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Impact of Antioxidant Supplementation to Maturation Medium on *In Vitro* Maturation of Rabbit Oocytes Collected from Cryopreserved Ovaries

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ABSTRACT

The aim of the present study was to evaluate the effect of adding two types of antioxidants, namely vitamin E (VE) or glutathione reduced (GSH) on *in vitro* maturation of New Zealand white (NZW) rabbit oocytes recovered from ovaries exposed to vitrification solution or vitrification as compared to fresh ovaries. A total of 36 non-parous NZW rabbit does, ranging 4.5-5 months of age and 2.25-2.55 kg LBW, were used in this study. Oocytes were recovered from ovaries exposed to vitrification solution, vitrified ovaries and fresh ovaries. Oocytes were *in vitro* matured in TCM-199 medium supplemented with 0, 200 µM VE or 200 µM GSH in CO_2 incubator (5% CO_2) at 37.5°C and high humidity for 18 h. Results show that maturation rate in term of percentage of oocytes reached MII stage was the highest ($P<0.05$) for oocytes recovered from fresh ovaries, moderate for those recovered from exposed ovaries and the lowest for oocytes recovered from vitrified ovaries (60.19, 37.16 and 29.05%, respectively). This trend was associated with an opposite trend in percentage of degenerated oocytes (7.88, 13.40 and 18.09%, respectively). Regardless cryopreservation method of rabbit ovary, the effect of both types of antioxidant (VE and GSH) supplementation on the proportion of maturation rate of rabbit oocytes is revealed that he percentage of oocytes reached MII stage was higher ($P<0.01$) for oocytes matured by antioxidant than by control medium. The percentage of matured oocytes was higher (50.66%) for oocytes matured by GSH than VE (43.96%) *versus* the lowest percentage of control oocytes at MII stage (31.77%). However, oocytes at other stages was significantly ($P<0.05$) decreased for oocytes matured by GSH as compared to control, but those matured by VE did not significantly differ from that in GSH and control oocytes. Analysis of variance revealed that the effect of interaction between oocyte preservation and antioxidant supplementation was not significant on percentage of oocyte at different stages after IVM. Maturation rate of fresh, exposed or vitrified oocytes increased when they were *in vitro* matured in medium supplemented with antioxidant as compared to un-supplemented medium, being higher with GSH than VE. In conclusion, based on the foregoing results, oocytes recovered from ovaries exposed to vitrification solution or vitrified showed lower maturation rate than those recovered from fresh ovaries.

Keywords: Rabbit, ovary, vitrification, in vitro maturation, antioxidant.

INTRODUCTION

The genetic material is relatively quick and provides an important reserve of genetic resources that can be used for wide variety of conservation and research interests (McClintock *et al.,* 2007). In the past few years, cryopreservation of ovarian tissue has become an established procedure proposed in many centers around the world in order to store a large amount of primordial follicles prior to gonadotoxic treatment (Moffa *et al*., 2007).

In animal experiments, grafting has commonly been used to examine the capacity of immature oocytes to mature sufficiently to be fertilized after cryopreservation. Some studies have shown follicle survival, growth, and birth of pups from grafted mouse ovaries cryopreserved by slow freezing (Sztein *et al*., 1998). Cryopreservation of the ovary has been studied in many different species, but production of live offspring from treated ovaries has been reported only from sheep and mice (Gunasena *et al.,* 1997). Ovarian tissue cryopreservation (OTC) has potential advantages over mature oocyte preservation in that large numbers of pre-antral follicles are preserved, because it preserves both steroidogenic and gametogenic functions (Shaw *et al.,* 2000). The OTC preserves the primordial and primary follicles, but not the immature oocytes within the antral follicles that do not survive the procedure. These oocytes could, however, be recovered and subjected to *in vitro* maturation (IVM) (Gosden, 2002). Many authors reported that healthy infants were born following IVM (Edwards, 2007). The OTC is an alternative technique to preserve the fertility of mammalian species. Many attempts have been made to improve cryopreservation conditions using simple, economical and efficient technique, vitrification (Bagchi *et al,* 2008). OTC ahead of any cytotoxic treatment, in comparison (Aubard *et al,* 2001), is a promising option to preserve fertility. Migishima *et al.* (2003) succeeded in obtaining of live birth from vitrified ovaries. Also, in humans, autografted ovarian tissues have been reported to contain viable primordial follicles for more than 3 months (Gook *et al.,* 2001).

