CHAPTER 8

8.0 GENERAL DISCUSSION

8.1 ESTABLISHMENT OF TECHNIQUES

The study has led to several accomplishments. One of those was the establishment of techniques and protocols in our laboratory. The new techniques being introduced were ICC and PZD protocols. Although the techniques were already applied elsewhere but the efficacy of each technique varies between laboratories, hence, its validity and accuracy must be tested. In Chapter 4, it was proven that the ICC protocols utilised were reliable and produced high recovery and produced comparable results with other laboratories. The establishment of ICC not only had help in obtaining the objectives of the study but also served as an important tool in cellular and molecular studies in future research. It provides a wider perspective for the analysis of the gametes and embryos, especially for local applications. Using the ICC technique, one could probe deeper into multifaceted cellular study not only in the physiology of reproduction but also in many other related fields. It acts as window to the intricacies of cellular and molecular events that may relate one system with another and also very useful for diagnostic purposes. The technique is simple and precise. In addition, it allows multi-staining features that makes ICC, the technique of choice in many laboratories. Accordingly, in this study four structures were analysed per oocyte which were the MS, MTOCs, CG and NM using the different fluorescent probes namely mouse anti tubulin-conjugated to FITC, LCA-conjugated to TRITC and Hoechst. As for the sperm, the PSA-conjugated to FITC or LCA- conjugated to TRITC as well as Hoechst were applied to stain acrosomes and nuclear materials, respectively. Actin was the additional features stained using Alexa-fluor 568. Other detailed structures and cellular

processes could be added in the future such as peroxidation and metabolic activities along with progressive development of both *in vivo* and *in vitro* development of the mammalian embryos. However, the limitation of these studies would depend on the types of excitation-emission filters assembled onto the microscope. The three filters commonly adopted in many laboratories were either the long-band pass or the short band pass filters for FITC, TRITC and DAPI/Hoechst. Being the pioneer to establish and utilise ICC technique in the field of reproduction in ABEL laboratory, several challenges occurred at the beginning of the study for establishing the methods. These had inevitably affected the final results. For examples, switching of microscopes from conventional to a computer aided system had produced two sets of images; the Kodak film set and the digital camera printed images with varied qualities. Also, inadequate fluorescent filter of the microscope had incurred duplication of works. For examples, two sets of oocytes or embryos were used for tagging actin and cortical granules separately, using the same excitation filter for rhodamine probes. This had incurred more time and energy spent for the preparation of the samples. Moreover, the results may not represent the accurate correlation of the structural distributions for the same cohorts of oocytes or embryos because the images were taken from the different batches of samples. Thus, it is recommended that additional filters for examples, yellow or orange fluorescence is to be assembled onto the existing microscope for future use. This would lower the cost of energy and time expenditures for preparing multiple sets of samples. Secondly, the first and foremost problem encountered in ICC was the poor adherent of oocyte or embryos onto a glass slide. This was due to the round shape of oocytes that would easily dislodge either during fixing, rinsing or staining. The modified ICC procedures discussed in Chapter 4 could salvage the dislodged cells by

transferring it manually using micropipettes or glass needles into the rinsing solution and subsequently into staining and mounting solutions. Result has shown that significant recovery of oocytes number ($p<0.01$) was obtained using the microdroplets preparation than the poly-l-lysine coated cover slips. Therefore, oocyte adherence is no longer the primary obstacle and the use of poly-l-lysine coated glass slide can be minimised. For future research, it is recommended to use the 1- or 2-well chambered glass slides for preventing the loss of samples (Tae Do, 2002) and ease the process of transferring oocytes during consecutive rinsing and staining steps.

The results from ICC experiments showed that methanol was a better agent for fixing the mouse gametes and embryos than formaldehyde. This was based on the appearance of the MS that was beautifully tagged by using both E7 antibody and FITC-anti mouse IgG. In some oocytes the presence of meiotic spindles and the nuclear materials could be revealed through the ordinary light of the microscope without exciting the dyes using fluorescent lamp. This was observed among the oocytes fixed with 2% formaldehyde (Figure 5.3). This showed that each fixative is unique in fixing the subcellular structures of the oocytes and embryos. As for the methanolic fixation, less rinsing hours was required during sample preparation compared with the amount used to remove formaldehyde residues. In addition, formaldehyde may cause quenching and reduce the contrast between samples and the background. On the other hand, the disadvantage of using methanol fixation was high kinetics of its molecules that trigger detachment of oocytes from coverslips or glass slides. Another important aspect in conducting ICC procedure is the information supplied by the manufacturer of the dyes and antibody used. The information would be useful for determining the optimum titre concentrations for the respective

antibody and stain used. Optimum results could be obtained with only minimum testings, hence, shortened the time spent for the preliminary procedures involved. As suggested by many laboratories, anti fade agent mixed with the mounting medium is inevitably required for preventing bleaching of fluorescence colour during observation. Also, prepared samples must be properly stored at low temperature and wrapped for keeping light away. This is crucial when observation need to be repeated. Most fluorescent dyes restore the emission colours if the samples are properly stored. Another significant finding of the ICC staining was high efficiency of the two lectin binding proteins namely *Pisum sativum* agglutinin and *Lens culinaris* agglutinin each conjugated to fluorescein and rhodamine, respectively, to stain the mouse sperm acrosome (Nooraain *et al*., 2006). The technique was also proven to be effective for staining bull sperm (Nor Fadillah *et al*., 2005) and goat sperm (Ana Aryani *et al*., 2006). The similar affinity was reported for the two plant lectins (Cox *et al*., 1998) to specifically bind with α-linked mannose sugar and their high saccharide-binding specificities (Magargee, 1988) for the acrosome membranes in higher mammals. In addition, the procedures worked well with both zona intact and zona free mouse oocytes. The results showed that there were high appearance of MS ($p<0.001$) among the zona intact than the zona free oocytes. This could probably due to two possibilities; disruption of MS and/or failure of meiotic progression among the zona free oocytes, during which exposure to acidic condition to remove zona pellucida had probably caused disruption and instability of the MS. Thus, further experimental design is required to specifically verify the effect of exposure hours in acid Tyrode's on MS integrity among the zona free oocytes.

