Influence of temperature and sperm preparation on the quality of spermatozoa

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Abstract This study investigated the effects of long-term (24 h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters. Semen samples ($n = 41$) were prepared both by density-gradient centrifugation (DGC) and the swim-up technique in order to compare the influence of sperm preparation on sperm quality after incubation. Progressive motility and morphology were significantly higher after incubation at RT compared with 35°C ($P < 0.001$ and $P < 0.01$, respectively). The proportions of acrosome-reacted, apoptotic and dead spermatozoa were significantly lower in samples incubated for 24 h at RT compared with 35°C ($P < 0.001$, $P = 0.01$ and $P < 0.001$, respectively). The number of motile, morphologically normal, non-acrosome-reacted and nonapoptotic spermatozoa recovered after sperm preparation was significantly higher in DGC compared with swim-up samples ($P < 0.001$). However, spermatozoa prepared by swim-up showed better survival after incubation compared with DGC-prepared spermatozoa, especially when incubated at 35°C. In conclusion, this study indicates a significantly better and longer preservation of sperm quality when incubation is performed at RT. These findings may convince laboratories to change the routinely used sperm storage conditions in order to maximize the quality of the prepared sperm sample.

KEYWORDS: acrosome reaction, apoptosis, flow cytometry, sperm preparation, sperm quality, temperature

Introduction

Sperm quality is a very important factor in the IVF laboratory since male infertility accounts for 20—30% of the infertility cases (ESHRE, 2012) and treatment options are mainly based on sperm-quality improvement techniques (Elder and Dale, 2011). The sperm-quality parameters routinely used in the IVF laboratory are concentration, motility, viability and morphology (Ombelet et al., 1997a; WHO, 2010). However, sperm-function assessments could additionally provide a valuable indication of sperm quality. Fertilization of an oocyte with an apoptotic spermatozoon has been shown to have detrimental effects on fertilization rate, implantation rate and embryo survival in assisted reproduction treatment (de Vantery Arrighi et al., 2009). Furthermore, occurrence of the acrosome reaction is essential to achieve fertilization in intrauterine insemination (Grunewald et al., 2006). Both of these sperm-function parameters can be easily determined by use of flow cytometry; however, this technique is not routinely available in the IVF laboratory.

The quality of the sperm sample is influenced by various laboratory factors, including: (i) use of different sperm preparation techniques (Boomsma et al., 2007; Chen and Bongso, 1999; Marchesi et al., 2010); (ii) temperature during sperm preparation (Franken et al., 2011; Otsuki et al., 2008); (iii) time interval from sperm preparation to IUI (Yavas and Selub, 2004); and (iv) temperature during long-term in-vitro incubation of prepared sperm samples (Aitken et al., 1996; Makler et al., 1981; Matsuura et al., 2010; Petrella et al., 2003). It is well known that the testis temperature is approximately 2—3°C below body temperature (Elder and Dale, 2011), as this is required for the production and maintenance of viable spermatozoa (Appell et al., 1977; Setchell, 1998). Despite the numerous articles published on the harmful effects of long-term in-vitro sperm incubation at body temperature, it is still current practice in most IVF laboratories to store prepared sperm samples at this unfavourable temperature prior to their use in assisted reproduction treatment (Matsuura et al., 2010).

Therefore, this study aimed to examine the effects of long-term (24 h) in-vitro sperm incubation at room temperature (RT; 23°C) versus testis temperature (35°C) on various sperm-quality parameters. In order to compare the influence of sperm preparation on sperm quality, native semen samples were split and prepared either by density-gradient centrifugation (DGC) or the swim-up technique. Conventional sperm-quality parameters such as concentration, progressive motility, normal morphology and viability (WHO, 2010) were taken into account. Additionally, sperm function was analysed by determining the proportions of spontaneously acrosome-reacted and apoptotic spermatozoa in the sample via flow cytometry.

