Egyptian J. Anim. Prod. (2017) 54(3):207-214

DEVELOPMENT OF EMBRYOS RECOVERED FROM DOE RABBITS TREATED WITH GREEN TEA EXTRACT AND CULTURED UNDER HIGH THERMAL CONDITIONS *IN VITRO*

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SUMMARY

This study aimed to evaluate whether orally treatment of doe rabbits with green tea extract (GTE) can eliminate the negative effects of hyperthermia on embryonic development in vitro. A total of 24 mature NZW does was randomly allotted into three groups, 8 in each. The 1st group was control, while the 2nd and 3th groups were orally treated with 1 ml distilled water contained 200 and 400 mg GTE/kg LBW, respectively, for one month and naturally mated with proven bucks. Embryos were recovered from slaughtered doe oviducts at blastocyst stage 72 h post-mating by flushing and morphologically evaluated. Only normal embryos at blastocyst stage were in vitro cultured at 38.5 °C for 48 h (normal temperature, NT) or at 41.5°C (hyperthermia, HT) for 6 h then at 38.5 °C for 42 h in 5% CO_2 and 95% humidity to develop to expanded and hatched blastocysts. Results showed that embryo recovery rate was not significantly affected by GTE. Embryos of does treated with both GTE levels were better (P<0.05) than control embryos. The GTE at 400 mg/kg showed the highest normality rates, hatched blastocyst proportion, and the lowest degenerated embryos. Formation rate of expanded and hatched blastocysts was higher (P<0.05) for embryos cultured in vitro at NT than at HT, while proportion of degenerated embryos showed an opposite trend. Effect of interaction between GTE treatment and thermal condition was significant on proportion of expanded, hatched and degenerated embryos (P<0.05), reflecting an increase in hatched embryos and decreasing degenerated embryos proportions by increasing GTE level at NT and HT, but GTE treatment showed the highest hatched and the lowest degenerated embryos proportions with level of 200 mg/kg at NT and with level of 400 mg/kg at HT. In conclusion, the negative effect of heat stress on embryonic development in vitro may be eliminated by GTE treatment of heat stressed does.

Keywords: Rabbit, green tea, embryo, hyperthermia, in vitro co-culture

INTRODUCTION

Reproductive efficiency of doe rabbits as mammals are very sensitive to confusion by high temperature in term of reduced yield and quality of embryos and subsequently affecting their fertility (Ondruska *et al.,* 2011). Under intensive rabbit production system, several attempts have been carried out toward elimination of the negative effect of hot season on reproductive process (Askar and Ismail, 2012). In rabbits, embryos are more sensitive to thermal condition at earlier stage compared with embryos at 6-day old during *in vitro* development (Wolfenson and Blum, 1988) and short-term exposure of pre-implantation embryos to high temperature (41.5° C) *in vitro* reduced development of embryos (Makarevich *et al.*, 2007). Also, in mice, *in vivo* embryo development at an early stage to blastocysts was lower in warm than in cold season (Ozawa *et al.,* 2004).

Evaluation of embryo developmental stage, basing on visual inspection of their morphology, is one of the useful criteria of pre-implantation embryo quality (kulíková *et al.,* 2012). Pre-implantation embryos are sensitive to various stressors such as oxidative, chemicals, and hypothermic conditions. These stressors may cause developmental arrest *in vitro* (Olexikova *et al.,* 2013). Also, these conditions may cause a reduction in embryo viability rate post-transfer (Al-Luhbi and Al-Bashan., 2013) as a result of cytoskeleton disorders, apoptosis (Makarevich *et al.,* 2007), chromatin abnormality and acumulating the droplets of lipid (Qian *et al.,* 2004 and Antonova, 2008). Under hyperthermia, reactive oxygen species (ROS) levels, as an oxidative stress, increase in association with damage of DNA (Alves *et al.,* 2013). Glutathione (GSH) maintains the intracellular redox status of embryos and is associated with their development and quality in rabbits (Wells *et al.,* 1997).

