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ANALYSIS OF DIFFERENT FACTORS AFFECTING THE INTRACYTOPLASMIC SPERM INJECTION (ICSI) YIELD IN PIGS

DOCTORAL THESIS

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MATERIAL AND METHODS

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Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

3.1 WASHING AND GAMETE MANIPULATION MEDIA

3.1.1 TRANSPORT AND WASHING OF OVARIES AND OOCYTES

Media used for ovary transport, washing and oocyte obtention were the following:

- Saline Solution (SS): 0.9% w/v NaCl with 100 mg/l of kanamicine sulphate.

- Cetrimide Solution (Hexadecil-trimetilamonie bromure: Cetab) 0.04% (w/v).

Both solutions were prepared with ultrapure water (Mili-RX) and were maintained at room temperature until use.

- Dulbecco modificated Phosphate saline (D-PBS) (Sigma D-8662) supplemented with 1mg/ml of alcohol polivinil and 0.005mg/l of phenol red as pH indicator. This medium was stored at 4ºC until use.

The medium used for matured oocyte washing and during the injection consisted of **supplemented D-PBS** with 10%(v/v) of foetal calf serum(FCS; Biological Industries®, Haemek, Israel).

3.1.2 DILUTION AND WASHING

The medium used for sperm dilution and washing was the *Beltsville Thawing Solution* (BTS) (Table 1):

TABLE 1. BTS extender for boar sperm (Pursel and Johnson, 1975)

Component	Concentration (mM)
Glucosa	0.0002
Na_2 -EDTA.$2H_20$	3.36
CO_3HNa	15
Citrate-Na_3.$2H_2O$	20
ClK	5

For ICSI sperm treatment, the medium used consisted of supplemented D-PBS.

3.2 CULTURE MEDIA AND SUPPLEMENTS

NCSU-37 stock medium. A stock solution was prepared in the laboratory (table 2) with ultrapure water (Milli-Q). Then, it was sterilized by filtration through 0’22 μm filters, and it was stored in sterile conditions at 4°C during two weeks.

For IVM, NCSU-37 stock medium was supplemented with cysteine, β-mercaptoethanol, insuline, dbAMPc, porcine folicular fluid (PFF), PMSG y HCG (Foligón, Chorulón; Intervet International B.V., Boxmeer, Holanda) as described by Funahashi *et al*. (1997) table 3.

TABLE 2. Composition of NCSU-37 stock medium (Peters and Wells, 1993).

Component	Concentration(mM)
NaCl	108.73
$NaHCO_3$	25.07
KCl	4.78
KH_2PO_4	1.19
$MgSO_4 \cdot 7H_2O$	1.19
$CaCl_2 \cdot 2H_2O$	1.70
Glucosa	5.55
D-Sorbitol	12.00
Penicillin G sodic	0.18
Streptomicin Sulphate	39.00 UI/ml

TABLE 3. NCSU-37 medium supplements for *in vitro* porcine oocyte maturation (Funahashi *et al*., 1997a).

Component	**Concentration (mM)**
Cysteine	0.57 mM
β-mercaptoethanol	50.00 μM
Insuline	5.00 mg/L
DbAMPc	1.00 mM
PFF	10% (v/v)
PMSG	10 UI/ml
HCG	10 UI/ml

TALP medium. Stock solution (table 4) was prepared in the laboratory with ultrapure water. Sterilization was done by filtration and the medium was stored under sterile conditions at 4°C during two weeks.

TABLE 4. Composition of TALP stock medium (Rath *et al*., 1999).

Component	Concentration(mM)
NaCl	114.06
$NaHCO_3$	25.07
KCl	3.20
NaH_2PO_4•H_2O	0.35
$MgCl_2$•$6H_2O$	0.50
Lactate Ca•$5H_2O$	8.00
Lactate sodic	10.00
Glucose	5.00
Cafeine	2.00
Kanamicin sulphate	0.17
Phenol red	0.003
PVA	1 mg/ml

For the culture post ICSI, TALP stock medium was supplemented with 3 mg/ml of bovine seric albumin (BSA-FAF) and 0.12 mg/ml of sodic piruvate (Rath *et al*., 1999). The pH was adjusted to 7.4 in the incubator at 5% of CO_2, 38.5°C and saturate humidity atmosphere for 20h before use.

