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Fibroblast growth factor 2 (FGF2) is present in human spermatozoa and is related with sperm motility. The use of recombinant FGF2 to improve motile sperm recovery

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SUMMARY

Fibroblast growth factors (FGFs) and their receptors (FGFRs) regulate several functions of somatic cells. In a previous work, we reported FGFR expression in human spermatozoa and their involvement in motility. This study aimed to evaluate the presence and localization of fibroblast growth factor 2 (FGF2) in human spermatozoa, to determine the relationship of FGF2 levels with conventional semen parameters and to assess the effect of recombinant FGF2 (rFGF2) on sperm recovery in a selection procedure. Western immunoblotting analysis using an antibody against FGF2 revealed an 18-kDa band in sperm protein extracts. The protein was immunolocalized in the sperm flagellum and acrosomal region, as well as in all germ cells. Sperm FGF2 levels, assessed by flow cytometry, showed a positive ($p < 0.05$) correlation with sperm concentration, motility, total sperm number and total motile cells per ejaculate. Moreover, samples with abnormal motility depicted diminished ($p < 0.01$) FGF2 levels compared to those with normal motility. Spermatozoa exposed to rFGF2 bound the protein, exhibited higher ($p < 0.05$) total and motile sperm recoveries, and increased ($p < 0.01$) kinematic parameters after the swim-up. Findings herein presented lead to consider sperm FGF2 level as a potential marker of sperm quality, and rFGF2 as a supplement for improving sperm recovery in selection techniques.

INTRODUCTION

Infertility affects nearly 50 million couples in reproductive age worldwide, and male factor contributes to approximately 50% of these cases (Agarwal *et al.*, 2015). Male infertility can be attributed to multiple factors, although sperm dysfunction (and particularly defects in sperm motility) is one of the most common causes of male infertility (Mascarenhas *et al.*, 2012; Kumar & Singh, 2015; Mehta *et al.*, 2016). To date, male infertility diagnosis is mainly based on the results of standard semen analysis; however, nearly one-third of men with normal values in routine semen parameters are unable to achieve pregnancy revealing the limited value of this analysis as a diagnostic tool (Ray *et al.*, 2012; Pizzol *et al.*, 2014). Regarding treatment, several assisted reproduction techniques have been developed, and all of them require the selection of motile spermatozoa from semen samples. Among the selection procedures, the swim-up is the most used method because of its simplicity and low cost, but its performance is poor in samples with reduced motility (Henkel & Schill, 2003). Therefore, the identification of novel molecular entities involved in sperm function and the development of alternatives in sperm selection techniques are essential to improve current diagnosis and treatment of male infertility.

Fibroblast growth factors (FGFs), their receptors (FGFRs), and the related signaling are involved in a variety of cellular processes, having a role in the development and maintenance of normal tissues, but also in tumor progression (Turner & Grose, 2010; Belov & Mohammadi, 2013; Ornitz & Itoh, 2015). Fibroblast growth factor 2 (FGF2) is the best characterized member of the FGFs family (Itoh & Ornitz, 2011), and five human FGF2

isoforms (of 18, 22, 22.5, 24 and 34 kDa) have been described (Sorensen *et al.*, 2006). The 18-kDa (also known as low molecular weight, LMW) isoform is mainly localized in the cytoplasm and also is secreted and conjugated to heparan sulfate proteoglycans (HSPGs) of the extracellular matrix (Vlodavsky *et al.*, 1996). The other FGF2 protein forms, known as high molecular weight (HMW) isoforms, are mostly intracellular and with nuclear localization (Sorensen *et al.*, 2006; Yu *et al.*, 2007; Chlebova *et al.*, 2009).

The FGF ligands bind to specific tyrosine kinase receptors (FGFRs); among them, the most studied are FGFR1, FGFR2, FGFR3, and FGFR4 (Powers *et al.*, 2000). Interaction of FGFs with HSPGs allows their binding to FGFRs, phosphorylation of the receptors and the activation of several intracellular transduction cascades. In somatic cells, components of these cascades translocate into the nucleus and activate the expression of FGF-target genes (Eswarakumar *et al.*, 2005).

The presence of FGFs and FGFRs has been reported in both female and male reproductive tissues, where they contribute to regulate the reproductive function (Cotton *et al.*, 2008; Chaves *et al.*, 2012; Jiang *et al.*, 2013; Price, 2016). However, the expression and role of FGFs/FGFRs in the gametes have not been completely described. In a recent study, we have identified FGFR1, 2, 3 and 4 in human spermatozoa and reported their localization in the acrosomal region and flagellum (Saucedo *et al.*, 2015). Sperm FGFRs are functional, as exposure to recombinant FGF2 (rFGF2) led to an increase in the phosphorylation of flagellar FGFRs and to the activation of ERK and Akt signaling pathways. Moreover, incubation of unselected spermatozoa with rFGF2 resulted in a significant increase in the percentage of motile cells, as well as in sperm kinematics, suggesting that the FGF2/FGFRs system is involved in motility regulation (Saucedo *et al.*, 2015). The current work aimed to evaluate the presence and localization of FGF2 in human sperm cells, to determine FGF2 levels in semen samples and its relationship with conventional semen parameters, and to establish whether rFGF2 can be used to improve sperm recovery in semen samples subjected to the swim-up procedure.

MATERIALS AND METHODS

Samples used in the study were obtained under donors' or patients' written consent, and protocols were approved by the Ethics Committee 'Dr. Enrique Segura' from the Instituto de Biología y Medicina Experimental (IBYME, Buenos Aires, Argentina).

