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FORMATION RATE AND CELLULAR COUNT OF BLASTOCYSTS OF RABBITS AS AFFECTED BY EPIDERMAL GROWTH FACTOR SUPPLEMENTATION TO CULTURE MEDIUM OF FRESH OR VITRIFIED MORULAE

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The aim of this study was to evaluate the effect of addition of epidermal growth factor (EGF) to culture medium of fresh and vitrified rabbit embryos on formation rate into blastocysts/hatched blastocysts and quality of the embryos. Total of 16 mature NZW rabbit does were superovulated by PMSG and hCG. Embryos were recovered 64-66 h post-mating by flushing from the oviducts of the slaughtered animals. Compact morulae were used in this study. Embryos were divided into two groups, fresh (n=200) and intended for vitrification (n=185). Fresh (n=200) or vitrified embryos (n=150) were in vitro cultured. Either fresh or vitrified embryos were in vitro cultured in medium supplemented with EGF (n=100 and 75) as compared to control medium (n=100 and 75), respectively, under mineral oil in CO_2 incubator at 38°C, 95% humidity and 5% CO_2 in air. Embryos were assessed daily to record the developmental competence of embryos in term of formation rate of embryos at blastocyst from morula stage, from blastocyst to expanded blastocyst stage or from expanded to hatched blastocyst throughout 5 days as culture period. Total cell number and intrazonal diameter of blastocysts were determined. Results showed that formation rate of blastocyst (BLs), expanded BLs and hatched BLs was the highest (P<0.05) for fresh embryos cultured with EGF (86.00, 94.11 and 88.92%), moderate for fresh embryos without EGF (74.00, 81.11 and 75.23%) or those vitrified with EGF (70.62, 80.67 and 73.89%), and the lowest for vitrified embryos cultured without EGF (57.76, 70.00 and 56.76%). Adding EGF in culture medium increased (P<0.05) total cell number and intrazonal diameter of blastocysts produced from fresh (121.20/BLs and 129.2 µm) or vitrified (118.2/BLs and

124.6 µm) morulae as compared to fresh (109.0/BLs and 124.2 µm) or vitrified (105/BLs and 117.6 µm) morulae cultured with free media.

***In conclusion**, supplementation of culture medium with 10 ng/ml epidermal growth factor (EGF) had beneficial effects of the developmental competence of fresh or vitrified embryos at morula stage to reach hatched blastocyst stage with good quality.*

Keywords: Rabbit, embryo, vitrification, growth factor, development, quality.

Cryopreservation is an important tool for creation of embryo banks for future use in animal breeding. This technique also enables to protect germ cells of rare or endangered species and strains of farm and wild animals. In certain cases optimization of cryopreservation protocols for more sensitive embryos is relevant (Chrenek *et al*., 2014). Cryobiologist and reproductive biologists have provided with a better understanding of the physical principals of cryopreservation techniques (Liu *et al*., 2012) and their short and long term biological effects on the embryo. Regardless of embryo production, all unused embryos are cryopreserved by vitrification (Do *et al*., 2014), which is an important tool for preservation of mammalian embryos (Chrenek and Makarevich. 2011), but require highly trained personnel for manipulating embryos in small volumes and in short equilibration times (Almiñana and Cuello, 2015). In the vitrification process, the embryo is exposed first to a low cryoprotectants (CPAs) concentration solution and then to a much more concentrated solution. Vitrification relies especially on 2 aspects that are closely linked: 1) A very high cooling rate (around 20000 ºC/min), which is achieved by plunging the sample in liquid nitrogen (-196ºC) and by using different devices or straws (Arav, 2014) that allow embryo vitrification in minimal volumes; and 2) the high viscosity of vitrification media, which depends on the concentration of the CPAs.

Regardless of the method of freezing employed, the freezing and thawing processes decrease embryo viability, an effect attributed to physical and chemical damage induced during cryopreservation (Bagusi *et al.*, 2000). Despite many reports on successful vitrification of mammalian embryos, the rate of development of vitrified-warmed embryos is still lower than that of non-vitrified embryos (Cseh *et al.*, 1999). On the other hand, as the embryo develops, factors present in medium surrounding the embryo can influence cell growth and development (Díaz-Cueto and Gerton, 2001).

