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Role of Spirulina Platensis as a Natural Antioxidant on in Vivo and *In Vitro* Embryo Development in Rabbits

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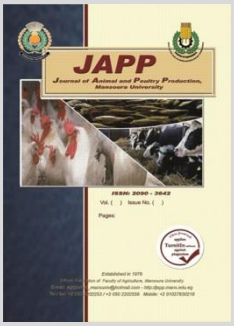

ABSTRACT

Various natural antioxidants, such as *spirulina platensis* (SP) as dietary additives, are beneficial to attenuate the detrimental effects of oxidative stress on fertility of females. The present study targeted to investigate:1) dietary SP effect (200 and 400 mg/kg LBW) on the reproductive performance, ovulatory responses and embryos development, and 2) effect of liquid SP addition to culture media (0.05 and 0.1 ml/ml) on *in vitro* embryos development of NZW rabbit does under heat stress. Results revealed that both dietary SP additions led to an increase (P<0.05) in ovarian weight, relative ovarian weight, total, antral follicles and CLs numbers, ovulation rate, and number and percentage of expanded blastocyst and hatched blastocysts, while decreased (P<0.05) number of bleeding follicles, number and percentage of degenerated embryos compared with control group. *In vitro* embryo developmental competence, in terms of number and percentage of hatched blastocysts increased (P≥0.05) by SP compared to control. Number and percentage of expanded blastocysts and degenerated embryos decreased (P≥0.05) in treatment groups as compared to control group. In conclusion addition of SP at a level of 400 mg/kg LBW in the diet of rabbit does improved reproductive traits in terms of increasing site and rate of ovulation, yield of acceptable embryos, and hatched blastocyst production. However, in vitro addition of SP in culture medium had no pronounced effect on the developmental competence of rabbit embryos.

Keywords: *Rabbit, blue-green algae, ovulation, embryo, hatched blastocyst*

INTRODUCTION

In Egypt, rabbit production has an vital and important portion to solve the shortage in animal protein requirement. Egypt is located within the humid tropics with high temperature and humidity, in particular in the summer (Mousa-Balabel *et al*., 2017). Heat stress causes several changes in blood constituents and reproductive performance of rabbits (Zeweil *et al*., 2009; Abdel-Hamid *et al*., 2015), because rabbits are characterized by a comfortable zone ranging from 18 to 21ºC (Fayez and Marai, 1994). Under high ambient temperature, the oxidative stress occurs by increasing reactive oxygen species (ROS) and other free radicals and reducing the cellular antioxidants (Ruder *et al*. 2009).

Exposing to heat stress condition causes a marked decrease in animal immunity and increasing of production of free radicals as well as lipid peroxidation of the cellular membranes (Mirzaie *et al*., 2018). Also, heat stress increases respiration rate, water intake, and corticosteroid levels, while decreases feed intake, and LH and FSH secretion, affecting the development of the ovaries and rate of ovulation (Chatterjee and Chatterjee, 2009; Arabameri *et al*., 2017). Moreover, the accumulation of oxidative damage under heat stress in distinct sub-cellular components, exerts impaired effects on DNA, proteins and lipids (Wang *et al*., 2017).

Under the normal environmental conditions, there are sufficient antioxidants against the free radical production (oxidants), of body metabolism. The oxidative stress occurs by exceeding oxidants production more than the capacity of body antioxidants (Roth, 2000). The simple strategy to inhibit the oxidation reactions is introduction of natural antioxidants in the diets (Botsoglou *et al*., 2004).

During in vitro fertilization ROS may increased and the collected gametes (oocyte quality, sperm function and, embryo development) may be impaired (Kiani-Esfahani *et al*. 2013; Lu *et al*. 2018; Agarwal *et al*. 2018). Excessive amount of ROS affects negatively influences the maturation and fertilization of oocytes (Agarwal *et al*. 2012; Wojsiat *et al*. 2017), together with a role in decreasing sperm motility, sperm number, and sperm–oocyte fusion (Cardoso *et al*., 2019), with deleterious impact on embryo development.

Enzymatic and dietary antioxidants are the major factors to prevent or inhibit the oxidative stress (Sikka *et al*. 1995). Non-enzymatic antioxidants, known as natural supplements in the diets, such as vitamins, minerals, and natural antioxidants, which have also biological effects (anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer properties) (Xu *et al*. 2017).