Materials and methods

Collection of semen samples

Following a 2—7-day abstinence period, semen samples (n = 41) were obtained through masturbation from patients presenting at the fertility centre for an initial diagnostic semen analysis. Inclusion criteria were a sperm concentration of ≥15 million/ml and a motility of ≥32% progressively motile spermatozoa, according to World Health Organization (WHO) reference limits for normal semen samples (WHO, 2010). Routine sperm analysis and sperm preparation were initiated after liquefaction at RT (23°C) and within 1 h of production. Ethical approval of the study was granted by the ethics committee of Ziekenhuis Oost-Limburg (reference 13/055U, approved 31 May 2013).

Experimental design

Figure 1 gives a schematic overview of the experimental design. The native semen sample was split and one half of the sample was prepared by DGC while the other half was prepared by the swim-up technique. Aliquots of DGC and swim-up prepared sperm samples were then incubated for 24 h at RT or 35°C. Prior to incubation, samples were gassed with a gas mixture (6% CO_2, 5% O_2 and 89% N_2) for 90 s in order to maintain the pH of the incubation medium. Samples incubated at 35 ± 0.5°C were placed in a Labotect transport incubator (Cell-Trans 4016; Labor-Technik, Göttingen, Germany); while the RT samples were incubated on a bench in the laboratory (23 ± 1°C). Although RT samples were exposed to light during incubation, in contrast to the samples incubated at 35°C, this should not have an influence on sperm quality (Makler et al., 1980). For the final hour of the incubation period, the samples that were stored at RT were placed in the Labotect transport incubator in order to restore sample temperature to 35°C and obtain an equivalent motility count for both samples (Birks et al., 1994). Analysis of sperm-quality parameters, routine as well as flow cytometry measurements, was performed in duplicate on the native sample, after sperm preparation and after incubation.

Sperm preparation

Earle's balanced salt solution (EBSS; E3024; Sigma; Origio, The Netherlands) was supplemented with sodium pyruvate (S8636; Sigma; Origio), penicillin—streptomycin 100 × solution (Life Technologies, Invitrogen, Belgium) and 5% human serum albumin (HSA; Red Cross, Belgium) for use in sperm preparation (Ombelet, 1998). A three-layer gradient (90%, 70%, 40%) was prepared by diluting PureSperm 100 (Nidacon International; Origio) with supplemented EBSS (Chen and Bongso, 1999). The 90% gradient layer of 1.5 ml was layered

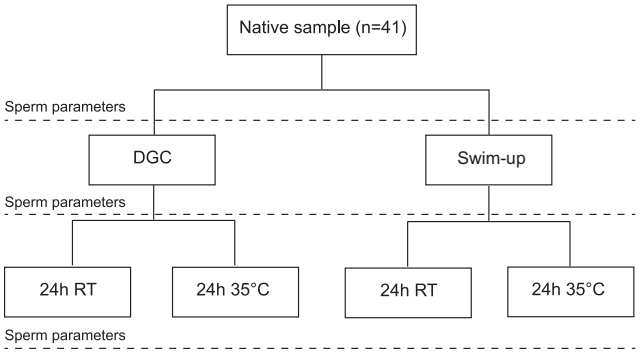

Figure 1 Schematic overview of the experimental design. DGC = density-gradient centrifugation; RT = room temperature (23°C).

with 1 ml of 70%, followed by 1 ml of 40% and a maximum of 1 ml of liquefied semen on top. This was centrifuged for 20 min at 310***g***. Spermatozoa were removed from the base of the 90% gradient layer and washed twice with 10 ml supplemented EBSS by centrifuging for 10 min at 350***g***. The final pellet (300 μl) was resuspended in 0.7 ml supplemented EBSS medium.

For sperm swim-up, semen samples (0.5 ml) were placed under a 2-ml layer of supplemented EBSS medium (Ombelet, 1998). The sample was incubated for 1 h at 35 ± 0.5°C at a 45° angle (Otsuki et al., 2008). After 1 h of incubation, the uppermost 1.5 ml of medium, containing highly motile sperm cells, was collected (Ombelet, 1998).

