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# Exogenous platelet-activating factor improves the motility of human spermatozoa evaluated with C.A.S.A.: Optimal concentration and incubation time

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**ABSTRACT. The objective of this study is to determine the optimal conditions for human semen incubation treated with exogenous platelet activating factor (ePAF) for intra-uterine insemination (IUI). This prospective study was carried out on 32 infertile men and each semen sample was processed with the ISolate Sperm Separation Medium, washed with sperm washing medium (SWM) and resuspended either in SWM alone (control samples), or with ePAF 0.1, 0.5, and 1.0 μM. Each concentration was subsequently incubated and evaluated at 5, 15, 30, and 60 min. The motility parameters were evaluated by the computer-aided sperm analysis (C.A.S.A.) system. Curvilinear velocity, straight line velocity, average path velocity, rapid and progressive motility significantly increased compared to control samples at an ePAF concentration of 0.1 μM (with at least 15 min of incubation). The best results were obtained with ePAF concentrations of: 0.1 μM (60 min of incubation) and 0.5 μM (30-60 min of incubation). In conclusion, results are enhanced when ePAF is added to standard semen preparation for IUI. An ePAF concentration of 0.1 μM, with an incubation time of 15 min, can be used for semen samples with normal motility. Whilst, for semen samples with poor motility, the ePAF concentration is best increased to 0.5 μM and/or the incubation time prolonged to 60 min.**

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## INTRODUCTION

Platelet-activating factor (PAF), 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine is a biologically active phospholipid mediator. Although PAF was initially named for its potential to induce platelet aggregation, further research has evidenced potent biological actions of PAF in a broad range of cell types and tissues (1).

PAF is present in human spermatozoa (2) and its endogenous content has been reported to have a significant and positive effect on both motility and pregnancy rate (3). Although the underlying mechanism of PAF action upon spermatozoa has not yet been fully clarified, it is known that this mechanism of action in other cell types is mediated through a specific receptor (4, 5). Immunoflourescent data have provided significant evidence that spermatozoa contain PAF receptors. Normal spermatozoa have the greatest concentration of PAF receptors in the neck region, followed by the mid-piece region (6). The neck region is the location of the proximal centriole which plays a critical role in pre-implantation and embryo development (7). The mid-piece is the site of mitochondria which are essential for spermatozoa motility (6).

Some authors have reported that exogenous PAF (ePAF) enhances sperm motility and fertilisation rates in mammals, including humans (3, 8-11). Moreover, preliminary studies have shown that there is a significant improvement in pregnancy rate with intrauterine insemination (IUI) after the separation of the spermatozoa with a medium containing ePAF (12, 13). However, the most effective ePAF concentration and incubation time that provides the greatest improvement in human spermatozoa motility parameters still remains to be established.

*In-vitro* methods for sperm preparation are designed to mimic the *in-vivo* process of separation between spermatozoa and seminal plasma and the selection of normal and highly motile spermatozoa (14). The outcome of these methods of sperm preparation is usually evaluated by their effect on sperm quality parameters e.g. spermatozoa motility and it has been demonstrated that the characteristics of spermatozoa motility play an important role in fertilization (14).

The advent and development of computer-aided sperm analysis (C.A.S.A.) systems have made it possible to carry out objective measurements of the characteristics of sperm motion (15). Such criteria include curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP), amplitude of lateral head displacements (ALH), tail beat cross frequency (BCF), linearity (LIN=VSL/VCL), and straightness (STR=VSL/VAP).

The aim of this study is:

- to determine the optimal conditions for human semen incubation with ePAF by testing the effect of 3 different ePAF concentrations, at 4 different incubation times, on the motility of human spermatozoa evaluated with the C.A.S.A. system;
- to evaluate the possibility of adding ePAF to our standard semen preparation for IUI, so as to enhance the motility parameters of our semen samples and increase our IUI pregnancy rate.

*Key-words:* A.R.T., C.A.S.A., human spermatozoa, platelet-activating factor, sperm motility.