NCSU-23 medium. Following the protocol of Peters and Wells (1993) the stock solution (Table 5) was prepared with ultrapure water, and it was further filtrated and stored at 4ºC during two weeks. At the time of using for EC, NCSU-23 stock was supplemented with 4 mg/ml of BSA-FAF, insuline 0.57mM, β-mercaptoethanol 50μM, 2% v/v BME (Aminoacids solution 50x) and 1% v/v MEM (non-essential amino acid solution 100x). Finally the solution was filtered and kept in the incubator for one day before use.

TABLE 5. Composition of NCSU-23 stock medium (Peters and Wells, 1993).

Component	Concentration (mM)
NaCl	108.73
$NaHCO_3$	25.07
KCl	4.78
KH_2PO_4	1.19
$MgSO_4{\bullet}7H_2O$	1.19
$CaCl_2{\bullet}2H_2O$	1.70
Glucose	5.55
Taurine	7.00
Hipotaurine	5.00
Penicillin G sodique	0.18
Streptomicin sulphate	39 UI/ml

R-enantiomer of roscovitine (2-[R]-[1-Ethyl-2-hydroxyethylamino]-6-benzylamino-9-isopropylpurine; R-7772) was solubilized in dimethyl sulfoxide before freezing at −20°C as a 10 mM stock.

Inositol 1, 4, 5-trifosfato ($InsP_3$, Sigma I-7012). $InsP_3$ was diluted to 0.5μM in a buffer solution (120mM KCl, 20mM Hepes, and pH 7.4) before freezing at -20ºC until use (Amano *et al*., 2003).

3.3 IN VITRO MATURATION (IVM)

The medium used for oocyte maturation was NCSU-37 (Peters and Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/ml insulin, 50 μM β-mercaptoethanol, 10 IU/ml eCG (Foligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), and 10% porcine follicular fluid (v/v).

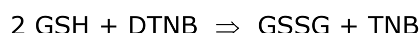
Within 30 min of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline containing 100 μg/ml kanamycin sulfate at 38°C, washed once in 0.04% cetrimide solution and twice in saline. Oocytes-cumulus cell complexes (COCs) were collected from antral follicles (3-6 mm diameter) by slicing, washed twice with Dulbecco's PBS supplemented with 1 mg/ml PVA and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO_2 in air. Only COCs with a homogeneous cytoplasm and a complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 μl maturation medium for 22 h at 38.5ºC under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG and hCG and cultured for an additional 20-22 h (Funahashi and Day, 1993). This in vitro maturation system is commonly employed in pigs for several years and it is based upon the results from Funahashi et al. in 1997.

In the experiments with roscovitine, around 45-50 oocytes were allocated in a petri dish with 500μl of NCSU-37 without PMSG, HCG and dbAMPc and with 50μM of roscovitine during a period of 20-28h. Then, these oocytes were matured in the same way as the others (Funahashy and Day, 1993).

3.4 INTRACELLULAR GSH CONTENT

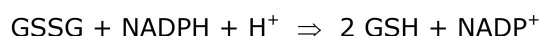
To analyse the content of intracellular gluthatione in the matured oocytes, the method described by Tietze (1969) with modifications (Anderson, 1985) was carried out. This method is based in the cyclic test of the enzyme 5.5-dithiobis-(2-acid nitrobenzoic)-glutathione reductase (DTNB-GSSG) that detects into the oocytes the reduced and oxided gluthatione.

This cyclic test occurred in two steps. In the first one, the reduced glutathione with DTNB is oxided:



$$2\ GSH + DTNB \Rightarrow GSSG + TNB$$

And in the second step the reductase enzyme DTNB-GSSG catalyzes the following reaction:



$$GSSG + NADPH + H^{+} \Rightarrow 2\ GSH + NADP^{+}$$

GSH: reduced glutathione.
GSSG: oxided glutathione.
DTNB: 5.5-dithiobis-(2-acid nitrobenzoic).
TNB: 5-thio-2-acid nitrobenzoic.
NADPH: reduced dinucleotid phosphate of nicotinamide and adenine.
$NADP^+$: oxided dinucleotid phosphate of nicotinamide and adenine.

The total content of glutathione can be estimated from the TNB production that is determined to 412 nm (UV) of absorbance.

