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GAMETE BIOLOGY

Lower sperm DNA fragmentation after r-FSH administration in functional hypogonadotropic hypogonadism

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Abstract
Purpose An observational clinical and molecular study was designed to evaluate the effects of the administration of recombinant human FSH on sperm DNA fragmentation in men with a non-classical form of hypogonadotropic hypogonadism and idiopathic oligoasthenoteratozoospermia.
Methods In the study were included 53 men with a non-classical form of hypogonadotropic hypogonadism and idiopathic oligoasthenoteratozoospermia. In all patients, sperm DNA fragmentation index (DFI), assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) in situ DNA nick end-labelling (TUNEL) assay, was evaluated before starting the treatment with 150 IU of recombinant human FSH, given three times a week for at least 3 months. Patients' semen analysis and DNA fragmentation index were re-evaluated after the 3-month treatment period.
Results After recombinant human FSH therapy, we did not find any differences in terms of sperm count, motility and morphology. The average DNA fragmentation index was significantly reduced (21.15 vs 15.2, $p<0.05$), but we found a significant reduction in patients with high basal DFI values (>15 %), while no significant variation occurred in the patients with DFI values ≤15 %.
Conclusions Recombinant human FSH administration improves sperm DNA integrity in hypogonadotropic hypogonadism and idiopathic oligoasthenoteratozoospermia men with DNA fragmentation index value >15 % .

Keywords Apoptosis · Gametogenesis · DNA · Sperm

Capsule Recombinant human FSH administration improves sperm DNA integrity but not sperm count, motility and morphology, in men with a non-classical form of hypogonadotropic hypo-gonadism and idiopathic oligoasthenoteratozoospermia with baseline DNA fragmentation index value >15 %.

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Introduction

It is well known that sperm DNA quality has been recognized as one of the most important markers of male reproductive potential [30, 42, 60], in contrast to standard semen parameters such as sperm density, motility and morphology that do not act as powerful discriminators between fertile and infertile men [14, 21].

DNA damage in the male germ line is a major contributor to infertility, miscarriage and birth defects in the offspring [2]. In animal models, it has been unequivocally demonstrated that the genetic integrity of the male germ line plays a major role in determining the normality of embryonic development [1].

In humans, several papers reported conflictual data concerning the effects of higher DFI on ICSI clinical outcomes. Morris et al. [39] showed that sperm DNA damage is associated with impaired embryo cleavage. Increased DFI seems to be associated with an higher miscarriage rates [14] and also with a significantly increased risk of pregnancy loss after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [67]. A reduced DFI seems to be related to increased ICSI outcomes [17, 33], other authors reported any interference of DFI on clinical outcomes [32, 66].

In particular, chances for pregnancy are close to zero if more than 30 % of spermatozoa present DNA fragmentation,

either by means of natural conception or intrauterine insemination [7, 57]. In addition, Greco et al. in 2005 reported a clinical pregnancy rate (PR) of 0 % with microinjection of testicular sperm in patients with DNA fragmentation levels in semen >15 %, as measured by TdTmediated-dUTP nick-end labeling (TUNEL) [19]. Higher percentage of fragmented chromatin in a seminal sample is associated with a reduced pregnancy and implantation rates after conventional IVF and it is predictive of failure of embryo development in couples undergoing ICSI cycles [17] confirming the "paternal effect" of sperm DNA on the embryo development, as demonstrated by several other authors [10, 41, 61].

Since there is a clear relationship between sperm DNA damage and poor assisted reproduction technology (ART) outcomes, efforts should be directed in developing treatments to improve sperm DNA quality that could be introduced into clinical use. Only a limited number of studies examined potential treatments to reduce sperm DNA damage and no therapy has been proven to be indicated in reduction of sperm DNA fragmentation [44]. There is growing evidence supporting the use of antioxidant therapy to improve DNA integrity [19], although many studies did not support these observations [12, 38]; moreover, some authors reported benefits using FSH administration but with conflicting results [4].

For infertile males patients with severe oligoasthenoteratozoospermia ICSI can be performed, but single sperm selection for oocyte microinjection is based on standard morphological criteria that have limited power in the determination of its embryogenic capacity [29], possibly because they cannot provide information about DNA integrity and thus impairing ICSI outcomes [3, 33]. In 2006, Berkovitz proposed a morphological sperm selection method, hypothesing that the presence of vacuoles in the sperm head was associated with chromatin alterations, demonstrating that high-magnification sperm selection could improve ICSI clinical outcomes (pregnancy, implantation, delivery and birth rates) [6].