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Table 1 - *Sperm parameters, evaluated with optic microscope, of 32 semen samples before preparation.*

| | Median | Range |
|---|---|---|
| Total sperm number (no. × $10^6$) | 234.0 | 118.5-840.0 |
| Sperm concentration (no. × $10^6$/ml) | 60.0 | 20.0-160 |
| Rapid motility (class A) % | 8.2 | 0.0-35 |
| Slow or sluggish linear or non linear motility (class B) % | 20.4 | 12.0-50.0 |
| Non progressive motility (class C) % | 22.8 | 2.0-50.0 |
| Immobile (class D) % | 43.8 | 17.0-65.0 |

The choice of ePAF concentrations tested in this study was based on literature data (12, 13, 16, 17). The C.A.S.A. system was chosen for the evaluation of the semen samples as it has the advantages of providing objective semen analysis data with increased accuracy in spermatozoa motility evaluation.

## MATERIALS AND METHODS

The study cohort included 32 men (mean age 35.0±7.1 yr, range 21-45 yr) who referred to our clinic (Assisted Reproduction Unit of the Department of Obstetrics and Gynaecology, University of Torino, Italy) due to couple infertility (duration of infertility ≥1 yr). None of these patients were affected by chronic diseases or on andrologycal therapy. Nine patients were smokers (28%). Infertility diagnoses included anovulatory, idiopathic, and male factor. None of patients' female partners were affected by tubal infertility.

Informed consent was obtained so as to use a part of their semen sample (32 samples) for our research. The samples were collected by masturbation after a period of 3-5 days of abstinence and allowed to liquefy for 30-60 min at 37 C. The semen samples were evaluated by a single operator before preparation, according to the World Health Organization method 1999 (18). These semen sample parameters are reported in Table 1. Two milliliters of each semen sample were adequately mixed and used for testing. Each 2 ml specimen was divided into 4 aliquots (0.5 ml) and each aliquot was placed in a conical test tube.

### *PAF solution preparation*

One milligram of lyophilised PAF (Calbiochem-Novabiochem, La Jolla, CA) was dissolved in distilled water at a concentration of 100 µM (stock solution). This solution was then divided into aliquots of 1 ml and maintained at a temperature of –20 C for no more than 3 days. The stock solution was first thawed and then diluted in sperm washing medium (SWM) (Irvine Scientific, Santa Ana, California, USA), in 3 concentrations (0.1 µM, 0.5 µM, 1 µM) and used for 1 working day only.

### *Semen processing*

The 4 aliquots of each semen sample were processed (300g, 30 min) through the ISolate® Sperm Separation Medium (Irvine Sci-

Table 2 - *Rapid and progressive motility and kinematic parameters, evaluated with the computer aided sperm analysis (C.A.S.A.) system, of 32 semen samples after treatment with exogenous platelet-activationg factor (ePAF) at 3 different concentrations and four incubation times as compared with the control samples by Friedman test.*