Hyperthermia is due to insufficient production of heat shock protein and GSH from the embryos (Alves *et al.,* 2013), but most important role in the protection of pre-implantation embryo against high temperature is production of heat shock proteins 70 in response to thermal or other stressors (Hansen, 2007). These findings may indicate direct and indirect relationships between hyperthermia and oxidative stress during the development of embryos. Therefore, antioxidant administration to control the intra- or extra-cellular redox status (both *in vivo* and *in vitro)* may be a way to decrease heat stress related oxidative stress to improve quality and developmental competence of embryos (Abdel-Khalek *et al.,* 2016 and Takahashi, 2012).

Recently, green tea is a very strong antioxidant and potent scavenger of free radicals and ROS in biological system. It also has properties for

improving reproductive performance of rabbit does (El-Ratel *et al.,* 2017). In mammals, influence of hyperthermic conditions on *in vitro* embryonic development depends on animal species, duration of embryo exposure, and embryonic stage (Makarevich *et al.,* 2007). The protocols for *in vivo* and *in vitro* production of embryos resisted to heat stress are needed to better understanding the physiological responses of cells, gametes and embryos submitted to hyperthermic condition (Shehab El-Deen *et al.,* 2010 and Payton *et al.,* 2011).

Therefore, this study aimed to evaluate the resistance of pre-implantation embryos during *in vitro* development to hyperthermic condition (41.5ºC) as affected by treatment of doe rabbits with green tea extract (200 or 400 mg/kg LBW).

MATERIALS AND METHODS

Animals:

In this study, total of 24 New Zealand White (NZW) mature doe rabbits as embryo donors were randomly allotted into three groups (8 in each). The first was a control group without treatment, while the second and third groups were orally administrated with 1 ml distilled water contained 200 and 400 mg green tea extract (GTE, Arab Company for Pharmaceuticals and Medicinal Plants, Mepaco-Medifood, Egypt) per kg live body weight, respectively, for one month prior to mating as a treatment period according to El-Ratel *et al.* (2017). Total of ten or twenty tablets (each tablet contained 300 mg green tea as a dry extract, 30% polyphenols) were dissolved in 15 ml distilled water for obtaining oral dose of 200 or 400 mg/ml of GTE, respectively.

At the end of treatment period, does were naturally mated with proven bucks (n=5) of the same breed. Then does were transported from farm 72 h post-mating to Physiology and Biotechnology Laboratory, Animal Production Department, Faculty of Agriculture, Mansoura University.

Experimental procedures:

Embryos were recovered from slaughtered does 72 h post-mating by flushing medium (phosphate buffer saline, PBS) containing 10% fetal calf serum (FCS) and 50µg gentamycin/ml. Number of embryos at stage was recorded (n=362) and morphologically evaluated for normal (n= 346, Plate 1) and abnormal (n=16, Plate 2) embryos under stereoscopic microscope, morphology of mucin coat, zona pellucidae, blastomeres, and refractive cytoplasm after washing for 3 times in PBS.

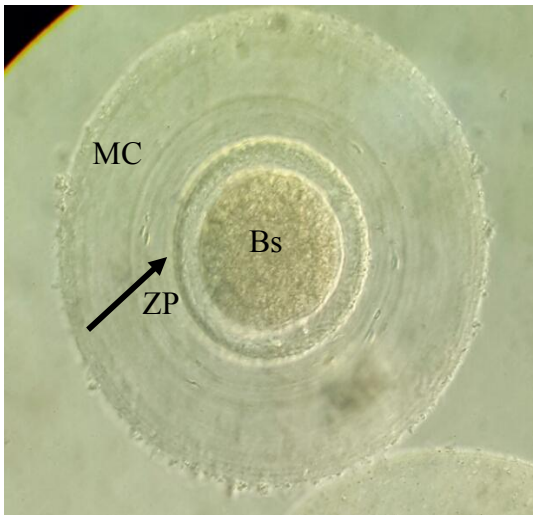

Plate 1. Normal embryo with intact mucin coat (MC), zona pellucidae (ZP)and blastomeres (Bs)

