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Effects of pentoxifylline and platelet activating factor on sperm DNA damage☆

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ABSTRACT

Objective: Pentoxifylline and platelet-activating factor (PAF) have been used to increase sperm motility in embryology laboratories. In the present study, we aimed to investigate whether these agents pose sperm DNA damage using DNA sperm chromatin dispersion (SCD) assay.
Study design: Following application of pentoxifylline and PAF, sperm samples of 50 individuals with different sperm parameters were compared to baseline in terms of DNA damage using SCD assay. Furthermore, the relationship between DNA damage and sperm parameters in predicting DNA damage was assessed.
Results and conclusions: Significant increase in DNA damage was observed following application of PAF and pentoxifylline. Furthermore, DNA damage was significantly increased with application of pentoxifylline compared to PAF. Sperm motility was observed to be a statistically significant indicator in predicting alterations in DNA damage in baseline and subsequent to application of PAF and pentoxifylline independent of sperm concentration and morphology.

Increased DNA damage was observed in both groups following application of pentoxifylline and PAF. Furthermore, the increase in DNA damage was higher in samples treated with pentoxifylline compared to samples treated with PAF. Thus, PAF seems to be more innocent in choosing viable sperm cells and in achieving sperm motility in the in vitro fertilization laboratory.

Introduction

Infertility is seen in about 15% of the married couples and about 30–50% of cases are due to male factors [1]. One of the main causes of male infertility is the samples with immotile sperms and improving sperm motility was reported to be associated with increased outcome either after intrauterine insemination or after in vitro fertilization [2].

In males with sperm motility problems, several agents have been defined to increase sperm motility [3–5]. These factors are essential in initiating and maintaining the motility. In vitro stimulation of the sperm motility depends on stimulation of sperm function via increased intracellular level of cAMP [6]. When such phosphodiesterase inhibitors as pentoxifylline are added to the semen in the laboratory, they increase intracellular level of cAMP, glycolysis, and generation of ATP [7] thereby increasing the rate of motile sperms and meanwhile initiating motility in the viable but non-motile spermatozoas. They have also been shown to stimulate motility of the frozen-thawed sperms [8].

Platelet-activating factor (PAF; 1-O-alkil-2-acetyle-sn-glycero-3-phosphoryl choline) is present in the human spermatozoa and its endogenous content has significant and positive relationship with

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rate of motility and pregnancy [9]. Although mechanism of action of PAF is not clear, its significance in reproductive function is obvious. Endogenous PAF may have role as a biomarker for normal sperm function [9]. A special advantage of using PAF is that it is a natural substance produced by the sperms [10]. PAF is considered to promote sperm capacitation either by distorting the sperm plasma membrane [11] or by PAF-PAF receptor mediated signaling [12].

As sperm DNA chromatin integrity is known to have an important effect on fertilization, sperm DNA damage may have a great impact on outcome of assisted reproduction [13–16]. Although using several agents to achieve sperm motility has recently drawn attention, there is no consensus on whether they have toxic effect on the sperm cells. In the present study, we aimed to investigate whether pentoxifylline, a commonly used agent to achieve sperm motility in the embryology laboratory, and platelet activating factor (PAF) use of which has recently begun to be reported, pose sperm DNA damage using DNA sperm chromatin dispersion (SCD) assay.

Materials and methods

The present study was approved by the Institutional Review Board of Bilim University and all patients gave informed consent form prior to the study.

Patient selection

The present study included 50 males aged between 24 and 48 years and was conducted at Genart Human Health and Biotechnology Centre, Ankara between January 2013 and June 2014. All patients were enrolled into the study regardless of the cause of infertility.

Semen sample collection

The semen samples were collected by masturbation after 2–5 days of sexual abstinence. After complete liquefaction of the sample, semen analysis was performed according to World Health Organization guidelines [17] and sperm morphology was analyzed following the Kruger strict criteria [18]. Sperm concentrations were determined using Makler counting chamber (Counting Chamber Makler, Sefi Medical Instruments, Israel). Each samples were treated with pentoxifylline and PAF and compared with baseline in terms of sperm DNA damage using sperm chromatin dispersion test.

Sperm washing procedure

All system components and samples were kept at room temperature or 37 °C. Sperm preparation was performed according to the density gradient centrifugation method recommended by the manufacturer (Vitrolife Sweden).

Use of pentoxifylline

Pentoxifylline was added to the samples (1.76 mM final concentration) collected after washout and the samples were incubated for 15 min.

Use of PAF

10 mL of sperm wash was added into PAF and it was vortexed vigorously for 1 min prior to use. 3 mL of PAF was added into the sperm wash medium and the pellet was re-suspended. Final concentration of PAF was 10^{-4} M. The suspension was centrifuged at 300*g* for 8–10 min following incubation at 37 °C for 15 min. The supernatant was removed and appropriate medium was added for the second washing step. Finally pellet was resuspended with suitable volume of appropriate medium.