The **employed reactive** were:

- Phosphoric acid (H_3PO_4).
- Phosphated sodic hidratated ($NaH_2PO_4H_2O$).
- Phospahted sodic (Na_2HPO_4).
- acid etilen-diamine-tetracetic (EDTA).
- DTNB.
- GSH.
- DTNB-GSSG reductase.
- NADPH.

The employed solutions were prepared with ultrapure water at the concentrations indicated in table 6.

TABLE 6.Solutions for glutathione assay.

SOLUCIONS	CONCENTRATION
H_3PO_4	1.25 M
$NaH_2PO_4H_2O$	0.2 M
Na_2HPO_4	0.2 M
EDTA	10 mM
DTNB	6 mM
Glutatione reductase	266 UI/ml

Solutions were prepared as follows: The buffered and NADPH stock solutions:

- Buffered stock solution: a 0.2 solution of sodic hydrated phosphate and 10 mM of EDTA (solution A) was prepared. Another similar solution but with sodic phosphate (solution B) was also prepared. Finally, the buffered stock solution was prepared by mixing both solutions A and B until reaching a pH of 7.2.
- NADPH solution: it consisted of a solution of 0.3 mg NADPH per ml of buffered stock solution.

Samples were prepared following the methodology describes by Funahashi et al. (1994b). Briefly, *cumulus oophorus* cells were removed by successive gently aspiration through an automatic pipette (Nichiryo). Denuded oocytes were aspirated into three times washed in a buffered stock and samples of 30 oocytes were aspirated into a volume of 5 µl. Samples were introduced at the botton of Eppendorf tube and 5 µl phosphoric acid 1.25 M were added. The tube was stored immediately at -20ºC until analysis.

The standards were prepared the same moment of the assesment with 5 GSH knonw solutions: 0.01, 0.025, 0.05, 0.1 and 0.5 mM. For each one of them it was mixed 5µl of the GSH solution respectively, and 5µl of phosphoric acid 1.25 M. The standard "cero" was prepared with 5µl of PBSDm and 5µl of phosphoric acid 1.25 M.

The oocytes samples were thawed at room temperature and after a volume of 700 µl NADPH solution, 100 µl DTNB solution and 190 µl purified water, was added to each one. The oocytes were maintained at room temperature during 15 minutes and then 10 µl of GSH reductase was added to start the reaction. Then, samples were introduced in a spectrophotometer (Beckman DU-40) where the TNB formation at 412 nm (UV) absorbance every 30 seconds during two minutes was assesed.

The GSH concentration of the samples was determine in every one by comparison with the Standard curve. Total quantity of glutathione was divided by the number of oocytes in the sample to obtain the total quantity of GSH per oocyte.

3.5 SPERM PROCESSING

Fresh semen was collected once a week from three stud boars of known fertility by the gloved hand method. The sperm rich fraction was collected, immediately transported to the laboratory and diluted in Beltsville thawing solution (BTS) at 15ºC. The fresh diluted spermatozoa were used the same day as collection.

DPBS treatment involved the centrifugation of 10 ml of the fresh semen samples at 1200 x*g* for 3 min. The pellet was then resuspended in DPBS supplemented with 10% FCS to reach a concentration of 5×10^5 spermatozoa/ml.

3.6 INTRACYTOPLASMIC SPERM INJECTION

Oocytes cultured for 44 h in maturation medium were mechanically denuded by gentle aspiration with a pipette. Denuded oocytes were washed twice in supplemented DPBS medium and transferred to ICSI drops. ICSI was conducted on a heated microscope at 200X magnification using a Nikon Diaphot 300 inverted microscope with attached micromanipulators. The ICSI medium used was DPBS supplemented with 10% FCS. Prior to ICSI oocytes were placed in a lid of Petri dish (1 oocyte per drop of 4µl of DPBS-FCS). In total 10-15 micro-drops were placed in each lid surrounding central sperm drops which contained 4 µl of DPBS-FCS and 1µl of the sperm suspension. The microdrops were covered with mineral oil (Sigma M-8410). ICSI was performed as described by Probst and Rath (2003). Briefly, one single sperm was immobilized by crushing the mid-piece with the tip of the injection pipette. The immobilized sperm was aspirated with the tail first. Thereafter, the injection pipette was moved into the drop containing the oocytes to be injected. A single oocyte was fixed with the holding pipette positioning the polar body in a 6 or 12 o'clock position. The injection pipette was pushed through the *zona pellucida* and the oolema into the cytoplasm at a 3 o'clock position. Small amounts of cytoplasm were sucked into the injection pipette in order to prove that the sperm was introduced through the oolema into the cytoplasm. Subsequently, the aspirate with the immobilized spermatozoon was released into the cytoplasm. The temperature was maintained at 38.5ºC through the whole procedure using

a heated microscopical stage. The injected oocytes were placed in TALP medium.

