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PROGESTERONE AND ESTROGEN SUPPLEMENTATION *IN VITRO* CULTURE MEDIA IMPROVE MORPHOGENESIS AND DEVELOPMENT GASTRULATION EMBRYOS RABBITS

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The objective of this study was to compare the development competence of in vitro embryo rabbit and gastrula under culture media supplemented with serum pregnancy does rabbit (SPDR). Eighty four rabbit embryos from 20 does at 6d.p.c; divided into two groups. The 1st group culture with stander media TCM 199 (control). The 2nd group culture with TCM 199 supplemented with (10% v/v) pregnancy serum rabbit does with the final concentration of progesterone 9ng/ml and 17β-estradiol 20pg/ml for three days.

Numerically viability rates were improved insignificantly according to SPDR additive to culture media. The increase rate significantly (P<0.05) in blastocysts dimension (area 74.29%, perimeter 32.80%) and in gastrula dimension (area 82.02%, perimeter 47.24%) at 48h of culture compared with control group. In the other hand, the rate of collapsed embryos elevated in the control group compared with the serum group. Furthermore, the present results were observed that significantly increased (P<0.05) in gastrulation stages after 8 d.p.c and 9 d.p.c, 65% and 100% of embryos treated with SPDR reached to stage 5 to 7 with strong brachyury expression along the entire primitive streak also in notochordal process .

***Conclusively**, overall progesterone and estrogen play an important role in the development and implantation of pre-gastrulation embryos rabbit.*

Key words: Progesterone, Embryogenesis, Morphogenesis, Gastrulation, *In vitro,* Development, Rabbit.

Rabbit blastocysts can be exactly staged in living embryos *in vivo* and *in vitro* and are amenable to experimentation under standard culture conditions (Halacheva *et al.* 2011).

Gastrulation in the rabbit starts at day 6 p.c., at a stage which is easily accessible as implantation has not yet started. Seven gastrulation stages have

been identified so far (Romia *et al*., 2010), using morphological and molecular methods (Viebahn *et al*., 1995; Schafer-Haas and Viebahn, 2000; Viebahn *et al*., 2002 and Viebahn, 2004). Early cell-lineage decision during embryonic development is another example where embryos of different mammals clearly vary between species, as recently emphasised by Rossant (2011).

Estrogen and progesterone play an important role in the development and implantation of preimplantation embryos. There is now general consensus that metabolism of steroid hormones in target tissues may play a significant part in their overall action in many types of tissues (Labrie *et al*., 2000). Its play key roles in the establishment and maintenance of pregnancy (Crosier *et al*., 2000). Elimination of these hormones from pregnant animals causes deleterious effects on the development and implantation of the embryos. It appears to be important to maintain the concentrations of estrogen and progesterone at appropriate levels relative to each other. Elevated ratios of estradiol to progesterone were shown to inhibit blastocyst metabolism and implantation (Safro *et al*., 1990).

Plasma concentrations of progesterone and 20α-dihydroprogesterone (Fuchs and Beling, 1974), and the rate of rise of plasma progesterone (Singh and Adams, 1978) have been reported to be higher in pregnant than in pseudopregnant rabbits during the immediate preimplantation period, Days 5-6 following mating. Crosier *et al*., (2000) showed that embryos treated with estrogen *in vitro* had significantly higher implantation rates when transferred to foster mothers receiving only progesterone, suggesting that estrogen acts directly on the embryo. This raises the question of whether the estrogens could also have a direct effect on the embryo's development.

To our knowledge, there is no report on the effect of progesterone and estrogen in the culture medium on gastrulation rabbit *in vitro* development from 6d.p.c till 9d.p.c.

Therefore, this study was carried out to investigate the effect of serum pregnancy does rabbit supplementation in culture medium on in vitro development of pre-implantation rabbit embryos. In order to know the possible effects of hormonally active agents on developing animals.

MATERIALS AND METHOD

Animals:

All experimental procedures involving animals were approved by the Research Ethics Committee of the UPV and licensed by European Community Directive 86/609/EC. Rabbit does used as donors and recipients belonging to the New Zealand White line from the Instituto de Ciencia y Tecnología Animal (ICTA) at the Universidad Politécnica de Valencia (UPV).

