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LOCALISATION OF CALCIUM-BINDING PROTEINS IN RAM SPERMATOZOA USING THE IMMUNOFLUORESCENCE TECHNIQUE

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ABSTRACT

Localization of two calcium-binding proteins (proteins A and B) believed to be involve in membrane fusion on whole spermatozoa were carried out in two stages; before and after the acrosome reaction, the reaction being a prerequisite to fertilization. Determination of the acrosome reaction and sperm viability is carried out using fluorescent dyes i.e., FITC-conjugated *Pisum sativum* agglutinin (PSA) and propidium iodide (PI) respectively. Polyclonal antibodies were raised in rabbits. Ejaculated semen was diluted in buffer and loaded into tubes. Acrosome reaction was induced with calcium ionophore A23187 at 39^{0}C. PI was added to the sub-samples at time 0 and 45 minutes. Excess PI, ionophore and seminal plasma was filtered out with a syringe. Smears were made on slides and air-dried. The cells were pemeabilised with ethanol and rinsed in PBS. Batch I slides were incubated with FITC-PSA in the dark while batch II slides were incubated in 1% sheep serum. Batch II slides were then rinsed in PBS twice and incubated in both antiserum and pre-immune serum (negative control). These slides were then incubated in FITC-conjugated secondary antibody (anti-rabbit IgG) and kept in the dark. After final washing and mounted, both batches of slides were viewed immediately using fluorescence microscope. Results obtained before acrosome reaction showed localization of both antibodies to the whole sperm head, along the midpiece and tail. The acrosomes were also intact and cells were viable. After the acrosome reaction, localization of both antibodies were observed at the post-acrosomal region, midpiece, tail and the equatorial segment with no binding to the acrosome. Cells were mainly acrosome-reacted and dying. No binding was observed with pre-immune serum. Results indicate that the antigens were present in the acrosome and the change in binding suggests that the antigens have been redistributed after commencement of the acrosome reaction. The findings suggest that the proteins may be involved in the acrosome reaction.

Keywords: calcium-binding proteins, acrosome reaction, polyclonal antibodies, immunofluorescence, fluorescent dyes

INTRODUCTION

The acrosome reaction occurring in spermatozoa, is an exocytotic process involving multiple sites of fusion between the outer acrosomal membrane and the overlying plasma membrane with release of acrosomal enzymes required for fertilization (1). This reaction, along with capacitation is two physiological processes that every spermatozoon should undergo before effecting fertilization. Natural inducers of the acrosome reaction are the zona pellucida (2, 3) and other egg associated materials. Artificial inducers of the acrosome reaction includes a variety of substances with calcium ionophore A23187 being one of the more common (4). Calcium influx is required for the acrosome reaction (5, 1) and the presence or absence of calcium can affect the occurrence of the acrosome reaction (6) and sperm-egg fusion (7). Influx of calcium occurs during capacitation, a species-specific, reversible and time-dependent phenomenon that alters plasma membrane permeability to calcium after loss of glycosaminoglycans, cholesterol and other elements from the plasma membrane (8). Modulation of calcium movement into and from the spermatozoon is via three systems: the Ca^{2+} - ATPase pump to pump Ca^{2+} out of the cell, the Na^{2+} - Ca^{2+} exchanger to pump Ca^{2+} out and Na^{2+} in, and Ca^{2+} channels to allow Ca^{2+} influx (9).

The acrosome reaction in ram spermatozoa commences anterior to the equatorial segment and proceeds upward (10). Calcium-binding sites is demonstrated to be localized in the area where vesiculation commences by using the calcium pyroantimonate technique (11). The non-random localization of the pyroantimonate precipitates suggests that calcium might be binding to a membrane-bound protein (12) that could be a calcium-modulated protein (5). Isolation of the outer acrosomal membranes of ram spermatozoa revealed membrane-bound proteins of which two proteins, A and B of molecular weight 33 kDa and 39.3 kDa respectively were found to bind calcium on ^{45}Ca autoradiography (13, 14). The proteins were purified to raise polyclonal antibodies in rabbits. Localization and distribution of the proteins before and after the acrosome reaction in the spermatozoon was carried out using the immunofluorescence technique. Fluorescent dyes i.e. *Pisum sativum* agglutinin was used to determine acrosomal status while propidium iodide was used to determine cell viability in ram spermatozoa (15).