Reagents and antibodies

All reagents were of tissue culture grade and molecular biology quality and purchased from Sigma Chemical Co. (St. Louis, MO, USA), Bio-Rad (Hercules, CA, USA), GE-Amersham Pharmacia (Piscataway, NJ, USA) and Invitrogen-Life Technologies (Carlsbad, CA, USA), unless indicated.

Polyclonal anti-FGF2 (sc-79) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-acrosin developed in rabbit (Furlong *et al.*, 2000) were used. Other antibodies used were anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Sigma) and with Alexa Fluor® 488 (A-11008) (Life Technologies).

Recombinant FGF2 was produced in a bacterial expression system and gently provided by Dr. A. Baldi and Dr. A. Góngora (IBYME, Buenos Aires).

Semen samples and testicular tissue

Fresh semen samples were provided by normozoospermic volunteers or by patients attending the Instituto Valenciano de Infertilidad (IVI, Buenos Aires). Samples were obtained by masturbation and subjected to routine analysis following WHO guidelines (World Health Organization, 2010). Sperm and round cell concentrations were determined using a Neubauer hemocytometer. Total sperm number was calculated by multiplying the sperm concentration by the whole ejaculate volume. Sperm motility (progressive + non-progressive) was assessed, and total motile spermatozoa per ejaculate were calculated by multiplying total sperm number by sperm motility. All parameters were determined in at least 200 cells in duplicate slides. Only samples containing $<1 \times 10^6$ round cells/mL were included in the study.

Human testicular tissue was obtained from adult patients undergoing orchiectomy as treatment for prostatic carcinoma, and not receiving any hormonal treatment prior to surgery.

Sperm protein extraction and Western immunoblotting

Semen samples containing 30×10^6 sperm cells were processed as described (Marín-Briggiler *et al.*, 2010). Samples were subjected to SDS-PAGE in 15% polyacrylamide gels and Western immunoblotting using the anti-FGF2 antibody (2 μg/mL), or the primary antibody pre-incubated with fivefold (by weight) excess of blocking peptide (sc-79P, Santa Cruz Biotechnology Inc.) following the manufacturer's indications. The reactive bands were detected by enhanced chemiluminescence (ECL kit, Amersham) using standard procedures. Recombinant FGF2 was included as a control.

Immunocytochemistry

Spermatozoa were fixed, processed as described (Marín-Briggiler *et al.*, 2010), and stained overnight with anti-FGF2 (20 μg/mL). As controls, the primary antibody was omitted or non-immune rabbit IgG was used. Alexa Fluor® 488-conjugated anti-rabbit IgG (20 μg/mL) was used as secondary antibody, and nuclei were stained with propidium iodide. Slides were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired with a Nikon laser confocal microscope C1, using an objective PlanApo 60x/1.40 oil, excitation/emission: 488 nm/515–530 nm and 544 nm/570 LP.

To quantify the FGF2 levels in spermatozoa, single in-focus plane images were acquired. Using the IMAGEJ program (NIH, NY, USA), an outline was drawn around each cell, and mean fluorescence was measured. The total corrected cellular fluorescence (TCCF) was determined, as described (McCloy *et al.*, 2014).

Immunohistochemistry

Small portions of human testis were fixed and immunostained as previously described (Marín-Briggiler *et al.*, 2008), using the anti-FGF2 antibody (4 μg/mL) and the LSAB + System HRP kit (K0690, Dako, Carpinteria, CA, USA). Specimens were counterstained with hematoxylin, dehydrated and mounted. Sections were evaluated at ×600 magnification using an Alphaphot-2 YS2 microscope (Nikon, Tokyo, Japan).

Flow cytometry

Spermatozoa (2×10^6 per condition) were fixed with 2% paraformaldehyde in PBS for 30 min and incubated in blocking

solution (5% bovine fetal serum (BFS) in cytometry buffer (CB: PBS supplemented with 1% BFS and 0.1% sodium azide) for 30 min. Spermatozoa were incubated for 30 min with anti-FGF2 or anti-acrosin antibodies resuspended in CB. As a control, primary antibodies were omitted. After washing, Alexa Fluor® 488-conjugated anti-rabbit IgG (10 μg/mL) was added for 30 min, and samples were maintained in CB at 4 °C until assessment. Evaluation was performed in a flow cytometer (FACS CANTO 2, BD, Franklin Lakes, NJ, USA), with the FACS DIVA software (BD, Franklin Lakes, NJ, USA), which was calibrated every day following the manufacturer's instructions, and the analysis was performed on at least 10000 spermatozoa, identified by their forward scatter and side scatter characteristics. Results were analyzed with FLOWJO 7.6 software (Ashland, OR, USA) and expressed as mean fluorescence intensity (fluorescence intensity obtained with the first antibody - fluorescence intensity when the first antibody was omitted).

To evaluate rFGF2 binding to spermatozoa, they were incubated in the presence of rFGF2 (0, 10, or 100 ng/mL) for 30 min and then washed with PBS to remove the unbound protein. Samples were further fixed and treated with anti-FGF2 as detailed below. As a control, the primary antibody was omitted.

Swim-up procedure

To determine the effect of sperm incubation with rFGF2 upon the swim-up procedure yield, semen samples were split into three aliquots that were supplemented with 0, 10, or 100 ng/mL rFGF2 and incubated for 30 min at 37 °C, 5% CO_2 in air. To select the motile cells, 1 mL of Biggers–Whitten–Whittingham medium (BWW; World Health Organization, 2010), supplemented with 0.3% BSA and 0, 10, or 100 ng/mL rFGF2, was gently added over the semen aliquots and incubated for an additional hour (World Health Organization, 2010). The upper fraction containing the motile cells was recovered, and concentration and motility of the selected cells were determined. Total sperm recovery ([concentration x volume of the recovered aliquot] / [concentration x volume of the used semen aliquot]), as well as motile sperm recovery ([concentration x volume x motility of the recovered aliquot] / [concentration x volume x motility of the used semen aliquot]), was calculated.

