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Correspondence:
Joaquín Gadea, Department of Physiology, School of Veterinary Medicine, University of Murcia, Campus Mare Nostrum, Murcia 30 100, Spain. E-mail: jgadea@um.es

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Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success

J. C. Martínez-Soto,*,† J. Landeras† and J. Gadea*

*Department of Physiology, School of Veterinary Sciences, University of Murcia, Murcia, Spain, and †IVI Murcia, Murcia, Spain

SUMMARY

There is a lack of information about the importance of fatty acid composition of the human sperm membranes and seminal plasma in the cryopreservation procedure. Our aims were to study the possible relationships between the fatty acid composition of human spermatozoa or seminal fluid before freezing, and the sperm quality, measured in terms of viability and motility, before and after freezing–thawing. A further objective of this study was to determine whether the antioxidant capacity (TAC) of the seminal plasma is related to fatty acid (FA) composition and to success of the cryopreservation process. Polyunsaturated fatty acids (PUFA), ω3 PUFAs and docosahexaenoic acid (DHA) in spermatozoa were significantly positively correlated with sperm viability and motility parameters before and after freezing. An inverse relationship was found for monounsaturated (MUFA), ratio ω6/ω3, ratio saturated saturated fatty acids/PUFA (SFA/PUFA) with the seminal parameters. Seminal plasma fatty acid composition was not related to viability. However, motility parameters before and after freezing were related to stearic acid (C18:0) and DHA. TAC in seminal plasma was directly related to PUFA, w3 and DHA. On the other hand, SFA, C22:0, C24:0 and MUFA in seminal plasma were inversely related to the antioxidant capacity. TAC was directly correlated with motion parameters after thawing,

We described a significant correlation between the fatty acid composition of the human spermatozoa or seminal plasma and the sperm parameters of the samples after thawing. PUFA, W3 and specially DHA are directly correlated with sperm motility and viability after freezing/thawing, and MUFA was inversely correlated. This means that in the future the fatty acid composition could be used as a predictor of the capacity of cryopreservation. On the other hand, we could design further procedures to modify the lipid composition or/and antioxidant capacity of ejaculate to make it more resistant to the cryopreservation process.

INTRODUCTION

Human semen cryopreservation represents a useful therapeutic option in the management of infertility with several possible applications (Anger *et al.*, 2003; Holoch & Wald, 2011). During cryopreservation, spermatozoa are exposed to physical and chemical stress that results in adverse changes in membrane lipid composition, sperm motility, viability and acrosome status (Alvarez & Storey, 1993; Hammadeh *et al.*, 1999; Schiller *et al.*, 2000; O'Connell *et al.*, 2002). All these changes reduce the fertilizing ability of human spermatozoa after cryopreservation (Brotherton, 1990; Oehninger *et al.*, 2000).

Mechanisms of cryodamage to human spermatozoa are thought to be multifactorial. Some authors have reported direct physical damage to sperm structure or function during cell freezing related to ice formation and high osmotic pressure during freezing (Mazur, 1984; Brotherton, 1990). On the other hand, sperm cryopreservation is associated with oxidative stress and reactive oxygen species (ROS) generation (Wang *et al.*, 1997). ROS-induced damage to spermatozoa is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids (PUFAs), leading to lipid peroxidation and inducing DNA damage (Aitken, 1989; Agarwal *et al.*, 2008). To counteract the harmful effects of ROS, spermatozoa and seminal plasma possess a number of antioxidant systems that scavenge ROS and prevent internal cellular damage (Alvarez *et al.*, 1987; Aitken, 1995; Gadea *et al.*, 2011).

Different antioxidants are present in seminal plasma and provide a very effective protection against oxidative stress (Agarwal

et al., 2006). This system includes enzymes such as catalase, glutathione peroxidase (GPx) or superoxide dismutase (SOD) and non-enzymatic compounds such as glutathione (GSH/GSSG), urate, ascorbic acid, vitamin E, carotenoids, ubiquinones, taurine, hypotaurine, etc (Li, 1975; Jeulin *et al.*, 1989; Therond *et al.*, 1996). The total antioxidant capacity (TAC) of seminal plasma has been defined as the sum of antioxidants. Low seminal TAC has been shown to be related to male infertility (Smith *et al.*, 1996; Lewis *et al.*, 1997; Lenzi *et al.*, 2002; Tavilani *et al.*, 2008) and TAC has been proposed as a biochemical predictor of male fertility (Mahfouz *et al.*, 2009; Adeel *et al.*, 2012).

It is well-known that the lipid composition of the sperm plasma membrane has a significant effect on the functional characteristics of spermatozoa (Poulos & White, 1973; Alvarez & Storey, 1995; Zalata *et al.*, 1998; Conquer *et al.*, 1999; Lenzi *et al.*, 2000; Gulaya *et al.*, 2001; Lessig *et al.*, 2004; Aksoy *et al.*, 2006; Tavilani *et al.*, 2006). Long-chain PUFAs have been detected at high concentration in human spermatozoa (Ahluwalia & Holman, 1969; Poulos & White, 1973) and the proportion of these unsaturated fatty acids in relation to the saturated fatty acids and cholesterol is closely correlated with sperm membrane fluidity (Lenzi, 1996). Owing to their large number of double bonds, PUFAs in the sperm membrane are particularly susceptible to peroxidative breakdown (Alvarez & Storey, 1995). This situation is especially important during cryopreservation, when the total ROS generated is increased (Wang *et al.*, 1997) and the antioxidant system is altered (Alvarez & Storey, 1992; Lasso *et al.*, 1994; Gadea *et al.*, 2011).

Membrane lipid composition is of great interest with respect to cryopreservation. Variations in PUFAs within sperm membranes have been associated with differences in cryotolerance in spermatozoa from different wild animal species (Miller *et al.*, 2004, 2005). Some previous studies, in human and domestic animals, have shown alterations in the plasma membrane lipid composition and a significant reduction in w3 PUFA fatty acids in the freezing/thawing process (Alvarez & Storey, 1992; James *et al.*, 1999; Schiller *et al.*, 2000; Maldjian *et al.*, 2005; Chakrabarty *et al.*, 2007). Other authors have shown that freezing/thawing induced a decrease in membrane fluidity (Giraud *et al.*, 2000; Peña *et al.*, 2004) and a relationship has been reported between lipid membrane composition and post-thawing spermatozoa parameters in boar and stallion spermatozoa (Waterhouse *et al.*, 2006; Macías García *et al.*, 2011).

