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Relationship between Contents of Lipocalin-Type Prostaglandin D Synthase on the Surface of Infertility Sperm and in Seminal Plasma

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Abstract—Lipocalin-type prostaglandin D synthase (L-PGDS) is localized in Leydig cells, sperm, and epithelial cells of the epididymis. The present study was to determine the correlation between content of this enzyme in seminal plasma and on the surface of sperm. We analyzed 90 semen samples. L-PGDS in seminal plasma was analyzed by an ELISA procedure. L-PGDS on sperm was analyzed by flow cytometry. The semen donors were categorized in three groups: normal, oligospermic, and azoospermic. According to results obtained, L-PGDS may have the ability to improve progressive motility of sperm, and L-PGDS in seminal plasma and on sperm surface may impact male fertility in the female reproductive tract.

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Lipocalin-type prostaglandin D synthase (L-PGDS) was first identified in rat brain as an enzyme that catalyzes the conversion to prostaglandin D2 (PGD_2) from prostaglandin H2 (PGH_2), the latter being a common precursor of all prostanoids [1]. Later, L-PGDS was found to be identical to β-trace [2, 3], which had been identified earlier as a major protein in human cerebrospinal fluid (CSF). PGD_2 is a major prostanoid in the brain, known as the most potent endogenous somnogenic substance so far identified, and is involved in various physiological events such as regulation of sleep and pain responses [4, 5]. Because human L-PGDS-overexpressing transgenic mice and L-PGDS-gene knockout mice showed abnormality in the regulation of non-rapid eye movement (NREM) sleep and pain responses, PGD_2 produced by L-PGDS is thought to play an important role in the regulation of NREM sleep and pain sensation in the central nervous system. Alternatively, L-PGDS binds small lipophilic molecules such as retinal and retinoic acid (K_d = 70-80 nM) [6] and biliverdin and bilirubin (K_d = 33-37 nM) [7] with affinities higher than those of other members of the lipocalin family. Therefore, L-PGDS is unique bifunctional protein acting as a PGD_2-producing enzyme and as a lipophilic molecule-binding protein.

The origin of L-PGDS in the male genital tract has not been unequivocally identified. In the human body, it seems to be secreted mostly by Sertoli cells [8] even if immunohistochemical studies show that it is also localized in Leydig cells, sperm [9], and epithelial cells of the epididymis [10, 11]. In other species, the distribution of L-PGDS is different: (i) the epididymis of the mouse [12], bull [13], stallion [14], and rat; (ii) in the Leydig cells of the mouse [15, 16] but not in other species; (iii) in the germ cells of the bull and rat [17].

This work has two main objectives: quantification on human sperm of L-PGDS and determination of correlations between L-PGDS on sperm and L-PGDS in seminal plasma.

Abbreviations: BSA) bovine serum albumin; CASA) computer assisted systemic analysis; CSF) cerebrospinal fluid; HRP) horseradish peroxidase; L-PGDS) lipocalin-type prostaglandin D synthase; NREM) non-rapid eye movement; PGD_2) prostaglandin D2; PGH_2) prostaglandin H2.

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MATERIALS AND METHODS

Materials. Ni-NTA was from Amersham Pharmacia (USA); goat anti-mouse IgG-conjugated with horseradish peroxidase (HRP) and Percoll were from Sigma

(USA); fluorescein isothiocyanate (FITC)-labeled affinity-purified goat anti-mouse IgG was from Kirkegaard & Perry Laboratories (USA); recombinant human L-PGDS and anti-human L-PGDS MAb were from Biolabs (China).

Clinical samples. Ninety semen samples were obtained from the diagnostic semen laboratories at Jinling Hospital, Nanjing, China. Provided with the samples were the results of computer assisted systemic analysis (CASA) including sperm density, percentage of motility, and ejaculate value. Based on sperm density, the semen samples were divided into three clinical groups. The normal group (normozoospermia) composed of samples with a total cell concentration greater than $20 \cdot 10^6$/ml. The azoospermic group consisted of samples with sperm density of zero. The oligospermic group consisted of the remaining samples with sperm characteristics between those defined for the normal and azoospermic groups. The classification criteria were adopted according to the standard criteria of the World Health Organization.

