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Gaurav Singhal[1]

[1] University of Adelaide

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Abstract

Problem: The sequential changes in the immunogenicity of spermatozoa in male reproductive tract and the effect of seminal vesicle secretions are long thought to act as central players in influencing immunological equilibrium in the male reproductive tract.Method of Study: Popliteal lymph nodes of mice were collected on the 8th day after sensitizing them with the testicular and epididymal spermatozoa of boar, weighed, dissociated into a cell suspension and the white blood cells were counted using haemocytometer.

Index terms— immunosuppression, popliteal lymph node, secondary immune response, seminal vesicle, testicle.

1 Introduction

he spermatozoon has an immune privileged status in the testis [1][2][3][4] . Once ejaculated in the female reproductive tract, spermatozoa act as the potential target for the female immune system due to their foreign nature 5 . Females exposed to spermatozoa have shown an increase in the weight of lymph nodes that drain the reproductive tract even though there is an immunosuppressive effect of seminal plasma 6 . However, in spite of the fact that single physiological exposure to semen by natural insemination initiates an immune response involving the lymph nodes which drain the uterus, a significant immune reaction rarely occurs in females even with frequent coital activity 7 , the reason for which is still not known. Although, factors like immune insult from bacterial infections 8,9 , and female sex hormones 10 have been shown to influence the viability of spermatozoa and immune response against them in females.

The secretions from the accessory sexual glands also affect the immunogenic property of spermatozoa in each ejaculation. The immunosuppressive components obtained from the seminal fluid have been found to reduce B lymphocyte activity to mitogens11. In addition, seminal proteins coating on sperms is essential for several processes in female reproductive tract, such as formation of the oviductal sperm reservoir, sperm capacitation, oocyte recognition and sperm binding to the oocyte **??**2. Indeed, seminal plasma, containing cytokines and prostaglandins, is believed to provide the physiologically protective environment to the highly antigenic spermatozoa in female reproductive tract **??**1,13-17. Dostalet al. found reduction in the number of white blood cells and decrease in the activity of plaqueforming cells after injecting the immunosuppressive components of boar seminal plasma into the rectum of female mice18. It has been suggested by researchers that this immunosuppressive effect of seminal plasma may also compromise the immune system in females for viral and bacterial attack **??**1,18-21. The immunosuppressive components of boar seminal fluid lead to the suppression of primary and secondary immune response and delay in the production of immunoglobulin G and immunoglobulin M antibodies to boar epididymal spermatozoa and to bacterial antigens **??**2. Researchers have also demonstrated that seminal leukocytes are responsible for the phagocytosis of morphologically abnormal spermatozoa in the semen **??**7,23. In some women, genital secretions and the serum showed the presence of sperm antibodies and this raises the question as to whether these sperm antibodies are produced in response to the immunogenicity of spermatozoa in reproductive tissues or it is a transudate from the serum 24 . However, the titre of the antibodies to spermatozoa is generally lower in serum than in genital secretions which supports the hypothesis that these antibodies are produced in response to spermatozoa in the genital tract and not in the serum 25 . Formation of anti-sperm antibodies has been established as an important cause of both male and female infertility, especially in humans **??**6,27. The aim of the current study is to investigate variations in the immunogenicity of spermatozoa, as they move from rete testis to different locations in epididymis, using

popliteal lymph node assay in mice. Estimation of the effect of seminal fluid on spermatozoa antigenicity and the secondary immune response to spermatozoa were also included during our work.

2 II.

3 Materials and Methods

4 a) Animals

Ethics approval to conduct research on animals was taken from the James Cook University (JCU) Animal Ethics Committee prior to the commencement of study (Approval number A 1191).