However, there is a lack of information about the importance of fatty acid composition of the human sperm membranes and seminal plasma in the cryopreservation procedure. This is the first study, in our knowledge, to report a significant relationship between the fatty acid composition of human spermatozoa or seminal fluid and the post-thaw sperm quality, measured in terms of viability and motility. We propose that in the future the fatty acid composition could be used as a predictor of the capacity of cryopreservation of a seminal sample.

A further objective of this study was to determine whether the antioxidant capacity (TAC) of the seminal plasma is related to spermatozoa and seminal plasma fatty acid composition and to explore whether TAC of the seminal plasma is directly related to the success of the cryopreservation process. If it is confirmed, we could design further procedures to modify the lipid composition or antioxidant capacity of ejaculate to make it more resistant to the cryopreservation process.

MATERIALS AND METHODS

Ethics

This study was conducted with institutional approval from Instituto Valenciano Infertilidad (IVI-Murcia, Spain) and informed consent from men who visited the centre for infertility screening.

Sample collection

Semen samples were obtained from 64 patients (n = 192 ejaculates, three ejaculates per patient with 5 weeks of difference between sampling) attending the clinic for infertility screening. Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction, semen samples were examined for volume, sperm concentration, morphology, viability and motility according to WHO, 2010; fifth edition guidelines (WHO, 2010).

Samples were categorized into the following groups: Normozoospermia (n = 103); Oligozoospermia (n = 21); Asthenozoospermia (n = 25); Oligoasthenozoospermia (n = 31), according to World Health Organization criteria (WHO, 2010). Oligozoospermia was considered when the total number of spermatozoa in the ejaculate was below 39×10^6 spermatozoa, Asthenozoospermia when percentage of progressively motile spermatozoa was below 32%.

Aliquots of each sample were centrifuged (3000 ***g*** for 10 min) and seminal plasma and spermatozoa were stored at −80 °C until antioxidant capacity and fatty acids composition were evaluated.

Cryopreservation of semen samples

Semen samples were frozen in pellets on the surface of dry ice using a glycerol-based cryoprotectant with egg yolk (Freezing Medium-Test yolk Buffer; Irvine Scientific, Santa Ana, CA, USA) as we previously described (Martinez-Soto *et al.*, 2010). All the pellets formed were transferred into a cryotube immersed into liquid nitrogen and then immediately moved to semen banks for long-term storage.

Thawing process

Three pellets per sample (total 150 μL) were transferred to a Falcon tube and maintained for 10 min at room temperature, followed by another 10 min period at 37 °C without medium. Finally, 300 μL of the medium (human tubal fluid, HTF; Irvine Scientific) supplemented with 10% human serum albumin (HSA; SAGE Coopersurgical, Trumbull, CT, USA) at 37 °C was added before viability and motility assessment (Martinez-Soto *et al.*, 2011).

Motion parameters measured by computer-assisted sperm analysis

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (ISAS, Valencia, Spain) (Martinez-Soto *et al.*, 2010, 2011). The CASA-derived motility characteristics studied were percentage of motility and progressive motility, curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear

trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross-frequency (BCF, Hz).

A 7 μL drop of the sperm sample was placed on a warmed (37 °C) slide and covered with a 24 × 24 mm cover slip that produced a depth of 10 μm. The setting parameters were as follows: camera velocity 50 frames/sec, evaluation of 100 frames in which spermatozoa had to be present in at least 25 to be counted, images were obtained at 200× magnification on a phase contrast microscope (Nikon, Tokyo, Japan). Spermatozoa with a VAP <10 μm/s were considered immotile. A minimum of five fields per sample were evaluated, counting a minimum of 200 spermatozoa per subsample.

Fatty acid analysis

Fatty acid analyses in seminal plasma and spermatozoa were evaluated using gas chromatography (Lepage & Roy, 1986) in samples before freezing. Aliquots of 300 μL seminal plasma were transferred into glass tubes for direct transesterification. To each sample 2 mL of methanol-benzene (4:1, v/v) was added with an internal standard (heptadecanoic acid, C17:0) and 0.01 % butylhydroxytoluene as an antioxidant. Samples were vortexed at low speed while slowly adding 200 μL of acetyl chloride, over a period of 2 min. Samples were then heated for 60 min at 100 °C in a heating block and shaken continuously. After the tubes had been cooled to room temperature, 5 mL of 6% (w/v) potassium carbonate was then added. The samples were vortexed for 30 sec and centrifuged at 900 ***g*** for 20 min at 15 °C. The fatty acid methyl esters (FAMEs) contained in the upper benzene phase were transferred to gas chromatography vials and stored at 4 °C until chromatograph evaluation.

In the case of spermatozoa, 2–10 × 10^6 cells were diluted with one volume of PBS and centrifuged at 900 ***g*** for 8 min. The pellet was resuspended in 400 μL of PBS. Then aliquots of 300 μL of sperm suspension were treated in the way previously explained.

The analysis was performed on a Varian CP-3900 Gas Chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a flame ionization detector, using a capillary column model CP9205-VF-WAXms (Agilent Technologies España S.L., Madrid, Spain). The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance were achieved through the use of calibration standard mix. Individual fatty acids were identified by order of elution and compared with known commercially prepared fatty acid standards GLC 566-C (Nu-Chek Prep Inc., Elysian, MN, USA). The percentage of each fatty acid class was expressed as molar percentage of total fatty acids. To compare differences in FA composition in a more holistic manner, the proportion of SFA, monounsaturated fatty acids (MUFA) and PUFA, total ω3 and ω6, ratios of ω6/ ω3 and SFA/PUFA were calculated.

Total antioxidant capacity measurement in seminal plasma

The study of the TAC (Rice-Evans & Miller, 1994) was carried out with a commercially available antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. TAC is a colorimetric assay that measures the combined antioxidant activities of all its constituents. TAC assay is based on the ability of antioxidant molecules to quench the long-lived ABTS+ radical, a blue-green chromophore with characteristic absorption at 750 nm, compared with Trolox, a water-soluble vitamin E analogue, used as standard reference for the calibration of TAC. In this assay, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate (ABTS) was incubated with metamyoglobin and hydrogen peroxide to produce ABTS+. The quantity of ABTS+ produced was measured for absorbance in a Bio-Rad microplate reader (Bio-Rad Laboratories, S.A., Madrid, Spain). The results were expressed as concentration of Trolox equivalents (nM).