Apoptosis plays an important role in spermatogenesis. It is indispensable in limiting the excessive germ cell proliferation to a number that can be adequately supported by Sertoli cells, thus ensuring normal spermatogenesis. Moreover, apoptosis seems to be responsible for the selective removal of abnormal or injured germ cells [51]. It has been shown that when good-quality sperm cells are exposed to oxidative stress, their removal is often triggered by apoptosis [40]. However, sometimes apoptosis is not able to completely destroy the damaged cells and these may differentiate into sperm that is still able to fertilize (abortive apoptosis) [51].

In the past few years our scientific interest has been focused on FSH, as its ability to support spermatogenetic process, and its role in controlling apoptosis in male germinal epithelium is well known [63]. Furthermore, several studies demonstrated that patients with FSH receptor or FSH-β subunit mutations show a defective spermatogenesis with different semen parameters alterations that can lead to a reduced sperm competence in producing embryos with higher implantation potential [43, 59].

Some studies demonstrated that in men with idiopathic oligoastenozoospermia, exogenous administration of FSH improves sperm structure [5, 58]. The efficacy of r-FSH therapy in oligozoospermic men, aiming at improving other sperm parameters such as motility, number and morphology, is still debated and published data remain controversial [13, 15]. In addition, recombinant human FSH (r-FSH) administration improves sperm DNA integrity in men with idiopathic oligoasthenoteratozoospermia (iOAT) with increased DNA fragmentation index (DFI) values [11]. Other studies, the treatment with rFSH in combination with human chorionic gonadotropin (hCG) was proved to be effective in stimulating spermatogenesis in gonadotropin-deficient men [36, 56, 64].

An impairment of gonadotropin secretion and, therefore, a reduced efficiency of spermatogenesis were reported to be frequently associated with conditions different from the classical causes of secondary hypogonadism. These conditions (metabolic, endocrine and eating disorders, physical exercise etc.) have been associated with a non-classical form of hypogonadotropic hypogonadism (HH) called "functional" HH. Functional HH differs from the classical one by the evidence that gonadotropin levels are in the low-normal range, but it is inadequate for the testosterone levels, that often are also in the low-normal range [28].

The aim of this observational study was to investigate the effects of r-FSH administration on sperm DNA fragmentation of functional HH patients undergoing ICSI due to normal/low levels of FSH and LH despite oligoastenozoospermia, comparing the DFI before and after 90 days of r-FSH therapy.

Materials and methods

Patients

Fifty-three functional HH iOAT men, with a median age of 33.6±7.6 years, referred to our clinics because of fertility problems after at least 2 years of natural attempts of conceiving, and undergoing rFSH therapy, were selected for the study. The inclusion criteria were: sperm count of $\leq 15\times10^6$/ml, total motility ≤40 %, normal morphology ≤4 %, normal BMI (20–25 Kg/m^2), low/normal plasma FSH (1–8 IU/L), LH(1–8 IU/L), prolactin (2–12 mg/L), testosterone (3–10 ng/ml) and inhibin B levels (50.0–250.0 pg/ml). Exclusion criteria were: presence of cryptorchidism, varicocele, genital infections, antisperm antibodies, Y chromosome micro

deletions, karyotype abnormalities, CFTR gene mutations and systemic diseases.

In all patients sperm DNA fragmentation was evaluated prior to treatment with 150 IU of recombinant human FSH (GONAL-f®, Merck Serono, Roma) three times a week for at least 3 months. All patients included in this study declared to have followed the treatment plane. Patients' semen analysis and DNA fragmentation were re-evaluated after the 3-month treatment period. The TUNEL test was conducted by blinded investigators.

This protocol was approved by the institutional review board of the Centre and all patients had previously given informed consent for the study.

Semen analysis and preparation

A single semen analysis was performed before the beginning and at the end of therapy, 90 days later. Semen samples, obtained by masturbation after 2–5 days of sexual abstinence, were examined within 60 min of ejaculation, following a 30' of incubation at 37 °C to allow liquefaction. Ejaculate volume, pH and sperm concentration, motility and morphology were evaluated according to World Health Organization guidelines [65].

All semen samples were prepared by swim-up after dilution in HTF medium supplemented with 5 % Human Serum Albumine and centrifuged at 300 g for 5–7 min. Swim up was performed stratifying 200 µl of HTF medium supplemented with 5 % of HSA on the pellets, and incubating for 30 min at 37 °C. The spermatozoa collected in the supernatant were smeared on poly-L-lysine coated slides for fixation before performing the TUNEL test to evaluate the DNA Fragmentation Index (DFI).

