DNA fragmentation (Erenpreiss et al., 2006). Oxidative stress is caused by an imbalance between the production of ROS and antioxidant capacity of the cell (Sharma and Agarwal, 1996).

Mumps is a viral disease caused by the mumps virus that is preventable with vaccination. Initial symptoms of mumps are non-specific and include fever, headache, malaise, muscle pain, and loss of appetite. These symptoms are usually followed by painful swelling of the parotid glands, called parotitis, which is the most common symptom of a mumps infection. Symptoms typically occur 16 to 18 days after exposure to the virus and resolve within two weeks. About one third of infections are asymptomatic

Oxidative stress appears to be the major cause of DNAdamage in the male germ line (Aitken et al., 2003a,b; Saleh et al., 2003). Furthermore, many studies have indicated a significant correlation between DNA damage and high levels of ROS in infertile patients (Barroso et al., 2000; Aitken and Baker, 2004).

DNA damage in the male germ line has been associated with poorsperm quality, low fertilization rates, impaired preimplantation development, increased abortion rates and an elevated incidence of disease in the offspring, including childhood cancer. The exactcauses of this DNA damage are still unclear but the major candidatesare oxidative stress and aberrant apoptosis (Lewis et al., 2008). The precise etiology of sperm DNA fragmentation is still poorly understood, but a relationship between mumps disease and increased DNA damage in infertile patients compared with non-patients has been demonstrated (Zenzes, 2000; Saleh et al., 2003). Several investigatorshave now found a link between oxidative stress and sperm DNA damage (reviewed by Tremellen, 2008).

Mumps orchitis disease has been associated with significantly increased levels of seminal ROS, which cause oxidative stress (Saleh et al., 2002). Mumps orchitis may inversely affect sperm quality and decrease the antioxidant activity in seminal plasma (Pasqualottoet al., 2008). mumps may induce alterations of the sperm plasma membrane and cause a high degree of DNA fragmentation (Churchand Pryor, 1985). Recent studies have shown that the spermatozoa of mumps patients have significantly higher levels of DNA fragmentation than those of non-patients (Sepaniak et al., 2006; Elshal et al., 2009).

The purposes of the present study were to determine the effect of mumps disease on sperm chromatin structure by directly quantifying sperm P1and P2 concentrations in patients and non- patients,and to evaluate the relationship between mumps disease and oxidative stress and the effect on DNA protamination and other sperm parameters, to find out and compare the levels of seminal plasma oxidative stress biomarkers (ROS,family history and mobile use) .

2.MATERIALS AND METHODS

2.1.Subjects

A total of 232 semen sample from male partners of couples facing infertility who attending assisted reproduction and andrology laboratory at the AL- hussein hospital teaching, fertility unit were included in this study. The men included 126 nonpatients106 patients. Information regarding bodyweight, height, smoking, consumption of alcohol and occupational exposures was obtained from a questionnaire. Patients were also asked about any history of genital or urinary tract infection varicocele, surgery, vasectomy-reversal surgery or history of any chronic illness such as tuberculosis.. In addition, samples with a sperm concentration of less than 10 × 106 ml were excluded because they offered insufficient material. This stringent selection was done to exclude as many known co-existing factors as possible from the study groups, since we aimed to study the impact of mumps disease on specific aspects of sperm characteristics

2.2.Sperm collection and preparation

Only one sample per patient was included in the study. Semen samples were collected by masturbation after 3–4 days of sexual abstinence. Samples were collected in sterile containers and allowed to liquefy at 378C for 30 min and processed immediately after complete liquefaction, according to World Health Organization (1999) guidelines as previously described by Hammadeh et al. (2006). Briefly, semen samples were examined for volume, pH, sperm concentration, sperm motility, sperm vitality, membrane integrity, sperm morphology and presence of agglutination, according to WHO guidelines (World Health Organization, 1999).

2.3.Assessment of chromatin condensationof spermatozoa

Non-condensed chromatin was measured by chromomycin A3 (CMA3) staining as previously described by Bianchi et al. (1993). Fluorochrome was examined using a Zeiss photomicroscope III using acombination of exciter dichromic barrier filter of BP 436/10: FT 580:LP 470.

2.4.DNA fragmentation analysis DNA fragmentation was assessed using the terminal deoxyribonucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay as previously described (Borini et al., 2006). The TUNELassay was performed using the in situ Cell Death Detection Kit following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany).

2.5.Extraction of sperm nuclear proteins

Tail dissociation

Sperm tail dissociation was performed as previously described by Balhornet al. (1977),

Nuclear protein extraction Sperm nuclear protein extraction of each sample (n ¼ 116) was performed by using an established protein extraction protocol described by Carrell and Liu (2001)

2.6.Production of a protamine standard

A human protamine standard was prepared as described by Mengual et al. (2003).

2.7.Protein extraction control sample

A quality control sample was applied to ensure that protamine quantification could produce valid and reproducible results; the preparation of these samples were as described (Aoki et al., 2005).

2.8.Acid-urea-page ,immunobloting and P1and P2 quantification

Protamine extracts were tested by acetic acid-urea polyacrylamide gel electrophoresis as described by Carrell and Liu (2001) The gels were pre-run at 200 V, 40 mA for 1.5 h.