To evaluate the motility parameters, sperm suspension was placed in a pre-warmed Makler chamber and recorded using a phase contrast Eclipse E 200 microscope (Nikon), coupled to a BASLER aca 780-75 gc videocamera (Basler AG, Ahrensburg, Germany) and a computer. Videos were acquired with the VIRTUALDUB 1.7.7 program (www.virtualdub.org), at a rate of 30 frames per second (fps), a gray scale of 8 bits and a resolution of 680 × 500 pixels. Sperm trajectories were analyzed with a CASA plugin for IMAGEJ (Wilson-Leedy & Ingermann, 2007). For each sample, at least four microscopic fields were analyzed and more than 200 motile spermatozoa were evaluated. The following kinematic parameters were measured: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN: VSL/VAP), wobble (WOB: VAP/VCL, a measure of sperm head side-to-side movement), progression (PROG, distance spermatozoa travelled on VAP path), and beat cross frequency (BCF, Hz).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The relationship between sperm FGF2 levels and seminal

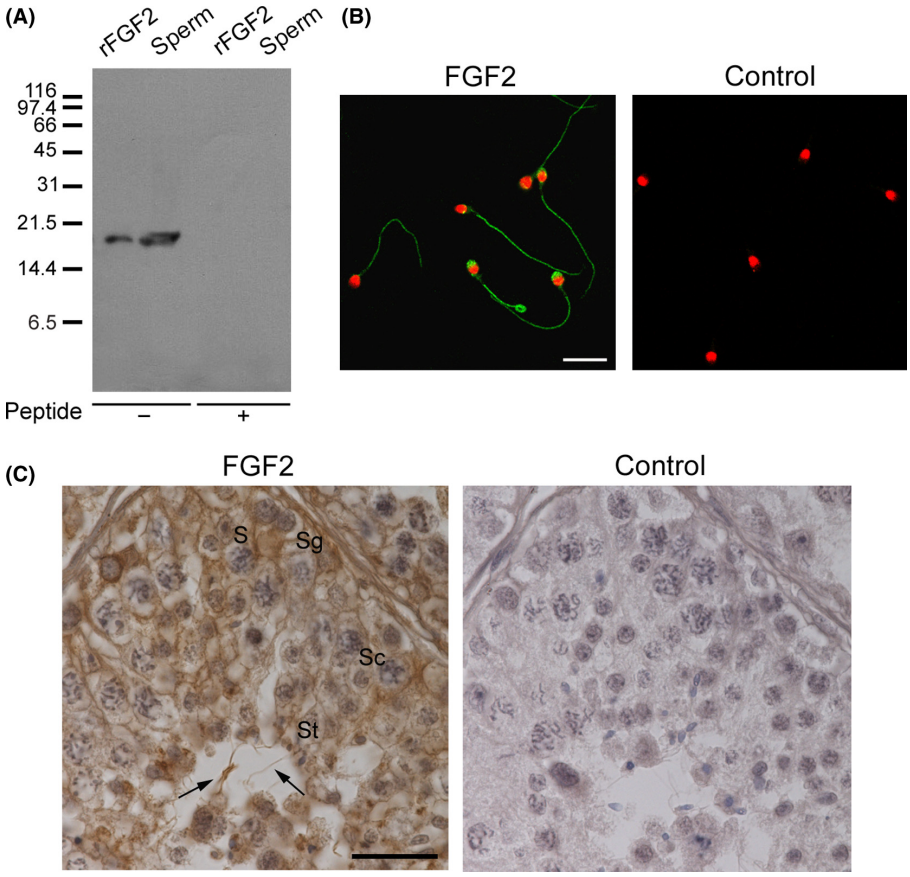

Figure 1 Presence and localization of FGF2 in human spermatozoa and in germ cells. (A) Detection of FGF2 using Western immunoblotting. Recombinant FGF2 protein (rFGF2, 1 ng) or protein extracts from human spermatozoa (30×10^6) were subjected to SDS-PAGE and Western immunoblotting using a polyclonal anti-FGF2 antibody. As a control, the primary antibody was pre-incubated with the blocking peptide (peptide +). Molecular weight markers are indicated on the left. (B) Localization of FGF2 in human spermatozoa evaluated by indirect immunofluorescence. Spermatozoa were stained with anti-FGF2 and a secondary antibody labeled with Alexa Fluor® 488. As a control, the primary antibody was omitted. Nuclei were stained with propidium iodide. Bar: 10 μm. (C) Immunohistochemical analysis of FGF2 in human testis using a polyclonal anti-FGF2 antibody. As a control, the primary antibody was omitted. The specimens were counterstained with hematoxylin. Images are shown at ×600 magnification. S: Sertoli cell, Sg: spermatogonia, Sc: spermatocyte, St: spermatid. The arrows indicate FGF2 immunoreactivity in the flagellum of elongating/elongated spermatids. Bar: 20 μm.

