RESULTS

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4.1 EFFECT OF CULTURE TIME IN TALP MEDIUM ON EMBRYO DEVELOPMENT AND ICSI

4.1.1 Effect of time culture in TALP medium on ICSI parameters

As shown in table 7, a possitive effect of oocyte culture in TALP medium was observed.

The activation and degeneration rates were not affected by the time culture in TALP in relation to the control group. The activation rate was aproximately 85% and there was only 18% of degeneration in the three groups. Although, the embryo rate was higher for 6h and 20h (9.72±3.92 and 10.68±3.06) in relation to the 0h group (0%) ($p<0.05$).

TABLE 7. Media and SEM for different fertilization parameters of the injected oocyttes cultured in different time periods in TALP medium, in 6 replicates. Evaluation at 22 hpi.

TALP h	Deg (%)	Act (%)	Pre-2PN* (%)	2PN* (%)	Embryos* (%)	Others* (%)
0h N=152	12.50±3.26	91.21±2.98	28.92±5.01	55.42±5.49	0 b	15.66±4.01
6h N=105	23.81±4.18	90.00±3.38	22.22±4.93	59.72±5.82	9.72±3.92 a	8.33±3.28
20h N=104	19.08±0.03	83.74±3.34	26.21±4.35	56.31±4.91	10.68±3.06 a	6.80±2.42
P	0.1076	0.2003	0.6393	0.8528	**0.0096**	0.1160

* In relation to the activated oocytes

a, b Different letters in the same column indicate values significantly different ($P<0.05$)

4.1.2 Effect of time culture in TALP medium on in vitro embryo development

As shown in table 8, no differences in any of the variables studied were found. Cleavage rate was aproximately 60% for the three groups with a low rate of blastocysts. The number of blastocysts was 3 for the control group(20h), seven for the 6h group and only one for the 0h group.

TABLE 8. Media and SEM for different *in vitro* embryo development parameters post-ICSI and different time culture in TALP medium, in 4 replicates. Cleavage rate evaluated at 48 hpi and blastocyst rate at 7 days post- injection.

TALP h	Cleavage (%)	Blastocysts (%)	N cells/blastocyst
0h N=91	54.55±5.71	4.76±3.33	50
6h N=90	64.44±5.07	12.07±4.31	41.71±8.32
20h N=77	68.13±4.91	4.84±2.75	37.66±8.69
P	0.1791	0.2424	0.8726

4.2 EFFECT OF ROSCOVITINE ON IN VITRO OOCYTE MATURATION ICSI AND IN VIVO EMBRYO DEVELOPMENT

4.2.1 Nuclear status of oocytes after 22h of culture in roscovitine

As shown in table 9, Roscovitine was effective to inhibit the meiotic resumption after 22 h of culture, giving a higher percentage of GV-I stage oocytes and a lower percentage of GV-III stages oocytes in ROS group than in A or B groups (Table 9). Oocytes just before culture and after treatment with roscovitine were similar for both nuclear stages. The percentage of oocytes reaching the Met-I stage was higher in the B group than in the remaining three groups.

TABLE 9. Nuclear status of pig oocytes after 22 h culture in NCSU-37 with or without hormonal supplements, or with 50 μM .

Nuclear stage (%)	Before culture	A	B	ROS	P
N	156	173	164	152	
GV-0	9.6 ± 2.4 a	0.6 ±0.6 b	0 b	0 b	<**0.001**
GV-I	60.9 ± 3.9 a	37.0 ±3.7 b	31.7 ± 3.6 b	63.2 ± 3.9 a	<**0.001**
GV-II	8.3 ± 2.2 a	37.0 ±3.7 b	26.2 ± 3.5 b	29.6 ± 3.7 b	<**0.001**
GV-III	7.0 ± 2.1 a	23.7 ±3.2 b	20.1 ± 3.1 b	7.2 ± 2.1 a	<**0.001**
GV-IV	7.0 ± 2.1 a	0 b	3.7 ± 1.5 ab	0 b	<**0.001**
Metaphase I	5.1 ± 1.8 ab	1.2± 0.8 a	13.4 ± 2.7 b	0 a	0
Anaphase I	0	0	1.2 ± 0.9	0	0.12
Telophase I	0.6 ± 0.6	0	2.4 ± 1.2	0	0.06
Metaphase II	1.3 ± 0.9	0.6 ± 0.6	2.4 ± 1.2	0	0.53

a, b Different letters in the same row indicate values significantly different ($P<0.05$)
A: 22 h culture in NCSU-37 with hCG, eCG, dibutyryl cAMP
B: 22 h culture in NCSU-37 without hCG, eCG, dibutyryl cAMP
ROS: 22 h culture in NCSU-37 without hCG, eCG, dibutyryl cAMP and with 50 μM

4.2.2 Effect of roscovitine on nuclear progression of oocytes

After maturation for 44 h, oocytes from ROS-IVM group resumed meiosis and reached Metaphase II stage at the same levels than IVM group, over 85% (Table 10).