| | Incubation time (min) | RM (%) median (range) | $Z_F$-value | PM (%) median (range) | $Z_F$-value | VCL (µm/sec) median (range) | $Z_F$-value | VSL (µm/sec) median (range) | $Z_F$-value | VAP (µm/sec) median (range) | $Z_F$-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control samples | 0 | 7.7 (0.0-44.2) | | 16.6 (0.0-56.5) | | 43.6 (17-57.8) | | 23.0 (1.9-34.0) | | 26.7 (10.4-38.5) | |
| SWM-ePAF 0.1 µM | 5 | 13.2 (1.3-59.0) | 2.60 | 28.6 (1.7-63.6) | 3.12 | 46.0 (36.2-72.8) | 2.31 | 26.2 (11.5-46.3) | 2.44 | 32.0 (18.9-50.3) | 2.31 |
| | 15 | 19.9 (2.2-50.0) | 5.94[d] | 33.3 (4.8-68.3) | 6.07[e] | 52.7 (36.8-74.9) | 4.78[c] | 32.5 (15.3-48.7) | 4.91[d] | 36.7 (22.5-55.0) | 4.75[c] |
| | 30 | 20.0 (2.0-54.6) | 7.25[e] | 38.5 (4.7-71.2) | 7.16[e] | 57.9 (39.6-83.1) | 7.35[e] | 31.9 (18.1-46.7) | 6.13[e] | 36.9 (22..9-52.3) | 6.23[e] |
| | 60 | 22.1 (1.6-63.5) | 7.69[e] | 37.2 (4.2-85) | 7.08[e] | 60.9 (43.4-88.2) | 8.87[e] | 36.0 (20.0-50.2) | 8.07[e] | 40.6 (23.5-57.5) | 8.51[e] |
| SWM-ePAF 0.5 µM | 5 | 15.0 (0.78-45.0) | 3.07 | 28.1 (5.1-60.8) | 3.66 | 49.3 (36.5-67.0) | 2.66 | 29.5 (16.4-43.2) | 3.63[a] | 32.7 (19.3-48.0) | 3.87[b] |
| | 15 | 16.9 (2.6-52.2) | 6.37[e] | 33.8 (6.6-70.6) | 5.90[d] | 54.8 (39.6-72.5) | 5.49[d] | 31.0 (18.9-49.0) | 6.07[e] | 35.9 (23.5-51.6) | 6.02[e] |
| | 30 | 24.4 (4.2-100.0) | 8.39[e] | 37.4 (9.1-100) | 7.43[e] | 61.4 (42.6-73.3) | 8.52[e] | 37.0 (17.3-51.6) | 8.55[e] | 40.8 (21.8-55.3) | 8.51[e] |
| | 60 | 24.7 (1.3-59.0) | 8.97[e] | 37.9 (5.7-75.3) | 8.42[e] | 58 (42.0-76.7) | 7.48[e] | 34.9 (21.9-50.0) | 8.31[e] | 38.5 (26.9-52.8) | 8.15[e] |
| SWM-ePAF 1.0 µM | 5 | 11.6 (1.44-55.9) | 3.45[a] | 26.6 (6.3-72.0) | 3.75[b] | 50.7 (34.1-63.7) | 2.66 | 29.5 (15.6-40.9) | 2.34 | 33.4 (20.3-43.8) | 2.28 |
| | 15 | 18.3 (3.3-45.3) | 5.47[b] | 30.3 (8.1-61.3) | 5.30[d] | 56.9 (37.6-71.8) | 5.91[d] | 34 (19.9-48.2) | 5.91[d] | 37.7 (23.9-58.5) | 5.97[d] |
| | 30 | 18.7 (3.6-50.7) | 6.50[e] | 32.2 (9.0-69.7) | 6.36[e] | 56.2 (40.1-88.7) | 7.19[e] | 34.4 (18.8-50.6) | 6.93[e] | 38.7 (21.6-57.1) | 6.97[e] |
| | 60 | 23.6 (1.6-56.0) | 8.15[e] | 36.1 (3.1-91.5) | 7.29[e] | 60 (43.9-81.6) | 8.54[e] | 35.5 (23.3-52.7) | 8.06[e] | 40.5 (28.0-59.1) | 8.22[e] |

SWM: sperm washing medium; RM: rapid motility, PM: progressive motility, VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; $Z_F$ value: Friedman Z value, [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, [d]$p<0.0001$, [e]$p<0.00001$.

Table 3 - *Kinematic parameters [linearity (LIN), straightness (STR), amplitude of lateral displacement of the head (ALH) and beat cross frequency (BCF)], evaluated with the computer aided sperm analysis (C.A.S.A.) system, of 32 semen samples after treatment with exogenous platelet-activationg factor (ePAF) at 3 different concentrations and 4 incubation times in comparison with the control samples by Friedman test.*

