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EFFECT OF SELENIUM PLUS VITAMIN E OR VITAMIN C ALONE ON *IN VIVO* FERTILITY, VIABILITY, FREEZABILITY AND CULTURE OF EMBRYOS IN RABBITS

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SUMMARY

In vivo fertility and embryos viability after cryopreservation is essential for the success of assisted reproductive techniques. The aim of this study was to investigate the impact of vitamin E (VE) +selenium (Se) or vitamin C (VC, as ascorbic acid) on in vivo fertility, embryos quality, freezing ability and culture of rabbit embryos. A total of 30 New Zealand White (NZW) rabbit does and 10 NZW fertile bucks were used in this study. Rabbits were kept under the same condition and individually housed in metal cages. Rabbit does were divided into three similar groups, (n=10 each). Does in the 1st group (G1) were considered as a control group without treatment. Does in the 2nd (G2) and 3rd (G3) groups were treated with 1% of VE+Se and 1% VC given in drinking water, respectively, for 15-18 days before mating. At the end of treatment period, experimental does were naturally mated by untreated bucks. After 72 h of mating, three does from each group were slaughtered and embryos were collected by flushing. At the end of pregnancy period, kindling rate (KR) and litter size (LS) and litter weight were recorded at birth and weaning. The recovered embryos from each group were washed, evaluated and preserved by verification method. Post-thawing embryos were in vitro cultured for assessing their ability to develop up to hatched blastocyst stage.

Results showed that KR and LS at birth and weaning was higher (P<0.05) in G3 (80%, 9.29 and 8.86) than in G2 (70%, 7.86 and 7.57) and G1 (70%, 7.0 and 6.57), respectively. Average bunny weight at birth and weaning was higher in G2 and G3 than in G1. Embryo recovery rate after collection was not affected by treatment, being 89.3, 87.9 and 83.9, for G1, G2 and G3, respectively. Embryos of G3 showed the highest (P<0.05) percentage (92.3%) of viable embryos after recovery, normal embryos post-verification (95.8%) and hatched embryos (73.9%) post in vitro culture as compared to G1 (84.0, 90.5 and 52.6%, respectively). The corresponding percentages of embryos of G2 did not differ significantly from those in G1 and G3, being 86.3, 80.0 and 55.0%, respectively.

In conclusion supplementing drinking water of rabbit does with 1% ascorbic acid within 15 days pre-conception is recommended to improve litter size and litter weight. Moreover it improves rabbit's embryos quality embryos.

Keywords Selenum ,Vitamin E ,Vitamin C IN VIVO Fertility, Viability, Freezability , Rabbit Embryos

INTRODUCTION

Aerobic metabolism utilizing oxygen is essential for energy requirements of the gametes and the free radicals play a significant role in physiological processes within the ovary. Many studies have demonstrated involvement of reactive oxygen species (ROS) in the follicular-fluid environment, folliculogenesis, and steroidogenesis (Sugino *et al*., 2004).

The oxidative stress reduces embryo quality and viability because of the peroxidation of membrane lipids and the modification of important molecules, such as proteins and nucleic acids, which results in cellular death through apoptosis (Van Soom *et al*., 2002). Cells contain antioxidants such as glutathione and superoxide dismutase to protect against the production of oxygen radicals. It has previously been demonstrated that adding antioxidant solutions to the *in vitro* culture systems for embryos stimulated development (Orsi and Leese, 2001) and improved ability of the embryos to survive post cryopreservation (Tarin and Trounson, 1993).

Selenium (Se) is essential trace element and may have antioxidant activity in biological systems (Gutteridge, 1986). It is a component of seleno-proteins and is involved in immune and neuropsychological function in the nutrition of animals (Meschy, 2000). Also, it is essential component in selenium-dependant glutathione peroxides, which helps in regulating hydrogen peroxide damage to cell membranes (NRC, 2007). Moreover, Se has a biological function related to vitamin E and Se deficiency induces oxidative stress because reduced fertility through reducing number and

differentiation of germ cells as reported in mice (Sánchez-Gutiérrez, *et al*., 2008).

Vitamin E (VE) requirement can be defined as the amount required preventing per oxidation in the particular sub cellular membrane which is most susceptible to per oxidation (Koyuncu and Yerlikaya, 2007). Vitamin E prevents oxidative damage to sensitive membrane lipids by suppressing hydro peroxide formation (Chow, 2001) and protects cellular membranes thus maintaining membrane integrity and reducing oxidative stress (Hogan *et al*., 1993). Culture of bovine embryos with VE enhanced development and increase the percentages of embryos developed to early and expanded blastocysts (Olson and Seidel, 2000). Also, blastocyst qualities of porcine somatic cell nuclear transfer and *in vitro* fertilization embryos were improved when embryo culture media was supplemented with VE (Jeong *et al*., 2006).