Statistical analysis

The data were firstly examined using the Shapiro–Wilk test to assess normality distribution. In view of the non-Gaussian distribution of most of the data gathered, non-parametric test was used.

Data expressed as mean value ± SEM, and compared by a non-parametric Wilcoxon Signed-Rank test to directly compare pairs of values. Kruskal–Wallis Test and a Conover–Inman test for all pairwise comparisons were used to compare groups of samples.

The Spearman non-parametric test was used to study the correlation between the analysis of the lipids of the spermatozoa and seminal plasma pre-freezing and sperm quality before and after freezing–thawing.

RESULTS

Sperm parameters before and after cryopreservation

The main semen parameters before and after cryopreservation are illustrated in Table 1. Cryopreservation is related to a significant decrease in the values of viability, motility and most of the motion parameters measured by CASA (Table 1, $p < 0.05$).

Fatty acid composition of spermatozoa and seminal plasma

Fatty acid compositions of spermatozoa and seminal plasma measured before freezing are given in Table 2. The fatty acid composition in sperm membranes is different from seminal plasma composition in most of the fatty acids evaluated

Table 1 Basic semen parameters before and after cryopreservation. Data expressed as mean value ± SEM, and compared by a non-parametric Wilcoxon signed-rank test

Parameter	Fresh	Frozen–thawed	*p*-value
Age	35.44 ± 0.37		
Volume (mL)	3.42 ± 0.11		
Sperm concentration (10^6 cells/mL)	30.25 ± 1.82		
Normal morphology (%)	4.18 ± 0.22		
Viability (%)	85.53 ± 0.83	36.93 ± 1.89	<0.01
Motility (%)	40.42 ± 1.51	20.30 ± 1.17	<0.01
Progressive motility (%)	33.76 ± 1.43	16.73 ± 1.02	<0.01
VCL	51.03 ± 0.97	47.51 ± 1.31	<0.01
VSL	27.30 ± 0.68	29.52 ± 1.10	0.73
VAP	35.95 ± 0.69	34.87 ± 1.09	0.09
LIN	53.83 ± 0.71	61.38 ± 1.13	<0.01
STR	72.90 ± 0.67	80.67 ± 0.91	<0.01
WOB	72.38 ± 0.46	73.64 ± 0.81	0.13
ALH	2.10 ± 0.04	1.80 ± 0.04	<0.01
BCF	7.41 ± 0.10	8.05 ± 0.17	0.21

CASA: Computer-assisted sperm analysis, VCL: Curvilinear velocity, VSL: Straight-line velocity, VAP: Average path velocity, LIN: Linearity of the curvilinear trajectory, STR: straightness, WOB: Wobble (VAP/VCL), ALH: Amplitude of lateral head displacement, BCF: Beat cross-frequency.

Table 2 Fatty acid composition of the human spermatozoa and seminal plasma before freezing. Fatty acid is presented as mole percentage. Data expressed as mean ± SEM, and compared by a non-parametric Wilcoxon signed-rank test

Fatty acid		Spermatozoa	Seminal plasma
Myristic acid	C14:0	0.92 ± 0.09	0.46 ± 0.04*
Myristoleic acid	C14:1	0.11 ± 0.03	0 ± 0
Palmitic acid	C16:0	28.87 ± 0.45	30.52 ± 0.16*
Palmitoleic acid	C16:1	1.27 ± 0.10	0 ± 0*
Stearic acid	C18:0	13.35 ± 0.18	15.94 ± 0.18*
Vaccenic acid	C18:1n7	2.57 ± 0.07	1.55 ± 0.03*
Oleic acid	C18:1n9	9.33 ± 0.43	14.43 ± 0.2*
Linoleic acid (LA)	C18:2n6c	5.67 ± 0.13	2.85 ± 0.09*
Linoleic acid (LA)	C18:2n6t	1.2 ± 0.14	1.47 ± 0.15*
α-Linolenic acid (ALA)	C18:3n3	0.81 ± 0.10	0.59 ± 0.07
γ-Linolenic acid	C18:3n6	0.95 ± 0.11	1.67 ± 0.1*
Stearidonic acid (SDA)	C18:4n3	0.30 ± 0.07	0.09 ± 0.01
Arachidic acid (AA)	C20:0	1.24 ± 0.09	5.16 ± 0.08*
Eicosenoic acid	C20:1n9	0.81 ± 0.05	1.47 ± 0.07*
Eicosadienoic acid	C20:2n6	1.50 ± 0.07	0.32 ± 0.03*
Eicosatrienoic acid (ETE)	C20:3n3	0 ± 0	0 ± 0
Dihomo-gamma-linolenic acid (DGLA)	C20:3n6	3.42 ± 0.08	2.28 ± 0.05*
Arachidonic acid (AA)	C20:4n6	2.73 ± 0.11	3.34 ± 0.06*
Eicosapentaenoic acid (EPA)	C20:5n3	0.43 ± 0.08	0.01 ± 0.01
Behenic acid	C22:0	0.90 ± 0.10	6.1 ± 0.1*
Erucic acid	C22:1n9	0.05 ± 0.03	0 ± 0
Eicosedienoic acid	C22:2n6	1.21 ± 0.15	0.43 ± 0.05
Eicosatrienoic acid	C22:3n3	0.04 ± 0.01	0.06 ± 0.01*
Docosatetraenoic acid	C22:4n6	0 ± 0	0.18 ± 0.02*
Docosapentaenoic acid (DPA)	C22:5n3	0.84 ± 0.05	0.07 ± 0.01*
Docosapentaenoic acid	C22:5n6	0 ± 0	0 ± 0
Docosahexaenoic acid (DHA)	C22:6n3	19.01 ± 0.65	4.28 ± 0.17*
Lignoceric acid	C24:0	1.42 ± 0.09	4.46 ± 0.08*
Nervonic acid	C24:1n9	1.01 ± 0.05	2.25 ± 0.04*
Saturated fatty acids	SFA	46.71 ± 0.58	62.63 ± 0.27*
Monounsaturated fatty acids	MUFA	15.14 ± 0.45	19.71 ± 0.24*
Polyunsaturated fatty acids	PUFA	38.14 ± 0.68	17.66 ± 0.34*
Omega-3 fatty acids	Total ω3	21.45 ± 0.69	5.11 ± 0.17*
Omega-6 fatty acids	Total ω6	16.69 ± 0.24	12.55 ± 0.23*
Ratio omega-6/omega 3 fatty acids	ω6/ω3	0.97 ± 0.06	2.76 ± 0.10*
Ratio Satured/ Polyunsaturated fatty acids	SFA/PUFA	1.32 ± 0.05	3.73 ± 0.08*

*$p < 0.05$.