Nucleoprotein samples were loaded onto the gel and then run at 200 V, 80 mA for 5–6 h. The gel was divided into three parts after electrophoresis. One-third of the gel was stained with Coomassie Brilliant Blue (0.2% Brilliant R250, 0.01% Brilliant G250, 50% methanol and 10% acetic acid) (Serva, Germany) and subsequently de-stained in 20% methanol and 10% aceticacid and scanned. The other two parts of the gel were used to identity of the protamine bands using western blot analysis. Gels were transferred toa PVDF-membrane (Roche, Germany) in 0.0009 N acetic acid at 150 mA, 75 V for 2 h and blocked in PBS, pH 7.4, 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk. One membrane was incubated with monoclonalanti-protamine 1 antibody (Hup 1 N) diluted 1:100000. The second membrane was incubated with monoclonal anti-protamine 2 antibody (Hup 2B) diluted 1:500000 overnight at 48C (Both Hup 1N and Hup 2B were generously provided by Prof. Dr Rod Balhorn). Membranes were incubated with the horseradish peroxidase-conjugated goat anti-mouse IgG (Dianova, Germany) antibody diluted 1:10 000 for 1 h at RT. Protamines were detected using the Lumi-light chemiluminescence kit (Roche, Germany).

Negative immunoblot controls were performed as above without theprimary antibody. Signals for protamines were visualized by using the Enhance Chemiluminescence system (Bio-Rad, Germany) (Fig. 1). The intensity of the bands corresponding to P1 and P2 were quantified. P1 and P2 concentrations were calculated from the standard curve generated from the human protamine standard as described above (Fig. 2). The P1/P2 ratio of each sample was calculated and the mean values were reported (all samples were tested in duplicate).

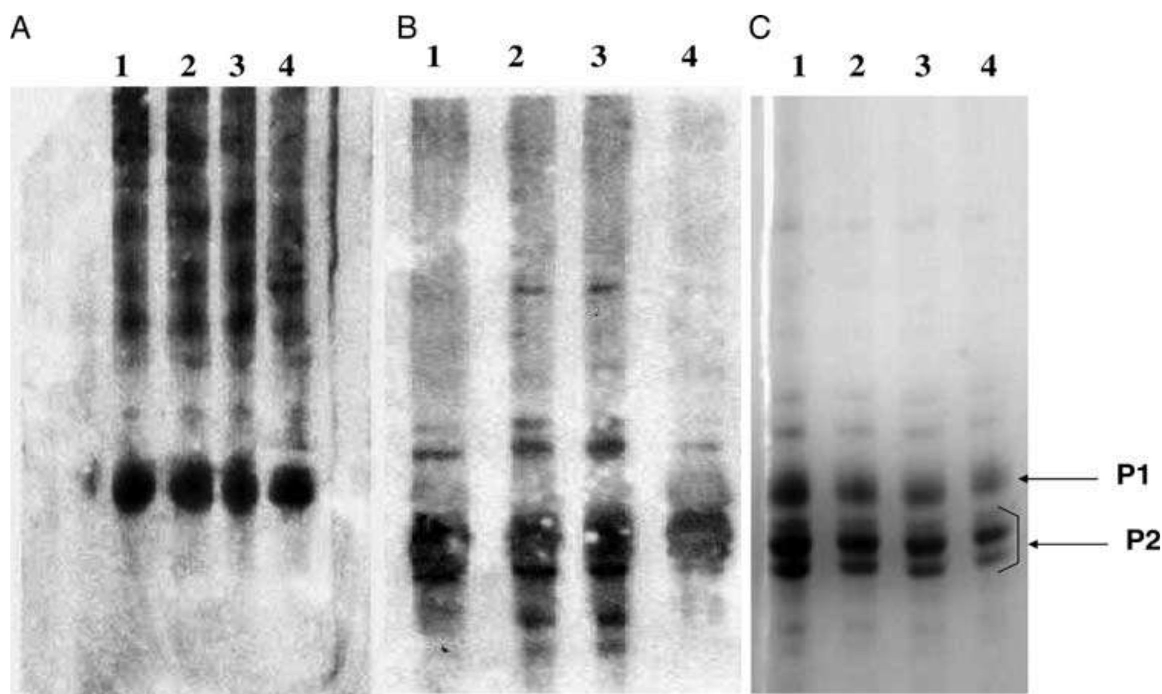


Figure 1 Analysis of protamine 1 (P1), protamine 2 (P2). (A) Western blot, corresponding to a replica of the gel shown in C, using an antibody specific for P1. (B) Western blot, corresponding to a replica of the gel shown in C, using an antibody specific for P2. (C) Proteins extracted from spermatozoa, separated on a polyacrylamide-acetic acid-urea gel and stained with Coomassie Blue. Lanes 1–4 correspond to decreasing amounts of a human protamine standard (2.00, 1.50, 1.00 and 0.50 mg) included in each lane.

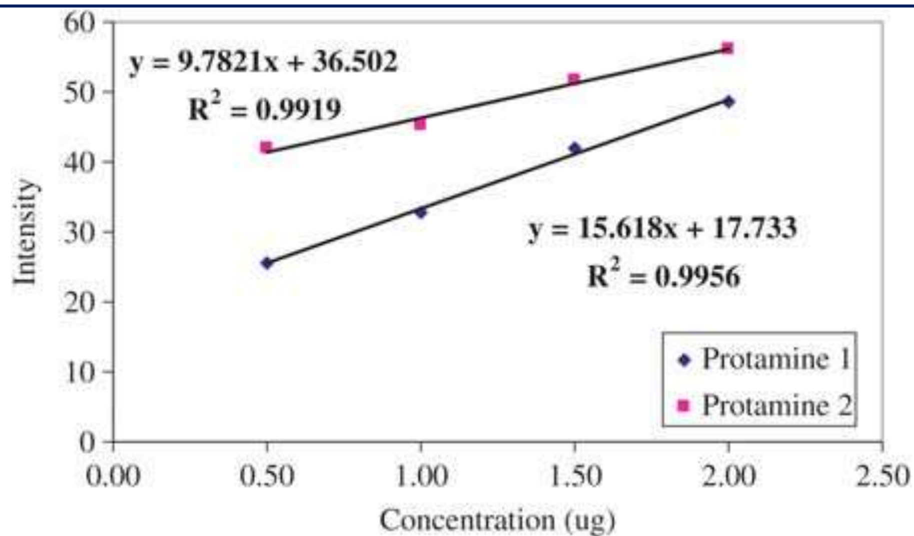


Figure 2 Standard curve of protamines1 and 2 (0.50, 1.00, 1.50 and 2.00 mg).

