Research Article

HUMAN SERUM ALBUMIN AND PROLACTIN INDUCIBLE PROTEIN COMPLEX ENHANCES SPERM CAPACITATION *IN VITRO*

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Abstract: Prolactin inducible protein (PIP) is a 17 kDa protein expressed in human body fluids. It interacts with several other proteins including fibrinogen, IgG, actin, CD4, ZAG, etc. and their association contributes to multifaceted molecular functions of PIP in diverse classes of biological processes. The roles of PIP are reported in immunoregulation, tumor progression, apoptosis and fertility. Recently, PIP was purified in complex with human serum albumin (HSA) from human semen. Here, we have reported comparative expression analysis of this complex in human seminal plasma samples of various categories of infertility conditions (oligozoospermia, azoospermia and asthenozoospermia) and fertile controls (normozoospermia). We have also evaluated sperm capacitation and acrosome reaction in presence of varying concentrations of this complex. Comparative expression analysis was performed under same experimental conditions by co-immunoprecipitation followed by western blot analysis and results highlighted that HSA-PIP complex is down-regulated in azoospermia as depicted by the intensity of protein bands on the blot. Assessment of sperm functions in presence or absence of this complex revealed that this complex acts as an inducer of *in vitro* sperm capacitation. It was observed that 87% sperm were capacitated after 4h incubation with HSA-PIP complex (100 μg/mL) in comparison to 60.33% in +ve control. On the basis of our findings, we conclude that this complex might be a positive regulator of sperm motility and capacitation *in vivo*.

Keywords: Acrosome reaction; capacitation; co-immunoprecipitation; HSA-PIP complex; western blotting.

Introduction

The work of several researchers across the world is integrated to define the process of fertilization that involves several pathways and associated biological processes. Reproduction is essential for survival of any species, but an interruption of any related process may result in fertilization failure. Routine semen tests are performed to screen fertility status of males in infertile couples. These tests are usually based on semen quality, such as liquefaction time, volume, pH and viscosity and sperm parameters, such as motility, count and morphology. However, these parameters fail to explain male inability in almost half of the cases. With all the parameters falling in normal range, there is no definite way to explain the reasons of infertility/sub-fertility in idiopathic cases (Kovac *et al.,* 2013).

Semen minus spermatozoa is called seminal plasma (SP), which is a mixed secretion originating from multiple accessory sex glands of the male reproductive system. It provides safe surrounding and nutrition to spermatozoa and helps in their maturation (Mann, 1978). SP is capable of protecting spermatozoa from acidic

environment in the vagina due to its high buffering capability (Pilch and Mann, 2006). In order to make a human sperm capable of fertilizing oocyte, it must undergo a series of essential modifications, collectively termed as capacitation, which prepares it for acrosome reaction (AR) (Yanagimachi, 1994). The sperm functions, including motility, sperm capacitation and AR are influenced by various components of SP including proteins (Burden *et al.*, 2006). SP is a quite rich source of proteins and contains more than 2000 proteins, including semenogelins, fibronectin, prostate specific antigen, albumin, lactoferrin, prostatic acid phosphatase, β-microseminoprotein, Prolactin inducible protein (PIP) and immunoglobulins (Batruch *et al.*, 2011).

Protein–protein interactions are vital as they are known to play significant roles, essentially required for functional organization of the cells. Their identification and characterization is crucial as molecular functions and biological activities of proteins are usually modulated by other protein(s) they interact with (Shah and Rao, 2016). PIP is a 17 kDa protein expressed abundantly in body fluids, including semen and saliva. It interacts with several other proteins including fibrinogen, immunoglobulin G, actin, CD4, zinc-alpha-2 glycoprotein (ZAG), etc. and their association contributes to multifaceted molecular functions of PIP in diverse classes of biological processes, specifically immunoregulation, tumor progression, apoptosis and fertility (Hassan *et al.*, 2009). PIP has also been reported to play a protective role for its binding partner ZAG against various denaturants (Hassan *et al.*, 2012). Recently, Kumar *et al.* (2012) have reported purification of native HSA-PIP complex from human seminal plasma and its biochemical characterization. The aim of this study was to perform comparative expression analysis of HSA-PIP complex in infertile SP samples and fertile controls and to evaluate its effect on sperm functions – capacitation and acrosome reaction.