Embryo collection and treatment

Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 6 days of pregnancy. Eighty four embryos were recovered by perfusion of each oviduct and uterine horn with 20 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% of Bovine Serum Albumin (BSA). The blastocysts were randomly distributed in round-bottomed microwell plates (Nunc, Roskilde, Denmark) with 750 uL of two mediums. The control medium was TCM-199 with 25mM Hepes buffer and antibiotics (100 IU of G sodium penicillin and 25ug per mL of dihydrostreptomycin). The second medium contained the same components as above described, but supplemented with 10% of rabbit serum (v/v); pre-filtered in a 0.2 µm pore diameter (TCM-199+S). The microwell plates were kept under a humified atmosphere of 5% CO2 at 38°C.

Serum preparation

Whole blood was collected with the aid of a Vacutainer-heparin tube (LH/Li Heparin Tube TAPVAL®, MonLab, SL. Barcelona, Spain) from 6 day-old blastocyst donor does. Blood was centrifuged (1500 g, 10 min at 4ºC) and sera were detoxified at 56 °C for 30 minutes before frozen and stored at -80 °C. Serum levels of progesterone (steroid C21, preg-4-ene-3,20-dione) and 17β-estradiol were determined by direct enzyme immunoassay technique following the manufacturer's instructions (Rabbit Progesterone Elisa Test, Endocrine Technologies, Inc. Newark, USA and Estradiol Elisa Ultra-Sensitive Kit, DRG International, Inc. Marburg, Germany). Sensitivities of the tests used were 0.1 ng/ml for progesterone and 1.4 pg/ml for 17β-estradiol). Estrogen and progesterone levels of serum was measured by the final concentration of 17β-estradiol 20 pg/ml and progesterone was 9 ng/ml.

Morphometry and gastrulation analysis

Embryos were morphological evaluated every 24 hours for their developmental progression during the in vitro culture, images of embryo and gastrula were recorded using stereo-zoom microscope (Nikon SMZ-1500) associated to photograph chamber (Nikon DS-U1) and software (Nikon ACT-2U). Qualitative variables such as gastrulation stage and viability were noted. Only hatched or collapsed embryos were stained with a differential vital dye (propidium iodide and SYBR-14, -Life technologies inc-), all collapsed embryos were died and all hatched were alive).

Gastrulation stages were assigned according to the cataloguing made by Viebahn (2004), Blum *et al*., (2007) and Hassoun *et al*., (2010). Gastrulation stages between 3 and 4+ were grouped together because both stages correspond with the beginning of the formation of gastrulation (early

stage) and the stages 5-, 5+, 6-, 6+ and 7 were grouped as one considering them and advanced gastrulation development (late stage).

Statistical analysis

Data was statistically analyzed using the SPSS program for windows (SPSS, 2006). A Chi-Square Test was used to analyze the effect of serum does rabbit on the viability and gastrula stages development rates according to Snedcore and Cochran (1982). Independent-Samples T-Test was used to compare measurements of embryos and gastrula cultured with or without serum doe rabbits. The statistical significance was considered at $P<0.05$.

RESULTS AND DISCUSSION

Three days of *in vitro* culture to study the viability rate, measurements and stages as a key event at the start of gastrulation were chosen. The viability rates were improved according to serum pregnancy does additive to culture media but with no significant effect (Table 1). The development of embryos and fetuses is influenced by circulating levels of progesterone, particularly during the pre and pre-implantation period. The mechanism by which this occurs is unclear but probably does not involve a classic, direct effect of progesterone on the developing embryo (Farin *et al*., 2000).Some of progesterone is picked up by the fetal circulation and used as substrate for fetal corticosteroids. The increasing of the viability according to increase of metabolic rate and energy of embryos as a result of the ratio of steroids hormones in the serum, are agreement with (Satterfield et al. 2006 and McNeill *et al*., 2006) The importance of progesterone levels in the immediate post-conception period (days 4–7) depends on subsequent pregnancy maintenance. In addition, progesterone is a highly potent antagonist of the mineralocorticoid receptor (receptor for aldosterone and other mineralocorticosteroids), Rupprecht *et al.,* (1993). Progesterone has a number of physiological effects that are amplified in the presence of estrogen. Estrogen through estrogen receptors upregulates the expression of progesterone receptors (Kastner *et al*., 1990). Also, progesterone prevent the apoptosis while progesterone and estrogen play as antioxidation.