2.9. ROS measurement

The concentration of ROS was measured by a colourimetric assay for the quantitative determination of peroxides in EDTA–plasma, serum and other biological fluids using Enzyme linker immunosorbant assay (ELISA) kit (Oxy Stat; Cat. No. BI-5007 Biomedica Medicine product GmbH & Co KG, Wien, Austria) as previously described by Hammadeh et al. (2008). Briefly, the peroxide concentration was determined by the reaction of the biological peroxides with peroxidase and a subsequent colourreaction using TMB (3, 3, 5, 5-tetramethylbenzidine) as a substrate. After the addition of the stop solution, the coloured liquid was measured photo metrically at 450 nm. A calibrator was used to calculate the concentration of circulating biological peroxides in the sample (one point calibrator). Measurement of the peroxide was performed following the manufacturer'sguidelines and the peroxide concentration was calculated according to the equation: Peroxide(mmol/l) = ([DOD(optical density)sample × (mmol/l)calibrator]/ DOD calibrator)
DOD = OD2 − OD1

2.10.8-Hydroxy-2-deoxyguanosine (8-OHdG) measurement

The 8-OHdG enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA) is a competitive assay that can be used for the quantification of 8-OHdG in urine, cell culture, plasma and other sample matrices. The EIA utilizes an anti-mouse IgG-coated plate and tracer consisting of an8-OHdG-enzyme conjugate. The 8-OHdG-antibody used in this assay recognizes both free 8-OHdG and DNA-incorporated 8-OHdG. Procedures applied were followed in sequence following the manufacturer'sguidelines,

3.STATISTICAL ANALYSIS

Data from 106 patients and 126 non- patients were expressed asmean+SD and statistically analyzed using SPSS v. 17.0 for Windows Software Package (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was used to verify a normal or non-normal distribution of values. The non-parametric Mann–Whitney U-test was used to examine differences between samples from patients and non- patients, and Spearman's test was used to calculate the correlations. The probability value of P , 0.050 was considered significant and P , 0.010 was considered highly significant

4.RESULTS

The results of various conventional seminal parameters measured in 106 infertile patients and 126 non-patients are summarized in Table I. The age of the patients, semen volume and sperm count did not differ significantly among the groups (Table I). mumps disease had no apparent effect on semen volume and sperm concentration but tended to reduce other semen parameters. Also, patients had significantly lower sperm vitality (30.8+16.5%), motility (23.0+8.0%),membrane integrity (36.2+18.2%) and morphology (23.5+10.2%) than non-patients (38.2+17.6%; P = 0.03; 31.4+13.7% P =0.001; 52.8+20.2% P = 0.001 and 36.2+9.1%, respectively).

4.1.P1, P2 and P1/P2 content

The levels of P1 and P2 of 106 patients and 126 non-patients samples were analyzed by acid-urea gel electrophoresis and the co-omassie blue-stained protamines bands were quantified by densitometry.

The P1/P2 ratios were determined by dividing the intensity of P1 by intensities of the P2 family. The levels of P1 in patients (411.81+108.81) were non-significantly lower (P . 0.050) than that of non-patients (420.3+95.9). However the levels of P2 were significantly lower (P , 0.050) in patients in comparison to that of non-patients (334.8+117.8 versus 388.9+107.3) (Table II, Fig. 3). The P1/P2ratios also showed a significant difference (P , 0.010) between patients (1.35+0.47) and non-patients (1.12+0.21) (Table II,
Fig. 4).

4.2.Correlations between P1, P2 and P1/P2 ratio with sperm chromatin integrity

The mean P1 levels were not found to be significantly (P . 0.050) correlated with non-condensed chromatin (CMA3 positive), and DNA fragmentation (TUNEL positive) (r ¼ 20.168, r ¼ 20.137, respectively). In addition, P2 levels showed a negative correlation with noncondensed chromatin (r ¼ 20.209, P , 0.050) but DNA fragmentation was not correlated (r ¼ 20.036, P . 0.050). P1/P2 ratios showed a significant positive (P , 0.050) correlation with noncondensed chromatin (r ¼ 0.183), but correlation with DNA fragmentation (Table III). Significant positive correlations (P , 0.01) were also observed between P1 and P2 (r ¼ 0.623) and negative correlationswere found between P2 and P1/P2 ratios (r ¼ 20.639) (Table III).

4.3.Correlations between P1, P2 and P1/P2 ratio with oxidative stress markers

Mumps disease is correlated with elevated oxidative stress (Saleh et al., 2002. The levels of oxidative stress and family history affectation in seminal plasma were analyzed in samples of 62 patients and 64 non-patients. In seminal plasma of patients, mobile using (9.02+1.47), ROS (mmol/l) (138.48+41.86), 8-OHdG (ng/ml) (2.98+1.83) and family history) (99.56+64.59) were all significantly higher (P , 0.010) than those values observed in the non-patients group (6.52+1.07, 65.75+30.17, 0.82+0.64 and 2.69+2.37, respectively) (Table II). These findings supported the finding about higher oxidative stress in semen samples of patients in comparison with that of non-patients

Table I Spermiogram of all patients (patients and non-patients).

