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RESEARCH ARTICLE

Quantification studies in human seminal plasma samples identify prolactin inducible protein as a plausible marker of azoospermia

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Abstract

Background: Prolactin inducible protein (PIP) is a ~17 kDa protein, which is known to play vital roles in immunoregulation, fertility, antimicrobial activity, apoptosis and tumour progression.

Objectives: This study reports quantification of PIP concentration in human seminal plasma (SP) samples.

Methodology: PIP was purified by immunoprecipitation and its concentration in human SP samples was quantified by ELISA method.

Results: Average concentration of PIP in normozoospermia, oligozoospermia and azoospermia was 290.3 ± 71.5 µg/mL, 306.4 ± 71.2 µg/mL and 60.5 ± 23.6 µg/mL respectively.

Conclusion: There was no significant variation in PIP levels in normozoospermia and oligozoospermia while its expression was down-regulated in azoospermia, indicating that PIP may be a plausible marker of azoospermia.

Keywords: ELISA, immunoprecipitation, infertility, normozoospermia, oligozoospermia

Introduction

Prolactin inducible protein (PIP) is a ~17 kDa glycoprotein present in various human body fluids. In seminal fluid, it is secreted from prostate gland, seminal vesicles or testis (Autiero et al. 1997; Yamakawa et al. 2007). Alternative names of PIP are gross cystic disease fluid protein 15, secretory actin-binding protein and gp17 (Hassan et al. 2009). It is known to play vital roles in immunoregulation, antimicrobial activity, apoptosis and tumour progression. PIP interacts with many proteins, such as fibrinogen, actin, keratin, myosin, tropomyosin (Schenkels et al. 1994), immunoglobulin G (Chiu & Chamley, 2003), zinc-α-2 glycoprotein (Hassan et al. 2008) and human serum albumin (Kumar et al. 2012). Due to large protein–protein interactome, various roles of PIP have been suggested in diverse biological processes including male fertility/infertility; however exact physiological function remains unclear to date.

The role of PIP in cancer proliferation is extensively studied. The mitogenic activity assays on breast-cancer cell lines suggested active role of PIP in tumor proliferation (Mazoujian et al. 1983; Cassoni et al. 1995). Its expression analysis in breast and prostate cancers identified it as a marker of these subtypes of cancer (Hassan et al. 2009). It had been found in various studies that PIP is differentially expressed in infertile and fertile sperm/seminal plasma (SP). But most of these studies were performed at preliminary stage and detailed relative expression quantification was not performed. Yamakawa et al. (2007) did comparative 2D-PAGE analysis of normozoospermic and azoospermic SP samples. They found that PIP levels were decreased/absent in azoospermia and suggested it as a candidate marker for the same. In another study, Martínez-Heredia et al. (2008) detected PIP with a lower amount in the asthenozoospermic samples when compared with the sperm donor control samples. In

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a recent study, Davalieva et al. (2012) also found that PIP was differentially expressed in azoospermia in comparison to oligozoospermia.

In our previous study, we isolated PIP as one of the Concanavalin A binding glycoproteins of human SP and found that PIP in this fraction is differentially expressed (down regulated) in infertility patients (oligozoospermia and azoospermia) in comparison to fertile subjects (normozoospermia) (Tomar et al. 2011). In this paper, we are reporting purification of PIP from human SP by immunoprecipitation, identification by MALDI-TOF/MS and quantification of its concentration in human SP samples from different conditions, including normozoospermia, oligozoospermia and azoospermia using Human PIP-ELISA kit.

Methodology

Experimental procedure is summarized in Figure 1.

Samples

Semen collection

Human semen samples were collected from Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India after a written consent form signed by the patients. This study was also approved by Institutional Ethics Committee. The World Health Organization (WHO) 2010 recommendations for semen analysis were followed and samples were categorized as normozoospermic (sperm count >15 million/mL, sperm motility > 40% & normal spermatozoa morphology), oligozoospermic (sperm count < 15 million/mL, sperm motility > 40% & normal spermatozoa morphology) or azoospermic (no spermatozoa).

Inclusion and exclusion criteria

The samples, included in this study, were selected using the following criteria – age of semen donors – from 20 to 40 years, normozoospermia (sperm count >20 million/mL & sperm motility > 60%), oligozoospermia (sperm count <10 million/mL & sperm motility >60%) and azoospermia (no sperm, non-obstructive). In case of oligozoospermia, samples with sperm count below the lower reference limit and all other parameters above WHO 2010 reference limit were used. Samples of related conditions, such as oligoasthenozoospermia, oligoasthenoteratozoospermia or oligoteratozoospermia were discarded. Further, the HIV positive samples and/or those contaminated with blood were not included in this study. For sample details, see Table 1.