Preparation for anti-human L-PGDS MAb. The coding region of the *L-PGDS* gene was amplified by PCR and inserted into the plasmid pPIC9 and transformed into *Pichia pastoris* GS115 cells. Methanol was used to induce the expression of the His-tag protein. The recombinant human L-PGDS was purified with Ni-NTA as described previously [18]. BALB/c mice were each given an injection of 100 μg of recombinant human L-PGDS in Freund's complete adjuvant. The same dose was repeated five weeks later in Freund's incomplete adjuvant. Splenocytes of the immunized mice, which has the highest antibody titer to recombinant human L-PGDS as determined by ELISA, were fused with mouse hybridomas cell line SP2/0-Ag14 in the presence of 1 ml of polyethylene glycol 4000. After being cloned three times, antibodies 1A4 and 4D7 were obtained. Antibody-producing hybridomas were identified by ELISA as described below. The native protein was purified as reported by Tokugawa [11]. Microtiter plates were coated for 2 h at 37°C and then overnight at 4°C with either native L-PGDS, recombinant human L-PGDS, or plasminogen at 5 μg/ml in 100 μl of 0.05 M sodium carbonate/bicarbonate buffer, pH 9.5. Plates were first washed with 0.1 M phosphate-buffered saline (PBS) containing 0.05% Tween-20 and quenched with 10% (v/v) bovine serum albumin (BSA) (Sigma) in the washing solution, and then incubated at 37°C for 1 h with 100 μl of tissue culture supernatant. After that, the plates were washed 5 times with 5% BSA in washing solution. After washing, 100 μl of diluted solution of goat anti-mouse IgG conjugated with HRP was added to each well and incubated at 37°C for 1 h. The wells were washed and color was developed by adding 100 μl of substrate (*o*-phenylenediamine dihydrochloride and hydrogen peroxide). After 10 min at room temperature, the enzyme reaction was stopped by adding 100 μl of 2 M H_2SO_4, and the absorbance of each well was read at 495 nm. Two hybridomas, 1A4 and 4D7, secreting antibodies that reacted with native L-PGDS and recombinant human L-PGDS (absorbance > 0.5) but not with plasminogen (absorbance < 0.15), were selected as specific MAbs.

Preparation of spermatozoa and seminal plasma. Fresh human semen was obtained from donors and was left 30-40 min at room temperature. We centrifuged the material (800*g* for 10 min) to harvest sperm. The supernatant was used to prepare ELISA. The pellet was suspended in 30 mM Tris containing 130 mM NaCl. The sperm were purified by layering on 70% Percoll and centrifuging at 7000*g* for 10 min. The pellet was then washed twice with 30 mM Tris containing 130 mM NaCl. The final pellet was suspended in the same buffer and immediately used for further procedures.

Sandwich ELISA. ELISA was performed as previously described [19].

Flow cytometry. For flow cytometric analysis, the sperms were washed and incubated with MAb 1A4 for 45 min at 37°C. Cells were then washed and incubated with FITC-labeled affinity-purified goat anti-mouse IgG for 30 min at 37°C. After incubation, the cells were washed twice with PBS (pH 7.2) and suspended in PBS. The suspensions were then filtered through a nylon mesh. Negative controls were sperm incubated with only the conjugate, and these cells were used to set the acquisition

Description of the variables in seminal plasma

Variable	Patients (*n*)	Mean (SD)
L-PGDS in seminal plasma (μg/ml)*		
azoospermic	14	1.3 (0.9)
oligospermic	38	1.8 (1.4)
normal	38	9.7 (3.5)
L-PGDS on sperm (fluorescence intensity units)*		
azoospermic	14	–
oligospermic	38	64.7 (65.3)
normal	38	256.5 (100.8)
Sperm density (10^6/ml)*		
azoospermic	14	0
oligospermic	38	10.0 (5.1)
normal	38	46.6 (27.3)
Progressive motility (%)**		
azoospermic	14	–
oligospermic	38	22.4 (19.7)
normal	38	27.9 (15.5)

* $P < 0.01$, ** $P < 0.05$. The significance of the differences between groups of the test population was evaluated by the Kruskal–Wallis test for non-normally distributed parameters and by the analysis of variance (ANOVA) test for normally distributed values.

parameters and were then analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, USA). For data acquisition, the flow cytometer was set as follows: detector threshold (FSC-H) – 32; FSC – E00; SSC – 368; parameters: FSC – 5.35 (linear) and SSC-H – 5.55 (linear) and FL1-H 588 (logarithmic scale).

