_____ DISCUSSION

5

DISCUSSION

Currently, there is considerable interest in producing large amounts of pig oocytes (by *in vitro* maturation techniques) and embryos (by *in vitro* fertilization (IVF) techniques) with the intention of progressing in both basic and biomedical research. The pig has become increasingly more important as a potential organ donor for xenotransplants, as well as a transgenic animal to produce specific proteins, given the biological similarities it has with human beings. Attempts of cloning and producing transgenic pigs by means of pronuclear injections require mature oocytes and zygotes, respectively. However, surgically obtaining oocytes and embryos from donor animals proves to be an expensive procedure, it is time-consuming, and the quantity of biological material obtained is limited. It is for these reasons that the effective use of ovaries originating from the abattoir in order to produce mature oocytes and embryos through *in vitro* techniques is crucial (Abeydeera, 2002).

As we have already stated, the general objective of this study was to increase the performance of the *in vitro* embryo production in pigs, which scarcely reaches 15% with the present-day methodology used. To achieve this objective, our first step consisted in looking for the possible reasons for this low performance from among the bibliography sources, and in applying the acquired knowledge with the intention of formulating solutions. The factors affecting this process were basically the following: the complications associated with the *in vitro* maturation process, polyspermy, and the inadequate embryo culture conditions. On the one hand, the polyspermy problem is solved by introducing the ICSI technique, since only one sperm is placed inside the oocyte. However, the already low blastocyst rate obtained through traditional IVF after the embryo culture, drastically drops

after ICSI. The reasons behind this failure may partially lie in the ICSI technique itself, and in this case, the problems associated with this methodology may not be entirely avoided (plasmatic oocyte membrane damage, microtubular system disruption with the injection pipette…). However, we can work on the causes related to the oocyte, to the sperm or to the zygote. With regard to the sperm factor, the sperm along with all of its membranes is placed inside the ovocyte during the ICSI technique, which does not occur in physiological fertilization. This factor was previously studied in our laboratory when fresh sperm as opposed to cryo-preserved sperm were used, and the differences mainly observed concerned both the boar and the preservation method used. However, the blastocyst rate only increased in one of the boars used (García-Roselló, 2003). For this reason, we decided to focus this study on both the oocyte and zygote, with the intention of increasing its developmental capacity. The first step was to optimize the *in vitro* fertilization sequence and the embryo culture (IVF, EC) media that are employed in most pig laboratories. Secondly, we attempted to improve the *in vitro* maturation system by introducing meiotic inhibitors, such as roscovitine, and the *in vivo* embryo development of the ICSI zygotes was assessed under these conditions. The optimal time for *in vitro* maturation was also studied in this experiment by comparing three commonly used IVM times (36, 40 and 44 h). Finally the InsP$_3$ effect on *in vitro* embryo development was studied to verify whether the problem actually lay in the necessity of artificially activating the oocyte-zygote during ICSI in order to facilitate calcium oscillations.

5.1 THE TIME EFFECT THAT THE CULTURE IN TALP MEDIUM HAS ON BOTH ICSI AND *IN VITRO* EMBRYO DEVELOPMENT PARAMETERS.

Given the expansion achieved cloning techniques over recent years, the *in vitro* culture media used to develop zygotes are shown to be still inefficient in most mammalian species. For this reason, a considerable number of research groups have been working on the identification of either beneficial or harmful factors present in the culture media affecting genome activation, blastomers cleavage capacity, blastocyst hatching, etc. Among other conclusions there is the well-known biphasic effect of the serum that inhibits the first cleavage, and yet it stimulates both the morula compactation and blastocyst formation (Lim *et al*., 1994). It has been observed that this additive, so commonly employed as a protein source, actually reduces blastocyst cryotolerance and disturbs mRNA expression

patterns (Rizos *et al*., 2002). On the other hand, as knowledge on the oviductal and uterine microenvironments increases, a common agreement arises regarding the need to use sequential media within which the components are changing at the same time as the developing embryo requirements are changing. An example of such are the culture media that have been designed in different species to eliminate glucose during the pre-compactation period, and to add it during the post-compactation period. These media are based on the fact that ATP production is very low during the first developmental stages, and also on the fact that the high glucose concentrations in these stages may be toxic for the embryo (Jiménez *et al*., 2003).

