Chapter 6

6.0 EXPERIMENT 3

TITLE: FLUORESCENCE IMAGING OF MOUSE SPERM

6.1 ABSTRACT

The protocol for detecting acrosome in mouse sperm was developed by using two types of fluorescent dyes namely fluorescein conjugated *Pisum sativum* agglutinin (FITC-PSA) and rhodamine conjugated *Lens culinaris* agglutinin (TRITC-LCA). The results showed that mean percentages of acrosome intact sperm incubated *in vitro* were considerably low. After one-hour incubation there were 43.20±2.83%, 31.81±8.44% and 30.90±4.15% for ICR, C57/6J and F_1 sperm, respectively. Intact acrosome in ICR sperm was significantly reduced ($p<0.05$) to 16.95±4.23% after two-hour incubation, however, insignificant reduction ($p>0.05$) was found for C57BL/6J and F_1 sperm, which were 30.03±2.06% and 20.93±3.81%, respectively. The results showed that both dyes FITC-PSA and TRITC-LCA suitably stained the mouse sperm and produced insignificant difference ($p>0.05$) in the percentages of acrosome intact sperm, which were 40.03±4.20% and 49.79±4.63%, respectively. One-hour incubation was adequate to capacitate sperm *in vitro*. Low acrosome intact due to premature acrosome reaction during extended period of incubation may compromise *in vitro* fertilisation rates.

Keywords: Sperm, acrosome, lectin, Pisum sativum agglutinin, insemination

6.2 INTRODUCTION

Fertilisation resulted from the union of both paternal and maternal nuclear materials to form a zygote. However, the processes that precede successful fertilisation are very crucial to the sperm cell so as to enable it to fertilise the egg. The sperm must undergo

capacitation, the physiological state at which the sperm is ready for the acrosome reaction (AR). In *in vivo* fertilisation (IVO), mammalian sperm must undergo AR upon contacting the egg's zona pellucida (Jaiswal *et al*., 1998). This involves the reaction of the acrosomal bodies that lie at the tip of the sperm head releasing its hydrolytic enzyme to digest the zona pellucida, the acellular layer through which the sperm would penetrate to fertilise the egg. Thus, intact acrosome is required before a sperm meets the egg's zona pellucida. Not every sperm is successful in maintaining its integrity through-out the journey prior to the fertilisation process. Many cannot survive the challenges imposed by the *in vivo*, as well as *in vitro* environment. One of the negative impacts concerning sperm capacitated *in vitro* is acrosomal loss. Acrosomal loss among mammalian sperm incubated *in vitro* has been widely reported, including mouse sperm. This is one of the examples of defective parameter reported in many cases of *in vitro* fertilisation failures. Fraser (1981) reported that approximately 30-35% of acrosomal loss incidences occurred in mouse sperm incubated *in vitro* in synthetic media and a premature acrosome reaction leads to the loss of zona pellucida recognition sites from the surface of the sperm (Lui and Baker, 1994). This would eventually compromise the number of egg being fertilised *in vitro* (IVF). A precise timing of the acrosome reaction was shown to be required for fertilisation (Green *et al*., 1999). It was suggested that optimum period of *in vitro* incubation of human sperm is at least 0.6-2.2 hours (Jaiswal *et al*., 1998), whereas capacitation can be achieved in mouse sperm incubated for 2 hours in the capacitation medium, at 37^{o}C in a 5% CO_2 environment (Toshimori *et al*., 1998).

Differences in the successful rate of IVF have been significantly observed in various strains of mice (Sztein *et al*., 2000). When morphologically similar sperm produced low rate of fertilisation, this would suggest the need for a more comprehensive

study to identify the inherent defects, which render certain sperm unable to fertilise. The hidden anomalies may be at the sperm membrane level or at the chromatin level which should also be evaluated. Numerous studies have sought a key factor that would be predictive for male infertility including acrosome status. Acrosome intact is not only important in IVO or IVF, but also in intracytoplasmic sperm injection (ICSI). Khalili *et al*. (1998) has reported total fertilisation failure when round headed acrosomeless human sperm were injected into the MII oocytes. They further assumed that acrosomes were necessary for oocyte activation after ICSI. In addition, sperm with small acrosomes are more susceptible to cell death and non-physiological acrosomal loss (Roelof *et al*., 2003). Roelof *et al*. (2003) also concluded that acrosome size reflects the physiological capability of sperm function and, therefore, male fertility potential.

