INTRODUCTION

Recent interest has grown for the use of green microalgae including *Dunaliella salina* (*D. salina*) as a novel feed supplement for nutritional purposes (Yaakob et al., 2014) or detoxification and chelating of toxic and heavy metals. *D. salina* grant various health effects to the humans and animals as well. *D. salina* can protect against CNS oxygen toxicity (Bitterman et al., 1994) and gastrointestinal inflammation (Lavy et al., 2003) through anticancer and antioxidant properties because of its content of high β-carotene (Krinsky, 1988). Moreover, *D. salina* has immunomodulatory and anti-inflammatory effects (Hemmingson et al., 2006; Abdel-Daim et al., 2015). *D. salina* improved body growth and reproduction (Nagasawa et al., 1989). Supplementation of algae or algal oil to dairy cattle increase n-3 percentage in milk fat without adverse effect on milk fat content (Stamey et al., 2012).

Stimulation of ovarian activity was widely explored through nutrition (Downing et al., 1995; Gifre et al., 2017), hormonal administration (Mandiki et al., 1993; Zarazaga et al., 1996), both nutritional and hormonal supplementation (Mohammed and Attaai, 2011). Because of the negative impacts of increased protein and energy in the diet on health and fertility (McEvoy et al., 1997; Dawuda et al., 2002) and the high cost of ration, it is important to search for non-traditional supplements with beneficial character-
The stimulatory effects of dietary green microalgae on reproductive performance has been confirmed. Conclusion of our work (Senosy et al., 2017) indicated that *D. salina* supplemented to Boer goats (10 g/day/head) accelerated ovarian follicle development and significantly increased follicle numbers (small, medium and large follicles). In addition, plasma metabolites and hormones (FSH, LH, estrogen and progesterone) were significantly increased upon supplementation of *D. salina*. Therefore, this study was conducted to explore beyond such information as development of oocytes and embryos. Kinetics of oocytes and embryos cleavages indicated differences either in vitro or in vivo (Mohammed et al., 2005; Mohammed and Attaa, 2011). First cleave of embryos and development was changed upon maturation of bovine oocytes in the presence of follicular fluid (FF) or fetal calf serum (FCS) (Mohammed et al., 2005). Because of the beneficial effects of *D. salina* on reproduction, the present work carried out to explore such improvement through the development of oocytes and embryos. Therefore, the objectives of the present research were to explore kinetics of oocyte and embryo cleavages in addition to reproductive performances upon feeding *D. salina* in mice.

**MATERIALS AND METHODS**

All organic and inorganic materials used in this work were obtained from Caisson Lab (USA), unless stated otherwise. All media were prepared fresh and sterilized through 0.2-µm syringe filter (Nalgene, Cat. No. 190-2520).

**SITE OF STUDY AND MANAGEMENT**

The study was conducted according to procedures approved by the Ethics Committee of Animal Experimentation of King Faisal University, Saudi Arabia. The study was conducted during the period from March to May 2017. Mice were kept controlled under cycle of 12h light and 12h dark. The controlled temperature and relative humidity during the experiment were 25 ±3°C and 50 ±10%, respectively. Mice are fed commercial pellets of basal control diet (Arasco, KSA) or basal control diet supplemented with *D. salina* (100g/kg diet) and had free access to food and water for at least 4 wk.

**ANIMALS AND FEEDING**

Sixty female albino mice of age 6-8 weeks were used during the study. The female were classified into two groups; the control group fed basal diet obtained from Arasco (KSA), which composed of 21.0%, 2.9% fat and 3.3% fiber, 1% mixture of vitamins and minerals (Table 1) and 3300 kcal/kg energy. In addition, *D. salina* algae added to basal diet (100 g/kg). *D. salina* microalgae were produced by Aqua-culture National Group (KSA). The chemical composition of the experimental *D. salina* algae using standard methods (AOAC, 1990) was 8.47% ± 0.12 moisture, 54.17% ± 0.47 crude protein, 0.80% ± 0.10 fiber, 11.42% ± 0.22 total lipid, 18.47% ± 0.26 ether extract and 6.67% ± 0.23 ash.

**Table 1: The mixture of vitamins and minerals added to the basal control diet**

<table>
<thead>
<tr>
<th>Vitamins mixture</th>
<th>Vitamins mixture</th>
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<tbody>
<tr>
<td>Vitamin A</td>
<td>2000000, IU/kg</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>300000, IU/kg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>40000, IU/kg</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>60000, mg/kg</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>1200, mg/kg</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>4000, mg/kg</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>4000, mg/kg</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>2400, mg/kg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>10, mg/kg</td>
</tr>
<tr>
<td>Niacin</td>
<td>24000, mg/kg</td>
</tr>
</tbody>
</table>

| Antioxidant      | 20000, mg/kg     |
| Cobalt           | 200, mg/kg       |
| Copper           | 1000, mg/kg      |
| Iron             | 4000, mg/kg      |
| Manganese        | 15000, mg/kg     |
| Selenium         | 20, mg/kg        |
| Zinc             | 15000, mg/kg     |