In pigs, NCSU-23 (Petters and Reeds, 1991) is the most widely used medium for embryo culture. It was used in our study with all the new modifications. This medium contains fatty-acid free albumin, but not serum, insulin as a growth factor, essential and non essential amino acids, as well as glucose, among other substances. In order to improve the *in vitro* blastocyst rate after zygote culture through ICSI, we designed an experiment to verify whether the direct transfer of the zygotes to this medium (NCSU) (which has been used in different research projects: Kim *et al.,* 1998; Kolbe and Holtz., 1999; Lai *et al.,* 2001; Lee *et al.,* 1998, 2003) is one of the causes of low performance obtained. According to our hypothesis, the fact that the ICSI technique suppresses steps in the fertilization process related to gamete recognition, sperm-ZP binding, and the induction of the acrosome reaction or the oolema fusion, does not mean that a recently injected zygote is found in a pronuclear state, nor that its culture requirements are equivalent to a two-cell embryo. For this purpose, we decided to compare the effect that the culture times in TALP medium has on embryo development results before the transfer to NCSU-23 medium takes place. The pronuclear formation is produced in the TALP medium during traditional IVF (Coy *et al.,* 2002; Matás *et al.,* 2003) and the zygotes are transferred to the NCSU medium after a variable period of time between 6 and 20 h. However, no references contemplating the ideal culture time sequence between TALP and NCSU media of the recent injected pig zygotes were found in our bibliographic revision , even though references do exist which show that the pronuclear formation takes place during the first six hours after injection (Lee *et al.,* 2002).

According to our hypothesis, as pronuclear formation has taken place during the first six hours after injection, the fact that the zygotes are present in the IVF medium, which is enriched with calcium, pyruvate and lactate, means that this situation might improve, as could the early stages of embryo development. Maintaining these zygotes in this medium for a lengthy period of time (20 h) may also prove beneficial as this would provide sufficient time for the first embryo cleavages to be produced, since this occurs in the oviduct, before they are transferred to NCSU-23.

These results partially confirm our hypothesis, since those zygotes which were cultured for 6 or 20 hours in TALP medium reached the two-cell stage more quickly than those zygotes which were directly transferred to NCSU did. The fact that these first cleavages may be observed during the first 18-20 h post-injection in TALP medium indicates that the viability of these embryos would possibly improve, despite the fact that no significant effect was observed in the blastocyst rate at 7 days of culture. As previously demonstrated, the moment of the first cleavage is an important indicator of further developmental capacity (Lonergan *et al.*, 1999). An alternative explanation might be a quicker preimplantational development of male embryos in relation to female embryos when glucose is present in the medium (Larson *et al*. 2001), as is our case. Under our experimental conditions however, this possibility would not explain the differences found since the glucose concentration in both TALP and NCSU-23 medium was the same.

With regards to the remaining assessed parameters, where no differences between the three experimental groups were found, they may be useful to gauge the validity of our methodology. In this way, oocyte activation which was around 83% in all cases, is comparable to the results obtained by Katayama *et al*. (2002) where recently injected oocytes were cultured in IVF medium (TCM-199); or to those by Lee *et al* (2003) with NCSU-23. In the same way, our pronuclear formation results at 22h, or our cleavage rates at 48h post injection, were similar to those obtained by other researchers (Kim *et al.,* 1998; Lai *et al.,* 2001; Probst and Rath, 2003).

To conclude, the results of this first experiment confirm our hypothesis that the culture of injected oocytes in IVF medium before they are transferred to EC medium is beneficial for the zygotes. However, no differences were found between 6 or 20 h. We believe that a greater

presence of calcium in TALP medium (necessary for pronuclear formation) may be one of the reasons for these results. On the other hand, as we have indicated, pyruvate and lactate are important energy supplements for early stages of embryo development *in vitro* (Kim *et al*., 2004). Recently, it has been reported that lactate and pyruvate supported greater development in terms of blastocyst formation rate and total cell number in PZM and NCSU-37 media (Kikuchi *et al*., 2002; Yoshioka *et al*., 2002). To this end, the greater proportion of embryos obtained with two cells in TALP may also be due to the presence of pyruvate and lactate in this medium (even though it is not an exclusive EC medium).