The second procedure utilised in the study was a simple manipulation of oocytes known as PZD. The zonae of the oocytes were successfully pierced manually by using needle, without adding high concentration of sucrose into the medium. Also, no holding needle was required to immobilise the oocytes although the process of cutting was relatively slow. The then viable PZD oocytes were used in IVF. The results showed varied effects of PZD on structural appearance among the different strains of mouse oocytes. This suggested that the conventional technique may be suited for one strain such as C57BL6J and ICR but not in another, such as the F_1 mice. Apparently, the C57BL6J strain was widely used in many laboratories as research model for testing the effects of PZD micromanipulation on rates of fertilisation (Nagakata, 1992; Kawase *et al.*, 2002) and establishment of transgenic mice (Nakagata *et al.*, 1997). Matson *et al*. (1997) stated that microdissection of the zona in cryopreserved F_1 embryos was not warranted since there was no effect observed on the implantation rate with that shown by the freshly oocytes. However, cryopreservation induced zona hardening among its matured oocytes. The negative impact of PZD on genetic abnormalities of the human oocytes underwent zona dissolution using acid Tyrode's was reported by Ng *et al*. (1990) but less damage was detected among the mechanically drilled oocytes. This suggested that exposure to high pH (2.0-2.5) would cause deterioration of the genetic configuration of an oocyte. Therefore, the procedures must be rapid and cautiously conducted. Faster zonae dissolution was accomplished in warmed medium. PZD procedures mostly utilised high sucrose concentrations (0.05 M or 0.1 M) to be added into the culture medium to induce shrinkage of the vitelline membrane. This would ease the process of zona cutting and subsequent washing in the culture medium which devoid of sucrose (Malter *et al.*, 1989). Although the

technique advances over a decade ago with major breakthrough, however, conventional PZD carved using needles is still valid especially for laboratories that depend on limited resources and apparatus to conduct their laboratory testing. Advanced micromanipulator such as piezo-micromanipulator had been used successfully to carve the zonae without the need to add hypertonic solution or subsequent successive washing procedures (Kawase *et al*., 2002). The potential use of PZD as a tool for ART have been well documented among domestic animals (Katayose *et al*., 1999) and in human (Yanagida *et al*., 2001). From this study, the results have shown that in ICR strain, PZD can be used as an alternative procedure for improving fertilisation rate (56.7±6.1% as compared with 47.6±4.0% of its zona intact oocytes) and improved percentages of subsequent embryonic development *in vitro*, including the F_1 strain. This could be due to easy penetration of essential substance such as glucose which is required for capacitation and *in vitro* culture. A study had showed a positive correlation between increasing glucose concentration during capacitation and fertilisation rate in mouse (Travis *et al*., 2003). The pitfall observed from this procedure was the inability to specifically define the size of a hole to be carved on the zona. A large hole may result in immature hatching of embryos. They then adhered onto each other and eventually assembled and fused as one embryo and resumed development until blastocyst stage as shown in Figure 7.0 (Part 7.9.1, Chapter 7). It is believed that such embryo would contain double chromosome numbers due to fusion of cells from two separate embryos. Therefore, it is important to give thorough observation on progress development of *in vitro* cultured embryos produced from PZD oocytes. Safeguards and precautious measures should be addressed while handling the PZD produced embryos. One option is to minimise the number of embryos cultured per droplet of medium and another is to reduce the size of

the hole made on zonae in order to prevent fusion of embryos and polyspermy. On the other hand, fusion of cells could be useful in other fields of studies such as for ooplasmic or nuclear transfer as well as genetic engineering. It was reported that PZD method was applied for transferring GV-containing karyoplasts into an enucleated GV oocytes. The reconstituted GV oocytes were cultured and the post-natal growth deficiencies were observed among the progeny (Cheng *et al*., 2008). Hence, the established PZD technique may provide an optional technique for manipulating oocytes in genetics and epigenetic studies. Besides, the technique of karyoplast transfer is current technology being practised by many laboratories to enhance embryonic development. This would widen the horizon of mammalian reproductive investigations in our laboratory and the conventional PZD method is reliable for the purpose.