Spirulina platensis (SP) is a photosynthetic, filamentous and blue-green algae and used as a dietary additives. It contains high level of protein (65-70%), essential amino and fatty acids, vitamins, minerals, carotene and photosynthetic pigments (Farag *et al*., 2016). It is considering as a natural antioxidant having a positive role in attenuating the deleterious impact of oxidative stress on fertilization (Macedo *et al*., 2014; Mirzaie *et al*., 2018). Moreover, SP contains antioxidant compounds such as flavonoids, alkaloids, phenoles and steroids, which gave SP antioxidant, anti-inflammatory and immune modulatory properties and hypo-lipidemic action (Deng and Chow, 2010). Generally, SP stimulates the activity of antioxidant enzyme, and decrease the production of free

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radicals, lipid peroxidation and DNA damage (Abdelkhalek *et al*., 2015; Anbarasan *et al.*, 2011).

Therefore, the present study targeted to investigate the effect of *Spirulina platensis* on ovulatory responses and embryo development of heat stressed NZW rabbit does in Egypt. The effect of liquid SP addition to culture media at a level of 0.05 and 0.1 ml/ml on *in vitro* developmental competence of embryos of rabbit doe was also studied.

MATERIALS AND METHODS

This study was conducted at a private commercial rabbit farm, Mansoura City, Dakahlia Governorate, Egypt. The laboratorial work was carried out at Physiology and Biotechnology Laboratory, Animal Production Department, Faculty of Agriculture, Mansoura University, Egypt.

Animals

Healthy and adult New Zealand White (NZW) rabbit Does (n= 18 in the 1st experiment and n= 18 in the 2nd experiment were used in this study. Average live body weight (LBW) and age of the experimental does were 3.05±0.22 kg and about 6 months of age. In a naturally ventilated and lighted rabbitry, does were housed individually in stainless steel cages batteries (40 × 50 × 35 cm) supplied with feeders and automatic fresh-water nipples.

Feeding system:

The experimental animals (n=36) were fed *ad libitum* on a commercial pelleted diet (17% crude protein, 14% crude fiber and 2850 Kcal digestible energy/kg), covering their daily nutritional requirements according to NRC (1977). Ingredients and chemical analysis of the commercial diet used in rabbit does feeding are presented in Table 1.

Table 1. Ingredients and chemical analysis of the diet fed to rabbit does in different experimental groups

Item	(%)
Ingredient:	
Clover hay	30.0
Soybean meal (44%)	18.0
Wheat bran	24.6
Barley grain	21.0
Molasses	3.0
Limestone	1.0
DL-Methionine	0.20
Common salt	0.50
Minerals[1]	0.15
Vitamins[2]	015
Di-Calcium phosphate	1.40
Total	100
Chemical analysis (on DM basis, %):	
Organic matter	93.15
Crude protein	18.15
Crude fiber	10.19
Ether extract	2.60
Nitrogen free extract	62.21
Ash	6.85

Experimental design:

The 1st experiment: (In vivo study)

Total of 18 rabbit does were divided into three groups, six in each. A basal complete feed diet in pelleted form was used in feeding the control group (G1), while does in G2 and G3 were fed the same diet with oral administration with *Spirulina platensis* (SP) in amount of 200 and 400 mg/10 ml distilled water/doe, respectively. The treatment period of rabbit does was 30 days before mating.

The 2nd experiment: (In vitro study):

All rabbit does (n = 18) were fed the commercial complete feed diet without any supplementation and kept under the same conditions of does in the 1st experiment. These does were used as embryo donors.

Antioxidant and chemical analysis of *Spirulina platensis*:

The *Spirulina platensis* (SP) in powder form was obtained from Alga Biotechnology Unit, National Research Center, Dokki, Egypt. Nutritional analysis and active composition of SP is summarized in Table 2.

All analyses were performed at the Regional Centre for Food and Feed (RCFF), belonging to Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. All analyses were expressed on the basis of 100 g edible portion of SP. The nutritional analysis of SP samples was determined according to AOAC (2006), while active antioxidant composition was determined according to AOAC (2000).

Table 2. Active antioxidant compounds and chemical analysis of *Spirulina platensis*.

Item	
Antioxidant compounds:	
Vitamin E (μg)	110
Total carotenoids (μg)	455
β-carotene (mg)	220
Chlorophyll (mg)	1.085
Phycocyanin (mg)	11.65
Superoxide dismutase (IU)	510.0
Chemical composition (% on dry matter basis)	
Crude protein	55.8
Crude fat	6.2
Crude fiber	4.9
Nitrogen free extract	23.0
Ash	10.1

Experimental procedures:

Mating:

In both experiments, all receptive does (with red vulva) were naturally mated with fertile bucks at a ratio of one male/3 females during 24 h as a mating period. In the 1st experiment five conceived does out of 6 mated does, were taken from each group, transported to Laboratory and slaughtered 72 h post-mating. The conceived does were determined by the presence of corpora lutea (CLs) on their ovarian surface, then ovulatory response and embryo recovery were achieved. In the 2nd experiment, does were slaughtered at 64 h post-mating to obtain embryos at morula stage.