(Table 2, $p < 0.01$). Higher values were found in sperm membranes than in seminal plasma for PUFA, ω3 PUFA and ω6 PUFA, and lower values in SFA, MUFA and ratios ω6/ω3 and SFA/PUFA in sperm membranes than in seminal plasma (Table 2, $p < 0.01$).

In sperm membrane, SFA represented 46.7%, MUFA 15.1% and PUFA 38.1% of the total fatty acids. In seminal plasma, these proportions were 62% for SFA, 19.7% for MUFA and 17.7% for PUFA. This means a significant difference for the ratio SFA/PUFA (1.32 in spermatozoa vs. 3.7 in seminal plasma) and ratio ω6/ω3 (0.97 in spermatozoa vs. 2.76 in seminal plasma).

In spermatozoa, the main SFA were palmitic (C16:0) and stearic acids (C18:0) that both together represented 90% of the total SFA. In seminal plasma, besides the presence of C16:0 and C18:0, it is important to note the presence of arachidic (C20:0), behenic (C22:0) and lignoceric acids (C24:0). The main MUFA in spermatozoa is oleic acid (C18:1n9) that contributed in more than 60% of total MUFA. In seminal plasma, the percentage of C18:1n9 was higher than in spermatozoa (9.3% vs. 14.4%, $p < 0.01$) and contributed to more than 70% of the total MUFA.

The main PUFA in spermatozoa was docosahexaenoic acid (DHA, C22:6n3), which contributed 50% of total PUFA. However, in seminal plasma DHA was significant lower than in spermatozoa and made up only 25% of the total PUFA.

Fatty acid composition of spermatozoa and seminal plasma in normo-, oligo-, astheno- and oligoasthenozoospermia

When the samples were classified according to (WHO, 2010) as normozoospermia, oligozoospermia, asthenozoospermia and oligoasthenozoospermia some differences were found for the seminal parameters and fatty composition in sperm membrane and seminal plasma (Table 3).

Most of the seminal parameters evaluated showed differences between groups ($p < 0.01$, Table 3a). A decreased antioxidant capacity of the seminal plasma is present in the oligozoospermia groups (oligozoospermic and oligoasthenozoospermic samples) compared with normozoospermic group ($p < 0.01$, Table 3a). However, TAC values are similar between normozoospermic and asthenozoospermic groups ($p > 0.05$, Table 3a).

In relation to fatty acid composition of the sperm membranes, normozoospermic samples showed lower SFA than asthenozoospermic and oligoasthenozoospermic groups (Table 3b; $p < 0.05$) and comparable values to oligospermic samples. MUFA composition of sperm membranes in normozoospermic was also lower than in the other groups (Table 3b; $p < 0.05$). PUFA was higher in normozoospermic group than in the others (Table 3b; $p < 0.05$). This increase in PUFA was related to an increase in ω3 fatty acid and mainly with higher values for C22:6n3 (DHA) (Table 3b, $p < 0.05$), whereas ω6 fatty acid values are similar in all the groups, ranging between 16.35 and 17.01%. Also, the ratios ω6/ω3 and SFA/PUFA were lower in normozoospermia than in other groups.

In seminal plasma, a similar picture was found in the values of normozoospermic vs. oligozoospermic and oligoasthenozoospermic groups (Table 3c). MUFA was reduced in normozoospermia in comparison with oligoasthenozoospermic samples, whereas PUFA, total ω3 fatty acid and C22:6n3 (DHA) were increased in normozoospermic in comparison with other groups (Table 3c, $p < 0.05$). No differences between groups were observed for SFA (Table 3c, $p > 0.05$). We did not find any significant difference in fatty acid composition of the seminal plasma between normozoospermic and asthenozoospermic groups (Table 3c).

Correlation between spermatozoa and seminal plasma fatty acids and sperm parameters before freezing

We calculated the Spearman correlations between fatty acid composition in spermatozoa and semen parameters before freezing (Table 4a, $p < 0.01$). Significant positive correlations between PUFA, Total ω3, DHA and Araquidonic acid (AA, C20:4n6) were observed with seminal parameters. On the other hand, an inverse relationship was found for SFA, MUFA, Total ω6, ratio ω6/ω3, ratio SFA/PUFA and some specific fatty acids (C20:0, C18:1n9) with the seminal parameters (Table 4a, $p < 0.01$). Morphology and total number of spermatozoa in the ejaculate are the seminal parameters with a highest number of significant correlations with the fatty acids.

The study of the correlation between fatty acid composition of seminal plasma and semen parameters before freezing showed that sperm viability of the fresh sample before freezing was not

Table 3 (a) Sperm parameters and fatty acid composition in sperm membrane in samples from men class classified according to WHO, 2010 in normozoospermic, oligo-, astheno- and oligoasthenozoospermic. (b) Fatty acid composition in sperm membrane before freezing, (c) Seminal plasma before freezing. Data expressed as mean ± SEM, and compared by a non-parametric Kruskal–Wallis Test and a Conover–Inman Test for All Pairwise Comparisons