| | Incubation time (min) | LIN (%) median (range) | $Z_F$-value | STR (%) median (range) | $Z_F$-value | ALH (µm) median (range) | $Z_F$-value | BCF (Hz) median (range) | $Z_F$-value |
|---|---|---|---|---|---|---|---|---|---|
| Control samples | 0 | 53.5 (8.0-62.9) | | 83.1 (18.3-94.9) | | 3.0 (0-5.7) | | 7.6 (4.9-11.1) | |
| SWM-ePAF 0.1 µM | 5 | 54.1 (31.7-67.9) | 0.61 | 81.8 (55.5-93.7) | 0.10 | 3.4 (2.0-8.6) | 1.11 | 7.6 (5.6-10.9) | 1.25 |
| | 15 | 57.9 (37.4-68.2) | 2.38 | 84.6 (60.9-90.5) | 0.32 | 3.6 (1.9-5.3) | 1.83 | 7.0 (5.7-9.8) | 2.34 |
| | 30 | 57.1 (38.9-75.8) | 2.71 | 83.7 (68.9-93.7) | 0.83 | 3.6 (1.7-7.0) | 2.86 | 6.6 (5.1-11.5) | 4.80[b] |
| | 60 | 56.3 (43.0-75.3) | 2.39 | 85.3 (64.0-91.0) | 1.75 | 3.8 (1.8-8.4) | 3.72[a] | 6.5 (5.2-11.5) | 4.65[b] |
| SWM-ePAF 0.5 µM | 5 | 56.1 (37.8-68.2) | 1.85 | 83.1 (76.0-94.4) | 1.11 | 3.3 (2.0-7.3) | 1.14 | 7.5 (5.9-9.6) | 2.29 |
| | 15 | 58.9 (37.7-70.9) | 4.20[a] | 85.2 (68.8-80.7) | 2.10 | 3.4 (2.3-6.7) | 1.46 | 6.8 (4.6-9.2) | 5.09[c] |
| | 30 | 60.0 (43.1-71.0) | 4.00[a] | 85.5 (76.8-92.0) | 1.88 | 3.5 (1.6-7.6) | 1.17 | 6.3 (4.6-9.9) | 6.19[d] |
| | 60 | 60.4 (49.0-76.9) | 4.59[c] | 86.0 (73.8-95.2) | 2.92 | 3.5 (2.0-6.1) | 3.11 | 6.4 (5.7-9.2) | 5.54[c] |
| SWM-ePAF 1.0 µM | 5 | 55.2 (37.7-65.7) | 0.93 | 82.8 (68.7-92.4) | 1.22 | 3.4 (2.3-6.5) | 1.52 | 7.4 (6.2-9.5) | 1.56 |
| | 15 | 56.8 (37.8-70.2) | 2.12 | 85.1 (74.6-94.5) | 1.70 | 3.4 (2.2-6.9) | 2.21 | 6.9 (5.0-9.5) | 3.84[a] |
| | 30 | 58.9 (41.6-69.9) | 2.54 | 83.7 (72.5-94.0) | 0.98 | 4.0 (1.3-10.4) | 3.95[a] | 6.7 (5.1-9.7) | 4.46[b] |
| | 60 | 57.9 (42.8-83.0) | 3.40 | 84.4 (73.2-82.0) | 2.20 | 3.5 (1.8-6.4) | 2.20 | 6.7 (4.6-8.9) | 4.93[c] |

SWM: sperm washing medium; $Z_F$-value: Friedman Z value. LIN=VSL / VCL × 100, STR=VSL / VAP × 100 (VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity). [a]$p$<0.01, [b]$p$<0.001, [c]$p$<0.0001, [d]$p$<0.00001.

entific, Santa Ana, California, USA), a 2-layer density gradient system for the separation of spermatozoa. ISolate® is a sterile, colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in a HEPES-buffered human tubal fluid medium. Control group: the first aliquot of each semen sample was washed with 1 ml of SWM, centrifuged (300g, 10 min) and resuspended in 0.5 ml of SWM. PAF group: the 3 remaining aliquots were resuspended in SWM with PAF 0.1 µM, 0.5 µM, and 1 µM. Each semen sample was incubated and evaluated at all concentrations at 4 different time points: 5, 15, 30, and 60 min.