Methodology

Expression analysis

Comparative expression analysis of HSA-PIP complex in human SP samples was performed by co-immunoprecipitation (Co-IP) followed by western blotting. Four types of SP samples were used, including oligozoospermia, azoospermia and asthenozoospermia (infertility conditions) and normozoospermia (fertile controls). Experiment set up is summarized in Figure 1.

Sample collection and processing

Human semen samples were collected from Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi (INDIA). Freshly ejaculated semen was left at room temperature for 30 minutes for complete liquefaction. Then, semen parameters such as volume, sperm concentration and sperm motility were evaluated. Samples were divided into four categories - normozoospermia, oligozoospermia, azoospermia and asthenozoospermia (according to WHO 2010 guidelines) and stored separately. The samples were centrifuged (5,000g, 20 min, 4 °C) to remove spermatozoa (pellet). SP was again centrifuged (10,000g, 20 min., 4 °C) to remove cell debris and other impurities. The supernatant obtained was collected and used for this study.

Co-immunoprecipitation

Co-IP experiment was performed as previously described (Tomar *et al.*, 2013). To pre-clear the samples from each condition, samples were incubated with Protein A-Agarose (Bio Basic Inc., Canada) beads for 1hr at 4°C, centrifuged at 10,000 g for 2 min. and supernatants were carefully separated from pellet. For each set up, sample was diluted to a final protein concentration of ~4 µg/µL with Co-IP buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 2mM DTT, 1%Triton X-100, 2mM EDTA, cocktail of protease inhibitors). In a centrifuge tube, 10µg PIP primary antibodies (Santa Cruz Biotechnology Inc. California, USA) were added to 500µL diluted protein sample and incubated at 4°C for 4 hrs on a rotating device. 50µL Protein A-Agarose beads were added to it and incubated at 4°C for 2 hrs. The mixture was centrifuged (2,000g, 4°C, 2 min.). The supernatant was discarded carefully and pellet was washed three times. After removal of complete buffer, beads were resuspended in 40µL Laemmli sample buffer (2X) for 30 min. at RT followed by boiling

at 95°C for about 5 min. The supernatant was collected after centrifugation at 10,000g for 5 min. In negative controls, samples were directly added to Protein A-Agarose beads without any PIP antibodies.

Electrophoresis and western blotting

Co-immunoprecipitated proteins were separated by 12% SDS-PAGE under reducing conditions (Laemmli, 1970) and transferred to nitrocellulose membrane for 2hr at 100V. After blocking the membrane with 5% (w/v) nonfat dry milk in phosphate-buffered saline (PBS) for 1 hr, HSA primary antibodies (1:2000) were added and incubated overnight at 4°C. After several washes with PBS containing 0.1% tween 20, appropriate HRP conjugated secondary antibodies (1:5000) were added and incubated at RT for 1hr. Blot was washed thrice and HRP activity was detected by DAB (3, 3'-Diaminobenzidine) substrate.

Evaluation of sperm capacitation in presence of HSA-PIP complex

HSA-PIP complex from human SP was purified as previously described by Kumar *et al.* (2012)

using various chromatographic methods. Assessment of sperm capacitation and acrosome reaction in presence of HSA-PIP complex was done by method proposed by De Jonge and Barratt (2013) with some modifications.

Sample collection and processing

Semen samples were collected from five healthy and fertile donors. After liquefaction at RT for about 30 minutes, semen was centrifuged at 1000 rpm to isolate spermatozoa. Sperm pellet was washed twice with bicarbonate-buffered culture medium (Ham's F-10 Nutrient Mix, Thermo Fisher Scientific), which was pre-warmed and equilibrated to 37°C in a 5% CO_2 incubator.