	Normozoospermia (n = 103)	Oligozoospermia (n = 21)	Asthenozoospermia (n = 25)	Oligoasthenozoospermia (n = 31)
(a)				
Motility (%)	52.34 ± 0.80[a]	51 ± 1.88[a]	25.26 ± 1.93[b]	18.9 ± 1.72[b]
Viability (%)	89.1 ± 0.66[a]	84.44 ± 1.95[b]	81.6 ± 2.39[b]	76.52 ± 3.28[b]
Morphology (%)	4.83 ± 0.31[a]	3.72 ± 0.46[a]	3.54 ± 0.48[a,b]	2.56 ± 0.36[b]
Volume	3.46 ± 0.14[a]	2.65 ± 0.21[b]	4.51 ± 0.36[c]	2.96 ± 0.26[a,b]
Concentration	43.34 ± 2.23[a]	7.51 ± 1.32[b]	25.83 ± 3.45[c]	6.45 ± 1[b]
Total sperm	144.19 ± 9.11[a]	16.47 ± 2.34[b]	109.81 ± 15.65[c]	15.91 ± 2.29[b]
TAC	1710.23 ± 27.85[a]	1678.49 ± 37.68[b]	1765.01 ± 34.86[a]	1625.07 ± 37.92[b]
(b)				
C22:6n3	22.39 ± 0.72[a]	15.31 ± 2.15[b]	17.50 ± 1.06[b]	13.64 ± 1.33[b]
SFA	45.82 ± 0.64[a]	46.34 ± 2.04[a,b]	48.8 ± 1.43[b]	48 ± 1.63[b]
MUFA	12.87 ± 0.46[a]	17.72 ± 1.09[b]	14.95 ± 1.26[c]	19.52 ± 1.07[b]
PUFA	41.31 ± 0.7[a]	35.94 ± 2.3[b]	36.24 ± 1.25[b]	32.48 ± 1.63[b]
Total ω3	24.58 ± 0.79[a]	18.93 ± 2.35[b]	19.55 ± 1.07[b]	16.13 ± 1.58[b]
Total ω6	16.73 ± 0.33[a]	17.01 ± 0.75[a]	16.7 ± 0.67[a]	16.35 ± 0.64[a]
ω6/ω3	0.77 ± 0.05[a]	1.17 ± 0.14[b]	0.9 ± 0.06[b]	1.41 ± 0.2[b]
SFA/PUFA	1.14 ± 0.04[a]	1.49 ± 0.2[b]	1.39 ± 0.08[b]	1.65 ± 0.14[b]
(c)				
C22:6n3	4.66 ± 0.19[a]	3.11 ± 0.31[b]	4.68 ± 0.5[a,b]	3.39 ± 0.45[a,b]
SFA	62.47 ± 0.34	63.78 ± 1.23	61.61 ± 0.48	63.46 ± 0.65
MUFA	19.35 ± 0.3[a]	20.11 ± 1.03[a,b]	19.65 ± 0.46[a,b]	20.62 ± 0.64[b]
PUFA	18.18 ± 0.44[a]	16.12 ± 0.83[b]	18.74 ± 0.71[a]	15.92 ± 0.93[b]
Total ω3	5.39 ± 0.19[a]	4.01 ± 0.52[b]	5.73 ± 0.48[a]	4.28 ± 0.49[b]
Total ω6	12.79 ± 0.33[a]	12.11 ± 0.5[a]	13 ± 0.37[a]	11.64 ± 0.58[a]
ω6/ω3	2.53 ± 0.09[a]	3.58 ± 0.44[b]	2.57 ± 0.24[a]	3.2 ± 0.27[b]
SFA/PUFA	3.59 ± 0.1[a]	4.11 ± 0.29[a,b]	3.39 ± 0.14[a]	4.28 ± 0.26[b]

Numbers within columns with different superscripts differ ($p < 0.05$).

associated with any lipid parameter (Table 4b. $p > 0.05$). However, C22:6n3 (DHA) was directly related to all the other seminal parameters studied (Table 4b, $p < 0.01$). MUFA was inversely related to morphology and total spermatozoa in the ejaculate. PUFA and total ω3 directly related to total spermatozoa. However, w6/w3 and SFA/PUFA ratios were inversely related to this parameter.

Correlation between fatty acid in seminal plasma and spermatozoa and antioxidant capacity measured in seminal plasma

Total antioxidant capacity of the seminal plasma was in mean 1703.08 ± 15.89 mM (trolox equivalent). TAC was directly related to PUFA, total w6, C18:2n6t, C20:3n6, total w3 and C22:6n3 (DHA) in the seminal plasma (Table 5, $p < 0.01$). On the other hand, SFA, C22:0, C24:0 and MUFA in seminal plasma were inversely related to the antioxidant capacity (Table 5, $p < 0.01$). However, when relationship between fatty acid composition of the spermatozoa and TAC in seminal plasma was evaluated, we observed a lower relation and in an opposite way than in the fatty composition of the seminal plasma. TAC was directly related to SFA and C22:2n6 in the spermatozoa and inversely related to C22:5n3 (Table 5, $p < 0.01$) .

Correlation between spermatozoa and seminal plasma fatty acid measured before cryopreservation and seminal parameters after freezing–thawing

Direct relationships between sperm parameters in frozen–thawed samples and fatty acid composition of spermatozoa and seminal plasma are shown in Table 6a and b.

As previously described for seminal parameters in samples before freezing, we observed significant positive correlations between C22:6n3 (DHA), PUFA and Total ω3 in sperm membrane with seminal parameters after thawing. An inverse relationship was found for some of the SFA as C16:0, C18:0, C20:0 and C22:0; MUFA, ratio w6/w3 and SFA/PUFA (Table 6a, $p < 0.01$).

The fatty acids composition in seminal plasma were not related to the viability of the frozen–thawed samples (Table 6b, $p > 0.05$). Some fatty acids as C22:6n3 (DHA), w6, C18:0 and ratio w6/w3 are directly related to motility and motion parameters (Table 6b, $p < 0.01$). On the other hand, C20:0, C22:0, C20:5n3 are inversely related to the motion parameters (Table 6b, $p < 0.01$).

To study in detail the consistency of the correlations previously obtained, the correlation between fatty acid composition of spermatozoa and seminal plasma and the variation in these sperm quality parameters before vs. after freezing–thawing were evaluated (Table 7a and b). This variation was calculated for each seminal parameter as value for frozen–thawed sample/value for fresh sample* 100. Although the level of the correlations changed with lower values for the Spearman correlations and p-Values in relation to the direct correlation only with frozen–thawed samples (presented in Table 6a and b), in general terms the data obtained are consistent.