Among the growth factors and receptors, epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) have been shown to enhance blastocyst development (Pan *et al*., 2105). EGF is a 6 kDa protein consisting

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of 53 amino acid residues and three intra-molecular disulfide bridges such as cell proliferation; promote cell differentiation, steroidogenesis and play a key regulatory role in preimplantation embryonic development (Babitha *et al.*, 2014). The *in vitro* effects of EGF on embryo development are reported in several mammalian species (Ahumada *et al*., 2013). In this respect, addition of EGF to culture medium promotes cleavage and blastocyst development and implantation rate after transfer to the uterus, and a deficiency in EGF results in deficiencies in placental structure and fetal growth (Chandra *et al.*, 2012). EGF binds with its receptor (EGFR) to enhance cell proliferation (Fujihara *et al.*, 2014). It has been shown that EGFR is expressed in the 2-cell to blastocyst stage in the mouse and from the 8-cell stage to the blastocyst stage in human (Kane *et al.*, 1997). EGF treatment significantly increased the blastocyst formation rate, the total number of cells per blastocyst, the cell ratio of the inner cell mass and the trophectoderm (Zeng and Harris, 2014).

Therefore, the objective of the present study was to evaluate the effect of adding EGF to culture medium on embryo development, in terms of formation rate of blastocysts / hatched blastocysts and number of cells of blastocyst for vitrified compared with non-vitrified (fresh) rabbit embryos.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Embryos recovery:

Total of 16 New Zealand White rabbit does (4-5 months of age, 3-3.5 kg live body weight) were superovulated using a single i.m. injection of 20 IU PMSG/kg LBW (Foligon, Intervet International B.V., Boxmeer), followed by 40 IU hCG/kg LBW (Pregnyl, Organon, Nile Co., Egypt), 48 h later on day of natural mating with fertile bucks belonging to the same rabbit breed. Embryos were recovered 64-66 h post-mating by flushing from the oviducts of the slaughtered animals using flushing medium, phosphate buffer saline (PBS) supplemented with 10 % fetal calf serum (FCS, sigma) and antibiotics, 100 IU penicillin and 100 µg/ml streptomycin (Sigma Chemical Co., St. Louis, Mo, USA) in Petri dishes. All recovered embryos were evaluated morphologically under inverted microscope and normal embryos (compact morula with intact mucin coat and zona pellucida were used in this study according to Mehaisen *et al.* (2015). Embryos were washed twice in fresh flushing medium and randomly divided into two groups, fresh (n=200) and intended for vitrification (n = 185).

Embryo vitrification and thawing procedures:

The vitrification of embryos was performed following the method developed by Mehaisen *et al.* (2006) with minor modifications. Briefly, the vitrification procedure was carried out in two steps at 25 ^{o}C. In the 1st step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (1.75 M DMSO, Sigma) and 12.5% (v/v) ethylene glycol (2.23 M EG, Sigma) in DPBS supplemented with 10% (v/v) of FCS. In the 2nd step, embryos were suspended for 1 min in a solution of 20% (v/v) DMSO and 20% (v/v) EG in DPBS supplemented with 10% (v/v) of FCS. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws (IMV, L'Aigle, France) between two drops of DPBS separated by air bubbles. Finally, the straws were sealed and plunged directly into liquid nitrogen.

After storage in liquid nitrogen for more than one week, straws were warmed up in 25 ºC water-bath for 10-15 sec. Then the content of the straw was expelled into an empty Petri dish and mixed by gently shaking the dish. The embryos were transferred to 0.5 M sucrose solution (S-PBS) for 5 min and then to 0.25 M sucrose solution (0.5 S-PBS) for 5 min and washed twice in DPBS supplemented with 20% FCS (Celestinos and Gatica, 2008).

Treatment and embryo culture:

Culture medium was tissue culture medium (TCM-199, Sigma) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma) and 50 µg/ml of Gentamicin sulphate. Two types of culture medium were used in this study for *in vitro* culture of embryos. These types included culture medium supplemented with 10 ng/ml epidermal growth factor (EGF, Sigma) as compared to control culture medium without supplementation).