**COLLECTIONS OF GERMINAL VESICLE OOCYTES**

Females were given 7.5 I.U. of pregnant serum gonadotropin (PMSG, Intervet). Female mice were killed through cervical dislocation 44-48 h after PMSG injection. Ovaries were removed from the donor females. The GV-stage oocytes of ovarian follicles were released by puncturing with 30G sterile needles under a stereomicroscope. The cumulus-enclosed GV oocytes were released into HEPES tissue cell culture medium (TCM 199) supplemented with 5% fetal bovine serum. The cumulus-enclosed GV oocytes were immediately collected using glass pipette, its tip diameter larger than the diameter of cumulus-enclosed GV oocytes. The collected oocytes were graded into cumulus-enclosed GV oocytes (good oocytes), partial cumulus-enclosed GV oocytes (fair oocytes) and denuded oocytes (Figure 1A, B, C) (Mohammed and Attaa, 2011).
Timing of Germinal Vesicle Breakdown (GVBD) and Extrusion of First Polar Body

Cumulus cells were removed from oocytes using glass pipette, its tip diameter was smaller than cumulus-enclosed GV oocytes diameter. The denuded oocytes were cultured in TCM199 supplemented with 10.0% FCS. The oocytes were investigated for timing of GVBD and extrusion of first polar body during in vitro maturation (Figure 1D, E, F) (Mohammed et al., 2008; Mohammed et al., 2010; Mohammed, 2014).

Quality of Collected Embryos

Embryos were flushed from the uteri at 4.00 days post hCG injection by using Dulbecco’s modified medium supplemented with 10% FCS. The collected embryos graded into three categories; good, fair and bad embryos (Figure 1 J, K, L).

Reproductive Performance

Females were checked for parturition 4 times/day since day 18 of hCG injection. The numbers and weight of pups per female were counted and weighted for each group.

Statistical Analysis

Data are presented as mean ± SEM. The results were subjected to statistical analysis with independent t-test (SAS 2008). Comparison was done between control and D. salina treated groups. Values of probability of less than 0.05 (P < 0.05) were considered significant.

Results

Oocyte Quality

The GV-stage oocytes of ovarian follicles collected 44-48 h after PMSG injection were graded into good fair and bad quality. Quality of oocytes was improved upon D. salina supplementation. Percentage of cumulus enclosed GV (good) oocytes were significantly (P < 0.05) increased whereas denuded (bad) oocytes were significantly decreased in D. salina group compared to control one. The percentage of good, fair and denuded oocytes in D. salina group was 71.6± 1.29% (43/60), 20.0± 0.85% (12/60) and 8.4± 0.43% (5/60), respectively versus 50.0±1.29% (30/60), 15.0± 0.43 (9/60) and 35.0 ±0.85 (21/60) in control one (Figure 2).

Collection of Cleaving Embryos

The females mice were super ovulated by 7.5 I.U. injection of equine chorionic gonadotropin (PMSG, Intervet) followed 48 h later by 7.5 I.U. injection of human chorionic gonadotropin (hCG, Intervet) and mated with fertile males. The same males were used to inseminate both females of control and DS group to exclude the paternal effect on embryo first cleavages. Late 1-cellstage embryo-
**Timing of Germinal Vesicle Breakdown (GVBD) and Extrusion of First Polar Body**

Sixty GV-stage oocytes cultured for *in vitro* maturation were investigated for timing of GVBD and extrusion of first polar body. No differences observed in timing of GVBD of *D. salina* and control oocytes, which occurred 2-3 hr. of starting maturation. Extrusion of 1st polar bodies of both *D. salina* and control oocytes occurred 9-12 h (Figure 3). It seems as *D. salina* supplementation slightly accelerated timing of polar body extrusion and did not change (P > 0.05) percentage of oocyte maturation (96.66%± 0.74;58/60 vs. 95.0%± 1.29; 57/60).

**Timing of Embryo Cleavage and Quality and Reproductive Performance**

Sixty late 1-cell stage embryos collected at 29-30 hr. of hCG injection of both *D. salina* and control were explored for first cleavages. Timing explored for first cleavage of late 1-cell stage embryos to two-cell stage embryos was not differed between *D. salina* and control group where all cleavages occurred within one hour and half. One hundred and thirty embryos were collected from ten females of *D. salina* group whereas one hundred and thirteen were collected from ten females of control group at day 4.0 of hCG injection. Percentage of embryos of good, fair and bad quality were significantly differed between *D. salina* and control groups. Percentage of embryos of good quality (62.16% ± 1.09 vs. 54.77% ± 1.38) and fair quality (23.51% ± 2.46vs. 18.97% ± 2.40) were increased in *D. salina* group compared to control one. On the other hand, Percentage of embryos of bad quality were decreased in *D. salina* group compared to control one (14.30% ± 1.84 vs. 26.24%± 1.72) (Figure 4).