Sperm chromatin dispersion (SCD) test

The semen sample was diluted with sperm washout solution to a final concentration of 5–10 mL/10^{-4}. SCD procedure was performed according to manufacturer's instructions of halosperm DNA damage assay kit (Halotech, Madrid, Spain). Spermatozoa having large or medium sized halos were considered as normal (non-fragmented), whereas sperm with small, no halo or with degradation were designated as significantly fragmented (Fig. 1). At least 500 spermatozoa were evaluated per examined sample and rate of the samples with DNA damage was determined.

Statistical analysis

Statistical analyses were performed using SPSS 15 for Windows package (SPSS, Chicago, IL). Normal distribution of discontinuous numerical variables was evaluated using Shapiro–Wilkinson test. Descriptive statistics were expressed as median (min–max) for the discontinuous numerical variables. Friedman's test was performed to determine whether significant difference exists in DNA damage among baseline and pentoxifylline and PAF. In the case of Friedman's test statistics being significant, the factor(s) causing the difference were determined using Wilcoxon's signed rank test.

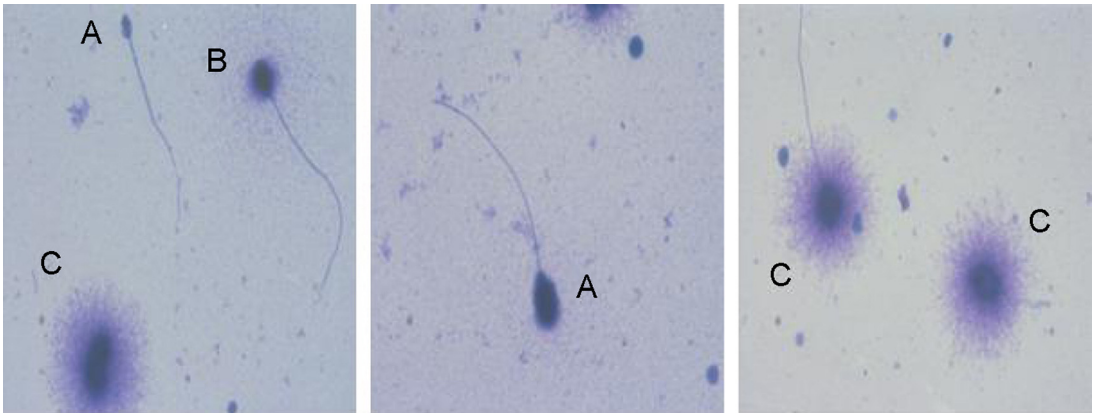

Fig. 1. Illustrations of DNA fragmentation in sperm cells treated with SCD test. (A) Spermatozoa without halo and degradated. (B) Spermatozoa with medium sized halo. (C) Spermatozoa with big halo assigned as without fragmentation.

Spearman's correlation test was performed to determine whether there were any correlations between DNA fragmentation and the conventional seminal parameters in three groups.

Multivariate linear regression analysis was performed to determine the clinical factor(s) which were deterministic in predicting the change in the rate of DNA damage. Regression co-efficient and 95% confidence interval was calculated for each variable. Logarithmic transformation was not performed in regression analysis since rates of DNA damage did not show normal distribution. Unless otherwise specified, p values <0.05 were considered to be statistically significant. Bonferroni adjustment was made in all possible multiple comparisons in order to control the type I error.

Results

Sperm parameters of all patients in the present study were described in Table 1. Compared to the baseline, sperm DNA damage was significantly increased when samples are treated with PAF and pentoxifylline (each $p < 0.001$). Furthermore, compared to PAF, significant increase in sperm DNA damage was observed in samples treated with pentoxifylline ($p < 0.001$) (Table 2). The proportions of sperm chromatin dispersion according to sperm parameters were given in Table 3.

Although a significant negative correlation was found between DNA damage and sperm parameters ($r = -0.399$, $p = 0.005$ for concentration, $r = -0.471$, $p < 0.001$ for motility and $r = -0.401$, $p < 0.001$ for morphology) subsequent to use of pentoxifylinne, motility was the only parameter to show a significant negative correlation with DNA damage following PAF use ($r = -0.423$, $p < 0.001$).

Based on univariate linear regression analysis, sperm concentration and morphology had no significant predictive role for alterations in the baseline DNA damage ($p = 0.085$ and $p = 0.172$, respectively) whereas rate of DNA damage significantly decreased as sperm motility increased ($B = -0.0166$; %95 CI: -0.0238 to -0.0095, $p < 0.001$) (Table 4). While sperm concentration subjected to Bonferroni's adjustment did not predict DNA damage after application of pentoxifylline, DNA damage was found to be significantly related with sperm motility ($B = -0.0164$; 95% CI: -0.0238 to -0.0090, $p < 0.001$) and sperm morphology ($B = -0.1258$; 95% CI: -0.2109 to -0.0408, $p = 0.005$).

