Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.11, 2013

Ion Exchange Fractionation of Rabbits Seminal Fluid: Recognizing a DNA Retardation Activity from the Main DNase Activity

Mohammed Baqur Al-Shuhaib[1], Ali Al-Saadi[2], Mahanem Noor[3], Mufeed Ewadh[4*]
1. Department of Biology – College of science for Women – University of Babylon
2. Department of Biology – College of Science – University of Babylon
3. School of Biosciences and Biotechnology – Faculty of Science and Technology – University Kebangsaan Malaysia (UKM)
4. Department of Clinical BioChemistry – College of Medicine – University of Babylon
*. Email: mewadh@yahoo.com

Abstract

The role of seminal proteins charge on the nature of seminal fluid inhibitory effect that exerted against exogenous DNA. Has been identified and an approach closely with more details to the nature of inhibitory activities available in rabbit seminal fluid proteins that prevent the entry of exogenous DNA into the head of sperm. After collection of rabbit's ejaculate and removing sperm cells, seminal fluid was incubated with fixed concentration of exogenous DNA. The seminal fluid – exogenous DNA mixture was analyzed by electrophoresis. Ion exchange chromatography was used to separate seminal proteins on the basis of their charge. Positively charged proteins were eluted, lyophilized, and their profile was characterized by SDS-PAGE and native-PAGE. After incubation of this eluted group with the same source of DNA, the same electrophoretic conditions were applied on this group.

According to our knowledge, this is the first paper in which ion exchange chromatography was used to separate two DNA counterfeiting activities of the seminal fluid using non-radioactive method in rabbits and even in other mammals. Thus, more than one inhibitory activity were identified and separated. DNA retardation activity (or DNA binding activity) which repressed DNA electrophoretic migration was the only activity that found to be available on the positively charged fractions while the DNase activity was found exclusively on the negatively charged group.

Key words : Seminal fluid **,** transgenesis techniques **,** DNA electrophoretic migration **,** DNase

1.Introduction

In addition to the main line of elucidating transgenesis techniques through sperm mediated gene transfer (SMGT), there was a parallel line of considerable importance with this field which was represented by the understanding the molecular level of the protective mode taken from the seminal proteins to prevent the entry of exogenous DNA .It was demonstrated that certain factor(s) in seminal fluid were found to be responsible about inhibiting the internalization process of exogenous DNA (Zani et al., 2005). The fact which refers to the existence of one or more factors in seminal fluid that able to block sperm permeability must be taken into account. This means, extensive washing steps of ejaculate to remove seminal fluid is necessary and should be made before incubating sperm with exogenous DNA. Lauria and Gandolfi reviewed that seminal fluid inhibitors have two ways of inhibition to exogenous DNA, either directly or indirectly (Lauria & Gandolfi, 1993). These seminal fluid inhibitory factors may prevent transfection of intact sperm by foreign DNA (Camaione et al., 1992). Gandolfi showed that there is a consensus on the experiments made on seminal fluid of the ejaculated spermatozoa of mammals in the impermeability of sperm cell to the aggression of foreign DNA as long as seminal fluid is not removed (Gandolfi, 2000).We think that this route of research is not less important than the main route since as much as researchers get more understanding to the nature of seminal proteins as much as they will be more close to elucidate all the natural facts that hamper the enhancement of SMGT.

Aim of study : This work is to evaluate seminal fluid natural defense mechanisms equally with the transgene entry mechanisms .

2. Materials & Methods

2.1 Materials

Kit; PCR SuperMix (Invitrogen – Cat. # 0572-014), PageSilver™ Silver Staining Kit (Fermentas - Cat

K0681), Ladders; TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen – Cat. #10488090), DNA size

marker; MassRuler™DNA Ladder Mix (Fermentas – Cat. # SM0403), Protein size Markers; PageRuler™ Unstained Broad Range Protein Ladder (Fermentas – Cat # 1881), Protein low molecular weight size marker (Amersham – code: 17-0446-01), Oligos; Forward primer (5´–CCATGCCCGAAGGTTATGTA–3´)and reverse primer (5´– GAAAGGGCAGATTGTGTGGA –3´) Invitrogen. Vector; gWiz-GFP (green fluorescent protein) vector (Aldevron – Cat. # 5006).

2.2 Experimental Animals:

Eight New Zeeland sexually mature healthy white rabbits were included in this study. New Zeeland white rabbits were raised in the animal house in the school of bioscience and biotechnology/FST/UKM. They were individually housed under controlled conditions of temperature (19 – 21 °C) and standard artificial light (12 hour light and 12 hours dark). A diet of grower rabbits pellets (ad libitum) and fresh water was provided. Animals were cared according to international standards management established for the care and use of laboratory animals in facilities approved by the University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

2.3 Methods

Collection of seminal fluid: Sperm was collected by home-made artificial vagina. Seminal fluid was separated from sperm cells by single centrifugation step .