5 i. Boars

Male pigs were purchased from a pig farmer at 3-4 weeks or 16 weeks of age and grown to 12 months of age using standard husbandry practices within the animal facilities of the School of Veterinary and Biomedical Sciences, James Cook University (JCU), Townsville.

ii. Mice Female Balb/c mice 12-15 weeks of age were used for the lymph node bioassay. The mice were obtained from the rodent facility of the School of Veterinary and Biomedical Sciences at JCU. Food was withheld for 12 hours and the boar pre-medicated with an intramuscular injection of atropine (Apex Laboratories Pty. Ltd., Somersby, New South Wales, Australia) at 5 mg/kg body weight. Surgical anaesthesia was induced with intramuscular injections of xylazine hydrochloride (Ilium xylazil-100; Troy Laboratories Pty. Ltd., Smithfield, New South Wales, Australia) at 1 mg/kg body weight and ketamine (Parnell Laboratories Pty. Ltd., Alexandria, New South Wales, Australia)at 6 mg/kg body weight. Once anaesthesia was induced, the scrotum was prepared aseptically and 5 mls of local anaesthetic (Lignocaine 20; Troy Laboratories Pty. Ltd., Smithfield, New South Wales, Australia)was injected under the scrotal skin along the intended site of incision. A vertical incision of about 8 cm in length was made on the skin of the scrotum. The incision was deepened through the subcutaneous tissue and spermatic fascia to reach the parietal vaginal tunic which was then excised to expose the testicle. The testicle with attached epididymis and spermatic cord was extruded out. A large haemostat was applied to the spermatic cord proximal to the pampiniform plexus and three simple interrupted sutures (6.0 metric chromic catgut) were applied to the spermatic cord. The spermatic cord was cut ventral to the sutures and the testicle removed by incising the spermatic fascia and the scrotal ligament. The testicle was held in a vertical position for 2-3 minutes in order to drain out as much blood as possible. Immediately after that, it was placed in an insulated box containing frozen cold blocks until spermatozoa were collected in the laboratory. Simple interrupted sutures (3.5 metric chromic catgut)were used to suture the parietal vaginal tunic and scrotal muscles and the scrotal skin was closed with mattress sutures (Vicryl 3.0 metric; Johnson and Johnson, North Ryde, New South Wales, Australia). The boar was given an intramuscular injection of 1200 mg oxytetracycline (Engemycin 100; Intervet Australia pty. Ltd., Bendigo, Victoria, Australia) in the neck muscles for preventing any post-operative infections. c) Collection of the second testicle and seminal vesicles Each boar was sent to the Charters Towers abattoir four to five weeks after the unilateral castration. The testicle and seminal vesicles were collected immediately after slaughter, placed in an insulated box containing frozen cold blocks and bought back to the laboratory at School of Veterinary and Biomedical Sciences, JCU. The interval between slaughter and collection of seminal vesicle fluid and spermatozoa was between two and two and half hours. Spermatozoa were collected from the caput, corpus and cauda epididymidis, as well as from the rete testis (Fig I) into sterile 15 ml graduated conical tubes (Falcon 2096; Beeton Dickinson Labware, Franklin Lakes, New Jersey, USA). Seminal fluid was also collected into Falcon tube by incising the seminal vesicle and aspirating the contents with a sterile pipette.

6 d) Collection of spermatozoa from testis and epididymis

Spermatozoa from the caput, corpus and cauda epididymidis, and rete testis were collected and suspended in normal saline at concentrations of 2x103, 2x105, 2x107/ml. The caput, corpus and caudal epididymal spermatozoa were collected by taking incisions on the caput, corpus and cauda, aspirating the contents and placing it into sterile Falcon tubes containing 1 ml of sterile normal saline. Spermatozoa were collected from the rete testis by excision of the mediastinum and aspirating the contents.

7 e) Determination of the concentration of spermatozoa

The concentration of spermatozoa was determined in each sample using a Hamilton Thorne sperm analyser. Half hour before the analysis, the HTM-IVOS analyser version 10 (Hamilton Thorne; Beverley MA, USA) was turned on in order to acquire the working temperature of 39ºC. The temperature of the four compartmented 20 micron deep analysis chamber (Standard count, Leja, Nieuw-Vennep, Netherlands) was set at 39ºC and then the chambers were loaded with the semen samples by capillary action. This was followed by the loading of the analysis chamber into HTM-IVOS analyser and the spermatozoa concentration in each sample was determined. The final calculations to obtain the required concentration were done manually using a calculator.

