**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

# Cinética de producción de $H_2O_2$ en embriones bovinos producidos *in vitro* y su efecto sobre actividad mitocondrial (Kinetics of $h_2o_2$ production and its effect on mitochondrial activity in bovine embryos produced *in vitro)*

**CESAR AUGUSTO SERRANO-NOVOA[(a)(1)] Médico Veterinario MSc.; MARTHA OLIVERA-ANGEL[(1)] Médico Veterinaria PhD Agr. Sci.; CARLOS VELEZ PARDO[(2)] Microbiólogo PhD; MARLENE JIMÉNEZ DEL RIO[(2)] Microbióloga PhD**

(1)Grupo de Fisiología y Biotecnología de la Reproducción – BIOGENESIS. Grupo de Neurociencias Universidad de Antioquia.

(a)Author to whom reprint request should be sent
cserranon@agronica.udea.edu.co A..A .1226, Medellín, Colombia
http://www.veterinaria.org/revistas/redvet/cserranon

**Resumen**

**Objetivos:** Determinar la cinética de producción de $H_2O_2$ y de alteración en la función mitocondrial durante el desarrollo temprano en embriones bovinos producidos *in vitro* (ebpiv) con alto y bajo potencial de desarrollo.

**Metodología:** De acuerdo a su potencial de alcanzar el desarrollo hasta el estado de blastocito, ebpiv que completaron su primer ciclo celular antes de 32 horas post-inseminación (hpi) fueron clasificados como competentes, mientras que aquellos que no lo habían hecho, se clasificaron como no competentes. Para cada grupo, se tomaron un mínimo de 20 embriones para cada tiempo de estudio: 32, 40, 50, 72 y 100 hpi para las pruebas de cinética de $H_2O_2$ y función mitocondrial. Los embriones fueron incubados con Dihidrorodamina 123 (DHR) (1μM) por 15 minutos, y con JC-1 (5μM) por 30 minutos. Una vez incubados los embriones con sus respectivos fluorocromos, fueron lavados de 3 a 5 veces en PBS para ser evaluados directamente al microscopio de epifluorescencia utilizando los filtros específicos para cada uno. Los embriones fluorescentes (verde) para DHR fueron considerados como positivos para niveles elevados de $H_2O_2$. La funcionalidad mitocondrial se determinó por las áreas de fluorescencia roja. Cada prueba se realizó durante tres cohortes diferentes, para un total de 362 embriones competentes y 345 no competentes evaluados para DHR y 292 embriones competentes y 312 no competentes evaluados para JC-1. Los datos

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

(promedio +/- D.E) entre los diferentes puntos de corte (32,40,50,72 y 100 hpi) y entre los grupos (competentes y no competentes) se analizaron mediante ANOVA.

**Resultados:** Se observó un aumento progresivo en la frecuencia de eventos positivos a DHR a partir de las 32 hpi, y al parecer su disminución tiene que ver más con el estado de desarrollo que con el tiempo de cultivo. Aunque esta tendencia se presenta en ambos grupos, se encuentran diferencias significativas ($p<0.01$) en los valores observados entre el grupo competente y no-competente para cada tiempo de estudio, siendo mayor el número de eventos positivos en éste ultimo grupo. No se encontraron diferencias significativas entre los tiempos de observación ni entre grupos en cuanto a función mitocondrial de ebpiv, pero esta funcionalidad se encontró muy deprimida durante el desarrollo temprano.

**Conclusiones:** La mayor proporción de embriones no competentes que presentan elevación en los niveles de $H_2O_2$, podría estar relacionada con el bloqueo en el ciclo celular observado en condiciones *in vitro* y por ende, con la menor proporción de éstos que alcanzan el estado de blastocito a los 7 días de cultivo. Dichas elevaciones en los niveles de $H_2O_2$ no parecen relacionarse con la actividad mitocondrial, ni ésta con la generación del H2O2. De otro lado, la actividad mitocondrial no parece ser un factor de competencia para superar la detención en el ciclo celular. Esta depresión en la función mitocondrial estaría relacionada con algunos arreglos metabólicos observados por otros investigadores.

Las fermentaciones en estado sólido han sido utilizadas ampliamente en el reciclaje de materiales voluminosos a través de tecnologías sencillas, con la que se logran incrementar los valores proteicos, mejorando el balance de aminoácidos y la digestibilidad de las materias primas empleadas (Matho *et al*. 1992; Pedroza *et al*. 1995 y Rodríguez *et al*. 2001a). No obstante los conocimientos benéficos que hoy se logran en Cuba con estas tecnologías, aún están limitados a un número reducido de sustrato.

**Palabras Clave:** $H_2O_2$, Mitocondria, EROS, Apoptosis, Desarrollo, Embrión, Bovino

## Abstract

**Objective**: The aim of this research was to determine the kinetic of $H_2O_2$ production and inner mitochondrial membrane potential during early development of *in vitro* produced bovine embryos (ivpbe) with high and low developmental potential.

**Methodology:** According to the potential to reach blastocyst stage, ivpbe were grouped as follows: Those that reach the two cell stage at 32 hours post-insemination (hpi) were considered competent, while those that did not, where considered non-competent. Embryos were taken from each group 32, 40, 50, 72 and 100 hpi in order to be evaluated for $H_2O_2$ and inner mitochondrial membrane potential

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

by epifluorescence. Half of the embryos in each pool were incubated with dihydrorhodamine 123 (DHR) (1μM) for 15 minutes, while the other half were stained

with JC-1 (5μM) for 30 minutes before direct observation through epifluorescent microscopy. Green fluorescent embryos after DHR staining were assumed to be positive for $H_2O_2$. Inner mitochondrial membrane potential were determined by red fluorescent dots. The data were analyzed (average +/-S.D.) between pools (32,40,50,72 and 100 hpi), and between groups (competent vs. non-competent), by ANOVA.