In addition to new techniques and protocols, the study has helped in readdressing laboratory accomplishment and performance from the established standard procedures for *in vivo* and *in vitro* embryo productions. It was apparent that the percentages of *in vitro* produced embryos declined sharply as compared with the previous achievement reported. For examples, it was noted that the production of 2-cell stage and blastocyst using $1.0x10^6$/ml sperm to inseminate COC oocytes in F_1 strain were 51.1±2.9% and 11.0±1.9%, respectively. These were far below the previous percentages reported which were 83.3±3.02% and 82.8±4.93% for the respective stages (Faridah, 1994). However, comparable results were observed for the low and high concentrations of sperm. The poor results indicated the occurrence of some factors that impede embryo production. Thus, appropriate action is inevitably required to address the situation. The IVF results could be influenced by many including the chemicals used, the condition of the experimental

animals, handling of samples, laboratory conditions and sterility of apparatus and equipment and many others. One of the factors is the condition of experimental animals. The age of male mice (adult) and the concentration of sperm ($1.0x10^6$/ml) are two of the vital criteria accounted for good performance of IVF outputs (Faridah, 1994). On the other hand, oocyte factors do play a major role in determining IVF performance in mice such as prolonged incubation of oocytes (*in vitro* aged-oocytes) might lead to poor embryo development (Takahashi *et al.*, 2008). There was a suggestion to shift the hormonal injection in the morning instead of afternoon to reduce chromosomal destruction among the mouse oocytes. Also, modification of the IVF procedure is recommended especially in the chemical composition of the media and sperm oocyte ratio per droplet for improving the IVF outputs. For example, Gil *et al*. (2004) had suggested an appropriate ratio of sperm to oocyte (8000:1) for a better fertillation rate and blastocyst formation. In addition, their earlier suggestion was to use low volume of IVF medium (0.1 ml) with 30-50 oocytes inseminated by 2000 sperm for improved *in vitro* embryo development in pigs (Gil *et al*., 2003). In addition, continuous assessment on the laboratory IVF performance is useful because the technique is the only tool for supplying *in vitro* embryo production and for advanced studies in embryology.

In the field of microscopy, the study had contributed for the establishment of improved imaging system. With the availability of the image analysis software and advanced image processing devices, detailed measurements of the gametes and embryos could be explored. Nevertheless, due to time constraints and delayed in supplies of equipment such analyses was not conducted in this study. It is hoped that future research would take the opportunity to utilise the established system for obtaining a comprehensive reports on images of the mammalian gametes and embryos.

8.2 COMPARATIVE STRUCTURAL ANALYSIS OF MOUSE GAMETES USING ICC TECHNIQUE

The mouse gametes understudy included both oocytes and sperm. The ICC technique established was applied to study structural integrity and distributions of selected organelles, through which their appearance may indicate specific cellular events of the oocytes and sperm. These included oocyte maturation and acrosomal integrity of the sperm. Among the mouse oocytes, structural distribution was associated with cytoplasmic and nuclear maturation. Therefore, the appearance of the selected structures of oocytes was used to extrapolate its cellular events and maturation stages. The related structures included the nuclear materials, the microtubules and microtubule organising centres that highlighted the meiotic stages and nuclear maturation of oocytes. The cortical granules play a role for assessing activation of oocyte. Oocyte maturation is one of the important criteria in embryology and ART. Availability of MII oocytes *in vivo* and *in vitro* may affect the end results of research in the related fields. In this study, various factors had been identified affecting oocyte maturation, which were strains, culture conditions and the time of the day at which oocytes were harvested from superovulated females. The study proved that *in vitro* environment had significant impacts over certain strains of oocyte to progress from MI to MII stage. This was observed among ICR and F_1 but not in C57BL/6J. However, activation of oocytes would not necessarily improve fertilisation rate *in vitro*, because oocyte activation is also associated with aging and thickening of the zona pellucida. Shape of oocytes was not of species specific criterion. However, variation was observed with round shape was commonly detected in most strains. Nevertheless, oval and potato shapes were obvious among the ICR strain. Although polarity of oocyte was prominently

identified among the MII oocytes by referring to the position of the polar body, but, among the MI, staining was the best procedure for determining the structural appearance and distributions. A detailed topography for the MI and MII oocytes such as eccentric position of the nuclear materials, granular appearance and compartmentalisation by actin filaments could be highlighted through ICC staining. In addition, abnormalities of aberrant structures in both the nuclear and cytoplasmic materials were ascertained. The most peculiar one was fragmentations of either/both nuclear materials or/and cytoplasm by forming lobes or patchy granules and disarranged vesicles. These represent the subquality oocytes that may jeopardise fertilisation rates of a species or a strain if being used in IVF. First polar body was claimed to be a reliable indicator of oocyte age and a well-shaped nonfragmented polar body was associated with increased pregnancy rates (Ebner *et al*., 1999).

Oocyte maturation in selected strains were extremely affected by the culture conditions. Apparently, *in vivo* oocytes showed indifferent percentages between mature and immature oocytes among the strains. However, the number of MII oocytes was significantly low in *in vitro* condition especially for the F_1 strains in contrast to high *in vitro* MII oocytes in ICR. The rank of order of *in vitro* MII oocytes production among the strains was ICR > C56BL/6J > F_1. This showed that in natural condition, intrinsic factor plays a major role in determining reproductive success of a species. However, susceptibilities among the strains upon exposure to *in vitro* culture condition varied. One possible reason could be due to precocious activation stimulates maturation induction to occur faster in ICR than the other strains (Nooraain *et al*., 2009). Also, possibly in some strains, the positive intrinsic factor is removed or masked by the culture medium that mimics natural ingredients of the body fluid. This however, could be tested by determining