MATERIALS AND METHODS

Semen was collected from trained Freisland rams housed at the Royal Veterinary College into an artificial vagina when the rams mounted the restrained teaser ewe. Semen was diluted 1:100 with HEPES glucose buffer (NaCl 139 mM, KCl 2.5 mM, HEPES 20 mM, glucose 10 mM and $CaCl_2$ 3 mM, pH 7.4). Diluted fresh ram spermatozoa were induced to undergo the acrosome reaction with calcium ionophore A23187 at 39^0C. Briefly, calcium ionophore A23187 was dissolved in dimethyl sulphoxide (DMSO) at a final concentration of 1 μM and added to 1 ml of diluted semen (10^8 spermatozoa/ml) and incubated at 39^0C. Subsamples were taken

at 0 and 45 minutes. Propidium iodide (PI) at 10 μg/ml was added to the sample for determination of cell viability. Excess PI, calcium ionophore and seminal plasma was removed using a novel technique of filtration (13, 15). Approximately 20 μl was smeared onto clean glass slides and slides were made in triplicates. Spermatozoa were permeabilised by flooding slides with 100% ethanol for 5 minutes. Excess ethanol was removed by washing slides in PBS. The slides were later air-dried and divided into batch I slides and batch II slides for subsequent staining.

Acrosomal status determination

Batch I slides were flooded with fluorescein-isothiocyanate *Pisum sativum* agglutinin (FITC-PSA) at 40 μg/ml in HEPES glucose buffer and kept in the dark for 10 minutes. Excess stain was removed by washing in deionized water with intermittent agitation. Slides were later air-dried.

Immunofluorescence binding

Batch II slides were incubated for 30 minutes in 1% sheep serum in PBS to block non-specific binding sites. Unbound sheep serum was washed away with PBS for 5 minutes twice. Incubation with 1 ml of primary antibody A and B and pre-immune serum (negative control) at 1:100 in PBS was carried out for 1 hour at room temperature. Slides were washed again in PBS and incubated with FITC-conjugated secondary antibody (anti-rabbit IgG) at a dilution of 1:64 in PBS for 1 hour in the dark. Unbound antibody was removed by washing slides in PBS.

Fluorescence enhancement

A drop of p-phenylenediamine in 9:1 glycerol:PBS was placed on all slides to enhance fluorescence, a coverslip added and the edges sealed with nail varnish. Slides were viewed within two hours under an Olympus BHS microscope equipped with standard FITC filter set. Two hundred cells were counted for each slide.

RESULTS

The localization of antibody A and B at time 0 of ionophore treatment showed binding to the whole sperm head, along the entire mid-piece and tail (Fig. 1i and Fig. 1ii). The acrosomal status at time 0 of ionophore treatment showed more acrosome-intact cells as compared to acrosome-reacted cells (Fig. 1iii). Spermatozoa at time 0 of ionophore treatment were also mostly viable (Fig. 1iv). Prolonged ionophore treatment at 45 minutes causes the binding of antibody A to disappear slowly from the sperm head but it remained at the post-acrosomal region, midpiece and tail and some showed binding to the equatorial segment (Fig. 2i). The binding of antibody B to the sperm head also decline with prolong ionophore treatment but remained post-acrosomally, along midpiece and tail and equatorial segment (Fig. 2ii). Binding of antibody A was stronger as compared to antibody B. No binding was observed at the acrosome for both antibodies after the commencement of the acrosome reaction.

Most of these cells were also acrosome-reacted (Fig. 2iii) but some remained viable or slowly dying (Fig. 2iv). Both pre-immune sera A (Fig. 3A, a) and B (Fig. 3B, b) showed no binding at all to the spermatozoon.

DISCUSSIONS

The localization of immunofluorescence at the acrosomal region of the spermatozoa for both antibodies showed that the antigens are present in the OAM. The staining that was observed in the equatorial segment with prolonged ionophore treatment was difficult to explain. However, calcium pyroantimonate deposits were observed in this region as a late development that occur long after vesiculation had commenced (16). This therefore suggests that the proteins have somehow become redistributed in the surrounding regions and may enter the equatorial segment. These antibodies are specific to the proteins, and therefore suggest that they follow the proteins. If this is the case, therefore, that will explain the binding seen in this region.