### *Semen analysis*

All semen samples were evaluated by the C.A.S.A system (CGA-WLJY-9000; CGA Distribution, Florence, Italy). The digital imaging system set-up was performed according to Mack et al. and Mortimer (19, 20). The image acquisition rate was 60 frames/sec and the track sampling time 400 msec (20 frames). Approximately 200 sperm were analysed for each sample and magnified at 10× (object lens). A MicronCell-20Micron (Conception Technologies, San Diego, CA, USA) counting chamber was used to carry out the analysis. A total of 6 random areas were selected and evaluated by the C.A.S.A. system at 37 C.

The following kinematic parameters were computed and calculated for the analysis of the sperm motility: VCL (µm/sec), VSL (µm/sec), VAP (µm/sec), LIN (VSL/VCL), STR (VSL/VAP), ALH (µm), and BCF (Hz). The motile spermatozoa were subdivided into these categories setting maximum velocity = 400 µm/sec: class A = rapid motility (RM); class B = slow or sluggish motility; class A+B = progressive motility (PM) (18).

### *Statistical analysis*

The non-parametric Friedman 2-way analysis of variance was performed as seminal spermatozoa kinematic values often do not have a normal distribution. This test is an extension of the sign test to more than 2 matched, or paired variables and is known as a randomized block design. In our analysis, for each kinematic parameter the rows were the blocks (32 semen samples) and the columns were the 13 treatment values: the controls value i.e. no ePAF treatment and 3 ePAF concentration values (0.1, 0.5, and 1 µM) for each of the 4 incubation times (5, 15, 30, and 60 min). The null hypothesis was that of no treatment differences. All results are expressed as median±range.

For multiple comparisons, let $R_i$ be the sum of the ranks for the $i^{th}$ variable in randomized block design, declare groups $i$ and $j$ to differ significantly at overall $\alpha$ if $\left| R_i - R_j \right| \geq z_{\alpha'/2} \sqrt{Nk\,(k+1)/6}$, where $\alpha' = 2\alpha / [k(k-1)]$ [the number of multiple comparisons being $k(k-1)/2 = 78$], $k$=13 is the number of variables, $N$=32 is the number of cases and $\alpha$=0.05. With 13 groups, the critical z value for overall $\alpha$ of 0.05 was 3.41. The Friedman Z-values, $Z_F = \left| R_i - R_j \right| / \sqrt{Nk(k+1)/6}$, with their $p$-values are reported in the text and in Tables 2 and 3.

The parametric 2-way analysis of variance was also considered, taking into account non-normal distribution of data (e.g. angu-

lar transformations of percentages were performed before analysis). As a 2-way analysis of variance and a non-parametric one produced similar results, only the Friedman 2-way analysis of variance results were reported.

Data were processed by the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA) and the multiple comparisons of the Friedman 2-way analysis of variance were performed by the Biomedical Data Package statistical software (BMDP, Statistical Solutions, Saugus, MA, USA).

## RESULTS

A total of 5 C.A.S.A parameters i.e. RM, PM, VCL, VSL, and VAP, were taken into consideration for the evaluation of the most effective ePAF concentration and incubation time for IUI, due to their significant correlation to hyperactivation after sperm separation (21), sperm binding capacity to the *zona pellucida* (14), pregnancy rate with IUI (22) and fertilization (23-25). The ALH, BCF, LIN, and STR in ePAF-treated samples in comparison to the control samples were also reported.

### *Comparison of ePAF-treated samples with control samples*

A general improvement of the kinematic and motility parameters examined were observed at computerized analysis of the ePAF-treated semen samples, taken at different concentrations and incubation times. Table 2 reports the comparison results of RM, PM, VCL, VSL, and VAP in the ePAF-treated samples with the control samples.

An increase in the percentage of RM was observed for all 3 ePAF concentrations (Fig. 1A, Table 2) when incubation times were increased. The PM percentage trend at 3 ePAF concentrations and 4 incubation times, are reported in Figure 1B.