the presence of protein receptors for the signaling pathways of the oocytes. Perhaps, the presence of activating or inhibiting kinases may relate the cellular activities of oocytes with maturation stages, which could be included in future research. Also, oil overlay culture had been reported to cause a delay in meiosis I progression in mouse oocytes (Segers *et al*., 2008). Perhaps, future research could ascertain this by using ICC staining method for determining various mechanisms involved in this calcium mediated signaling pathway of the mouse oocytes. It was interesting to note that specific structural appearance differed between the IVM and IVO group. For example, NM and MS were greater among the IVM group but high MTOCs were among the IVO counterpart. Factors such as medium compositions could be tested for its effect on structural formation of the oocytes. Thus, some criteria are to be tested such as the formation of gap junction, the density of the protein receptors on cell membrane, as well as the phosphatase and kinases concentrations each for down regulation and activation of cell cycle progression, respectively. Patchy cytoplasmic granules were higher among the IVM than the IVO group for both MI and MII oocytes. This was probably associated with premature activation of the oocytes such as premature cortical release (Vincent *et. al*., 1990) induced by the chemical compositions of the culture medium had consequently produced aged oocytes. Fragmented nuclear materials were also an indication of apoptotic oocytes (Han *et al*., 2004). The ICR strain normally showed good response to superovulation and being categorised as good ovulators as compared to other species of mice (genus *Mus*) (Martin-Coello *et al*., 2008). It also normally produces prodigious number of offspring through natural mating. However, cell blocks among its *in vitro* embryos were commonly observed. Meanwhile, superior cellular structures were shown by C57BL/6J for both *in vivo* and *in vitro* condition. Undoubtedly,

it is therefore the strain of choice in many laboratories as research model for *in vitro* produced embryos and for manipulation purposes such as PZD. Thus, fertilising capability of an oocyte is a complex phenomenon which relies upon various factors, among which is maturation competency. Maturation is the stage determined by series of physiological and structural responses. These responses varied accordingly in the different environment and between the strains. In addition, oocyte collection time plays a role for improving the number of MII oocytes, especially in the F_1 strain. It was shown that delaying the collection time of oocytes had improved occurrence of meiotic spindle and MTOCs but reduced the cortical granules in both *in vivo* and *in vitro* conditions. This explains the slower pace of oocyte maturation through natural environment, perhaps, to ensure appropriate accumulation of growth factors needed during oocyte development. This also reflects the intrinsic role of oocytes to attain maturation. It was also suggested that extending the period of hCG priming time may improve subsequent pregnancy outcome for IVM treated human oocytes (Son *et al*., 2008).

The ICC technique had successfully determined and compared the characteristics of the different strains of mouse sperm cultured *in vitro*. The results may highlight some criteria which can be associated to fertilising capabilities in the different strains of mice. Fertilising capabilities of mouse sperm depend upon multitude of factors among which are the quality of genetic materials, capacitation and acrosome reaction. Capacitation and acrosome reaction are the complex physiological changes sperm must acquire, prior to be able to fertilise an egg. This is also known as sperm activation, which would occur during both *in vivo* and *in vitro* conditions. The process is manifested by various changes such as membrane reorganisation and loss of acrosome. These physiological responses of sperm

were shown and compared from the different strain of mice incubated *in vivo* and *in vitro* conditions and the results were used to correlate with their fertilising capabilities. In the study, matured intact sperm were analysed through the appearance of the genetic materials in the head region that was stained with Hoechst 34324 and acrosome intactness was detected using PSA-FITC conjugates. The general morphology of the sperm were analysed between strains for both *in vivo* and *in vitro* conditions. The ICR mice were generally noted as prolific breeders *in vivo* but its fertilising performance *in vitro* is questionable. This study had proved the significant reduction in percentage of acrosome intact sperm in ICR males during long exposure hours in *in vitro* condition. This could explain for its poor IVF performance, which was due to sperm factor. In mice, strain was proven to be the main factor that determined IVF performance (Kaneko *et al*., 2006). The fertilisation rates using long term cryopreserved sperm of C57BL/6J, DBA/2N, BALB/cA, C3H/HeJ, B6D2F_1 and B6C3F_1 varied among the strains (66.4%, 92.3%, 72.8%, 32.9%, 60.3% and 53.7%, respectively). In some strains, loss of fertilising capabilities is more likely to be caused by certain manipulation procedure such as long term of cryopreservation which resulted in higher susceptibility towards oxidation of its mitochondria (Gray and Magnuson, 2009).

8.3 GAMETES AND FERTILISATION

The superiority of the *in vivo* condition upon producing higher fertilisation rates than the *in vitro* conditions has been reported in animals including mice (Ibanez *et al*., 2005) and bovine (Rizos *et al*., 2002). However, the rates differ between animals and strains. This study (Chapter 7) showed insignificant difference of *in vivo* fertilising capacity among the different strains of mice. In contrast, for *in vitro* fertilisation the F_1 hybrids showed higher fertilising capacity than the ICR. This quality is crucial for ART and embryo manipulation