**Results:** A progressive increase on $H_2O_2$ positive embryos was observed from 32 hpi. Although this tendency was maintained among both groups, there was a significant difference ($p<0.01$) between the number of positive events in each group: the number of $H_2O_2$ positive embryos was greater in the non-competent group at each observation time. There was not significant difference between groups nor between pools on inner mitochondrial membrane potential, but great proportion of mitochondrial clusters showed depolarization in early embryos. **Conclusions:** The large number of non-competent embryos showing high $H_2O_2$ levels, maybe related with the cell cycle arrest observed under culture conditions on ivpbe, and it is consistent with poor blastocyst production in delayed embryos. Increase production of $H_2O_2$ does not seem to be related to mitochondrial depolarization, and additionally mitochondrial depolarization does not seem to be a factor involved in the cell cycle arrest. Other investigators have previously reported metabolic rearrangements which may be compatible with the low mitochondrial function observed by us.

**Key words:** $H_2O_2$, Mitochondria, ROS, Apoptosis, Development, Embryo, Bovine

**Introduction:**

The study of developmental biology of mammals through *in vitro* fertilization have had a main obstacle, the developmental block frequently observed during embryo culture (6). *In vitro* production of embryos (ivpe), despite overcome some critical aspects about culture conditions, has been limited by this alteration, characterized by cell cycle arrest during a particular developmental stage which coincides with embryonic genome activation and with the beginning of glucose metabolism (3) occurring at 8 to 16 cells stage passage in bovine. In fact, the poor rate of blastocysts production (25 -30%), prevents to generate the enough quantity of biological material to supply the livestock market (25).

*In vitro* cell cycle arrest has been related with reactive oxygen species (ROS) production (9,11). ROS increase on ivpe, seems to be an independent phenomenon about either, cell cycle or embryonic genome activation , since the use of cell cycle or transcription inhibitors, did not avoided it (11), however, the use of these inhibitors

could induce the accumulation of ROS themselves. On the other hand, the generation requires the oocyte activation, either for fertilization or partenogenic stimulus, maybe involving a cascade of metabolic events through interactions with external environmental conditions (11,19).

As ROS sources under *in vitro* conditions, have been described like high oxygen tension, light exposition, as well as oxidative metabolism alteration (increase of glycolysis and pentose phosphate pathway, and decrease of Kreb´s Cycle and Oxidative phosphorilation) and mitochondrial function disturbance (6).

It has been possible to determine that ivpe that are not able to overcome the observed cell cycle arrest, show disturbances on their mitochondrias at level of duplication, distribution and functionality (2,21), alterations on nucleolar maturation processes (12), a delay of three hours in their cleavage rate during the first three cycles and of 5 hours during the fourth, with respect to those of high developmental competence, reaching these last, their first division before the 32 hours post-insemination (10). It is relevant that some authors have related the time to the first division with the relative quantity of certain molecules, as Glutathione, which are involved in defense mechanisms against ROS (7), suggesting that non-competent embryos present transcriptional failures that prevents them to defend against certain harmful substances, like ROS, during a time of permissive transcription before embryonic genome activation(7,14,20). Besides, mitochondrial function has been considered as very important factor, since they are responsible for ATP contribution during pre-implantatory development (26).

On this way, seems to be that non-competent embryos (those who do not overcome the cell cycle arrest), suffer an oxidative stress (imbalance between oxidants and reductives molecules in favor of the first ones), decreasing their ROS scavenger capacity and showing high levels of ROS which make them more susceptible to cellular damage (lipid peroxidation of membranes, proteins denaturalization and DNA break); in fact, some authors have demonstrated a clear relationship between processes of oxidative stress and DNA damage (24).
Of another side, *in vitro* cell cycle arrest, has been also associated with a type of cellular death called apoptosis (4), characterized by chromatine condensation, DNA breaking and plasmatic membrane contraction that, in contrast with necrosis, happens without damage to the neighboring cells.

The alterations on mitochondrial function could be associated to this event, since it has been documented, a mitochondrial signaling way for apoptosis (23). In human embryos, apoptosis detected by TUNEL, has been associated with high ROS levels, as $H_2O_2$, when adding it to the culture medium during different stages of the development; the experimental results have corroborated the presence of this phenomenon in bovine embryos starting from the state of 9 to 16-cells stage, in relationship to the moment of embryonic genome activation. The function of this apoptotic wave could be to eliminate embryos that have not activated its genome

efficiently, or to eliminate non viable cells on development embryos, since most of the embryos, show some degree of cellular death (27).

According to Yang et al. (27) the apoptotic index is inversely related with blastoyst cellularity, indicating that after an initial activation, the cellular death diminishes with the blastocyst expansion. Their results demonstrate that blastocysts with a reduced number of cells, and consequently, with a reduced potential for the development, have a higher and variable apoptosis incidence.

It has been suggested that when apoptosis index reaches certain threshold, it is detrimental for the future development of the embryo, as mechanism of eliminating non viable embryos. Comparing the apoptosis extension among the embryos that complete their first cellular cycle before 30 hpi and those who did not, indicates that in the later ones, apoptosis incidence results in reduced competence to reach the blastocyst stage (4).

Although has been found that ROS induces apoptosis, the molecular mechanisms have not still been clarified. Some authors have indicated that ROS induces topoisomerase II activation (28), p53, p66 adaptor protein (17), and p85 subunit of phosphatidyl inositol 3- kinase (PI3K) (16).