Parameters	All patients	Non- patients	patients	P-value
Samples	464	264	212	
Age (years)	37.8+5.8	37.2+5.8	38.9+5.7	0.185
Volume (ml)	3.3+1.8	3.5+1.7	3.2+1.9	0.169
pH	8.8+0.5	8.8+0.5	8.8+0.5	0.923
Count (mill/ml)	61.5+31.2	62.2+31.2	60.7+31.5	0.904
Motility (% motile)	27.5+12.1	31.4+13.7	23.1+8.1	0.001
Sperm vitality (Eosin) (%)	34.8+17.4	38.2+17.6	30.8+16.5	0.030
Membrane integrity (HOS) (%)	45.2+20.9	52.8+20.2	36.2+18.2	0.000
Morphology(%)	29.5+12.8	36.2+9.1	23.5+10.2	0.000

P , 0.050 was considered significant and P , 0.010 was considered highly significant

Table II Sperm and seminal plasma parameters of non-patients and patients

Parameters	All patients	Non- patients	patients	P-value
Samples	464	264	212	
Protamine 1 (ng/106 sperm))	416.31+101.71	420.3+95.9	411.09+108. 81	0.750
Protamine 2 (ng/106 sperm)	363.61+114.08	388.9+107.3	343.8+117.8	0.030
P1/P2 ratio	1.23+0.37	1.12+0.21	1.35+0.47	0.000
Mobile use	7.83+1.78	6.52+1.61	9.02+1.47	0.000
Reactive oxygen species (ROS) (mmol/l)	103.86+5.69	65.75+30.17	138.48+41.86	0.000
Family history	53.43+67.34	2.69+2.37	99.56+64.59	0.000
	1.96+1.77	0.82+0.64	2.98+1.83	0.000

8-Hydroxy-2-deoxyguanosine (8-OHdG) (ng/ml)				
Non-condensed chromatin (positive CMA3) (%)	32.8+8.3	29.9+7.2	36.5+8.2	0.000
DNA fragmentation (positive TUNEL) (%)	14.3+5.7	11.4+4.3	17.5+5.4	0.000

P , 0.050 was considered significant and P , 0.010 was considered highly significant

Table 111 Correlation coefficients of sperm non-condensed chromatin and DNA fragmentation with oxidative stress parameters of patient samples (n 5 116).

Parameters	Protamine 2 (ng/106 sperm)	Protamine 2 (ng/106 sperm)	P1/P2 ratio	P-value AND CORRELATIO N
Non-condensed chromatin (positive CMA3) (%)	-0.168 0.075	-0.209* 0.026	0.181 0.051	*r* *p*
DNA fragmentation (positive TUNEL) (%)	-0.137 0.147	.-0.036 0.712	0.063 0.509	*r* *p*
Mobile use	-0.035 0.788	-0.188 0.138	0.346** 0.007	*r* *p*
Reactive oxygen species (ROS) (mmol/l)	-0.123 0.343	-0.298* 0.018	0.369** 0.004	. *r* *p*
Family history	-0.048 0.7023	-0.269* 0.035	0.412** 0.002	*r* *p*
8-Hydroxy-2-deoxyguanosine (8-OHdG) (ng/ml)	-0.162 0.207	-0.302* 0.018	0.375** 0.004	*r* *p*
Protamine 1 (ng/106 sperm)	1.000	0.624** 0.0001	0.106 0.262	*r* *p*
Protamine 2 (ng/106 sperm)	0.624 0.001	1.000	-0.639** 0.000	*r* *p*
P1/P2 ratio	0.106 0.262	-0.639** 0.000	1.000	*r* *p*

r =correlation and *p*=*p*-value p=0.010 was considered highly significant (**)

Table IV Correlation coefficients of sperm non-condensed chromatin and DNA fragmentation with oxidative stress parameters of patient samples (n 5 116).

Parameters		Non-condensed chromatin (positive CMA3) (%)	DNA fragmentation (positive (TUNEL) (%)	Family history	Mobile use	Reactive oxygen species (ROS) (mmol/l)
Mobile use	*r* *p*	0.36.2** 0.005	0.518** 0.000	0.618** 0.000	1.000 0.000	0.596** 0.000
Reactive oxygen species (ROS) (mmol/l) (n = 113)	*r* *p*	0.511** 0.000	0.403** 0.000	0.734** 0.000	0.596** 0.000	1.000 0.734**
Family history	*r* *p*	0.452** 0.000	0.501** 0.000	1.000 0.000	0.618** 0.000	0.000 0.503**
8-Hydroxy-2-deoxyguanisine(8-	*r*	0.434**	0.499**	0.76189	0.000	0.000

OHdG)ng/ml	p	0.000	0.000	0.000	0.591**	

r =correlation and *p*=*p*-value p=0.010 was considered highly significant (**)

The levels of P2 were inversely (P , 0.050) correlated with levels ofROS (mmol/l) (r ¼ 20.298), 8-OHdG (ng/ml) (r ¼ 20.302) and the mumps disease marker family history (r ¼ 20.269, P , 0.05) (Fig. 5 a–c), whereas a non-significant correlation was detected between the concentrations of P2 and mobile use (r ¼ 20.188, P . 0.050). Significant positive correlations (P , 0.010) were found between the P1/P2ratios and mobile use (r ¼ 0.346), ROS (r ¼ 0.369) and 8-OHdG (r ¼0.375) and family history (r ¼ 0.412) (Table III). The levels of oxidative stress biomarkers did not show correlations (P . 0.050) with the levels of P1 Mobil use (r ¼ 20.035), ROS (mmol/l) (r ¼ 20.123), 8-OHdG (ng/ml) (r ¼ 20.162) or family history (r ¼ 20.048).