Adding ascorbic acid into the culture media prevents follicular apoptosis in rat and mouse follicles, and also improves the blastocyst production in mouse (Eppig *et al*., 2000). There are reports about embryotrophic effect of L-ascorbic acid (Wang *et al*., 2002 and Kitagawa *et al*., 2004) but no information is available about the effect of *in vivo* treatment of females with antioxidants on embryo quality.

Therefore, the present study aimed at evaluating the effect of *in vivo* rabbit doe treatment with antioxidants (vitamin E + selenium or vitamin C alone on the ability of recovered embryos to cryopreserve and culture for advanced embryonic stages.

MATERIALS AND METHODS

This study was carried out at Sakha Animal Production Research Station, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt during the period from October 2010 to May 2011.

Animals:

A total of 30 New Zealand white (NZW) rabbit does of about six months of age and 3.25 - 3.50 kg live body weight (LBW) was used as embryo donors. Ten NZW bucks between seven and eight months of age and 3.6-4.1 kg LBW were used for mating of the experimental does. Experimental rabbits were kept under the same condition and were individually housed in metal cages (40x50x 60 cm) provided with feeders and water nipple for drinking. Rabbits were fed *ad libitum* on a commercial pelleted concentrate diet.

Rabbit does were divided randomly into three similar groups, 10 does each. Does in the first group (G1) were considered as control, while those of the 2nd (G2) and 3rd groups (G3) were given 1% (w.v.) from a combination of vitamin E (200.000 IU/l) and selenium (500 mg/l) and 1% (w.v.) of vitamin C (ascorbic acid) added to drinking water, respectively.

Does in G2 and G3 were subjected to adaptation period for five days to be able to drink water with Se and vitamin E and then for 15 days as a treatment period, before mating. Mating of does in all groups was done within seven days. After 72 hrs of mating, three does from each group were slaughtered for embryo collection. The rest of the does in each group were allowed to complete the pregnancy term and the diagnoses of the pregnancy was taken place 10-12 days after mating. Kindling rate was recorded on the bases of pregnancy of all does (n=7) in each group. After kindling, litter size and average bunny weight in each group were recorded at birth and weaning.

Preparation of flushing medium:

Phosphate buffer saline (PBS) medium was prepared according to Gordon (1994). The pH value of the media and osmolarity of the medium were adjusted to be 7.2-7.3 and 280-300 mOsmol/kg, respectively. The medium was filtrated by 0.22-µm millipore filter (milieux GV, millepore, Cooperation Bedford MOA).

Embryo recovery:

Embryos were recovered from uterine horns (right or left) using flushing technique using pre-warmed PBS (38°C), containing 2% bovine serum albumin (BSA) antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and 0.3 mM sodium pyruvate. The flushing medium was collected in sterile plastic Petri dishes. The embryos were washed three times with PBS and evaluated under a stereomicroscope (X 20–40).

Verification procedure:

The basal solution used for verification was PBS supplemented with 4 % BSA antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). The verification procedures employed throughout this experiment were according to Vicente *et al*. (1999) with some modification. Verification solution 1 (VS1) contained 12.5% (v:v) ethylene glycol, 12.5% (v:v) dimethyl- sulfoxide (DMSO) and 0.5 M sucrose in basal medium. While, verification solution 2 (VS2) contained 20% (v:v) ethylene glycol, 20% (v:v) DMSO and 0.5 M sucrose in basal medium.

Verification procedure was carried out in two steps: firstly, embryos were transferred

into VS1 medium in disposable sterile Petri dish and left in this medium for two minutes; secondly, 2-3 embryos were suspended in VS2 medium and loading in as small volume as possible (< 1.0 µl) on to the inner surface of the open edge of hemi-straws (HS) prepared from 0.25 ml plastic insemination straws (IVM L' Aigle, France) using a fine glass capillary pipette. The HS were plunged vertically into liquid nitrogen (LN2) and placed into a 0.5 ml cryostraws held under liquid nitrogen. Then, the cryo-straws were plugged with a plastic colourcoded plug before cryostorage (Vanderzwalmen *et al*. 2003).