Other authors, also agree with the fact that apoptosis is not evidenced in bovine embryos until late stages of early development (starting from 8 to 16-cells stage), not being apparent in early stages of cleavage, consequently, they don't associate it clearly to development arrest (29). The same authors, studied the expression and localization of p53 using reverse transcription of polimerase chain reaction (RT-PCR), finding that, although p53 is expressed, its localization is citoplasmic and not nuclear, consequently, since p53 translocation is a faulty process in undifferentiated cells, a p53 independent form of apoptosis, could be occurring on bovine embryos (29).

In fact, it has been implied the citoplasmic fraction of Ab-1 (c-Ab1) participation, a pro-oncogene with either, nuclear and citoplasmic localization, which have tirosine kinase activity, which in turn, is activated by genotoxics, and that it could be the link connector between ROS production and mitochondria-mediated apoptosis activation. In fact, additional studies, demonstrate that citoplasmic form and not the nuclear form, of c-Ab1, is activated in answer to $H_2O_2$, which in turn, induces citocromo-c release, driving toward apoptosis in a c-Ab1 dependent way (22).

All these evidences have been registered under culture media intervention by addition of diverse ROS inducers, and since this studies have been carried out in cellular models different to those of bovine embryos, even when it seems not to be differences among tissues (22), additional studies should be made in order to clarify if during the pre-implantatory development, this mechanism is active.

Although it has not been possible to establish the apoptosis ROS-mediated way on bovine embryos during early development, have been possible to characterize, on murine fibrobastic cells, a mitochondrial-mediated apoptosis that implies citocromo-

c release induced by citoplasmic c-Ab1 from ROS stimulus (22), which suggests that more than source of ROS, mitochondria is a target of the same ones.

Nevertheless all the previous evidences, it has not been clearly determined that ROS act as inducers to *in vitro* cell cycle arrest, until now, it has not been possible to demonstrate that non-competent embryos have higher ROS levels than competent embryos, and, although $H_2O_2$ concentrations on *in vitro* mouse embryos are higher than *in vivo* produced ones at the time corresponding to embryonic genome activation (19), differences have not been determined among the *in vitro* bovine embryos which stop their development and those who don't make it, previous to the activation of the genome.

There are no studies about kinetics of production of ROS, as $H_2O_2$, on bovine ivpe during the previous time to the activation of the embryonic genome, which allow to demonstrate differences among competent (first cleavage <32 hpi) and non-competent (first cleavage >32 hpi) embryos. Besides, it´s not been clarify yet the role of mitochondria in ROS generation; it means about, if the mitochondria is the source or the target of ROS.

The aim of this work is to determine the kinetics of $H_2O_2$ production and inner mitochondrial membrane potential pattern during the early development, to clarify the role of mitochondria on *in vitro* cell cycle arrest and ROS generation in whose last case, would be key point in the signaling of apoptosis. Additionally, it is sought to determine if differences exist in the production of ROS, previous to arrest, in embryos that overcome it and those that don't make it, determining association among these two events.

## Materials and Methods:

### *In vitro* production of bovine embryos:

*In vitro* production of bovine embryos was carried out in agreement with that described by Camargo et. al (5), briefly:

Ovaries from slaughterhouse were recovered and transported to the laboratory in physiologic saline solution at 38° C within 2 to 3 hours after their recovery. Once in the laboratory the ovaries were washed 4 to 5 times in saline solution and maintained in a water bath at 38° C until their follicles (between 2 and 7 cms) were punctured; the fluid recovered by this procedure was maintained within a 50 cc Falcon tube in water bath at 38°C for 15 minutes. Then, the button was recovered with a pasteur pipette and deposited into a petri dish to oocyte search and recovery using a stereoscope. Once recovered, oocytes with a homogeneous cytoplasm, integrity of their zona pellucida and having more than 3 to 4 layers of granulose cells were selected. These oocytes were placed on 95μL drops (25 to 30 embryos per drop) of ivm media (TCM 199 suplemented with 10% Bovine Fetal Serum) previously prepared

and stabilized, and incubated for 24 hours at 5%CO2, 95% humidity and at 38°C environment. After this time, oocytes were retired of ivm media and washed 3 times in HEPES and transferred to drops of 85μL ivf media (IVF Talp) previously prepared and stabilized. Once transferred to ivf media the oocytes were inseminated with 4μL of semen, previously capacited by swim-up, at a concentration of 1x106 spermatozoas/mL, and then supplemented with 4 μL of Heparine (0.5 mg/mL) and 4 μL of PHE (Penicillamine -2mM -, Hipotaurine -1mM -, Epinephrine - 250 μM). After 18 hours, the zygotes were washed 3 times in HEPES to be transferred to 95 ml drops of development (CR1) media, previously prepared and stabilized, media in which the zygotes/embryos remained during the following 6 to 7 days until the end of the experiment.

**Experimental design:**

After 32 hours post - insemination (hpi), the zygotes were evaluated in relation to their first cellular division and they conformed two groups: the first one, the Competent group, the zygotes that reached the 2-cells stage; the second, the Non-competent group, those that were not divided at this time.

Development embryos from 2-cells until blastocysts stage were randomly selected in each group at 32, 40, 50, 72 and 100 hpi, to $H_2O_2$ and inner mitochondrial membrane potential tests and to determine the kinetics of these events.

This procedure was carried out during three cohorts; since there was not significant differences among the different cohorts, they were analyzed as repetitions. A total of 362 competent embryos and 345 not competent evaluated for $H_2O_2$ and 292 competent embryos and 312 not competent evaluated for inner mitochondrial membrane potential.

**Zona digestion:**

All the embryos were treated with acidic Tyrode's solution for 15 to 20 seconds to digest the zona pellucida, after this, they were washed 5 times in PBS, before being incubated with their respective fluorophores.