The localization of both proteins in the acrosomal region showed similarity with other proteins and enzymes i.e., 32.5 kDa calelectrin in bull spermatozoa (17), calmodulin in rabbit spermatozoa (18) and boar spermatozoa (19) and protein kinase C in human spermatozoa (20). The sperm protein SP-50 from hamster spermatozoa, which possess annexin-like function (21), were also localized in the acrosomal region and might be related to proteins A and B. Proteins A and B may play a role in the initiation of the acrosome reaction but their exact function is not clearly understood. The development of polyclonal antibodies may prepare the way for further studies to elucidate functions.

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REFERENCES

1. Yanagimachi R (1994) Mammalian fertilization. In: *The Physiology of Reproduction. Vol. 1.* Eds: : Knobil E and Neill J. Raven Press Ltd, NY pp 189-317.
2. Bleil JD and Wassarman PM (1980) Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zona pellucidae possessing receptor activity for sperm *Cell* **20**: 873-882
3. Wassarman PM (1995) Sperm egg recognition mechanisms in mammals *Current Topics in Developmental Biology* **30**: 1-19
4. Shams-Borhan G and Harrison R (1981) Production, characterization, and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete Research* **4**: 407-432.
5. Kretsinger RH (1980) Structure and evolution of calcium-modulated proteins *CRC Critical Reviews of Biochemistry* **8**: 119-174

6. Yanagimachi R and Usui N (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa *Experimental Cell Research* **89**: 161-174

7. Yanagimachi R (1978) Calcium requirement for sperm-egg fusion in mammals *Biology of Reproduction* **19**: 949-958

8. Fraser LR (1995a) Cellular biology of capacitation and the acrosome reaction *Human Reproduction* **10**: 22-30

9. Fraser LR (1995b) Ionic control of sperm function *Reproduction Fertility and Development* **7**: 905-925

10. Flechon JE, Harrison RA, Flechon B and Escaig J (1986) Membrane fusion events in the Ca^{2+} ionophore-induced acrosome reaction of ram spermatozoa *Journal of Cell Science* **81**: 43-63.

11. Plummer JM and Watson PF (1985) Ultrastructural localization of calcium ions in ram spermatozoa before and after cold shock as demonstrated by a pyroantimonate technique *Journal of Reproduction and Fertility* **75**: 255-263

12. Watson PF and Plummer JM (1986) Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa *Journal of Experimental Zoology* **238**: 113-118

13. Sukardi, S. (1996) Studies on the isolation and partial characterisation of the outer acrosomal membrane of ram spermatozoa *PhD thesis*, University of London

14. Sukardi, S. and Watson, PF (1995). Isolation and partial characterisation of the calcium-binding proteins in the outer acrosomal membrane of ram spermatozoa *J. Reprod. Fert. Abst. Series.* **15**

15. Sukardi, S and Watson, PF (1993). Simultaneous detection of acrosomal status and sperm viability of ram spermatozoa with fluorescent *markers J.Reprod. Fert. Abst. Series 12*

16. Watson PF, Plummer JM, Jones PS, Bredl JCS (1995) Localization of intracellular calcium during the acrosome reaction in ram spermatozoa *Molecular Reproduction and Development* **41**: 513-520

17. Sudhof TC, Ebbecke M, Walker JH, Fritsche U and Boustead C (1984) Isolation of mammalian calelectrins: a new class of ubiquitous Ca^{2+} regulated proteins *Biochemistry* **23**: 1103-1109

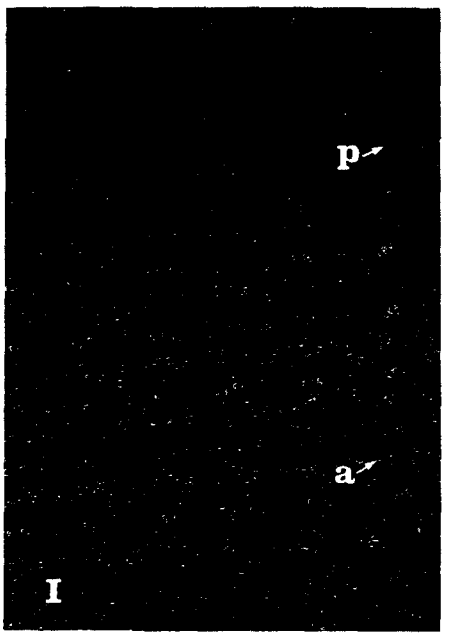
18. Jones HP, Lenz RW, Palevitz BA and Cormier MJ (1980) Calmodulin localization in mammalian spermatozoa *Proceedings of the National Academy of Science United States of America* **77**: 2772-2776