4.4.Correlations between sperm chromatinintegrity and oxidative stress parameters

The percentages of sperm with non-condensed chromatin (CMA3positive %) and DNA fragmentation were significantly and positively correlated (P , 0.010) with the concentrations of oxidative stress biomarkers in seminal plasma, mobile use (r ¼ 0.352; r ¼ 0.518), reactive oxygen species (ROS, mmol/l) (r ¼ 0.511; r ¼ 0.403) and (8-OHdG, ng/ml) (r ¼ 0.433; r ¼ 0.498), with the mumps disease marker family history (r ¼ 0.452; r ¼ 0.501) (Table IV). These findings show the negative effect of oxidative stress and mumps disease on sperm chromatin integrity. Furthermore, the mumps disease marker family history was correlated significantly and positively (P , 0.010) with the oxidative stress parameters Mobile use (r ¼ 0.618), ROS (mmol/l) (r ¼ 0.734) and8-OHdG (ng/ml) (r ¼ 0.762) (Table IV). These results indicate that mumps disease induces oxidative stress in seminal plasma. Moreover, the concentrations of ROS showed a significant positive correlation (P , 0.010) with Mobile use (r ¼ 0.596), and 8-OHdG (r ¼ 0.503). A similar correlation was observed between mobile use and 8-OHdG (r ¼ 0.591, P , 0.010).

DISCUSSION

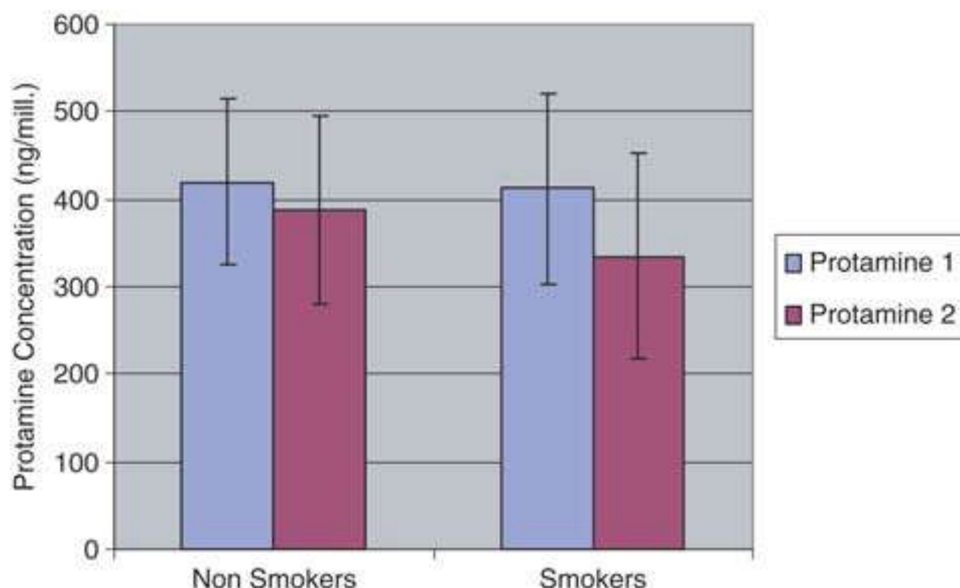
The study of semen parameters such as sperm motility, sperm count and sperm morphology have conventionally been used to assess these men quality of an individual. However, none of these measures, alone or in combination, can be considered diagnostic of infertility (Guzick et al., 2001). The effect of mumps disease on classical sperm parameters and fertility has been under investigation for decades (Soares and Melo, 2008). Ku¨nzleet al. (2003), when evaluating many men attending a fertility clinic, found that mumps disease was associated with reductions insperm concentration, motility and normal morphology. Gaur et al. (2007) showed that motility is one of the first sperm parameters affected and asthenozoospermia may be an early indicator of reduced semen quality in light smokers (Gaur et al., 2007). They reported significantly high teratozoospermia in achronic disease compared with non-patients.

In a study conducted on fertile men, it was observed that fertile men who were patients showed reductions in semen volume in comparison with non-patients, and this reduction in semen volume was in proportion to the number of patients injury (Pasqualotto et al., 2006). The largest cross-sectional study, including 2542 healthy males, onthis issue was carried out and published by Ramlau-Hansen et al. (2007) and it showed that with increasing patients injury, a 20–30%reduction in sperm count, volume and motile spermatozoa were observed. A study on voluntary males of reproductive age showed that after ejaculation, sperm motility deteriorated much more rapidly in heavy patients injury in comparison to controls (Suleiman et al., 1996).

However, the exact effect of mumps on male fertility remains controversial. Toxic metabolites which constitute during disease, such as stress ,heating and others have been demonstrated to be negatively associated with the normal development ofmale and female gametes and embryos (Kumosani et al., 2008; Thompson and Bannigan, 2008).