2.3.1 Exogenous DNA - seminal fluid incubation:

gWizGFP (6µg) vector was incubated with increasing concentrations of rabbit's seminal fluid (ranging from 1 to 10µl) and completed to 30µl with sterile deionized water. The incubation was lasted for 60 min at room temperature. Mixtures were analyzed by agarose gel electrophoresis (fig. 1).

2.3.2 Fractionation with ion exchange chromatography (IEC):

Seminal fluid proteins were fractionated according to Teixereira et al., 2006 and Villemure et al., 2003 with modifications

2.3.2.1 Resin selection: DEAE cellulose (5 g) (Sigma Aldrich – USA) was applied as anion exchanger for rabbit seminal fluid fractionation .

2.3.2.2 Regeneration of DEAE-cellulose: Preparative steps were conducted in 500ml capacity flask. DEAE-cellulose (anion exchanger) was purchased from Sigma. 5 g DEAE-cellulose was slowly added to 300 ml 0.1M sodium hydroxide with gentle stirrer for 30 min (pH reached to 13). The sodium hydroxide solution was discarded and the resin was washed with double distilled water until pH reached to pH 8.0. Then the solution was replaced with 0.1 M hydrochloric acid with stirring for 30 min (pH reached to 1.0). The resin was washed with double distilled water until pH reached to 3.0. The distilled water was discarded and replaced with 500mM 10X tris buffer pH 8.0 with stirring for 30 min. The 10X buffer was discarded and then the resin was equilibrated with 50 mM tris HCl pH 8.0. After removing more fines, the suspension of DEAE-cellulose resin was transferred into a glass column (2x20 Bio-Rad – USA). Length of resin after its packing with 0.01M PBS was only 4cm. the packed resin was stored in 4ºC until loading samples .

2.3.2.3 Loading sample: The pH of DEAE cellulose effluent was checked up before seminal fluid was applied. Gravity flow was applied. One ml of seminal fluid containing 3mg of protein was dissolved in 0.02 M phosphate buffer, pH 7.3, and loaded onto an ion exchange chromatography column (DEAE-cellulose, 2 x 20 cm), which was previously equilibrated with the same buffer .

2.3.2.4 Elution of positively charged proteins: The column was washed and eluted with 0.01M phosphate buffer pH 7.3. The column effluent was collected with manual fractionation, 3ml per fraction (fig. 2). Protein concentrations were spectrophotometrically determined (Spectrophotometer – Shimadzu/Japan). According to proteins concentration measurement data, the first three fractions were discarded. SDS – Polyacrylamide Gel Electrophoresis (SDS – PAGE) of the other eluted positively charged proteins was taken place (fig. 3). Laemmli

discontinuous method (Laemmli, 1970) for protein electrophoresis was considered, gel dimensions were 10-cm wide by 8-cm long and 0.1-cm thick (provided by Bio-Rad – USA). The resolving gel concentration used in these experiments was 14%. While the stacking gel concentration was 4%. Silver Staining was taken place according to Fermentas instruction manual (Cat # K0681). Picture was taken by 7.2 Mp digital camera (Sony – China).

2.3.2.5 Elution of negatively charged proteins: After eluting of positively charged proteins, the other proteins were eluted from the glass column with 100ml linear gradient of 0-1M NaCl in 0.01M phosphate buffered saline. Protein concentrations were spectrophotometrically determined (fig. 2).

2.3.2.6 Lyophilization of positively charged rabbit's seminal fluid protein fractions:

Each five fractions 1 to 5, 6 to 10, 11 to 15, and 16 to 20 were freeze dried (Lyotrap – UK) and SDS-PAGE (figure 4) and native PAGE (figure 5) were performed for these fractions (fig. 3). Gels were stained by silver nitrate. Pictures were taken by 7.2 Mp digital camera (Sony – China).

Incubation of eluted and lyophilized positively charged fractions with gWizGFP vector: Fixed concentration (2µg) of gWizGFP DNA was incubated with variable concentrations of DEAE cellulose positively charged fractions (ranging from 1µl to 10µl) for one hour at room temperature. After incubation, each sample was analyzed by agarose gel electrophoresis (fig. 6). Picture was taken by photodocumentation unit (Alpha Innotech – USA).