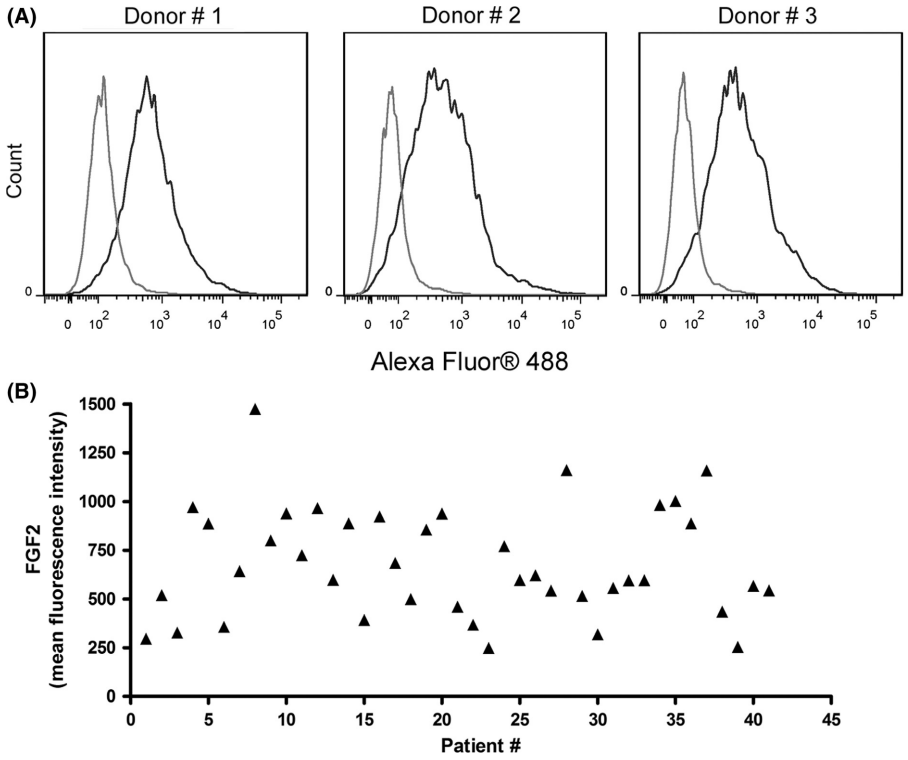

Figure 2 Quantification of sperm FGF2 by flow cytometry. (A) FGF2 levels in semen samples from three different normozoospermic donors. The histograms show the fluorescence signal for each sample with anti-FGF2 and a secondary antibody labeled with Alexa Fluor® 488 (black line) and in the control, omitting the anti-FGF2 antibody (gray line). (B) FGF2 mean fluorescence intensity obtained in spermatozoa from a group of patients. Each triangle represents a patient (n = 41).

parameters was evaluated by the Spearman correlation test. FGF2 and acrosin levels in samples with normal and abnormal motility, as well as TCCF values, were compared by Mann–Whitney U-test. The recovery yields and kinematic parameters of spermatozoa exposed to different concentrations of rFGF2 were compared by Friedman test and Dunn's multiple comparison test. Statistical analyses were performed using the GRAPHPAD INSTAT program (GraphPad Software, San Diego, CA, USA). A p value of <0.05 was considered statistically significant.

RESULTS

Presence and localization of FGF2 in human spermatozoa and germ cells

Presence of FGF2 in human spermatozoa was assessed by Western immunoblotting using a polyclonal anti-FGF2 antibody. An 18-kDa single band was observed in human sperm protein extracts that comigrated with rFGF2 used as control. Immunodetection was specific, as no signal was obtained when the primary antibody was pre-incubated with a blocking peptide (Fig. 1A).

The same antibody was used to localize FGF2 in the human male gamete and germ cells. FGF2 was detected along the flagellum (middle and principal pieces) of all the spermatozoa, and in the acrosomal region of 71 ± 10% of the cells (n = 3) (Fig. 1B). Moreover, the protein was immunolocalized on all germ cells of the seminiferous epithelium, including the flagellum of elongating/elongated spermatids (Fig. 1C). Immunoreactivity was also observed in Sertoli cells, tubular walls, and testicular microvessels.

Quantification of sperm FGF2 by flow cytometry and its relationship with semen parameters

To quantify the FGF2 levels present in human spermatozoa, flow cytometry was carried out using anti-FGF2, as a control, and the primary antibody was omitted. Figure 2A shows the histograms of FGF2 quantification in three donors; in these samples, a mean fluorescence intensity of 1048 ± 56 was obtained.

FGF2 levels were also determined in a group of patients attending an infertility clinic. This population comprised a total of 41 semen samples from different men (age: 39 ± 1 years, range 28–50); average semen parameters are listed in Table 1. They showed a mean FGF2 fluorescence intensity of 680 ± 44, with high variability among the samples (minimum: 248, maximum: 1475) (Fig. 2B). The relationship between FGF2 levels and conventional semen parameters was also analyzed, and a positive significant correlation was observed with sperm concentration (r = 0.468, p < 0.01), total sperm number per ejaculate (r = 0.349, p < 0.05), sperm motility (r = 0.498, p < 0.001), and total motile spermatozoa per ejaculate (r = 0.466, p < 0.01) (Fig. 3). No association was found with semen volume (r = 0.070), sperm vitality (r = 0.102), or morphology (r = 0.269) (not shown).

Relationship between sperm FGF2 levels and motility

To further analyze the relationship between sperm FGF2 and motility, FGF2 levels were compared in semen samples classified as with normal motility (NM, > 40% of motile spermatozoa) or abnormal motility (AM). The characteristics of these samples are depicted in Table 2. Spermatozoa from AM samples showed reduced (p < 0.01) FGF2 levels in comparison with normal ones (AM: 486 ± 42 vs. NM: 751 ± 53). Contrasting, similar levels of acrosin, a protein present in the acrosome, were observed in spermatozoa from these samples (AM: 658 ± 117 vs. NM: 600 ± 90) (Fig. 4A).