Fresh (n=200) or vitrified embryos (Survival viable, n =150) were *in vitro* cultured in a one-well embryo culture dish (NUNC A/ S, Thermo Fischer Scientific, Roskilde Site, Denmark), containing 100 µl drops of culture medium. Either fresh or vitrified embryos were *in vitro* cultured in medium supplemented with EGF (n= 100 and 75) as compared to control medium without supplementation (n=100 and 75), respectively, under mineral oil in CO_2 incubator at 38°C, 95% humidity and 5% CO_2 in air. Embryos were assessed daily to record the developmental competence of embryos in term of formation rate of embryos at blastocyst from morula stage, from blastocyst to expanded blastocyst stage or from expanded to hatched blastocyst throughout 5 days as culture period.

Cellular count and intrazonal diameter of embryo at blastocyst stage:

Five intact embryos in case of fresh or vitrified cultured, with or without EGF, were randomly selected on the second day of cultivation (at blastocyst stage) were stained as described earlier by Koruji *et al.* (2004). Briefly, embryos were incubated in 0.5% sodium nitrate solution at 37ºC for 30 min. Then the embryos were removed using a Pasteur pipette, placed on a clean slide and the excess sodium nitrate was removed from the slide. Before drying the embryos, a few fixatives (mixture of acetic acid and methanol, 1: 3) were added to the blastocysts. After air drying, the slides were stained with 5% Giemsa (Sigma, USA) in PBS for 30 min and washed with distilled water. Nuclei of the blastomeres were count using a light microscope with magnification of 100x.

Embryonic intrazonal diameter at blastocyst stage was measured light microscopy and eyepieces.

Statistical analysis

Data were analyzed by Analysis of Variance (ANOVA) using computer program of SAS (1998). ANOVA was performed after arcsine transformation of original data expressed as proportions. The significant differences among group means were preformed using Duncan Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of EGF on in vitro culture of fresh or vitrified rabbit morulae:

Results shown in Table (1) revealed significant ($P<0.05$) effect of adding epidermal growth factor (EGF) in culture medium on formation rate of embryos at blastocyst (BLs), expanded blastocyst (EBLs) and hatched blastocyst (HBLs) stages after culture period of 5 days of fresh or vitrified embryos (at morula stage). The formation rate of BLs, EBLs and HBLs was significantly ($P<0.05$) the highest for fresh embryos cultured with EGF (86.00, 94.11 and 88.92%), moderate for fresh embryos without EGF (74.00, 81.11 and 75.23%) or those vitrified with EGF (70.62, 80.67 and 73.89%), and the lowest for vitrified embryos cultured without EGF (57.76, 70.00 and 56.76%).

It is interesting to observe that formation rate into all blastocyst stages was higher for fresh than vitrified embryos, regardless EGF addition. In accordance with the present results, Chrenek et *al.* (2014) showed that after cryostorage and de-vitrification almost 73% of rabbit embryos survived and developed to advanced blastocyst stage versus 96% in the intact control. Also, Chrenek and Makarevich (2011) reported significantly lower developmental rate of vitrified/thawed transgenic embryos reached hatching blastocyst stage (68%)

Table (1): Formation rate of fresh or vitrified rabbit morula into blastocyst stages in culture medium with or without EGF.

Group	Treatment Groups	Vitrified embryos	Development rate		
			Blastocyst	Expanded Blastocyst	Hatching Blastocyst
Fresh morulae	Without EGF	100	74.00±1.87[b]	81.11±1.23[b]	75.23±5.17[b]
	With EGF	100	86.00±1.87[a]	94.11±1.86[a]	88.92±1.16[a]
Vitrified morulae	Without EGF	75	57.76±3.31[c]	70.00±4.45[b]	56.76±3.16[c]
	With EGF	75	70.62±2.58[b]	80.67±6.09[b]	73.89±4.38[b]

Means denoted within the same column with different superscripts are significantly different at P<0.05.

than the control embryos (100%). In the same line, Makarevich *et al.* (2008) showed significantly lower ability (P<0.05) of hatching blastocyst formation when intact (97.0%) were compared with vitrified rabbit embryos (63%).

