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SHORT COMMUNICATION

HOMBURG/SAAR, GERMANY

Relationship between nuclear chromatin decondensation (NCD) in vitro and other sperm parameters and their predictive value on fertilization rate in IVF program

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***Purpose*:** The purpose of this study was to determine the in-vitro induced nuclear chromatin decondensation (NCD) of human spermatozoa and its value in combination with routine semen analysis in predicting the outcome of in-vitro fertilization (IVF).
***Methods*:** The ejaculate of 52 couples, undergoing IVF, was incubated with lithium diidosalicylic acid (LIS) and dithiothreitol (DTT) (G.1) or with heparin and sodium dodecyl sulphate (SDS) (G.2) to induce chromatin decondensation (NCD). Smears were made at 30, 60, and 120 min after incubation.
***Results*:** NCD was evaluated by morphometrical detection of the surface area of the spermatozoa using a semi-automatic image analysis system (IBAS). In both groups, the sperm heads showed a significant enlargement after 30, 60, and 120 min incubation in comparison to the initial size. However, no correlation was found between NCD at various periods of time and the fertilization rates. The mean area of the sperm heads in the native sample in the G.1 was $9.45 \pm 1.33\,\mu m^2$ and $9.02 \pm 1.15\,\mu m^2$ in the G.2. This area increased after incubation for 30, 60, and 120 min to 10.92 ± 1.48, 12.26 ± 2.16, 13.54 ± 3.14, and $15.35 \pm 7.78\,\mu m^2$ in the first group (G.1) and to 10.29 ± 1.15, 11.23 ± 1.85, 11.46 ± 1.97, and $11.27 \pm 2.82\,\mu m^2$ in the second group, respectively.
***Conclusions*:** NCD in vitro after incubation with LIS + DTT or heparin + SDS could not be recommended as a predictive parameter for IVF outcome.

KEY WORDS: Chromatin decondensation; human spermatozoa; image analysis; morphometry; surface area.

INTRODUCTION

During spermiogenesis, histones of round spermatids are replaced first by transition proteins (1) and then by the arginine- and cysteine-rich protamines (2). The cysteine residues in these protamines form intramolecular and intermolecular disulfide bonds which stabilize the protamine-DNA complex (3).

The condensation and stabilization of the chromatin is regulated during different stages at the elongation of the spermatid (4), epididymal transport (5) as well as by seminal fluid after ejaculation (6). Furthermore, the chromatin stability increases with time after ejaculation and appears to be caused by the formation of excessive disulphide bridges within the chromatin (7). Hyperstability of human sperm chromatin is interpreted variously as detrimental to the delivery of the male genome into the oocytes (8) or as essential to avoid chromosomal damage and thus leads to successful fertilization (9). In addition, the stabilization of chromatin seems to compensate for the lack of DNA-repair enzyme in spermatozoa (10).

During fertilization, the decondensation process is characterized by the degradation of protamines, synthesis of histones, and binding of the histones to DNA leading to restoration of the paternal genome and its transcriptionally active conformation (3). Reduction of these—SS-bonds by ooplasmic glutathione (GSH) is an essential preliminary to sperm nucleus decondensation in mammals. However, glutathione is not sufficient for full decondensation of sperm nuclei (11). Perhaps, nucleoplasmin is also necessary to remove protamines from DNA (12). Nucleoplasmin is a major sperm nucleus-decondensing factor, which is released from the germinal vesicle (GV) into ooplasma at GV breakdown (13). However, the failure of sperm decondensation in the oocytes may be a consequence of a subtle sperm abnormality that is unrecognizable by conventional analysis (14) such as a structural or biochemical defect associated with chromatin packaging or organization during spermatogenesis (15).

Nevertheless, human sperm heads can be decondensed in vitro using a combination of detergents and disulphide bond (S—S) reducing agents such as sodium dodecyl sulphate/dithiothreitol (SDS/DTT) and heparin (16). The in vitro decondensation test can be used to determine the fertilization potential of spermatozoa, since chromatin decondensation induced in vitro by activation of intrinsic mechanisms (17) can resemble that observed in spermatozoa entering the oocytes (18,19). Therefore, this study was undertaken in order to i) investigate and compare the chromatin decondensation ability after incubation either with lithium diidosalicylic acid + dithiothreitol

LIS + DTT or with heparin + SDS as evaluated by an interactive image analysis system (IBAS) and ii) to determine the relationship between the extent of chromatin decondensation in vitro, other sperm parameters, and fertilization rate after IVF therapy.