FGF2 levels, assessed both by flow cytometry and immunocytochemistry, were also compared in two individual cases: (i) a semen sample classified as NM (PM19, with 76% of motile cells)

Table 1 Standard semen parameters of samples used for FGF2 quantification by flow cytometry (n = 41)

	Mean ± SEM	Range
Volume (mL)	2.8 ± 0.2	1.0–6.0
Concentration (10^6/mL)	82.9 ± 10.1	9.5–280.0
Motility (%)	55.8 ± 2.9	29.0–90.0
Vitality (%)	84.0 ± 0.9	74.0–94.0
Normal morphology (%)	6.3 ± 0.8	1.0–14.0

and (ii) a semen sample classified as AM (LM108, with 33% of motile spermatozoa). While FGF2 mean fluorescence intensity for PM19 sample was 1475, LM108 depicted a mean fluorescence intensity of 520 (Fig. 4B). Moreover, quantification of FGF2 immunofluorescence signal in individual spermatozoa (assessed by IMAGEJ) in LM108 sample was significantly reduced ($p < 0.01$) in comparison with that of PM19 sample (TCCF: 37070 ± 3116 and 53845 ± 3892, respectively) (Fig. 4C).

Effect of sperm incubation with recombinant FGF2 on the recovery of motile spermatozoa in the swim-up procedure

To analyze if human spermatozoa are able to bind additional FGF2, cells were incubated for 30 min with rFGF2 (10 and 100 ng/mL) and assessed by flow cytometry using anti-FGF2. Results show an increase in the fluorescent signal in both conditions (Fig. 5A). Next, we aimed to determine whether rFGF2 could be used to improve sperm recovery in the swim-up procedure. Semen samples were incubated for 30 min in the presence of 0, 10, or 100 ng/mL rFGF2, subjected to the swim-up, and the yields and sperm kinematics of the recovered aliquots were compared (n = 9). Samples exposed to 10 ng/mL rFGF2 showed a significant increase ($p < 0.05$) in both total and motile sperm recoveries in comparison with those incubated with 0 and 100 ng/mL rFGF2 (Fig. 5B). Additionally, spermatozoa selected in the presence of 10 ng/mL rFGF2 depicted higher VCL, VSL, and LIN values ($p < 0.01$) than those corresponding to the other conditions (Table 3).

DISCUSSION

While the expression and function of the FGFs/FGFRs system in somatic cells have been extensively evaluated, there is scarce evidence of its presence and role in the gametes. Results from our study have shown the presence of an 18-kDa FGF2 form in human sperm protein extracts. This isoform would correspond to the LMW form described in other cells, which is mainly secreted and exerts an autocrine and paracrine activity by binding to HSPGs and FGFRs (Sorensen *et al.*, 2006; Yu *et al.*, 2007; Chlebova *et al.*, 2009). The current study also showed FGF2 localization in the flagellum (middle and principal pieces) of all

Figure 3 Relationship between sperm FGF2 levels and semen parameters. Correlation analysis between FGF2 levels (assessed by flow cytometry) and sperm concentration, total sperm number, sperm motility, and total motile spermatozoa. n = 41 samples. Sperm concentration: r = 0.468, $p < 0.01$; total sperm number: r = 0.349, $p < 0.05$; sperm motility: r = 0.498, $p < 0.001$; total motile spermatozoa per ejaculate: r = 0.466, $p < 0.01$.