Routine sperm analysis

The laboratory has an accreditation according to the ISO15189 standards. Routine sperm analysis was performed by a single person and included concentration, motility, viability and morphology assessments, according to WHO guidelines (WHO, 1999, 2010). In order to evaluate sperm motility, the progressive movement of spermatozoa was graded A, B, C or D, with A + B motility representing the progressively motile sperm population (WHO, 1999). Viability of the spermatozoa was estimated by assessing the membrane integrity of the cells using eosin dye exclusion (WHO, 2010). An eosin B (Merck, Belgium) 0.5% (w/v) solution was added to a semen aliquot in a 1:1 ratio, mixed and left to stabilize for 30 s. The number of stained and unstained spermatozoa was counted under a microscope equipped with negative phase-contrast optics. Sperm morphology was scored by classifying spermatozoa as normal or abnormal according to strict criteria (Kruger et al., 1986) after Papanicolaou staining (Ombelet et al., 1997b,c, 1998; WHO, 2010). According to the WHO (2010) guidelines, 200 spermatozoa were counted per slide and in duplicate if a sufficient amount of spermatozoa was present. In total, 41 patients were evaluated. Therefore, group averages represented a count of approximately 16,400 spermatozoa per group ($200 \times 2 \times 41$).

Flow cytometry

The general staining protocol from BD Biosciences was adapted in order to stain human spermatozoa for flow cytometry assessment of apoptosis (annexin V), acrosome reaction (CD46; Grunewald et al., 2006) and viability status (7-aminoactinomycin; 7-AAD). A total of 100,000 spermatozoa were washed with 500 μl Dulbecco's Ca^{2+} Mg^{2+}-free phosphate-buffered saline (BioWhittaker, Lonza, Belgium). Cells were pelleted at 380***g*** for 10 min, supernatant was then removed and cells were resuspended in 100 μl 1 × annexin-V-binding buffer (BD Biosciences, Belgium). The annexin-V allophycocyanin (APC; BD Biosciences, Belgium) conjugate was 1:3 diluted with 1 × annexin-V-binding buffer. Subsequently, 5 μl of the 1:3 diluted annexin-V-allophycocyanin, 5 μl mouse anti-human CD46 fluorescein isothiocyanate (FITC; BD Biosciences), and 5 μl 7-AAD (BD Biosciences) were added for detection of apoptotic, acrosome-reacted and dead spermatozoa, respectively. Cells were then incubated for 15 min at RT in the dark. In a final step, cells were pelleted at 380***g*** for 10 min, the supernatant was discarded and cells were resuspended in 400 μl 1 × annexin-V-binding buffer prior to flow cytometry analysis (BD FACS Canto II).

The spermatozoa population was gated on the forward versus side scatter dot plot, in order to exclude the interference of debris and clumps during analysis. Fluorescence data were recorded for a minimum of 10,000 sperm cells using a high flow rate. The APC signal was obtained via the 633 nm excitation laser and the red fluorescence channel (650—670 nm filter range), whereas the FITC and 7-AAD signals were obtained via the 488 nm excitation laser and the green (515—545 nm filter range) and red (≥670 nm filter range) fluorescence channels, respectively. Compensation settings for the fluorochromes were performed by labelling cells with each fluorochrome separately and measuring the spectral overlap. Flow cytometry results were analysed using BD FACS Diva software (version 6.3.1).

Statistics

For each parameter, normality of distribution was tested using the D'Agostino and Pearson omnibus normality test. Statistical analysis was performed using the paired sample t-test for Gaussian distributed samples and the Wilcoxon signed rank test for matched pairs was used in case of a non-Gaussian distribution of the values. Statistical significance was established at $P < 0.05$ and power calculations revealed an overall power of >85%. Statistical analysis was performed using Predictive Analytics SoftWare (PASW version 17.0 for Windows). Data were represented as box and whisker plots, whereby boxes depict the 25th and 75th percentiles with indication of the median value, and whiskers depict the 10th and 90th percentiles.