PCR primers design for gWizGFP gene: Two specific primers for the transgene green fluorescent protein (GFP) in gWiz-GFP vector were designed according to Genamics Expression software. A PCR fragment of 364 bp was chosen for amplification which extended within the open reading frame of the recombinant GFP; from 2156 bps into 2520 bps. PCR amplification was taken place using conventional thermal cycler (Eppendorf Master Cycler - USA). Upstream and forward primers and DNA template were added to the PCR Super Mix. The PCR tubes were placed on ice and all the components were added to make 50µl final reaction volume. Thirty cycles (denaturation at 95ºC, annealing at 52 ºC, extension at 75 ºC) of amplification were performed .

Incubation of eluted and lyophilized positively charged fractions with 364bp PCR fragment: Fixed concentration of 364bp PCR fragment was incubated with different increasing concentrations of DEAE cellulose positively charged fractions (through 1µg, 5µg, 10µg and 15µg) for one hour at room temperature. After incubation, each sample was analyzed by agarose gel electrophoresis (fig. 7). Picture was taken by photo documentation unit.

3. Results & Discusion

Several papers were demonstrated the inhibitory role of seminal fluid against the entry of exogenous DNA (Lavitrano et al., 1992 and 1997; Zani et al., 1995). Several proteins were involved in this inhibition. Accordingly, the seminal proteins that inhibit the entry of exogenous DNA should somehow bind with it. This interaction was detected usually using radioactively labeled isotopes or other cost effective labeling method such as foot-printing in which the DNA bound protein was retarded during its migration through PAGE compared with its natural unbound form. DNase activity profile of rabbit's seminal fluid after its incubation with gWizGFP vector: Although several results were focused on the DNase activity of seminal fluid that taken from several mammals (Carballada & Esponda, 2001), no one mentioned the existence of another activity in the same fluid. In this study, DNA binding or retardation activity of the seminal fluid was taken into consideration .

After simple incubation of gWizGFP vector with seminal fluid, two significant groups were identified easily in seminal fluid. The first one; was the expected DNase activity, which was highlighted as a known exogenous DNA counterfeiting mechanism in several mammals such as mice (Carballada & Esponda, 2001), hamster (Sotolongo et al., 2005), bulls (Tanigawa et al., 1975), human (Singer et al., 1983), and even in birds (Sato et al., 2003). This mechanism could be described as a natural defense barrier against the entry of gWizGFP vector – or any exogenous DNA – into the sperm cells. DNase mechanism, as it was shown in figure 1, was increased

significantly as seminal fluid concentration increased with exogenous DNA. As long as DNase mechanism was not specific one, it was not

necessary to change the type of exogenous DNA to be incubated with this fluid. Although, the exogenous DNA used was hydrolyzed relatively with seminal fluid when the later used in low concentration, but this mean that the seminal fluid – even with low concentration- was capable of commencing degradation of exogenous DNA. The proportional increase in this hydrolysis was expected since some researches were indicated such activity (Abdorrahman and Foster, 2005).

The second observed outstanding mechanism was a second group of seminal proteins owning a distinct exogenous DNA counterfeiting mechanism. The action of this group of seminal proteins was observed even when seminal fluid was used in low concentrations (fig. 1). This wasn't reflecting the abundance of only DNase activity of one seminal group, but, this reflecting the abundance of DNA binding activity of another seminal group as well. According to our knowledge, this binding mechanism was never highlighted by any research before this one. This could be attributed to many reasons; the first one could be represented by the field at which seminal fluid was placed, in which, almost all researchers were concerning only on the reproduction aspects. Or, nobody thought that simple DNA – seminal fluid incubation and direct agarose gel electrophoresis could not lead to more than a DNA hydrolysis.

This research was not the only one demonstrated the presence of more than one activity in the seminal fluid of mammals, since this data was demonstrated previously, without focusing on DNA incubation nature of the positively charged seminal fractions (Carbellada & Esponda, 2001). Some papers was referred indirectly to the occurrence of certain forms of polyamine molecules in seminal fluid (Setchell & Brooks, 1988), these molecules were contributed somehow in the binding with exogenous DNA provided to explain how such binding was taken place (Camaioni *et al.*, 1992).

It was deserve to be noted that these results of clear DNase activity of seminal fluid were not in agreement with the results obtained after incubation of porcine seminal fluid with DNA for the same time and conditions since no significant DNase activity was observed in the seminal fluid after incubation (Horan *et al.*, 1991). Moreover, no DNA neutralizing ability was found in the same paper after studying the porcine seminal fluid – exogenous DNA interaction. This may be attributed possibly to the fact that the difference in species may be responsible about such vast different results obtained between this research and the research of Horan *et al.*, (1991) or to unknown reason. Since two seminal activities were identified according to figure 1, our efforts were directed toward separation of these two activities according to their charge. It was simply speculated that the second activity (the DNA binding activity) was represented by a positively charged group. Therefore, ion exchange chromatography was applied to accomplish such purpose.