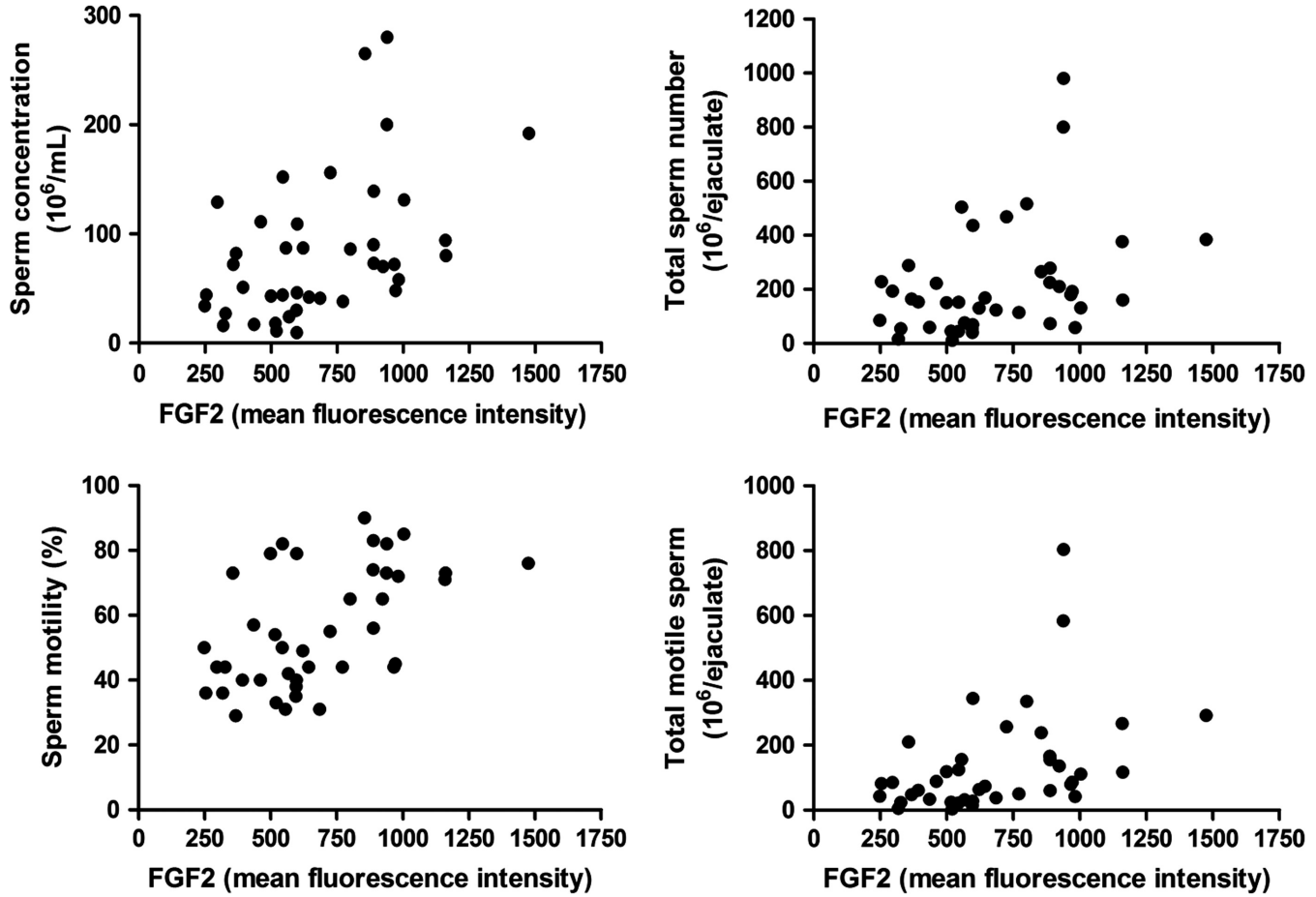

Table 2 Standard semen parameters of samples with normal motility (NM) and abnormal motility (AM)

	Mean ± SEM	Range
NM (n = 30)		
Volume (mL)	2.9 ± 0.2	1.0–6.0
Concentration (10^6/mL)	95.7 ± 12.6	17.0–280.0
Motility (%)	63.3 ± 2.8	42.0–90.0
Vitality (%)	84.2 ± 1.0	74.0–93.0
Normal morphology (%)	6.3 ± 0.6	1.0–14.0
AM (n = 11)		
Volume (mL)	2.6 ± 0.4	1.0–5.8
Concentration (10^6/mL)	48.1 ± 9.9	9.5–111.0
Motility (%)	35.4 ± 1.2	29.0–40.0
Vitality (%)	83.6 ± 2.3	75.0–94.0
Normal morphology (%)	4.6 ± 0.7	2.0–8.0

the ejaculated human spermatozoa and in the acrosomal region of more than 70% of the cells. Such result is consistent with the detection of FGFs in sperm cells (Sugihara *et al.*, 2013) and with our findings of FGFR1, 2, 3 and 4 localization in the human male gamete (Saucedo *et al.*, 2015). Interestingly, HSPGs have also been described in the human spermatozoa (Ceballos *et al.*, 2009), so their role mediating FGFs and FGFRs interaction can be postulated.

Immunohistochemical analysis of testicular sections revealed FGF2 expression in all germ cells, including elongated/elongating spermatids, with a similar localization to that found for the FGFRs (Saucedo *et al.*, 2015). The presence of FGF2 and FGFRs in the human seminiferous epithelium has been previously reported, but particularly FGF2 has been immunodetected only in spermatogonia (Steger *et al.*, 1998). The discrepancy with our findings would be attributed to the antibodies used, or to other methodological differences.

In the present study, sperm FGF2 levels were quantified by flow cytometry, a highly sensitive and objective technique that has been recently used to measure other sperm proteins (Tamburrino *et al.*, 2015; Gianzo *et al.*, 2016). The analysis performed