After fertilization, the one cell egg undergoes a series of cleavage divisions, progressing through 2-cell, 4-cell, 8-cell, 16-cell, mulberry-shaped 20- to 30-cell mass (morula) to the formation of blastocyst, which is composed of trophoblast and inner cell mass that eventually develop into placenta and embryo, respectively. Accumulated evidence suggests that growth factors are necessary components in early embryonic development *in vitro* and *in vivo* (Park and Han, 2009). Successful development of embryo is dependent on genomic expression, regulatory hormone, energy source and growth factors and cytokines secreted from uterine tube, uterus and embryo itself (Kim *et al.,* 1999).

According to the present results, adding EGF enhanced formation rate into all blastocyst stages in rabbits, regardless cryopreservation. In this respect, Koruji *et al.* (2004) concluded that the higher dose of EGF (10 ng/ml) can improve the development rate of mouse morula. Also in mouse, Głąbowski *et al.* (2005) found a higher blastocyst rates as well as an augmented uptake of nutrients by blastocyst cells in the media enriched with EGF. The present results and findings previously reported indicated that EGF plays an important role in embryo developmental form as early as pre-implantation (Dadi *et al.,* 2007). Finally, EGF has been shown to promote pre-implantation embryo growth (Grazul-Bilska *et al.,* 2003), as well as trophoblast invasion and post implantation embryo growth (Haimovici and Anderson, 1993).

Effect of EGF on cellular contents and intrazonal diameter of cultured blastocysts:

Results presented in Table (2) cleared that adding EGF in culture medium significantly (P<0.05) increased average cell number and intrazonal

Table (2): Developmental rate of rabbit embryos fresh or vitrified at morula stages and quality of rabbit embryo in culture media with or without EGF.

Group	Culture medium	Blastocyst quality	
		Total cell number	Intrazonal diameter (µm)
Fresh morulae	Without EGF	109.00± 2.92[b]	124.2±1.3[b]
	With EGF	121.20± 2.99[a]	129.2±1.24[a]
Vitrified morulae	Without EGF	105.00± 2.74[b]	117.6±0.8[c]
	With EGF	118.20± 2.03[a]	124.6±0.6[b]

Means denoted within the same column with different superscripts are significantly different at P<0.05.

diameter of blastocysts produced from fresh (121.20/BLs and 129.2 µm) or vitrified (118.2/BLs and 124.6 µm) morulae as compared to fresh (109.0/BLs and 124.2 µm) or vitrified (105/BLs and 117.6 µm) morulae cultured with free media. However, impact of EGF addition was pronounced on intrazonal diameter more than on average cell number of BLs. Such finding may be attributed to increasing cell volume or interstitial space in-between.

It is worthy noting that average cell number and intrazonal diameter of blastocysts produced from fresh morulae was higher than those of vitrified ones, regardless EGF supplementation.

The observed increase in fresh in comparing vitrified morulae regarding cellular number was indicated by Chrenek *et al.* (2014), who showed that total cell number in the vitrified embryos was significantly lower than in the intact control (117 *vs.* 135), but they found no significant differences between the vitrified and intact embryos in the embryo diameter (123.2 *vs.* 129.85 µm). Also, Chrenek *et al*. (2011) showed that vitrification procedure caused decrease in total cell numbers of vitrified embryos compared to the control group (117 *vs*. 141/BLs).

In association with the obtained results, Głąbowski *et al.,* (2005) stated that the culture media enriched with EGF showed an accelerated growth, improved blastocoel formation, trophoectoderm expansion and protein metabolism. Also, Koruji *et al.* (2004) showed significant impact of EGF on cell number of BLs from fresh embryos (97 vs. 72.5/BLs, P<0.01), but insignificantly affect cell number of BLs from vitrified embryos (79 vs. 72/BLs).

In general, EGF treatment significantly increased the total blastocyst cell numbers compared with the control group due to increases in the numbers of inner cell mass (Zeng and Harris, 2014 and Lee *et al.,* 2005).

In the embryos, the cryoprotectant comes out gradually from the embryonic cells and so makes the environment unappreciated for embryo development. The beneficial effect of EGF addition to culture medium may induce some biochemical changes especially for vitrified embryos (Koruji *et al.*, 2004).