Sample preparation

The samples were centrifuged at 5000*g* for 20 min at 4°C to remove spermatozoa (pellets) from SP (supernatant). SP was further centrifuged at 10,000*g* for 20 min. Supernatants obtained were pooled and stored at –20°C for isolation of PIP. For quantification of PIP concentration, semen (n = 50 for each type of sample) from individual donors was collected, centrifuged and SP (supernatant) was stored separately.

Purification and identification of PIP

Immunoprecipitation

PIP from SP was purified by immunoprecipitation using human anti-PIP primary antibody. To pre-clear the sample, it was incubated with Protein A-Agarose beads for 30 min. at 4°C. It was centrifuged at 10,000*g* for 5 min, pellet was discarded and supernatant was kept for immunoprecipitation. Pre-cleared SP was

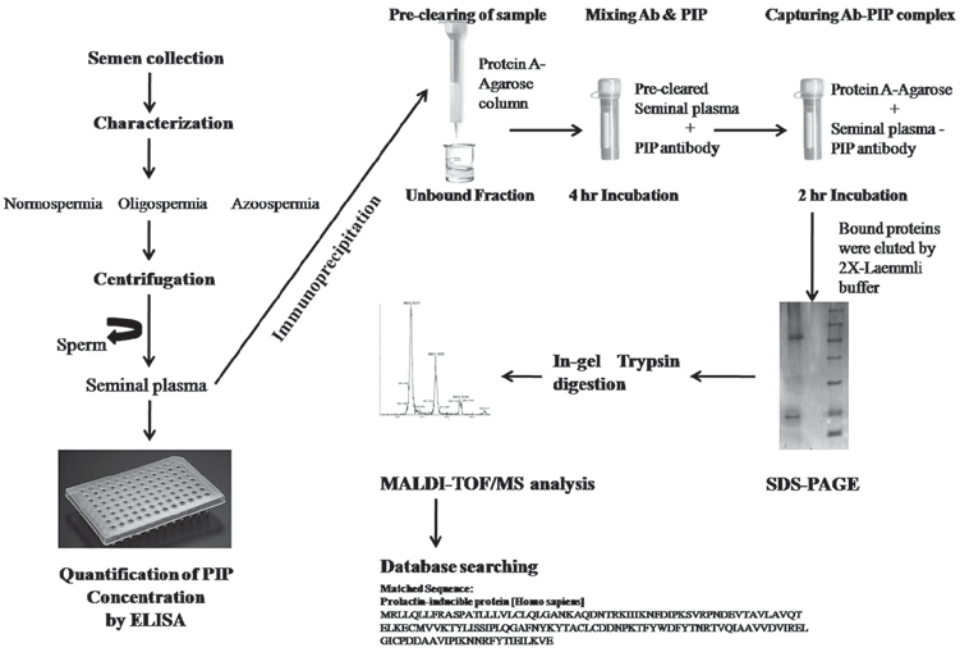


Figure 1. Methodology.

diluted with immunoprecipitation lysis buffer (IP buffer, containing 25 mM Tris-HCl pH-7.5, 150 mM NaCl, 2 mM DTT, 1%Triton X-100, 2 mM EDTA, 0.2% SDS & cocktail of protease inhibitors) to a final protein concentration of ~2 µg/µL. In a centrifuge tube, 5 µg anti-PIP primary antibody (Santa Cruz Biotechnology Inc. California, USA) was added to 250 µL protein sample and incubated at 4°C for 4 h on a rotating device. Then, 40 µL Protein A-Agarose beads were added to it and incubated at 4°C for 2 h. The mixture was centrifuged at 2000*g* for 2 min. and supernatant was discarded. The pellet was washed thrice with IP buffer, resuspended in 30 µL 2X Laemmli sample buffer for 30 min. and boiled for about 5 min. Finally, it was centrifuged at 2000*g* for 2 min and supernatant was collected carefully so that beads did not mix with it.

Gel electrophoresis

Supernatant, collected in last step of immunoprecipitation experiment, was acetone precipitated and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel under reducing conditions as previously described (Laemmli 1970). Protein bands on gel were visualized by Coomassie Brilliant Blue G-250 staining.