Ovulatory response: (in vivo study)

Ovaries were isolated immediately after slaughter, then weighed and relative ovarian weight (ROW) to pre-slaughter weight was calculated. The antral follicles (AF), hemorrhagic follicles (HF), and CLs numbers were counted on the ovarian surface per dose. Also, ovulatory response in term of ovulation rate (OR) was computed as the following: *OR = (No. of CLs/No. of HF and AF) x 100.*

Embryo recovery:

Also, reproductive tract was separated and all ova (fertilized and unfertilized) were recovered from the slaughtered does by Flushing in Petri dishes with phosphate buffer saline (PBS) supplemented with 10% fetal calf serum (FCS) and gentamycin (50 μg /ml). The Petri dishes were examined by stereoscopic microscope for searching embryos (fertilized ova). These embryos were counted, then embryo recovery rate (ERR) was calculated as the following: ERR = *No. of embryos/No. of CLs x100.* The collected embryos were

morphological evaluated after washing 3 times in PBS into acceptable and abnormal embryos according to El-Ratel and Gabr (2019).

Embryo culture: (*in vitro* study)

After slaughtering of does in the 2nd experiment 64 h post-mating, embryos were recovered and only acceptable embryos (n=45) at morula stage were taken. Embryos were cultured in control medium (M1) containing TCM-199 (100 ml) supplemented with 10% FCS and 50 µg gentamycin/ml. The control medium were supplemented with SP in liquid form (Alga Biotechnology Unit, National Research Center, Dokki, Egypt) at a level of 0.05 ml/ml (M2) or 0.1 ml/ml (M3). A total of 45 embryos were cultured M1, M2 and M3 (15 embryos in each) in CO2 incubator (38.5°C, high humidity and 5% CO2 in air) under mineral oil for 5 days for determining the developmental competence to embryos at blastocyst, expanded and hatched blastocyst stages.

Statistical analysis:

One-way ANOVA design (Completely randomized design) was used within the software package of SAS (2004) was used for the statistical analysis of the obtained data. The statistical model was $Y_{ij} = \mu + G_i + e_{ij}$, where μ = the overall mean, G_i = group (1-3), and e_{ij} = residual error. Duncan's multiple range test was used for separating the significant differences among means at P<0.05 according to Duncan (1955).

RESULTS AND DISCUSION

Effect of *in vivo Spirulina platensis* treatment on:

Ovulatory response:

Data shown in Table 3 clear that ovarian weight and relative ovarian weight were significantly (P<0.05) higher in treatment groups (G2 and G3) than in G1 (control). The effect of SP treatment on number of total and antral follicles was not significant, but this effect was significant on number of bleeding follicles, being significantly (P<0.05) lower in treatment groups than in control one. Treatment with SP increased number of CLs and ovulation rate but the differences were not significant.

It is of interest to note that increasing the absolute and relative ovarian weight was in association with increasing number of total follicles and CLs on the ovarian surface of does in treatment groups. Also, the ovulation rate was in a negative relationship with number of bleeding follicles, and positive with number of total follicles as affected by SP treatment (Table 3).

These results reflected positive impact of SP treatment on the ovulatory response of doe rabbits, being the highest for addition of 200 mg/kg LBW. In agreement with the present results, El-Ratel and Gabr (2019) reported that SP treatment showed marked increase in absolute and relative weight ovarian weight of rabbit does. This trend is in association with increase in number of CLs and decrease in number of follicles at different stage of development in treatment groups. In accordance with the present results, Mirzaiel *et al.* (2018) reported significant improvement in performance traits of broiler chickens fed diet supplemented with SP under heat stress as previously proved on reproductive parameters of does (El-Ratel, 2017) and mice (Pankaj, 2015) under normal conditions. Moreover, similar results of SP was reported on enhancing the ovulatory response by Abadjieva *et al.* (2018) in pigs and Nikodémusz *et al.* (2010) in birds.

Table 3. Effect of *Spirulina platensis* on ovulatory response and ovulation rate of does in the experimental groups.