**$H_2O_2$ test:**

$H_2O_2$ tests, was performed by fluorescence determine of Dihydrorhodamine 123 (DHR). DHR in presence of peroxide and the cell endogenous enzyme peroxidase, is oxidized to cationic rhodamine 123, which produces green fluorescence detected by epifluoresecence microscopy using the specific filter.

The embryos were incubated by 15 minutes in presence of DHR (1μM) in PBS, prepared from stock solution immediately preceding staining ( Stock: 14.4 mM (5 mg/ml) in DMSO maintained at -20°C and protected of the light). After incubation, the embryos were washed with PBS 3 times after direct evaluation by epifluorescence.

**Inner mitochondrial membrane potential test:**

It was performed by fluorescence determine of 5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethylethylbenzimidazolo-carbocyanide iodide (JC-1). JC-1 is a cationic carbocianine that concentrates according to inner mitochondrial membrane potential.

This fluorophore stain is as a monomere that a low concentrations shows a green fluorescence and at high concentrations, it forms red fluorescent aggregates. On this way, according to their capacity to concentrates this stain, mitochondrias with high polarized inner membrane (functional mitochondrias) will show a red sataining pattern while low polarized (non-functional mitochondrias) will not.

Embryos were incubated for 30 minutes in presence of JC-1 (5 μM) in PBS, prepared from stock solution unmediately preceding staining (Stock: 1.5mM in DMSO, maintained at -20°C and protected of the light). After incubation they were washed 3 times in PBS after direct evaluation by epifluorescence using the specific filters.

$H_2O_2$ positive controls, were performed adding to staining drop (100μL) 17 μL of peroxide (30 μg/ml) at the incubation time, to reach a final concentration of 150 μM; the negative controls were carried out without addition of DHR.

Positive controls to inner mitochondrial membrane potential were performed staining ivm oocytes with JC-1 while negative controls were achieved incubating the embryos without JC-1. Previously, matured oocytes were treated with azida 1% (an inhibitor of oxidative phosphorilation) before JC-1 incubation showing a decrease of red fluorescent pattern.

All the reactives used in *in vitro* production of bovine embryos were provided from Sigma Chemical Co. (P.O. Box 14508 St. Louis, MO 63178 USA) except FSH (Schering Plough A.A. 4597 Bogotá, Colombia).

Fluorophores used in H2O2 and Mitochondrial functionality tests were provided from Molecular Probes Inc. (PO Box 22010 29851 Willow Creek Road, Eugene, OR 97402-0469 USA).

Plastic disposables dishes were provided from Nalge Nunc (75 Panorame Creek Drive, Rochester NY 14625-2385) and Falcon tubes were provided from BD Biosciences (Tel 905.542.8028 Fax 905.542.9391, Canada canada@bd.com).

**Samples preparing and evaluation:**

All the embryos were aspirated with a minimal amount of the mounting medium (PBS) and transfer to a slide expelling just enough mounting medium to cover the cells. Slowly lower a cover-slip onto drop with embryos. Then, they were evaluated by direct observation by epifluorescence microscope (Carl Zeiss AXIOLAB) using specific filters (Set 09 and 14 for JC-1; Set 09 for DHR 123).

All the observations were carried out by the same observer and the data registered immediately.

**Statistical Analysis:**

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

Datas from $H_2O_2$ tests (number of fluorescent embryos) were expressed as average ± SD. Inner mitochondrial membane potential were determined as the relative red fluorescent area according to positive control and the datas (number of embryos) were registered within ranges (0 to 25%; 26 to 50%; 51 to 75%). The number of embryos registered within each range were expressed as average ± SD.

Datas were analysed (Satatistic 7.0) by 2-way ANOVA with interaction ($p<0.01$) whose statistical model was: $Y_{ijk} = \mu + H_i + C_j + HC_{ij} + l_{ijk}$; where:

$\mu$ = Populating media

$Y_{ijk}$ = Number of embryos
$H_i$ = Hour effect
$C_j$ = Competence effect
$HC_{ij}$ = Interaction effect
$l_{ijk}$ = Experimental mistake

Aditionally, a Tukey probe ($p<0.05$) was performed to determine statisticals differences.

**Results:**

As control group, embryos were preserved during the whole period of study and their cellular division rate was:

At 32, 40, 50,72 and100 hpi, the great majority of the competent embryos (95%) showed their normal expected developmental stage, this is: 2,4,8 and 16-cells stage respectively. Non-competent embryos showed a delayed development being most of them at 32 hpi in 1-cell satge; at 40 hpi in 2-cells stage, at 50 hpi in 4-cells stage, at 72 hpi in 5 to 8-cells stage , condition that was maintained until 100 hpi, with low percentage (8.22%) of them in 9 to 16-cells stage, which reflect the so called cell division arrest.