8 f) Washing of spermatozoa

The samples were then added to sterile normal saline to make a final volume of 14 1200 rpm (207.24 g) for 10 minutes. The supernatant was discarded and the sperm pellet re-suspended and washed in 14 ml of normal saline and centrifuged again. The spermatozoa were then re-suspended in normal saline to the required three concentrations.

9 g) Injection of mice and collection of popliteal lymph nodes

Fifty µl of each sample were injected subcutaneously with a 25 G needle and a 1ml syringe just above the right hock of the mouse. Three mice were used for each sperm concentration, source of spermatozoa, diluent and time period. A control injection of 50 µl of sterile saline was injected subcutaneously above the left hock. At four, eight and twelve days after the injection, the mice were killed with CO2 gas and both popliteal lymph nodes were carefully removed, placed in normal saline, adhering fat removed under a stereomicroscope, blot dried and weighed in Sartorius analytical balance (maximum capacity = 120 g; readability = 0.1 mg; repeatability = 0.1 mg; linearity = 0.2 mg; weighing units = g, mg, kg, oz t, ct).

10 III.

Full Experimental Protocol a) Primary immune response Spermatozoa were collected from the rete testis and caput, corpus and cauda epididymidis from ten testes, prepared, re-suspended in normal saline and injected in mice as described in previous sections. The mice were killed eight days later and the popliteal lymph nodes weighed as described above. The lymph nodes were then dissociated into a cell suspension in 1.5 ml conical eppendorf tubes by meshing it with a sterile cell strainer in 1 ml normal saline and the number of white blood cells enumerated using a haemocytometer. The response to the lymph nodes was calculated as a stimulation index based on weights of test and control lymph nodes as well as a stimulation index based on the number of cells in the test and control lymph nodes. The repeatability of the response between the two testes and epididymis of each boar was also examined.

11 b) Secondary immune response

The secondary immune response to spermatozoa from four boars was examined. Groups of three mice were injected with spermatozoa from the rete testis and caput, corpus and cauda epididymidis. When the boar was slaughtered four to five weeks later, the mice were injected again near the popliteal lymph node and killed eight days later. The stimulation indices based on the weight and cell numbers in the lymph nodes were calculated as above. c) Influence of seminal vesicle fluid on the primary immune response Fluid from the seminal vesicles was collected from seven boars and kept at room temperature until sperm samples were being prepared. In the first group of experiments, spermatozoa were prepared in normal saline as well as seminal vesicle fluid and injected into mice as described previously. In a second group of experiments, 2x107 spermatozoa were incubated in 1 ml of seminal fluid for 15 minutes at 390 C. The samples were then centrifuged at 207.24 g for 10 minutes, the supernatant removed and spermatozoa re-suspended in 14 ml of normal saline. The process was repeated twice before suspending spermatozoa in 1 ml normal saline for injection. In the third group of experiments, seminal vesicle fluid from six boars was injected into groups of four mice with sterile normal saline as control to determine the response to seminal vesicle fluid alone. The stimulation indices based on the weight and cell numbers in the lymph nodes were calculated as above.

12 d) Statistical analyses

A descriptive analysis was carried out on the data obtained using Microsoft excel and SPSS software. A parametric or non-parametric test was performed depending upon the nature of sampling distribution and the satisfaction of basic assumptions of the tests. One way ANOVA or Kruskal-Wallis test were used to find the significant differences among various samples in a group or among groups. Linear regression was used to find the relationship between mean lymph node weight stimulation index and mean cellularity index for all the groups. The results were expressed as Mean ± Standard Error and the p value was calculated at 95 % confidence interval i.e., p? 0.05.

13 IV.

14 Results

15 a) Immunogenic effect of spermatozoa in normal saline

Irrespective of the boar, the overall mean lymph node weight stimulation index value for the four samples declined from rete testis towards the corpus epididymidis before it increased to maximum for the cauda epididymidis (Table **??**).