3.1 Ion-exchange chromatogram of rabbit seminal fluid on a DEAE-cellulose column

It was noticed that DNA binding to spermatozoa is not random process: since DNA molecules have a preferred binding site localized in the post-acrosomal region of sperm head in most species. The main property that seems to be regulated by the negative charge of the DNA molecule (Lavitrano *et al.*, 1992). Although the speculated DNA binding activity observed in rabbit's seminal fluid was not necessary to be exclusively available only in positively charged proteins, but, this activity was not far away from the charge aspects. It might be rational to say that such activity was either sequence specific, or non-sequence specific. In the second case, it was possible to include charge in this suggestion since seminal fluid, in general, was designed to prevent any outsider from the entry inside the sperm cell. This activity, in turn, was not far away from the charge calculations because the "natural defense" and "natural charge" characteristic features had generalized meaning instead of the specific counterpart. DEAE cellulose ion exchanger was applied according to these aspects on seminal fluid as an inevitable tool to separate seminal proteins on the basis of their charge (fig. 2).The direct application of the eluted protein fractions on SDS-PAGE and the utilization of the highly sensitive silver nitrate on this gel was answered several questions about the nature of the positively charged resolved fractions (fig. 3). This direct application of the eluted fragments could be taken place without undergoing the classical pre-electrophoresis spectrophotometric assay. This free-spectronic protocol was applied for several technical reasons; such as to sum up time, which was however, a crucial factor.

The direct run of the eluted fractions was capable to give more elaborated information about the nature of the electrophoresed proteins. Added to that, this protocol was much simpler. The direct run of the eluted fractions was capable to give more elaborated information about the nature of the analyzed proteins (Bollag *et al*., 1996).Therefore, to get much more accurate data about the nature and the number of bands of the eluted fraction, direct SDS-PAGE and silver nitrate dye were applied (Bonner, 2007). In spite of the relative differences obtained in the SDS-PAGE resolved positively charged fractions but such differences might not be exist because this relative difference didn't belong to the difference in the protein profile of the resolved fractions as much as to the difference in the development time used in silver staining kit (Fermentas) to get the desired picture of the resolved bands (fig. 3). This suggestion was supported by the absence of any clear differences in the band profile of all the fractions after being lyophilized (fig. 4). After the elution of all positively charged fractions of the seminal fluid, several low molecular weight bands were not eluted with the profile of the positively charged fractions. This profile was clear in SDS-PAGE (fig. 4) and in native-PAGE (fig. 5).

3.2 SDS – Polyacrylamide Gel Electrophoresis (SDS – PAGE) of eluted and lyophilized positively charged fractions:

It was found that is it was necessary to visualize the lyophilized positively charged seminal proteins on one dimentional native PAGE in addition to SDSPAGE as well (Garvin, 1990). This was done in order to completely characterize the positively charged fractions (Marques *et al.,* 2000). Since SDS PAGE sometimes did not give full electrophoretic information so the deformation taken place in the proteins conformations as a result of denaturation (Laemmli, 1970). Add to that, as it was observed (fig. 4) that there were no clear distinctions between the profile of the whole seminal fluid profile and the profile of the eluted positively charged proteins. That's why further data had to be provided by directly analyzing the same fraction on a gel without losing any of deformation.

Nondenaturing – Polyacrylamide Gel Electrophoresis (Native – PAGE) of eluted and lyophilized positively charged fractions: More data were found in figure 5, in which much more clear distinguishing between the positively charged fractions and the seminal fluid. These differences were clearer than differences obtained in figure 4. Since some low molecular weight bands observed between 15kd and 20 kd were found to be absent in the positively charged fractions compared with the whole seminal fluid profile. In figure 4, it was found a certain ambiguity in the profile of the analyzed proteins on native PAGE. This might be attributed to the obvious positively charged characteristic feature observed in these fractions which made the electrophoresis much more difficult because the fierce resistance of these positively charged fractions to the electrical current applied. Several native PAGEs before this one were failure to run these fractions because all of these lanes were analyzed in parallel and continuous electrical current. Accordingly, as it shown in figure 5, an empty lane (lane 4) was placed between adjacent lanes and several on – off orders were used by power supply with time interval rests to alleviate the fierce resistance of these fractions to the electrical current applied (Westernmeier et al., 2005).