19. Camatini M, Anelli G and Casale A (1986) Immunocytochemical localization of calmodulin in intact and acrosome-reacted boar sperm *European Journal of Cell Biology* **41**: 89-96

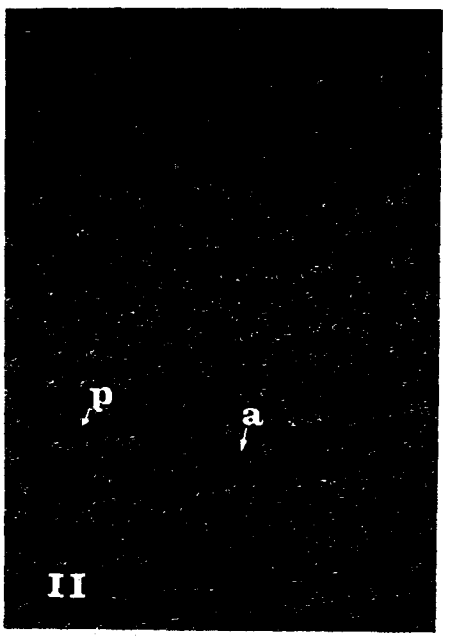
20. Kalina M, Socher R, Rotem R and Naor Z (1995) Ultrastructural localization of protein kinase C in human sperm *Journal of Histochemistry and Cytochemistry* **43**: 439-445

21. Hernandez EO, Roaespitia AL, Trejo JC and Mujica A (1996) Sperm protein (sp50) binds to acrosome and plasma-membranes in a Ca^{2+} - dependent manner - possible role in acrosome reaction *Molecular Reproduction and Development* **43**: 366-375

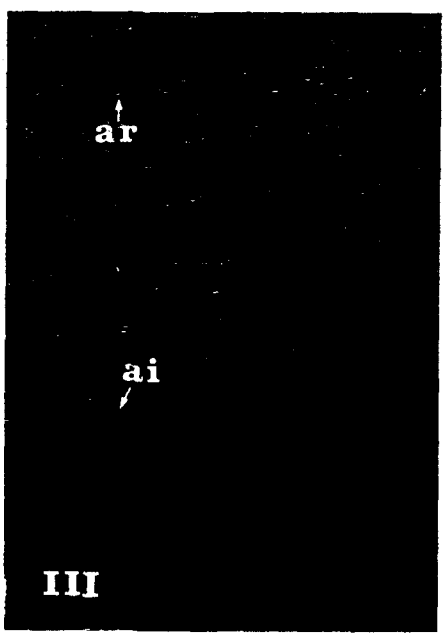
Figure I: **Localization of antibodies A and B, and acrosomal status and viability of spermatozoa at time 0 of ionophore treatment**



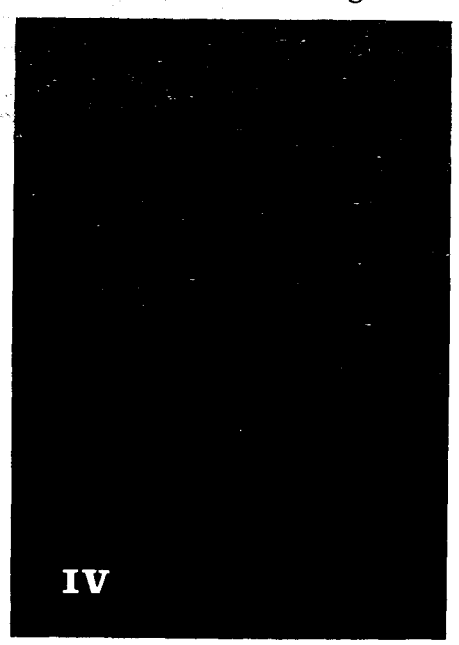
I - antibody A binding
p- post-acrosomal binding



