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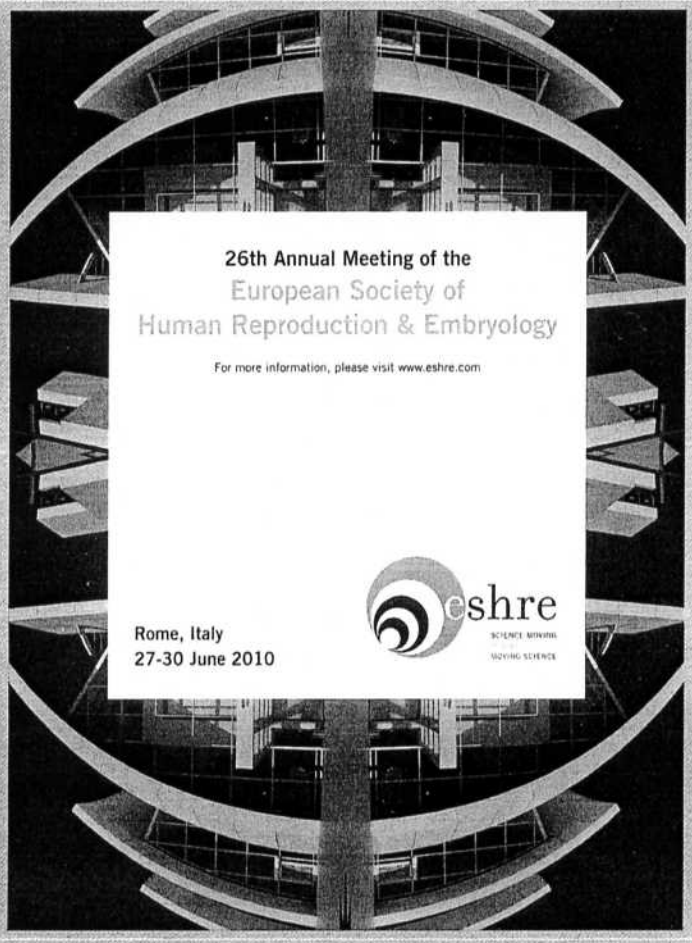

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DNA damage has been associated adversely with fertilization rates, embryo quality and pregnancy rates after ART. As there is little correlation between conventional semen parameters and sperm DNA damage, men with idiopathic infertility can have significantly high levels of sperm DNA damage which remain undetected. In this study we assessed the effect of sperm DNA on embryo quality in couples with male, female and idiopathic infertility and the impact of oocyte quality when sperm DNA damage remained constant.

Materials and Methods: A total of 133 couples attending for IVF were identified and of these, 89 couples (51% female, 17% male and 32% idiopathic infertility) who were having embryo transfer on day three were included in this study. Couples with failed fertilization and men with antisperm antibodies in their semen were excluded from the study. Semen analysis was performed according to WHO guidelines (1992). Native semen and sperm prepared by density gradient centrifugation were analysed for DNA damage by the alkaline Comet assay. The embryo score was calculated by multiplying embryo grade (A = 4, B = 3, C = 2 and D = 1) with the number of blastomeres for each embryos and then they were grouped into three categories low (< 12), moderate (12-24) and good (> 24) embryo grades. Similarly, sperm DNA damage was categorized into three levels of fragmentation [low (0-40%), moderate (41-70%) and high (71-100%)]. Chi square analysis was performed to evaluate the differences in embryo quality obtained from couples with male, female and idiopathic infertility. Duncan's test for multi-group comparison was performed to analyse embryo quality within each category of DNA damage in couples with male, female and idiopathic infertility.

Results: A total of 678 embryos were generated, of which 53.7% were low, 27.8% were moderate and 18.5% were good quality embryos. There were no correlations between embryo quality and any conventional semen parameters. However, a significant inverse correlation was observed between embryo quality and sperm DNA damage ($r^2 = 0.131$, $P = 0.001$). When sperm DNA damage was separated into the three categories, there was a significant inverse correlation between embryo quality (9.27 ± 0.51, 10.71 ± 0.92 and 12.73 ± 1.01) and sperm with high, moderate and low DNA damage respectively, ($P = 0.006$). High sperm DNA damage resulted in poor quality embryos (57%, 60% and 70%), in male, female and idiopathic groups of patients, respectively. Also, in the group with moderate sperm DNA damage, there was no difference in the percentage of low and moderate quality embryos in patients with male, female or idiopathic infertility. Surprisingly, couples with idiopathic infertility and low sperm DNA damage had significantly less embryos of high quality (13%, embryo grade 8.96 ± 1.44) than the other two groups of patients (female infertility 28%, embryo quality 14.11 ± 1.49; $P = 0.05$ and male infertility 38%, embryo quality 18.44 ± 2.45; $P < 0.001$).