At 22 hours after ICSI, samples of oocytes/zygotes were fixed with acetic alcohol, stained with 1% (w/v) lacmoid and examined at 400x magnification under a phase contrast microscope. Oocytes with at least one pronucleus (PN) were classified as activated. Stages with two pronuclei were classified as fertilized in the sperm injected group and as parthenogenetically activated in the sham group. Oocytes with non-classifiable forms were named as "other".

3.7 EMBRYO CULTURE

After 22h in TALP medium, putative zygotes were washed three times in NCSU-23 previously equilibrated overnight, transferred in a 4-well Nunc multidish containing 500 μl of NCSU-23 per well, and incubated at 38.5ºC and 5% CO_2 in 100% humidified air. Forty eight and 144 h after fertilization, cleavage rate and blastocyst formation, respectively, were evaluated under a stereomicroscope. Blastocysts were then placed on a slide, air-dried, and fixed in absolute ethanol for 24 h. Nuclei were counted under an epi-fluorescence microscope by staining with Hoechst 33342 (10 μg/ml in 2.3% sodium citrate).

3.8 *VALORATION OF RESULTS*

3.8.1 ASSESMENT OF NUCLEAR STATUS

The nuclear status were assesed following the technique described by Chang (1952) with minor modifications (Coy, 1992). Briefly, after the fixation period in ethanol-acetic (1:1) during 24h, the oocytes were stained with lacmoid.

3.8.2 ASSESMENT OF PRONUCLEAR FORMATION

After the culture in TALP medium during 22h, the injected oocytes were evaluated following two methods in relation to the experiment; Experiment 1 and 2.4 were realized following the technique described by Chang (1952) with minor modifications (Coy, 1992). And experiments 2.6, 2.7 and 3 were evaluated with Hoecsth fluorocrome. Oocytes were fixed for 15 min (2% glutaraldehyde in PBS), stained for 15 min (1% Hoechst 33342 in PBS) and finally washed in PBS containing 1 mg/ml polyvinyalcohol and mounted on glass slides.

Oocytes were examined under an epifluorescence microscope at 200X and 400X(Leika®). The oocytes with the metaphase plate were considered as not activated, and the others were considered as activated (with female pronucleus, two cells stage or others). The oocytes with two pronuclei (with or without visible PB in the stain) and two cells embryos were considered fertilized.

The variables analized after ICSI were:

- Rate of degenerated oocytes (**% Degeneration)** in relation to the total injected oocytes.
- Rate of activated oocytes (**% Activation)** in relation to the not degenerated oocytes. The oocytes in the stage previous to two pronucleus 2PN (1PN and one compact or descondensed sperm head; **% Pre-2PN)**, oocytes in the two pronuclear stage or two cells embryos (**% 2PN and % Embryos**), the oocytes without classification (**% others**).

3.8.3 ASSESMENT OF EMBRYO DEVELOPMENT

Cleavage embryos were considered those that appear in two-four cells (homogeneous blastomeres) after 48h post injection. Following 7 days of culture, the zygotes that were visually like blastocyst were isolated under stereomicroscope and were put in warm PBS. Then, embryo quality was assesed. Number of blastomeres per embryo was counted (Dobrinsky *et al*., 1996). Briefly, the oocytes were fixed in alcohol during 24h, afterwards,

they were stained with Hoescht 33342 solution with 1% in PBS to stain the nucleus. Finally, they were visualized in a floorescent microscope at 400X and 495 nm wavelength. All nuclei were counted like the total number of nucleus per blastocyst.

The variables were:

- Rate of cleaved embryos (**% Cleavage**): The numbers of embryos in 2-4 cells stage were counted in relation to the total embryos injected and cultured.
- Rate of blastocyst (**% Blastocyst**): the number of blastocyst in relation to the total embryo cleaved.
- Average number of cells per blastocyst (**N cells/blastocyst**): the average number of stained nucleus per blastocyst.