Thawing and culture of vitrified embryos:

Thawing embryos were performed under LN2 and with forceps. The HS were pulled out of the larger straw and the tip of the straw holding the embryos was immediately immersed into a Petri dish containing PBS with 4 % BSA and 0.33 M sucrose for one min, followed by serial dilutions in PBS and 0.175 M sucrose solution for two min. The embryos were washed three times in PBS solution for five min per time to remove cryoprotectants at room temperature (Vanderzwalmen *et al.*, 2003).

Viable post-thawed embryos were *in vitro* cultured in 100 µl drops of tissue culture medium 199 supplemented with 15% heat inactivated fetal calf serum and 50 µg /ml of gentamicin sulphate under oil at 38°C, 95% humidity and 5% CO_2 in air for 72 h. Embryo survival was assessed by their ability to develop to the expanded and hatched blastocyst stages following *in vitro* culture.

Statistical analysis:

Data were statistically analyzed by General Linear Model (GLM) procedures using statistical software SAS (2004) to evaluate the effect of treatment as one way analysis. The significant differences among groups were tested by the multiple range test (Duncan, 1955).

RESULTS

In vivo fertility of doe rabbits:

Results presented in Table (1) revealed that kindling rate (KR), litter size (LS) and litter weight (LW) of rabbit does at birth and at weaning were affected (P<0.05) by treatment. KR and LS at birth and weaning were higher (P<0.05) in G3 than in G2 and G1. However, these traits did not differ significantly in G2 compared to G1. On the other hand, bunny weight at birth and weaning was higher (P<0.05) in both treatment groups (59.4 g in G2 and 59.3 g in G3) than in (G1 (54.3 g).

Recovery rate and viability of embryos:

Results presented in Table (2) showed that percentage of viable embryos was higher (P<0.05) in G3 (92.3%) and G2 (86.3%, P≥0.05) than in G1 (84.0%), although recovery rate was higher G1 than in G2 and G3, but the differences were not significant. The highest percentage of viable embryos in G3 was associated with the lower (P<0.05) unviable embryos (7.7%) as compared to G2 (13.7%) and G1 (16%). These results indicated positive effect of ascorbic acid treatment on viability of recovered embryos.

Survival rate and quality of vitrified embryos:

Results of post-verification presented in Table (3) showed similarity in survival rate of post-vitrified embryos in all experimental groups (100% for all), with significant (P<0.05) differences in their quality. It is of interest to note that the percentage of post-vitrified normal embryos was higher (P<0.05) in G3 (95.8%) than in G2 (80.0%) and both groups did not differ significantly from that in G1 (90.5%). The observed reduction in percentage of normal embryos post-verification in G2 was attributed to the highest (P<0.05) percentage of abnormal embryos (20.0%) as compared to the control (9.5%). However, G3 showed the lowest percentage of abnormal embryos (4.2%, P<0.05). These results indicated also impact of ascorbic acid treatment on quality of post-vitrified embryos.

In vitro culture of vitrified embryos:

Results of *in vitro* post-thawing-culture for 72 h presented in Table (4) revealed that post-vitrified embryos recovered from does in G3 showed higher (P<0.05) percentage at hatched blastocyst stage than those of G2 and G1 (73.9 *vs.* 55.0 and 52.6%, respectively). However, does in G1 showed higher (P<0.05) percentage of embryos at blastocyst stage.

DISCUSSION

In according with the present results of *in vivo* fertility in term of increasing litter size and litter weight of rabbit does, many studies demonstrated intensified lipid peroxidation in the preovulatory Graafian follicle **(**Jozwik *et al.*, 1999). Also, during embryo development the intrinsic metabolism generates reactive oxygen species (ROS) in its extracellular surroundings (Guerin *et al*., 2001) because the lumen of the female reproductive tract contains oxygen level between 3 and 7% (Fischer and Bavister, 1993). The ROS can block or retard normal development of the embryo leading to apoptotic events (Salas-Vidal *et al*., 1998) and increasing concentrations of H_2O_2 reflects in higher apoptosis incidence (Yang *et al.*, 1998)

and negatively quality of pre-implantation embryo (Moss *et al*., 2009). Moreover, *in vitro* handling and culture conditions exposed embryos to oxidative stress resulting from light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates (Agrawal *et al*., 2003).

The embryos also produce endogenous ROS by various enzymatic actions and metabolic pathways (Riley and Behrman, 1991). Oxidative stress is involved in defective embryo development and retardation of embryo growth (Agarwal *et al*., 2003) that is attributed to induced cell-membrane damage, DNA damage, and apoptosis. Therefore, reactive oxygen species must be continuously inactivated in order to keep only a small amount necessary to maintain normal cell functions.

