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Sperm DNA adducts impair fertilization during ICSI but not during IVF

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Abstract: Many studies emphasize the influence of the status of spermatozoal nucleus on fertilization, mainly with regard to DNA fragmentation. This study was undertaken to analyze the influence of DNA adducts content in spermatozoa on fertilization during assisted reproduction. Ovarian hyperstimulation, oocyte retrieval and laboratory work-up in 61 IVF (in vitro fertilization) and 118 ICSI (intracytoplasmic sperm injection) first cycles were performed according to the same protocol. Semen analysis was made according to WHO Manual (1999). DNA adducts assay in spermatozoa was performed by ^{32}P-postlabeling method. In total 331 fertilizable oocytes were obtained during IVF and 659 during ICSI. Both groups differed significantly by sperm count, motility and morphology but not by the concentration of DNA adducts in spermatozoa (0.0306 ± 0.0217 in IVF versus 0.0373 ± 0.0321 in ICSI). The fertilization rate during IVF was significantly influenced by sperm count ($p = 0.0002$) and motility ($p=0.0037$) but not by DNA adducts concentration ($p=0.30528$), whereas during ICSI was positively influenced by sperm motility ($p=0.04669$) and negatively by DNA adducts concentration ($p=0.00796$). DNA adducts concentration in spermatozoa significantly negatively influences fertilization rate during ICSI, but not during IVF.

Key words: Assisted reproduction - DNA adducts - Fertilization - Spermatozoa

Introduction

Nowadays the male factor in assisted reproduction is discussed intensively. Numerous papers dealing with the correlation of DNA damage in sperm and the results of assisted reproduction have been published. Special attention was paid to DNA fragmentation [1-9] and many of these papers published suggest that DNA damage in sperm does not affect fertilization. DNA damage in sperm may also be present in covalent DNA modifications - DNA adducts. Some exogenous compounds (*e.g.* polycyclic aromatic hydrocarbonates) causing various disturbances or after penetration into cells and also exogenous factors (*e.g.* estrogens) may be after metabolic activation transformed and covalently bonded to DNA bases or sugar compound. This, in turn, if not repaired, may cause malfunction of some genes, forming in some cases oncogenes from protooncogenes, cell dysfunction and even tumorogenesis, which is well documented, for example in the respiratory tract epithelial cells in smokers [10]. Because the coincidence of testicular tumors and male sterility is well known, the question arises as to what is linking these two facts. It may be hypothesized that DNA adducts are involved in both. The next question arises, if these DNA adducts may influence the fertilization potential of spermatozoa. Investigations through assisted reproduction, visualizing immediately the fertilization process, seemed to be the most appropriate model.

The influence of DNA adducts in sperm in the course of assisted reproduction has not yet been investigated. Only one paper [11] considers the possibility of transmission of spermatozoal DNA adducts to offspring. The DNA damage in spermatozoa by various exo- or endogenous genotoxic agents is considered as a factor lowering male fertility though ICSI (intracytoplasmic sperm injection), in contrast to classical IVF (*in vitro* fertilization), enables overcoming the poor quality of semen as a cause of sterility. The purpose of this work is to study the influence of the DNA adducts in spermatozoa on fertilization of human oocytes during assisted reproduction. The literature on the DNA adducts in male gametes mostly does not deal with relations concerning assisted reproduction and, to

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Table 1. The age of IVF and ICSI patients (p=0.5285).

Age	IVF	ICSI
≤ 30 years	14	39
31-35 years	23	38
36-40 years	20	31
41-45 years	4	10

Table 2. The age of husbands of IVF and ICSI patients (p=0.4965).

Age	IVF	ICSI
≤ 30 years	6	18
31-35 years	27	40
36-40 years	20	37
41-45 years	6	19
> 45 years	2	4

our knowledge, this is the first study assessing the influence of DNA adducts concentration in spermatozoa on results of assisted reproduction, especially on the fertilization ability of spermatozoa. Preliminary results of our investigations were presented for the first time at the ESHRE Annual Meeting in Copenhagen in 2005 [12].