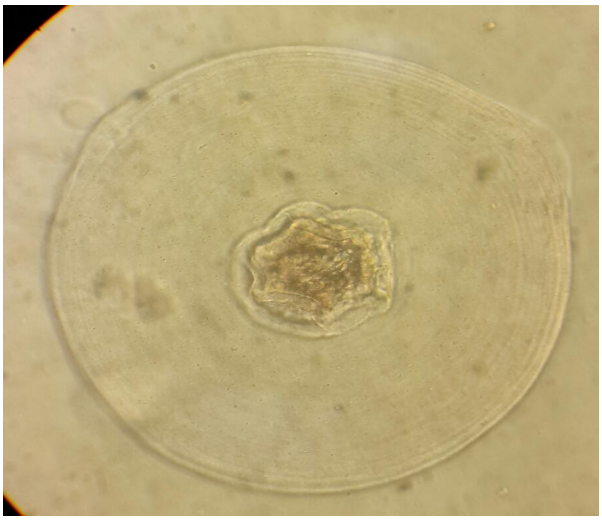

Plate 2. Abnormal embryo

Embryo culture in vitro:

Only normal embryos at blastocyst stage (n=346) were cultured *in vitro* under two thermal conditions (normal at 38.5 °C and hyperthermic at 41.5°C) in CO_2 incubator (95% humidity and 5% CO_2 in air) according to Olexiková *et al.* (2007). Embryos were cultured at normal temperature for 48 h (NT, n=174) or at hyperthermic condition (41.5°C) for 6 h and at normal temperature for 42 h (HT, n= 172). All embryos were *in vitro* cultured in 500 µl drops of tissue culture medium (TCM 199, Sigma) supplemented with 10% FCS and 50 µg of Gentamicin sulphate/ml under mineral oil to develop into embryos at expanded (Plate 3) and hatched blastocyst (Plate 4) stages.