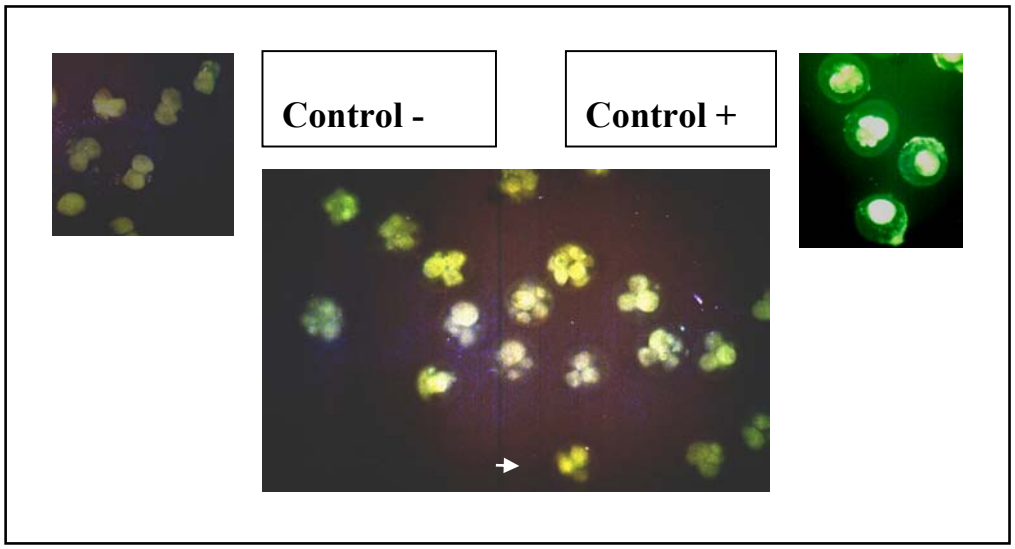
**$H_2O_2$ test:**

A total of 64, 70, 74, 76 and 78 competent embryos and 61, 72, 74, 69 and 69 non-competent embryos, were evaluated at 32, 40, 50, 72 and 100 hpi respectively for $H_2O_2$ determining. The average ± SD of embryos positives to DHR were shown in Table 1. ANOVA analysis determines a significant ($p<0.01$) effect of quality of embryos (Competent vs. Non-competent) and of time of evaluation (hours) without interaction between them.

For each time of study, a higher number of positive embryos were observed in Non-competent group. It was observed in Competent embryos a gradual increase in the number of embryos starting from the 2-cells stage (32 hpi) that although reaches their maximum value to the 8-cells stage (50 hpi), it is maintained during the period of the fourth cellular cycle in the step from 8 to 16-cells stage (72 hpi), diminishing,

until the culmination of this (100 hpi), to values found in earlier stages. Non competent embryos, showed a similar tendency observed along the time of study, however, in an earlier fashion, it was observed that the maximum values are maintained during 2 and 4-cells stages (40 and 50 hpi respectively) to decrease about 4 and 8-cells stages (72 hpi). According with their cellularity, was observed that during earlier developmental stages (2 and 4 cells), the average of positive embryos are higher than their contemporaries in the competent group, diminishing, in the same way, as they reaches the 8-cells stage. Typical observations could be noticed in Figure 1.

**Figure 1. DHR-Positive embryos, during early embryo development of *in vitro* bovine embryos.**

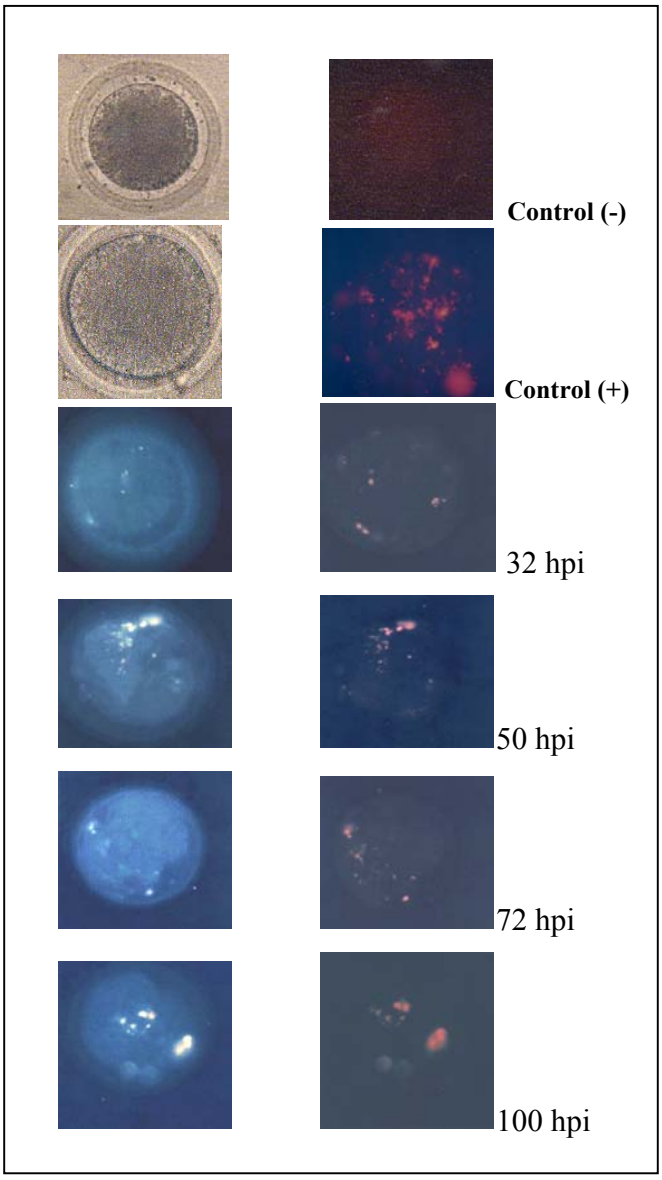



Competent embryos at 72 hours post-insemination, showing DHR-positive reaction (white arrows) by oxydation of DHR 123 to fluorescent cationic form, Rhodamine (Emission at 529 nm), due to high $H_2O_2$ levels .

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

Figure 2. Inner mitochondrial membrane potential during early embryo development of *in vitro* bovine embryos.



Left photos were shoot with white light for controls and filter 09 was used for experimental embryos to check green fluorescence of JC-1 monomer (Emission at 525 nm); Right photos were shoot with filter 14 to check red fluorescence of JC-1 aggregated (Emission at 590 nm), corresponding to the same embryo at the same post-fertilization time. Embryo cell morphology is lost because cover-slip pressure. Regardless observation time, it was observed a mitochondrial depolarization during early bovine embryo development, determined by a relative loss of red fluorescence area compared with positive control (*in vitro* matured oocyte).