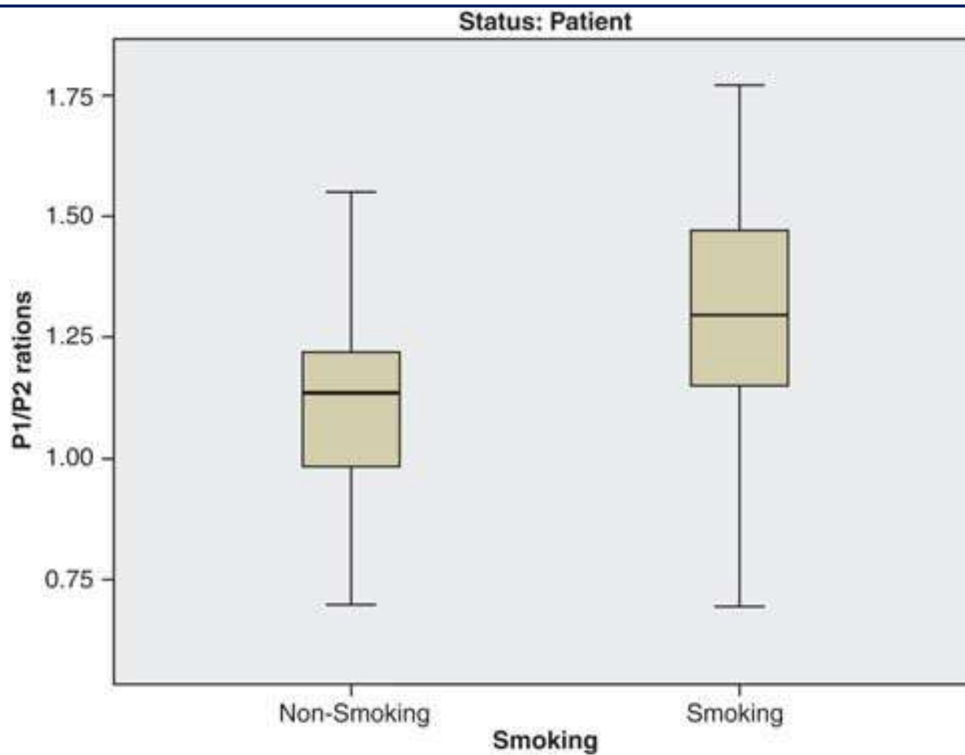
thus producing a negative effect on the viability and morphology of spermatozoa (Koksal et al., 2003). In the present study, it was found that mumps patients had no apparent effect on semen volume and sperm concentration but tended to reduce other semen parameters. Mumps patients had significantly lower sperm vitality, motility and membrane integrity than did non-patients. In addition, the percentage of sperm cells with normal morphology in patients was significantly lower than in non-patients. The exact molecular mechanisms for these effects are not well understood (Arabi and Mosthaghi, 2005). Animal models using rats linked the reduction of sperm developmental processes and fertility with mutagenic components of smoke (Sorahan et al., 1997). These metabolites may cause deficiencies in spermatogenesis and chromatin condensation and reductions in sperm motility and the number ofgerm cells (Yamamoto et al., 1998), as well as induction of apoptosisin the genital cells of the rat testes (Rajpurkar et al., 2002), a secretory deficit of Leydig and Sertoli cells (Kapawa et al., 2004) or impairment of testicular histology (Ahmadnia et al., 2007). Different groups have studied sperm DNA fragmentation in patients and non-patients men from infertile couples. An increased rate of sperm DNA fragmentation in smokers was found in pre and post-swim-up samples (Sepaniak et al., 2006; Viloria et al., 2007). The protamine deposition can also be incomplete in patients, resulting in ratios of histone to protamine and of protamine1 to protamine 2 that differ from the normal. Both types of defects in spermiogenesis are associated with subfertility or infertility (Aoki et al., 2006a,b; Oliva, 2006). Numerous

studies, however, have demonstrated that male infertility is associated with an abnormal histone to protamine ratio (Zhang et al., 2006) and an aberrant P1/P2 ratio at both theprotein level in ejaculated spermatozoa (Belokopytova et al., 1993) and at the mRNA level in testicular spermatids (Steger et al., 2003). In the present study the concentrations of P2 were lower in patients than in non-patients. In contrast, the P1/P2 ratio was significantly higher in the patients. These results show a negative effect o mumps disease on the process of protamination. Under-expression of P2seems to be the critical change with smoking and the reason for the high P1/P2 ratios in mumps disease.



Non-patients
patients

Figure 3 Protamines 1 and 2 contents in the sperm of patients and non- patients. P1 in patients and non-patients (411.81+108.81 versus 420.3+95.9, P . 0.050). P2 in patients and non-patients (334.8+117.8 versus 388.9+107.3, P , 0.050).



Non-patients - patients mumps disease

Figure 4 Box-plots showing the mean, median and range of P1/P2ratios of both patients and non-patients of patients. P1/P2 ratio was significant higher in patients in comparison to non-patients (1.35+0.47, 1.12+0.21, P , 0.010).