Fluoresceinated plant lectins, have been widely used to study mammalian sperm surface and organelles because of their ability to specifically bind with α-linked mannose sugar (Cox *et al*., 1998) and high saccharide binding specificities (Magargee, 1988). In general, *Pisum sativum* agglutinin (PSA) (Chan *et al*., 2002) is the lectin of choice in many laboratories to stain acrosome of mammalian sperm. To date, there is no specific report of usage of conjugated *Lens culinaris* agglutinin (LCA) for staining mouse acrosome. Although Tiziana *et al*. (2005) has reported that LCA could stain the acrosome of porcine sperm as well as oocyte cortical granules, but because of interspecific variations in membrane constituents, its use on mouse sperm requires a preliminary validation.

Much attention was given on oocyte parameters as the key factors to IVF performance. Nevertheless, sperm quality is equally important while carrying out IVF such as the intactness of acrosome prior to insemination. This attempt is very important especially when IVF results were severely affected. The IVF protocol in mice generally

uses 0.5 hour for capacitation followed by 3 to 4 hours for insemination in fertilising medium. However, there was little information reported on cellular integrity of sperm being exposed to varying periods of insemination hours in different strains of mice. It is believed that sperm of different strains of mice would have different survival and fertilising capabilities especially in *in vitro* environment. Eventually, sub-quality sperm may affect IVF end results. Hence, in this study, our aims were; to develop the fluorescence staining protocol for the detection of mouse sperm acrosome; to compare the efficiency of using two types of fluorescent dyes namely fluorescein conjugated *Pisum sativum* agglutinin (FITC-PSA) and rhodamine conjugated *Lens culinaris* agglutinin (TRITC-LCA) for staining the acrosomes and to determine the effect of *in vitro* incubation duration on acrosome integrity (intact or loss) of mouse sperm. We hypothesised that differences in IVF results between inbreds and outbreds counterparts could be due to their varied physiological tolerance of the sperm in *in vitro* culture. General anomalies of sperm were also observed in this experiment.

6.3 MATERIALS AND METHODS

The materials and procedures included in this experiment were described in the following subsections.

6.3.1 Animals

Inbred male mice consisted of ICR (albino) and C57BL/6J (ebony) and the outbred counterpart (F_1) from the crossed between CBA X C57/6J, aged 8-16 weeks were used in this study. It is hypothesised that the poor IVF records from the inbreds as compared to that of the outbred counterpart could be due to their varied physiological tolerance of the sperm in *in vitro* culture. The animals were maintained *ad libitium* in the Animal House of

Institute of Biological Sciences, Faculty of Science, Universiti Malaya. Several males were sacrificed for the study. However, for the result purposes only the sperm preparations that produced high quality fluorescent images obtained from 7 ICR, 4 C57BL/6J and 5 F_1 hybrids males were included. A 2x3x2 replicates was prepared for each male in which two collections each from the left and right caudas were separately incubated in triplicates and each sample was stained in two replicates for each stain.

6.3.2 Sperm Preparation

The sperm was collected from the cauda epididymides of the mature male mice. The dense sperm mass was squeezed off the cauda and minced into 400 µl of Toyoda, Yokoyama and Hoshi (TYH) medium (Toyoda *et al*., 1971) covered with silicon oil (R&M Chemicals). The medium was pre-incubated in 5% $CO_{2,}$ at 37^{o}C overnight. The sperm were allowed to disperse for 1 hour and then diluted further into three separate microdrops of TYH medium each containing approximately 2.0x10^{6} sperm/ml. The sperm were subsequently incubated for another one and two hours, separately inside the CO_2 incubator until staining.

6.3.3 Fluorescence Staining

Prior to staining, sperm suspension was diluted by transferring 10 µl of sperm into 100 µl TYH medium. Of the reconstituted sperm 10 µl was then transferred onto a pre-cleaned cover slip and allowed to dry on a slide warmer at 37^{o}C. The cover slip was then immersed into 3 ml of paraformaldehyde (2%) for two hours. The cover slip with fixed sperm was then washed in 0.1 M phosphate buffer solution (PBS) containing TritonX-100 (0.5%) inside a 6-well culture dish five times, five minutes each. Excess solution was dabbed with tissue papers. The sperm was then permeabilised in 3 ml of TritonX-100 (1%) for one hour.