Conclusion: Sperm DNA damage impacts adversely on the quality of day three embryos in couples with male and female infertility. However, poor quality embryos in couples' with idiopathic infertility and low DNA damage suggests that an oocyte defect may be a contributory aetiological factor.

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P-023 Use of high-magnification microscopy for the assessment of sperm preparation methods

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Introduction: It has long been accepted that sperm preparation methods, density-gradient centrifugation and swim-up are necessary to produce samples with fertilization capacity. But which of the current preparation methods is more effective? Studies conducted in the past based on assessment of conventional semen parameters have not been conclusive. In recent years, a method called IMSI (Intra-cytoplasmic Morphologically-selected Sperm Injection) has been developed. This method is based on the observation of unstained motile sperm with high magnification (8000X) microscopy and the selection of normal cells applying strict morphologic criteria. Despite of some controversy, sperm morphology evaluation has also played a crucial role in the diagnosis of male fertility potential. Using IMSI, it has been shown that presence of large nuclear vacuoles in sperm is associated to DNA damage, poor embryo quality and early pregnancy lost.

It is, therefore, of great importance to select a preparation technique that minimizes possible paternal effects on embryo development.

With this aim we performed nuclear-vacuole analysis at high magnification to compare the quality of sperm preparations obtained by density gradients and swim-up techniques.

Material and Methods: Prospective study on sperm samples from 53 patients. Whole semen was analysed according to WHO guidelines. Each sample was prepared by density gradient centrifugation and swim-up techniques and analysed under high magnification microscopy at 8000 X. Spermatozoids were counted and classified into categories according to the vacuoles present: Grade I, absence of vacuoles; Grade II, maximum of two small vacuoles; Grade III, more than two small vacuoles or at least one large vacuole; Grade IV, large vacuoles in combination with abnormal head or other abnormalities.

Results: Samples were capacitated by two different techniques and several variables were analyzed in the pre- and post-capacitated samples. Regarding the degree of vacuolization of spermatozoids, there were significant differences in the proportion of sperm carrying low number of small vacuoles in the prepared semen in comparison with the whole semen. An increment in grades I and II, the less vacuolated types, was observed after preparation, with the correspondent decrease in grades III and IV. Direct comparison of the results obtained with both methods revealed that the increment in groups I and II was considerable higher in the swim-up group than in the density gradient, 59.29% vs. 15.67%, clearly showing that this method has an advantage over the gradient to generate samples that are enriched in sperm suitable for ICSI.

Conclusions: In the present analysis we found that the degree of vacuolization of sperm was lower after the preparation methods, suggesting that they both allow the selection of less vacuolated sperm cells and presumably with less DNA fragmentation. Most importantly, we could show that swim-up preparation technique was significantly more effective. Therefore, we suggest that swim-up should be the preparation technique of choice used prior to ICSI/IMSI, particularly for those couples with a history of early abortion that might be caused by the presence of a defective male genetic contribution.

P-024 Increased body weight is associated with decreased conventional and non conventional flow cytometric sperm parameters in otherwise healthy men

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Introduction: Obesity has been reported to negatively affect male reproduction. The most common aspects investigated in these patients are hormonal and sexual dysfunction, oxidative stress, and scrotal hyperthermia. As far as seminology, the studies thus far conducted have only examined the effect of obesity on conventional sperm parameters (density, motility and morphology), whereas little is known about the effects of body weight on sperm mitochondrial function, chromatin compactness and apoptosis. Therefore, this study was undertaken to evaluate conventional and non conventional sperm parameters in healthy, normal, non smoking overweight and obese men.

Materials and Methods: A total of 24 men with increased body weight were enrolled in this study. They were divided into two groups: 1) overweight (n = 11) with a body mass index (BMI) of 25-29.9 kg/m^2; and 2) obese (n = 13) with a BMI >30 kg/m^2, according to the WHO 1995 criteria. Normo-weight healthy non smoking men (n = 16) with a BMI of 18.5-24.9 kg/m^2 were recruited as controls. A complete general and andrological clinical, laboratory and instrumental diagnostic work-up was conducted on each subject. Men with systemic diseases, male accessory gland infection, history positive for cryptorchidism or varicocele, microrchidism, alcohol and/or drug abuse, endocrine diseases and recent hormonal treatment were excluded. Semen samples were collected after 3-5 days of sexual abstinence and analyzed according to the WHO 1999 criteria. The remaining spermatozoa were used for the evaluation of the following non conventional parameters, by flow cytometry: mitochondrial membrane potential (MMP), following JC-1 staining, phosphatidylserine (PS) externalization, after annexin V and propidium iodide (PI) staining, chromatin compactness, after PI staining, and DNA fragmentation, by TUNEL assay. Results were analyzed by 1-way analysis of variance (ANOVA) followed by the Duncan test. Correlation analysis was conducted by Pearson test. A statistically significant difference was accepted when the p value was lower than 0.05.