3.9 EMBRYO TRANSFER

Multiparous crossbred sows physiologically synchronized were used to surgical embryo transfer. Oviducts from animals which had showed heating 72 h before embryo transfer were exposed through mid-ventral incision under general anesthesia, as we have previously described (Coy et al., 1993). Over 50 putative embryos (immediately after injection) were introduced in one oviduct by a Tom Cat Catheter (Kendall Co., Mansfield, MA, USA) connected to an insulin syringe containing PBS at 37ºC. The catheter was later observed under stereomicroscope in order to check that all embryos were transferred into the oviducts. The sows were kept under the usual farm conditions and 25 to 28 days after transfer pregnancy diagnosis was carried out by ultrasonography (Echoscan T-100).

3.10 EXPERIMENTAL DESIGN

EXPERIMENT 1. EFFECT OF CULTURE TIME IN TALP MEDIUM ON ICSI AND IN VITRO EMBRYO DEVELOPMENT.

***Experiment 1.1:* Effect of time culture in TALP medium on ICSI parameters**.

The assesment of fertilization was realized after 20-24h of injection. Part of the injected oocytes were fixed and stained with lacmoid.

This experiment was realized in 6 replicates and the total oocytes used were 361.

***Experiment 1.2*: Effect of time culture in TALP medium on in vitro embryo development**

The assesment of embryo development was done after culture of the zigotes in NCSU-23.

This experiment was realized in 4 replicates and the total oocytes used were 258.

***EXPERIMENT 2*. EFFECT OF ROSCOVITINE ON *IN VITRO* OOCYTE MATURATION, ICSI AND *IN VIVO* EMBRYO DEVELOPMENT.**

Before running the experimental work, 831 oocytes in 4 replicates were stained just after collection with BCB to approve the growth stage and to check if culture in roscovitine for 22 h inhibited the last step of growing.

***Experiment 2.1*: Nuclear status after 22 h culture in roscovitine**

This experiment was carried out to know if the culture of oocytes in NCSU-37 medium without dibutyryl cAMP, eCG and hCG and with 50 μM roscovitine for the first 22 h of maturation (ROS group, n=152) kept the nuclear stage at the same level than that found in the oocytes just after recovery (Before culture group, n=156). As control groups we employed oocytes cultured for 22 h in supplemented NCSU-37 medium under usual IVM conditions, which included dibutyryl cAMP, eCG and hCG (A group, n=173) and oocytes cultured for 22 h in the same NCSU-37 medium without dibutyryl cAMP, eCG and hCG (B group, n=164). Doing so, we were able to see if the inhibitory effect of roscovitine on the nuclear progression was similar or not to that demonstrated by dibutyryl cAMP, which blocks only oocytes at stages beyond the GV II-stage [28] and to compare the

nuclear stage at 22 of inhibited oocytes (ROS and A groups) and those under supposed spontaneous resumption of meiosis (B group).

After culture, oocyte nuclear status (GV-0 to Metaphase II) was recorded in all groups. This experiment was performed in 4 replicates.

***Experiment 2.2**: Nuclear progression after IVM with a prematuration period in roscovitine.*

In order to assess the reversibility of the treatment with roscovitine, COCs were cultured 44 h under permissive maturation conditions in each of 3 replicates with (ROS-IVM group, n=153) or without (IVM group, n=161) a previous culture period of 22 h in presence of 50 μM roscovitine. Nuclear stage was recorded by Hoechst staining.

***Experiment 2.3**: Oocyte glutathione content after roscovitine treatment.*

This experiment was designed to know the effect of roscovitine on the intracellular GSH content of the oocytes just after collection, at 22 h of culture with or without roscovitine, and after maturation with or without a preculture in roscovitine. In seven replicates, 960 COCs were collected and allotted in five groups: Before culture group (oocytes processed just after collection), A group (oocytes cultured in supplemented NCSU-37 medium with dibutyryl cAMP, eCG and hCG), ROS group (oocytes cultured 22 h in supplemented NCSU-37 medium without dibutyryl cAMP, eCG and hCG an with 50 μM roscovitine), IVM group (oocytes cultured for 44 h under the described step-wise in vitro maturation system) and ROS-IVM group (oocytes cultured for 22 h as the ROS group and for a further 44 h as the IVM group). GSH content was measured as above described.