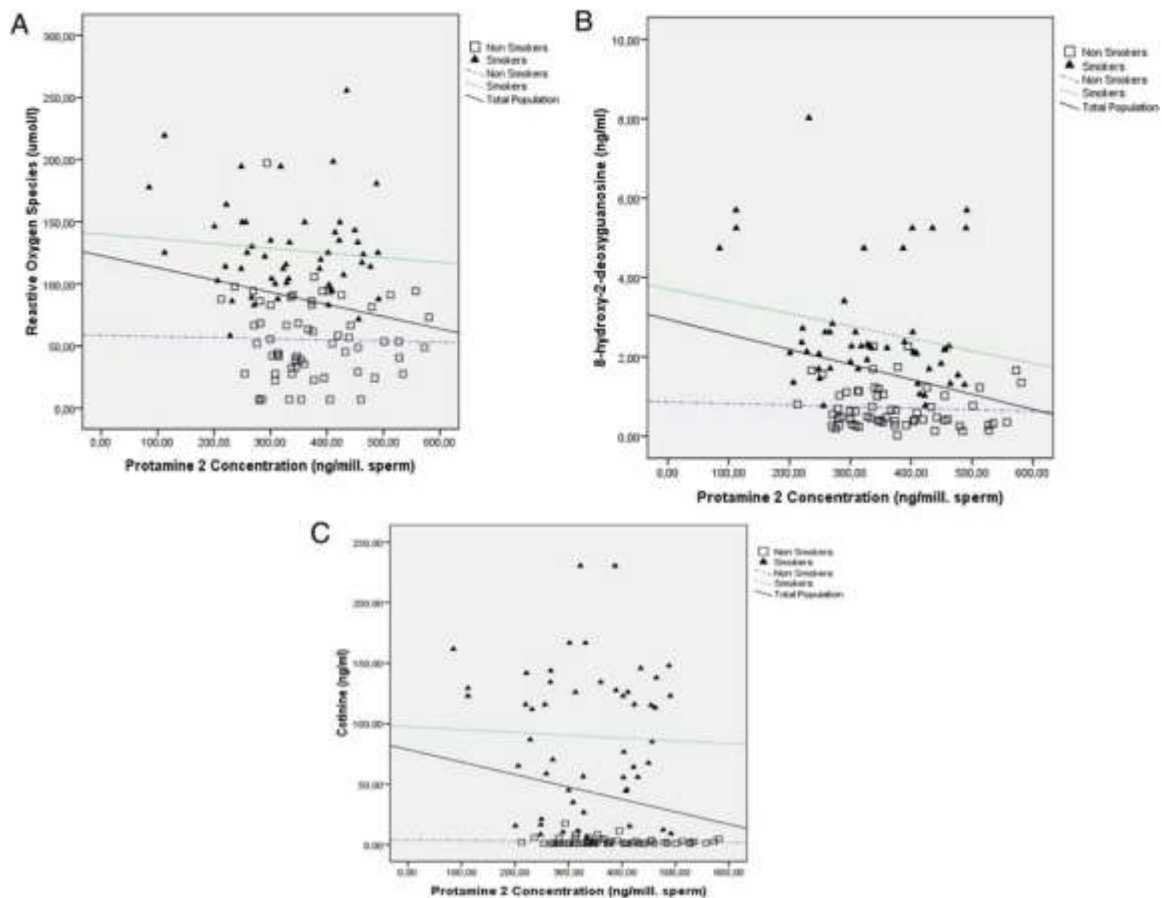


Figure 5 (A–C) Scatter plot of correlation between concentrations of ROS (mmol/l), 8-hydroxy-2-deoxyguanosine (8-OHdG) and family history, respectively, in seminal plasma and the concentration of protamine 2 (ng/106 sperm) in the sperm of mumps patients, non-patients and total population. In both groups, significant negative correlations were illustrated (r ¼ 20.298, r ¼ 20.302, r ¼ 20.269, respectively P , 0.050).

Table II Sperm and seminal plasma parameters of non-patients and patients

Parameters	All patients	Non- patients	patients	P-value
	464	264	212	
Protamine 1 (ng/106 sperm))	416.31+101.71	420.3+95.9	411.09+108. 81	0.750
Protamine 2 (ng/106 sperm)	363.61+114.08	388.9+107.3	343.8+117.8	0.030
P1/P2 ratio	1.23+0.37	1.12+0.21	1.35+0.47	0.000
Mobil use	7.83+1.78	6.52+1.61	9.02+1.47	0.000
Reactive oxygen species (ROS) (mmol/l)	103.86+5.69	65.75+30.17	138.48+41.86	0.000
Family history	53.43+67.34	2.69+2.37	99.54+64.59	0.000
8-Hydroxy-2-deoxyguanosine (8-OHdG) (ng/ml)	1.94+1.77	0.82+0.64	2.98+1.83	0.000
Non-condensed chromatin (positive CMA3) (%)	32.8+83	29.9+7.2	36.5+8.2	0.000
DNA fragmentation (positive TUNEL) (%)	14.3+5.7	11.4+4.3	17.5+5.4	0.000

P , 0.050 was considered significant and P , 0.010 was considered highly significant

These results support the hypothesis that subfertility in general, and mumps disease in particular, increases sperm susceptibility to chromatin abnormalities and fragmentation, which is in agreement with other studies. Some reports have shown that abnormal elevation of theP1/P2 ratio of infertile male populations is due to reduction of P2 expression in infertile men (Carrell and Liu, 2001; Aoki et al., 2005). De Yebra et al. (1993) and Carrell and Liu (2001) showed a complete absence of P2 in some cases of infertile men. Findings from the present study demonstrate that the concentrations of ROS were significantly higher in patients compared with non-patients. Also, ROS concentrations in seminal plasma showed significantly positive correlations with P1/P2 ratios and with the mumps disease marker family history on seminal plasma, but a negative correlation with P2 in spermatozoa. Armstrong et al. (1998) showed that seminal oxidative stress is correlated with impairment of sperm metabolism, motility and fertilizing capacity. Oxidative stress occurs in seminal plasma of male mumps disease with increased levels of seminal ROS (Saleh et al., 2002) and with increases in the concentrations of cadmium and lead (Kiziler et al., 2007) and with decreases in the concentration of ascorbic acid and the activity of other components of the antioxidant defense mechanism (Mostafa et al., 2006; Pasqualotto et al., 2008). Infertile men who has mumps disease injury have higher levels of seminal oxidative stress indicators than infertile non-patients (Saleh et al., 2002). Oxidative stress has been shown to be a major cause of male infertility; a large proportion of infertile men were shown in one study to have elevated levels of seminal ROS (Agarwal et al., 2005). ROS causes oxidative damage to normal sperm DNA, proteins and lipids, which maybe related to sperm abnormalities (Moustafa et al., 2004; Aitken andBaker, 2006). Moreover, plasma membranes of spermatozoa, whose physically properties and functional integrity largely determine motility and fertilizing ability, hold a large amount of polyunsaturated fatty acids, prone to oxidative injury, whereas the cytoplasm contains low concentrations of antioxidant enzymes (Velando et al., 2008). LPO of the high levels of polyunsaturated fatty acids in the sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage, leading to decreased sperm motility, viability and increased morphology defects, with deleterious effects on sperm capacitation and the acrosome reaction (De Lamirande and Gagnon, 1995; Dandekar et al., 2002; Agarwal et al., 2005; Aitken and Baker, 2006). In the present study, the finding that Mobile were significantly higher for patients than for non-patients and that mobile use significantly correlated with levels of family history, ROS and 8-OHdG suggests that higher effect of Mobile use r esulted from mumps injury-induced ROS. This is in agreement with other previous reports by Mostafa et al. (2006). In addition, a negative correlation between MDAwith P2 levels and a positive correlation of mobile use with P1/P2 ratios demonstrates the inverse relationship between LPO and protamination of sperm chromatin and indicates that P2 is more affected by mobile use than is P1. Oxidative stress (ROS) may cause several forms of sperm