II - antibody B binding
a - acrosomal binding

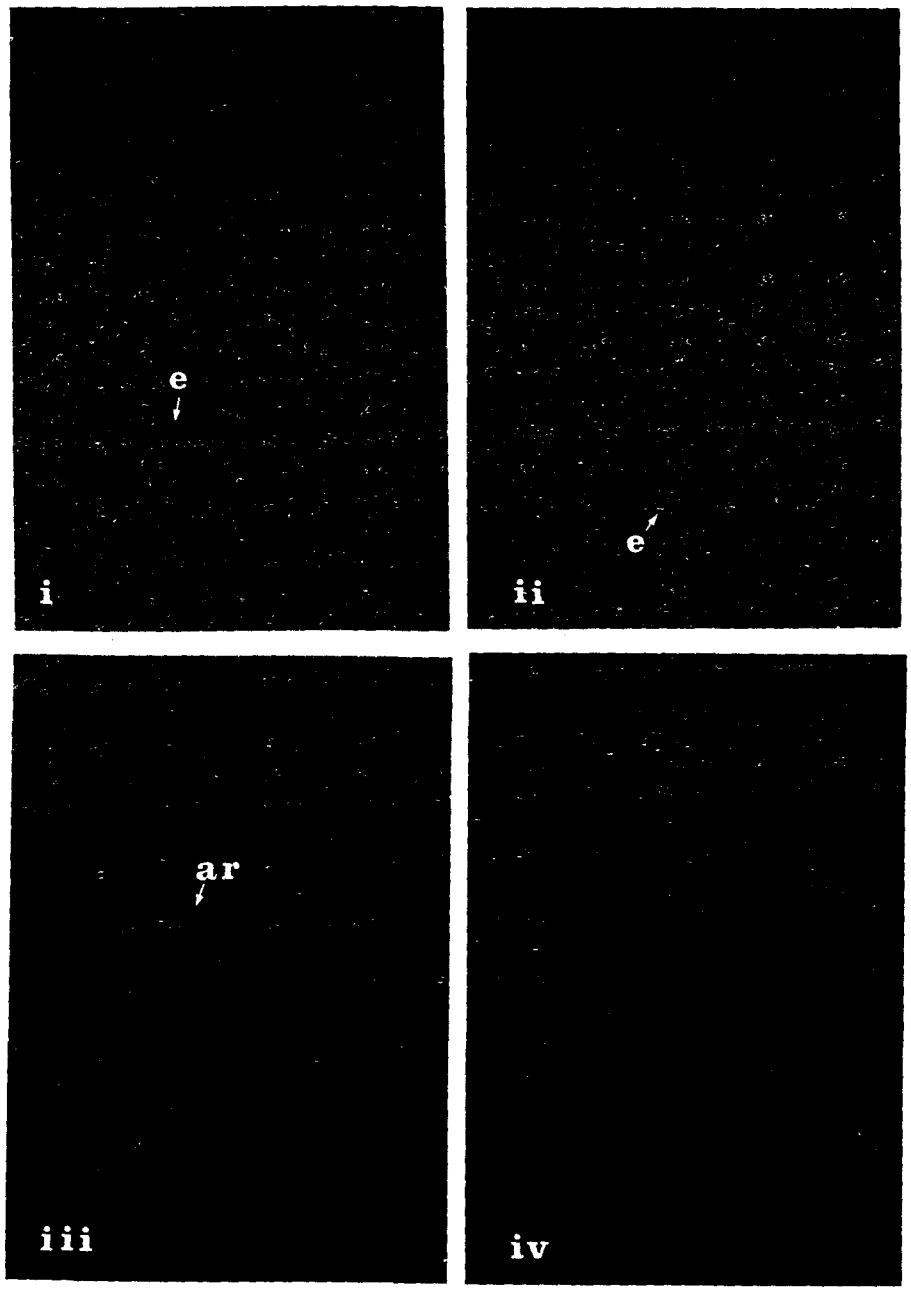


III - acrosomal status
Ai - acrosome-intact cells
Ar - acrosome-reacted cells



IV - No PI staining (viable)

Figure 2: **Localization of antibodies A and B, and acrosomal status and viability of spermatozoa with prolonged (45 minutes) ionophore treatment**