***Experiment 2.4**: ICSI and full development of roscovitine treated oocytes.*

Just after recovering, oocytes were cultured in presence of 50 μM of roscovitine for 22 h. Following this period of time, oocytes were washed and allowed to mature for 44 h under permissive conditions (ROS-IVM group). Other COCs were also collected and introduced in the IVM system at the

same time than ROS-IVM group. Matured oocytes from both groups were then injected and after 20-24 h post-injection samples of the putative embryos were processed to assess the ICSI variables. The remaining cells were transferred into the oviduct of physiologically synchronized sows (one sow per group and replicate, ten sows as a total). Pregnancies were assessed by ultrasonography 25-28 days after transferring.

This experiment was realized in xx replicates and the total oocytes used were 254.

***Experiment 2.5*. Effect of IVM time on roscovitine treated oocytes and fertilized by ICSI.**

This experiment was carried out to evaluate the maturation time in different fertilization (ICSI) parameters. COCs were premadurate in roscovitine (50 µM), and then were matured following normal maturation culture during 36h, 40h and 44h. Next, putative embryos were transferred to TALP medium during 20-24h and then were stained with Hoechst 33342.

This experiment was realized in 7 replicates and the total oocytes used were 456.

***Experiment 2.6:* Effect of roscovitine pre-maturation and maturation during 36h.**

Just after recovering, oocytes were cultured in the presence of 50 µM roscovitine. Then, oocytes were washed and allowed to mature for 36 h under permissive conditions (ROS-IVM group). Other COCs were also collected and introduced in the IVM system at the same time as ROS-IVM group. Matured oocytes from both groups were then injected and 20-24 hpi, samples of the putative embryos were processed to assess the ICSI variables.

This experiment was realized in 3 replicates and the total oocytes used were 192.

EXPERIMENT 3. EFFECT OF InsP$_3$ ON IN VITRO EMBRYO DEVELOPMENT.

The object of this experiment was to increase the oocyte activation and as a result the final blastocyst rate through the InsP$_3$ injection together with the sperm. COCs were matured following the normal protocol of IVM during 40h. The InsP$_3$ injection was realized following the same protocol as the ICSI. The microinjection pipete was filled with InsP$_3$ medium or buffer solution. The volume of the IP$_3$ was aproximately the length of the sperm. Four groups of injection were realized:

- **Control group**; sperm injected oocytes.
- **InsP$_3$ group**: sperm and InsP$_3$ injected oocytes
- **Sham-buffer group**; buffer injected oocytes. The InsP$_3$ or buffer activation capacity was evaluated in this way.
- **Sham-InsP$_3$ group**: InsP$_3$ injected oocytes. The InsP$_3$ activation capacity without the sperm was evaluated in this way.

Embryo development was evaluated after NCSU-23 culture, as explained in the embryo culture chapter. The following were evaluated:

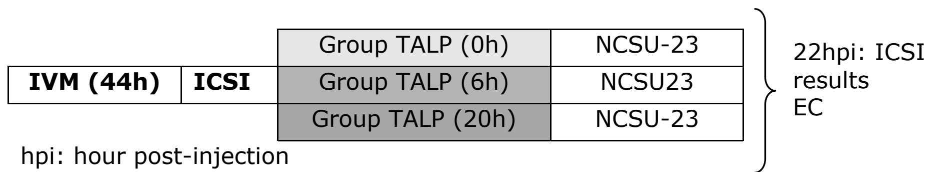
- Cleavage rate, blastocyst rate and number of cells per blastocyst.

This experiment was realized in 3 replicates with a total of 215 oocytes.

EXPERIMENTAL DESIGN

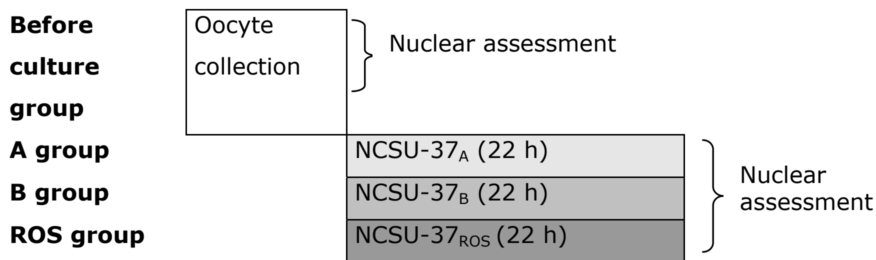
NCSU-37_A: NCSU-37 supplemented with eCG, hCG y $dbAMP_C$
NCSU-37_B: NCSU-37
NCSU-37_{ROS}: NCSU-37 supplemented with 50μM roscovitine
IVM: 22 h in NCSU-37_A following 22 h in NCSU-37_B
EC: embryo culture