research. Therefore, the F_1 hybrids were commonly used in many laboratories for obtaining *in vitro* produced embryos in mice. It has been shown in this study that *in vitro* fertilising competency of the ICR mice was low. This could be related to several factors. First, the barrier developed by zona pellucida that impedes penetration of sperm during *in vitro* condition. This was proven by high rates of fertilisation observed among its PZD cohorts of oocytes, through which a channel was made across ZP to ease sperm penetration. This had eventually increased not only the rate of fertilisation but also cleavage development to the blastocyst stage. The rates were higher (56.7±6.1%, 8.3±3.5%, respectively) than those obtained from the normal IVF groups (47.6±4.0%, 2.4±1.2%, respectively). PZD had also proven to increase blastocyst in $F_{1,}$ which we believe it facilitates mobilisation of essential substances into the embryos toward later development in the culture medium. Second, the incompetency of ICR sperm in *in vitro* environment was proven too. The study (Chapter 6) had clearly indicated high percentage of acrosomal loss among the *in vitro* cultured ICR sperm. The incidence was higher with longer exposure in *in vitro* condition. Premature disintegration of acrosome would joepardise binding of sperm onto membrane receptor, preventing sperm attachment on the ZP3 layer of the zona and eventually attributed to failure of fertilisation. Both reasons were evidenced by having low rates of fertilised denuded oocytes produced as compared with higher IVF rate of denuded oocytes among the F_1 strain. These clearly indicated zona impenetration and sperm incompetency of the ICR strain affected their fertilising capability. The effect of culture medium on zona hardening in various strains of mice could be verified in future research by measuring the rate of zona dissolution over time using either acid Tyrode's or chymotrypsin. Conversion of the zona pellucida glyoprotein

ZP2 to ZP2f by a protease from precociously released oocytes' cortical granules appears to be a major contributory factor of zona pellucida hardening (Ducibella *et al*., 1990) especially in serum-free medium of culture condition (Schroeder *et al*., 1990). Although low concentration of sperm (0.5 mill/ml) showed no significant effect on the rates of fertilisation in ICR but the result was significantly lower for denuded oocytes of this strain than the COC and PZD oocytes in F_1 with low sperm concentration. The rate was further reduced when denuded oocytes were used in combination with high sperm concentration (5.0 mill/ml). Thus, fertilisation rate was severely affected with high concentration of sperm and denuded oocytes. Fertilisation rate was not affected among the normal COC group. This showed the importance of cumulus cells and the role they played to maintain high fertilisation rate using high concentration of sperm. Here it clearly indicated the intricacies of factors such as sperm count, oocyte factor, strains, culture's condition and cumulus cells for determining successful fertilisation. Therefore, it was obviously observed that in IVF, a complex interaction of factors affected the success rate which included the gametes and the strains. Manipulation of oocytes did affect the fertilisation results as shown by highest rate of fertilisation for the denuded oocytes with 1 mill/ml sperm and improved fertilisation rate for the PZD oocytes in ICR. Although maturation rate *in vitro* was greater among ICR than F_1 oocytes (Chapter 5) but this was not the only factor that determined IVF rate. Oocyte viability and structural integrity for the IVM oocytes were comparable among the strains except for a remarkably low CG in ICR oocytes. This could be due to activation of oocytes to trigger premature cortical reaction upon exposure to *in vitro* condition that may lead to zona hardening.

Oocyte cytoplasmic integrity affects subsequent development during fertilisation and pronuclear formation, especially in mice. This is because post fertilisation survival of the embryos depended upon the maternal factors provided by oocytes not sperm. In was noticed that the MS appearance among the IVF group of ICR embryos was the lowest among all the strains. The most superior MS *in vitro* was detected in C57BL6J followed by F_1 embryos. On the contrary, MS of *in vivo* fertilised oocytes was greatest in ICR and low in others. Thus, meiotic spindle appearance was one of the structural integrity related to pronuclear formation and mitotic division such as polar body formation and 2-cell stage. This is especially crucial for *in vitro* produced embryos. The roles of MS were critical for ensuring completion of the meiotic division to produce haploid oocyte that then becomes the female pronucleus when the sperm penetrates and form the male pronucleus. Hence, MS is the indicative of a good quality oocyte. The slower pace of *in vivo* maturation than the *in vitro* counterparts explains the importance of oocyte being equipped with adequate necessity for ensuring subsequent development during fertlisation and cleavage formation especially when in mice it is maternal origin. It was also noted that meiotic progressions among the oocytes varied in which not all oocytes produced two polar bodies upon establishment of sperm pronucleus at fertilisation. In most of the strains, there were variations in the number of polar body and pronuclei formed. Only C57BL/6J showed the presence of two polar bodies and two pronuclei from the total zygotes stained where as others showed a different combinations of polar body and pronucleus count with a majority displayed one polar body and two pronuclei. Thus, this provides an insight on nonsynchronous activity of cellular cycle among the oocytes to halve the chromosome number. Unfortunately, there was no clear evidence to implicate such activity with

fertilising capacity and cleavage development among the strains of mice. However, as shown in the results, the groups which having two polar bodies regardless of the culture condition ended up with high percentage of 2-cell stage. Undoubtedly, this trend was found among the F_1 and C57BL/6J mice. Progressive movement at approximation of the pronuclei would depend on the cytoplasmic machineries including the MTOCs and microtubules. A study had revealed the maternal inherited centrosome serving as microtubule-organising centre in mice in contrast with other mammals which are paternally inherited (Schatten *et al*., 1991). Both structures were detected to have high appearance during migration of the pronuclei towards the centre part of an oocyte. At the end of cytokinesis microtubules was found to surround the nuclear materials in both the polar bodies and the daughter cells of the first cleavage. Further development of the MS was shown to be perpendicular to the axis between the two cells to distribute the nuclear materials equally during the second cleavage. The MS which separate female pronucleus from the second polar body were also detected in many *in vitro* produced embryos especially from the C57BL/6J mice which showed the highest MS among all. However, to use this as the criterion of a highly competence embryo *in vitro* was ambiguous since the amount of 2-cell stage embryos produced from the strain was lower than those of F_1. It is believed polymerisation and depolymerisation of MS and MTOCs were rapid, thus, at certain stages they were found missing and not traceable at all. Constriction point equidistant between the daughter nuclear materials was obvious not only during polar body formation but also at cleavage development. Perhaps, this point acted as the central axis for cellular divisions from 2-cell stage to morula. Low CGs was detected among the fertilised

oocytes especially during pronuclei approximation, which indicated its specialised function that is confined during the early stage of fertilisation.