The acrosomes were directly stained with FITC-PSA (12.5 µg/ml) or TRITC-LCA (10 µg/ml). Both were supplied by Vector Laboratory. A 50 µl of either stains was added onto the sperm-containing cover slip, and covered with a petridish to prevent dehydration and incubated inside a humidified incubator for one hour. Following the final washing step, the nuclear materials of the sperm was stained with bis Benzimide Hoechst 33258 (10 µg/ml) (SIGMA) for 30 minutes, followed by series of washing and finally mounted onto a drop of 1:4 glycerol and water that contained antifade agent (1%) of 1,4-diazabicyclo-[2,2,2]octane (DABCO, SIGMA) on a glass slide. At each time, the sperm was stained in duplicates. The coverslips were sealed with nail polish, labeled and wrapped with aluminium foil. They were stored at 4^{o}C inside a refrigerator. However, for high quality images, immediate observation is recommended under the fluorescent microscope. Approximately, a total of 200 sperm were counted at random in at least five different fields per slide. The percentage of the acrosome intact sperm were determined and averaged by counting either red (TRITC-LCA) of green (FITC-PSA) fluorescence emissions over the total numbers detected from the blue (Hoechst) fluorescence.

6.4 MICROSCOPIC ANALYSIS

Fluorescence images were mostly observed and analysed using Nikon Opthipot microscope that is equipped with BA520 and XF102-2 filters for green and red fluorescence emissions, respectively. Meanwhile, a compatible computer generated image analysis software Image-Express Pro-Plus of the Carl Zeiss inverted fluorescent microscope system was also used towards the end of the experiment.

6.5 STATISTICAL ANALYSIS

Descriptive analysis and one-way ANOVA of the results were conducted using SPSS software, version 11.0. Results are presented as mean of percentages ± SEM and significant test between means was executed using post-hoc Duncan multiple range test at the significant level $p<0.05$.

6.6 RESULTS

The galleries of fluorescence images of the mouse sperm are depicted in Figures 6.1 and 6.2. In general, all sperm cells that contain nuclear materials emitted blue fluorescence when stained with Benzimide Hoechst 33258. This has enabled us to detect and count the total numbers of sperm available as well as distinguish between morphologically normal and abnormal sperm heads (Figure 6.1d). In normal sperm, the acrosome was revealed as a thick ridge of apple green or red fluorescent along the convex contour of the sperm head when stained with FITC-PSA or TRITC-LCA, respectively (Figure 6.2). The ridge was not detected in acrosomeless sperm. The abnormal sperm heads included collapsed and triangular shape, absent or deformed acrosomes and nuclear materials (Kishikawa, 1999). Of the same cohorts, only some sperm revealed the acrosome structure as detected by TRITC-LCA or FITC-PSA dye. Hence, the percentages of acrosome intact and acrosomeless sperm could be determined. From the fluorescence images, the efficiency of using two different dyes namely FITC-PSA and TRITC-LCA was evaluated by counting the lectin-bound to total sperm ratio to obtain mean percentages of acrosome intact sperm (Table 6.1). The number of FITC-PSA and TRITC-LCA bound sperm were found to be insignificantly different ($p>0.05$), which were 40.03±4.20% and 49.79±4.63 %, respectively.