However, due to the low blastocyst rate obtained (below 10%), we decided to continue working on our second hypothesis, which proposed the introduction of changes to the IVM system, with an increase of cytoplasmic maturation, initially by using meiotic inhibitors, and secondly through the changes made in the IVM duration.

5.2 EFFECT OF THE ROSCOVITINE ON *IN VITRO* MATURATION, ICSI AND ON *IN VIVO* EMBRYO DEVELOPMENT.

Several ICSI studies conducted on pigs suggest the incomplete terminal differentiation of the oocyte as the main cause for the low capacity of *in vitro* matured oocytes developed after fertilization (Kolbe and Holth, 1999; Probst and Rath, 2003). In order to attempt solving these maturation problems, several meiotic inhibitors which maintained the oocyte at the GV stage have been employed. In this way, an attempt to mimic the internal *in vivo* conditions of the follicle is carried out, whereas an increase of the cytoplasmic maturation period is also attempted. Roscovitine (Meijer *et al.,* 1997) is one of the most effective MPF inhibitors; it has been proved that this may inhibit a lower quantity of kinases than 6-DAMP, but with more specificity in the ATP pocket binding of cdc2. Moreover, this has proved to be one of the inhibitors with less prejudicial effects, and although there are slight indications of a possible beneficial effect of preculture in roscovitine on further embryo development (Coy *et al.,* 2003; Marchal *et al.,* 2001; Mermillod *et al.,* 2000), interpretations of these results must be taken very cautiously since an evident effect of roscovitine on the inhibition of RNA synthesis has also been demonstrated (Ljungman and Paulsen, 2001). Furthermore, roscovitine does not prevent most of the modifications in phosphorilations patterns of the proteins observed during maturation

(Vigneron *et al*., 2004a), and several cellular events involved in the maturation regulation of the bovine oocytes seem to be independent of both MPF activation and the resumption of meiosis (Vigneron *et al*., 2004b).

The first step of this second experiment was to study the inhibitor effect of the roscovitine on nuclear oocyte status after 22 h of culture and after IVM. Next, we studied the effect this inhibitor had on cytoplasmic maturation by assessing the intracellular GSH content and the pronuclear formation after ICSI. Finally, the injected zygotes were directly transferred to the oviduct of recipient gilts to allow for *in vivo* development, given the EC results obtained in the previous experiment.

In our study, doses of 50µM roscovitine were sufficient to block the GVBD in more than 90% of oocytes after a 22-hour culture with similarities between control and ROS groups being evident for the GV-I and GV-III stages. On the contrary, when the IVM medium with no roscovitine was employed, oocytes started to spontaneously resume meiosis. Moreover, when the NCSU-37 medium without dibutyryl cAMP was used, the progression to stages beyond GV-IV was faster, since the inhibitory effect of dibutyryl cAMP (Funahashi *et al*., 1997a) was absent. These data demonstrate that the progression of porcine oocytes to the GV-III stage in the presence of 50μM roscovitine is differently inhibited than in the presence of dibutyryl cAMP, or in the absence of inhibitors, since they remain at nuclear stages that are similar to those in the follicles, which were assessed just after recovery.

The effect of roscovitine on the resumption of meiosis has been previously showed in pigs. Ju *et al*. 2003 found that levels of 80-120μM of roscovitine were necessary to inhibit germinal vesicle breakdown (GVBD) in 83-91% of oocytes. However, their study assessed the nuclear stage of oocytes 44 h after beginning to culture in roscovitine, when the inhibitor activity could have decreased in the medium. In fact, roscovitine has an approximate 24-hour lifespan in blood plasma concentration (Meijer and Raymond, 2003). Likewise, Mc. Clue *et al*. (2002) observed that the effect of roscovitine peaked between 8 and 24 hours after the culture commenced.

The reversibility of the roscovitine effect on the nuclear maturation has been corroborated in our study by the high levels of Metaphase II stage oocytes reached after a 22-hour inhibition following a 44-hour culture in the

conventional IVM system. Similar results with 25µM roscovitine were obtained by Mermillod *et al.* (2000) in cattle. These authors demonstrated that GVBD was inhibited with this concentration, after a 24-hour culture. This was indeed a reversible effect, where more than 89% of the oocytes resumed meiosis when they were cultured without the inhibitor.