Furthermore, average of offspring number(9.9 ± 0.26 & 8.2 ± 0.30) and weight (12.01 ± 0.31 &9.84 ± 0.37)at birth of ten deliveries were increased significantly (P<0.05) in the *D. salina* group compared to control one (Figure 5).

**DISCUSSION**

In the current study, the effects of *D. salina* supplementation on development of oocytes and embryos in addition to reproductive performance of mice were explored. *D. salina*, as an important type of green microalgae, supplementation improved oocyte and embryos development. Furthermore, reproductive performances including number and weight at birth were improved significantly (P < 0.05) upon *D. salina* supplementation. Nutritional supply, *D. salina*, may be used as an alternative to hormonal treatments to enhance reproductive performance. *D. salina* has high levels of beta-carotene, glycerol, protein and other fine chemicals (Gouveia et al., 2008; Ghasemi et al., 2011; Wichuk et al., 2014; Cuellar-Bermudez et al., 2015; Gong and Bassi, 2016).

**Oocyte Quality**

There are significant increases of cumulus-enclosed GV-oocytes (71.6% ± 1.29 vs. 50.0% ± 1.29) and partial cumulus-enclosed GV-oocytes (20.0% ± 0.85 vs. 15.0% ± 0.43), respectively in *D. salina* group compared to control...
one. *D. salina* has high levels of beta-carotene, glycerol, protein and other fine chemicals (Gouveia et al., 2008; Ghasemi et al., 2011; Wichuk et al., 2014; Cuellar-Bermudez et al., 2015; Gong and Bassi; 2016), which might improve the quality of oocyte and support further embryonic development. Conclusion of our work (Senosy et al., 2017) indicated that *D. salina* supplemented to Boer goats (10 g/day/head) accelerated ovarian follicle development and significantly, increased follicle numbers (small, medium and large follicles). The improvement of oocyte quality in *D. salina* group compared to control one might be related to high levels of beta-carotene and elevation of plasma metabolites and hormones levels. Short-term beta-carotene supplementation in goat affects positively on serum insulin concentrations and ovarian activity (Meza-Herrera et al., 2013). Several studies suggest a direct role for insulin action on female reproduction. Senosy et al. (2017) found that *D. salina* supplementation to Boer goats resulted in significant increase of plasma FSH hormone levels, which rescued cumulus cell apoptosis and enhanced developmental capacity of buffalo oocytes (Jain et al., 2016).

**Timing of Germinal Vesicle Breakdown (GVBD) and Extrusion of First Polar Body**

No differences observed in timing of GVBD of *D. salina* and control oocytes, which occurred 2-3 hr. of starting maturation. Timing extrusion of 1st polar bodies of both *D. salina* and control oocytes occurred 9-12 hr. It seems as *D. salina* supplementation slightly accelerated timing of polar body extrusion and did not change percentage of oocyte maturation (96.66% ± 0.74 vs. 95.0% ± 1.29). Timing of GVBD and extrusion of first polar body affects arrangements of chromosomes during maturation and further embryonic development (Mohammed et al., 2008; Mohammed et al., 2010; Mohammed, 2014). It seems that the timing occurred of germinal vesicle breakdown and polar body extrusion supported further embryonic development and reproductive performance.

**Timing of First Embryo Cleavage and Embryo Quality and Reproductive Performance**

Although timing explored for first cleavage of late 1-cell stage embryos to two-cell stage embryos was not differed between *D. salina* and control group, percentage of embryos of good quality (62.16% ± 1.09 vs. 54.77% ± 1.38; P < 0.05) and fair quality (23.51% ± 2.46 vs. 18.97% ± 2.40; P > 0.05) were increased in *D. salina* group compared to control one. The improvement of developmental capacity of oocytes and embryos due to supplementation of female mice with *D. salina* (100g/kg diet) confirmed by the significant (P < 0.05) increase of number of litter size (9.9 ± 0.26 Vs. 8.2 ± 0.30; P < 0.023) and weight (12.02 ± 0.31 Vs. 9.84 ± 0.37; P < 0.023) at birth compared to control group. The improvement of number of litter size and weight might be attributed also to the beneficial effects of

**Conclusion**

Supplementation of *D. salina* could increase reproductive performance of mice through the improvement of oocyte quality and preimplantation embryo development. Further in vitro studies are still required to investigate the developmental competence of oocytes and embryos upon *D. salina* supplementation.

**Acknowledgments**

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**Conflict of Interest**

No conflict of interest for author to declare.
All authors contributed equally.

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embryonic nuclei in the G1, S, or G2 stages of the cell cycle.