The increased of the viability was associated with the increased of blastocysts and gastrula size (area and perimeter) of treated group than the control group (Table 2), but the significant increased ($P<0.05$) was observed at 48h of culture for blastocysts (area 22.57µm, perimeter 16.64µm) and gastrula (area 1.62µm, perimeter 4.8µm) than the non treated group blastocysts (area 12.95 µm, perimeter 12.53 µm) and gastrula (area 0.89 µm, perimeter 3.26µm). The results indicated that a decline in the number of

Table 1: Compared between control and serum supplementation *in vitro* culture on the viability rate.

Treatment	Initial No.	Viability rate, % (n)		
		24h	48h	72h
Control	37	48.6 (18)	24.3 (9)	10.8 (4)
Serum	47	66.0 (31)	42.6 (20)	25.5 (12)

Control TCM199 culture, Serum: TCM199+ serum does rabbits.

Table 2: Embryos and gastrula dimension *in vitro* as affected by additive serum does on culture media for three days of culture (Means ± SE).

Culture time	Treatment	N	Embryo (Means ± SE) µm		Gastrula (Means ± SE) µm	
			Area	Perimeter	Area	Perimeter
0h	Control	37	7.44 ± 0.50	9.59 ± 0.40	0.96 ± 0.07	3.60 ± 0.18
	Serum	47	8.62 ± 0.38	10.18 ± 0.25	0.94 ± 0.06	3.42 ± 0.12
24h	Control	18	14.17 ± 1.32	13.16 ± 0.63	1.52 ± 0.19	4.30 ± 0.31
	Serum	31	16.01 ± 0.92	14.06 ± 0.41	1.49 ± 0.12	4.44 ± 0.18
48h	Control	9	12.95^{a} ± 1.51	12.53^{a} ± 0.81	0.89^{a} ± 0.21	3.26^{a} ± 0.37
	Serum	20	22.57^{b} ± 1.86	16.64^{b} ± 0.76	1.62^{b} ± 0.21	4.80^{b} ± 0.36
72h	Control	4	14.60 ±1.35	13.53 ±0.62	2.107 ±0.73	3.417 ±1.73
	Serum	12	18.04 ±9.92	14.62 ±4.10	1.767 ±0.89	4.307 ±1.79

Control TCM199 culture, Serum: TCM199+ serum does rabbits.
Values with different letters in the same column are significantly different ($P < 0.05$).

culture embryos to 4 in control group and 12 in treated group at 72h. The lost of biggest embryos may be attributed to more sensitive and explosion neozona with any touch, for with insignificant differences between the two groups with stay the numerically (4 vs. 12) and dimension (14.60 vs. 18.04 µm) of control group and treated group, respectively.

Early embryonic development seems to be particularly susceptible to the effects of progesterone, administration of supplemental progesterone to bovine females between day 1 and 6 after breeding resulted in conceptuses that were significantly lengthened compared to controls (Garrett *et al.*, 1988). Fetuses resulting from embryos exposed to exogenous progesterone

between day 1 and 3 or 1 and 6 of gestation were significantly larger at midgestation than untreated controls (Kleemann *et al*., 1994). Progesterone treatment between day 1 and 3 of gestation was also associated with an increase in the proportion of embryos with blastomeres on day 3 of gestation (Walker *et al*., 1996).

The additive of serum pregnancy does to culture media increased significantly (P<0.05) on development rate of gastrula to reached advanced stage more than the non-treated group after 48h and 72h (Table 3). After 48h of culture 64.5% of gastrula reached to stage (5-7). As a result of gastrulation the notochordal process begins to emerge from Hensen's node along the anterior midline of the embryonic disc (Fig 1) and, in parallel, the anterior half of embryonic disc begins to elongate along the anterior-posterior axis to accommodate the neural plate as the first step of neurulation.