Despite our study demonstrating the reversibility of ROS by obtaining a high rate of MII oocytes, we cannot deduce whether the cytoplasmic maturation has actually increased. However, the fact that nuclear and cytoplasmic maturations are not always correlated is well documented (Coy *et al.*, 1999). The resumption of meiosis in oocytes after removal from roscovitine presumably re-starts MPF activation, yet it is not a prerequisite to ensure the cytoplasmic maturation of such oocytes. However, intracellular GSH content and the oocyte´s ability to form the male pronucleous, feature among the commonly accepted parameters used to assess the cytoplasmic maturation in porcine oocytes (Coy *et al.*, 1999; Funahashi *et al.*, 1995; Sawai *et al.*, 1997).

GSH is dependent on the amount of cysteine made available to the cell (Meister and Tate, 1976). Cysteine is commonly added to the pig oocyte maturation medium at a 0.57mM concentration, and as a substrate for GSH synthesis, to promote male pronuclear formation (Yoshida *et al.*, 1993), and this was our case for NCSU-37. In our study, the GSH content of immature oocytes just after removal from follicles was lower than that found after 22 or 44h of culture in the IVM medium. The values obtained in the latter case were similar to those obtained by Brad *et al.* (2003). This indicates that the IVM system we employed was effective for the oocyte in GSH synthesis.

Interestingly enough, the GSH content was higher at 22h of culture than it was at 44h, which differs to what Yoshida *et al.* (1993) found, who reported a continuous increase in GSH content during IVM. On the one hand, these authors employed TCM-199 for IVM, which is a rich culture medium with readily available GSH precursors (cysteine, cystine, glutamic acid, glutamine and glycine) and even glutathione. However, NCSU-37 was used in our study which is only supplemented with cysteine and glutamine, where the former auto-oxidized to cystine in the maturation medium within 1 h (de Matos and Furnus, 2000), therefore it cannot be incorporated as easily as cysteine can (Yoshida & Takahashi, 1998). On the other hand, it has been reported that GSH synthesis is related to the acquisition of the

sperm nuclear decondensing ability during initial and mid phases of porcine oocyte maturation (Yoshida, 1993), and that GSH synthesis is responsible for high GSH levels in mature oocytes during the initial stages of hamster oocyte maturation (Perreault *et al.*, 1988). These observations suggest a strong GSH synthesis during the first half of oocyte maturation, and they would be in accordance with our results which showed a GSH peak after 22h of culture.

Another interesting finding in our study shows that those oocytes which were precultured in roscovitine for 22h had higher GSH levels than those cultured with dibutyryl cAMP for the same period of time. Cystine uptake activity is abolished by the mechanical disruption of gap junctional communication (Yoshida and Takahashi, 1998), and it has been suggested that GSH synthesis may be impaired due to the uncoupling of *cumulus* cells (de Matos *et al.*, 1998). Since roscovitine-treated oocytes showed no *cumulus* expansion as the dibutyryl cAMP-treated ones showed (Coy *et al.*, 2004; Marchal *et al.*, 2001; Schoevers *et al.*, 2004), the closest cooperation between the *cumulus* cells and oocyte would be maintained for a longer time, which would partly explain the higher GSH content observed in roscovitine-treated oocytes.

Our first data reveal that roscovitine does not affect the male pronuclear formation through ICSI fertilization. Moreover, no differences were found in any of the fertilization parameters assessed at 22 hours post injection (hpi). These results were expected given the similar GSH levels we found for *in vitro* matured oocytes precultured either with or without roscovitine, since it has been reported that male pronuclear formation is related to intracellular GSH content (Funahashi *et al.*, 1995; Yoshida *et al.*, 1992). The activation and putative zygotes rate were similar to those results obtained by other researchers who did not use roscovitine (Kim *et al.*, 1998; Lee *et al.*, 2003). As far as we are aware, no references to pig oocytes that had been prematured with roscovitine and fertilized by ICSI are found. Recently, Franz *et al.* (2003) worked with mare oocytes that had been prematured in ROS and fertilized by ICSI. They demonstrated that ROS is capable of increasing the development capacity when *cumulus*-enclosed oocytes are used, and a higher cleavage rate was also obtained. Our study has also demonstrated that the cleavage kinetics was higher in ROS oocytes, and that the cleavage rate assessed at 22hpi was higher in those oocytes. Our hypothesis was partially confirmed, as the moment the

first cleavage takes place is an important indicator of further embryo development capacity (Lonergan *et al*., 1999), as we have already mentioned. However, this ROS effect cannot be demonstrated in cattle (Mermillod *et al.,* 2000).