**Table 1. Average ± S.D. of number of Competent and Non-competent embryos positives to DHR, according hour and quality. $H_2O_2$ test.**

| **Hour ± S.D.**[(1)] | **Competent** | **Non-competente** | **Average** |
|---|---|---|---|
| 32 | 2.33±0.81 | 3.33±0.81 | 2.83± 1.68[b] |
| 40 | 6.67±2.94 | 15.00±9.79 | 10.83±14.45[a] |
| 50 | 10.33±4.54 | 16.33±7.25 | 13.33±11.28[a] |
| 72 | 9.33±4.54 | 11.66±6.37 | 10.50± 8.33[a] |
| 100 | 7.33±2.94 | 9.33±3.55 | 8.33± 5.22[ab] |
| Average[(1)] | 7.20[a] | 11.13[b] | |

(1) Tukey ($p<0.05$). Average with different letter were different between them.

Inner mitochondrial membrane potential test:

A total of 67, 67, 56, 60 and 40 Competent embryos and 71, 71, 68, 58 and 42 Non-competent embryos, were evaluated at 32, 40, 50, 72 and 100 hpi respectively for inner mitochondrial membrane potential test. The average ± S.D. of embryos within each range of relative red fluorescence to JC-1 respect to the positive control, were shown in Table 2 for Competent group and in Table 3 for Non-competent group. Was not found effect ($p<0.01$) neither, hour nor quality of embryos nor interaction between them by ANOVA within each range of fluorescence, however, it was found significant differences between ranges by Tukey ($p<0.05$).

Regardless of studied variables, even cellularity, in vitro produced bovine embryos showed mitochondrial depolarization along the early development. Typical observations could be noticed in Figure 2.

Table 2. **Average ± S.D. of number of Competent embryos according hour of study at each range of relative red fluorescence to JC-1. Inner mitochondrial membrane potential test.**

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

| Range | Hour 32 | 40 | 50 | 72 | 100 | Average[(1)] |
|---|---|---|---|---|---|---|
| **0 a 25% 12.0±14.4[a]** | **14.3±9.4** | **11.0±8.6** | **11.3±4.1** | **12.3±1.6** | **11.0±1.4** | |
| **26 a 50% 7.67±10.4[b]** | **8.0±3.7** | **9.6±3.5** | **6.3±5.3** | **6.3± 5.3** | **8.0±1.4** | |
| **51 a 75%** | **------** | **1.6±0.8** | **1.0±1.4** | **1.3±1.6** | **1.0±0.0** | **1.00±3.1[c]** |
| **Average[(1)]** | **7.44** | **7.44** | **6.22** | **6.67** | **6.67** | |

(1) Tukey ($p<0.05$). Average with different letter were different between them.

Tabla 3. **Average ± S.D. of number of Non-competent embryos according hour of study at each range of relative red fluorescence to JC-1. Inner mitochondrial membrane potential test.**

| Range | Hour 32 | 40 | 50 | 72 | 100 | Average[(1)] |
|---|---|---|---|---|---|---|
| **0 a 25% 14.06±14.1[a]** | **17.0±9.2** | **13.6±7.7** | **14.0±2.8** | **13.3±2.1** | **12.3±2.1** | |
| **26 a 50% 7.13±9.8[b]** | **6.6±4.1** | **9.0±6.1** | **8.3±3.5** | **5.3± 1.6** | **6.3±0.8** | |
| **51 a 75%** | **------** | **1.0±1.4** | **0.3±0.8** | **1.3±1.6** | **2.0±0.0** | **0.93±3.6[c]** |
| **Average[(1)]** | **7.89** | **7.89** | **7.55** | **6.67** | **7.11** | |

(1) Tukey ($p<0.05$). Average with different letter were different between them.

**Revista Electrónica de Veterinaria REDVET -** ISSN 1695-7504
**http://www.veterinaria.org/revistas/redvet**

Vol. VI, Nº 5, Mayo 2005 –
http://www.veterinaria.org/revistas/redvet/n050505.html

## DISCUSSION

It has been described that as fertilization occur *in vitro*, produced zygote begins to generate peroxide. According to our results, the number of Competent embryos that presents high levels of $H_2O_2$ increases from 2-cells stage (32 hpi), maintaining their higher values between 8 to 15-cells stage (50 and 72 hpi). At 16-cells stage (100 hpi), the number observed decrease, maybe as an effect of the beginning of the synthesis of some substances that acts as ROS scavenger (for instance, glutathion)(18), once the embryonic genome has been activated; this would be valid for the competent embryos that shows a synchronous development, however, Non-competent embryos, which shows a delayed development with positive embryos number decrease about 8-cell stage (72 to 100 hpi), without genome activation at the same time, the explanation could be, as discuted by Morales (18), that when peroxide stimulus is given on earlier stages (1 to 2-cells stages), the damage is more devastating than when it is given in more advanced stages, maybe as a result of the effect of cell number in relation to the biochemical capacity to defend against peroxide, in other words, the decrease in the number of positive embryos would be due to an increase in their cellularity.

Since the number of positive peroxide embryos increase before 8-cell stage, it could be said that peroxide causes the cell division arrest, more than to be the result of the same. However, although was showed differences between both groups about peroxide positive embryos, explaining the highest proportion of Non-competent embryos arrested, cannot be discarded that the problem is not the higher or lower production of $H_2O_2$, but the capacity to defend against them.