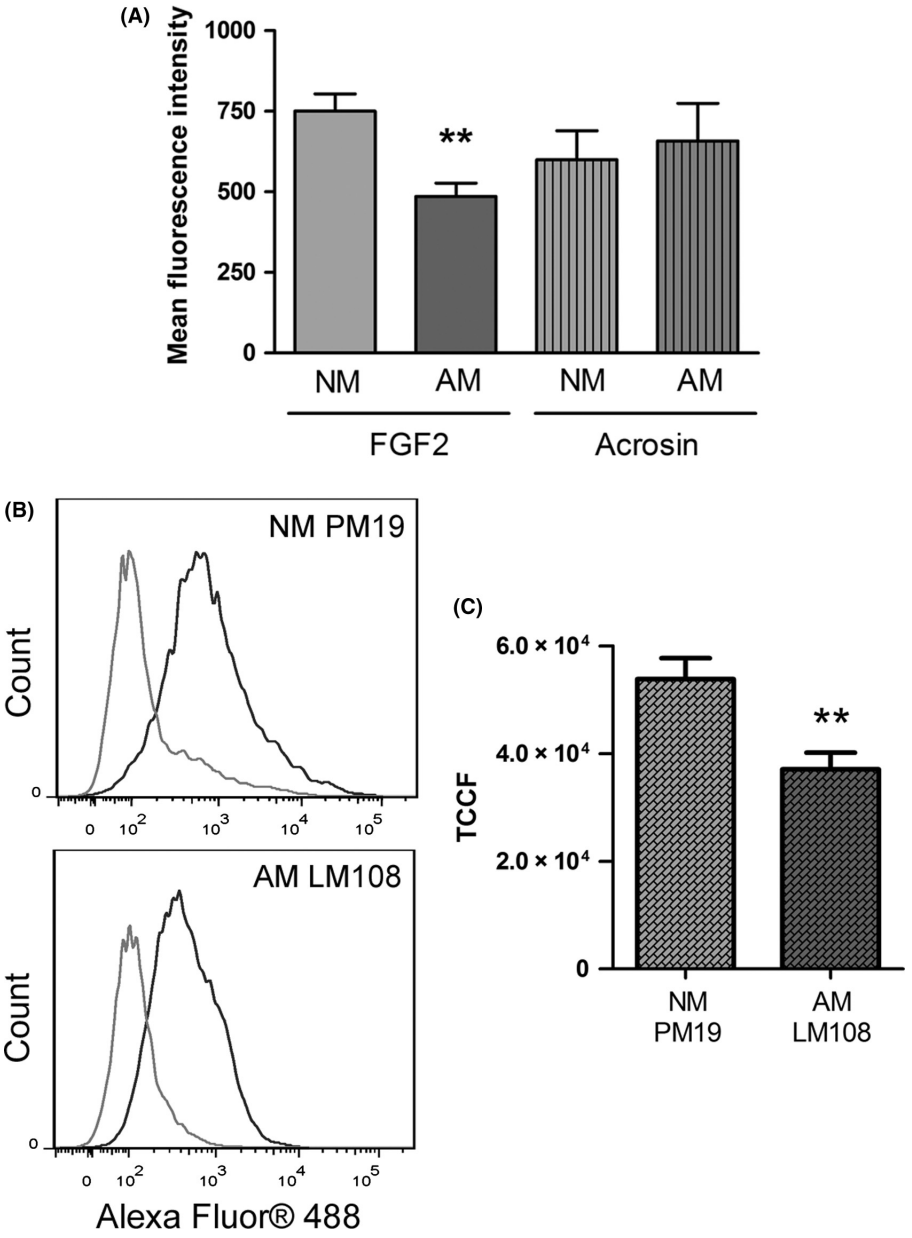

Figure 4 FGF2 levels in human spermatozoa and its relationship with motility. (A) Comparison of FGF2 and acrosin levels in semen samples with normal and abnormal motility (NM and AM, respectively). FGF2 and acrosin levels were determined by flow cytometry, using specific antibodies. n = 30 samples for NM, and n = 11 for AM. Results are expressed as mean ± SEM; $^{**}p < 0.01$ vs. NM for FGF2. (B) FGF2 mean fluorescence intensity obtained for PM19, a patient with normal motility (NM), and LM108, a patient with abnormal motility (AM). The histograms show the fluorescence signal for the samples with anti-FGF2 and a secondary antibody labeled with Alexa Fluor® 488 (black line) and in the control, omitting the anti-FGF2 antibody (gray line). (C) Total corrected cellular fluorescence (TCCF) for spermatozoa from PM19 (NM) and LM108 (AM) samples. Results are expressed as mean ± SEM; $^{**}p < 0.01$. $n > 50$ spermatozoa.

on semen samples from patients attending an infertility clinic showed a great variability in FGF2 levels among different individuals. Correlation studies indicated a relationship between sperm FGF2 levels and sperm concentration, as well as total sperm number per ejaculate. These results suggest a biological role for the FGF2/FGFRs system in the regulation of human spermatogenesis, as described for some animal species (Cotton *et al.*, 2008; Jiang *et al.*, 2013). In particular, FGFs and their receptors have been involved in maintaining undifferentiated spermatogonia (Hasegawa & Saga, 2014), and in regulating male germ cells apoptosis and survival (Xu *et al.*, 2016).

Our findings also revealed a significant positive correlation between sperm FGF2 levels and the percentage of motile cells, and total motile spermatozoa per ejaculate, indicating the involvement of the FGF2/FGFRs system in the regulation of human sperm motion. Moreover, FGF2 levels in semen samples with abnormal sperm motility were significantly reduced in comparison with those of normal motility, suggesting that FGF2 could be an indicator of sperm quality. Based on these results and on our previous observations of the beneficial effect of rFGF2 on human sperm motility (Saucedo *et al.*, 2015), in this study we also determined whether rFGF2 can be used to improve the swim-up yield. Results showed that incubation with 10 ng/mL rFGF2 led to an increase in both total and motile sperm recoveries, and in sperm VCL, VSL and LIN values in comparison with 0 or 100 ng/mL rFGF2. The biphasic effect in sperm response to rFGF2 is in agreement with previous observations in somatic cells (Kanodia *et al.*, 2014). These authors propose that at low/intermediate FGF2 concentrations, there are sufficient free FGFRs available for the formation of the FGF2-HSPG-FGFR signaling unit, and that at high FGF2 concentrations, FGFR binding sites become saturated, inhibiting the complex formation. Such biphasic effect was not observed in our previous study, in which spermatozoa were incubated for 2 h with rFGF2, and higher percentages of motile cells and kinematic parameters were obtained with 100 ng/mL rFGF2 in comparison with 10 ng/mL rFGF2 (Saucedo *et al.*, 2015). Differences between the results of both reports may reside in the characteristics of the sperm populations exposed to the recombinant protein (washed unselected samples vs. spermatozoa subjected to the swim-up). Moreover, in the present study, rFGF2 was added to semen samples and the influence of seminal plasma components cannot be ruled out. The underlying mechanisms by which FGFs regulate sperm motility warrant further investigation.