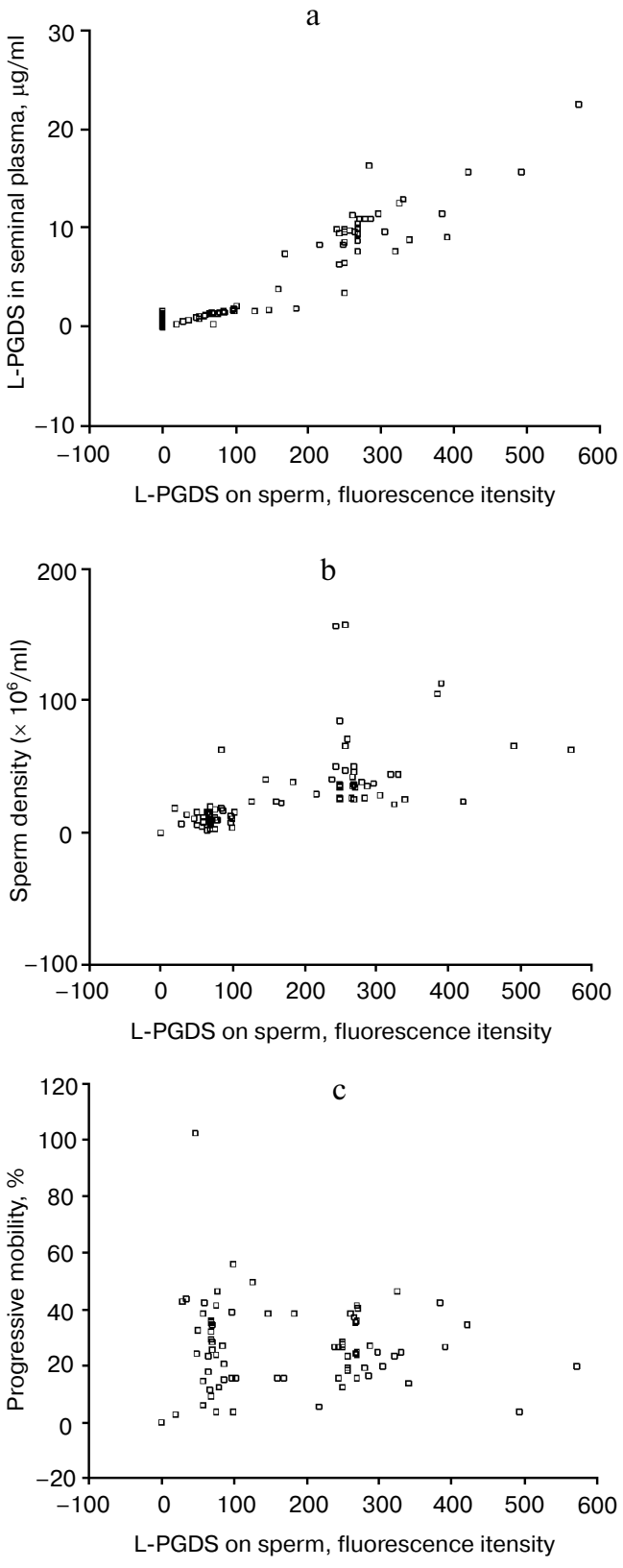
Statistical analysis. The data were evaluated using the Statistics Package for Social Sciences (SPSS) 11.0 software for Windows (SPSS Inc., USA). The Kolmogorov–Smirnov test was done to see how well it fits the normal or non-normal distribution of values. The significance of the differences between groups of the test population was evaluated, respectively, by the Kruskal–Wallis test for non-normally distributed parameters and by the analysis of variance (ANOVA) test for normally distributed values. To explore statistically the relationship between ranked data, Spearman's coefficient of correlation was calculated, and mean values and standard deviations were also obtained as measured results.