Based on multivariate linear regression analysis, sperm motility was found to be a significant indicator in predicting the alteration in baseline DNA damage independent from sperm concentration and morphology ($B = -0.0176$; 95% CI: -0.0263 to -0.0088; $p < 0.001$). Sperm motility was found to be a statistically significant indicator independent from sperm concentration and morphology in predicting the alterations in DNA damage after application of pentoxifylline and PAF ($B = -0.0137$; 95% CI: -0.0226 to 0.0048, $p = 0.003$; $B = -0.0175$; 95% CI: -0.0261 to -0.0088, $p < 0.001$ respectively) (Table 4).

Discussion

Pentoxifylline inhibits phosphodiesterase and increases intracellular cAMP concentration and phosphorylation of tyrosine at the tail level [19]. Increasing intracellular concentration of cAMP usually causes increase in sperm motility as well as in agonist-induced acrosome reaction (AR) and fertilization [20]. In assisted reproduction technology, effect of pentoxifylline on sperm motility and fertilization capacity has been approved in astenozoospermia [20]. Another agent, PAF, has been shown to induce sperm capacitation and AR in many species [21–23]. Additionally, some authors have reported that exogenous PAF enhances sperm motility and fertilization rates in human [9,21]. Although the use of both agents in order to improve sperm motility has drawn attention in recent years, there is no consensus on whether they have toxic effect on the sperm cells. Therefore, in the present study we aimed to investigate whether pentoxifylline, an agent commonly used in the embryology laboratory to achieve sperm motility, and platelet activating factor (PAF), whose use has been recently reported, cause sperm DNA damage using sperm chromatin dispersion (SCD) assay and we showed that both

Table 1
Baseline characteristics of sperm parameters in all cases.

Variables	$n = 50$
Volume (ml)	3.3 (2.3–4.1)
Concentration (10^6/ml)	17.3 (0.1–61.1)
Total count (10^6)	52 (0.3–200)
Motility (%)	58.5 (0–85)
Morphology (%)	2 (0–8)
Sperm concentration	
Excessive oligospermia	3/50 (6.0)
Oligospermia	11/50 (22.0)
Normospermia	36/50 (72.0)
Sperm motility	
Abnormal	17/50 (34.0)
Normal	33/50 (66.0)
Sperm morphology	
Abnormal	40/50 (80.0)
Normal	10/50 (20.0)

Values for continuous variables are median (min–max). Values for categorical variables are number/total number of cases (%).

Table 2
Proportions of spermatozoa with DNA damage, as assessed by sperm chromatin dispersion test, subsequent to treatment with PAF and pentoxifylline.

Groups	SCD rate Median (min–max) %
Baseline	8 (3–35)[a,b]
PAF	10 (2–47)[a,c]
Pentoxifylline	15 (2–49)[b,c]

Abbreviations: PAF, platelet activating factor; SCD, sperm chromatin dispersion.
[a] The difference between baseline and PAF was statistically significant ($p < 0.001$).
[b] The difference between baseline and pentoxifylinne was statistically significant ($p < 0.001$).
[c] The difference between PAF and pentoxifylinne was statistically significant ($p < 0.001$).

Table 3
Proportion of spermatozoa with DNA damage, as assessed by sperm chromatin dispersion test, according to sperm parameters.

	Baseline	PAF	Pentoxipylline	p value
Sperm concentration				
Oligospermia	11.5 (3–35)[a,b]	20 (9–47)[c]	25.5 (11–49)	<0.001
Normospermia	7 (3–34)[a,b]	9 (2–39)[c]	13 (2–42)	<0.001
Sperm motility				
Abnormal	13.5 (5–35)[a,b]	21.5 (9–47)[c]	27.5 (9–49)	<0.001
Normal	6 (3–19)[a,b]	9 (2–22)[c]	12.5 (2–40)	<0.001
Sperm morphology				
Abnormal	8 (3–35)[a,b]	10.5 (2–47)[c]	16.5 (2–49)	<0.001
Normal	6.5 (3–12)[a,b]	9 (6–19)	10.5 (5–24)	0.032

All values were given as median (min–max) %.
[a] The difference between baseline and PAF was statistically significant.
[b] The difference between baseline and pentoxipylline was statistically significant.
[c] The difference between PAF and pentoxipylline was statistically significant.

Table 4
Univariate and multivariate analyses of sperm parameters in predicting DNA damage alterations subsequent to use of PAF and pentoxipylline. Bold values: (a) The difference between baseline and PAF was statistically significant. (b) The difference between baseline and pentoxipylline was statistically significant. (c) The difference between PAF and pentoxipylline was statistically significant.