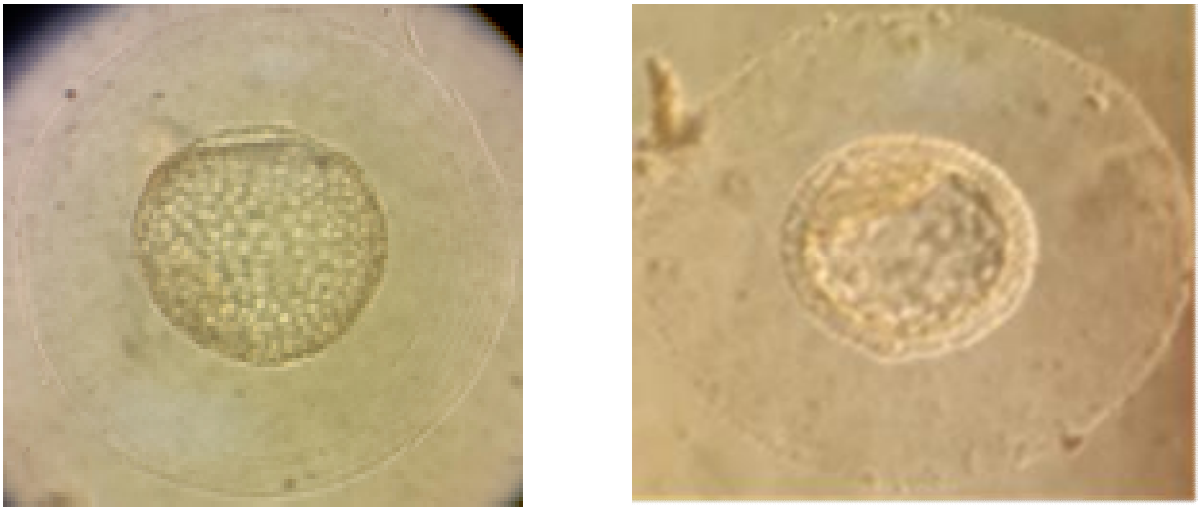

Plate 3. Embryos at expanded

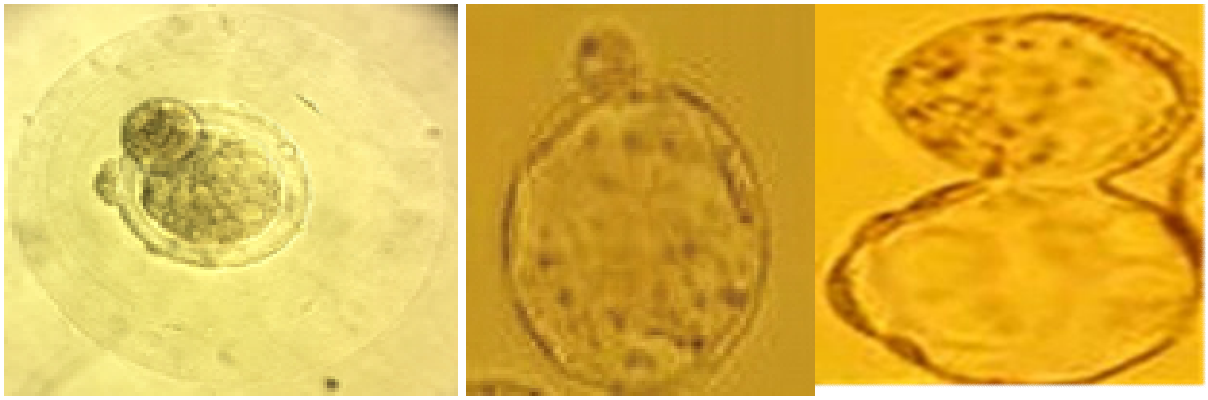

Plate 4. Embryos at hatched blastocyst stages

Statistical analysis:

The obtained data after arcsine transformation of original values expressed as precentages, including embryo recovery rate, normality and abnormality were statistically analyzed by one way design (NOVA), while the effect of GTE level and thermal condition on embryo development was performed using factorial design (3 GTE levels x 2 temperature condition) using a software package (SAS, 2004). The significant differences among means were tested using Duncan's Multiple Range Test (1955).

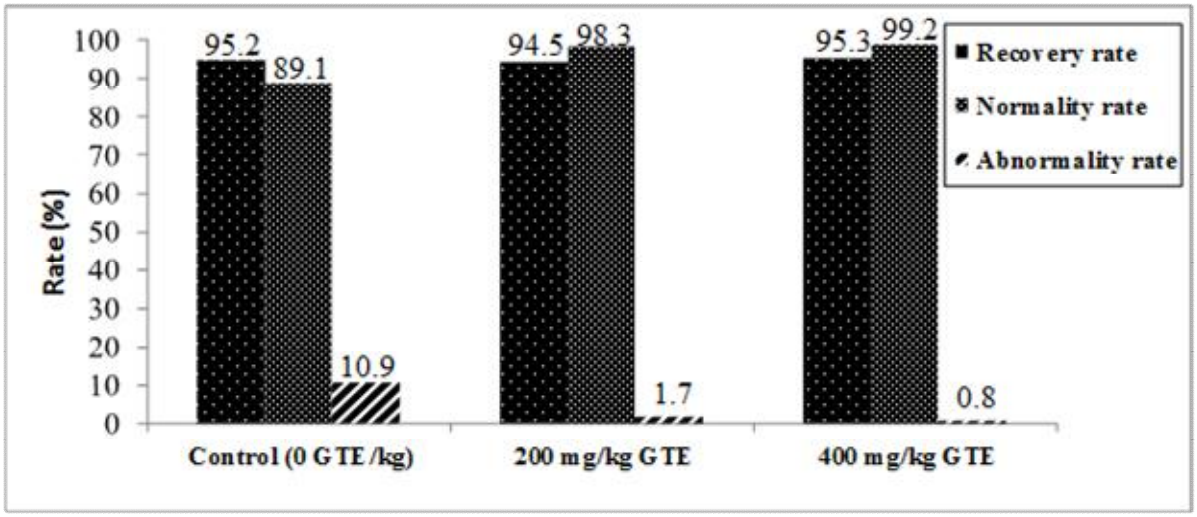

Fig 1. Rate of recovery, normality and abnormality of rabbit embryos as affected by green tea extract (GTE) orally treated doe rabbits.

RESULTS AND DISCUSSION

Recovery rate and embryo quality of doe rabbits treated with GTE:

Although recovery rate of embryos was not significantly affected by GTE treatment, its quality significantly (P<0.05) differed, being better for embryos recovered from does treated with both levels of GTE than those recovered from control does. Generally, does treated with GTE at a level of 400 mg/kg showed the highest normality rates (Fig. 1). These results indicated beneficial effect of GTE on quality of recovered embryos as compared to control.