We chose to place the zygotes in the best possible physiological medium immediately after the injection, such us the oviduct of recipient females, in order to achieve the best possible viability. The results of the *in vivo* experiment showed that the ICSI embryos premature with ROS and transferred to female receptors, were capable of establishing pregnancies, yet they were not capable of reaching full term. The same results were observed for the control group.

Those few studies conducted with IVM-ICSI and ET showed that the number of piglets obtained was very low (Kolbe and Holtz, 2000; Lai *et al.,* 2001; Martin, 2000; Nakai *et al.,* 2003; Probst and Rath, 2003). Only one group obtained three piglets with IVM oocytes *(*Nakai *et al.,* 2003*),* and the success rate seemed to increase when *in vivo* matured oocytes are employed (Probst and Rath, 2003), the result of which was 6-7 piglets per litter. Therefore, in spite of the beneficial effect of ROS during early development stages, ICSI zygotes demonstrated difficultly in developing to term even in the most favorable culture conditions (in the pig oviduct).

It is known that only around 20-30% of the transferred embryos survive with traditional IVF, despite the improvements in pig IVP techniques (Abeydeera, 2002), and that only around 6 piglets are obtained from the transferred female (Abeydeera *et al.,* 1998). With a view to demonstrating that ROS was not detrimental for embryo development, and that live offspring could be obtained, a parallel study was carried out in our laboratory with traditional IVF (not presented in this study). Between 10-12 piglets were obtained for the control and ROS groups, respectively. This is the first study conducted that proves that live offspring can be obtained through IVM oocytes prematured with ROS (Coy *et al.,* 2004, 2005).

Similar results may be obtained by using ROS-prematured oocytes without the inhibitor. The use of cultured pig oocytes under meiotic inhibitor conditions with no decrease in development capacity is feasible, and it permits timetable flexibility in complex experiments. Therefore, not only would it prove to be a most useful tool for ovary collection in laboratories

which have no slaughterhouse in their vicinity, but it could also be a useful tool to study the influence of exogenous factors on oocyte terminal differentiation.

With this strategy (slaughterhouse oocytes kept with ROS for a few hours to adjust our protocol), we decided to study the effect that IVM duration has on ICSI performance. A well-known critical factor is oocyte age, and aged oocytes have a limited development capacity. Moreover, a lower MPF quantity exists in these oocytes. MPF is involved in p220 phosphorylation that binds tubuline and affects the microtubular formation, thus aged oocytes have a greater degree of fragmentation than the rest of oocytes do (Kikuchi, 2000). We decided it would be suitable and necessary to carry out this experiment since no references comparing IVM duration and ICSI fertilization in pigs exist.

Three different *in vitro* maturation times of common use in pig IVM were used, 36h (Ka *et al*., 1997; Yoshida *et al*., 1993), 40h (Galeati *et al*., 1991; Liu *et al*., 1997) and 44h (Coy *et al*., 2003; Galeati *et al*., 1991;). A preculture in ROS permits an adjustment to the injection timetable to be made, as previously mentioned. A previous experiment showed that ROS prematured oocytes and further 44-hour maturation obtained the two-cell stage more quickly than the control oocytes did. On this occasion, we attempted to investigate whether IVM duration could be reduced to increase embryo development post-ICSI with prematured oocytes.