Results

Sperm-quality parameters after incubation at RT compared with 35°C

The motility of spermatozoa was significantly higher in DGC-prepared samples incubated at RT compared with 35°C ($P < 0.001$, **Figure 2**A). Furthermore, in both DGC and swim-up samples, the proportion of morphologically normal spermatozoa was significantly higher in samples incubated at RT compared with 35°C ($P < 0.001$ and $P = 0.004$, respectively, **Figure 2**B). For the DGC-prepared samples, the proportion of $CD46^+$ spermatozoa was significantly lower in samples incubated at RT compared with 35°C ($P < 0.001$, **Figure 2**C). However, swim-up samples incubated at RT showed a significantly lower proportion of total annexin-V^+ spermatozoa compared with samples incubated at 35°C ($P = 0.01$, **Figure 2**D). Finally, in both DGC and swim-up samples, the proportion of $eosin^+$ and 7-AAD^+ spermatozoa was significantly lower after incubation at RT compared with 35°C ($P < 0.001$, **Figure 2**E and F).

DGC and swim-up sperm preparations for selection of improved-quality spermatozoa

Both DGC and swim-up yielded significantly higher proportions of motile and morphologically normal spermatozoa

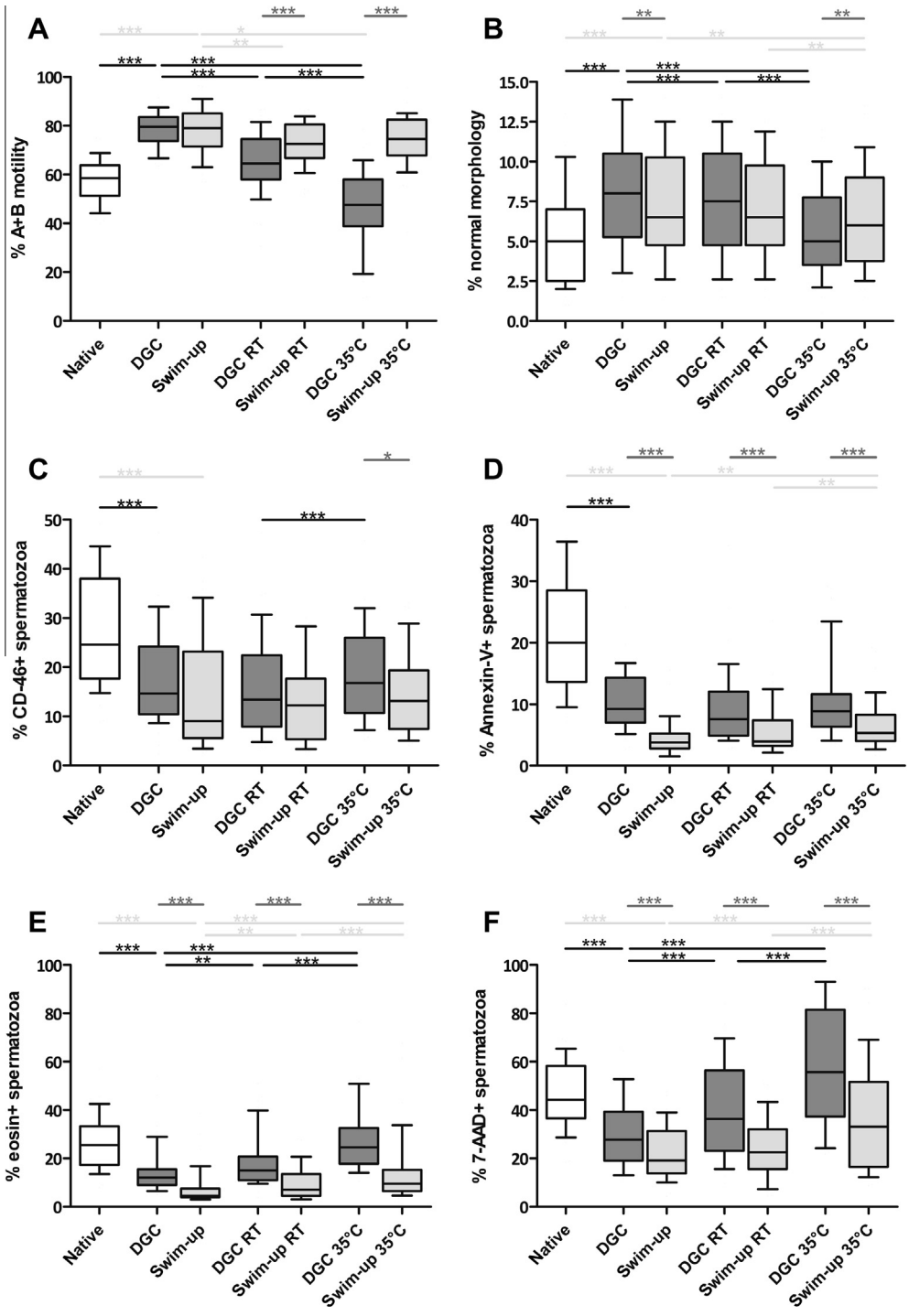

Figure 2 Sperm-quality parameters for DGC and swim-up samples after incubation at RT compared with 35°C: (A) Progressively motile spermatozoa (A + B motility); (B) normal morphology (strict criteria); (C) acrosome-reacted spermatozoa ($CD46^+$); (D) apoptotic spermatozoa (annexin-V^+); (E) dead spermatozoa ($eosin^+$); (F) dead spermatozoa (7-aminoactinomycin$^+$; 7-AAD^+). Boxes depict the 25th and 75th percentiles with indication of the median, and whiskers depict the 10th and 90th percentiles. White = native semen samples; light grey = swim-up samples; dark grey = DGC samples. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. DGC = density-gradient centrifugation; RT = room temperature (23°C).

when compared with the native sample ($P < 0.001$, **Figure 2**A and B). Furthermore, both techniques signifi-cantly decreased the proportion of $CD46^+$, annexin-V^+, $eosin^+$ and 7-AAD^+ spermatozoa in the sample ($P < 0.001$, **Figure 2**C–F). DGC and swim-up selected equally for progressively motile spermatozoa. Additionally, there was no significant difference for the proportion of total $CD46^+$ cells between both sperm preparations. Swim-up samples

contained significantly less total annexin-V$^+$ spermatozoa compared with DGC ($P < 0.001$, **Figure 2**D). Total CD46$^+$ and total annexin-V$^+$ spermatozoa represent the sum of the CD46/7-AAD$^-$ and CD46/7-AAD$^+$, and annexin-V$^+$/7-AAD$^-$ and annexin-V$^+$/7-AAD$^+$, subpopulations of spermatozoa, respectively. In addition, the proportions of eosin$^+$ and 7-AAD$^+$ spermatozoa were also significantly lower in swim-up samples compared with DGC ($P < 0.001$, **Figure 2**E and F). On the other hand, DGC selected better for normal morphology than swim-up ($P = 0.01$, **Figure 2**B).