DNA binding activity profile of rabbit DEAE cellulose positively charged seminal fractions after their incubation with gWizGFP DNA. Although the same incubation experiments shown in figure 1 were repeated on the positively charged fractions but the result in figure 6 was different in several interesting aspects. As long as no DNA hydrolysis happened in all lanes even with the lanes that had high concentrations of the positively charged fractions that incubated with the same DNA concentrations, so, there was no DNase activity in all the positively charged fractions. But the observed low level of DNA binding activity of the positively charged fractions (fig. 6) was less than what it was observed with the intact, non-fractionated seminal fluid (fig.1). This could be attributed to several reasons such as the loss of considerable amount of DNA binding activity during the process of ion exchange chromatography (Villemure et al., 2003), or during the process of lyophilization (Matejtschuk, 2007). The other reason that might explain such phenomenon was the possibility of existing of another significant DNA binding activity on the negatively charged fractions.

Unfortunately, this site of view was never focused on during undergoing such experiments for several reasons such as the DNase activity was a classic and non-surprising activity observed usually observed in seminal fluid and it was not necessary to rediscover it. The second important reason of not taking negatively charged fractions in consideration with respect to new discovered seminal DNA binding activity was the apparent DNA binding activity observed in another DNA – seminal fluid incubation experiment (fig. 7). The number of papers that dealt with the DNA binding ability of the chromatography separated positively charged fractions of seminal fluid were not exist, but it was suggested that factor(s) in seminal fluid might prevent potentially dangerous molecules such as foreign DNA (which might not be present in the genital tracts from bacterial or viral sources) from gaining access to spermatozoa (Camaioni *et al.*, 1992).

3.3 DNA binding activity profile of rabbit DEAE cellulose positively charged seminal fractions after their incubation with 364bp PCR fragment:

The significant DNA binding activity observed in figure 7 was very clear and don't require extended discussion since as much as the concentration of the positively charged fractions increase as much the retardation of the electrophoretic movement of the bound DNA was observed. This was attributed to the electrostatic attractions or ionic interactions taken place between DNA and proteins (Reece, 2004). But the only two things that deserve to be mentioned was the absolute absence of any DNase activity even when very high concentrations (15µg) of the positively charged fractions were used. The sort of

DNA retardation (fig. 7) that extended in high concentration of these fractions even to the right DNA marker don't represent any "specific" mode of DNA binding. This, in turn, affirms the general protective behavior performed by such fractions as one of the inevitable barriers against the entry of any bacterial or viral agent into the sperm cells (Camaioni *et al.*, 1992).

Conclusion : In addition to the naturally available DNase activity of seminal fluid, there is an undiscovered DNA binding activity available in positively charged seminal proteins. These two main activities could be separated by ion exchange chromatography. Accordingly, exogenous DNA was not only hydrolyzed by seminal proteins but its charge was largely neutralized by second group of seminal proteins have a positive charges in physiological pH. According to ion exchange chromatography, seminal DNase activity is exclusive to the negatively charged fractions.

Acknowledgements

We would like to thank Ms. Qumaria , Ms. Habiba ,and Mr.muhammed Ewadh for their support and cooperation .

References

Alghamdi A. S., and Foster D. N. (2005). Seminal DNase Frees Spermatozoa Entangled in Neutrophil Extracellular Traps. Biol.Reprod. 73, 1174–1181 .

Baccetti, B. and Spadafora, C, (2000). 'Conclusions'. Molecular Reproduction & Development, 56; 329-330.

Bollag D. M., Rozycki M. D., Edelstein S. J. (1996). Proteins Methods. Jhon Wiley and Sons Publications.

Bonner L R. (2007). Protein Purification (the basics). Taylor and Francis group.

Camaioni, A., Russo, M.A., Odorisio, T., Gandolfi, F., Fazio, V.M., and Siracusa, G. (1992). Uptake of exogenous DNA by mammalian spermatozoa: Specific localization of DNA on sperm heads. Journal of Reproduction & Fertility 961: 203-212.

Carballada R, Esponda P. (2001). Regulation of Foreign DNA Uptake by Mouse Spermatozoa. Experimental Cell Research, 62, 104–113.

Coligan J, Kruisbeck A, Margulies D, Shevach E, Strober W. (2005). Current Protocols in Immunology. New York: John Wiley and Sons, Inc.

Gandolfi F. (2000). Sperm-mediated transgenesis. Theriogenology, 53, 127-37.

Garvin D E. (1990). One-dimentional gel electrophoresis. From methods in enzymology, v 182, guide to protein purification, edited by Murray P. Deutscher.

Horan R, Powell R, McQuaid S, Gannon F, Houghton J. A. (1991). Association of foreign DNA with porcine spermatozoa. Arch Androl 26 :83-92.