Materials and methods

179 couples with primary sterility entering for the first time treatment by assisted reproduction (61 by IVF and 118 by ICSI) performed in the Clinical Department of Obstetrics and Gynecology were investigated. The mean age of women was in the IVF group 33.6±4.5 years and in the ICSI group 33.5±4.8 years. Their husbands' age was 35.3±4.6 years in the case of IVF and 35.9±5.2 years in the case of ICSI. The age of patients is shown in Table 1 and the age of their husbands is shown in Table 2.

There were no statistically significant differences in respect of age and its distribution as well as lifestyle, dietary and smoking habits between men and women in both the groups.

The indications for IVF was tubal factor in 73.8% and unexplained sterility in 26.2% but for ICSI the male factor (oligo-, astheno-, terratospermia or combined sperm defect, assessed according to WHO criteria) was the exclusive indication irrespective of its cause. The cases with abnormal karyotype and antisperm antibodies either in men or in women were not included in the study.

Controlled ovarian hyperstimulation was performed in IVF group in 47.5% using long protocol and in 52.5% using short protocol. In ICSI group 45.8% of patients were stimulated using long and 54.2% using short protocol. Nafarelin (Synarel, Searle, Pharmacia-Upjohn) nasal spray at the daily dose 400μg was applied from the first day of the cycle and, using the long protocol, human menopausal gonadotrophin (HMG) (Humegon - Organon) from the eleventh day of the cycle, after pituitary desensibilization, at the daily dose 150-375 IU depending on the expected response with regard to the age of the patient and results of her hormonal assays before stimulation. Using the short protocol, HMG was administered from the second day of the cycle. From the fifth day the dosage of HMG was diminished by 75 units in both the stimulation protocols. When leading follicle achieved the diameter 20 mm and serum estradiol was 200-300 pg/ml/follicle of diameter ≥14 mm human chorionic gonadotrophin (HCG) was administered. The oocyte retrieval was performed 36 hours thereafter by ovarian punction using transvaginal ultrasound probe and 18G needle in general intravenous anesthesia. The follicular fluid was immediately transferred in the embryologic laboratory. Laboratory procedures were performed in all the cases according to the same established protocol. Sperm for IVF was prepared by swim-up technique and oocytes were inseminated 2 hours after retrieval, using about 100,000 spermatozoa per one oocyte. ICSI was performed 2 hours after oocyte retrieval by spermatozoa prepared by swim-up technique or simply by sperm-wash. Fertilization was assessed after 19 hours.

Semen analysis was performed according to the fourth edition of WHO Manual (1999), using WHO criteria for sperm morphology and peroxydase method for leukocytes. Part of the semen sample was after liquefaction stored in liquid nitrogen for DNA adducts assay in spermatozoa performed by ^{32}P-postlabeling method according to Gupta with later modifications. It is described in detail elsewhere [13]. Briefly, sperm cells washed in PBS were incubated with proteinase K. DNA was then purified by 2-3 repeated extractions with phenol/chloroform and recovered by ethanol precipitation, dissolved, incubated with RNAse A, then re-purified by phenol/chloroform extraction and ethanol precipitation. DNA concentration was measured spectrophotometrically and its integrity examined by agarose gel electrophoresis. Thereafter DNA was digested to mononucleotides and the non-modified mononucleotides were removed by digestion with nuclease P1. Modified nucleotides were radiolabeled with ^{32}P-γ-ATP and T4 polynucleotide kinase, then applied to PEI-cellulose thin layer chromatography plates, and separated by chromatography. The adduct spots were visualized by autoradiography, and radioactivity of appropriate areas of plates was counted to calculate the level of DNA adducts.

Statistics. Statistical work-up was made using package STATISTICA PL version 6.0. The normality of distribution of variables was analyzed using Shapiro-Wilk test. If the distribution was not normal the analysis was performed after logarithmic transformation (ln) of data. One step logistic regression was used to assess the chance of fertilization of the oocyte in dependence on particular semen parameters. To compare which of the parameters investigated was of higher prognostic value during fertilization process, multiple regression analysis was performed and standardized dependence coefficient BETA was calculated. Results were considered significant if p was <0.05.