Was not evidenced a clear kinetic pattern of mitochondrial depolarization along early development, neither was appreciated significative differences among Competent and Non-competent embryos, which would indicate that mitochondrial function doesn't represent a competition factor to overcome *in vitro* cell cycle arrest of bovine embryos. It is probable that acquisition of competence will be centered earlier, in the relative quantities of ATP stored during oocyte maturation, more than in later mitochondrial function after fertilization.

Seems to be clear that inner mitochondrial membrane potential still depressed along early development of *in vitro* produced bovine embryos without relationship to their capacity to overcome the cell cycle arrest.

Some authors, mainly Thompson (26), working with mitochondrial inhibitors and uncouplers, have said that, although partial inhibition of mitochondrial function during peri-compaction (from 16-cells stage) is beneficial to embryo development, this inhibition in earlier stages (1 to 8-cells stages) is detrimental, to conclude, that mitochondrial function during this period is essential for optimal early development. However, this study has not been showed mitochondrial function. It could also be said, that although it is true that mitochondrial function is required during this time, we don't know what proportion of it is needed. That is to say, the partial inhibition

during an early stage, will generate inhibition of the little mitochondrial function, perhaps, beyond the necessary threshold to guarantee the development.
In fact, some authors(15,26), have described that bovine embryos don't depend at all, of oxidative phosphorylation within mitochondria, but they present an active repertory to replace their energy requirements (v.g. phosphate pentose way and glycolysis). This is also supported in previous evidences that relate a higher lactate production in *in vitro* versus *in vivo* produced embryos (8,13), and that some components of the mediums, as the serum (1) or certain environmental conditions related to culture, could be exacerbating a metabolic change of embryo metabolism, driving them to glycolysis, what would explain mitochondrial depression under culture.

Nevertheless the above-mentioned, still not clear, if mitochondrial function is also depressed in *in vivo* produced embryos, in a similar way to that observed, that which would mean a physiologic phenomenon in the bovine embryos, or if as counterpart, this depression is due strictly to culture conditions.

If culture conditions alter energy metabolism of *in vitro* embryos, as already have been said, the question is if metabolism disfunction is a consequence of mitochondrial disturbance due to elevation in peroxide or if the decrease in the mitochondrial functionality is an effect of preferential routes of energetic metabolism under culture. This metabolic changes during peri-compaction stage, obeys, according to the authors (15,26), to embryo necessity to adapt oneself to an hypoxic environment during early implantation, however, the fact that *in vitro* embryos during earlier stages, shows this inhibition, it is not necessarily a detrimental event, for instance, it could be a metabolic arrangement to avoid ROS generation during a period of poor oxygen uptake while there are high oxygen availability (atmospheric tension of 20% Vs. oviductal and uterine tension of 4 to 5%) in fact, in other species, it has been possible to determine that the oxygen consumption is very low until the period of blastocyst, even in species of earlier embryonic genome activation as mouse whose genome is activated toward 2-cells stage.

It could be said that the role of mitochondria during e pre-implantatory stage is more related with its capacity to produce ATP during oocyte growth and maturation, enough to guarantee its availability until embryonic genome activation. Seems to be clear that a threshold of minimal mitochondrial function is required like it has already been said, but that the restitution of this energy source could be relegated to other metabolic routes.

But, if it is not the mitochondria, or their alteration, the responsible for ROS generation, in this case peroxide, where they can be generating?. Previous evidences can suggest that alterations at another levels can be driving, not only peroxide generation, but to a decrease in defense capacity against him. For example, it has been possible to relate the capacity to reach blastocyst stage with the relative quantity of some molecules like glutathion and the enzyme HGPRT (hipoxantine-guanine phosphorribosil transferase); the first one, perhaps the main enzymatic defense

molecule against ROS, the second, enzyme responsible of purines rescue way, way that when being depressed, forces to xantine catabolism, which by the enzyme xantine oxidase produces peroxide. Additionaly, the pentose phosphates way can conduce to ROS production. On the other hand, nucleolar alterations during post-fertilization development, it has also evidenced direct relationship with developmental arrest, and although this organele comes to be active during embryonic genome activation, it has been possible to demonstrate a permissive transcription (20) during previous stages, being able to suppose that nucleolar disturbance could alter this transcription that has been related strongly with the capacity of the embryo to respond to environmental insults (for instance, oxidative stress or thermal shock). thus, a relationship of peroxide production and mitochondria disfunction not necesarely exists (even to the inverse one).

Concluding, under culture conditions, bovine Non-competent embryos, presents, in a higher proportion, high levels of $H_2O_2$ compared with Competent embryos; this not seems to be related with mitochondrial depolarization. The role of mitochondria as ROS producer remains without clarifying by the light of these results, moreover, mitochondrial depolarization, beyond being constituted in a competition factor among the embryos that overcome cell cycle arrest and those that don't make it, seems to be an adaptative arrangement of *in vitro* produced bovine embryos, being the responsibility of competence acquisition in another place.

**Acknowledgements:**

The authors offers special thanks to COLCIENCIAS for financial support.