Variables	Regression coefficient	Univariate 95% Confidence interval		*p* value	Regression coefficient	Multivariate 95% Confidence interval		*p* value
		Lower limit	Upper limit			Lower limit	Upper limit	
Baseline								
Sperm concentration	−0.0034	−0.0073	0.0005	0.085	0.0004	−0.0036	0.0044	0.836
Sperm motility	−0.0166	−0.0238	−0.0095	**<0.001**	−0.0176	−0.0263	−0.0088	**<0.001**
Sperm morphology	−0.0615	−0.1506	0.0276	0.172	0.0124	−0.0743	0.0990	0.775
PAF								
Sperm concentration	−0.0038	−0.0077	0.0001	0.059	0.0004	−0.0036	0.0043	0.854
Sperm motility	−0.0173	−0.0243	−0.0103	**<0.001**	−0.0175	−0.0261	−0.0088	**<0.001**
Sperm morphology	−0.0794	−0.1677	0.0088	0.076	−0.0056	−0.0911	0.0799	0.895
Pentoxipylline								
Sperm concentration	−0.0044	−0.0083	−0.0005	0.028	−0.0001	−0.0042	0.0039	0.946
Sperm motility	−0.0164	−0.0238	−0.0090	**<0.001**	−0.0137	−0.0226	−0.0048	**0.003**
Sperm morphology	−0.1258	−0.2109	−0.0408	**0.005**	−0.0643	−0.1520	0.0234	0.147

agents but especially pentoxifylline led to DNA damage compared to baseline.

It has been reported that detecting DNA damage may have more diagnostic and prognostic importance than morphology, concentration, and motility [24]. However, several studies examining the relationship between the standard semen parameters and values of DNA fragmentation indices are conflicting. Mehdi et al. [25] showed a positive correlation between DNA damage and solely sperm morphology in infertile men while Zini et al. [26] reported that rates of spermatozoa containing denaturated and fragmented DNA showed a significant relationship with deterioration in sperm parameters and that those rates were higher in infertile men compared to fertile men. In a similar study, Fernandez et al. [27] found more DNA damage in sperm using SCD method in infertile men than in fertile men. In the present study, although the subjects were not grouped based on their indications, selection bias was avoided by exposing their sperms to both agents separately. As an important finding, rate of DNA damage was found to be only related to motility in the present study unlike Zini et al. [26] Although Zini et al. [26] demonstrated that DNA fragmentation is negatively correlated with all standard semen parameters (concentration, motility, and morphology), the strongest correlation being with sperm motility, the present study showed that normal concentration and morphology did not imply low rate of sperm DNA damage. To summarize, "no need to detect DNA damage" in the subjects with normal sperm parameters in terms of morphology and concentration, may not be the most appropriate approach.

When the detrimental effect of external agents such as pentoxifylline and PAF on sperm DNA integrity are taken into account; other viability testing methods like mechanical sperm selection and HOST are still attractive approaches. Stanger et al. [28] showed that the application of hypoosmotic swelling test (HOST) may be a valuable tool in the routine identification and selection of viable, DNA-intact individual spermatozoa for ICSI. Therefore, the use of HOST or other mechanical methods such as sperm tail flexibility test instead of using PAF and pentoxypylline may be more effective in selecting optimum spermatozoa in patients with total immotile sperm parameters [29]. In our laboratory, we use mechanical viable sperm selection methods to avoid increase in sperm DNA damage rate.

Although, to our knowledge, this is the first study assessing the sperm DNA damage after the use of PAF and pentoxifylline to induce sperm motility, the results of the study are preliminary. The oocytes are known to repair sperm DNA damage to some extent immediately after fertilization [30]. However, increased damage load by other factors such as age or smoking as well as use of PAF and pentoxifylline may influence repair mechanisms, resulting in miscarriage or serious disease in the offspring, including birth defects and cancer [31–33]. Therefore, prospectively designed studies are needed to show to what extent the oocytes can handle DNA damage repair in the presence of these risk factors. In addition, embryo quality, fertilization rates and pregnancy outcomes should be warranted in further studies subsequent to exposure to these agents.

In conclusion, it was found that rate of DNA damage was increased in both groups compared to the baseline following application of pentoxifylline and PAF. However, higher rate of DNA damage was found in the pentoxifylline group compared to PAF group. Therefore, in regards to toxicity, dose and duration of exposure to these agents should be evaluated in detail prior to application of these agents. In addition to that, new methods may be designed to detect undamaged DNA prior to ICSI with immotile sperm following use of these agents. Large-scale well-designed studies are necessary including pre-implantation genetic diagnosis, fetal genetic examination on abort material, and major and minor malformations of the infants due to the risk of embryotoxicity following IVF/ICSI subsequent to treatment with these agents.

Conflict of interest

There is no conflict of interest.

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