Ability of embryos to cryopreservation obtained in the present study is in agreement with the findings of Tarin and Trounson (1993) and Orsi and Leese (2001). Presence of antioxidants are important for the development of the blastocyst inner cell mass (Van Soom *et al*., 2002) as well as improving *in vitro* blastocyst development (Burton *et al*., 2003), which agree with the present findings. *In vivo* ascorbic acid treatment may cause both the follicular and oviductal fluid to be rich in oxygen scavengers which protect embryos from oxidative damage (Lapointe *et al*., 1998) and reduce susceptibility of embryos to reactive oxygen species during developmental stages (Ali *et al*., 2003). The antioxidant activity of ascorbic acid in preventing free radical-initiated peroxidative tissue damages is accepted by most investigators and is believed to be the primary free radical scavenger in mammalian cell membranes as for VE (Droge, 2002). Also, the antioxidants delay or inhibit oxidative damage to cellular molecule (Gutteridge and Halliwell, 1994 and Wang et al., 2002)

No embryotrophic effect of VE+Se is in the present results come in agreement with several authors found no beneficial effect of administration of supplemental VE alone or in combination with Se (Kappel *et al*., 1984).

CONCLUSION

Treating rabbit does with ascorbic acid have positive effect on litter size and litter body weight at birth and weaning. Also, it has enhancement effect on *in vitro* embryonic development and survival rate after vitrified/thawed process. The present study recommend to supplement drinking water of rabbit does with 1% ascorbic acid within 15 pre-conception to obtain high quality embryos having high freezing ability and developmental competence post-vitrification.

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Table 1. Kindling rate, litter size and litter weight of does as affected by treatment at birth and weaning

Treatment group	KR (%)	Litter size/doe		Average bunny weight (g)	
		At birth	At weaning	At birth	At weaning
G1 (Control)	70[b]	7.00±0.308[b]	6.57±0.300[b]	54.3±2.020[b]	937.3±12.420[b]
G2 (Vit. E+Se)	70[b]	7.86±.0404[b]	7.57±0.368[b]	59.4±1.525[a]	1032.7±15.129[a]
G3 (Vit. C)	80[a]	9.29±0.359[a]	8.86±0.260[a]	59.3±1.148[a]	1059.0±21.015[a]

[a and b:] Means denoted within the same column with different superscripts are significantly different at (P<0.05).

Table 2. Effect of treatments (Vit. E+ Se or Vit. C) on recovery rate and viability of rabbit embryos

Treatment	Number		Recovery rate (%)	Viable Embryos		Unviable Embryo	
	CL	Embryos		n	%	n	%
G1 (Control)	28	25	89.3	21	84.0[b]	4	16.0[a]
G2 (Vit. E+ Se)	33	29	87.9	25	86.3[ab]	4	13.7[ab]
G3 (Vit. C)	31	26	83.9	24	92.3[a]	2	7.7[b]

[a and b:] Means denoted within the same column for each effect with different superscripts are significantly different at (P<0.05).

Table 3. Effect of treatments (Vit. E+ Se or Vit. C) on survival rate and quality of vitrified/thawed embryos

Treatment	Vitrified embryos (N)	Post-vitrification		Normal embryos		Abnormal embryos	
		n	%	n	%	n	%
G1 (Control)	21	21	100	19	90.5[ab]	2	9.5[b]
G2 (Vit. E+ Se)	25	25	100	20	80.0[b]	5	20.0[a]
G3 (Vit. C)	24	24	100	23	95.8[a]	1	4.2[b]

[a and b:] Means denoted within the same column for each effect with different superscripts are significantly different at (P<0.05).

Table 4. Effect of oral treatments (Vit. E+Se or Vit. C) on *in vitro* development of embryos post-vitrified/thawing for 72h

Treatment	Survival Embryos (N)	Embryonic stage of *in vitro* culture for 72 h							
		Early Blastocyst		Expanded blastocyst		Hatched blastocyst		Degenerated embryos	
		N.	%	N.	%	N.	%	N.	%
G1 (Control)	19	5	26.3[a]	3	15.8	10	52.6[b]	1	5.3
G2 (Vit. E+Se)	20	4	20.0[a]	4	20.0	11	55.0[b]	1	5.0
G3 (Vit. C)	23	2	8.30[b]	4	17.4	17	73.9[a]	0	0

[a and b:] Means denoted within the same column for each effect with different superscripts are significantly different at (P<0.05).