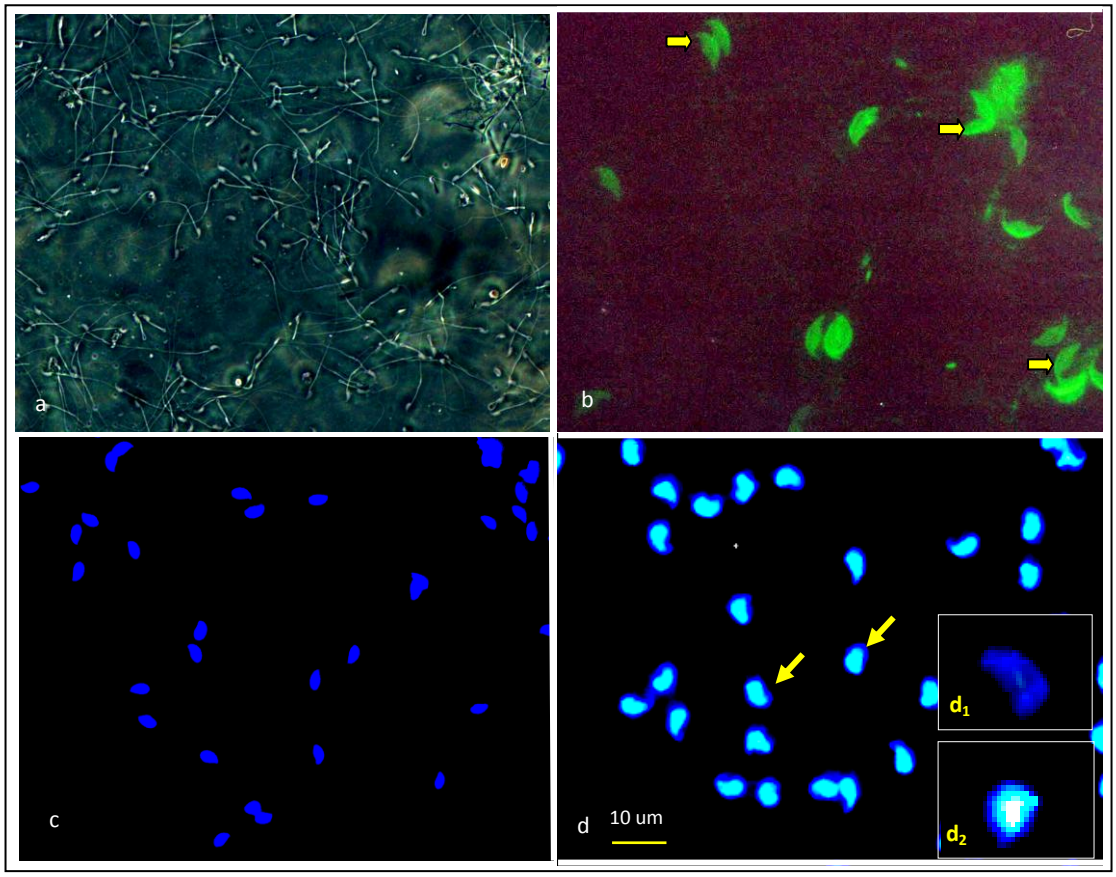

Figure 6.1: Sperm morphology. (a) Phase-contrast. (200x), (b) FITC-stained acrosomes, shown by arrows. (1000x), (c) Normal sperm heads stained with bis Benzimide Hoechst 33258. (1000x), (d) Abnormal sperm heads (arrows) (1000x). Insets (d_1 and d_2) were enlarged images of abnormal sperm heads (not according to the actual scale) showing irregular shape and deformed nuclear materials. Scale bar = 10 µm.