Reactive oxygen species (ROS) are produced in cells during the course of ascorbic metabolism (Guerin *et al*., 2001). *In vitro* culture results in higher oxygen concentrations than *in vivo* environments, leading to an increased level of ROS (Luvoni *et al.,* 1996). Polyunsaturated lipids of cellular membranes are very sensitive to peroxidation by reactive oxygen species (ROS). It has been observed that alpha-tocopherol, the most active form of vitamin E, is present in cellular membranes and acts as a protective liposoluble agent against lipoperoxidation by removing peroxyl and alcoxyl radicals, generating the poorly reactive tocopheryl radical (Liebler, 1993).

Oocytes and embryos produce endogenous ROS by various enzymatic actions and metabolic pathways. ROS can damage a wide range of macromolecules, possibly leading to cell death, if not effectively and rapidly removed from cells. Therefore, ROS must be continuously inactivated in order to maintain physiologically tolerable levels that play a role in normal cell functions (Harvey *et al.,* 2002). Both enzymatic and non-enzymatic mechanisms have evolved to protect cells from ROS induced damage during *in vivo* development. These mechanisms include antioxidants such as ascorbate, glutathione and tocopherols, as well as ROS-scavenging enzymes such as superoxide dismutase

(SOD), catalase and peroxidase (Sharma and Buetner, 1993).

When oocytes and embryos are cultured *in vitro*, they are challenged to oxidative stress as the recovery and culture involves exposure to light, elevated oxygen concentrations, and disturbed concentrations of metabolic substrates (Guerin *et al*., 2001). It is important to protect embryos against oxidative stress during *in vitro* culture; one approach is to supplement the medium with antioxidant compounds.

Therefore, the aim of this experiment was to evaluate the effect of adding two antioxidants, vitamin E (α-Tocopherol, 200 µM) and (GSH reduced, 200 µM) to maturation medium on *in vitro* maturation of NZW rabbit oocytes recovered from ovaries exposed to vitrification solution or vitrification as compared as fresh ovaries.

MATERIALS AND METHODS

This study was carried out at the Laboratory of Physiology and Biotechnology, Animal Production Department, Faculty of Agriculture, Mansoura University, in cooperation with the International Livestock Management Training Center (ILMTC), Kafrelsheikh Governorate.

All chemicals used in this study were purchased from Sigma (Sant Luis, MO, USA), unless otherwise indicated.

A total of 36 New Zealand white (NZW) rabbit does was used in this study. All does were non-parous ranging 4.5-5 months of age and 2.25-2.55 kg having live body weight. Immediately after slaughtering of rabbits does, ovaries were removed, washed by saline solution (0.9% NaCl), dried by cleaning paper and transferred to laboratory within 10-15 min.

The basal medium used for vitrification was phosphate buffer saline (PBS) prepared according to Gordon (1994). The basal medium was supplemented with 20% doe rabbit serum (DRS) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). Rabbit ovaries were vitrified by two steps. The first step, vitrification solution 1 (VS1) was 10% (v:v) from ethylene glycol (EG), 10% (v:v) from dimethyl sulphoxide (DMSO) and 0.5 M sucrose in the basal medium for 15 min at room temperature. The second step, vitrification solution 2 (VS2) was 20% EG, 20% DMSO and 0.5 M sucrose in the basal medium for 4 min at 4C°. After that ovaries were placed into 1.5 ml eppendorf tubes and plunged immediately into LN2 (-196°C) or exposed to VS1 and VS2 without plunged into LN2.

After cryopreservation of the ovaries for at least 3 weeks in LN2, vitrified ovaries were warmed by holding the eppendorf tubes for 30 sec. in air and then agitating them in water bath at 20°C for at least 30 sec. To remove of intracellular cryoprotectants from exposed and vitrified ovaries, ovaries were transferred to solution of PBS supplemented with 10% DRS and 0.5 M sucrose, then in the same medium with 0.25 M sucrose, the same medium with 0.125 M sucrose and finally in the same medium without sucrose for 5 min per solution.

After warming the ovaries, oocytes were collected using slicing technique into glass Petri dishes containing 4 ml of PBS, supplemented with 2 mg bovine serum albumin (BSA)/ml, 100 IU sodium penicillin G/ml (Misr Co. for Pharm., Egypt) and 100 µg streptomycin/ml. The pH values of all media were adjusted to 7.2-7.4 and osmolarity of 280-300 mOsmol/kg. The medium was filtered by 0.22-µm millipore filter (Milieux GV, milpore, Cooperation Bedford MOA).