Several authors indicated beneficial effects of *in vivo* antioxidant treatment of females on embryo quality. In rabbits, Abdel-Khalek *et al.* (2016) showed that daily oral administration of doe rabbits with Coenzyme Q10 or L-Carnitine as antioxidants can improve quality of recovered embryos. In human, dietary supplementation with Coenzyme Q10 may improve embryo quality (Bentov *et al.,* 2010 and Scott, 2013). In mouse, *in vivo* administration of the antioxidant epigallocatechin gallate (EGCG) was found to improve embryo quality (Roth *et al.,* 2008). In cattle, Amaral (2003) found that animals injected with vitamin A on the 1st day of the superovulation can increase number of viable embryos as compared to untreated animals.

In vivo embryonic development may be influenced by the ROS produced in the female genital system (Bedaiwy *et al.,* 2002). According to Du Plessis *et al.* (2008), *in vivo* embryos rely on mitochondrial oxidative phosphorylation for energy, a process which is subsequently accompanied by ROS generation (Du Plessis *et al.,* 2008). The antioxidant capacity of the embryos against the harmful assault of oxidation, because the fast developing embryo produces energy via ATP generation through mitochondrial oxidative phosphorylation and glycolysis (Agarwal *et al.,* 2014). As it develops, the embryo is capable of producing ROS through several pathways, namely oxidative phosphorylation, NADPH and xanthine oxidase systems (Guerin *et al*., 2001).

This finding concurs with the results observed in the current study may be justified the establishment of supplementation antioxidants (GTE) promoted equilibrium between oxidative agents and the antioxidative system, which may have improved embryo quality.

Developmental competence of embryos recovered from does treated with GTE and cultured at normal and hyperthermic condition:

Effect of GTE treatment:

Regardless co-culture temperature, treatment of does with GTE at a level of 400 mg/kg LBW yielded significantly (P<0.05) the highest frequency distribution of hatched blastocysts, and the lowest distribution of degenerated embryos, while distribution of expanded blastocysts was not affected significantly by GTE treatment (Table 1).

Table 1. Developmental competence of embryos recovered from doe rabbits treated with green tea extract (GTE)

	Total number of embryos	Developmental stage				Degenerated embryos	
		Expanded blastocyst		Hatched blastocyst			
		N	%	n	%	n	%
Control	106	52	49.06	30.00	28.30[b]	24	22.64[a]
GTE (200 mg/kg)	118	56	47.46	42	35.59[ab]	20	16.95[b]
GTE (400 mg/kg)	122	62	50.82	44	36.07[a]	16	13.11[b]
±SEM			1.77		2.51		1.79
Significance			NS		*		**

[a and b]: **Means with different letters in the same column differ significantly at P<0.05.**
NS = not significant. * Significant at P<0.05. ** Significant at P<0.01.

In accordance with the present results, Roth *et al.* (2008) reported that *in vivo* administration of EGCG in GT as antioxidant improved developmental competence of mouse embryos. Also, Cerri *et al.* (2009) found that increased selenium of the re-productive tract in dairy cows may improve competence of embryo development, pregnancy and fetal development. This was indicated in the current study in doe rabbits treated with GTE. Furthermore, impact of different types of antioxidant in *in vitro* culture medium, on developmental competence of embryos was proved by several workers. In this line, addition of melatonin at a level of 10^{-6} M for rabbit embryos (Mehaisen and Saeed, 2015), GT polyphenols for bovine embryos (Wang *et al*., 2013), and L-Carnitine (Abdelrazik *et al.,* 2009) and vitamin C at a level of 50 µmol/ L for mouse embryos (Wang *et al.,* 2002) markedly improved embryo development rate *in vitro*.