The antioxidant capacity of the seminal plasma was directly related to some motility parameters (Table 6b, $p < 0.01$). In particular, TAC was directly related with average path velocity (VAP) and straight-line velocity (VSL), which means that ejaculates with higher antioxidant capacity in the seminal plasma showed spermatozoa with higher velocity.

Table 4 Spearman correlations between composition of fatty acids of the (a) human spermatozoa, (b) seminal plasma measured before freezing and sperm parameters before freezing ($p < 0.01$)

	Motility	Viability	Morphology	Total spermatozoa
(a)				
SFA	−0.22	−0.20	−0.12	−0.22
C16:0	–	–	–	–
C18:0	–	–	–	−0.24
C20:0	−0.22			−0.44
C22:0			−0.27	
MUFA	−0.32	−0.11	−0.26	−0.63
C18:1n7			0.24	
C18:1n9	−0.30	–	−0.29	−0.58
PUFA	0.36	0.23	0.23	0.57
Total ω6	–	−0.24	−0.19	–
C18:2n6c		−0.26	−0.25	
C20:3n6				0.42
C20:4n6	0.19	0.25	0.28	0.37
Total ω3	0.43	0.28	0.27	0.63
C20:5n3				0.19
C22:6n3	0.44	0.28	0.26	0.67
ω6/ω3	−0.42	−0.31	−0.32	−0.58
SFA/PUFA	−0.31	−0.24	−0.19	−0.44
(b)				
SFA	–	–	–	–
C16:0	–	–	–	–
C18:0	0.22			
C20:0				
C22:0				−0.18
MUFA	–		−0.25	−0.21
C18:1n7				
C18:1n9				−0.20
PUFA	–			0.27
Total ω6	–			
C18:2n6c	–	–	–	–
C20:3n6	–	–	–	–
C20:4n6	–	–	–	–
Total ω3	–			0.30
C20:5n3				0.19
C22:6n3	0.19	–	0.22	0.52
ω6/ω3	–			−0.23
SFA/PUFA	–			−0.26

Table 5 Spearman correlations between composition of fatty acids of the spermatozoa or seminal plasma and total antioxidant capacity (TAC) of the seminal plasma

	Seminal plasma – Antioxidant capacity	Spermatozoa – Antioxidant capacity
SFA	−0.22	0.23
C22:0	−0.22	
C24:0	−0.26	
MUFA	−0.19	
PUFA	0.30	
Total ω6	0.25	
C18:2n6t	0.25	
C20:3n6	0.20	
C22:2n6		0.24
Total ω3	0.28	
C22:5n3		−0.28
C22:6n3	0.33	
SFA/PUFA	−0.30	

Fatty acid in sperm membrane in bad vs. good frozen samples

The samples were grouped as good or bad frozen according to cryotolerance. Frozen samples were considered good if motility of frozen sample was >50% of the motility of the fresh semen. Good frozen samples are characterized by higher values for PUFA, w3 and DHA than bad frozen samples. On the other hand, they presented lower SFA, ratio w6/w3 and SFA/PUFA (Table 8, $p < 0.05$).

DISCUSSION

Freezing is associated with damage to sperm functions affecting those processes required for successful in vivo and in vitro fertilization of the oocyte (Alvarez & Storey, 1993; Hammadeh *et al.*, 1999; Schiller *et al.*, 2000; O'Connell *et al.*, 2002). As it was expected, in this study we observed a significant reduction in the viability and motility after freezing. Mechanisms for cryodamage to human spermatozoa are thought to be multifactorial, but excessive ROS production during freezing and thawing and oxidative stress have been previously demonstrated to be significant contributing factors (Wang *et al.*, 1997; Thomson *et al.*, 2009). During freezing, two important processes have been reported: (i) production of ROS that can induce changes in membrane function and structure (Wang *et al.*, 1997; Chatterjee & Gagnon, 2001); and (ii) an alteration in antioxidant defence systems (Alvarez & Storey, 1992; Lasso *et al.*, 1994; Gadea *et al.*, 2011).

Long-chain PUFAs have been detected at high concentration in human spermatozoa (Ahluwalia & Holman, 1969; Poulos & White, 1973). Owing to their large number of double bonds, PUFA in the sperm membrane is particularly susceptible to peroxidative breakdown (Alvarez & Storey, 1995). In addition, PUFA presence in sperm membrane brings a greater plasticity useful in the cryopreservation process.

In this study we have explored the relation between the fatty acid composition with the cryotolerance of the seminal samples.

Fatty acid composition in spermatozoa and seminal plasma

The lipid composition of the sperm membrane has a significant effect upon the functional characteristics of spermatozoa (Zalata *et al.*, 1998; Conquer *et al.*, 1999; Lenzi *et al.*, 2000; Gulaya *et al.*, 2001; Calamera *et al.*, 2003; Lessig *et al.*, 2004; Aksoy *et al.*, 2006; Tavilani *et al.*, 2006). Previously, some authors have reported high amounts of DHA and the predominance of 16:0 and 18:0 among SFA of spermatozoa (Ahluwalia & Holman, 1969; Alvarez & Storey, 1992; Zalata *et al.*, 1998; Aksoy *et al.*, 2006). These authors found relatively different amounts of every specific fatty acid in comparison with our results, which may be explained by differences in the methods of sperm preparation used to analyse.

We confirmed that the fatty acid composition in sperm membrane and seminal plasma measured before freezing is related to the spermatozoa structure and function. So normozoospermia samples presented higher level of PUFA, w3 and specifically DHA than oligospermia, asthenospermia groups, as previously reported in other studies (Zalata *et al.*, 1998; Conquer *et al.*, 1999; Calamera *et al.*, 2003). In relation to the seminal plasma we have shown that TAC values in normozoospermia samples are higher than in oligospermia and oligoasthenospermia groups, but similar to asthenospermia (Table 3a). This situation is similar for the fatty acid composition of the seminal plasma (Table 3c). This fact suggests the high level of correlation between antioxidant capacity and fatty acid composition of the seminal plasma as later was shown in Table 5. On the contrary,

Table 6 Spearman correlation of (a) spermatozoa lipid composition and (b) Seminal plasma measured before freezing and viability and motility of frozen–thawed spermatozoa ($p < 0.01$)