Table 3: Gastrula stages development as affected by additive serum pregnancy does rabbit on culture media for three days of culture.

Culture time	Treatment	N	Gastrula stages (from 3to 7), %		Collapsed embryos (died embryos)
			Category 3-4	Category 5-7	
24h	Control	37	100 (37)	0 (0)	0% (0/37)
	Serum	47	100 (47)	0 (0)	0% (0/47)
48h	Control	18	77.8[a] (14)	22.2[a] (4)	37.8% (14/37)
	Serum	31	35.5[b] (11)	64.5[b] (20)	31.9%(15/47)
72h	Control	9	44.4[a] (4)	55.6[a] (5)	54.0% (20/37)
	Serum	20	0[b] (0)	100[b] (20)	48.9%(23/47)

Control TCM199 culture, Serum: TCM199+ serum does rabbits.
Values with different letters in the same column are significantly different (P <*0.05*).

The increasing length of the notochordal process can be determined easily in dorsal views during early neurulation and is, therefore, used here to divide the beginning of neurulation into 3 stages: Stage 5 covers the period after the first and prechordal mesoderm cells leave Hensen's node (end of stage 4) until the notochord acquires the same length as the primitive streak; during stage 6, the notochordal process elongates further and its posterior part becomes nearly twice as wide as its anterior part; this is in contrast to the posterior half of the embryonic disc which becomes narrower than its anterior half, and at stage 7, the first somites start to form on either side about equidistantly from the two extremities of the notochordal process, i.e. next to the anterior extremity of the posterior, wide part of the notochordal

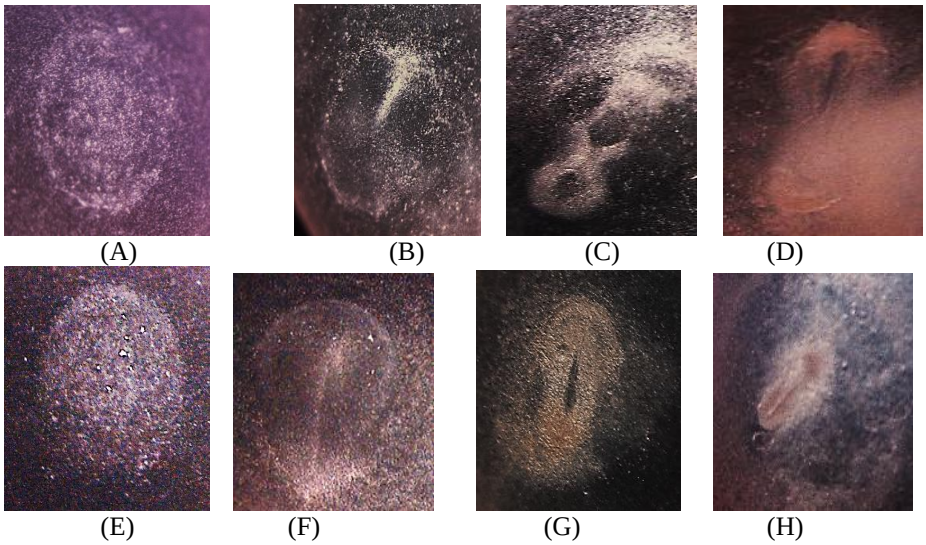

Figure 1. Early gastrulation stages of the rabbit embryo in control and serum treated group. (A) control group, (E) serum treated group whole blastocyst at a pre-primitive streak stage (6d.p.c.); the plane of focus is on the blastocyst wall containing the embryonic disc, (B) control group, (F) serum treated group with primitive streak (s) 7 d.p.c. in the midline of the PGE. The former posterior margin is still visible (arrows). (C) Control group, (G) serum treated group in late stage 4 with node fully formed and strong Brachyury expression along the entire primitive streak at 8d.p.c. (D) Control group, (H) serum treated group 9 d.p.c Stage 5 and 6 with Brachyury expression also in notochordal process. Scale bars: 250 mm.

process (Hassoun *et al* 2010). After 72h of culture 100% of gastrula reached to stages (5- 7) and 65.5% reached to stages (5-7) after 48h in treated group, while only 22.2 % reached to stages (5-7) at 48h of culture and 55.6% reached to (5-7) after 72h of culture in control group. That it may be to the effect of esetroids hormone which increased the metabolic rate.