The progressive development of nuclear activities among the fertilised embryos was successfully revealed from the study. It ranged from penetration of sperm, formation of pronuclei, approximation and fusion of pronuclei, until mitotic events. The ICC technique revealed varied pronuclei appearance that represented several stages of nuclear maturation prior to pronuclei fusion. It began with solid structure of nuclear materials of approximately equal size which then expanded and unwound with male pronucleus was always bigger than the female. Varied orientations of the pronuclei relative to one another were observed during their migration towards the centre with different angles relative to the position of the polar body. There were some pronuclei that aligned well in one line paralleled with the polar axis while others showed horizontal arrangement perpendicular to the polar axis. To implicate this criterion with fertilising competency of the embryos would need further research in which emphasis would be given on the migration pathways of the marked pronuclei (male or female) by tagging them separately using green fluorescent protein or other fluorescent stains that are suitable for live embryos. However, this study had showed that the presence of two pronuclei (2PN) in the embryos was independent of polar body number and a majority of the embryos possessed unequal sized of pronuclei. Also, most embryos showed a single nucleolar precursor body (NPB) within the pronuclei except the ICR which was observed to posses many NPBs. Embryos with nonpolarised PN was reported to have slower rate of cleavage formation (Kahraman *et al*., 2002). Scoring of embryos at pronuclear stages (Baczkowski *et al*., 2004) can be focussed among the different strains of mice in future studies. This approach may enhance the process of

determining genetic susceptibility of their nuclear materials in the different culture conditions.

The images obtained from the study also reflected the formation of linkages developed between the male and female pronulcei during decondensed state. Unfurled pronuclei resembles the image of substance spun from a centre point unleashed the nuclear materials to the surrounding region (Figure 7.6 a-d) displayed cytoplasmic halo. However, this needs to be verified in further study. Perhaps, the application of X-ray diffraction device may give the insight of such distribution. Disappearance of the nuclear materials is believed to represent the actual state of nuclear fusion at which the nuclear materials were fully unwound. This is the crucial stage of fertlisation during which the paternal and maternal chromosomes mix and exchange the genetic codes. The unwound compacted pronuclei began when one or a few small nucleoli appeared within the nuclear materials from both sexes. The nucleoli can be distinguished from the main circular structure of the nuclear expansion. Nucleoli orientation was reported to determine polarity of the embryos and can be graded according to their distribution, numbers and orientation (Tesarik and Greco, 1998). Pronuclei with equal size of nucleoli aligned at the centre of the zygote would result in cleavage development. Approximately at 3-4 hours post fertilisation *in vitro*, a clear shape and size of the pronuclei would be revealed. However, towards a later hour the size grew larger and the structure became blurry. The unwound nuclear materials at decondensed stage were observed and the size of nuclear expansion may fill about a third of the entire oocyte size. The time taken for the NM to unwind was considerably long. This was accounted for interphase stage during which the embryos would be scrutinised during cell cycle checkpoints before advances to mitotic stage. The pronuclei were

structurally linked by the lamellae to display interaction or attraction occurred between their molecular components (Figure 7.6). Some incidences of eccentric PN was observed especially among the IVF group which may undergo irregular cleavage (Garello *et al*., 1999).

Manipulation of oocytes such as PZD could disrupt MTOCs formation when most strains showed reduction in the appearance of the structures especially the ICR mice. Similarly the MS were detected to be low in frequency among the PZD oocytes than the other IVM groups. But NM consistently maintained at high percentages for all the strains regardless of the manipulation procedure. Besides, ICR showed the lowest MS appearance in both the PZD group and *in vitro* fertilised oocytes as compared with highest frequency found in C57BL/6J and F_1 mice. However, their relationship with fertilising competency remained unclear because ICR with low MS and MTOC produced high fertilisation rate with PZD oocyte, meawhile the C57BL/6J were highly competent during IVF of COC oocyte but not PZD group, although the frequency of MS was greater in the later. Mitotic rate was assumed to be rapid among the F_1 since it produced the highest percentage of 2-cell as compared with others. Unequal rates of nuclear maturation were obvious among the strains. The number of polar body was not consistent with most possessed only one polar body with two pronuclei in the zygotes, in which the mechanism is unclear for the completion of meiotic division of an oocyte. Thorough studies on the temporal separation of the chromosomes by using different tags on the chromatids are highly recommended. This may help in distinguishing their distributions during fertilisation stage. It was shown in the study on temporal mitotic progression in F_1 which revealed varied categories of pronuclei development throughout the first 12 hours of post

fertilisation period *in vivo*. Assuming that mating took place right after mixing the male and female mice, it was detected that it took about 12-14 hours for the fusion of pronuclei to complete (Figure 7.6). Then, mitotic event commenced with prophase at 20 hours and followed by metaphase stage at 24 hours post mating. This was subsequently followed by anaphase and telophase stages and soon after a 2-cell stage could be detected. There appeared to be variations in the orientation of chromosomes at the metaphase plate as shown in Figure 7.6. The chromosomes were either paralleled to the polar axis or arranged in the perpendicular direction relative to the polar body. As shown by the telophase stage in Figure 7.6n cleavage occurred at the centre point equidistant from the animal pole and the vegetal pole. However, it was claimed that the sperm entry position (Piotrowska and Zernicka-Goetz, 2001) and the site of previous meiotic division marked by the second polar body (Piotrowska and Zernicka-Goetz, 2002) predicts the plane of initial cleavage of the mouse egg. Intensive works for staining the sperm entry position in various strains may show correlation between cleavage plain and developmental competent in mice though fertilisation cone could observed in some oocytes, which indicates the point of sperm entry. Also, further research is recommended to trace the factors that contribute to fertilisation failures among the gametes. It would be advantageous to separate indentity of the female and male components of the zygote and trace the events they involved throughout fertilisation process.