TABLE 10. Nuclear status of pig oocytes after in vitro maturation with (ROS-IVM) or without (IVM) a prematuration period of 22 h in 50 µM roscovitine.

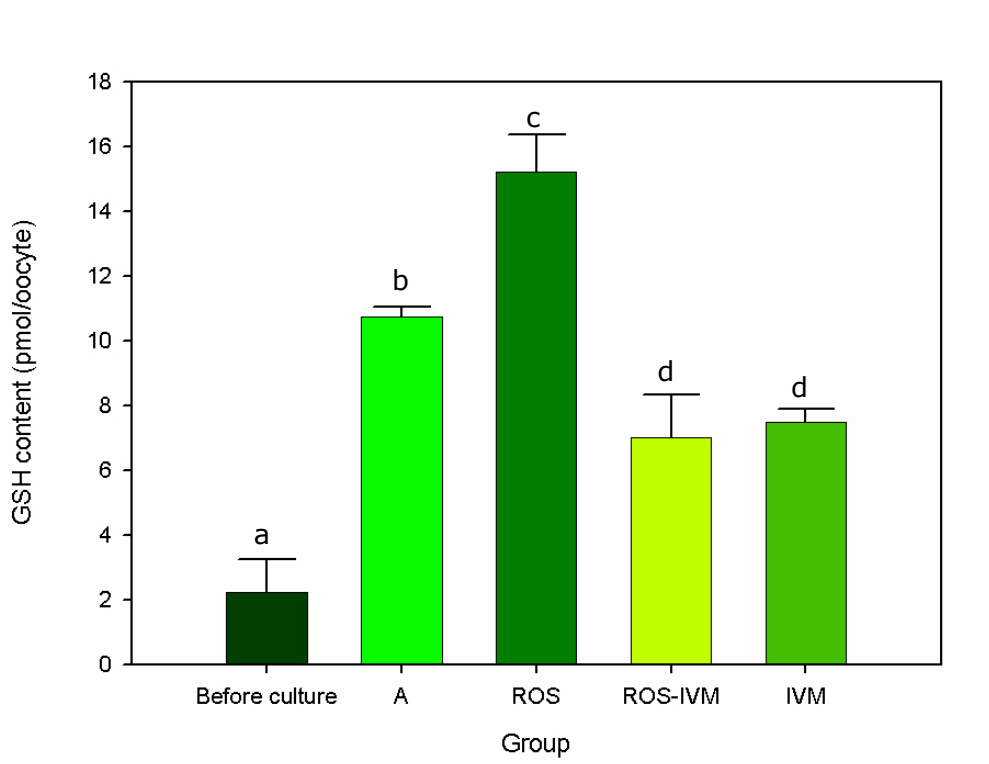
Nuclear Stage (%)	IVM	IVM-ROS	P
N	161	153	
GV-0	0.6 ± 0.6	0	O.33
GV-I	6.2 ± 1.9	4.6 ± 1.7	0.52
GV-II	1.9 ± 1.1	0	0.09
GV-III	1.2 ± 0.9	1.3 ± 0.9	0.96
GV-IV	0 a	6.5 ± 2.0 b	<**0.001**
Metaphase I	1.9 ± 1.1	0.6 ± 0.6	0.34
Anaphase I	0	0	1
Telophase I	0.6 ± 0.6	1.3 ±0.9	0.53
Metaphase II	87.6 ± 2.6	85.6 ± 2.8	0.61

a, b Different letters in the same row indicate values significantly different ($P<0.05$)

4.2.3. Effect of roscovitine on intracelullar GSH content

As shown in figure 12, intracellular GSH content increased after oocytes were cultured. When oocytes were kept for 22 h in culture, GSH content increased from 2.24 to 10.74 pmol/oocyte, and this amount was still higher when roscovitine was present during culture (15.23 pmol/oocyte). After IVM, no differences were observed independently of a 22 h prematuration period with (ROS-IVM) or without (IVM) roscovitine, being 7.02 and 7.48 pmol/oocyte, respectively. However, both groups showed a lower amount of GSH than ROS group.

FIGURE 12. Intracellular GSH content in pig oocytes before culture, after 22 h culture in NCSU-37 with with hCG, eCG and dibutyryl cAMP (A group); without hCG, eCG, dibutyryl cAMP and with 50 µM roscovitine (ROS group) and after a conventional IVM (22 h A + 22 h B) with (ROS-IVM) or without (IVM group) preculture in roscovitine.



a, b, c, d Different superscripts represent significant differences

(P<0.001)

4.2.4. Effect of roscovitine on fertilization by ICSI

The results of this experiment show that the activation and degeneration rates were not affected by the inhibitor. Activation rates was around 80% and degeneration rates was around 15% (table 11). The 2PN rates was around 50% for both groups. An effect was observed for the

variable embryos, the ROS group was higher in embryo rate than the control group (22.22% and 7.14%, p<0.05, for ROS and control, respectively). The others rate was higher for the ROS group than for the control group (0% vs 7.78% respectively) (p<0.05).

TABLE 11. Media and SEM for different ICSI parameters with or without Roscovitine prematuration and 44h of IVM, in 7 replicates. Evaluation at 22hpi.