Results

The total numbers of oocytes and oocytes in metaphase of the second meiotic division (MII oocytes) obtained in IVF group was 375 and 331 respectively and in ICSI group 872 and 659 respectively. No significant differences between both the groups analyzed in respect of number of all the oocytes per patient (6.14 in IVF versus 7.39 in ICSI) as well as MII oocytes per patient (5.43 in IVF versus 5.58 in ICSI) were observed.

No adduct pattern that would be common to all sperm samples was obtained. However, several more "typical" patterns were detected in the majority of the sperm samples (78.2%) treated with nuclease P1 (spot "S"), different from those obtained from leukocytes. This was described precisely in our previous work [14]. These spots contained 10-90% of spermatozoal

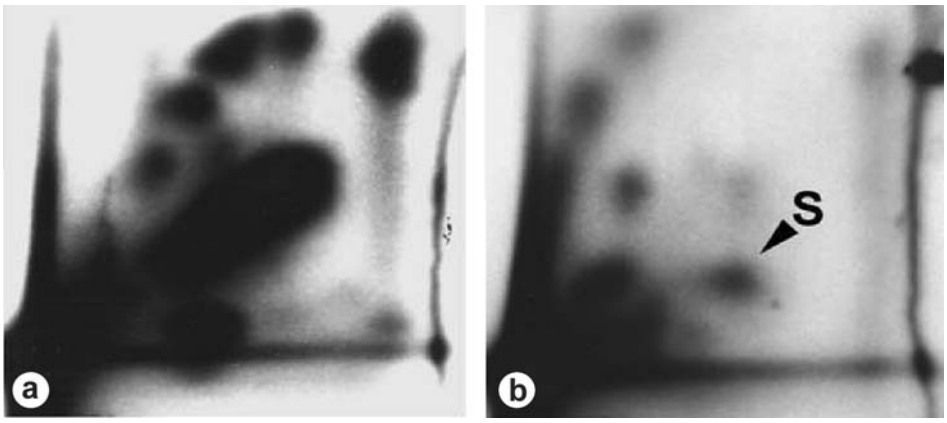

Fig. 1. DNA adducts from (**a**) leukocytes, (**b**) spermatozoa. Sperm specific spot of DNA adducts from spermatozoa. It is not probable that the adducts assayed in our study could originate from sources other than spermatozoa. The spot "S" (indicated by arrowhead) is absent in chromatograms from other cells and tissues with highest content of labeled compound, lack of any correlation with leukocyte concentration and even relatively high levels in ejaculates without leukocytes.

DNA adducts (Fig. 1) and their migration during chromatography suggests more polar character than in adducts recognized by *e.g.* anti-BPDE-DNA (benzo(a)pyrene diol epoxyde-DNA) antibodies.

The mean values ±SD of sperm count, motility and morphology as well as DNA adducts concentration in spermatozoa in both the groups analyzed are shown in Table 3. Despite expected significant differences in sperm count, motility and morphology between both the groups, the concentration of DNA adducts in spermatozoa was only slightly higher in the ICSI group.

The dependence of fertilization rate in the IVF group on the sperm count, motility, morphology and concentration of DNA adducts in spermatozoa is presented in Table 4.

As expected, the chance of fertilization of oocytes was evidently positively dependent on sperm count ($p<0.001$) and motility ($p<0.01$). What is surprising is that no evident correlation of fertilization rate and sperm morphology was observed. The concentration of DNA adducts in spermatozoa did not significantly influence the fertilization rate during classical IVF.

The most important parameters influencing fertilization rate during IVF in our study were the sperm count (BETA 0.142417) and motility (BETA 0.101107). Sperm morphology (BETA 0.008282) and DNA adducts concentration (BETA 0.020231) were not so important. The fertilization rate in the dependence on the same parameters as above (IVF) in the ICSI group is shown in Table 5.

Table 3. The mean values of sperm count, motility, morphology and concentration of DNA adducts in spermatozoa.

	IVF (n = 61)	ICSI (n = 118)
Sperm count (n)*	71 901 639 ± 37 941 931	32 050 000 ± 33 861 638
Motility (%)*	41.6 ± 11.8	19.4 ± 13.5
Morphology(%)*	40.9 ± 17.2	29.2 ± 14.6
DNA adducts concentration (fmol/μg DNA)	0.0306 ± 0.0217	0.0373 ± 0.0321

*$p<0.05$.