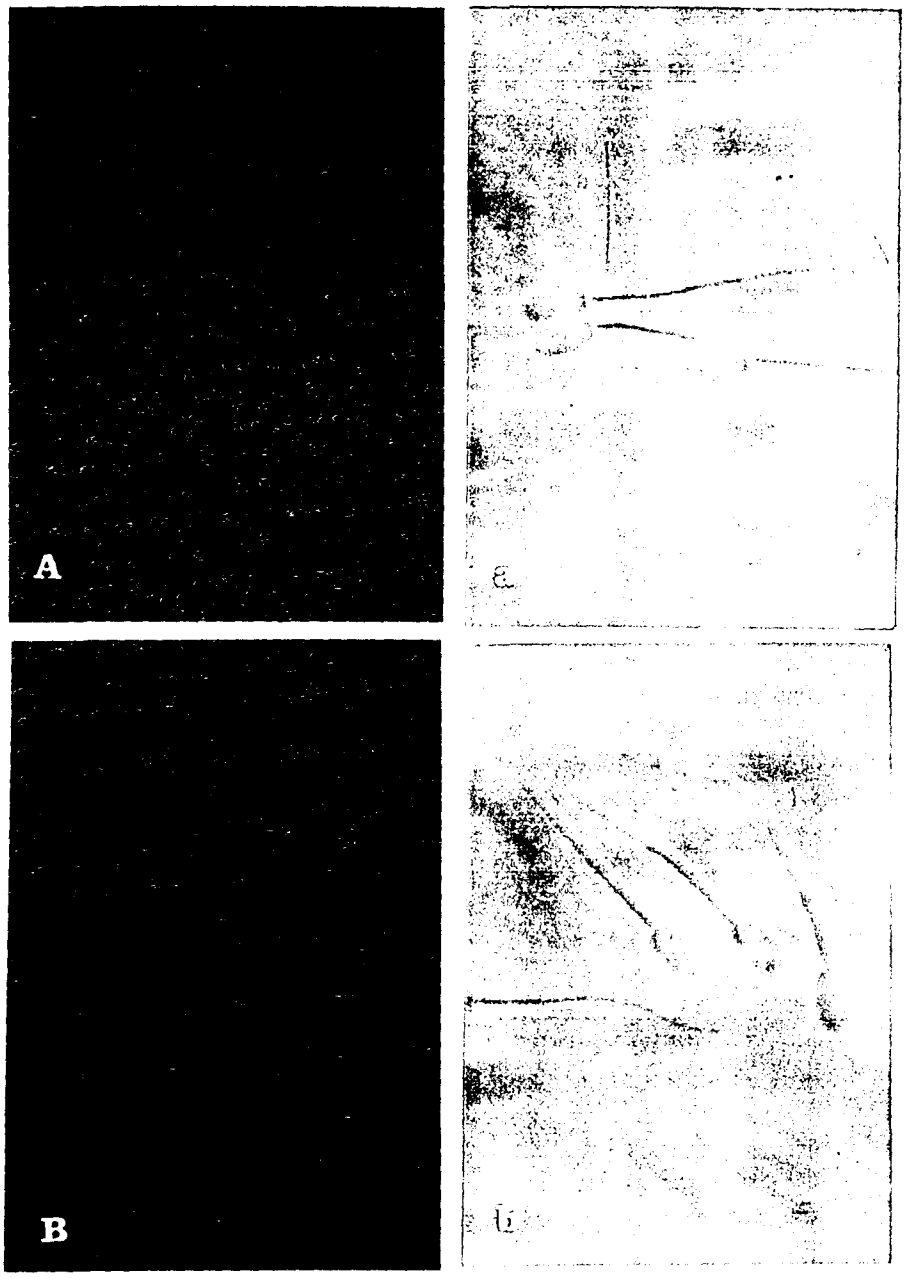


I - antibody A binding
e- equatorial segment binding
III - acrosomal status

II - antibody B binding

IV - Slight PI staining (dying cells)

Figure 3: Localization of pre-immune sera A and B to spermatozoa



A - negative pre-immune serum A binding
B- negative pre-immune serum B binding

a - corresponding phase contrast picture
b - correponding phase contrast picture