A statistically significant improvement in RM, PM, VCL, VSL, and VAP in comparison to the control samples was observed even at an ePAF concentration as low as that of 0.1 μM with 15 min of incubation. Nevertheless, the best $Z_F$, for both PM and RM in comparison to the control samples, was obtained with a ePAF concentration of 0.5 μM at 60 min of incubation (Table 2). The best $Z_F$, for VCL was obtained by an ePAF concentration of 0.1 μM at 60 min of incubation and for VSL values with an ePAF concentration of 0.5 μM at 30 min of incubation. The best $Z_F$ for VAP values were obtained both by an ePAF concentration of 0.1 μM at 60 min and by an ePAF concentration of 0.5 μM at 30 min of incubation (Table 2).

Table 3 reports the values of LIN, STR, ALH, and BCF in the ePAF treated samples and the $Z_F$ compared to the control samples.

### *Comparison of all incubation times*

The statistically significant differences were:

- an increase of VCL and VAP values at 60 min with the ePAF concentration of 0.1 μM compared to values at 15 min also after correction for multiple comparisons (respectively $Z_F$=4.09; $p$<0.01 and $Z_F$=3.76; $p$<0.01).
- the test showed a significant increase in VSL and VAP values with an ePAF concentration of 0.5 μM at 30 and 60 min of incubation, when compared to the values at 5 min (for VSL respectively $Z_F$=4.93; $p$<0.0001 and $Z_F$=4.69: $p$<0.001 and for VAP respectively $Z_F$=4.64; $p$<0.001 and $Z_F$=4.28; $p$<0.01).
- the test for multiple comparisons demonstrated a significant improvement in RM and PM at 60 min of incubation, compared to the values at 5 min ($Z_F$=4.70; $p$<0.001 and $Z_F$=3.55; $p$<0.05 respectively) with an ePAF concentration of 1 μM.

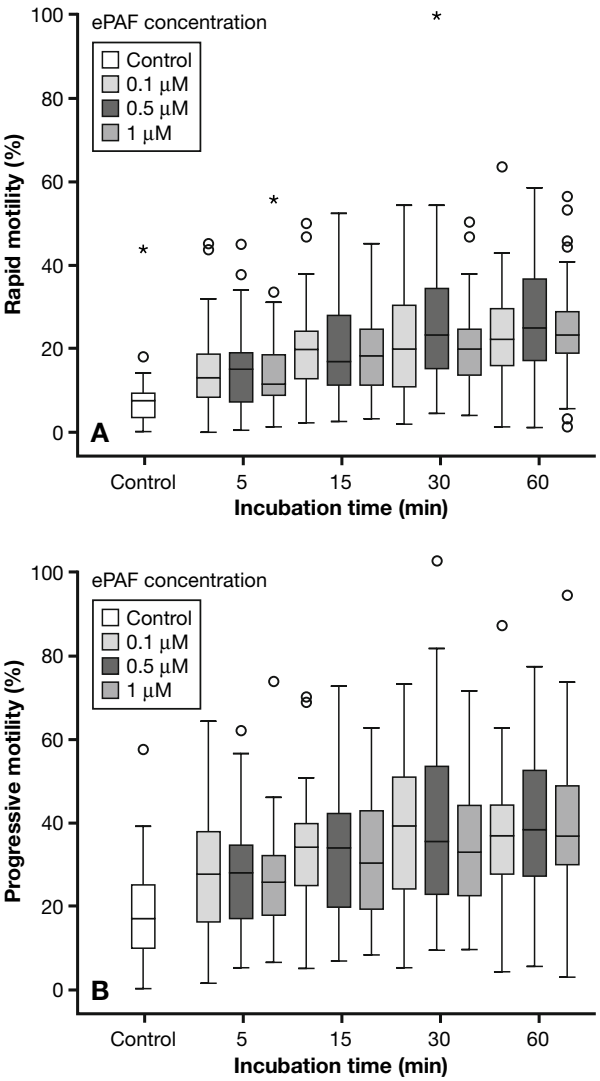


Fig. 1 - *Rapid (panel A) and progressive (panel B) motility after treatment with exogenous platelet-activationg factor (ePAF) at 3 different concentrations and 4 incubation times in comparison with the control samples. The boundaries of the boxes are Tukey's hinges. The median is identified by a line inside the box. The length of the box is the interquartile range (IQR) computed from Tukey's hinges. Whiskers represent the 5th percentile and the 95th, respectively. Values >3 IQR's from the end of a box are labeled as extreme and marked with an asterisk (*). Values >1.5 IQR's but <3 IQR's from the end of the box are labelled as outliers (○).*