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Table ?? : The mean (± SEM) lymph node weight stimulation index of murine popliteal lymph nodes stimulated by porcine spermatozoa from the rete testis, caput epididymidis, corpus epididymidis and caudaepididymidis in five groups

Similarly, looking into the cellularity index values (Table ??I), the mean cellularity index increased from the corpus epididymidis to the cauda epididymidis.

However, unlike the weight stimulation index values, the cellularity index values for caput epididymidis was higher than the cellularity index values for the rete testis.

Table ?? : The mean (± SEM) cellularity index of murine popliteal lymph nodes stimulated by porcine spermatozoa from the rete testis, caput epididymidis, corpus epididymidis and caudaepididymidis in five groups b) Immunogenic effect of spermatozoa in seminal fluid Irrespective of the boar, the overall mean for the lymph node weight stimulation index was almost the same for the four samples of spermatozoa in seminal fluid (Table ??). Still, the highest mean lymph node stimulation index is seminal fluid groups was observed for spermatozoa from the caput epididymidis and the minimum was for spermatozoa from the corpus epididymidis.

The mean cellularity index among spermatozoa in seminal fluid groups decreased from the rete testis to the corpus epididymidis before increasing again for the cauda epididymidis (Table ??I).

17 c) Immunogenic effect of spermatozoa incubated in seminal fluid

In contrast to all the previous findings, the lymph node weight stimulation index was least for the cauda epididymidis (Table ?? (D D D D) G

On analyzing the mean values for cellularity index in case of four samples of spermatozoa incubated in seminal fluid (Table ??I), we found that the mean cellularity index was almost same for all the four samples. d) Immunogenic effect of seminal fluid Seminal plasma from six boars was used to test the immunogenic effect of seminal plasma alone compared to saline controls.

The mean lymph node weight stimulation index value of seminal plasma alone was higher than for spermatozoa suspended in the normal saline and for spermatozoa incubated in the seminal fluid but lower than for spermatozoa suspended in the seminal fluid (Table ??). The mean cellularity index value also followed the same pattern (Table ??I). e) Immunogenic effect of spermatozoa in secondary immune response group Irrespective of the boar, the overall mean for the lymph node weight stimulation index among secondary immune response groups (Table ??) was least for the rete testis and increased to highest for the cauda epididymidis.

The mean cellularity index followed the same trend as the mean lymph node stimulation index (Table ??I) except that corpus epididymidis had lower mean cellularity index value than caput epididymidis.

In all of the above experiments, few findings were similar:

a) The popliteal lymph node weight stimulation index and cellularity index were highly variable for spermatozoa from rete testis but variance was least in case of the spermatozoa from cauda epididymidis being almost half of the rete testis. b) A positive relationship can be seen between the mean lymph node weight stimulation index and mean cellularity index indicating that the samples with a higher popliteal lymph node weight index also have higher cellularity index.

V.

18 Discussion

The results from the normal saline group suggest maximum immunogenicity of the caudal epididymal spermatozoa and least of the corpus epididymal spermatozoa among 4 groups. The immunogenicity of spermatozoa seems to decrease from the rete testis to corpus epididymidis before increasing for cauda epididymidis which is evident by the mean lymph node weight stimulation index as well as the mean cellularity index. The highly variable immunogenicity of spermatozoa taken from the rete testis indicates that some factors in the process of formation of spermatozoa in testis also determine the immunogenic trait of spermatozoa and this needs further evaluation.It is also clear that the groups with higher lymph node stimulation index also have a higher cellularity index. Some workers however have described the cellularity index attribute as more sensitive, informative and accurate than lymph node stimulation index ??8-30. The role of seminal fluid as an immunosuppressive agent to spermatozoa has been described by many workers in the past 11,15,17,18,20,22,31 . But the extent to which seminal fluid is responsible for the overall immunosuppressive effect on spermatozoa among many other probable factors has not been described before. The increase in the mean lymph node weight stimulation index from the corpus epididymidis to cauda epididymidis again confirms greater immunogenicity of spermatozoa in the cauda epididymidis. The seminal fluid alone does not seem to have any immunosuppressive effect which is clear from the results obtained. Instead, the results suggest that the seminal fluid is responsible for the increase in immunogenicity of spermatozoa.