	Deg (%)	Act (%)	Pre-2PN* (%)	2PN* (%)	Embryos* (%)	Others* (%)
Control N=115	21.74±3.96	80.46±4.28	34.29±5.71	55.71±5.98	7.14±3.10**a**	0**a**
ROS N=139	16.55± 3.16	81.31±3.79	21.11±4.33	48.89±5.30	22.22±4.41**b**	7.78±2.84**b**
P	0.8818	0.2946	0.0628	0.3945	**0.0090**	**0.0169**

* In relation to the activated oocytes

a, b Different letters in the same column indicate values significantly different (*P*<0.05)

4.2.5 Effect of roscovitine on in vivo embryo development

As the results show (table 12), ICSI embryos were capable to began pregnancies when they were transferred to female receptors. Sixty percent and 80% of transferred sows showed delayed estrus more than 23d for both groups. 20% of sows showed possitive pregnancy diagnosis with the presence of embryo vesicles between 21-25d. Ten percent had a visible abortion in both groups.

TABLE 12. Results of *in vivo* embryo development for prematured in roscovitine oocytes and fertilized by ICSI, in 10 replicates.

	N	Delayed estrus (>23d)	Embryo vesicles	Visible abortus
CONTROL	10	6	2	1
ROS	10	8	2	1

4.2.6 Effect of IVM time on ICSI oocytes prematured in roscovitine

It can be observed in this experiment that no significant differences exists in activation and degeneration rates for the different groups of oocytes prematured in roscovitine and matured at different times (83.61% vs 81.68 vs 90.0%) and (19.21% vs 17.61% vs 17.12%) for activation and degeneration rates respectively (table 13).

Howewer, a significant effect is observed for the Pre-2PN rate (18.63%; 29.91%; 41.28% $p<0.05$) and 2PN rate (73.53%; 61.68%; 55.05 % $p<0.05$) for 36, 40 and 44h respectively. Table 13.

TABLE 13. Media and SEM for different ICSI parameters in oocytes matured during different IVM times and preincubated in roscovitine, in 7 replicates. Evaluation at 22hpi.

IVM	Deg (%)	Act (%)	Pre-2PN* (%)	2PN* (%)	Others* (%)
36h n= 151	19.21±3.21	83.61±3.36	18.63±3.84a	73.53±4.39a	7.84±2.67
40h n=159	17.61±3.03	81.68±3.39	29.91±4.47ab	61.68±4.72ab	8.41±2.69
44h n=146	17.12±3.12	90.08±2.72	41.28±4.73b	55.05±4.78b	3.67±1.39
P	0.152	0.887	**0.002**	**0.019**	0.312

* In relation to the activated oocytes

a, b Different letters in the same column indicate values significantly different ($P<0.05$)

4.2.7 Effect of roscovitine and 36h of IVM on fertilization by ICSI.

No significant effect of roscovitine prematuration and the posterior IVM of 36h was observed in this experiment for any of the analized variables. Activation rates was around 80% for both groups. Degeneration rates were around 10% for both groups (table 14).

The other analizedavariables were not affected by the treatment. Embryo rate was aroun 70% for both groups (72.13% vs 72.00%) (table 14).

TABLE 14. Media and SEM for different ICSI parameters with or without roscovitine prematuration and 36h of IVM, in 4 replicates. Evaluation at 22hpi.

36hIVM	Deg (%)	Act (%)	Pre-2PN* (%)	2PN* (%)	Others* (%)
Control N= 90	14.14±3.72	79.22±4.65	13.11±4.35	72.13±5.78	14.75±4.57
ROS n= 102	7.84±2.67	79.79±4.16	22.67±4.86	70.67±5.29	6.67±2.90
P	0.928	0.145	0.155	0.852	0.125

* In relation to the activated oocytes

4.3 EFFECT OF $InsP_3$ ON IN VITRO EMBRYO DEVELOPMENT

As show in table 15 significant differences are found for the cleavage variable. Sperm injected oocytes groups (control and $InsP_3$) were higher than sham groups (buffer and IP_3). Cleavage rate was around 60% for sperm injected oocytes and 10% for sham groups. Blastocysts rate was very low for the four groups (8 for the control group, 3 for $InsP_3$ group and only one for sham- $InsP_3$ group), thus the results can not be inferred.

TABLE 15. Media and SEM for *in vitro* embryo development parameters in sperm injected oocytes (control and $InsP_3$) and sham injected oocytes (buffer and InsP3), in 3 replicates. Cleavage rate evaluated at 48 hpi and blastocyst rate at 7 days post- injection.

	Cleavage (%)	**Blastocysts (%)**	**NºCell/Blast**
Control n=58	68.97±6.12a	13.79±4.56a	31.25±5.35
$InsP_3$ n=54	59.26±6.67a	5.56±3.14ab	22.33±7.88
Sham-buffer n=55	10.42±4.44b	0b	0
Sham-$InsP_3$ n=48	20±5.44c	1.82±1.81b	21
P	**<0.001**	**0.008**	0.610