Sperm Capacitation

Control and experimental tubes were prepared, each containing approximately 2×10^6 motile spermatozoa in 1 ml of culture medium. In control tubes, spermatozoa were incubated in alone Ham's F-10 medium (-ve control) or with heparin, a capacitation inducing agent (+ve control). To four experimental tubes, HSA-PIP complex in varying concentrations was added (10 µg/mL,
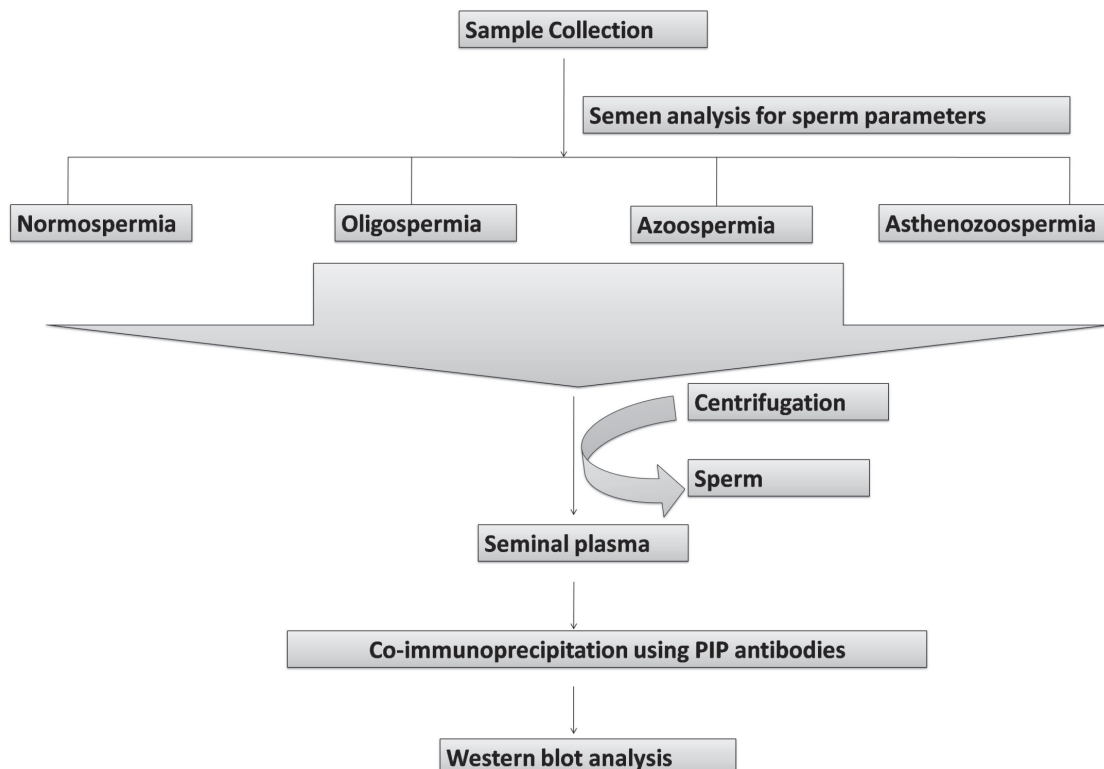

Figure 1: **Experimental setup for expression analysis of HSA-PIP complex**

100μg/mL, 500μg/mL, and 1mg/mL). After 4 hr of incubation, 60 µg/mL lysophosphatidylecholine (LPC) was added to each tube and again incubated for 15 min in a 5%CO_2 incubator at 37°C. Capacitation was estimated indirectly by the percentage of acrosome reacted spermatozoa as LPC is a fusogenic agent that induces AR only in capacitated spermatozoa. Spermatozoa were then stained by fluorescein isothiocyanate pea nut agglutinine (FITC-PNA) staining. About 1.5 cm long smears were prepared using 20µL aliquots from each control and experimental tubes. Fresh smears were checked for even cell distribution using phase-contrast microscope. All the smears were air dried and fixed in 95% (v/v) ethanol for 30 min. An antifading agent, 1, 4-Diazobicyclo-2,2,2-octane (DABCO) was added to smear before putting cover slip over it. Finally smears were analyzed under fluorescence microscope. Spermatozoa were categorized for acrosomal status as the following: (i) Acrosome intact: bright and uniform fluorescence is present in more than half of the sperm head, (ii) Acrosome-reacted: fluorescence localized to the equatorial segment or no fluorescence. Two hundred spermatozoa were analyzed per slide and classified as acrosome reacted or acrosome intact. The process was repeated, values were averaged and percentage of acrosome intact and acrosome reacted spermatozoa was calculated.

Results and Discussion

For differential expression analysis of naturally occurring HSA-PIP complex in seminal plasma of various infertile conditions in comparison to normal fertile seminal plasma, Co-IP experiments by human PIP primary antibodies followed by western blot analysis using human HSA primary antibodies were performed. Co-IP by HSA primary antibodies was not performed due to the fact that albumin is a high abundant protein and could have hindered the immune-capturing of complex resulting in false expression results. Comparative expression analysis was performed under same experimental conditions and different samples containing equal amount of total protein were used to capture HSA-PIP complex. The strength of HSA band on the blot was used to compare the probable expression of complex in different categories. We did not observe any band on the blot in negative controls. In comparison to normozoospermia, expression of HSA-PIP complex was reduced in azoospermia while no significant variation was observed in oligozoospermia and asthenozoospermia (Figure 2). The reduced expression in azoospermia is suggestive that formation of this complex might be important for sperm physiology and related processes.