تأثير المعاملة بالسلينيوم +فيتامين هـ أو فيتامين ج علي الخصوبه والحيوية والقابلية للتجميد والتطور للأجنة فى الأرانب

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يعتبر الحفاظ علي حيوية الأجنة من العوامل المساعدة المحددة لنجاح تكنولوجيا التناسل لذلك أجريت هذه الدراسة بهدف بحث تأثير فيتامين هـ+ السلينيوم أوفيتامين ج منفردا (حمض الأسكوربيك) على الجودة، القدرة علي التجميد والتطور لأجنة الأرانب. تم استخدام 30 آم من أرانب النيوزيلاندي الأبيض بمتوسط عمر5.7-5.9 شهر ومتوسط وزن جسم 3.25- 3.5 كجم كآمهات مانحة للأجنة واستخدم 10 ذكور من نفس السلالة بمتوسط عمر 7- 8 شهر ومتوسط وزن جسم 3.6-4.1 كجم لاستخدامها في التلقيح الطبيعي للآمهات. تم الاحتفاظ بالآمهات والذكور تحت ظروف معيشية ورعائية واحدة ، حيث تم إسكان الآمهات والذكور في أقفاص معدنية بمساحة (40× 50× 60سم). تم تقسيم آمهات الأرانب إلي 3 مجموعات بمعدل (10 أمهات في كل مجموعة). الآمهات في المجموعة الأولي أحتفظ بها كمجموعة كنترول بدون أي معاملة. بينما الآمهات في المجموعة الثانية والثالثة تم معاملتها بـ 1% فيتامين هـ+ السلينيوم و1% فيتامين ج، علي التوالي، أضيفت لهم في ماء الشرب لمدة 15-18 يوم كفترة معاملة قبل التلقيح. في نهاية فترة المعاملة تم تلقيح كل الآمهات في كل المجاميع طبيعياً بواسطة الذكور الغير معاملة. بعد 72 ساعة من التلقيح تم ذبح ثلاثة أمهات من كل مجموعة لجمع الأجنة بطريقة ألـ flushing, وتم تقدير معدل الاخصاب فى الامهات المتبقية فى كل مجموعة.فى نهاية التجربة تم تقدير عدد الخلفة وحجم الخلفة عند الميلاد وعند الفطام (فى عدد 7 أمهات لكل مجموعة تجريبية) الأجنة التي تم تجميعها من كل مجموعة تم غسلها وتقييمها وتجميدها. بعد اسالة الأجنة المجمده معملياً تم تقييمها لتقدير حيويتها ومدي قدرتها علي الوصول إلي مرحلة blastocyst. أظهرت النتائج أن عدد الخلفة وحجم الخلفة عند الميلاد والفطام كان عالى المعنوية عند مستوى(0.05) فى المجموعة الثالثة (80% ,9.29 و8.86) مقارنة بالمجموعة الثانية (70%, 7.86 و7.57) والمجموعة الاولى (70% , 7.0 و 6.57) على التوالى ,وكان متوسط وزن الخلفة عند الميلاد والفطام أعلى فى كلا من المجموعة الثالثة والثانية مقارنة بالمجموعة الاولى . كما أن معدل استرداد الأجنة بعد الذبح لم تتأثر معنويا بالمعاملات وكان 89.3 و87.9 و 83.9 للمجموعه الأولي والثانية والثالثة، علي التوالي. كما أظهرت أجنة المجموعة الثالثة (فيتامين ج)ارتفاعا معنوياًعند مستوي معنوية(0.05) لحيوية الأجنة (92.3%) بعد الجمع و أعلى معدل للأجنة الطبيعيه بعد عملية التجميد (95.8%) وكذلك أعلى معدل(73.9%) للأجنه التى وصلت الى مرحلة blastocyst بعد زراعتها معملياً لمدة 72 ساعة عند مقارنتها بالمجموعة الأولي (84.0 و 90.5 و 52.6% علي التوالي). بينما كانت قيم المجموعة الثانية (فيتامين هـ+ السلينيوم) هى 86.3 و 80.0 و 55.0، علي التوالي ولم تختلف معنويا عن المجموعة الأولي والمجموعة الثالثة.

توصى نتائج الدراسة الحالية أن إضافة فيتامين ج إلي ماء الشرب لمدة 15 يوم قبل التلقيح تؤدي إلي تحسن فى حجم ووزن الخلفة فى أمهات الارانب و الحصول علي أجنة عالية الجودة وذات قدرة عالية علي التجميد والتطور الى مراحل جنينيه بعد عملية التجميد .