Experiment 1. Effect of culture time in TALP medium on embryo development and ICSI

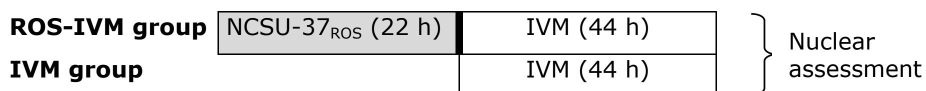


Experiment 2. Effect of roscovitine on *in vitro* oocyte maturation, ICSI and *in vivo* embryo development

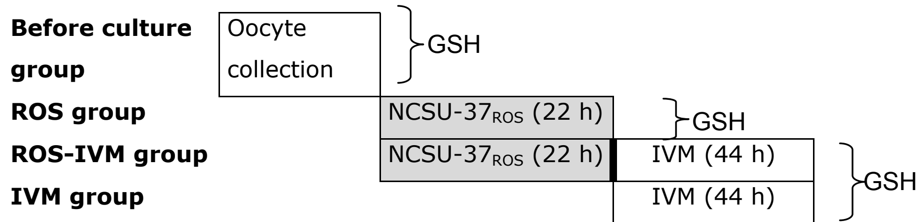
2.1 *Nuclear Status After 22 h Culture in Roscovitine*



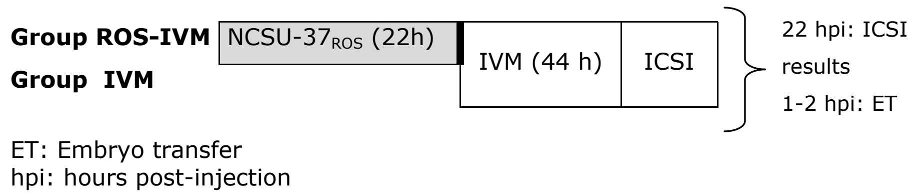
2.2 Nuclear Progression After IVM With a Prematuration Period in Roscovitine



2.3. Oocyte Glutathione Content After Roscovitine Treatment

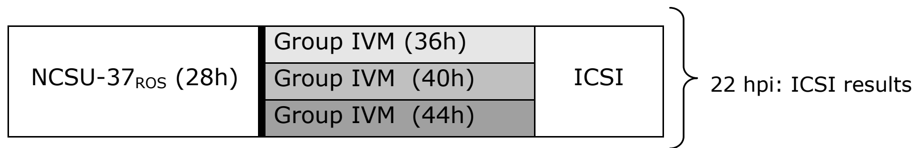


2.4 and 2.5. ICSI and Full Development of Roscovitine Treated Oocytes

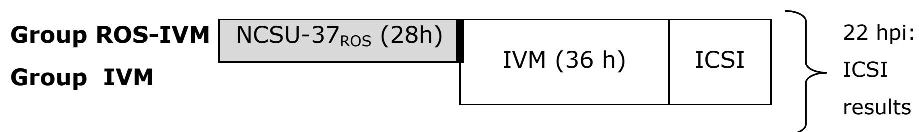


ET: Embryo transfer
hpi: hours post-injection

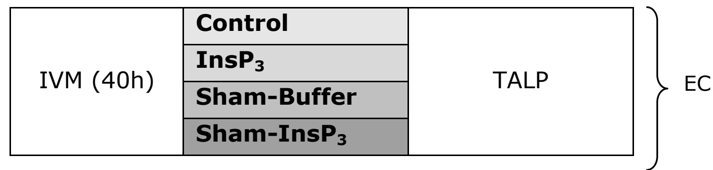
2.6. E*ffect of IVM duration on roscovitine treated oocytes and fertilized by ICSI*



2.7. Effect of roscovitine pre-maturation and maturation during 36h



Experiment 3. Effect of $InsP_3$ injection on in vitro embryo development.



3.11 STATISTICAL ANALYSIS

For the statistical analysis SPSS 11.5 for Windows was used. Data are presented as the mean ± SEM and all percentages were modelled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way ANOVA. When ANOVA revealed a significant effect, values were compared by the Tukey test. A p value <0.05 was taken to denote statistical significance.