### *Comparison of all concentrations*

The statistically significant differences were:

- The VSL values with an ePAF concentration of 0.5 μM at

30 min of incubation were significantly better than those with an ePAF concentration of 0.1 μM at 15 min ($Z_F$=3.64; $p$<0.05).
- Likewise, the VAP values were significantly better with an ePAF concentration of 0.5 μM at 30 min than with an ePAF concentration of 0.1 μM at 15 min ($Z_F$=3.76; $p$<0.01).

Even if not statistically significant, the VCL values obtained at 60 min of incubation with an ePAF concentration of 0.1 μM, seemed to give better results than did values with an ePAF concentration of 0.5 μM at 15 min of incubation (borderline value: $Z_F$=3.39; $p$=0.051).

In conclusion, the statistical analysis of our data evidenced that the best results were observed with an ePAF 0.1 μM at 60 min of incubation, or with an ePAF concentration of 0.5 μM at 30-60 min of incubation. It was observed that an increased ePAF concentration to 1 μM did not produce any further improvement in the kinematic and motility parameters in comparison to the other 2 ePAF concentrations in our semen samples.

## DISCUSSION

Mortimer and Mortimer studied the subpopulations of capacitating spermatozoa after direct swim-up from semen technique in semen of proven fertile donors and reported different motility patterns which included: forward progressive (FP), transition phase (TP), and hyperactivated motility (HA) (26). They observed that VCL increased significantly from FP to TP and HA (26), whilst both VAP and VSL decreased significantly and a significant reduction was also observed for both LIN and STR (26). Although our data differ from the observations made by Mortimer and Mortimer (26), some possible explanations for this discrepancy may include: the kinematic parameters for the detection of hyperactivation are dependent on the image-sampling rate (27), hyperactivation is an episodic, rather than a continuous condition of human spermatozoa (28), proven fertile donors may have different kinematic patterns than do infertile asthenospermic men and, lastly, different methods for separating spermatozoa may produce different subpopulations of spermatozoa (29).

McCann and Chantler observed with Percoll, a discontinuous density gradient method for the separation of spermatozoa, that there is a significant increase in the percentage of motile and progressively motile spermatozoa, compared to unprepared semen, evaluated with the C.A.S.A. system (30). These authors also observed a significant increase in VSL, VCL, and VAP average values with Percoll, when compared to the unprepared semen samples (30). Yogev et al. observed a significant increase in some of the variables evaluated with C.A.S.A. system, after different sperm preparation techniques (14). A statistically significant positive, although low correlation, was observed between sperm binding capacity to the *zona pellucida*, after swim-up preparation and VAP as well as with VSL (14). With mini-Percoll, another discontinuous density gradient method, these authors observed an increase in VAP, VSL, and VCL values in comparison to the values obtained with the swim-up preparation (14). There was also a statistically significant increase in the hemizona assay index with this technique, even if the authors did not observe any correlation between the increase in the kinematic parameters obtained with mini-Percoll and the hemizona assay index (14).

In 2004, Chantler et al. observed that swim-up produced subpopulations of sperm that differed significantly from those obtained with Percoll (29). Furthermore, it was observed that the two procedures revealed fundamental differences in the kinematic properties of sperm and that these differences were independent from hyperactivation, whilst the proportion of sperm population that exhibited hyperactivated motility was low for both treatments (6.1% for swim-up and 3.5% for Percoll gradient) (29). Although the relative clinical value of swim-up and Percoll, or mini-Percoll is a still debated question, *in-vitro* sperm prepared using Percoll frequently gives better results than does swim-up (31) and similar results have also been reported for fertilization rates if sperm are dysfunctional (32).