Mass spectrometry

A protein band on SDS-PAGE corresponding to ~17 kDa was manually excised from gel. To remove the dye, it was gently washed with Acetonitrile (ACN): H_2O (50%, v/v) containing 25 mM Ammonium bicarbonate. The in-gel Trypsin digestion was carried out as previously described (Alam et al. 2010). The reconstituted in-gel digested sample (2 µL) was mixed with 1 mL of matrix solution in 50% ACN & 0.1% TFA and spotted on to a MALDI target plate. MALDI-TOF/MS analysis was performed using Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Peptides from mass spectra were matched against protein databases, NCBInr and MSDB using Mascot search engine (Matrix Sciences, London, UK) for peptide-mass-fingerprinting.

Quantification of PIP concentration

PIP concentration in human SP samples from normozoospermia, oligozoospermia and azoospermia (n = 24 for each) was quantified by human PIP Elisa kit (Cusabio Inc., China) as per manufacturer's instructions. Briefly, 50 µL of standards and SP from various conditions, i.e. normozoospermia, oligozoospermia and azoospermia were added to the wells pre-coated with anti-PIP antibodies to capture available PIP in solution. 50 µL of HRP-Conjugate was added to each well, mixed well and incubated for 1 h at 37°C. Each well was washed three times with 200 µL wash buffer. Then, 50 µL of Substrate A and 50 µL of Substrate B was added to each well and incubated for 15 min at 37°C. Finally, 50 µL stop solution was added to each well and optical density of each well was read at 450 nm using a micro plate reader. Additionally, we also pooled 26 SP samples from each above mentioned conditions separately and quantified PIP concentration in those pooled samples using the same method.

Table 1. Sample details*.

Semen	Age of donor (years)	Sperm count (million/mL)	Motility (%)
Normozoospermia	29.8	54.6	80.6
Oligozoospermia	32.2	5.0	78.3
Azoospermia	30.7	–	–

*All values are average of 50 semen samples.

Statistical analysis

Statistical assessment was carried out using Microsoft Office Excel 2007. A standard curve was constructed by plotting the mean absorbance (optical density) of standards on the x-axis against their concentration on the y-axis and a best fit curve was drawn through the points of the graph. This curve was used to calculate concentration of PIP in various SP samples. Paired t test was performed to identify statistically significant variation in PIP concentration among different types of samples. p value <0.01 was considered statistically significant. Graphs were also generated using this program only.

Results

Isolation of PIP

PIP was purified by immunoprecipitation using anti-Human PIP primary antibodies. Purification was analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining (Figure 2). Three bands corresponding to

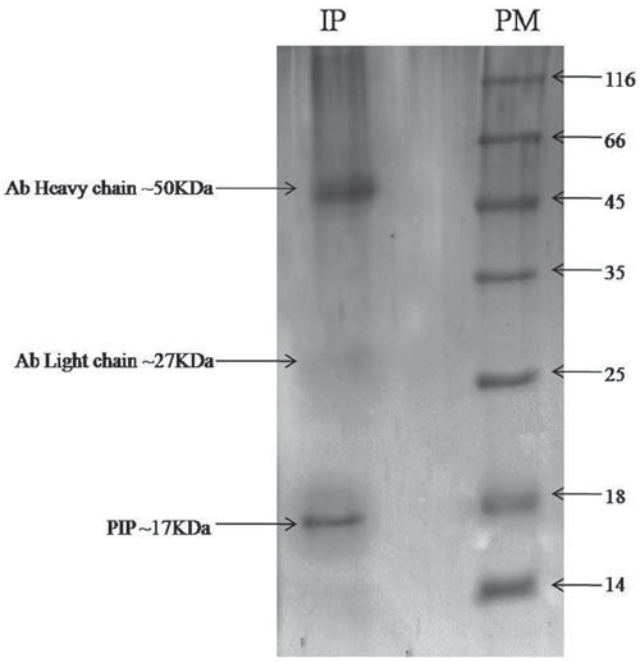


Figure 2. 12% SDS-PAGE profile of immunoprecipitation of prolactin inducible protein (PIP). Lane PM: Molecular weight markers; Lane IP: Immunoprecipitation elution, antibody-PIP complex captured by Protein A-Agarose beads; Ab, Antibody.