Many had reported on the structural integrity of gametes and the effects of culture medium on fertilising competency in mice. However, continuous monitoring on the said factors is required because any slight change may cause deleterious effects. The output from a laboratory is crucially determined by how it's physical and work environment are

controlled, maintained or improved. Thus, routine procedure must be strictly complied especially when dealing with assisted reproduction. This covers from breeding and maintaining stage of the experimental animal, supply of materials, preparation of culture media and ultimately the micromanipulation procedures. Sperm and oocyte capacity need to be consistently recorded in order to save energy and expenditure on preliminary screening steps especially genetic screening of strains to be used in the procedure although sometimes the results can be very puzzling. For examples, in a report produced by Martin-Coello *et al*. (2008), *in vitro* matured oocytes had resulted in high proportion of fertilisation from three species of mice including *mus musculus* the albino mice. Thus, a new challenge appears in determining the effect of strains on gamete compatibility and their susceptibility in various culture conditions among the mice. Also, their reproductive performance during different hours of incubation periods should be measured by altering the sperm count and manipulated oocytes. This could lead to new finding such as the contribution of gametes to free radicals present during IVF. High concentration of sperm during IVF had been reported to give negative impacts on fertilisation (Faridah, 1994). Perhaps gamete competency was affected due to excess toxic liberated by high concentration of sperm used in IVF. As for the manipulated oocytes, the structural profiles especially the MS for the PZD oocytes in ICR was four-folds higher than the F_1, low in MTOCs and no different for NM. Thus, possibly it was the spindle formation during maturation process that was crucial during *in vitro* fertilisation. Undoubtedly, that the nuclear materials of the sperm are more important than any other factors for fertilisation to ensue. The only problem is how to bring them to the inside of an oocyte. Structural development associating with fertilisation process had been successfully revealed through the ICC staining. This ranged

from the establishment of sperm head within the nucleas, formation of two polar bodies, development of two pronuclei, approximation of pronuclei, formation dan distribution of nucleoli, mitotic division of fertilised oocyte and cleavage formation. Synchronuous nuclear development among the IVF produced zygotes were observed and this proved the significant effect of *in vitro* condition in triggering simultaneous cellular development among the zygotes due to availability of adequate energy sources within the microenvironment of the culture medium. Meanwhile, nonsynchronous nuclear materials development among the *in vivo* group could be due to nonhomogenous growth factors and energy source distributed among the zygotes during fertilisation. Capability to fertilise would also depend upon the integrity of the structural intactness such as acrosome intact, viability of the NM, formation of microtubules and union of the pronuclei. *In vitro* environment which simulate the *in vivo* condition may not warranty the success rate of fertilisation to take place because the environment can be rapidly disturbed and fluctuated. Monitoring of the situation requires high maintenance and commitment of the personnel involved.

Genetic influence has a role in determining oocyte and embryo competencies in mice. The F_1 hybrids possessed a mixture of genetic makeup from the two strains (CBA x C57BL/6J). They inherit quality traits from both parents. Hence, their competencies in the different culture conditions are undoubtedly high. The C57BL/6J is well established for many micromanipulations of gametes and embryos research. In addition the combined genomes would result in unique modifications of the membrane proteins that are crucial for signaling and cellular communication for cellular responses. The variation of membrane proteins between the inbred and hybrids could be compared through freeze-fracture

technique as well as ICC staining in future research. The presence of membrane proteins during fertilisation and embryonic development may be indicative of cellular responses during important events in fertilisation. In addition variation in lipid compositions of the membrane may play a role in assimilation of substances between cells and their environment, hence, affecting the rate of transporting substances across it. However, structural assembly of the oocytes among strains revealed insignificant different. Meanwhile, the sperm factor and zona impenetration had been identified as the main caused for low fertilisation of the ICR strain. This was shown by reduced percentage of its intact acrosome during prolonged exposure *in vitro*. Intact acrosome is vital for ZP3 receptor of the zona pellucida to recognise and bind onto sperm. Immature loss of acrosome while exposed to *in vitro* culture had jeopardised fertilisation rate in this strain but not F_1 and C57BL/6J. The effect of sperm concentration on fertilisation rate between F_1 and ICR further support the finding when reduced percentages were found in ICR using 1 mill/ml sperm mixed with denuded oocytes. However, IVF of PZD oocytes had improved the fertilisation rate. However, using low concentration of sperm (0.5 mill/ml) with PZD oocyte of ICR did not improve fertilisation rate. Therefore, the results verified the importance of using the right concentration of sperm in IVF and acrosome intactness is the vital criterion for determining successful fertilisation. The ability of sperm to bypass the barrier possessed by zona pellucida (PZD) had improved fertilisation rate in ICR, although no significant effects were shown in other strains. Superior IVF quality was shown by the F_1 sperm that capable of penetrating the zona pellucida of denuded oocytes.