Conclusively, supplementation of culture medium with 10 ng/ml epidermal growth factor (EGF) had beneficial effects of the developmental competence of embryos at morula stage to reach hatched blastocyst stage with good quality in terms of higher cellular number and intrazonal diameter. Also, EGF addition is essential tool for reaching higher formation rate of embryos at hatched blastocyst, particularly for cultured vitrified embryos.

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معدل التشكل والعدد الخلوى لأجنة الارانب في مرحلة البلاستوسيست تحت تأثير اضافة عامل النمو الأبدرمى لبيئة زراعة الاجنة الطازجة أو المجمدة بالتزجج فى مرحلة الموريولا

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تهدف هذه الدراسة الى تقييم تأثير اضافة عامل النمو الابدرمى لبيئة زراعة اجنة الارانب الطازجة والمجمدة بالتزجج فى مرحلة الموريولا على معدل تشكل الاجنة الى مرحلتى البلاستوسيت و الفقس وعلى جودة الاجنة. استخدم فى هذه الدراسة ١٦ من امهات الارانب النيوزيلاندى البيضاء الناضجة والمعاملة هرمونيا بـ (hCG, PMSG) لاحداث التبويض الفائق. تم جمع الاجنة بعد من ٦٤-٦٦ ساعة من التلقيح بعمل غسيل لقناتى الرحم بعد ذبح الامهات.استخدمت الاجنة فى مرحلة الموريولا فى هذه الدراسة. تم تقسيم الاجنة الى مجموعتين:- الاولى (اجنة طازجة وعددها ٢٠٠جنين) والثانية (اجنة معدة للتجميدة بالتزجج وعددها ١٨٥). وتم زراعة كلا من الاجنة الطازجة والاجنة بعد التجميد فى بيئة زراعة الاجنة مضاف اليها ١٠نانو جرام/ملل من عامل النمو الابدرمى ثم مقارنتها بمجموعة الكنترول (بدون اضافة عامل النمو الابدرمى) . وتم قياس معدل قدرة الاجنة فى مرحلة الموريولا على التطور والوصول إلى مرحلتي البلاستوسيست والفقس يوميا ولمدة ٥ ايام. و تم قياس العدد الخلوى وقطر الزونا الداخلى للأجنة فى مرحلة البلاستوسيست.

اوضحت النتائج ان معدل تشكل الاجنة فى مراحل البلاستوسيست المختلفة (blastocyst, expanded and hatched) كانت اعلى معنويا للأجنة الطازجة التى تم زراعتها فى بيئة تحتوى على عامل النمو الابدرمى(٨٦,٠٠ و٩٤,١١ و٨٨,٩٢%) ومتوسطة للاجنة الطازجة بدون اضافة عامل النمو الابدرمى(٧٤,٠٠ و٨١,١١ و٧٥,٢٣%) او مع الاجنة المجمدة بالتزجج فى بيئة زراعة الاجنة مضاف اليها عامل النمو الابدرمى (٧٠,٦٢ و ٨٠,٦٧ و٧٣,٨٩ %) وكانت اقل للاجنة المجمدة بالتزجج فى بيئة زراعة الاجنة بدون اضافة عامل النمو الابدرمى(٥٧,٧٦ و٧٠,٠٠ و٥٦,٧٦%). ووجد ان اضافة عامل النمو الابدرمى الى بيئة الزراعة ادى الى زيادة معنوية للعدد الخلوى وقطر الزونا الداخلى للأجنة فى مرحلة البلاستوسيست المنتجة من اجنة الموريولا الطازجة (١٢١,٢٠/خلية و١٢٩,٢ ميكرون) او المجمدة بالتزجج (١١٨,٢/خلية و١٢٤,٦ ميكرون) مقارنة باجنة الموريولا الطازجة (١٠٩,٠/خلية و١٢٤,٢ ميكرون) او المجمدة بالتزجج (١٠٥/خلية ١١٧,٦ ميكرون) المنزرعة فى بيئة خالية من عامل النمو الابدرمى.

التوصية: نستخلص من هذه الدراسة ان اضافة ١٠نانو جرام /ملل من عامل النمو الابدرمى الى بيئة زراعة الاجنة كان مفيد فى عملية تطور الاجنة الطازجة والمجمدة بالتزجج فى مرحلة الموريولا الى مرحلة الفقس مع تحسين جودة الاجنة.