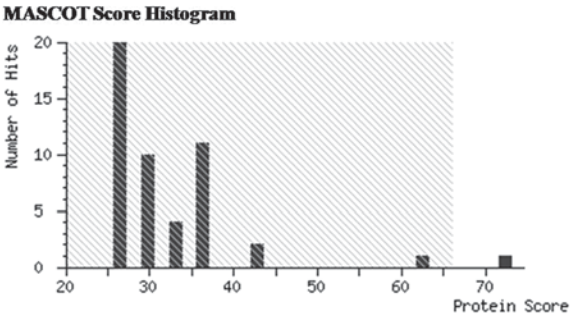
approximate molecular weights of approximate 50, 27 and 17 KDa were visible on gel. First two bands represent heavy and light chains of antibody. The ~17 KDa protein band was excised from gel, in-gel digested by Trypsin and identified as PIP by MALDI-TOF/MS analysis and database searching (Figure 3).

Quantification of PIP concentration

To quantify PIP concentration, 50 semen samples were collected from each group. PIP concentration in 24 samples was quantified individually while remaining 26 samples were pooled before quantification step. PIP concentration in different sample pools is summarized in Figure 4A & 4B. In brief, PIP levels in normozoospermia and oligozoospermia displayed no significant variation (p value = 0.48, paired t test) and average values were 290.3 ± 71.5 µg/mL and 306.4 ± 71.2 µg/mL respectively. In azoospermia, PIP expression was found to be down-regulated in comparison to normozoospermia (differences were highly statistically significant as p value <<0.0001, paired t test) and average concentration was 60.5 ± 23.6 µg/mL. In pooled samples, PIP concentration in normozoospermia, oligozoospermia and azoospermia was 267.70 µg/mL, 270.38 µg/mL and 53.55 µg/mL respectively.

Discussion

Among ~20% infertile couples worldwide, male factors account for 40–50% infertility cases due to low sperm count, poor sperm quality or both. The recent advances in this field focus on identification of differentially expressed proteins in sperm/SP and their functional aspects for better understanding of the related biological pathways towards discovering new biomarkers of infertility. SP is considered as a potential source of biomarkers for many disorders of the male reproductive system (Yamakawa et al. 2007; Tomar et al. 2012) as SP proteins play critical roles in sperm maturation and are essentially required for sperm function mainly for their interactions with the various environments of the tubular genital tract and the oocyte (Rodríguez-Martínez et al., 2011). Some of them may even influence the relative intrinsic fertility of the male and may be sometimes the sole reason of infertility in a large population of infertile couples. Few preliminary but very vital studies had been performed to identify differentially expressed proteome of SP among fertile and infertile subjects (Yamakawa et al. 2007; Bai et al. 2007; Wang et al. 2009; Bai et al. 2010). In most of these studies, PIP was identified as one of the key protein of SP associated with male reproductive disorders, i.e. different conditions of spermatogenic impairment when compared to normal samples. Its expression was found altered in fertile and infertile SP; however, exact levels were never quantified. The differences were either identified by 2D-PAGE profiling, mass spectrometry or intensity of western blot bands. This is the first study in which we quantified the amount of PIP present