The higher variability for rete testis spermatozoa further indicates that some factors involved in the formation of spermatozoa are responsible for variable immunogenicity. As these spermatozoa moves from the rete testis towards the cauda epididymidis, the immunogenicity seems to decrease initially until the corpus epididymidis and then it again increases for the cauda epididymidis. One possible cause for this increase might be the metabolic

activities that are taking place in spermatozoa while stored in the cauda epididymidis temporarily 32 change the antigenic proteins on the surface of spermatozoa during storage ??2-36. Immunosuppressive fractions of seminal fluid have already been isolated before by some of the workers and their immunosuppressive effect on spermatozoa has been demonstrated 12 . The effect of incubation on spermatozoa is immunosuppressive which is evident from the results obtained. But the values are slightly higher for each location than the normal saline group indicating the residual immunogenic effect of seminal proteins even after two washings with normal saline. However, the values were much lower than for spermatozoa suspended in seminal fluid indicating that two washings of spermatozoa in normal saline removed most of the adherent antigenic seminal proteins.

The results obtained show a high variance value for the caput epididymidis for the spermatozoa incubated in seminal fluid. But the variance for other rete testis and caput epididymidis followed by a progressive decrease towards the corpus epididymidis and the cauda epididymidis.

sites is less following the same decreasing trend from the rete testis to the cauda epididymidis. This perhaps indicates that spermatozoa with highly variable immunogenicity in the rete testis acquire almost the

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Year 2015 same immunogenicity level while stored in the cauda epididymidis though lower than the rete testis and caput epididymidis but higher than the corpus epididymidis. The spermatozoon after incubation in seminal fluid has the least immunogenicity for the cauda epididymidis suggesting that caudal spermatozoa loses maximum immunogenicity, more than corpus spermatozoa in seminal fluid.

The seminal fluid alone seems to be more immunogenic then spermatozoa in normal saline and spermatozoa incubated in seminal fluid by both the mean lymph node weight stimulation index and mean cellularity index. Conversely, the seminal fluid alone is less immunogenic then spermatozoa suspended in the seminal fluid. This could probably be due to the additive effect of immunogenicity of spermatozoa on the immunogenicity of seminal fluid. Since the spermatozoa incubated in seminal fluid are less immunogenic then seminal fluid alone, it indicates that the twice washing with normal saline has probably eliminated most of the immunogenic proteins of seminal fluid. Spermatozoa left after incubation and washed with normal saline were less immunogenic then the spermatozoa in seminal fluid possibly due to the immunosuppressive effect of some of the components of seminal fluid on spermatozoa during incubation.

The secondary immune response could be important for determining the fertility in both males and females. This is because after the first few intercourses, the predominant immune response in females with only one male partner will be the secondary immune response. On the other hand, the primary immune response could be important for the animals with multiple partners.The results obtained for the secondary immune response are contrary to earlier results in terms of the mean lymph node weight stimulation index and mean cellularity index. The immunogenicity of spermatozoa increases from the rete testis to cauda epididymidis; however the highest immunogenicity is for the spermatozoa from caput epididymidis than the spermatozoa from rete testis. However, the results obtained for secondary immune response were not statistically significant and also there was no linear relationship observed between the lymph node weight stimulation index and cellularity index. In addition, a lower immunogenic response was seen for secondary immune response then for spermatozoa in normal saline and seminal fluid. This was probably due to the occurrence of peak immunogenic response in mice at earlier than eighth day so that on the eighth day, the immune response was in the decline phase.

Overall, it is clear that the mean lymph node weight stimulation index and mean cellularity index among five groups are in the following order:Spermatozoa in seminal fluid group > seminal fluid only group > normal saline group > incubated seminal fluid group ? secondary immune response VI.