Houdebine LM. (2003). Animal transgenesis and cloning. West Sussex, UK: Wiley & Sons.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lauria A, Gandolfi F (1993): Recent advances in sperm cell mediated gene transfer. Molecular Reproduction & Development 36:255–257.

Lavitrano, M., French, D., Zani, M., Frati, L., and Spadafora, C. (1992). The interaction between exogenous DNA and sperm cells. Molecular Reproduction & Development 31: 161-169.

Lavitrano, M., Maione, B., Forte, E., Francolini, M., Sperandio, S., Testi, R. & Spadafora, C. (1997). The interaction of sperm cells with exogenous DNA: a role of CD4 and major histocompatibility complex class II molecules. Experimental Cell Research 233, 56—62.

Marques V A., Goulari L. R., and Silva F. (2000). Variations of protein profiles and calcium and phospholipase A2 concentrations in thawed bovine semen and their relation to acrosome reaction. Genetics and Molecular Biology, 23, 4, 825-829.

Matejtschuk P. Lyophilization of Proteins. (2007). From Cryopreservation and Freeze- Drying Protocols.Methods in Molecular Biology, Second Edition.Edited by John G. Day. © 2007 Humana Press Inc.

Reece, R.J. (2004). Analysis of Genes and Genomes. John Wiley and Sons Ltd. p361-366.

Sato M, Tanigawa M, Kikuchi N, Nakamura S, Kimura M, (2003). Efficient gene delivery into murine ovarian cells by intraovarian injection of plasmid DNA and subsequent in vivo Electroporation. Genesis 35 (3); 169–174.

Singer R, Sagiv M, Allalouf D, Levinsky H, Servadio C. (1983). Deoxyribonuclease activity in human seminal fluid. Arch Androl; 10: 169-72.

Sotolongo B., Huang T., Isenberger E., & Ward S. (2005). An Endogenous Nuclease in Hamster, Mouse, and Human Spermatozoa Cleaves DNA into Loop-Sized Fragments. Journal of Andrology, 26 (2); 272 – 280 .

Tanigawa Y, Yoshihara K, Koide SS. (1975). Endonuclease activity in bull semen, testis and accessory sex organs. Biol. Reprod., 12: 464-70.

Teixeira D., Melo L., Gadelha C., Cunha R., Bloch C., Rádis-Baptista G., Cavada B., and Freitas V. (2006). Ion-exchange chromatography used to isolate a spermadhesin-related protein from domestic goat (Capra hircus) seminal plasma. Genetic Molecular Researches, 5 (1): 79-87.

Villemure M., Lazure C. and Manjunath P. (2003).Isolation and characterization of gelatin-binding proteins from goatseminal plasma. Reproductive Biology and Endocrinology, 1 (39); 1 – 10 .

Westernmeier R., Gronau S., Becket P., Bulles J., Scchickle H. and Thebling G. (2005). Electrophoresis in practice. Wiley-VCH Verlang GmbH and Co KGaA.

Zani M, Lavitrano M, French D, Lulli V, Maione B, Sperandio S, Spadafora C. (1995). The mechanism of binding of exogenous DNA to sperm cells: factors controlling the DNA uptake. *Experimental Cell Researches* 2 17: 57-64.

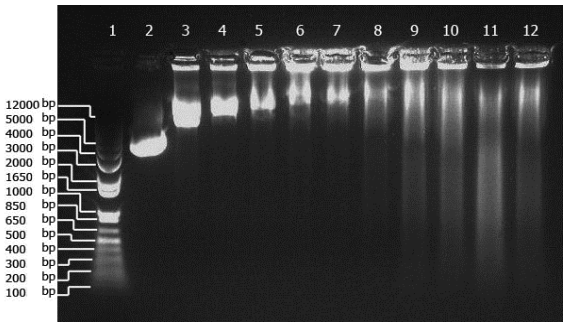

Figure 1. DNase activity profile of rabbit's seminal fluid after its incubation with gWizGFP vector .Lane 1: 20µl size marker (Invitrogen). Lane 2: 2µg gWizGFP DNA. Lane 3 to lane 12:10µl taken from incubation of 6µg gWizGFP DNA with (1, 2, 3, 4, 5, 6, 7, 8, 9, & 10)µl seminal fluid. Electrophoresis conditions: agarose concentration 1%, power applied: 4.1 V / cm, time of run: 1 hr.