Number of spermatozoa after incubation at RT compared with 35°C

Due to the significant difference in concentration between DGC and swim-up samples ($P < 0.0001$, results not shown), data were also corrected for concentration to indicate the actual number of spermatozoa (in 10^6/ml) present in the samples. A calculation was performed by multiplying the concentration of the sperm sample with the proportion of motile spermatozoa, proportion of normal morphology, proportion of non-acrosome-reacted spermatozoa and the proportion of nonapoptotic spermatozoa in the samples in order to become an overview of the 'ideal' spermatozoa population. This showed that the number of motile, morphologically normal, CD46$^-$/annexin-V$^-$ spermatozoa was significantly higher in DGC compared with swim-up samples ($P < 0.001$, **Figure 3**). Furthermore, the number of motile, morphologically normal, CD46$^-$/annexin-V$^-$ spermatozoa in DGC-prepared samples showed such a strong decline that the significant difference between DGC and swim-up samples still seen after incubation at RT ($P < 0.001$) was completely lost when samples were incubated at 35°C (**Figure 3**).

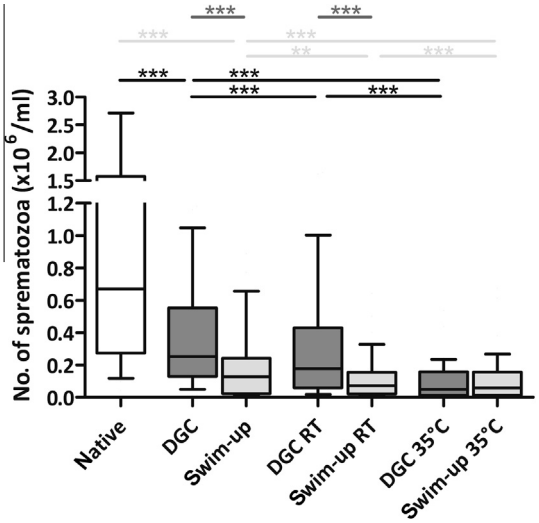

Figure 3 Number of motile, morphologically normal, CD46$^-$/annexin-V$^-$ spermatozoa in DGC and swim-up samples after incubation at RT compared with 35°C. Boxes depict the 25th and 75th percentiles with indication of the median, and whiskers depict the 10th and 90th percentiles. White = native semen samples; light grey = swim-up samples; dark grey = DGC samples. $^{**}P < 0.01$, $^{***}P < 0.001$. DGC = density-gradient centrifugation; RT = room temperature (23°C).

Discussion

The purpose of this study was to investigate the changes in sperm-quality parameters after long-term (24 h) in-vitro sperm incubation at RT (23°C) versus testis temperature (35°C). Parameters that were taken into account to define a high-quality sample were concentration, progressive motility, normal morphology, viability, acrosome reaction and the presence of apoptotic spermatozoa in the sample. A total of 41 semen samples, with a sperm concentration of ≥15 million/ml and progressive motility of ≥32% (WHO, 2010), were analysed.

The results presented in this study showed a significantly better and longer preservation of sperm quality when samples were incubated at RT compared with 35°C. This was in accordance with results proposed by Schuffner et al. (2002), who reported a significant loss of motility and an increased incidence of apoptosis after incubation at 37°C. Furthermore, Aitken et al. (1996) showed a significant decline in the motility of spermatozoa after incubation at ambient temperatures (i.e. 22°C), although incubation had no effect on the viability of the spermatozoa or their potential to undergo the acrosome reaction. It is assumed that when spermatozoa are incubated at lower temperatures, they adopt a resting state, which allows them to preserve their energy. In 2009, Gallup proposed 'the activation hypothesis' as a mechanism of spermatozoa capacitation *in vivo*. He postulated that the rise in temperature when spermatozoa enter the female reproductive tract could act as a trigger for the activation of spermatozoa, making them hyperactive (Gallup, 2009). This hypothesis possibly explains the diminished survival of spermatozoa at 37°C compared with lower temperatures. Finally, in addition to the previous sperm parameters, normal morphology of the spermatozoa also decreased significantly after incubation and especially after incubation at 35°C. The change in morphological characteristics has not been recorded during this study. However, an indication for this was found in a study by Peer et al. (2007), who reported that in-vitro incubation of sperm samples for ≥2 h at 37°C caused the appearance of large nuclear vacuoles, an effect which was not seen after incubation at 21°C.