Assessment of sperm DNA fragmentation by TUNEL

Sperm DNA fragmentation index (DFI) was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) in situ DNA nick end-labelling (TUNEL) assay.

Briefly, spermatozoa were smeared on poly-L-lysine coated slides, air dried and fixed with 3.7 % paraformaldehyde for 60 min. Then paraformaldehyde was removed and PBS-glycine was added (0.1 M glycine in PBS and 0.3 mg/ml BSA), the spermatozoa were washed for 5 min in PBS (137 mM NaCl, 2.68 mM KC1, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and permeated for 10 min on ice in TRITON X100 0.1 % and sodium-citrate 0.1 % in PBS; then, 3 washes were made using PBS for 5 min each at room temperature. The spermatozoa were then incubated for 60 min at 37 °C in a humidified chamber in 50 µl of a mixture containing 5 µl of nucleotide mix, 1 µl of TdT enzyme, and 45 µl of Equilibration Buffer (DeadEnd™ Fluorometric Tunel System, Promega Italia srl).

Control samples were incubated with the same mixture without the TdT enzyme (negative control) or pre-treated for 5 min with 100 µl of DNAse buffer, and then treated for 10 min with a DNAse buffer solution containing 10 unit/ml of DNAse I (positive control).

Reaction was blocked with SSC 2X for 15 min. Three 5 min washes were carried out in PBS. The spermatozoa were observed under a fluorescent microscope. Approximately 300 cells were counted and DFI was calculated as a ratio of the total number of spermatozoa evaluated by bright field and the positive TUNEL spermatozoa: an increased sperm DFI was defined for values >15 %, while a DFI ≤15 % was considered normal.

Statistical analysis

Data are expressed as mean ± SD and were analysed using the paired *t*-test and chi-square as appropriate. A p-value <0.05 was considered statistically significant.

Results

In the study we used an in situ TUNEL assay to detect fragments of DNA in human spermatozoa of the 53 iOAT patients before and after 3 months of treatment with r-FSH.

The fields of fixed semen samples on the slide were analysed, after TUNEL assay, by fluorescence and light microscopy and then recorded with the specific software (NIS-Element BR 2.30, Nikon), which allowed us to capture separately fluorescence images, light images and the combination. The advantage of having these three images of the same field is that it is possible to estimate the number of cells with DNA fragmentation from the fluorescence images, while the light images discriminates, for each field, the spermatozoa from the other cells that could interfere in calculating the apoptotic rate. It should be noted that the fluorescent cells include both sperm cells and somatic cells (leukocytes, epithelial cells, round spermatids) so that only with the fluorescent microscopy associated to light microscopy it was possible to distinguish sperm apoptosis from somatic cells DNA fragmentation. The merging allowed us to realize a specific counting of spermatozoa and to observe, at the same time, their morphology and the molecular damage associated, so to perform a real statistical evaluation limited to sperm cells only.

Our results showed that after 3 months of r-FSH treatment, no significant differences were observed between baseline and post therapy semen sample in the total patients evaluated as far as sperm count (6.8×10^6/ml±4.9 vs. 7.1×10^6/ml±3.4), total motility (21.2 %±11 vs. 20.4 %±12.3),

and normal morphology (3.6 %±0.2 vs. 3.4±0.2) were concerned. However, the percentage of sperm DNA fragmentation in the total patients dropped from 21.15±9.3 to 15.2±7.7 (Table 1, $p<0.05$). Interestingly, if patients were divided in two groups according to their basal level of DFI, no statistical difference was found in sperm DFI for patients ($n=14$) with a baseline DFI ≤15 % (10.5±4.2 vs. 11.4±4.5) (Table 2), but an evident and statistically significant DFI reduction in patients ($n=39$) with sperm baseline DFI value >15 % was found (24.4±9.6 vs. 15.4±4.6) (Table 3). No differences were found in the seminal parameters pre and post therapy for the two group divided according to their DFI baseline rate (Tables 2 and 3). In Fig. 1 the images of a field of a seminal sample captured by light microscopy (A), by fluorescent microscopy (B) and the merge of images (C), are shown as an example.