After slicing, oocytes recovered from ovaries were washed three times with harvesting medium (PBS) in Petri dishes. Oocytes were examined under stereomicroscopy and classified according to Ravindranatha *et al.* (2003) based on their compaction, number of cumulus cell layers and homogeneity of ooplasm. Only compact-cumulus oocytes (COCs) were used in this study.

Maturation medium was tissue culture medium (TCM-199 HEPES Modification, With Earl's salts-Glutamine without sodium bicarbonate. TCM-199 medium was resolved in autoclaved dionezed double distilled water and supplemented with 2.2 g/l sodium bicarbonate and (sodium penicillin G, 100 IU/ml and streptomycin, 100 µg/ml) (Misr Co. for Pharm. Egypt), then filtered and kept in refrigerator at 4 °C for one week as stock. On the day of maturation TCM-199 medium stock was supplemented with 10% heat-inactivated DRS, Na Pyruvate, 20 mMol final concentration and Sodium Penicillin G, 100 IU/ml and Streptomycin, 100 µg/ml). Three types of TCM medium including TCM without supplementation, TCM with 200 µM α-tocopherol, and TCM with 200 µM GSH were used for IVM.

About 200 µl from prepared medium was placed into sterile Petri dish (30 x 60 mm) and covered by sterile mineral oil. Dishes used in maturation were previously incubated in CO_2 incubator (5% CO_2) at 37.5°C and high humidity for one hour at least for equilibration. Oocytes were washed three times in each of PBS plus 3% Bovine Serum Albumin (BSA) and two time in prepared maturation medium (TCM-199) to remove substances which prevent maturation. Thereafter, oocytes were placed in the medium in Petri dish prepared previously and incubated for 18 h at 37.5°C, 5% CO_2 and high humidity.

After 18 h as a maturation period, oocytes were washed using PBS containing 1 mg /ml hyaluranidase to remove the cumulus cells, and then washed two times in PBS supplemented with 3% BSA and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) overnight. Thereafter, oocytes were stained with 1 % orcein in 45% acetic acid and examined for maturation under phase-contrast microscopy as the following: Matured oocytes: Chromosome at metaphase II (MII) and polar body exhausted in previtilline space, oocytes with germinal vesicle (GV): Chromosomal in disk in cytoplasmic with intact membrane of nuclei, oocytes with germinal vesicle breakdown (GVBD): Chromosomal in disk in cytoplasmic but intact membrane of nuclei is

breakdown, oocytes at metaphase 1 M1 and metaphase MII and degenerated oocytes: Oocytes were vaculated or cytoplasmic shrinked or chromatin condensed (Shamiah, 2004).

The experiment was replicated 5 times for both cryopreservation ovarian and antioxidant supplementation. Data were statistically analyzed as a factorial design using analysis of variance (ANOVA) after arcsine transformation. Dunacn's Multiple Range Test was followed for test the significant differences among treatments (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of cryopreservation method:

Results presented in Table (1) revealed that the maturation rate in term of percentage of oocytes reached M II-stage was significantly (P<0.05) the highest for oocytes recovered from fresh ovaries, moderate for those recovered from exposed ovaries and the lowest for oocytes recovered from vitrified ovaries (60.19, 37.16 and 29.05%, respectively). It is of interest to note that there is a wide variation in percentage of oocytes at MII stage when oocytes were recovered from fresh as compared to exposed or vitrified ovaries. This trend was associated with an opposite trend in percentage of degenerated oocytes (7.88, 13.40 and 18.09%, respectively).

Despite its infancy to preserve fertility and lack of studies, ovarian tissue cryopreservation has several potential advantages, such as the presence of many primordial follicles with oocyte arrest in diplotene of prophase of the first meiotic division, primordial follicles being theoretically less cryosensitive than mature oocytes, and preservation of the endocrinal function of the ovary.