**References:**

1. Abe H, Yamashita S, Satoh T, Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol Reprod Dev 2002; 61(1): 57-66
2. Barnet DK, Bavister BD. What is the Relationship Between the Metabolism of Preimplantation Embryos and Their Developmental Competence? Mol. Reprod. Dev. 1995; 43: 105 – 133
3. Betteridge KJ. Phylogeny, Ontogeny and Embryo Transfer. Theriogenology 1995; 44: 1061 – 1098
4. Byrne AT, Sothgate J, Brison DR and Leese HJ. Analysis of Apoptosis in the Preimplantation Bovine Embryo using TUNEL. J. Of Reprod. And Fert. 1999; 117: 97 – 105
5. Camargo O, Sierra R, Berdugo JA, Olivera-Angel M. Obtención de Embriones Bovinos in vitro. Rev. Col. Cienc. Pec. 1997; 10(Suplemento):
6. Camargo O, Ramirez JL, Olivera-Angel M.. Radicales Libres y Desarrollo Embrionario. Rev. Col. Cienc. Pec. 1999; 12 (2): 108 – 118

7. Edwards JL, King WA, Kawarsky SJ, Ealy AD. Responsiveness of Early Embryos to Environmental Insults: Potential Protective Role of HSP70 and Glutathione. Theriogenology 2001; 55 (1): 209 – 223
8. Gardner DK. Embryo Metabolism. Colorado Center for Reproductive Medicine, Englewood Colorado. Serono Symposium, Nov. 1997
9. Got Y, Noda Y, Mori T, Nakano M. Increased Generation of Reactive Oxygen Species in Embryos Cultured in vitro. Theriogenology 1993; 15: 69 – 75
10. Holm P et al. Developmental Kinetics of the First Cell Cycle of Bovine in vitro Produced Embryos in Relationship to their in vitro Viability and Sex. Theriogenology 1998; 50: 1285 – 1299
11. Johnson MH, Nasr-Esfahani MH. Radical Solutions and Cuyltural Problems: Could Free Oxygen Radicals be Responsible for the Impaired Development of Mammalian Embryos in vitro?. Bioessays 1994; 16: 31 – 38
12. Laurincik J et al. A Detailed Analysis of Pronucleus Development in Bovine Zygotes in vitro. Cell Cycle Chronology and Ultrastructure. Mol. Reprod. Dev. 1998; 50: 192 – 199
13. Leese HJ. Metabolism of the preimplantation mammalian embryo. En:Oxford Reviews of Reproductive Biology V 13. 1991
14. Lonergan P, Gutierrez-Adan A, Pintado B, Fair T, Ward F, Fuente JD, Boland M. Relationship Between Time of First Cleavage and the Expression of IGF-1 Growth Factor, its Receptor and Two Housekeeping Genes in Bovine Two-cell Embryos and Blastocysts Produced in vitro. Mol. Reprod. Dev. 2000; 57 (2): 146 – 152
15. Machaty Z, Thompson JG, Abeydeera LR, Day BN, Prather RS. Inhibitors of mitochondrial ATP production at the time of compaction improve development of in vitro produced porcine embryos. Mol Reprod Dev. 2001; 58(1):39-44
16. Matwee C, Betts DH, King WA. Apoptosis in the Early Bovine Embryos. Zygote 2000; 8(1):57 – 68
17. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 1999; 402(6759): 309-313
18. Morales H, Tilquin P, Rees JF, Massip A, Dessy F and Van Langendonckt A. Pyruvate Prevents Peroxide-Induced Injury of In Vitro Preimplantation Bovine Embryos. Mol. Reprod. And Dev. 1999; 52: 149 – 157
19. Nars-Esfahani MH, Aitken RJ, Johnson MH. Hydrogen Peroxide Levels in Mouse Oocites and Early Cleavage Stage Embryos Developed in vitro or in vivo. Development 1990; 109: 501 – 507
20. Plante L, Plante C, Shepherd DL, King WA. Cleavage and 3H-Uridine Incorporation in Bovine Embryos of High in vitro Developmental Potential. Mol. Reprod. Dev. 1994; 39: 375 – 383
21. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalvez PB and Wolf E. Mitochondrial Distribution and Adenosine Triphosphate Content of Bovine Oocytes Before and After In Vitro Maturation:

Correlation with Morphological Criteria and Developmental Capacity After In Vitro Fertilization and Culture1. Biology of Reprod. 2001: 64: 904 - 909

22. Sum X, Majunder P, Shiova H, Kumar S, Weichselbaum R, Kharbanda S and Kuffe D. Activation of the Cytoplasmic c-Ab1 Tyrosine Kinasa by Reactive Oxygen Species. J. Biol. Chem. 2000; 275(23): 17237 – 17240
23. Susin SA, Zamzami N and Kroemer G. The Cell Biology of Apoptosis: Evidence for the Implication of Mitochondria. Apoptosis 1996;1:231 – 242
24. Takahashi M, Keicho K, Takahashi H, Ogawa H, Shultz RM and Okano A. Effect of Oxidative Stress on Development and DNA Damage in in-vitro Cultured Bovine Embryos by Comet Assay. Theriogenology 2000; 54: 137 – 145.
25. Thompson JGE, Simpson AC, Pugh PA, Donnelly PE and Tervit HR. Effects of Oxygen Concentration on in vitro Development of Preimplantation Sheep and Cattle Embryos. J. Reprod. Fert. 1990; 89: 573 – 578
26. Thompson JG, Mc McNaughton C, Gasparrini B, McGowan LT, Tervit HR Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J Reprod Fertil. 2000; 118(1):47-55
27. Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW and Oh KS. Detection of Reactive Oxygen Species (ROS) and Apoptosis in Human Fragmented Embryos. Human Reprod. 1998; 13(4): 998 – 1002.
28. Yin Y, Solomon G, Deng C and Barret JC. Differential Regulation of p21 by p53 and Rb in Cellular Response to Oxidative Stress. Mol. Carcinog. 1999; 24(1):15 – 24
29. Yuan, Z. M., Huang, Y., Fan, M. M., Sawyers, C., Kharbanda, S., and Kufe, D. J. Genotoxic drugs induce interaction of the c-Abl tyrosine kinase and the tumor suppressor protein p53. J. Biol. Chem. 1996; 271(43): 26457-26460

Trabajo recibido el 11.02.05 nº de referencia 050522_REDVET. Enviado por su autor principal. cserranon, miembro de la Comunidad Virtual Veterinaria.org ®. Publicado en REDVET® el 01/05/05.