Item	Experimental group			P – Value
	G1	G2	G3	
Doe weight (kg)	3046.67±21.55	3041.67±27.38	3060.00±31.83	0.890
Ovarian weight (OW, g)	0.583±0.019[b]	0.713±0.038[a]	0.717±0.019[a]	0.004
Relative OW (g kg-1)	0.019±0.0007[b]	0.023±0.003[a]	0.023±0.0006[a]	0.008
Antral follicles/doe (n)	20.67±0.33	21.50±1.232	22.67±1.28	0.418
Bleeding follicles/doe (n)	3.00±0.58[a]	0.83±0.31[b]	1.50±0.43[b]	0.012
Total follicles	23.67±0.760	22.33±1.145	24.17±1.195	0.462
Corpora lutea/doe (n)	17.00±1.155	19.67±1.229	20.33±1.909	0.268
Ovulation rate (%)	72.01±47.88	88.83±57.70	84.74±75.43	0.165

Means denoted with different superscripts in the same row are significantly different at P<0.05.

Based on the obtained improvement in ovulation rate, antioxidant addition, in term of SP supplementation had a critical role in balancing the redox in supporting the normal function of the ovaries and embryonic development (Wang *et al.*, 2017).

Recovery and embryo production:

Results in Table 4 indicated insignificant effect of SP treatment on embryo recovery rate, although it was higher in treatment groups than in control group. However, total number of embryos/doe, number and percentage of acceptable embryos, significantly (P<0.05) increased, while number and percentage of abnormal embryos significantly (P<0.05) decreased in treatment groups (G2 and G3) as compared to the controls (G1). Similar results were observed by El-Ratel and Gabr (2019), who found that the embryo quality was improved by SP treatment than untreated groups, which may reflect positive effects of SP on in vitro embryo production.

Table 4. Effect of *Spirulina platensis* on recovery rate, and quantity and quality of embryos of does in the experimental groups.

Item	Experimental group			P - Value
	G1	G2	G3	
Embryo recovery rate	91.63±72.45	97.50± 93.11	98.43±95.8	0.52
Embryo/doe (n)	15.17± 0.40[b]	19.17± 0.25[a]	20.00±1.86[a]	0.044
Acceptable embryos (n)	13.00±0.258[b]	18.50± 1.232[a]	18.83±1.887[a]	0.011
Acceptable embryos (%)	86.13±33.64[b]	96.56±18.19[a]	93.88±17.22[a]	0.022
Abnormal embryos (n)	2.17±0.60[a]	0.67±0.33[b]	1.17±0.31[b]	0.075
Abnormal embryos (%)	13.87±3.362[a]	3.44±1.818[b]	6.12±1.722[b]	0.022

Means denoted with different superscripts in the same row are significantly different at P<0.05.

Embryonic stages:

Treatment of SP had insignificant effect on the number and percentage of embryos at expanded blastocyst stage. Meanwhile, number and percentage of hatched blastocysts were significantly (P<0.05) higher in treatment groups (G2 and G3) than in control one (G1). Number and percentage of degenerated embryos showed an opposite trend, being higher in control than in treatment groups, but this effect was significant (P<0.05) only on percentage of degenerated embryos (Table 5).

Our results are in agreement with data obtained by El-Ratel and Gabr (2019) noting that the percentage of expanded and hatched blastocysts increased by SP treatment, while percentage of degenerated embryos decreased in all treatment groups as compared to control.

Table 5. Effect of *Spirulina* on embryonic stage of does in the experimental groups.

Item	Experimental group			P – Value
	G1	G2	G3	
Expanded blastocyst (n)	4.67±0.67	5.33±0.62	4.83±0.60	0.74
Expanded blastocyst (%)	35.67±4.78	29.21±3.48	25.55±2.52	0.18
Hatched blastocyst (n)	5.50±0.34b	10.83±1.20a	12.00±1.18a	0.001
Hatched blastocyst (%)	42.40±2.81b	58.19±4.07a	63.87±1.55a	0.00
Degenerated (n)	2.83±0.65	2.33±0.49	2.00±0.52	0.58
Degenerated (%)	21.93±5.12a	12.60±2.61ab	10.58±2.32b	0.08

Means denoted with different superscripts in the same row are significantly different at P<0.05.

It is well known that LH level reduction may impair rate of ovulation (Chatterjee and Chatterjee, 2009), fertility, developmental competence of embryos (Silva *et al*., 2013; Avila *et al*., 2016), implantation failure, embryo fragmentation, impaired placentation, and abortion by excessive ROS production (Agarwal *et al.*, 2006).

Effect of SP treatment on *in vitro* embryo developmental competence:

After co-culture of embryos at morula stage for 5 days, the number and percentage of hatched blastocysts increased in treatment groups as compared to control (G1), but the differences were not significant. However, the effect of SP treatment on number and percentage of expanded blastocysts and degenerated embryos insignificant decreased in treatment groups as compared to control group (Table 6).