Differences in the distribution of cells to the inner cell mass vs. trophoblast in embryos may also occur as a result of progesterone treatment (Walker *et al*., 1996).The mechanisms through which pre-implantation concentrations of progesterone regulate embryo survival and growth are not well investigated but are thought to be mediated by secretions from the endometrium.

Conclusively, current research is focused upon understanding the importance of progesterone and estrogen levels mechanisms that enable the early embryo to initiate its normal developmental program. The effect of 17β-estradiol 20 pg/ml and progesterone 9ng/ml on morphogenesis and

development gastrula rabbit *in vitro* culture was clear on the numerical blastocyst viability, dimension and development rate. It should help to better define the limits of the early mammalian embryo developmental autonomy and is necessary to design healthier *in vitro* conditions, and perhaps to the development of new strategies to modify individual phenotypes.

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تأثير إضافة كلا من هرموني البروجستيرون و الأستروجين في بيئة الاستزراع علي تحثين الشكل المورفلوجي و معدلات نمو أجنة الأرانب المحضنة معمليا في مرحلة ما قبل تكون الجاستيرولا

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تهدف هذه الدراسة إلي مقارنة الدور الحيوي والعلاقة بين نسبة هرموني البروجستيرون والأستروجين الموجود في سيرم أمهات أرانب وحيوية أجنة الأرانب وما يترتب علية من استمرار الحمل أو فقد الأجنة قبل عملية الأنغراس. ومن هنا تم تصميم التجربة معمليا بتحضين أجنة أرانب مجموعة قبل مرحلة الأنغراس وإضافة هرموني البروجستيرون والأستروجين الموجود في سيرم أمهات أرانب علي بيئة التحضين الأجنة المجمعة في مرحلة ما قبل الجاستيرولا.

تمت الدراسة علي ٤٨ جنين مجمعة في عمر ٦ أيام من التلقيح و تم تحضنها لمدة ثلاث أيام معمليا تحت درجة حرارة ٣٩م ورطوبة ١٠٠% و نسبة ٥% ثاني أكسيد الكربون. قسمت هذه الأجنة إلي مجموعتين:

المجموعة الأولى: control بيئة الزراعة المعتادة TCM 199بدون إضافات.

المجموعة الثانية: وبها تم إضافة سيرم الأرانب الحوامل بمعدل ١٠% حجم / حجم وكان التركيز النهائي لهرموني البروجسترون $9x10^{-6}$mg/ml والاستيروجين $20x10^{-9}$mg/ml وذلك لمدة ثلاثة أيام.

و قد أظهرت النتائج المتحصل عليها ما يلي:

- تم ملاحظة تحسن في حيوية الأجنة النامية المجموعة الثانية ولكن بدون تأثير معنوي.

- لوحظ زيادة معنوية في المجموعة الثانية بعد ٤٨ ساعة من التحضين في أبعاد البلاستوسست والجستريولا نتيجة المعاملة بالهرمونات. بلغت الزيادة في أبعاد البلاستوسست ٣٢،٨% للمحيط الخارجي و ٧٤،٢٩%للمساحة الكلية.أما في الجستريولا بلغت الزيادة ٤٧،٢٤%للمحيط الخارجي و ٨٢،٠٢% للمساحة الكلية مقارنة بالمجموعة الضابطة.
- تم دراسة الشكل المورفولوجي للجاسترولا و التغيرات الحادثة بها نتيجة المعاملة بهرمونات البروجسترون والاستيروجين و قد لوحظ زيادة معنوية في معدلات نمو الجاسترولا ووصولها إلي مراحل متقدمة من ٦٥% الي ١٠٠% قد وصل إلي المرحلة الخامسة والسابعة من النمو و التي تتميز بهجرة جلايا البارنشيم وتكون الخط الظهري للجنين.
- **التوصية:** إجمالا البروجسترون والاستيروجين يلعبا دورا هاما في تطور و نمو أجنة الأرانب في مرحلة ما قبل الانغراس التي تمثل أهم المراحل في النمو الجنيني.