20 Concluding Remarks

Our study is the first evidence to suggest that there is a well-developed mechanism in the male reproductive tract to suppress the antigenicity of spermatozoa before ejaculation.This is also the first instance when an effort has been made to determine the immunogenicity of spermatozoa in different parts of the testes and epididymis. While higher values for the spermatozoa in seminal fluid group could probably be due to additive effect of antigenicity of seminal proteins and spermatozoon surface proteins, the higher value for the seminal fluid only group could be due to the antigenic effect of only seminal proteins. Similarly, the marginally higher values for spermatozoa incubated in seminal fluid could be due to the residual immunogenic effect of seminal proteins along with the immunogenic effect of spermatozoon surface proteins. Finally, the lowest value for secondary immune response group among all samples could probably be due to the initiation of immunogenic mechanism and recovery phase at the earlier stage than in the primary immune response. Although, decrease in the antigenicity of spermatozoa is evident in the male reproductive tract, substantial evidence are still required to confirm the hypothesis that seminal and spermatozoa surface proteins play a role in this process.

21 VII.

22 Future Aspects

Further studies are required for determining the type and the strength of immune response in females to spermatozoa during both primary and secondary immune response, the role of humoral and cellular immune system during this process and the factors responsible for altering the immunogenicity of spermatozoa in female reproductive tract. In addition, more studies are required to completely understand the immunogenicity of spermatozoa and its variability as it moves from the cauda epididymidis to the exterior at ejaculation. These studies may play an important role in understanding the exact role of immunological response to spermatozoa on fertility in mammals.

23 VIII. Conflict of Interest Statement

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. [1] [2]

Figure 1:

Year 2015

Figure 2: T

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Site		N	Mean±SEM	Minimum	Maximum	Range	Variance
RT+NS		10	2.3965± 0.26724	0.89	3.4		
CPT+NS		10	2.1699± 0.21106	1.13	3.3		
CPS+NS		10	1.8752± 0.25619	0.77	3.23		
CDA+NS		10	2.4773± 0.19306	1.66	3.54		
RT+SF		7	3.3131 ± 0.51335	2.12	6.28		
CPT+SF		7	3.3629 ± 0.46752	2.14	5.89		
CPS+SF		7	3.0621 ± 0.63132	1.5	6.3		
CDA+SF		7	3.1139 ± 0.44144	1.7	4.88		
RT+ISF		5	2.1094 ± 0.20466	1.48	2.75		
CPT+ISF		5	2.3892 ± 0.80524	1.1	5.56		
CPS+ISF		5	1.7604 ± 0.26991	0.93	2.54		
CDA+ISF		5	1.5972 ± 0.14358	1.06	1.88		
RT(SIR)		3	1.6190 ± 0.31072	1.11	2.18		
CPT(SIR)	N	4	1.7748 ± 0.19161 1.6608 ±	1.45	1.05	1	1.54 Minimum Maximum 2.27 2.16 3.23 4.5
CPS(SIR)		4	0.24145 1.9650 ± 0.47160				
CDA(SIR)		4	2.8367 ± 0.39930 Mean ±				
NS and SF		6	SEM				
Site							

RT+NS	10	25.3610 ± 4.04052	7.47	50.39
CPT+NS	10	26.0980 ± 4.46078	11.07	50.63
CPS+NS	10	18.9400 ± 3.12247	5.99	34.52
CDA+NS	10	24.1870 ± 3.53647	12.03	45.28
RT+SF	7	40.3614 ± 10.6637	20.07	102.46
CPT+SF	7	39.8729 ± 7.2426	21.86	68.63
CPS+SF	7	33.9429 ± 4.37092	19.05	52.88
CDA+SF	7	36.1857 ± 10.39975	11.98	94.72
RT+ISF	5	19.152 ± 3.40075	10.93	31.42
CPT+ISF	5	20.0760 ± 4.40577	9.18	32.24
CPS+ISF	5	19.0460 ± 2.50037	10.55	23.95
CDA+ISF	5	20.8120 ± 5.31957	4.65	32.57
RT(SIR)	3	14.8467 ± 0.77102	13.74	16.33
CPT(SIR)	4	17.1850 ± 2.63916	12.61	22.42
CPS(SIR)	4	15.4825 ± 2.97052	8.96	22.12
CDA(SIR)	4	21.2275 ± 5.13919	12.8	36.17
NS and SF	6	33.385 ± 4.76468	14.19	44.86

Figure 3:

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