Improving the developmental competence of embryos produced from doe rabbits treated with GTE may be attributed to that exogenous antioxidants activities of phenolic compounds in GT are due to their structure and particularly ability to donate a hydrogen ion to the peroxy radical generated as a re-sult of lipid peroxidation (Bisby *et al*. 2008). Also, Agarwal *et al.* (2003) mentioned that *in vitro* blastocyst formation is suboptimal, and supplementation with antioxidants may improve blastocyst development. According to the present results and the previous findings, antioxidants such as GTE, are likely to play a significant role in

preventing subsequent loss or damage to the embryo (Abdelrazik *et al.,* 2009). Finally, Barakat *et al.* (2014) showed that GTE acts as a direct scavenger of toxic oxygen derivatives and has the ability to reduce the formation of ROS and promotes DNA synthesis of embryos and intracellular.

Effect of co-culture temperature:

Regardless GTE treatment, the formation rate of expanded and hatched blastocysts was significantly (P<0.05) higher for embryos cultured *in vitro* in normal temperature (38.5 °C) than at hyperthermic condition (41.5 °C). However, frequency distribution of degenerated embryos showed an opposite trend (Table 2).

These results indicated hyperthermia conditions (41.5 °C) during co-culture did not stop progress of embryos to hatched blastocyst stage, but significantly decreased the developmental competence of embryos by about 31.07% and increased embryo degeneration by about 203.4%. Although, Ealy *et al.* (1995) reported that the development of 2-16 cell stage or more cell stage of bovine embryos was damaged and not cleavage following heat shock at 41 °C for 3 h, exposing the rabbit embryos in the present study to hyperthermia conditions at 41.5 °C allowed the embryos to develop up to hatched blastocysts, but at lower proportion than at normal temperature. However, increasing hyperthermia condition to 42.5 °C decreased development of rabbit embryos to pre-implantation stages, and all embryos were arrested only at early blastocyst stage (Olexiková *et al .,* 2007). Also, hyperthermia reduced the proportion of mouse pre-implantation embryos at different stages and changed the developmental capacity (Zhu *et al.,* 2004). In accordance with the obtained results in rabbits, it was found that hyperthermia conditions at 41.5 °C (Silva *et al.,* 2013) or 41 °C (Sakatani *et al.,* 2004) decreased developmental rates of bovine embryos.

In similar trend with the observed increase in proportion of degenerated embryos as affected by hyperthermia, several authors showed that the chronic elevation of temperature above the normal culture temperature (38.5-40 ºC) *in vitro* resulted in a higher incidence of embryo death that was evident shortly after embryo hatching (Makarevich *et al.,* 2007; Ryan *et al.,* 1992).

Table 2. Developmental competence of rabbit embryos as affected by *in vitro* co-culture temperature

Temperature	Total number of embryos	Developmental stage				Degenerated embryos	
		Expanded blastocyst		Hatching blastocyst			
		N	%	n	%	n	%
Normal	174	90.00	51.72[a]	69.00	39.65[a]	15.00	8.62[b]
Hyperthermia	172	80.00	46.51[b]	47.00	27.33[b]	45.00	26.16[a]
±SEM		1.44		2.05		1.47	
Significance		**		**		***	

[a and b]: **Means with different letters in the same column differ significantly at P<0.05.**

**** Significant at P<0.01. *** Significant at P<0.001.**

Effect of interaction:

Effect of interaction between GTE treatment and thermal condition was significant (P<0.05) on proportion of expanded, hatched and degenerated embryos. These effects reflected in increasing proportion of hatched embryos and decreasing degenerated embryos by increasing GTE level at both normal and hyperthermia conditions, but GTE treatment showed the highest hatched and the lowest degenerated embryos proportion with level of 200 mg/kg at normal condition and with level of 400 mg/kg at hyperthermia condition. In addition, proportion of expanded embryos decreased at the normal condition and increased at hyperthermia condition by increasing GTE level (Fig. 2).

Such results indicated more beneficial effects of GTE at a level of 200 mg/kg at the normal condition and the significant impact of increasing this level at hyperthermia condition up to 400 mg/kg.