Table 6. Effect of *Spirulina platensi* addition in culture medium on *in vitro* developmental competence of embryos.

Item	Experimental group			P - Value
	G1 (control)	G2 (SP)	G3 (SP)	
Normal (n)	15	15	15	-
Expanded blastocyst (n)	7.67±0.89	6.00±0.58	5.67±0.33	0.14
Expanded blastocyst (%)	51.11±58.79	40.00±38.49	37.77±22.22	0.14
Hatched blastocyst (n)	6.00±1.16	7.33±0.88	8.67±0.33	0.17
Hatched blastocyst (%)	40.00±7.70	48.89±5.88	57.78±2.22	0.17
Degenerated (n)	1.33±0.33	1.67±0.33	0.67±0.33	0.18
Degenerated (%)	8.89±5.12	1.11± 2.61	4.44±2.32	0.18

Recently, the usage of natural additives instead of synthetic vitamins must be put in attention towards animal production. Most compounds in natural sources of antioxidant is polyphenols, which have important physiological functions (Ebrahimzadeh *et al*., 2018). Female reproductive system is very sensitive to oxidative stress, then reduction in production and secretion of gonadotropins (LH and FSH) that are necessary for vital growth and development of ovarian follicles (Arabameri *et al*., 2017). Furthermore, a reduction in GSH of embryos, and elevation of ROS level leading to DNA damage were observed in *in vivo* heat stressed mice (Ozawa *et al*., 2002). Based on these findings, heat and oxidative stress are in a direct and indirect relationship with development of embryos.

In our study, the beneficial effects of SP addition may be in relation with improving the enzymatic antioxidants, which required for ovarian folliculogenesis, CLs formation, and embryo development. Also, SP as an antioxidant, acts as ROS scavenger contributing to maintain the luteal cell integrity and extension of CLs life span (Wang *et al*., 2017). Finally, SP contained several antioxidant compounds, such as carotenoids, chlorophyll, phycocyanin and superoxide dismutase (Table 2).

In conclusion addition of 400 mg/kg LBW in the diet of rabbit does improved reproductive traits in terms of increasing site and rate of ovulation, yield of acceptable embryos, and hatched blastocyst production. However, in vitro addition of SP in culture medium had no pronounced effect on the developmental competence of rabbit embryos. Further studies needed for studying different levels of SP added to maturation, fertilization and embryo development

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دور طحلب الاسبيرولينا كمضاد طبيعي للأكسده على تطور الإجنة داخل الجسم ومعمليا في الارانب

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مضادات الأكسدة الطبيعية المختلفة، مثل طحلب سبيرولينا بلاتنسيس كأضافات غذائية مفيدة للتخفيف من الآثار الضارة للإجهاد التأكسدي على خصوبة الإناث. هدفت الدراسة الحالية إلى تقييم: 1) تأثير أضافة الطحالب فى عليقة الأرانب (200 و 400 مجم / كجم من وزن الجسم) على الأداء التناسلي، واستجابات التبويض وتطور الأجنة ، و 2) تأثيرها فى صوره سائله إلى بيئة الأستزراع (0.05 و 0.1 مل / مل) على تطور الأجنة في معمليا فى أرنب النيوزيلاندى الأبيض تحت الإجهاد الحراري. أوضحت النتائج أن كلا من إضافات الطحالب الى العليقه أدت إلى زيادة معنويه في وزن المبيض، والوزن النسبي للمبيض وإجمالي عدد الحويصيلات الناميه وعدد الأجسام الصفراء، ومعدل التبويض، عدد ونسبة الأجنه فى مرحلة البلاستوسست المتمدده والفاقسه، بينما انخفضت عدد الحويصلات النازفة, عدد ونسبة الأجنة المتحللة مقارنة مع مجموعة الكونترول. زادت الكفاءة التطوريه للأجنه معمليا غير معنويا من حيث عدد ونسبة الأجنه فى مرحلة البلاستوسست مقارنةً بالكونترول وانخفض عدد ونسبة الأجنه فى مرحلة البلاستوسست المتمدده والأجنة المتحللة عند أضافة الطحاب الى بيئه الأستزراع معمليا مقارنة بمجموعة الكونترول. وتوصى الدراسه بإن إضافة الطحالب عند مستوى 400 مجم / كجم وزن جسم في غذاء الأرانب يؤدي إلى تحسين الكفاءه التناسليه من حيث زيادة معدل التبويض، وإنتاج الأجنة داخل الجسم. إضافة الطحالب الى بيئه الأستزراع يكن لها تأثير واضح على الكفاءة التطوريه لأجنة الأرانب.