Discussion

The present study shows that 90 days of rFSH administration improves sperm DNA integrity, by reducing DFI values in the group of patients with functional HH and iOAT showing a baseline DFI >15. The origins of DNA damage in the male germ line during either the production or the transport of sperm cells are probably due to multiple causes: (i) apoptosis during the process of spermatogenesis; (ii) DNA strand breaks produced during the remodelling of sperm chromatin in the process of spermatogenesis; (iii) post-testicular DNA fragmentation induced, mainly by oxygen radicals, during sperm transport through the seminiferous tubules and the epididymis; (iv) DNA fragmentation induced by endogenous caspases and endonucleases; (v) DNA damage induced by radiotherapy and chemotherapy; and (vi) DNA damage induced by environmental toxicants [49].

In particular, during spermatogenesis some strand breaks are caused physiologically by topoisomerase to reduce the torsional stresses that are created when DNA is condensed and packaged in the sperm head, during the late phase of differentiation [35, 50]. Normally, breaks are physiologically reassembled before spermatozoa are released from germinal epithelium [27], but, as a consequence of testicular injuries, including heat, exposure to toxicant [26, 31, 34] radiation [37], withdrawal of hormonal support [22, 55, 61], other testicular pathologies [18] or life habits (smoke, drug use), the repair process could be impaired, so that spermatozoa show high levels of DNA damage [8, 49].

Post-testicular DNA fragmentation mainly induced by oxygen radicals, seems to be the main cause of apoptosis in human germ cells [2]. Mammalian spermatozoa seem to be highly sensitive to free radical attack, with the consequent induction of a lipid peroxidation process that disrupts the integrity of the plasma membrane and impairs sperm motility [24, 25]. Mitochondrial DNA is also particularly vulnerable to free radical attack because it is essentially unprotected [52]. All these processes greatly destabilize the DNA structure and may ultimately result in the formation of DNA strand breaks [2].

FSH seems to play a pivotal role in spermatogenesis and in sperm DNA maturation process. FSH is a strong anti-apoptotic factor and during seminiferous tubule maturation its synergistic action with testosterone and estradiol support germ cell survival, while estradiol alone has an inhibitory, pro-apoptotic effect [63]. FSH is a key regulator of testis function, required for the establishment of full complements of Sertoli and germ cells during postnatal testis development and for the maintenance of spermatogenesis in the adult. FSH plays an important role in germ cell survival rather than proliferation during the window between 14 and 18 days of testicular development, which coincides with the cessation of Sertoli cell proliferation and the onset of germ cell meiosis during the first wave of spermatogenesis. FSH suppression induces spermatogonial apoptosis predominantly via

Table 1 Seminal characteristic and DFI pre and post therapy in the 53 iOAT patients

N° patients: 53	Baseline	Post therapy
Total sperm count (x10^6/ml)	6.8±4.9	7.1±3.4
Total motility (%)	21.2±11	20.4±12.3
Normal morphology (%)	3.6±0.2	3.4±0.2
DFI (%)	21.15±9.3[a]	15.2±7.7[a]

[a] p-value<0.05

Table 2 Seminal characteristic and DFI at baseline and post therapy in patients with DFI ≤15 %

N° patients: 14	Baseline	Post therapy
Total sperm count (x10^6/ml)	8.2±1.2	7.5±2.3
Total motility (%)	23.2±6.1	18.4±10.1
Normal morphology (%)	3.5±0.3	3.2±0.2
DFI (%)	10.5±4.2	11.4±4.5

Table 3 Seminal characteristic and DFI at baseline and post therapy in patients with DFI >15 %

N° patients: 39	Baseline	Post therapy
Total sperm count (x10^6/ml)	8.6±0.9	7.9±1.7
Total motility (%)	24.1±4.4	22.4±6.2
Normal morphology (%)	3.7 %±0.1	3.4±0.2
DFI (%)	24.4±9.6 [a]	15.4±4.6 [a]