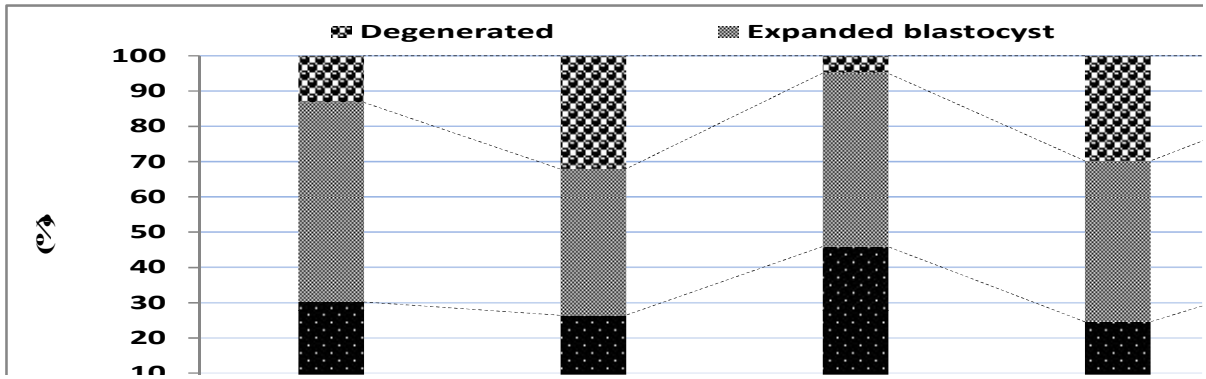

Fig 2. Frequency distribution of embryos at hatched, expanded and degenerated stages cultured at normal (NT) and hyperthermic condition (HT) and recovered from doe rabbits treated with green tea extract (GTE).

Thermo-tolerance of embryos is generally affected by many factors, such as heat shock duration, temperature degree, embryonic developmental stage and animal species (Makarevich *et al.,* 2007). In bovine, embryo developmental capacity was decreased under hyperthermia condition of 43 ºC (Ju *et al.,* 1999), and the development to

The sensitivity of pre-implantation embryos to different stress conditions (oxidation, toxicity and hypothermia) causes various types of embryo damage, in terms of mitochondrial and lysosomal changes as well as accumulation of lipid droplets (Olexikova *et al.,* 2013). During heat shock, free radical production had been suspected leading to promote oxidation events in the cell (Ara Ahmed *et al*., 2016). *In vivo* or *in vitro* heat stress caused a decrease in GSH of embryos, and elevated ROS levels associated with DNA damage in mice (Ozawa *et al*., 2002).

Addition of exogenous antioxidants during the development of bovine embryos is important to provide hyper-thermo resistance to overcome the negative effects (Rynkowska, 2011), which increase the chance of embryos, even those of fair quality, to develop to blastocysts. Supplementations of antioxidants at optimal levels have been demonstrated to have a positive effect on embryo development (Ara Ahmed *et al.,* 2016). In this respect, melatonin administration to heat-stressed mice alleviated hyperthermia-induced early embryonic death (Matsuzuka *et al.,* 2005). Also, vitamin E protects the early embryo from the effects of ambient heat stress (Arechiga *et al.,* 1995). In rats (Ishibashi *et al.,* 1997) and rabbits (Wells *et al.,* 1997), GSH maintains the intracellular redox status of embryos and was associated with their development and quality. The GSH can improve the thermo-tolerance of embryos (Arechiga *et al.,* 1995). Generally, under heat stress condition, *in vitro* antioxidant administration improved bovine embryo development, which was associated with intracellular ROS and GSH synthesis (Sakatani *et al.,* 2008).

During embryonic development, the current study indicated direct and indirect relationships between *in vitro* hyperthermia condition and oxidative stress. Using GTE as antioxidant to control the intracellular or extracellular redox status both *in vivo* and *in vitro* may be a way to reduce heat stress-related oxidative stress. Therefore, the deleterious effects of heat stress, either *in vivo* or *in vitro,* in terms of reduction in embryo viability (Al-Luhbi and Al-Bashan., 2013) and pregnancy rate, and increasing embryonic losses (Block and Hasen., 2007) was previously reported.

