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 3rd 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% CO2 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 (kuliková 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-Luubi and Al-Bashan., 2013) as a result of cytoskeleton disorders, apoptosis (Makarevich et al., 2007), chromatin abnormality and accumulating 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 (41.5°C) 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 50 μg 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 pellucida, blastomeres, and refractive cytoplasm after washing for 3 times in PBS.

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 CO₂ incubator (95% humidity and 5% CO₂ 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 1. Normal embryo with intact mucin coat (MC), zona pellucidae (ZP)and blastomeres (Bs)
Plate 2. Abnormal embryo
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 percentages, 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).

![Graph showing embryo recovery, normality and abnormality rates](image)

**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)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of embryos</th>
<th>Developmental stage</th>
<th>Degenerated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expanded blastocyst</td>
<td>Hatched blastocyst</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>106</td>
<td>52</td>
<td>49.06</td>
</tr>
<tr>
<td>GTE (200 mg/kg)</td>
<td>118</td>
<td>56</td>
<td>47.46</td>
</tr>
<tr>
<td>GTE (400 mg/kg)</td>
<td>122</td>
<td>62</td>
<td>50.82</td>
</tr>
<tr>
<td>±SEM</td>
<td></td>
<td>1.77</td>
<td>2.51</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>NS</td>
<td>*</td>
</tr>
</tbody>
</table>

*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 reproductive 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 peroxo radical generated as a result 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

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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 °C) 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**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Total number of embryos</th>
<th>Developmental stage</th>
<th>Degenerated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Expanded blastocyst</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Normal</td>
<td>174</td>
<td>90.00</td>
<td>51.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>172</td>
<td>80.00</td>
<td>46.51&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>1.44</td>
<td>2.05</td>
</tr>
<tr>
<td>Significance</td>
<td>**</td>
<td>**</td>
<td>**</td>
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</tbody>
</table>

<sup>a, b</sup> Means with different letters in the same column differ significantly at P<0.05.

**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).](image-url)
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 °C (Ju et al., 1999), and the development to blastocyst and proliferation reduced under heat stress at an early stage (Sakatani et al. (2004). Under hyperthermia condition (43-45.5 °C), disturbance in the development of pig embryos was proved after short-term exposure (10-60 min), while at 42 °C 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.

REFERENCES


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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
تطور الأجنة المستمرة من أمواج الأذناء المعلقة مستخلص الشاي الأخضر والمزرعة تحت درجات حرارة مرتفعة معملية

إبراهيم طلعت الرطل
قسم إنتاج الدجاج، كلية الزراعة، جامعة دمياط، دمياط، مصر

هدف هذه الدراسة هو تقييم ما إذا كانت المعلقة بمستخلص الشاي الأخضر يمكن أن تحدث عند التأثير السلبي للحرارة العالية على تطور الأجنة مفعلاً. استخدمت هذه الدراسة 24 أميةً لإجراء أربعة جرعة معملية مختلفة بين ثلاث جماعات (A) و (B) و (C). تم تعبئة الشاي الأخضر على التوالي لمدة شهر وفقاً للجرعة المطلوبة من نفس النوع. تم نقل الأجنة ببطء بعد 24 ساعة من التنقيح وعمل عسل لقناة المبيض والفصل على أجزاء البقول البلاستوسية وقيمها مورفولوجياً. تم زرع الأجنة الطبيعية فقط معملاً على درجة حرارة 30.5 درجة مئوية (حرارة طبيعية) لمدة 48 ساعة أو زراعتها على درجة حرارة 41.5 درجة مئوية (حرارة مرتفعة) لمدة 6 ساعات ثم نقلها إلى رطوبة درجة مئوية درجة 30.5 درجة مئوية درجة 22 درجة مئوية في 5% ثاني أكسيد الكربون و95% رطوبة تطور الأجنة من مرحلة البلاستوسية إلى مرحلة البلاستوسية المتقدمة ومرحلة الفقس. وقد أظهرت النتائج: عدم تأثير معدل الاجنة معملاً بمستخلص الشاي الأخضر. كانت جودة أجنة الأمهات المعلقة بالشاي الأخضر أفضل مع (P<0.05) مقارنةً بمحبة معدة الفطر. أتت الأجنة بـ 400 ملليج/كمج وزن حي مسحوق الشاي الأخضر إلى الإفراز في معدل الأجنة الطبيعية ونسبة الأجنة التي وصلت إلى مرحلة انساب نسبة الأجنة المصححة. كان معدل تكاثر الأجنة في مرحلة البلاستوسية الممتدة والمرحلة القصيرة معبأة على عينك (P<0.05) للاجنة التي تم زراعتها مع (P<0.05) من التفاعل. أما منتجةحرارة ببطء مثل هذه الحرارة المرتفعة، ن.Condition تسير السيارة مرة أخرى، حيث أن TEMATI للشاي الأخضر والجزء الفعال لزراعة الأجنة، مما عكس تأثير مختلف لكل عامل من العامل السلبي، حيث أن التفاعل مع الأجنة الطبيعية. و zostać معلقة مسحوق الشاي الأخضر إلى إفراز نتيجة البلاستوسية عند مستوى 400 ملليج/كمج وزن حي مع درجة الحرارة الطبيعية وعند مستوى 400 ملليج/كمج وزن حي مع درجة الحرارة المرتفعة. استلزم من هذه الدراسات أن التأثير السلبي للإجهاد الحراري على تطور الأجنة مفعلاً يمكن أن يحد مع نجماً إصابات الأجنة الواقعة تحت هذا التأثير مستخلص الشاي الأخضر.