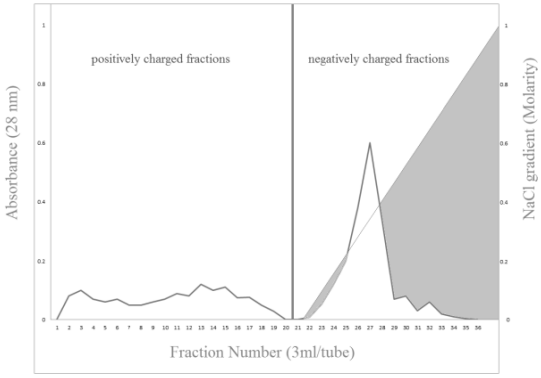

Figure 2. Ion-exchange chromatography of rabbit seminal fluid on a DEAE-cellulose column. The 4cm length of DEAE cellulose was washed with 0.02 M phosphate buffer, pH 7.3, at a flow rate of 3ml/fraction. The first 3 fractions were discarded. Thus no. 1 in this diagram refers to first fraction submitted to SDS-PAGE. Once protein concentration was reduced, linear gradient of 0 to 1 M NaCl was used to elute negatively charge fractions. Ion exchange chromatography conditions: column type: conventional glass column, flow force: gravity, flow rate partitioning: 3ml/tube each.

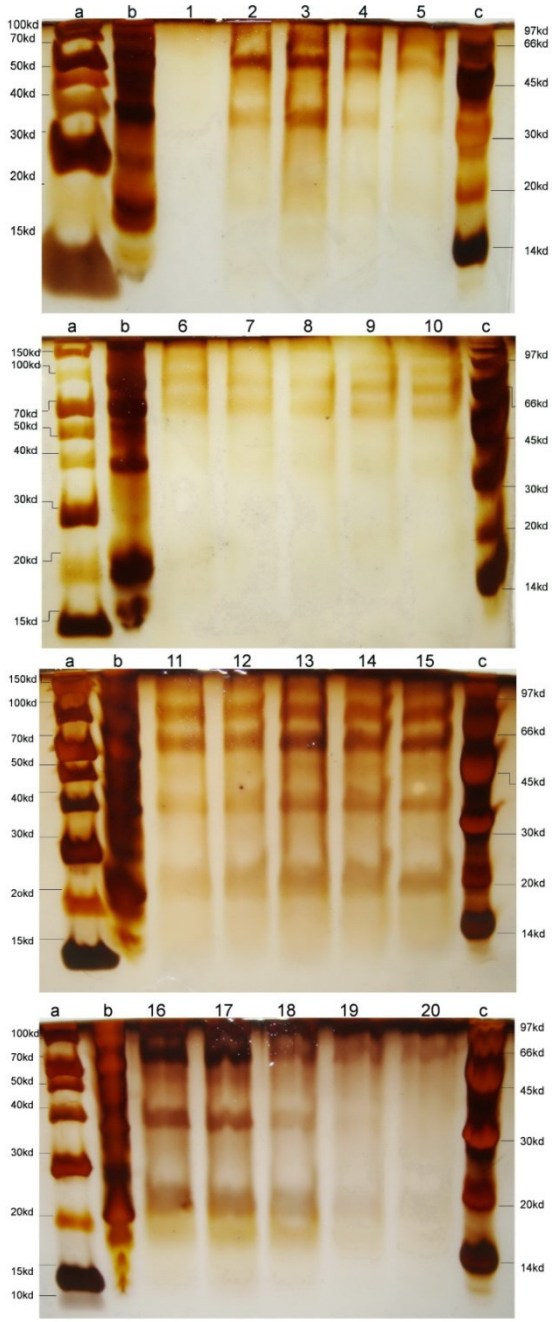

Figure 3. Silver stained positively charged proteins of rabbit seminal fluid electrophoresed on SDS-PAGE after being fractionated by DEAE anion exchanger. Lane a: protein size marker (Fermentas - 10µl). Lane b: rabbit seminal fluid (4.5µl). Lane 1 to 20: 15 µl from fraction 1 to fraction no. 20. Lane c: protein low molecular weight marker (Amersham - 7µl). SDS PAGE electrophoresis conditions: polyacrylamide concentration 14% separating gel and 4% stacking gel. Voltage applied: 11.54 V / cm.
run time: 75 min.