The assessment of capacitation and acrosome reaction highlighted that HSA-PIP complex influences sperm capacitation as depicted from higher rate of acrosome reaction in comparison to that in case of positive control (Figure 3). Sperm capacitation was measured indirectly from percentage of acrosome reaction as LPC induces AR only in capacitated spermatozoa. We observed a dose dependent effect on AR. In the beginning of experiment (time 0h), acrosome intact spermatozoa were 88% and they reduced to 73.67% after 4 hr incubation due to spontaneous capacitation (26.33% acrosome reacted spermatozoa) in absence of any inducer. While in positive control set, acrosome intact spermatozoa reduced to 39.67% and acrosome reacted spermatozoa raised to 60.33%. In presence of HSA-PIP complex, spermatozoa showed an increased chance to undergo capacitation/ acrosome reaction and this positive effect on sperm function was dose dependent. At a low concentration (10µg/mL) of this complex, effect is comparable to positive control. The number of acrosome reacted spermatozoa in presence of HSA-PIP complex was 60% in comparison to 60.33% in control. The optimum rate of AR (87%) was observed in presence of 100 µg/mL HSA-PIP complex. The rate of AR showed a decreasing trend with further increase in HSA-PIP complex concentration, 76.33% at 500 µg/mL and 57.33% at 1mg/mL.

It is well known that cholesterol acceptors, including albumin facilitate cholesterol efflux from sperm and promote capacitation (Go and Wolf, 1985; Langlais *et al.*, 1988; Furimsky *et al.*, 2005). HSA improves the recovery of high quality spermatozoa because of its powerful anti-oxidant properties and prevents oxidative stress-induced damage; thus, considered useful in processing of
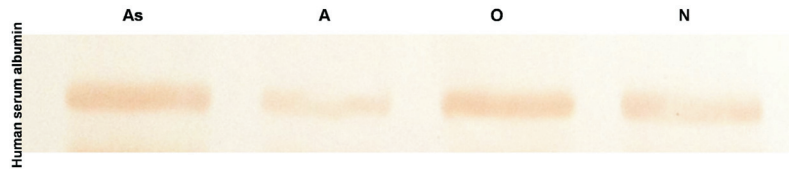
Figure 2: Western blot analysis of co-immunoprecipitate (As →Asthenozoospermia; A→Azoospermia; O→Oligozoospermia; N→Normozoospermia). 100μL pooled seminal plasma diluted with Co-IP buffer was co-immunoprecipitated with PIP primary antibodies. Co-immunoprecipitation elution was run on 12% SDS-PAGE and transferred to nitrocellulose membrane for western blot analysis using HSA primary antibodies. Blot was developed by 3,3' -diaminobenzidine (DAB).
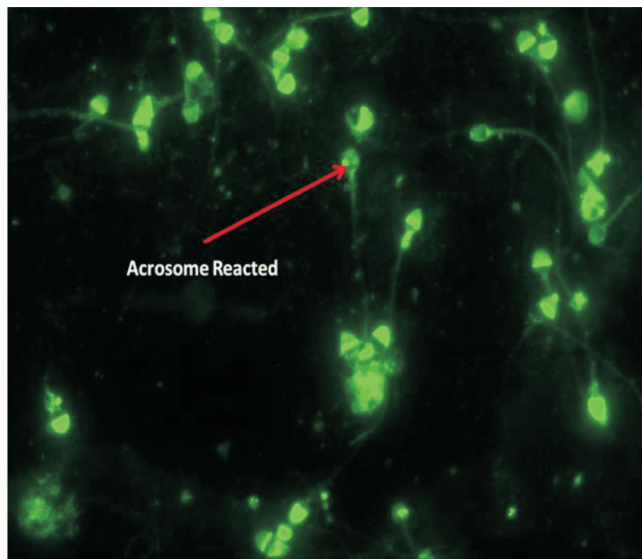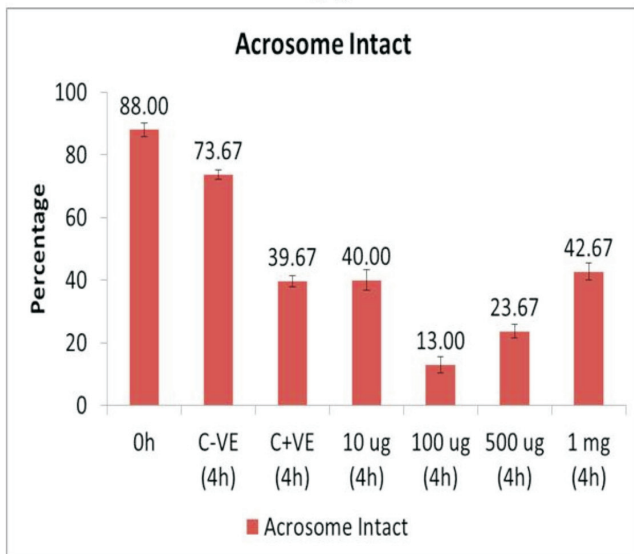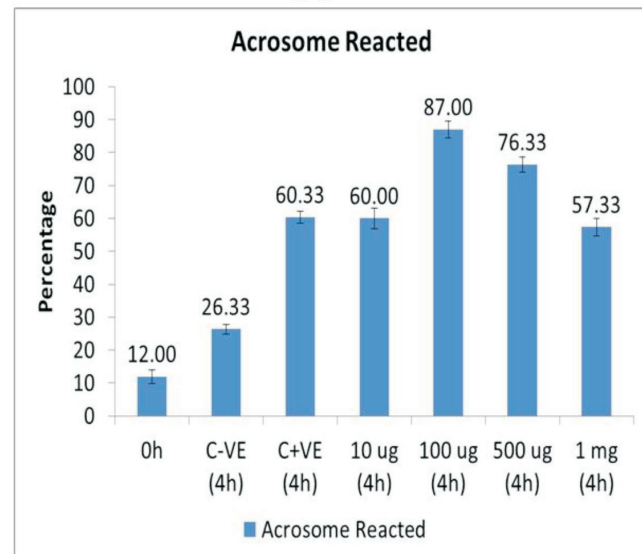