The results confirmed our hypothesis. When the IVM time was reduced, the putative embryo rates were significantly higher than in the other groups. Embryo development and male pronuclear formation were slower when oocytes underwent a maturation of either 40 or 44h, rather than 36h. We found no bibliographic references comparing this IVM duration time and further ICSI fertilization. IVM times vary by 42 and 50h (Kim *et al*., 1998; Lai *et al*., 2001; Lee *et al*., 2003). However, the putative embryo rates in all these studies did not reach the rates obtained in our study (73.5%). We believe that most of the differences could be due to the different exogenous oocyte activation treatments involved, and also to both the oocyte quality and IMV system. The ovaries in our study came from hybrid commercial females weighing 85-95kg (8-9 months) which were close to puberty. Moreover, the slaughterhouse is only a an hour's distance from our laboratory, so the ovaries arrive in good condition. Oocytes are

obtained by slicing antral follicles. Oocyte collection is known to affect the posterior development. In 1997, Liu and Moor obtained higher nuclear maturation and cleavage rates in oocytes harvested by dissection rather than by aspiration. In our study, only the best follicles were sliced, so we selected most of the oocytes at this level. A second more thorough selection was performed under the stereomicroscope. Only COCs and a complete and dense *cumulus oophorus* were used for the experiments. This factor is also important in IVP systems (Coy and Romar, 2002)

Another crucial factor could be the age of oocytes, as previously verified. Aged oocytes have less fertilization ability than normal ones have due to the lower H1kinase amount, and also to the lower quantity of activated MPF (Kikuchi *et al.*, 2000). Funahashi *et al*. (1997a) demonstrated that around 47% of the oocytes reached MII at 36h of IVM, so most oocytes were old at the time of the *in vitro* fertilization.

In cattle however, it has been demonstrated that roscovitine prematurated oocytes reached the MII stage earlier than the non prematurated oocytes did. This fact is not yet demonstrated in pigs. In order to verify that the differences observed in our study had no relation with roscovitine prematuration, we designed an additional experiment where the oocytes were matured either with or without roscovitine for 36h.

As the results showed, no significant differences were found for any of the variables analyzed. Furthermore, the putative embryo rate reached 70% once more. This fact indicated that early embryo development was affected with a 44-hour IVM period, regardless of the fact that an inhibitor, such as roscovitne, had been used or not. This was not observed in shorter IVM periods (i.e. 36h); where the rate of zygotes showing 2PM were higher than the remaining groups after ICSI.

After analyzing these results, the possibility that the failure of embryo development after ICSI was related to an inappropriate activation after the microinjection still remained to be investigated, as suggested by other research groups (Lai *et al*., 2001; Lee *et al*., 2003*;* Nakai *et al*., 2003; Probst *et al*., 2003).

5.3 EFFECT OF InsP$_3$ INJECTION ON *IN VITRO* EMBRYO DEVELOPMENT.

With the previous ICSI results obtained throughout this study, we can affirm that the exogenous activation of the oocyte is not needed to obtain high percentages of putative embryos. The sperm stimulus is sufficient to both activate the oocyte, and initiate embryo development. However, given the low number of embryos reaching the blastocyst stage (although they were no lower than they were in other research groups), we posed the possibility that the activation was inadequate, and therefore, the oocyte was incapable of further embryo development for the blastocyst stage to be reached.

Inositol 1, 4, 5-triphosphate (InsP$_3$) is one of the oocyte activation messengers (Wu *et al.,* 2001). Its action mechanism consists of inducing an increase in calcium concentration from the endoplasmic reticulum (ER). Although the mechanism through which the sperm-oocyte binding promotes calcium oocyte oscillations is not altogether elucidated, it is known that an activation of phospholipase C is observed during the phosphoinositol activation pathway, which mediates in the PIP$_2$ hydrolysis to produce DAG and InsP$_3$. InsP$_3$ then goes on to produce calcium liberation from the ER. In 1993, Miyazaky *et al*., demonstrated the InsP$_3$ function during the fertilization process. They observed an InsP$_3$ liberation blocking when InsP$_3$-R inhibitor antibodies were injected.

At first we thought that the calcium liberation produced by the sperm injection was not similar in either its duration and intensity to that produced during normal fertilization because, although an oocyte activation was produced, further embryo development was defective. So, we decided to continue with our third hypothesis: an attempt to increase oocyte activation through an InsP$_3$ injection together with sperm.