MATERIALS AND METHODS

52 couples undergoing IVF treatment were included in this study. A routine semen analysis according to WHO guidelines (20) was done after semen liquefaction for 30 min. Each semen sample was divided into two aliquots: the first aliquot was processed by PureSperm for routine IVF as previously described (21). The second aliquot was mixed (1.1) with detergents and reducing substances, either 10 mM LIS + and 1 mM DTT (Sigma-Aldrich Chemicals GmbH; Munich, Germany) (G.1; $n = 26$) or with 5000 USP/mL heparin+1% SDS (Sigma-Aldrich Chemicals GmbH) in borate solution (G.2, $n = 26$) and incubated for 120 min. The reaction was stopped by adding equal volumes of 3.5% glutaraldehyde in 0.05 M borate buffer (pH 9) to the incubation mixture.

A total of 25 smears were made from each semen sample, five smears before and 20 after semen incubation with detergent and reducing agent at various time intervals (0, 30, 60, and 120 min, five smears at each time point). The smears were stained with a modified Papanicolaou staining for spermatozoa as described in the WHO guidelines (20) the morphology was evaluated according to strict criteria described by Krüger *et al.* (22). The decondensed nuclear chromatin can easily be recognized by phase contrast microscopy as spermatozoa having a swollen opaque head with reduced light refractivity. The nuclear chromatin in spermatozoa that did not decondense in vitro appeared normal. The surface area of spermatozoa was detected morphometrically: semiautomated image analysis was performed using the IBAS 2.5 analysis system (Zeiss-Vision Co., D-85399 Hallbergmoos, Germany). The Spermatozoa for image analysis were taken from different areas of the smear. Areas of spermatozoa were evaluated on the analyzer screen after interactive demarcation of the circumference of the spermatozoa, and surface areas were automatically calculated by utilizing the enclosed pixel areas. For each slide at least 100 spermatozoa were analyzed. By applying this system, sperm chromatin decondensation could be evaluated by observing changes in size within the different time intervals after incubation of spermatozoa with detergents and reducing agents (native, directly, and at 30, 60, and 120 min after incubation with the reagents). The female partners underwent ovarian stimulation using the long protocol as described earlier (23).

Statistical Analysis

The significance of differences between G.1 and G.2 and the enlargement of the areas were evaluated with Wilcoxon test for nonparametric tests. The ability of sperm to decondense in vitro was compared with their ability to fertilize human oocytes in vitro. The number of retrieved, fertilized, and transferred oocytes, and pregnancies were correlated with the mean percentage of spermatozoa with decondensed chromatin using spearman rho test. Statistical analysis concerning surface areas—as evaluated with the semiautomated image analysis—was carried out using Excel and SPSS 11.0.

RESULTS

The results of this study are summarized in Tables I and II. The mean age of the patients was 28.31 ± 8.06 years, the mean sperm concentration was 61.5 mill/mL. The mean area of the native spermatozoa in the first group, as evaluated by the semiautomated image analysis system IBAS 2.5, was $9.45 \pm 1.33\,\mu m^2$. This value increased after incubation with LIS+DTT for 30, 60, and 120 min to $12.26 \pm 2.16\,\mu m^2$, $13.54 \pm 3.14\,\mu m^2$, and $15.35 \pm 7.78\,\mu m^2$, respectively (Table I). The enlargement of the native sperm heads within the 120 min of incubation was highly significant ($p = 0.001$).

In the second group, incubated with heparin + SDS, the chromatin decondensation increased

Table I. Surface Area of the Spermatozoa After Chromatin Decondensation Induced by In-Vitro Incubation with Either LIS + DTT (G.1) or Heparin + SDS (G.2) at Various Time Intervals

	LIS + DTT (G.1; $n = 26$) [μm^2] $M \pm$ SD	Heparin + SDS (G.2; $n = 26$) [μm^2] $M \pm$ SD
Native samples	9.45 ± 1.33	9.02 ± 1.15
Immediately after incubation	10.91 ± 1.48	10.29 ± 1.15
30 min after incubation	12.26 ± 2.16	11.23 ± 1.85
60 min after incubation	13.54 ± 3.14	11.46 ± 1.97
120 min after incubation	15.35 ± 7.78	11.27 ± 2.82

Table II. Outcomes of IVF-Procedures in the G.1 and 2

		LIS + DTT (G.1; $n = 26$) $M \pm$ SD	Heparin + SDS (G.2; $n = 26$) $M \pm$ SD
Number of retrieved oocytes	(475)	9.42 ± 6.584	9.62 ± 6.0
Number of fertilized oocytes	(233)	4.96 ± 4.49	4.08 ± 4.35
Number of transferred oocytes	(90)	1.85 ± 1.01	1.69 ± 1.05
Number of pregnancies	(14)	0.35 ± 0.49	0.20 ± 0.41

similarly from $9.02 \pm 1.15\,\mu m^2$ in the native sample, to $11.23 \pm 1.85\,\mu m^2$, $11.46 \pm 1.97\,\mu m^2$, and $11.27 \pm 2.82\,\mu m^2$ after incubation for the different time intervals (Table I). The area of the sperm heads as measured after 120 min incubation was significantly ($p = 0.001$) larger than that of the native ones. In the first group, the mean number of retrieved, fertilized, and transferred oocytes was 9.42 ± 6.58, 4.96 ± 4.49, and 1.85 ± 1.01, respectively. The pregnancy rate in the G.1 was $35.0 \pm 4.9\%$. The corresponding values of retrieved, fertilized, and transferred oocytes in the G.2 were 9.62 ± 6.0, 4.08 ± 4.35, and 1.69 ± 1.05 with $20.0 \pm .4.1\%$ pregnancies (Table II). There was no positive correlation between semen parameter (count, motility) and nuclear chromatin decondensation in vitro. Furthermore, no correlation could be found between nuclear chromatin decondensation after incubation with LIS + DTT (G.1) or heparin + SDS (G.2) and the fertilization rate after IVF treatment.