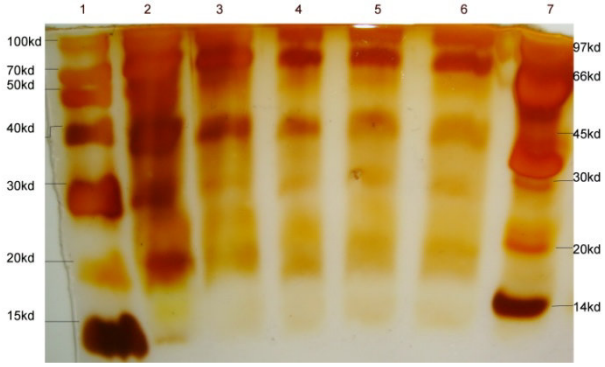

Figure 4. Silver stained lyophilized positively charged proteins of rabbit seminal fluid electrophoresed on SDS-PAGE after being fractionated by DEAE anion exchanger. Lane 1: protein size marker (Fermentas - 10µl). Lane 2: rabbit seminal fluid (4.5µl).Lane 3: fraction number 2-5 (1µg each). Lane 4: fraction number 6-10 (1 µg each). Lane 5: fraction number 11-15 (7 µl each). Lane 6: fraction number 16-20 (1 µg each). Lane 7: protein low molecular weight lyophilized marker (Amersham - 4µl). SDS-PAGE electrophoresis conditions: polyacrylamide concentration 14% separating gel and 4% stacking gel. Voltage applied: 11.54 V / cm. run time: 90 min.

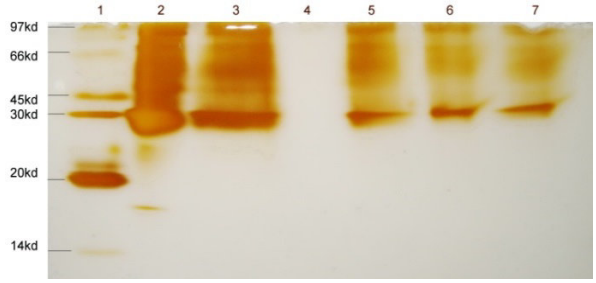

Figure 5. Silver stained lyophilized positively charged proteins of rabbit seminal fluid electrophoresed on native-PAGE after being fractionated by DEAE anion exchanger. Lane 1: protein low molecular weight lyophilized marker (Amersham - 4µl). Lane 2: rabbit seminal fluid (4.5µl). Lane 3: fraction number 2-5 (10µg). Lane 4: empty. Lane 5: fraction number 6-10 (7µg). Lane 6: fraction number 11-15 (7µg). Lane 7: fraction number 16-20 (7µg) Native-PAGE electrophoresis conditions: polyacrylamide concentration 14% separating gel and 4% stacking gel. Voltage applied: 11.54 V / cm. run time: 90 min.

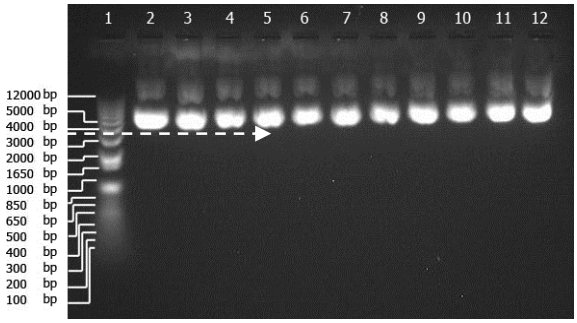

Figure 6. DNA retardation activity profile of rabbit DEAE cellulose positively charged seminal fractions after incubation of variable concentration of fractions with gWizGFP DNA. Lane 1: 12µl DNA size marker (Invitrogen). Lane 2: 2µg gWizGFP vector. Lane 3 to lane 12: 2µg gWizGFP vector with (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) µg of DEAE seminal fractions. Electrophoresis conditions: agarose concentration 1%, power applied: 4.1 V / cm, time of run: 1 hr.

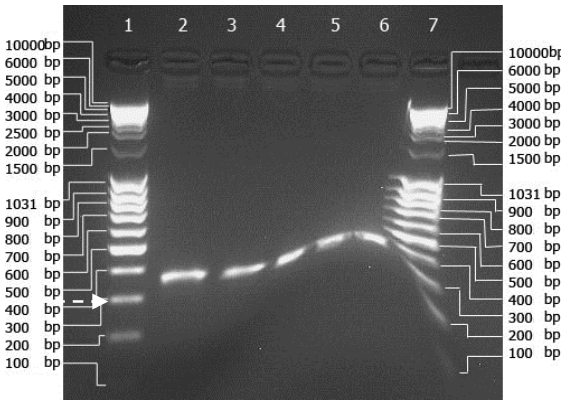

Figure 7. DNA retardation activity profile of rabbit DEAE cellulose positively charged seminal fractions after incubation of variable concentrations of these fractions with 364bp PCR fragment. Lane 1: 1.5µg DNA size marker (Fermentas). Lane 2: 0.5 µg 364bp PCR fragment. Lane 3 to lane 6: 6 µg 364bp PCR fragment incubated with (1, 5, 10, 15)µg DEAE cellulose eluted positively charged proteins respectively. Electrophoresis conditions: agarose concentration 2%, power applied: 5.5 V / cm, time of run: 1 hr.