Table (1): Maturation rate of oocytes recovered from fresh, exposed and vitrified rabbit ovary

Ovary	Total oocytes	CV		GVB		MI		MII		DG	
		n	%	n	%	N	%	n	%	n	%
Fresh	297	23	7.87^{b}	36	12.42^{b}	36	11.64^{b}	178	60.19^{a}	23	7.88^{c}
Exposed	290	27	9.22^{b}	52	18.31^{a}	63	21.91^{a}	110	37.16^{b}	38	13.40^{b}
Vitrified	292	45	15.93^{a}	48	16.57^{b}	61	20.37^{a}	84	29.05^{c}	54	18.09^{a}
±SEM	879	-	1.51	-	1.82	-	1.32	-	1.8	-	1.2

a, b and c: Means denoted within the same column with different superscripts are significantly different at P<0.05.

In accordance with the results obtained in rabbits, Özdaş *et al.* (2013) found that the percentage of ovine oocytes reached to MII stage ranged from 6.48 to 7.25%, while the percentage of oocytes reached to MI stage ranged between 30.65 and 33.33% for *in vitro* matured oocytes obtained from frozen-thawed cortex of sheep ovary by slow rate frozen methods. In comparable with the present results in rabbits, Wang *et al.* (2009) reported 77% matured *in vitro* oocytes with or without cumulus cells recovered from vitrified ovarian grafts, 84% matured *in vitro* oocytes recovered from slow-cooled grafts *versus* 83% *in vitro* matured fresh oocytes. No significant difference was found in the incidence of oocyte maturation between control and different cryopreservation treatments. However, Wang *et al.* (2011) found in 62.2% of *in vitro* matured oocytes, when they were recovered from the vitrified-warmed murine ovarian tissues, being significantly lower than the control group, for which the MII formation rate was 86.4%. The vitrification group yielded a significantly higher proportion of oocytes (29.1%) in GVB and at MI stage than that from controls (8.6%), but GV rates were not different between groups (vitrification: 8.7%; control: 4.9%). However, Hasegawa *et al.* (2004) showed that the maturation rate of oocytes at MII and the fertilization rate were not significantly reduced by cryopreservation.

The observed decline in IVM of cryopreserved ovarian tissues may be due to ultrastructural damages to the oocytes deleterious effects on chromosome and other cytoplasmic structure during cryopreservation seen in mouse (Van der Elst *et al.,* 1992) and human (Park *et al.,* 1997) oocytes, which may provide an rational explanation for the lower development rates of verified oocytes. Moreover, some authors reported that exposure to cryoprotectants or lower temperature may cause damage in microtublar spindle formation of MII as observed in mouse (Pickering *et al*., 1990) and human (Van der Elst *et al.,* 1988) oocytes.

Effect of antioxidant type in maturation medium:

Regardless cryopreservation method of rabbit ovary, the effect of both types of antioxidant (VE and GSH) supplementation on the proportion of maturation rate of rabbit oocytes is presented in Table (2). The percentage of oocytes reached MII stage was significantly (P<0.01) higher for oocytes matured by antioxidant than by control medium. In comparing both types of antioxidants, the percentage of matured oocytes was significantly (50.66%) higher for oocytes matured by GSH than VE (43.96%) versus the lowest percentage of control oocytes at MII stage (31.77%). However, oocytes at other stages was significantly (P<0.05) decreased for oocytes matured by GSH as compared to control, but those matured by VE did not differ significantly from that in GSH and control oocytes.

De Matos *et al.* (2003) and Tatemoto *et al.* (2000) concluded that the addition of antioxidants compounds to culture media could have different effects depending on the concentration used, the species and types of oocytes and medium. The recorded improvement in IVM of oocytes in medium supplemented with VE or GSH was explained by Tao et al. (2004), who showed that α-tocopherol promotes the meiotic maturation of denuded oocytes, especially from MI to MII; however, this effect was not observed in cumulus enclosed oocytes. Also, many authors reported that GSH has many functions, such as, protecting the cell from oxidative damage (Downs and Verhoeven, 2003; Urdaneta et al., 2004), formation of the male pronucleus (Perreault *et al.,* 1988), transporting amino

acids, synthesis of DNA and protein and reduction of disulfides (Funahashi, 2005). Glutathione has been shown to play an important role in oocyte maturation. The process of oocytes cytoplasmic maturation involves numerous molecular events, including synthesis of biochemical compounds, protein phosphrylation and activation of particular metabolic pathways (Eppig, 1996). Supplementing TCM maturation medium with GSH as antioxidant improved the expansion rate of buffalo oocytes either of good quality or of poor quality. The best improvement in this concept occurred when the maturation medium was supplemented with GSH at a level of 200 µM, being higher in poor (14.7%) than in good quality (9.5%) oocytes (Abdel Rahman *et al.,* 2014).