In vitro, Amano *et al*. (2004) used this molecule as a parthenogenetic oocyte activator in pigs. They demonstrated that the InsP$_3$ injection promotes oocyte activation, cleavage, and even blastocyst formation. This capacity could be positively related to the calcium increase produced by InsP$_3$.

The blastocyst rate results obtained showed no improvement after the InsP$_3$ injection in comparison to the control group (sperm injected

224

without InsP$_3$). As far as we are aware, the InsP$_3$ injection together with sperm has not been used to improve ICSI performance in pigs. Nonetheless, Kurokawa and Fissore (2003) have demonstrated that ICSI fertilization modifies the start of calcium oscillations, and that it provokes a lower persistence and duration in these oscillations than during traditional IVF, this being the main cause of low embryo developmental capacity of ICSI zygotes in mice. The results could be explained as Barenzes *et al*. (2004) proposed, since we did not properly mimick the calcium oocyte dynamics after fertilization, which consists of sequential activation and inhibition function series. These authors achieved mice offspring (67%) after the oocytes underwent 24 electric pulses every 8 minutes for three hours, that corresponded to the 24 calcium oscillations provoked through the intracellular calcium release. Possibly, if we had sequentially injected the InsP$_3$ into either the oocyte or the medium in our study, we would have seen some improvement. However, we cannot make a categorical interpretation of our results due to the low blastocyst number obtained in this experiment. In 2001, Lai *et al*. obtained a 30% blastocyst rate when electrical activation was employed, and only a 4.6% rate was obtained without this artificial activation, which matches our results.

The cleavage rate was also similar between both groups of injected sperm (with or without InsP$_3$). However, differences were found among the sham groups; sham-IsnP$_3$ displayed a higher cleavage rate than the sham-buffer group did, which reveals InsP$_3$-oocyte activation. In all cases, the rates were lower than those obtained by Amano *et al*. (2004). We believe that differences could lie in the InsP$_3$ concentration used, as we always worked at concentrations below 500μM. All in all, in our experiment we observed that the effect that sperm had on the cleavage was superior to the InsP$_3$ effect, indicating that sperm stimulus is sufficient to obtain cleavage rates that are similar to others groups which use activating agents.

Nevertheless, research has still to discover the information and knowledge in the void that exists between the sperm injection and the start-up of all the appropriate mechanisms that lead to the zygote becoming a blastocyst.

FURTHER RESEARCHES

6

FURTHER RESEARCHES

METODOLOGICAL NEWS

- This is the first study comparing a sequence of employment of two culture media post-ICSI to identify its effect on the final ICSI yield.
- Another new procedure is the use of a two steps in vitro maturation system, first step with a meiotic inhibitor, in which the intracellular GSH content and fertilization ICSI parameters were assessed.
- It is the first study to assess $InsP_3$ injection together with sperm in pigs.

FURTHER RESEARCHES

Further researches should be directed to improve the embryo culture media through cellular cocultures (oviductal and endometrial cells) or through modifications in their composition. An important effect that should be studied is the effect of the oviductins on the oocyte and further embryo development.

It would be interesting to evaluate different $InsP_3$ concentrations and times and analyse the intracellular calcium oscillations through intracellular calcium measurements. These ICSI oscillation patterns might be compared with IVF patterns and we could be able to explain the low development of ICSI zygotes.

Finally, we could study microtubular and microfilament system after ICSI in order to detect if they are altered. In this case, part of the low developmental ICSI capacity will be explained.

_____ CONCLUSIONS

7

CONCLUSIONS

1. ICSI derived pig zygotes achieve rates of two cells stage at 22h in higher proportion if they are cultured 6 or 20 hours previously in TALP medium than if they are directly transferred to NCSU-23 medium.

2. The culture of pig oocytes in 50µM roscovitine during 22 hours inhibits meiosis and increases intracellular glutathione content. This is a reversible effect after the in vitro maturation procedure and it increases the rate of two cells stage embryos after ICSI.

3. ICSI derived zygotes are able to establish pregnancies when they are transferred into female receptors nevertheless if they are treated or not with roscovitine.

4. A 36 hours period of in vitro maturation with or without roscovitine increases the putative embryos rate at 22h post-injection.

5. The inositol triphosphate injection together with the sperm does not increase the final ICSI performance in pigs.