[a] p-value<0.05

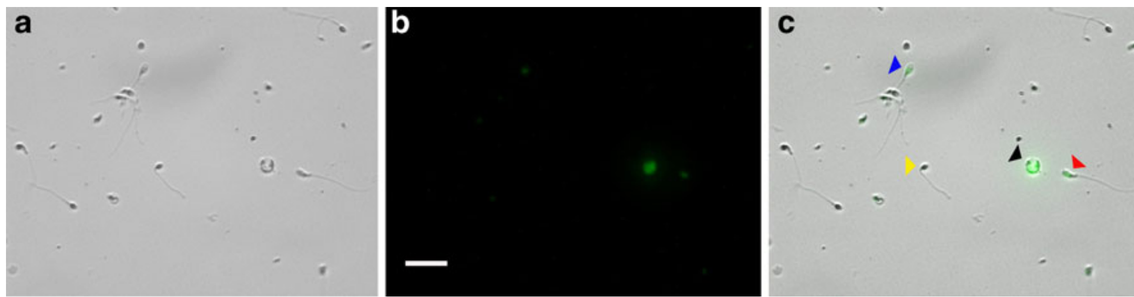

Fig. 1 TUNEL assay on spermatozoa samples, images captured by microscopy in the same field. Light image (**a**), fluorescent image (*green*) (**b**), merge of images (**c**). *Red arrowhead*: abnormal sperm (*flattened head*) with DNA fragmentation; *Yellow arrowhead*: abnormal sperm (*angled neck*) with DNA integrity; *Blue arrowhead*: morphologically normal sperm with DNA fragmentation; *Black arrowhead*: other cell. Scale bar=30 μm

the intrinsic pathway, while spermatocyte apoptosis occurs via both the intrinsic and extrinsic pathways [45].

Tesarik and colleagues [62] evaluated caspase activity and DNA integrity in germ cells within in-vitro cultured segments of human seminiferous tubules after induction of apoptosis by FSH or testosterone withdrawal. FSH withdrawal increased the incidence of DNA fragmentation in spermatocytes and spermatids, in a caspase-independent pathway.

Recently, Colacurci et al. [11] demonstrated that r-FSH administration improves sperm DNA integrity in iOAT men with increased DFI values, but other studies showed controversial results [4, 9].

To investigate the effects of r-FSH administration on sperm DNA fragmentation of functional HH and iOAT patients undergoing ICSI, we compared DFI before and after 90 days of FSH therapy. In order to demonstrate a positive effect of the hormone in improving the late spermatogenesis process, we performed a method that simultaneously highlights the morphology and molecular damage of spermatozoa.

Our findings suggest that r-FSH administration may improve sperm DNA integrity in functional HH and iOAT men with increased DFI values. The r-FSH treatment seems to be more effective in the patient where baseline DFI was higher than 15 %, while it seems to have no effect in those patients showing a baseline DFI less than 15 %, according to the recent paper by Colacurci et al. [11]. Differently from other papers we calculated DFI using three images of the same field (bright field, fluorescent and merge) so that we estimated the number of cells discriminating, for each field, the spermatozoa from the other cells that could interfere in the calculation of the apoptotic rate.

The positive effects of r-FSH in functional HH patients could be explained by a modified biological activity of endogenous FSH, requiring the support of exogenous FSH. In fact, it has been demonstrated that the presence of FSH receptors polymorphisms and of FSH receptor defects might be involved in some form of male infertility [46, 47, 53]. In addition, it has been demonstrated that men with FSH receptor mutations demonstrate variable spermatogenic activity, ranging from oligospermia to normal sperm counts [54].

Thus, we can speculate that exogenous FSH could have a positive role in controlling apoptosis and all the pathways involved in the process of spermatogenesis.

Our data shows that r-FSH administration did not lead to an improvement of semen parameters; the differences emerging from analysis of the literature can, in part, be justified by various factors, such as criteria adopted in the selection of the patients, the interpretation of the seminal parameters, the treatment dose and its length [16].

Reducing the apoptotic rate in a seminal sample means to increase the probability, in an ICSI cycle, to inject sperm showing chromatin integrity. Several papers demonstrated increased clinical outcomes when sperm DFI is in a physiological range [17, 33, 48]. Recently some tools have been proposed to try to select, prior to perform ICSI, a mobile, normo-morphologic sperm showing chromatin integrity, using hyaluronic acid–binding method based on the presence of a hyaluronic acid receptor [23], sperm magnetic sorting with annexin V microbeads based on apoptotic markers such as the presence of externalized phosphatidylserine to the surface membrane of spermatozoa [20], but none were considered sensitive and specific.

In conclusion, in our experience, FSH acts as a strong anti-apoptotic agent in reducing DNA fragmentation in functional HH and iOAT patients without modifying the semen parameters, as sperm count motility and morphology. The therapy may be a specific treatment for infertile male partners of couples undergoing ICSI, specifically in the case that basal DFI is higher than 15 %, reducing the percentage of spermatozoa with DNA integrity anomalies, suggesting a positive effect on the reproductive outcome. Further studies will need to verify if the DFI reduction after r-FSH therapy increases ICSI clinical outcomes and to investigate the specific apoptotic pathways involved in DNA fragmentation in human sperm, as well as the membrane receptors and the molecular cascades activate by exogenous FSH administrated.

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