Figure 3: Effect of HSA-PIP complex on sperm function tests (C-VE →Negative control, containing media only; C+VE→ Positive control, containing capacitation inducing agent; 10μg, 100μg, 500μg and 1mg → final concentration (per mL) of HSA-PIP complex). [a] Acrosome intact spermatozoa at 0 h; [b] Acrosome reacted spermatozoa at 4 h; [c] %age of acrosome intact spermatozoa in presence of HSA-PIP complex; [d] %age of acrosome reacted spermatozoa in presence of HSA-PIP complex.

semen samples for ART (Armstrong *et al.*, 1998; Parekattil and Agarwal, 2012). Albumin is known to preserve sperm motility and a possible reason might be the formation of HSA-PIP complex (Kumar *et al.*, 2012). The sperm function tests in presence of HSA-PIP complex in our study are indicative that this complex influences sperm functions, specifically capacitation which, in turn, helps spermatozoa to prepare for acrosome reaction. These findings can also be correlated with lower expression of this complex in azoospermic seminal plasma samples. Like most of the protein-protein complexes, formation of this complex in seminal plasma is also dynamic in nature. In some western blots, we have observed absence of this complex in azoospermia samples (results not shown). As there are no spermatozoa in semen of azoospermia patients, activities like capacitation that prepare spermatozoa to acquire their fertilization powers would also silent. In addition, factors that influence sperm maturation and motility would also be less active. As we speculate that this complex is required to help spermatozoa in motility and capacitation, its lower expression in azoospermia is justified. However, *in vitro* experiments performed here are limited and do not reveal how exactly this complex influences the sperm functions. Also, this complex may have other related roles in fertility related processes and more studies are needed to explore exact role of this complex in sperm physiology.

Conclusions

On the basis of our findings, we can conclude that HSA-PIP complex might be playing important roles in sperm motility and capacitation. Also, its down-regulation in azoospermia suggests that it can further be explored as one of the causes of infertility in idiopathic cases.

Abbreviations

AR: Acrosome reaction; Co-IP: Co-immunoprecipitation; HRP: Horseradish peroxidase; HSA: Human serum albumin; PBS: Phosphate buffer saline; PIP: Prolactin inducible protein; SP: Seminal plasma; ZAG: Zinc alpha-2 glycoprotein

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