Table 4. The dependence of fertilization rate on sperm count, motility, morphology and concentration of DNA adducts in spermatozoa during IVF.

	Sperm count	Sperm motility	Sperm morphology	DNA adducts concentration
Odds ratio	13.43669	3.907526	1.095820	1.823720
p	0.00020*	0.00366*	0.85663	0.30528

* statistically significant

Table 5. The dependence of fertilization rate on sperm count, motility, morphology and concentration of DNA adducts in spermatozoa during ICSI.

	Sperm count	Sperm motility	Sperm morphology	DNA adducts concentration
Odds ratio	0.855089	1.790666	0.818220	0.271678
p	0.68055	0.04669*	0.59421	0.00796*

* statistically significant

The rate was significantly positively influenced by sperm motility and negatively by the level of DNA adducts in spermatozoa. As can be seen, the chance of fertilization of oocytes was evidently positively dependent on progressive sperm motility (BETA 0.038234) but still more negatively on the concentration of DNA adducts in spermatozoa used for ICSI (BETA -0.109159). Sperm count (BETA -0.003094) and morphology (BETA -0.003245) did not practically influence the results.

Discussion

The correlation between DNA fragmentation and results of assisted reproduction is relatively well documented. Some authors are of the opinion that sperm DNA fragmentation negatively influences the fertilization rate; however, most authors consider that does not influence but actually has a negative effect on further development of embryos in humans as well in animals. Sakkas *et al.* [15] and Lopes *et al.* [8] stated distinct negative influence of DNA fragmentation on the fertilization rate during ICSI. Cebesoy *et al.* [2] found a significant negative association between the percentage of sperm with DNA fragmentation and the fertilization rate in ICSI patients. Hř st *et al.* [6,7] showed better correlation of IVF results with degree of DNA breakings, than with classical seminal parameters, and no such relation was stated in the case of ICSI. It was demonstrated that spermatozoa containing damaged DNA are able to penetrate the oocyte and fertilize it [4, 5] but the DNA fragmentation in spermatozoa distinctly negatively influences the results of assisted reproduction. Feliciano *et al.* [3] in turn analyzed the status of spermatozoal nucleus and its correlation with ICSI results. They stated the negative correlation of DNA fragmentation index with concentration of spermatozoa in the ejaculate, an evident correlation with man's age and no correlation with ICSI results. Also Zini *et al.* [9] are of the opinion that DNA fragmentation has no impact on fertilization rate during ICSI. In animal models Ahmadi and Ng [1] found no significant differences in fertilization rates between spermatozoa with DNA strand breaks from mice exposed to gamma radiation and spermatozoa from their control group.

The lesions of genetic material of spermatozoa are present also as DNA adducts. The ^{32}P-postlabeling method is the most common way of quantitative adduct assay because of its extreme sensitivity (detects one adduct per 10^6-10^{10} nucleotides) and universality (detects theoretically every kind of adduct irrespective of its chemical nature) and requires microgram amounts of DNA, comparing with spectrophotometry detecting tenths and hundreds of nanograms [16]. This method is useful especially detecting mostly lipophilic adducts, but of all the methods has the broadest spectrum. Other methods such as immunofluorescence and histochemistry or flow cytometry are of limited universality detecting only some types of DNA adducts. For these reasons the 32P-postlabeling method seems to be best as the first line of investigative methods. Such was successfully used to detect DNA adducts in other types of cells [13] and, moreover, this method was applied by other authors to detect DNA adducts in germ cells. Nivard *et al.* [17] also recently used this method to detect DNA adducts in Drozophila male germ cells. Unfortunately, it is not the method enabling determining the strict chemical nature of the adducts assayed. The chemical nature of the adducts analyzed in this work is not strictly known and ought to be stated in future studies using other methods. It is not probable that the adducts assayed in our study could originate from cellular sources other than spermatozoa (Fig. 1). According to our previous findings [12,14] the concentration of DNA adducts in spermatozoa was dependent on the age of the husband and correlated well with decreased sperm count and decline in progressive motility but not with morphology, leukocyte concentration, as well as other factors (occupational hazard, residence, smoking cigarettes or drinking coffee or alcohol).