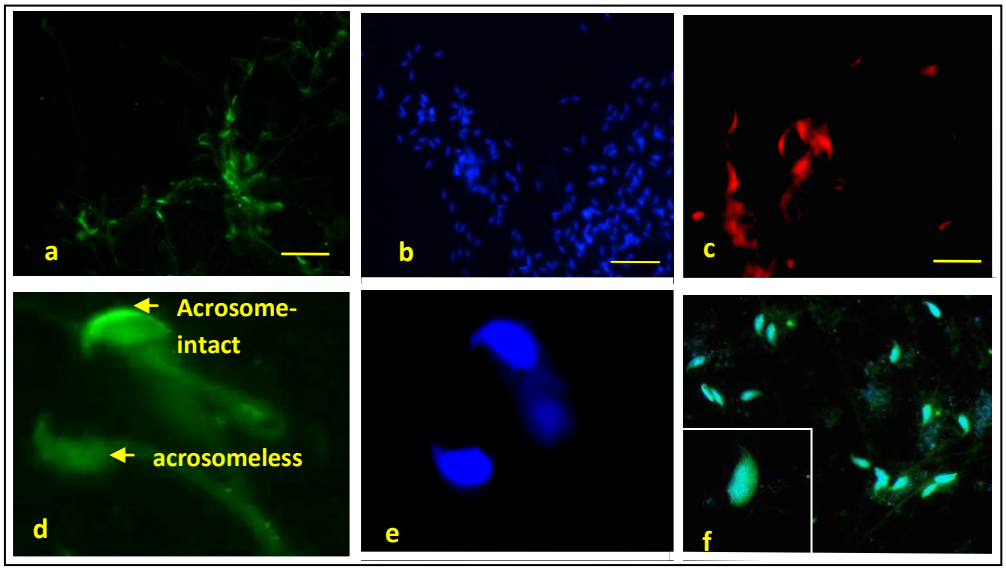

Figure 6.2: (a), (d) Acrosome intact and acrosomeless sperm (arrow heads) stained with FITC-PSA. (b), (e) nuclear materials stained with bis Benzimide Hoechst 33258 (SIGMA). (c) TRITC-LCA stained sperm. (f) Composite image of FITC-PSA and bis Benzimide Hoechst and inset (at bottom left) shows a digitally enlarged sperm image. a, b, c, f (400x); d, e (1000x). Scale bar = 10 µm.

Table 6.1: Mean percentages of acrosome-intact in ICR sperm stained with two types of fluorescent dyes during one-hour incubation

Fluorescent dye	Replication	Total Count	Acrosome intact (%) (Mean ± SEM)
FITC-PSA	27	4387	40.03 ± 4.20
TRITC-LCA	13	980	49.79 ± 4.63

Figure 6.3 highlighted the effect of incubation hour on sperm acrosome among three strains of mice. The values of acrosomes intact sperm are given as mean percentages ± SEM. For the purpose of easy comparison the values were tabulated in Table 6.2. At the end of the first hour of incubation, there were 43.20±2.83%, 31.81±8.44% and 30.90±4.15% of acrosome intact, respectively, in ICR, C57BL/6J and F_1 mice. However, the percentages were found to be lowered after the second hour of incubation period, which were 16.95±4.23% in ICR as compared with 30.03±2.06% and 20.93±3.8% in C57BL/6J and F_1, respectively. This was a significant reduction ($p<0.05$) for ICR sperm but not significantly different in the other two strains of mice ($p>0.05$).

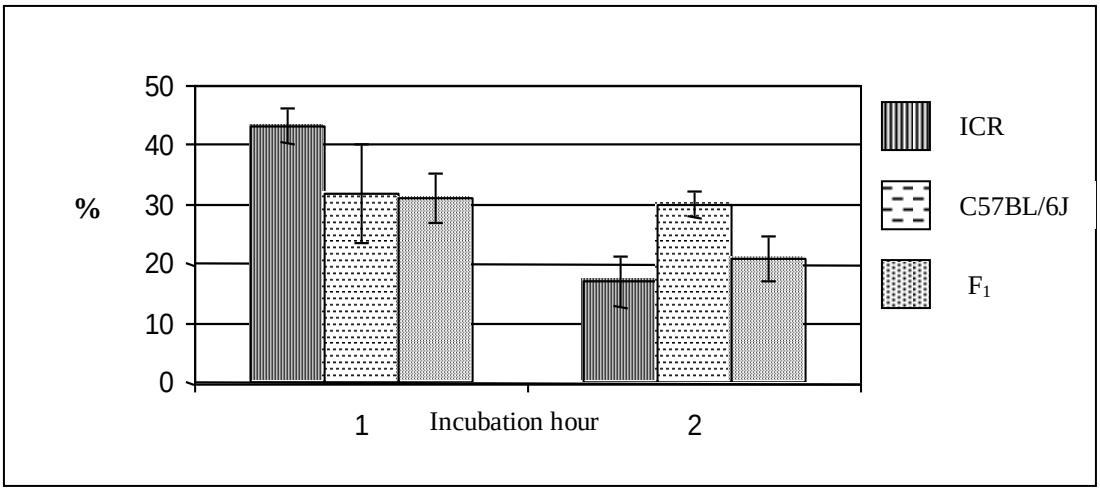

Figure 6.3: Mean percentages of acrosome intact sperm in various strains of mice.