	Viability	Motility	Mot progr.	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
(a)											
SFA									−0.22		
C16:0									−0.22	−026	
C18:0	−0.37										
C20:0			−0.22		−0.23		−.025	−.026	−0.25		
C22:0	−0.37	−0.24	−0.24		−0.24	−0.23					−0.22
MUFA	−0.39	−0.31	−0.31	−0.31	−0.35	−0.35				−0.25	−0.28
C18:1n7				−0.21							
C18:1n9	−0.46	−0.28	−0.28	−0.31	−0.35	−0.35				−0.26	−0.29
PUFA	0.30	0.32	0.37		0.32	0.31	0.26		0.33		
Total ω6											
C18:2n6c											
C20:3n6											
C20:4n6				0.32	0.29	0.30				0.26	0.30
Total ω3	0.43	0.38	0.41		0.28	0.28	0.23		0.31		
C20:5n3									0.22		
C22:6n3	0.43	0.41	0.45		0.28	0.29	0.20		0.28		
ω6/ω3	−0.44	−0.35	−0.35			−0.20					
SFA/PUFA		−0.28	−0.32		−0.22	−0.21	−0.27		−0.34		
(b)											
SFA	–	–									
C16:0	–	–									
C18:0		0.43	0.44		0.23		0.27	0.27	0.26		
C20:0		−0.31	−0.31	−0.25	−0.24				−0.25		
C22:0		−0.25	−0.25								
MUFA	–										−0.23
C18:1n7	–										
C18:1n9	–										
PUFA	–										
Total ω6	–						0.28	0.27	0.29		0.25
C18:2n6c											
C20:3n6								0.25			
C20:4n6	–		0.24								
Total ω3											
C20:5n3		−0.25	−0.23								
C22:6n3	–	0.30	0.30	–	–	–			0.24		
ω6/ω3	–				0.23			0.24			0.23
SFA/PUFA	–										
TAC					0.24	0.23		0.24			0.29

the relationship between TAC in seminal plasma and fatty acid composition of the spermatozoa is less tight.

The results of studying the relationships between fatty acid composition and seminal parameters before freezing are similar to previous studies. In all of them, DHA and PUFA are directly correlated with seminal parameters, and MUFA are inversely correlated and discrepancies are found for SFA (Nissen & Kreysel, 1983; Zalata *et al.*, 1998; Aksoy *et al.*, 2006; Tavilani *et al.*, 2006). Nevertheless, the fatty acid composition is also influenced by the dietary intake (Conquer *et al.*, 2000; Safarinejad, 2011; Attaman *et al.*, 2012); this fact could mask the real relationship between fatty acids and sperm function.

The presence of fatty acid in the seminal plasma could modify sperm function by the alteration of the antioxidant status. We have shown that the presence of PUFA in seminal plasma is directly related to the antioxidant capacity, whereas SFA and MUFA are inversely related to antioxidant capacity of the seminal plasma. Some previous studies have suggested that the addition of the omega-3 polyunsaturated fatty acids in the diet is relate to an increase in the GSH content and SOD activity in blood serum and a reduction in the lipid peroxidation (Romieu *et al.*, 2008). In the same way, some authors have reported a relationship between the presence of PUFA and gene expression involved in different pathways. These include oxidative stress response and antioxidant capacity (Lapillonne *et al.*, 2004). However, in our knowledge it is the first study that correlated fatty acid in seminal plasma and antioxidant capacity, and further studies must confirm these results and explore the causes of this relationship.

In contrast, when relationships between fatty acid composition of the spermatozoa and TAC in seminal plasma were evaluated, we observed a weaker and opposite relationship than in the fatty composition of the seminal plasma (Table 5). Different studies have previously evaluated the relationship between fatty acid composition of the spermatozoa and the antioxidant capacity or activity of the enzymes present in the seminal plasma, with different results (Calamera *et al.*, 2003; Tavilani *et al.*, 2008; Safarinejad *et al.*, 2010). Tavilani *et al.* (2008) reported a positive correlation between SOD activity and polyunsaturated fatty acid composition of spermatozoa. On the other hand, Safarinejad *et al.* reported a direct relationship between omega-3 fatty acid in spermatozoa and SOD and catalase in seminal plasma (Safarinejad *et al.*, 2010) and an inverse relationship between omega-6 fatty acid in spermatozoa and SOD and catalase in seminal plasma. Calamera *et al.* (2003) offered an interesting point of view when reporting that the

Table 7 Spearman correlation of (a) spermatozoa (b) lipid composition measured before freezing and the variation in the viability and motility before and after freezing–thawing ($p < 0.01$)

	Viability	Motility	Mot progr.	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
(a)											
SFA									−0.21		
C16:0	0.30								−0.23		0.29
C18:0		−0.21		−0.22		−0.22					
C20:0	0.22	−0.23	−0.25	−0.24	−0.37	−0.30	−0.22		−0.23		−0.23
C22:0		−0.24	−0.24								−0.28
MUFA	−0.33	−0.23	−0.22								−0.37
C18:1n7											
C18:1n9	−0.38										−0.39
PUFA		0.28	0.29		0.24	0.23			0.26		
Total ω6											
C18:2n6c	−0.26				0.22						
C20:3n6					0.21			0.236			0.27
C20:4n6											0.27
Total ω3		0.25	0.27						0.28		
C20:5n3									0.29		
C22:6n3		0.26	0.28						0.27		
ω6/ω3											
SFA/PUFA		−0.27	−0.27								
(b)											
SFA											
C16:0	0.42										
C18:0		0.46	0.44				0.25		0.29		
C20:0		−0.35	−0.30								
C22:0		−0.29	−0.27								
MUFA											
C18:1n7											
C18:1n9											−0.25
PUFA				0.25		0.24					
Total ω6				0.29	0.27	0.31					
C18:2n6c											
C20:3n6	−0.24										
C20:4n6											
Total ω3											
C20:5n3	−0.26										
C22:6n3		0.33	0.28								
ω6/ω3	−0.25										
SFA/PUFA						−0.24					

Table 8 Fatty acid composition in sperm membrane measured before freezing in samples classified according to cryotolerance. Good frozen is considered if motility of frozen sample is >50% of the motility of the fresh semen

	Good freezer	Bad freezer
SFA	42.29 ± 1.66	45 ± 0.76*
MUFA	13.04 ± 1.3	14.71 ± 0.62
PUFA	44.67 ± 2.09	40.29 ± 0.75*
Total ω6	16.63 ± 0.66	16.71 ± 0.36
Total ω3	28.04 ± 2.64	23.58 ± 0.77*
DHA (C22:6n3)	25.28 ± 2.3	21.19 ± 0.69*
ω6/ω3	0.66 ± 0.08	0.82 ± 0.06*
SFA/PUFA	0.98 ± 0.07	1.17 ± 0.05*

*$p < 0.05$.

relationship between unsaturated fatty acid (MUFA and PUFA) in spermatozoa and antioxidant SOD activity is different in normozoospermic samples and have higher SOD content than astheno- or polyzoospermic samples.