It is still not clear if DNA adducts in spermatozoa are of an exogenous or endogenous origin. The excessive proliferation of early spermatogenetic cells is regulated through apoptosis controlled by Sertoli cells, producing in due time Fas ligand, which reacts with membrane receptor Fas on the spermatogenic cells surface, so the spermatozoa containing damaged DNA in an ejaculate are those which avoided apoptosis (so-called "abortive apoptosis"). Fas and other apoptosis markers were found in higher concentrations in pathological ejaculates. Thus in an ejaculate there might be two kinds of defective spermatozoa: one, with poorly packed chromatin, open to subsequent DNA damage, *e.g.* by oxidative stress, and a second, with fulfilled damage [15]. It has been shown that DNA repair induced by several chemicals and radiation occurs early during gametogenesis (spermatocytes and early spermatids) but not in mature spermatids and spermatozoa [18], leaving the possibility of accumulation of non-repaired DNA damage during spermiogenesis. The barrier blood-testis is usually functioning well and the majority of different compounds are not able to cross it. There are suggestions that promutagenic adducts could be produced during natural metabolic processes, *e.g.* in the cascade of arachidonic acid catalyzed by cyclooxygenase [19].

Zenzes *et al.* [20] found strict correlation between cigarette smoking and detection of polycyclic aromatic hydrocarbonate (PAH)-DNA adducts in spermatozoa. On the other hand Gallagher *et al.* [21], using the ^{32}P-postlabeling technique in 36 healthy volunteers

revealed no differences between smokers and non-smokers. However, adducts levels detected in this study were at the sensitivity limit of the method applied. Gaspari *et al.* [22] stated no correlation and dose dependence between cigarette smoking and PAH-DNA adducts level in spermatozoa in 205 infertile men but alcohol consumption was connected with lowering of this level. Since there are such differences between various studies, including ours, detecting less non-polar adducts, it seems that the results depend to a great extent on the method used.

The question arises as to whether there is the possibility of transmission of DNA adducts to children born after assisted reproduction. Very interesting in this context is the work of Zhu *et al.* [23] suggesting an association of advanced paternal age with an excess occurrence of some specific congenital malformations in offspring. Zenzes *et al.* [11] are concerned with the possibility of the transmission of DNA adducts to children through the spermatozoa of men smoking cigarettes. However, the possibility of transmission of these adducts through oocytes of women living in the same room as a smoking husband could not be excluded. Their own data confirm this fact showing identical content of cotinine in the follicular fluid in actively and passively smoking women and the concentration of DNA adducts correlated best with the concentration of cotinine in the follicular fluid [24]. The concentration of DNA adducts was also similar in both cases.

The distinct influence of adducts on fertilization during ICSI and lack of it during IVF might be partially explained by insufficient action of DNA adducts in lower concentrations. They impair fertilization not before reaching sufficient concentration. This was higher, though insignificantly, in the ICSI group. On the other hand, the preparation of sperm for IVF by swim-up technique may produce the sufficient count of spermatozoa with possibly lowest content of DNA adducts and best motility. During the penetration of zona pellucida and fertilization a natural selection of spermatozoa occurs additively. This was not the case during ICSI. Though the possibilities mentioned above cannot be excluded it seems that DNA adducts concentration in spermatozoa significantly negatively influences fertilization rate during ICSI in concentration dependent manner. Sakkas *et al.* [25] in their former work stated that sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. In the case of IVF it seems to play a less negative role, because the more limiting factor is probably the ability of spermatozoa to penetrate the zona pellucida. Irrespective of the origin and nature of the DNA adducts in spermatozoa and the role they really play, the high concentration of DNA adducts in spermatozoa should be taken into account in cases of unexpectedly low fertilization rate during ICSI, especially in elder men. Because the influence of sperm DNA adducts on fertilization during ICSI and IVF has not yet been investigated and the strict chemical nature of the adducts is not known, additional research is needed to confirm the results, to state the influence of DNA adducts in sperm on reproduction outcome and to investigate the chemical nature of DNA adducts (spot "S").

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