Table 6.2: Mean percentages of acrosome intact in mouse sperm during *in vitro* incubation

Strain	Incubation hour	Replication	Total Count	Acrosome intact (%) (Mean ± SEM)
ICR	1	20	2265	43.20 ± 2.83^{a}
	2	9	702	16.95 ± 4.23^{b}
C57BL/6J	1	8	1472	31.81 ± 8.44^{ab}
	2	5	542	30.03 ± 2.06^{ab}
F_1	1	13	850	30.90 ± 4.15^{ab}
	2	5	501	20.93 ± 3.81^{ab}

Similar superscripts within columns indicated insignificant difference of results ($p>0.05$). (SEM – standard error of means). Superscripts^{a,b} within column are significantly different at $p<0.05$.

6.7 DISCUSSION

The ICC protocol for sperm staining has been successfully established using both FITC-PSA and TRITC-LCA. The results clearly showed that both dyes were equally suitable for staining the mouse acrosomes and might be having equivalent affinity with the acrosomal contents or similar membranous reaction. The finding validated the application of LCA, which was commonly used to stain cortical granules of oocytes, to detect mouse acrosomes as well. Hence, PSA and LCA can be categorised as galactose-positive lectins as *Arachis hypogaea* lectin (PNA), *Ricinus communis* lectin (RCA) and *Triticum vulgaris* lectin that strongly bound the anterior head region of epididymal sperm (Magargee *et al*., 1988), where acrosomes were localised.

Some degrees of acrosomal loss were detected in mouse sperm capacitated *in vitro.* The results revealed that more than 50% of the sperm were acrosomeless due to unknown reason. It could be due to defective factors such as premature acrosome reaction while *in*

vitro environment or incomplete morphological development. Perhaps, a majority of the sperm failed to attain maturation stage. The percentages were insignificantly different among the strains during the first hour of incubation. However, a strain-specificity of *in vitro* capacitation was observed after longer capacitation hour in TYH medium. A substantial amount of acrosomal loss in ICR sperm probably indicated its physiological intolerance to prolonged hours of *in vitro* incubation. This could be in line with variations in the membrane compositions of the sperm that triggered premature reaction of the acrosomes as reported by Yanagimachi (1989). A significant reduction of progressive movement was also reported in ICR sperm after 2.5 hours of *in vitro* incubation as compared with CBA and C57BL/6J sperm (Nor Hasma, 2006). Perhaps some modifications in the composition of the capacitation medium may be useful to certain strain of male mice during prolonged incubation period so as to reduce premature reaction of the acrosomes. A main change in medium composition has been reported to alleviate developmental block among *in vitro* produced mouse embryos (Biggers, 1998; Hadi *et al*., 2005). This could be one of the parameters attributed for the low percentages of acrosome intact sperm detected.

6.8 CONCLUSIONS

In conclusions, the findings of the study may be exploited not only to define a suitable fluorescent dye to stain mouse acrosomes but also as research model in sperm acrosome detection in other mammalian species. Mouse acrosome can be detected using either PSA or LCA conjugated fluorescent dye because both of them produced insignificant difference of results. In this study, it was found that one-hour incubation was appropriate to capacitate the sperm as shown by the highest mean percentages of acrosome intact as

compared with two-hour incubation in all the mouse strains. Prolonged incubation duration had severely reduced the percentages of acrosome intact in ICR sperm, which reflected a strain-specificity in *in vitro* capacitation. Among others, morphologically abnormal sperm might also have contributed to the low percentages of acrosome intact sperm incubated *in vitro*, eventually causing low rates of *in vitro* fertilisation. Therefore, sperm morphology reflects its physiological capability and function, thus male fertility potential (Roelof *et al*., 2003). In the Animal Biotechnology-Embryo Laboratory (ABEL), our IVF protocol requires the sperm to be capacitated one hour in CO_2 incubator prior to insemination. This had previously produced 90% fertilisation rates in selected strains, especially in hybrids mice (Faridah, 1994). However, we recently experienced reduction in fertilisation rates (Nooraain and Abdullah, 2003), in most of IVF attempts and one speculation was that the quality of sperm that had been jeopardised. The established ICC protocol had successfully proven the relationship between incubation hour and structural integrity of mouse sperm acrosomes, which could account for such results. Further study on other sperm parameters such as mitochondrial viability and membranous protein receptors during *in vitro* environment of sperm is highly recommended for a better understanding on sperm competency and fertilising capability.

REFERENCES

Biggers, J.D. 1998. Reflections on the culture of the preimplantation embryo. International Journal of Developmental Biology. 42:879-884.