Results: Overweight and obese men had lower sperm density, total sperm count, progressive motility (grade a + b) and normal forms than controls. Serum LH, FSH, prolactin and testosterone levels in overweight and obese men did not differ significantly from those found in controls, whereas a significantly higher

serum levels of 17ß-estradiol and SHBG were found. Overweight and obese patients had a significantly higher percentage of spermatozoa with low MMP and 31.9 ± 9.3%, respectively) and a significantly lower percentage of alive spermatozoa (56.5 ± 6.9% and 54.1 ± 5.8%, respectively) compared to controls (low MMP = 2.8 ± 1.1%; alive = 77.8 ± 1.9%). The percentage of spermatozoa with PS externalization (10.7 ± 3.7% and 16.1 ± 3.4%, respectively), an early sign of apoptosis, or with abnormal chromatin compactness (20.0 ± 2.3% and 20.6 ± 3.1%, respectively) was significantly higher in overweight and obese patients compared to controls (PS externalization = 2.7 ± 0.5%; chromatin abnormality = 13.9 ± 0.7%). Lastly, the percentage of patients with fragmented DNA was higher in both overweight (4.1 ± 0.8%) and obese (5.6 ± 1.3%) patients compared to controls (2.3 ± 0.7%), but the difference reached the statistical significance only in the latter. Correlation analysis showed that the BMI correlated negatively with total sperm count, progressive motility, normal forms and percentage of viable spermatozoa and positively with spermatozoa with low MMP, PS externalization and DNA fragmentation, but not with chromatin compactness.

Discussion: These results showed that healthy overweight and obese men have worst conventional sperm parameters, sperm mitochondrial function and chromatin/DNA integrity. All these parameters, but chormatin abnormality, correlated significantly with BMI. The lack of andrological, systemic and/or endocrinological diseases suggest that the increased body weight has a negative impact on these parameters. Given their relevant role played on couple's fertility, we suggest to include a body weight losing program among the therapeutic strategies of male infertility.

P-025 Effects of density gradient centrifugation and swim up techniques on sperm DNA integrity

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Introduction: A wide variety of techniques for sperm preparation are currently available, the most commonly used are density gradient centrifugation and *swim up*. Although these techniques appear to be effective in selecting functional sperm for assisted reproductive technologies (ART), to date, there is no consensus about which is the best method for isolating functionally competent spermatozoa and neither about the effect of these semen processing techniques on sperm DNA integrity.

Sperm DNA damage has been linked with poor semen quality and reduced fertility. Since there is concern about the effects of DNA fragmentation in ART success and embryo quality, the evaluation of the DNA damage caused by some of the protocols of semen processing is of interest. In this study we aim to examine and compare the effect of density gradient centrifugation and *swim up* processing techniques on DNA integrity within populations of spermatozoa from normozoospermic and non normozoospermic subjects.

Material and Methods:

Sperm preparation

One hundred and forty nine human semen samples were used in this study. Samples were obtained by masturbation after 3-5 days of sexual abstinence. After liquefaction of semen, standard semen parameters were obtained according to WHO guidelines. All of the semen samples used had a minimum concentration of 5 million spermatozoa/ml and absence of leukocy-tospermia.

Swim up. Samples diluted in IVF plus (Vitrolife, Göteborg, Sweden) were centrifuged at 1400 rpm for 10 minutes, the sperm pellet resuspended in IVF plus and incubated for 1h at 37°C under an atmosphere of 5% CO_2 in air. After the incubation period, the entire supernatant was aspirated and analysed.

Density gradient centrifugation. Semen samples were placed on top of a 50%-90% Spermgrad (Vitrolife, Göteborg, Sweden)/IVF plus gradient, centrifuged for 20 minutes at 1200 rpm and the pellet resuspended in IVF plus. Subsequently, the *swim up* protocol described above was performed.

Sperm DNA fragmentation analysis

Sperm Chromatin Dispersion (SCD) test. DNA fragmentation index (DFI) and DNA degradation index (DDI) of the samples were determined by using the Halosperm® kit (Halotech DNA SL, Madrid, Spain).