RESULTS AND DISCUSSION

The components of human seminal plasma may profoundly impact male fertility in the female reproductive tract. In our initial studies we analyzed the concentration of L-PGDS in human seminal plasma of normal, obstructive, and non-obstructive azoospermia infertility patients, and found that it reduced progressively from normal group to non-obstructive and to obstructive azoospermia patients, and the differences in L-PGDS concentration among the three groups were very significant. In this study, we measured the concentration of L-PGDS in human seminal plasma of normal group, oligospermic, and azoospermic infertility patients.

Quantification on human sperm of L-PGDS. The table reports the quantification of L-PGDS on human sperm using flow cytometry. The quantification of L-PGDS on human sperm was described by the mean of fluorescence intensity. When the samples were divided into normal group, azoospermic group, and oligospermic group according to the standard criteria (World Health Organization, 1999), the normal range of L-PGDS on sperm (mean ± SD) in the samples with normal parameter values was 256.5 ± 100.8 units of fluorescence intensity, and the determination in patients with oligospermic revealed mean values of 64.7 ± 65.3 units of fluorescence intensity. It reduced progressively from normal to oligospermic and to azoospermic patients. Significant differences were found among the three groups.

Correlations between L-PGDS on sperm and L-PGDS concentration in seminal plasma. Analysis of variance (ANOVA) was performed on the means of the three groups for each variable. The results are listed in the table. The Spearman correlation coefficients were also calculat-



Correlation between content of L-PGDS on sperm and L-PGDS concentration in seminal plasma (r = 0.925, $^*P < 0.01$) (a), sperm density (r = 0.700, $^*P < 0.01$) (b), and progressive motility (%) (r = 0.316, $^{**}P < 0.05$) (c)

ed to examine correlations between the parameters. We found that the correlations were significant at the level of $P < 0.01$. L-PGDS on sperm was positively associated with both L-PGDS concentration in seminal plasma ($r = 0.925$, * $P < 0.01$), progressive motility ($r = 0.316$, ** $P < 0.05$), and sperm density ($r = 0.700$, * $P < 0.01$). The identified correlations between L-PGDS concentration, sperm density, percentage of motility, and L-PGDS on sperm are shown in the figure. According to the results presented, the concentration of L-PGDS reduced progressively from normal group to oligospermic and azoospermic infertility patients. These data were consistent with our previous study [19], and it was further confirmed that the components of seminal plasma may therefore profoundly impact male fertility in the female reproductive tract [20].

The sequential sperm transformations that occur in the anterior part of the epididymis induce changes in sperm activity that become fully expressed in the corpus of the epididymis, e.g. increase in the percentage of motile sperm or in the ability of sperm to agglutinate or to bind eggs. In the posterior region, the new glycopeptides on the sperm surface appeared simultaneously with the occurrence of forward progressive motility of the gametes [21]. In our previous study and in this study, we found that whether in seminal plasma or on sperm surface, L-PGDS was associated with progressive motility. From these results, we propose tentatively that L-PGDS is a main protein to improve progressive motility of sperm.

Most investigators agree that sperm surface proteins mediate the sperm–egg interactions by binding with high affinity and specificity to the glycoconjugate receptor(s) present on egg *zona pellucida*. The evidence for the presence of several binding proteins on the surface of mouse sperm supports the argument that more than one receptor is involved and that the recognition and binding of gametes are perhaps regulated by the sum of all molecular interactions. Whether L-PGDS is involved in mediating the sperm–egg interactions is unclear, and further studies are warranted to understand the mechanisms of all the interacting molecules on the opposite gametes and the structural basis for these interactions.

In conclusion, a significant reduction of the L-PGDS on sperm was observed in severe oligozoospermic patients compared to normozoospermic subjects, and a significant correlation between L-PGDS on sperm and L-PGDS in seminal plasma and sperm density was found. Further studies are warranted to evaluate the possible diagnostic and pharmacological applications of these observations.

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