MASCOT Search Results

gi|4505821 Mass: 16847 Calculated pI: 8.26

Score: 71 Expect: 0.019 Matches: 5 Protein sequence coverage: 55%

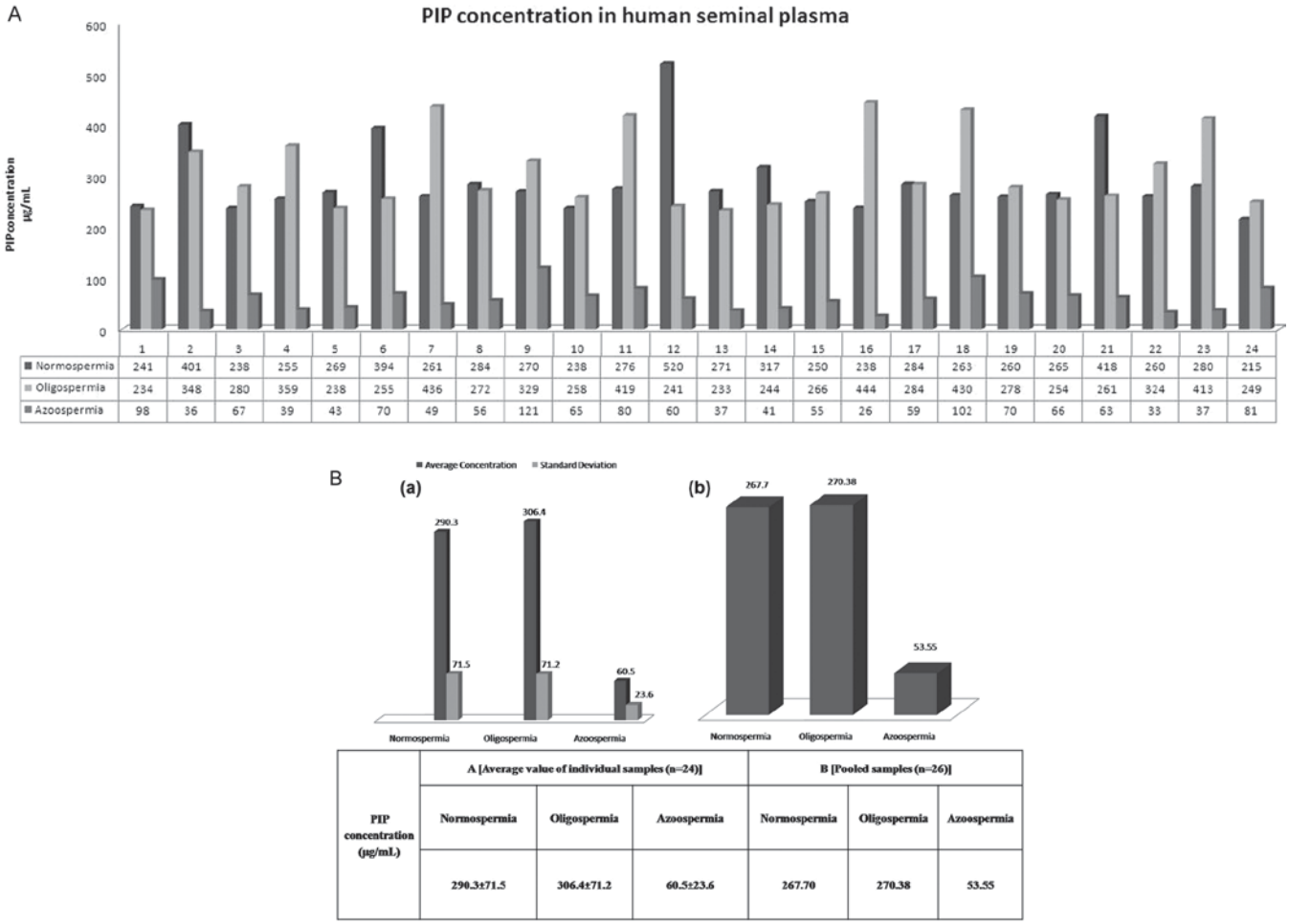
Prolactin-inducible protein precursor [Homo sapiens]

Observed	Mr (expt)	Mr (calc)	Start	End	Peptide
1254.65	1253.64	1253.69	137	146	R.FYTIEILKVE.-
1283.75	1282.74	1282.76	107	118	R.TVQIAAVVDVIR.E
1814.89	1813.89	1813.96	70	85	K.TYLISSIPLQGAFNYK.Y
1994.94	1993.93	1994.02	119	136	R.ELGICPDDAAVIPIKNNR.F
2815.19	2814.19	2814.46	45	69	K.SVRPNDEVTAVLAVQTELKECMVVK.T

Matched peptides are shown in bold.

1	MRLLQLLFRA	SPATLLLVLC	LQLGANKAQD	NTRKIIIKNF	DIPKSVRPND
51	EVTAVLAVQT	ELKECMVVKT	YLISSIPLQG	AFNYKYTACL	CDDNPKTFYW
101	DFYTNRTVQI	AAVVDVIREL	GICPDDAAVI	PIKNNRFYTI	EILKVE

Figure 3. MASCOT search result. Prolactin inducible protein was identified by MASCOT database searching of peptides identified by MALDI-TOF/MS analysis.



PIP concentration (µg/mL)	A [Average value of individual samples (n=24)]			B [Pooled samples (n=26)]		
	Normospermia	Oligospermia	Azoospermia	Normospermia	Oligospermia	Azoospermia
	290.3±71.5	306.4±71.2	60.5±23.6	267.70	270.38	53.55

Figure 4. (A) PIP concentration (µg/mL) in normozoospermic, oligozoospermic and azoospermic human seminal plasma samples. Total number of samples from each category (n) = 24. PIP concentration in each sample is adjusted to nearest Integer. (B) Average PIP concentration (µg/mL) a. Average PIP concentration in individual samples and standard deviation (n = 24 for each category). b. PIP concentration in pooled samples (n = 26 for each category).

in human SP in three states – normozoospermia, oligozoospermia & non-obstructive azoospermia (NOA) by ELISA method and compared the differences.