2 tailed (2T) Comet assay. Single stranded (ss) and double stranded (ds) DFI of the samples were determined by using the 2T comet assay (Enciso et al. 2009).

Results: SCD test DNA damage analysis revealed that neat non normozoospermic semen samples presented a significantly (U Mann Whitney, $p < 0.05$) higher mean DFI and DDI than normozoospermic samples.

After semen processing with density gradient and *swim up* techniques, mean sperm DFI and DDI, assessed by the SCD test, were significantly reduced (U Mann Whitney, $p < 0.05$) in both *swim up*-treated and density gradient-treated spermatozoa in both groups of subjects. Similarly, the percentage of spermatozoa with ds DNA breaks assessed by the 2T comet assay, was also significantly reduced (U Mann Whitney, $p < 0.05$) in both *swim up*-treated and density gradient-treated spermatozoa in both groups of subjects. However, the percentage of spermatozoa with ss DNA breaks assessed by the 2T comet assay, was significantly (U Mann Whitney, $p < 0.05$) reduced only in density gradient-treated but not in *swim up*-treated spermatozoa in the groups of subjects analysed.

Conclusions: The semen processing techniques *swim up* and density gradient centrifugation recover spermatozoa with improved DNA quality. Both methods are efficient in eliminating highly DNA damaged and double stranded DNA damaged spermatozoa but the density gradient centrifugation method is more efficient than the *swim up* technique in eliminating single stranded DNA damaged spermatozoa.

Reference:

1 Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. Reproductive BioMedicine Online, Vol. 18 (5): 609-616. 2009.

P-026 Can prepubertal human testicular tissue be cryopreserved by vitrification?

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Introduction: Cryopreservation of testicular tissue from prepubertal boys with cancer has emerged as an ethically acceptable strategy to preserve their fertility, since sperm banking cannot be considered in these patients. Controlled slow-freezing (SF) with dimethylsulfoxide (DMSO) as a permeating cryoprotectant (CP) has proved to be a promising approach to preserve immature human testicular biopsies. Nevertheless, an efficient cryopreservation protocol has not yet been established. Vitrification (V) might constitute a better approach by avoiding ice crystal formation and subsequent freeze injuries. Using a long-term in vitro organotypic culture system, our aim was to evaluate the efficiency of V to preserve spermatogonial cell (SPGc) survival and seminiferous tubule (ST) integrity of prepubertal human testicular tissue, since these parameters are essential for initiation of spermatogenic processes.

Material and Methods: Testicular tissue was obtained from two patients (6 and 12 years of age) before starting gonadotoxic treatment. Controlled SF was performed with a programmable freezer using a freezing solution containing DMSO (0.7 mol/l) and sucrose (0.1 mol/l). Increased concentrations of CP and faster cooling rates were used for V. After dehydration in DMSO (2.8 mol/l) and ethylene glycol (2.8 mol/l) solution, samples were placed in open cryostraws and directly plunged into liquid nitrogen. Fresh tissue (FR), used for control purposes, and thawed and warmed biopsies were cultured for 10 days. ST integrity was evaluated by light microscopy on stained sections of tissue fixed in Bouin's solution. SPGc survival and proliferation were evaluated by immunohistochemistry using, respectively, MAGE-A4 and Ki67 antibodies on sections of tissue fixed in formalin.

Results: Similar ST morphology was observed in both cryopreserved tissues and FR controls after long-term organotypic culture. Histological characteristics of SPGc and Sertoli cells were preserved, as well as cell-cell cohesion and cell adhesion to the basement membrane in all three groups (FR, SF and V). Pyknotic nuclei were found in the ST of FR and cryopreserved cultured tissue retrieved from the oldest boy. These cells were more frequently seen after cryopreservation, but no difference was noted between the SF and V methods. Survival of SPGc evidenced by MAGE-A4-positive immunostaining was confirmed in all cryopreserved tissue and FR controls after long-term culture. The ability of SPGc to proliferate after cryopreservation and culture was proved in all three groups by positive Ki67 immunostaining.

Conclusion: Vitrification is a convenient method for cryopreservation of immature human testicular tissue, since the process appears to be faster and cheaper than SF. As this technique preserves ST integrity and allows survival and proliferation of human SPGc in long-term organotypic culture, V might be considered as an alternative to SF, and thus a promising strategy to preserve the reproductive capacity of young boys. The functional characteristics of cryopreserved SPGc should be further evaluated through xenotransplantation experiments to determine whether SF and V are equally efficient approaches.