On the other hand, Dalvit *et al.* (2005) showed that addition of alpha-tocopherol and/or ascorbic acid to the maturation medium failed to modify meiotic maturation percentage regarding the control. No significant differences were observed among the different supplements. COCs incorporated additional amounts of alpha-tocopherol during bovine oocyte IVM.

Table (2): Maturation rate of oocytes obtained from fresh, exposed and vitrified rabbit ovary as affected by antioxidants

TCM supplementation	Total oocytes	CV		GVB		MI		MII		DG	
		n	%	n	%	n	%	n	%	n	%
Control	283	39	13.88^{a}	53	19.07^{a}	55	19.59	90	31.77^{c}	46	15.71^{a}
Vitamin E	298	31	10.36^{ab}	44	15.03^{ab}	55	17.92	130	43.96^{b}	38	12.73^{ab}
GSH	298	25	8.77^{b}	39	13.21^{b}	50	16.43	153	50.66^{a}	31	10.93^{b}
±SEM	879	-	1.51	-	1.82	-	1.32	-	1.8	-	1.2

a, b and c: Means denoted within the same column with different superscripts are significantly different at P<0.05.

Effect of interaction:

Data in Table (3) revealed that the effect of interaction between oocyte preservation and antioxidant supplementation was not significant on percentage of oocyte at different stages after IVM. Although maturation rate (oocytes at MII stage) was higher for fresh than both exposed and vitrified ovaries, and higher for exposed than vitrified oocytes, the observed insignificant effect the interaction on percentage of oocytes at MII was reflected on increasing maturation rate of fresh, exposed or vitrified oocytes when they were *in vitro* matured in medium supplemented with antioxidant as compared to un-supplemented medium, being higher with GSH than VE (Fig. 1).

Table (3): Maturation rate of oocytes recovered from fresh, exposed and vitrified rabbit ovary as affected by antioxidant supplementation.

Oocytes	Maturation medium	Total oocytes	CV		GVB		MI		MII		DG	
			n	%	n	%	n	%	N	%	n	%
Fresh	Control	98	11	11.48	16	16.67	10	9.85	53	54.04	8	7.96
	Vit. E	92	7	7.43	11	12.21	9	9.65	56	60.68	9	10.03
	GSH	107	5	4.68	9	8.39	17	15.43	70	65.85	6	5.64
Exposed	Control	82	9	10.37	18	22.22	21	26.23	19	23.29	15	17.88
	Vit. E	109	10	9.12	19	17.54	25	23.01	42	38.43	13	11.91
	GSH	99	8	8.16	15	15.18	17	16.52	49	49.75	10	10.40
Vitrified	Control	103	19	19.79	19	18.31	24	22.66	18	17.97	23	21.27
	Vit. E	97	14	14.53	14	15.33	21	21.10	32	32.79	16	16.25
	GSH	92	12	13.47	15	16.07	16	17.34	34	36.38	15	16.74
±SEM		879	-	2.62	-	3.15	-	2.29	-	3.12	-	2.08

In conclusion, based on the foregoing results, oocytes recovered from ovaries exposed to vitrification solution or vitrified showed lower maturation rate than those recovered from fresh ovaries. Although oocytes are adversely affected by low temperatures, freezing, and cryoprotective substances, they do not completely lose their capacity to develop oocytes from the germinal vesicle to MII stage. Supplementation of maturation medium with antioxidant, in particular GSH slightly improved the maturation rate oocytes recovered from cryopreserved ovaries, but still at lower level than that occur for fresh oocytes. The current study can suggest further studies on an appropriate freezing and thawing processes of the ovaries to avoid factors which lead to accelerate apoptosis.

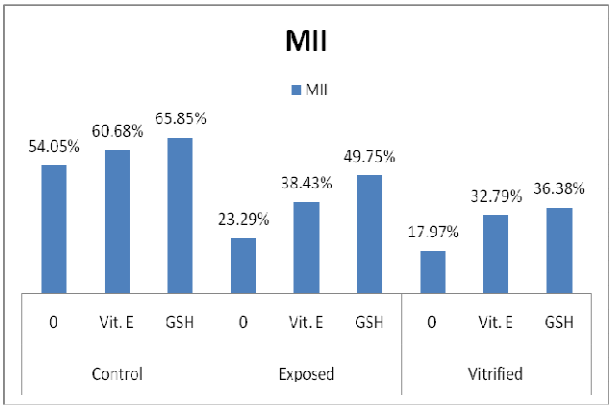

Fig. (1): Percentage of oocytes at MII stage as affected by the interaction between ovarian cryopreservation and antioxidant supplementation.