Oligozoospermia is one of the major causes of male sub-fertility worldwide, a condition in which semen contains less number of sperms than the reference value of WHO 2010 semen analysis manual (<15 million sperm/mL). Excluding sperm count, all other semen parameters are same as reference values for normozoospermia. Azoospermia is characterized by absence of sperm in the ejaculate and is one of the most severe forms of male infertility which is diagnosed in 5–20% infertile men (Kolettis, 2002). It may be categorized as pre-testicular azoospermia, NOA (due to testicular failure) and obstructive azoospermia (OA, due to congenital bilateral absence or blockage of the vas deferens or epididymis). In general, detailed patient evaluation is performed to differentiate semen samples into OA and NOA. The initial evaluation includes complete medical history of patient, physical examination and measurement of various hormone levels. If needed, testis biopsy is also performed for the final confirmation. Usually OA can be treated and reversed with microsurgery. Contrary to this, NOA is non-treatable in most of the cases. It is mainly caused by impairment of spermatogenesis and is very critical due to idiopathic nature in most cases. In order to improve the treatment efficacy we essentially need to identify precise factors at molecular level which may provide better insight of the processes related to impairment of spermatogenesis, leading to NOA. Thus, we believe that identification of proteins which are differentially expressed in NOA in comparison to normozoospermia may be vital in developing better therapeutic solutions.

Two datasets were prepared for PIP concentration quantification in 150 (50×3) human SP samples. In first set, 24 samples from each condition were taken separately and in another setup, 26 samples from each condition were pooled. PIP levels in normozoospermia and oligozoospermia showed no significant differences and average concentration was 290.3±71.5 µg/mL and 306.4±71.2 µg/mL respectively, indicating that there is no correlation between sperm numbers and PIP levels. While in comparison to these, PIP expression

was highly down regulated in NOA with average concentration 60.5 ± 23.6 μg/mL, hinting that PIP might be playing an important role in spermatogenesis. Similar PIP levels were reported in pooled SP samples too, with 267.70 μg/mL, 270.38 μg/mL and 53.55 μg/mL values in normozoospermia, oligozoospermia and NOA respectively. Reduced expression levels of PIP in azoospermia have been reported in various studies and are in good agreement with our quantification results (Yamakawa et al. 2007; Davalieva et al. 2012).

Previously, we isolated Concanavalin A binding proteins of human seminal plasma and identified ten of them by MALDI-TOF/MS analysis, including PIP. We also performed preliminary studies to examine the differential expression profile of this fraction in oligozoospermia and azoospermia in comparison to normozoospermia and observed that PIP was down regulated in both conditions. To further investigate and evaluate these differences, we quantified PIP levels in human SP samples and analyzed that PIP expression was down regulated in azoospermia; however, we surprisingly found that PIP levels were not altered in oligozoospermia. The possible reason might be that PIP has various molecular forms in SP (Akiyama & Kimura 1990) and its glycosylated form was down regulated in oligozoospermia but total PIP was not. Furthermore, equivalent PIP expression in normozoospermia and oligozoospermia may be due to normal spermatogenesis in both cases and supporting our view that PIP plays a critical role in spermatogenesis and reduced levels are indicative of impaired spermatogenesis in case of NOA. Any obstruction to reproductive tract may add to reduction of overall PIP in semen and in spite of normal spermatogenesis, there are high chances of reduced levels of PIP in OA too; however possibility of reduction of PIP levels in OA is more likely to be dependent on the type of reproductive tract obstruction and the levels may differ from those in NOA.

In summary, we immunoprecipitated PIP from human SP and quantified its concentration in normozoospermic, oligozoospermic and azoospermic SP samples. Results of this study highlight the statistically significant down-regulated expression of PIP in azoospermia when compared to normozoospermia and oligozoospermia. There appears no correlation between sperm number and PIP levels in SP; however, reduced levels of PIP may be considered as one of the critical factors causing mutilation of spermatogenesis. Thus, this particular study is of vast significance in reproductive physiology towards better understanding of male fertility/infertility.

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Declaration of interest

The author reports no conflicts of interest.

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