CONCLUSION

The negative effect of heat stress on embryonic development *in vitro* may be eliminated by GTE orally treated doe rabbits, in particular at a level of 400 mg/kg. However, treatment of doe rabbits with blastocyst and proliferation reduced under heat stress at an early stage (Sakatani *et al.* (2004). Under hyperthermia condition (43-45.5 ºC), disturbance in the development of pig embryos was proved after short-term exposure (10-60 min), while at 42 ºC the embryos had even a higher cell number and diameter than at control temperature (Kojima *et al.* 1996).
GTE at a level of 200 mg/kg is sufficient at the normal thermal condition.

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تطور الأجنة المستردة من أمهات الأرانب المعاملة بمستخلص الشاى الأخضر والمنزرعة تحت درجات حرارة مرتفعة معملياً

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تهدف هذه الدراسة الى تقيم ما اذا كانت المعاملة بتجريع امهات الارانب بمستخلص الشاى الاخضر يمكن ان يحد من التأثيرالسلبى للحرارة العالية على تطور الأجنة معمليا . استخدم فى هذه الدراسة ٢٤ من امهات الارانب النيوزيلاندى البيضاء الناضجة، قسمت عشوائيا الى ٣ مجموعات (٨ امهات فى كل مجموعة). المجموعة الاولى كنترول بينما جرعت المجموعة الثانية والثالثة بـ٢٠٠ و٤٠٠ مللى جرام/كجم وزن حى بمستخلص الشاى الأخضر على التوالى لمدة شهر وتلقيحها طبيعيا بذكور ناضجة من نفس النوع. تم ذبح الأمهات بعد ٧٢ ساعة من التلقيح وعمل غسيل لقناة المبيض والحصول على اجنة فى مرحلة البلاستوسيست وتقيمها مورفولوجيا. تم زراعة الاجنة الطبيعية فقط معمليا على درجة حرارة ٣٨.٥ درجة مئوية (حرارة طبيعية) لمدة ٤٨ ساعة أو زراعتها على درجة حرارة ٤١.٥ درجة مئوية (حرارة مرتفعة) لمدة ٦ ساعات ثم نقلها الى درجة حرارة ٣٨.٥ درجة مئوية لمدة ٤٢ ساعة فى ٥% ثانى اكسيد الكربون و٩٥% رطوبة لتطور الاجنة من مرحلة البلاستوسيست الى مرحلة البلاستوسيت الممتدة ومرحلة الفقس . وقد اظهرت النتائج :- عدم تأثر معدل استرداد الاجنة معنويا بمستخلص الشاى الأخضر. كانت جودة أجنة الأمهات المعاملة بمستخلص الشاى الأخضر افضل معنويا ($P<0.05$) مقارنة بمجموعة الكنترول. أدت المعاملة بـ٤٠٠ ملجرام/كجم وزن حى بمستخلص الشاى الأخضر إلى إرتفاع فى معدل الأجنة الطبيعية ونسبة الأجنة التى وصلت إلى مرحلة الفقس مع إنخفاض نسبة الأجنة المضمحلة. كان معدل تكوين الأجنة فى مرحلة البلاستوسست المتمددة ومرحلة الفقس أعلى معنويا ($P<0.05$) للأجنة التى تم زراعتها معمليا على درجة حرارة طبيعية مقارنة بالحرارة المرتفعة، بينما سجلت الأجنة المضمحلة عكس هذا الاتجاه. كان هناك تأثيرمعنوى ($P<0.05$) للتداخل ما بين المعاملات بمستخلص الشاى الاخضر ودرجة الحرارة لزراعة الأجنة، مما عكس تأثير مختلف لكل عامل من العوامل السابقة، حيث أدت المعاملة بمستخلص الشاى الأخضر إلى إرتفاع نسبة الأجنة التى وصلت إلى مرحلة الفقس مع إنخفاض نسبة الأجنة المضمحلة عند مستوى ٢٠٠ملجرام/كجم وزن حى مع درجة الحرارة الطبيعية وعند مستوى ٤٠٠ملجرام/كجم وزن حى مع درجة الحرارة المرتفعة. نستخلص من هذه الدراسة ان التأثير السلبى للإجهاد الحرارى على تطور الاجنة معمليا يمكن ان يحد بمعاملة امهات الارانب الواقعة تحت هذا التأثير بمستخلص الشاى الأخضر.