In conclusion, this study documented the presence of FGF2 in the ejaculated spermatozoa. Evidence on FGF2 expression in germ cells leads us to suggest the testicular origin of sperm FGF2, but additional FGF2 may bind or be incorporated to spermatozoa during maturation or after contact with the seminal plasma. In vivo, FGF2 would also bind to ejaculated spermatozoa during their transit through the female reproductive tract, as FGFs have been described in the uterus, oviduct, oocyte and cumulus cells (Fujimoto *et al.*, 1996; Gabler *et al.*, 1997; Malamitsi-Puchner *et al.*, 2001; Möller *et al.*, 2001). Binding of additional FGF2 would depend on the exposure of FGFRs or on the availability of HSPGs on the sperm surface. Moreover, our findings about the relationship between FGF2 levels and sperm concentration and motility suggest the relevance of the FGF2/FGFRs system in human spermatogenesis and in the regulation of sperm motion. In-depth knowledge of the entities involved in the maintenance of sperm physiology will help establishing new

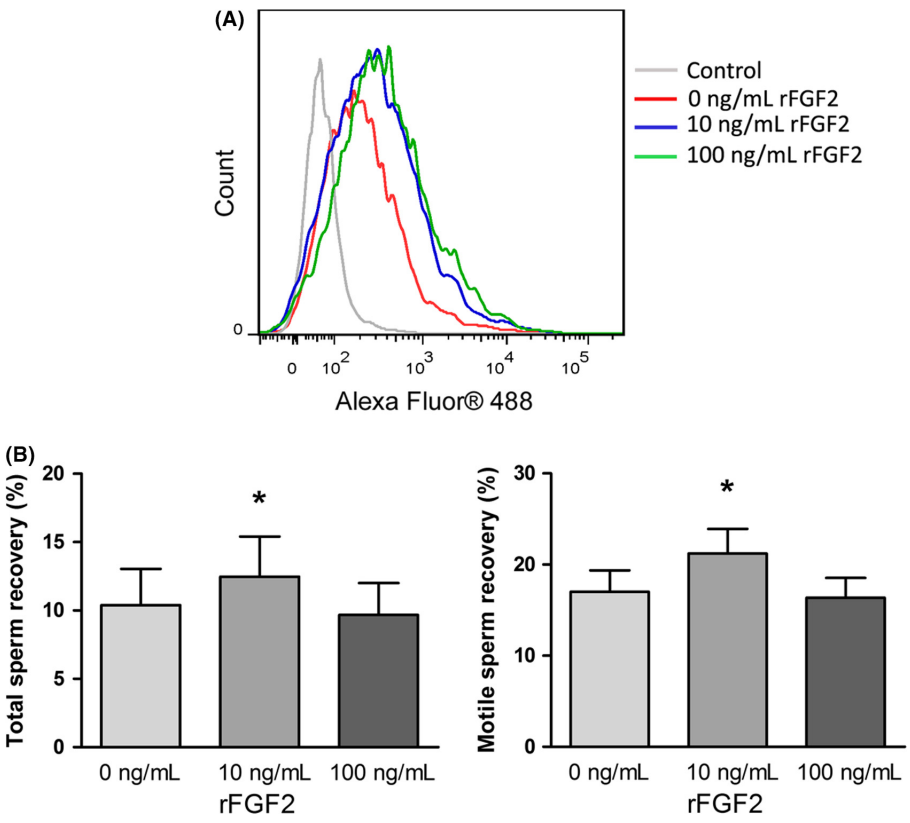

Figure 5 Effect of recombinant FGF2 (rFGF2) on sperm recovery in a selection procedure. (A) Binding of recombinant FGF2 (rFGF2) to the human spermatozoa. Cells were exposed for 30 min to 0, 10, or 100 ng/mL rFGF2 and analyzed by flow cytometry using anti-FGF2 antibody and a secondary antibody labeled with Alexa Fluor® 488. The control in which the first antibody was omitted is shown. The experiment was performed three times with different samples; a typical result is shown. (B) Effect of recombinant FGF2 (rFGF2) on the recovery in the swim-up technique. Spermatozoa were incubated for 30 min in the presence of 0, 10, or 100 ng/mL rFGF2 and subjected to the swim-up. Total sperm and motile sperm recoveries in each condition were calculated. Results are expressed as mean ± SEM, n = 9. *p < 0.05 vs. other conditions.

Table 3 Kinematic parameters of human spermatozoa subjected to the swim-up procedure in the presence of rFGF2

	0 ng/mL rFGF2	10 ng/mL rFGF2	100 ng/mL rFGF2
VCL (μm/sec)	90 ± 12	96 ± 12**	91 ± 12
VSL (μm/sec)	34 ± 4	39 ± 5**	35 ± 5
VAP (μm/sec)	45 ± 3	47 ± 4	47 ± 4
LIN (VSL/VAP)	0.7 ± 0.1	0.8 ± 0.1*	0.7 ± 0.1
WOB (VAP/VCL)	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
PROG (VSL/VAP)	8.3 ± 0.4	8.4 ± 0.3	9.1 ± 1.1
BCF (Hz)	13 ± 1	13 ± 2	13 ± 2

Computer-assisted sperm analysis of spermatozoa incubated with rFGF2. Kinematic parameters measured were as follows: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), wobble (WOB), progression (PROG), beat cross frequency (BCF). Results are expressed as mean ± SEM, $n = 9$. *$p < 0.01$, **$p < 0.001$ compared with 0 ng/mL rFGF2.

sperm biomarkers of male fertility potential. Results obtained in the present study can also be relevant for the clinical practice, as rFGF2 would be used to improve the quantity and quality of motile spermatozoa, especially in samples that need to be subjected to low-yield selection procedures and/or to low complexity assisted reproduction techniques.

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CONFLICT OF INTEREST

All authors declare no conflict of interests.

AUTHORS' CONTRIBUTIONS

C.I.M.B. and M.H.V.L. conceived and designed the study. C.I.M.B. prepared the manuscript, and M.H.V.L. contributed to its writing and revision. D.J.G.A. and L.S. performed the experiments. D.J.G.A., L.S., C.I.M.B. and M.H.V.L. analyzed the data. S.G., M.L.M., M.G.B. and F.N. contributed reagents/materials/analysis tools.

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