The semen samples obtained from infertile asthenospermic men in our study were processed with the discontinuous density gradient method ISolate® Sperm Separation Medium and were then resuspended in SWM at different concentrations of ePAF. There was a significant increase of VCL, VSL, and VAP values, along with a significant increase of rapid and progressive motility compared to the control samples which had not been treated with ePAF. This technique of sperm preparation is similar to the one used by McCann and Chantler (30) and to the mini-Percoll used by Yogev et al. (14). We are of the opinion that probably all discontinuous medium density gradients may produce similar behavior in the kinematic sperm parameters, evaluated with C.A.S.A. and that such behavior can be further stimulated with the addition of ePAF.

VSL and VAP are indicators of linear motility and, thus, represent sperm cells that have not completed capacitation. De Geyter et al. reported that VSL and VAP may indicate sperm cells that are either not yet capacitated, or are in the early process of membrane modification (33). However, they may still have the potential to undergo capacitation and, consequently, bind to the *zona pellucida* (33). Indeed, these movement parameters were found to predict fertilization (23-25). Also other authors, such as Moohan and Lindsay are of the opinion that an increase in VAP, VCL, and VSL, after Percoll technique, indicating hyperactivation and suggests that exposure to Percoll is able to accelerate capacitation (21).

A series of studies on the origin, secretion and function of PAF and PAF-acetylhydrolase suggests that PAF is a candidate for capacitation factors, whereas PAF-acetylhydrolase is regarded as a decapacitation factor (34). If sperm exposure to the discontinuous medium density gradients accelerates capacitation, then the addition of ePAF could further improve these changes. Previous studies have demonstrated that normal sperm morphology, assessed using strict criteria before sperm separation and 5 C.A.S.A. parameters after sperm separation, including rapid and progressive motility, VAP, VCL, and VSL, are able to predict pregnancy by IUI (22). Roudebush et al. noted that the cumulative pregnancy rate was

significantly higher ($p<0.05$) for patients with no asthenospermic semen in the ePAF-treated group (14/26; 53.90%) than in the control group (10/35; 28.60%) (12). Moreover, they noted that if the semen samples were deficient in motility, the improvement in pregnancy rate was not statistically significant (12). They treated their semen samples with an ePAF concentration of 0.1 μM at an incubation time of 15 min, which confirms our findings that, at this ePAF concentration and incubation time, there is a statistically significant improvement of RM, PM, VCL, VSL, and VAP. Nevertheless, our data showed that the best results can be obtained with an ePAF concentration of 0.1 μM by extending the incubation time to 60 min, or by the use of an ePAF concentration of 0.5 μM at 30-60 min of incubation. Ricker et al. observed that when human spermatozoa were treated with ePAF at different concentrations, they exhibited a statistically significant increase in motility compared to the control samples that had not been treated with ePAF and that this increase was significantly greater in the group with the lowest initial motility (16). The lack of improvement of the pregnancy rate in the asthenospermic semen samples observed by Roudebush et al. (12) could be due to high an ePAF dilution and/or to an inadequate ePAF incubation time.

It can be concluded that:

- it is useful to add ePAF to our standard semen preparation for IUI in as much as this treatment significantly improves motility and kinematic sperm parameters, evaluated with the C.A.S.A. system;
- if the semen sample shows a normal motility before preparation, after processing it through the ISolate® Sperm Separation Medium, ePAF should be added to the SWM at a concentration of 0.1 μM, with a 15-min incubation time;
- if the observation of the semen sample, before preparation, shows a slight or mild asthenospermia, then the same ePAF concentration can be maintained, but the incubation time is to be extended to 60 min. Alternatively, a higher ePAF concentration (0.5 μM) with an incubation time of 30-60 min can be used to obtain the greatest improvement of RM, PM, and kinematic parameters.

Although further clinical studies are required to confirm whether the addition of ePAF to the standard semen preparation for IUI, or *in-vitro* fertilization increases the pregnancy rate both in normospermic and in asthenospermic semen samples, these preliminary results are promising and in-vivo studies are ongoing in our clinic to this aim.

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