DNA damage such as chromatin cross-linking, chromosome deletion, mutations, DNA strand breaks, base oxidation and other lethal genetic effects (Agarwal et al., 2003; Tominga et al., 2004). ROSmay also induce apoptosis through cytochrome c release from mitochondria and caspases 9 and 3 activation, which result in high frequenciesof single- and double-stranded DNA breaks (Agarwal and Allamaneni, 2004; Said et al., 2004). Therefore, in the context of male infertility, seminal plasma oxidative stress, sperm DNA damageand apoptosis are linked and constitute a unified pathogenic molecular mechanism (Agarwal et al., 2005). DNA damage in sperm may arise from several sources: firstly, improper packaging and ligation during sperm maturation (Sailer et al., 1995); secondly, oxidative stress (Agarwal et al., 2003) and the increased levels of specific forms of oxidative damage such as 8-hydroxydeoxyguanosine in sperm DNA supports such a theory (Shen and Ong, 2000; Aitken and De luliis, 2010) and thirdly, induction of DNA fragmentation due to apoptosis (Sakkas et al., 2002, 2010). In the present study, the finding that 8-OHdG levels (an oxidativestress biomarker) were significantly higher for patients than for non-patients and that 8-OHdG significantly correlated with cotinine, ROS and MDA suggests that higher levels of 8-OHdG resulted from mumps injury-induced ROS, that is associated with abnormal chromatin organization and DNA strand breaks leading to male infertility. Moreover, a significant negative correlation between 8-OHdG and P2 levels and a positive correlation with P1/P2 ratios demonstrated the relationship between DNA oxidative damage induced by mumps disease and abnormalities in the protamination of sperm chromatin. In human sperm DNA, substantial oxidative modification in 8-oxo-2′-deoxyguanosine (8-OxodG), at the level of 2–4 per 100 000 deoxyguanosineshas been demonstrated (Fraga et al., 1996, Shen and Ong, 2000).
The level of 8-OxodG in sperm DNA has been reported to be increased in patients and the level correlated negatively with the seminal plasma concentration of vitamin C (Fraga et al., 1996; Shen et al., 1999). This modification could be a marker of oxidative stressin sperm which could also have negative effects on sperm function (Shen et al., 1999; Shen and Ong, 2000). The levels of DNA damage recorded in both unselected donors and patients attending an assisted conception clinic were highly correlated with the appearance of a marker for oxidative DNA damage, 8-hydroxy, 2′-deoxyguanosine (De luliis et al., 2009). DNA damage in the male germ line is the result of a programmed senescence pathways, oxidative DNA base damage and unresolved DNA strand breakage (Aitken and De luliis, 2010). Damage to the sperm nuclear proteins may prevent binding of protamines to sperm DNA increasing the susceptibility of the DNA to damage (Aoki et al., 2006a). Moreover, oxidative stress may affect the levels of protamine through influencing the spermatogenesis process. Proteins are one of the prime targets for oxidative damage (Jung et al., 2007), and cysteine residues are particularly sensitive to oxidation because the thiol group (2SH) in cysteine can be oxidized (Eaton, 2006). Disturbances of the redox ratios in plasma increase the vulnerability of thiol groups to oxidative damage (Kemp et al., 2008). Due to the fact that protamines are rich in cysteine which is rich in 2SH groups, they are susceptible to oxidation, and high levels of oxidative stress components may affect the formation of inter- and intramolecular disulfide bonds, resulting in less compaction of the sperm chromatin and a higher incidence of DNA damage. Family history affection on seminal plasma are considered as a biomarker for mumps disease injury. A significant negative correlation was detected with P2 levels in spermatozoa, and a significant positive correlation was detected with P1/P2 ratios. This is study conducted to evaluate the effect of smoking on the protamination process. It demonstrates the negative effect of mumps disease on protamination of sperm chromatin and indicates that the reason for higher P1/P2 ratios in patients is the under-representation of P2, suggesting that mumps disease may affect P2 expression greater than P1. Lewis et al.
(2003) studied the evolution of protamines and found that P2 geneis more recently derived than P1 and highly variable within the mammalian genome. Also, incomplete post-transitional processing of protamines may result in an under-representation of P2 and an increase inP2-precursors (De Yebra et al., 1998; Carrell and Liu, 2001). Mumps disease affects either the structure of protamines or their binding to DNA or affects sperm DNA directly through increasing oxidative damage. Mumps disease itself may be a source of ROS and oxidative stress through its metabolites which result in increases in leukocyte production (Potts et al., 1999). Activated leukocytes are capable of producing100-fold higher amounts of ROS than non-activated leukocytes (Plante et al., 1994). Toxic metabolites from mumps disease injury also may affect the spermatogenesis process directly (Barratt et al., 2010).

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CONCLUSIONS: The current study evaluate the effect of mumps disease on protamines. Abnormal elevation of the P1/P2 ratio appears to be associated with aberrant P2 expression in mumps patients. These results suggest that induced oxidative stress by mumps disease may have significant inverse effect on the protamination process by disrupting P2. In conclusion, attention should be focused on the possible role of mumps disease on protamine concentrations and ratios in spermatozoa. Taken together, the results of the present study suggest a negative biological effect of mumps disease on spermatozoa DNA integrity and protamine distribution. Mumps disease male partners of couples facing infertility should be counseled to stop disease.

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