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تأثير إضافة مضادات الأكسده الى بيئة الإنضاج المعملى لبويضات الأرانب المجمعة من المبايض المجمدة.

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كان الهدف من هذه الدراسه هو تقييم تأثير إضافة نوعين من مضادات الاكسده وهم فيتامين هـ (VE) والجلوتاثيون (Gsh) على الإنضاج المعملى لبويضات الأرانب النيوزيلندى الأبيض (NZW) التى تم استردادها من المبايض المعرضه لمحلول التجميد والمجمده مقارنة بالمبايض الطازجة. تم استخدام ٣٦ أنثي أرنب نيوزيلندى أبيض لم تلد تتراوح اعمارها مابين ٤.٥-٥ شهور ووزنها ٢.٢٥-٢.٥٥ كجم فى هذه الدراسه. البويضات تم تجميعها من المبايض المعرضه لمحلول التجميد والمبايض المجمده والمبايض الطازجه. البويضات تم انضاجها معملى فى بيئه Tcm-199 مضاف اليها ٢٠٠ ميكرومول من فيتامين هـ ، ٢٠٠ ميكرومول من الجلوتاثيون فى حضان CO_2 (٥% ثانى اكسيد الكربون) ودرجه حراره ٣٧.٥ °م ورطوبه عاليه لمده ١٨ ساعه. تشير النتائج الى أن النسبة المئوية لمعدل الإنضاج للبويضات التى وصلت لمرحلة الطور الاستوائى الثانى كانت عاليه ($P<0.05$) للبويضات التى تم تجمعها من المبايض الطازجه ومتوسطه للبويضات التى تم تجمعها من المبايض المعرضه لمحلول التجميد ومنخفضه للبويضات التى تم تجمعها من المبايض المجمده (٦٠.١٩, ٣٧.١٦, ٢٩.٥ % على التوالى). وكان ذالك على العكس فى نسبة البويضات المدمرة (٧.٨٨, ١٣.٤٠, ١٨.٠٩% على التوالى) بغض النظر عن طريقة الحفظ بالتبريد لبويضات الأرانب. بالنسبة لتأثير كلاً من نوعي مضادات اللأكسده المضافه على نسبة معدل انضاج بويضات الأرانب فقد كانت نسبة البويضات التى وصلت لمرحلة الطور الأستوائى الثانى ($P<0.01$) للبويضات الناضجه باضافة مضادات الأكسده عاليه عن البيئه الكنترول. النسبه المئويه للبويضات الناضجه فى الجلوتاثيون كانت عاليه (٥٠.٦٦%) عن فيتامين هـ (٤٣.٩٦%) مقارنة بالنسبه الأقل للبويضات الكنترول (٣١.٧٧%) فى مرحلى الطور الأستوائى الثانى. مع ذالك كانت البويضات فى المراحل الخرى معنويه عند ($P<0.05$) انخفضت للبويضات الناضجه من قبل الجلوتاثيون بالمقارنه بالكنترول. ولكن الناضجه من قبل فيتامين هـ لم تظهر اى اختلاف معنوى لكلاً من بويضات الحلوتاثيون والكنترول وكشف تحليل التباين ان تأثير التفاعل مابين حفظ البويضات واضافة مضادات الاكسده لم يظهر اختلاقا معنوياً فى النسب المئويه فى المراحل المختلفه للبويضات بعد الأنضاج المعملى.معدل الآنضاج للبويضات الطازجه, المعرضه لمحلول التجميد, والمجمده زاد عندما تم انضاجها فى بيئه مضاف اليها مضادات اكسده مقارنة بالبيئه الغير مضاف إليها وكانت عاليه مع اضافة الجلوتاثيون عن فيتامين هـ. فى النهايه تشير النتائج إلى أن البويضات المجمعه من المبايض المعرضه لمحلول التجميد أو المجمده اظهرت انخفاض فى معدل الأنضاج عن المجمعه من المبايض الطازجه.