On the other hand, the presence of some fatty acids could modify the ROS generation. Interestingly, it has been reported that incubation of human spermatozoa in the presence of linoleic acid (C18:2n6) or DHA were effective at stimulating ROS generation by human spermatozoa. In contrast, the SFA in human spermatozoa (C16:0 and C18:0) had no effect on ROS generation by these cells (Aitken *et al.*, 2006).

Fatty acid composition and cryopreservation

Membrane lipid composition is of considerable interest with respect to cryopreservation (reviewed by White, (White, 1993). Some changes in lipid composition have been detected during freezing. Alvarez and Storey reported a reduction of some FA in human spermatozoa after freezing, with a reduction in PUFA C18:2, C20:3, C20:4 and DHA (C22:6n3) and an increase of the SFA C:16 and C:18 after freezing (Alvarez & Storey, 1992). Later, Schiller *et al.* (2000) confirmed that cryopreservation induced a diminution of phosphatidylcholines (16:0, 22:6 and 18:0, 22:6) and SM (16:0) and the appearance of lysophosphatidylcholines (16:0 and 18:0) and ceramide (16:0). These authors suggested the release or activation of both phospholipase A2 and sphingomyelinase in human spermatozoa owing to the freezing/thawing cycle (Schiller *et al.*, 2000).

These changes in lipid composition means that cryopreservation has a rigidifying effect on the membrane, the average anisotropy increased in the freeze/thaw spermatozoa compared to fresh semen (Giraud *et al.*, 2000). In the same way, cryopreservation induced a reduction in the lipid diffusion in the membrane of all the regions of the spermatozoa (James *et al.*, 1999).

This is the first study, to our knowledge, to describe a significant correlation between the fatty acid composition of the human spermatozoa or seminal plasma and the sperm parameters of the samples after thawing. We confirmed that the molar percentage of PUFA and specifically DHA is directly related to the sperm functionality of the frozen–thawed samples.

The susceptibility of spermatozoa to rapid cold shock has been associated with a high ratio of PUFA/SFA membrane fatty acids and with low levels of cholesterol within the sperm membrane (White, 1993). As PUFAs can influence membrane fluidity, it is not surprising to see that membrane fluidity is a predictor of cryogenic success in humans (Giraud *et al.*, 2000). Giraud *et al.* (2000) concluded that 'the higher the membrane fluidity was before freezing; the better was the response of spermatozoa to cryopreservation'. Our results confirmed this concept, because PUFA, W3 and specially DHA are directly correlated with sperm motility and viability after freezing/thawing, and MUFA was inversely correlated.

Variations in PUFAs within sperm membranes, in particular Docosapentaenoic acid (DPA) and DHA, have been associated with differences in cryotolerance in spermatozoa from different wild animal species as Asian and African elephants (Swain & Miller, 2000), common wombats, grey kangaroos and koalas (Miller *et al.*, 2004) and blue foxes and silver foxes (Miller *et al.*, 2005). On the other hand, using boar semen Waterhouse *et al.*, 2006 demonstrated a significant relationship between post-thaw amounts of DPA and DHA in the sperm plasma membranes and survival rate as measured by plasma membrane integrity. Recently it has been reported in stallion spermatozoa that saturated fatty acids were correlated with the incidence of membrane-damaged spermatozoa. In contrast, the percentage of highly unsaturated fatty acids was positively correlated with intact membranes post-thaw (Macías García *et al.*, 2011).

Miller *et al.* (2005) suggested that if the possession of long-chain PUFAs, such as DHA and DPA, contributes to increased sperm membrane fluidity, then the relationship between cryogenic success and membrane fluidity may be biphasic. As they suggested in this model, firstly, a certain degree of membrane fluidity may be required for the insertion of cryoprotectants into sperm membranes and may facilitate dehydration prior to cryogenic freezing. However, too much membrane fluidity may also jeopardize sperm integrity (Miller *et al.*, 2005).

Individual differences in seminal cryopreservation capacity are well-known (Centola *et al.*, 1992), However, the possible causes of this fact are not well defined. Some studies have associated the differences between 'good' vs. 'bad freezers' with differences in genomic DNA (Thurston *et al.*, 2002) and sperm morphology; another effect linked to gene expression (Thurston *et al.*, 2001). Others have suggested that the importance of sperm DNA stability after freezing/thawing is related to unbalanced protamine-1 to protamine-2 ratios (Gosalvez *et al.*, 2011). In this study we have detected a difference in fatty acid composition between good and bad freezers, however, further studies must be developed to explore whether lipid differences that ultimately depend on genetic differences could be used as a good index of cryotolerance in combination with other sperm parameters.

CONCLUSIONS

We described a significant correlation between the fatty acid composition of the human spermatozoa or seminal plasma and the sperm parameters of the samples after thawing. PUFA, W3 and specially DHA are directly correlated with sperm motility and viability after freezing/thawing, and MUFA was inversely correlated. This means that in the future the fatty acid composition could be used as a predictor of the capacity of cryopreservation of a seminal sample. On the other hand, we could design further procedures to modify the lipid composition or antioxidant capacity of ejaculate to make it more resistant to the cryopreservation process (Agarwal & Sekhon, 2010; Safarinejad, 2011; Zini & Al-Hathal, 2011).

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DISCLOSURES

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AUTHORSHIP

J. C. M. S and J. G designed the research study and performed the research; J. L. has selected the patients; J. G. analysed the data and wrote the manuscript. All authors participated in the interpretation and commented on the manuscript.

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