DISCUSSION

The ability of spermatozoa to fertilize oocytes depends on a sequence of events ending ultimately in the decondensation of the sperm chromatin. Studies have indicated that the mechanism of decondensation probably involves both a loss of zinc from the sperm (24) and a reduction in the disulphide bonds of the sperm nucleus by glutathione reductase localized in the ooplasma (25). Sperms have been shown to undergo in vitro nuclear decondensation characterized by swollen sperm heads and dissolution of the head in the presence of sodium dodecyl sulphate and a sulphydryl reducing agent (5).

In vitro sperm nuclear decondensation has been achieved by adding, in addition to thiol, a detergent (26), a protease, and a high salt concentration (27), and a heparin (28). Using microscopy several groups have also suggested that defective chromatin decondensation can be found in some cases of infertility (9,29). Determination of the degree of decondensation of a sperm is arduous, since decondensation is dynamic and progressive (17,24) and a resulting classification remain subjective (25).

Several groups have measured sperm chromatin decondensation by observing changes in size and shape of the sperm head by microscopic examination (9,30–32). These methods tend to be somewhat subjective because it is difficult to decide what degree of sperm head enlargement is to be the criterion of decondensation. Samocha-Bone *et al.* (33) measured sperm chromatin decondensation in vitro by acridine orange staining followed by flow cytometry.

Zucker *et al.* (34) induced decondensation in rat and hamster spermatozoa using sodium dodecyl sulphate and dithiothreitol as a reducing agent and evaluated decondensation by increasing light scatter. Evenson *et al.* (35) evaluated decondensation in mouse and human sperm nuclei using acridine orange staining and plotting green fluorescence versus pulse width of the green signal, which is related to cell size.

In the present study, two combinations of decondensing agents, LIS + DTT in the G.1 and heparin + SDS in the G.2 were investigated. The nuclear chromatin decondensation was evaluated after modified Papanicolaou staining by measurement of the surface area of spermatozoa by semiautomated image analysis using the IBAS 2.5 analysis system (Zeiss-Vision Co., D-85399 Hallbergmoos, Germany). Areas of spermatozoa were evaluated on the analyzer screen. After interactive demarcation of the circumference of the spermatozoa, the surface areas were automatically calculated by utilizing the enclosed pixel areas.

The results from G.1 showed an increase of nuclear chromatin decondensation between all time points, and the second group had the similar results. So both combinations of decondensing agents are able to induce an efficient chromatin decondensation that reached its maximum at 120 min incubation. Nevertheless, no positive correlation could be found either between chromatin decondensation and fertilization rates, or between semen parameters such as sperm account, and motility in the native semen samples and nuclear chromatin decondensation.

These findings are in accordance with those of Liu *et al.* (36) who found a negative correlation between the mean percentage of motile spermatozoa, motility index, and in vitro chromatin decondensation using SDS/DTT, whereas sperm concentration

and morphology showed no correlation with chromatin decondensation. Rosenborg *et al.* (8) found no relationship between nuclear chromatin decondensation and fertilization rate in IVF programme. Another study from Gopalkrishnan *et al.* (37) found that spermatozoa showing a decondensation >70% with SDS+EDTA are able to fertilize oocytes, but when decondensation was less than 70%, no fertilization took place.

CONCLUSION

The mean percentage of sperm chromatin decondensation increased after incubation with time in comparison to the value in the native semen sample. However, no correlation could be observed between the chromatin decondensation and other semen parameters or fertilization rates either by using LIS + DTT or SDS + Heparin. Therefore, the chromatin decondensation test in vitro using LIS + DTT or SDS + Heparin could not be recommended for predicting the fertilization potential of spermatozoa in an IVF program. Additional studies concerning chromatin decondensation and fertilization rates both in IVF- and ICSI-programs are under investigation in our laboratories.

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Mohamed E. Hammadeh,[1,2] Alexandra Bernardi,[1] Theodosia Zeginiadou,[1] Ahmed Amer,[1] and Werner Schmidt[1]

[1] Department of Obstetrics and Gynaecology, University of Saarland, Homburg/Saar, Germany.
[2] To whom correspondence should be addressed; e-mail: frmham@uniklinik-saarland.de; mehammadeh@yahoo.de.