Comparison between DGC and swim-up samples after incubation showed a superior preservation of sperm quality in swim-up samples. A possible explanation could be that the centrifugation steps performed during DGC sperm preparation rendered the spermatozoa more vulnerable to certain incubation conditions compared with the more natural selection of spermatozoa by the swim-up technique. Additionally, the higher concentration of spermatozoa, and therefore the higher number of dead sperm cells, in DGC-prepared samples compared with swim-up could have resulted in greater amounts of reactive oxygen species present in DGC-prepared samples. High reactive oxygen species production may induce peroxidative damage and a loss of sperm function (Calamera et al., 2001; WHO, 2010). DNA damage in both the nuclear and mitochondrial genomes may also be generated, leading to a more rapid decline in sperm viability. Therefore, when sperm samples are to be

incubated in-vitro for an extended period of time (i.e. 24 h) it is recommended to use the swim-up technique for preparation of the sample.

The results indicated that both preparation techniques provided a significantly better sperm population compared with the native sample. However, no solid conclusion could be drawn whether one technique provided a qualitatively better sperm population compared with the other. Firstly, both techniques selected equally for progressively motile spermatozoa and DGC-prepared samples showed higher proportions of morphologically normal spermatozoa compared with swim-up. This was in contrast to results presented by Evliyaoglu et al. (1996) and Ng et al. (1992), who demonstrated a higher proportion of progressively motile spermatozoa in Percoll-selected samples and more morphologically normal spermatozoa in swim-up compared with Percoll-selected samples. Secondly, in accordance with the results presented by Evliyaoglu et al. (1996) and Ng et al. (1992), DGC yielded a significantly higher amount of spermatozoa compared with swim-up, whereby swim-up-selected samples showed a tendency towards a higher proportion of intact acrosomes. Thirdly, swim-up preparations also resulted in a significantly higher proportion of nonapoptotic and viable spermatozoa in the sample compared with DGC. The swim-up technique is a low-cost procedure and is less time consuming in comparison to DGC. However, selection of spermatozoa by swim-up is based on motility and consequently dependent on a sufficient number of motile spermatozoa in the sample. Therefore, in assisted reproduction treatment it is usually recommended to use DGC as it can also be modulated to prepare severe quality sperm samples (Nijs, 2009).

Various reports (Jackson et al., 2010; Lachaud et al., 2004; Petrella et al., 2003) showed no significant changes in sperm parameters evaluated after 4—6 h of incubation at both room and body temperatures. However, sperm parameters did change significantly after 24 h of storage in all three investigations. Furthermore, long-term in-vitro incubation of spermatozoa could serve multiple purposes. Firstly, according to Eskandar (2002), zero 24-h sperm motility is related to a lower fertilization rate and a higher incidence of failed fertilization. Secondly, preservation of sperm quality for up to 24 h could be used for in-vitro maturation of immature metaphase I oocytes for intracytoplasmic sperm injection to be performed the following day (Strassburger et al., 2004). Thirdly, Aitken et al. (1996) suggested 24-h storage of sperm samples at ambient temperature (i.e. 22°C) to allow transportation to a centralized, accredited, diagnostic laboratory in order to standardize laboratory assessments of semen quality in the context of multicentre clinical trials.

In conclusion, the results of this study show a significantly better preservation of sperm quality when samples are incubated for 24 h at RT compared with 35°C. However, further investigation is needed to confirm whether these results would also translate into an improvement in pregnancy rates if prepared sperm samples were to be stored at RT before their use in assisted reproduction treatment. Currently, a study is being performed in this study centre whereby prepared sperm samples are alternately incubated at RT or 37 ± 0.5°C before their use in IUI. Finally, the findings presented in this study may convince laboratories to change the routinely used sperm storage conditions in order to maximize the quality of the prepared sperm sample.

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