Chan, J.Z., M. Krause and C. Bohring. 2002. Computer-assisted analysis of sperm morphology with the aid of lectin staining. Andrologia. 34: (Abstract).

Cox, J.F., P. Fernandez, F. Saravia and M.V. A. Santa Maria. 1998. Use of propidium iodide and *Pisum sativum* for fast assessment of acrosome integrity in goat spermatozoa. Archivos de Medicina Veterinaria. 30:(Abstract).

Faridah, M. 1994. Kesan usia mencit jantan dan kepekatan sperma keatas prestasi IVF mencit (*Mus musculus*) hasil kacukan CBA/ca X C57BL/6J. B Sc. (Hons) Thesis. Zoology Department, University of Malaya.

Fraser, L.R. 1981. Dibutryl cyclic AMP decreases capacitation time *in vitro* in mouse spermatozoa. Journal of Reproductive Fertility. 62:63-72.

Green, S., S. Fishel and P. Rowe. 1999. The incidence of spontaneous acrosome reaction in homogenous populations of hyperactivated human spermatozoa. Human Reproduction. 14(7):1819-1822.

Hadi, T., M. Hammer, C. Algire, T. Richards and M. Baltz. 2005. Similar effects of osmolarity, glucose, and phosphate on cleavage past the 2-cell stage in mouse embryos from outbred and F_1 hybrid females. Biology of Reproduction. 72(1): 179-187.

Jaiswal, B.S., A. Cohen-Dayag, I. Tur-Kaspa and M. Eisenbach. 1998. Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction. FEBS Letters. 427:309-313.

Khalili, M.A., S.M. Kalantar, S. Vahidi and M. Ghafour-Zadeh. 1998. Failure of fertilisation following intracytoplasmic injection of round-headed sperm. Annual Saudi Medicine. 18(5): 408-411.

Kishikawa, H., H. Tateno and R. Yanagimachi. 1999. Chromosome analysis of BALB/c mouse spermatozoa with normal and abnormal head morphology. Biology of Reproduction. 61(3): 809-812.

Liu, D.Y. and H.W. Baker. 1994. Tests for human sperm-zona pellucida binding and penetration. *In* Tesarik, J. (ed.), Male factor in human infertility. Frontiers in Endocrinology, Ares-Serono Symposia, Rome. 8:169-185.

Magargee, S.F., K. Elaine and R.H. Hammerstedt. 1988. Changes in lectin-binding features of ram sperm surfaces associated with epididymal maturation and ejaculation. Biology of Reproduction. 38:667-685.

Nor Hasma, H. 2006. Effects of different hours of *in vitro* incubation on sperm in different strains of mice. B.Sc (Hons) Thesis. Faculty of Applied Sciences, Universiti Teknologi MARA.

Nooraain, H. and R.B. Abdullah. 2003. Production of mouse embryos using partial zona dissection (PZD) method. Proceeding for short term research seminar (Vote F). Project number F173/2002B. Institute of Management, Research and Consultation (IPPP), University of Malaya.

Roelof, M., E. Yehia, S. Wolf-Bernhard and H. Ralf. 2003. Relationship between sperm morphology and acrosomal function. Journal of Assisted Reproduction and Genetics. 20(10):432-438.

Sztein, J.M., .J.S. Farley and L.E. Mobraaten. 2000. *In vitro* fertilisation with cryopreserved inbred mouse sperm. Biology of Reproduction. 63(6):1774–1780.

Tiziana, A.I., R. Vassena, C. Francisei and F. Gandolfi. 2005. Role of adenosine triphosphate, active mitochondria and microtubules in the acquisition of development competence of parthenogenetically activated pig oocytes. Biology of Reproduction. 72: 1218-1223.

Toshimori, K., D.K.S. Tanii and K. Yoshinaga. 1998. An MN9 antigenic molecule, equatorin is required for successful sperm-oocyte fusion in mice. Biology of Reproduction. 59 (1): 22.

Toyoda, Y., M. Yokoyama and T. Hoshi. 1971. Studies on the fertilisation of mouse eggs *in vitro*. Japan Journal of Animal Reproduction 16: 147–157.

Yanagimachi, R. 1989. Sperm capacitation and gamete interaction. Journal of Reproductive Fertility. 38: 27-33.