Maturation of cytoplasm and nuclear materials of an oocyte may determine the ability of oocyte to be involved in the subsequent cellular process or development. It was

reported that a number of ultrastructural and molecular changes occurring during oocyte development have been linked to its developmental competence (Hyttel *et al*., 1997). Developmental competence of the *in vivo* matured oocytes was greater than the *in vitro* matured oocytes and successful fertilisation would produce higher number of blastocyts among the *in vivo* matured than the *in vitro* matured oocyte (Ward *et al*., 2002). *In vitro* condition has been implicated with high incidence of abnormalities in the oocyte (Hyttel *et al*., 1989). Failure in cytoplasmic maturation among the oocytes may interfere with the formation of the pronuclei after penetration of sperm, despite of normal germinal vesicle (GV) breakdown and extrusion of the first polar body (Abeydeera, 2002). Thus, research can be furthered on determining optimal time of fertilisation among the different strains of mouse oocytes in *in vitro* condition. Meanwhile, glucose requirement for oocyte maturation differed between species (Funahashi *et al*., 2008) and perhaps the different requirements are established between the strains of mice. In the mean time, the study on microfilaments of ocytes and embryos in the different strains of mice was restricted due to several constraints. First of all it was due to the limitation of excitation filter of the fluorescent microcope that cause delaying in results obtained. Secondly was inadequate supply of oocytes and embryos towards the end part of the study. However, several images of actin filaments were successfully captured among the oocytes and embryos. They were mostly observed around the nuclear material especially around the polar bodies of the fertilised oocytes (Figure 7.6f). Also, their occurrence was mostly detected at the animal pole of the oocytes (Figure 5.10). This defines the role played by the microfilament in various events during oocyte maturation and fertilisation in which actin mediate peripheral

nucleus migration and polar body emission during oocyte maturation (Sun and Schatten, 2006).

In conclusion, ICC technique was successfully utilised for studying mouse gametes and embryos and any attempt to improve ICC results should be directed towards an improvement in the basic protocols for imunolabeling such as the optimum temperature, pH and incubation period for staining. Improvement would also be necessary on the visualisation or imaging aspect of the microscopy technique for the nature of the study rely strictly on the quality of images captured from a sample. The study had also provided useful information on strain competencies in manipulation and IVF procedures in which ICR suited well with PZD methods. Meanwhile, F_1 and C57Bl/6J oocytes fertilised well *in vitro* using fresh and denuded oocytes, respectively. The application of PZD or ZP thinning is frequently practiced in the human embryos (Yano *et al*., 2007) and the technique is recommended if no harm was detected for successful hatching and improved implantation rates in ART embryos. Above all, intrinsic quality of the oocytes is the key factor for determining the fertilising and developmental capacities in mice. The additional extrinsic factors such as culture condition and manipulation treatments may either give positive stimulation or vice versa. A comprehensive study using ICC should be extended at the different stages of embryonic development including follicular oocytes and blastocyst stage. However, noninvasive method must be applied for ensuring no deleterious effect that could harm their progressive developments.

8.4 RECOMMENDATIONS

The outcomes of the study depend upon various factors including the animals, laboratory conditions, apparatus and many others. Below are some recommendations to be considered

upon for expansion of the research. Besides, additional search on the study as below may provide a comprehensive insight on the competency and fertilising capacity of mouse gametes.

i. Additional structures of gametes and embryos such as mitochondria and membrane junction should be included in future studies using ICC technique. Beside, some studies could be expanded into physiological processes such as metabolic function and level of reactive oxygen species in *in vitro* and *in vivo* conditions in the different strains of mice.

ii. The use of specific fluorescent dyes which could tag the maternal and paternal structural inheritance of the embryos would help to differentiate the male from the female counterparts. This may give definitive structural incompetency of between the sexes.

iii. This type of study requires prodigious supply of oocytes and embryos. The success of the study depends upon availability and continuous supply of the samples. Hence, a professional mouse breeding strategies would be advantageous in order to maintain pure strains of mice.

iv. Genetic screening of the resultant embryos should be included in the experiment in order to trace chromosomal aberrant of the embryos.

v. The use of glass-chambered slides is recommended in future studies to prevent sample loss and reducing the use of solvents and buffered medium. This would also speed up the staining process.

vi. The ICC technique can be used to trace multiple staining per sample. However, the limiting factors would be the number of excitation-emission filters assembled onto the microscope. Thus, adding filter sets onto the microscope would be advantageous, so that

more structures and cellular processes could be detected at once in order to establish relationship between structural fertilising competencies of gametes and embryos.

vii. Maturation stages of oocytes may be associated with cellular communication between oocytes and the cumulus cells. Future studies may include structural analysis of cumulus and correlate them with oocytes maturation stages and competency. For example, protein receptors for ligands and gap junction may give some insights on cellular communication between cumulus and oocyte maturation stages.

viii. Comprehensive analysis on the embryonic structural development ranging from the 1-cell to balstocyst stage should also be included in the different strains of mice. Thus, the transition of structural development could be compared between *in vivo* and *in vitro* systems.

ix. A study can also focus on determining the scoring of embryos among the different strains by using ICC technique based on pronuclei formation. The score can be used to determine fertilising and developmental competencies among the different strains of mouse embyos.

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