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EFFECT OF *Nigella sativa* SEED*S* DIETARY SUPPLEMENTATION ON OOCYTE MATURATION AND EMBRYO DEVELOPMENT IN MICE

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SUMMARY

The present study aimed to investigate developmental competence of oocytes and embryos upon dietary Nigella sativa seed (N. sativa) supplementation. Developmental competence of oocytes and embryos of mice supplemented with N. sativa seeds (N=25; 5.0%) compared to control (N=25; not receive N. sativa) were investigated. Female mice were injected with 7.5 IU of pregnant mare serum gonadotropin (PMSG) followed by 7.5 IU of human chorionic gonadotropin (hCG) after 48h and mated with males of proven fertility. Immature GV oocytes were harvested from ovaries after 48h of PMSG injection for investigating oocyte quality, timing of germinal vesicle breakdown (GVBD) and polar bodies extrusion and maturation rate (%). Late one-cell stage embryos were harvested from oviducts after 29-30 h of hCG and followed for cleavage injection whereas blastocyst embryos were harvested from uteri at 96 h of hCG and evaluated. The results indicated improvement of oocyte quality in N. sativa group whereas GVBD and maturation (%) were not differed between N. sativa and control groups. Although N. sativa seeds did not change timing of cleavage to two-cell stage embryos, it significantly (P<0.05) increased the quality of embryos in N. sativa group. Offspring number (9.2 ± 0.34 & 8.1 ± 0.29) and weight (11.71 ± 0.41 & 9.72 ± 0.36) of litter size at birth were significantly (P<0.05) increased in the N. sativa group compared to control. In conclusion, supplementation of N. sativa could increase reproductive performance of mice through the improvement of oocyte quality and preimplantation embryo development.

Keywords: Nigella sativa, oocytes, embryos, development, reproductive performance

INTRODUCTION

Interests have grown in the use of herbal medicinal plants as feed additives to animal rations in order to improve their productive, reproductive and therapeutic performances. *N. sativa* seeds and their purified constituents have shown beneficial effects in several studies on such aforementioned performances (Mohammed and Al-Suwaiegh, 2016). There are a wide range of studies, which proved the safety of *N. sativa* upon dietary administration in animals and humans (Babazadeh *et al*., 2012 and Dollah *et al*., 2013). The effects of *N. sativa* on reproductive performances of females have been reported concerning ovarian follicle development and pregnancy. The beneficial effects of *N. sativa* in gynecologic disorders have been reported in several studies. Parhizkar *et al*. (2016) investigated the effect of *N. sativa* on menopausal parameters of ovariectomized rats. The finding indicated the possible beneficial role of *N. sativa* in the treatment of postmenopausal symptoms and possibility of using *N. sativa* as an alternative to hormone replacement therapy for post menopause in humans. El-Harairy *et al*. (2006) studied the effect of level of feeding (80% or 100 of NRC) and replacing 50% of concentrate feed mixture protein by *N. sativa* meal protein on reproductive performance of Rahmani ewe lambs. They concluded that feeding Rahmani ewe lambs on 80% dietary CP or diets with or without 50% replacement of concentrate feed mixture by *N. sativa* proteins had beneficial effect on estrous activity in terms of length of estrous cycle and estrus duration as well as on conception rate. Zanouny *et al*. (2013) studied the effect of supplementing *N. sativa* at rates of 100 and 200 mg/kg body weight on Ossimi male lamb performance. The results indicated that supplementation of *N. sativa* increased serum testosterone concentrations. In a study carried out on male broiler breeder birds fed diet containing 0.5% and 1% *N. sativa* oil and seeds showed that the addition of either seed or oil resulted in best semen characteristics. The treated groups showed an increase in ejaculation volume, sperm mass motility, progressive motility, count, and total sperm output as well as viability percentage. On the other hand, breeders exhibited a decrease in time of ejaculation and sperm abnormalities. In the second part of this study, inclusion of *N. sativa* in the diet of cocks significantly improved the fertility and hatchability of the treated cock groups, as compared to non-black cumin diets (Abdulkarim and Al-Sardary, 2009). In general, the surplus availability of nutrients is the reason of reproductive enhancement upon *N. sativa* administration.

Despite the well-known properties of *N. sativa* seeds and oil, only few reports on biological activity on oocytes and embryos development in mammals have been published. The stimulatory effects of *N. sativa* on ovarian follicle development has been confirmed. Therefore, this study was conducted to explore beyond such information as development of

oocytes and embryos. Our previous studies (Mohammed *et al*., 2005and Mohammed and Attaai, 2011) indicated differences of oocytes maturation and embryos development either *in vitro* or *in vivo*. Therefore, the present work was carried out to explore oocytes maturation and embryos development in addition to reproductive performances upon supplementing female mice with 5.0% *N. sativa* seeds.

MATERIALS AND METHODS

Animals, rations and management:

The experiment was carried out for 4-weeks following the procedures approved by the Ethics Committee on Animal Production Department of Assiut University, Faculty of Agriculture. Fifty female albino mice of 6-8 weeks of age were randomly divided into two groups (25 animals each). Group 1 was considered as a control and fed a basal diet consisting of concentrate mixture. Group 2 was fed the basal diet supplemented with 5% *N. sativa* seeds. The chemical composition of concentrate mixture and *N. sativa* seeds is shown in (Table 1).The chemical analysis of concentrate mixture and *N. sativa* were accomplished according to the procedures of Association of Official Analytical Chemists (AOAC, 1999) using duplicate samples. The female mice were kept under controlled conditions with 12h light and 12h dark, cycle starting at 7 a.m. The controlled temperature and relative humidity during the experiment were 25 ± 3 ºC and 50 ± 10%, respectively. Clean fresh water was available free of choice and feed was offered once daily at 8.00 a.m. All inorganic and organic compounds used in this study were purchased from Caisson Lab (USA), unless otherwise stated. All media were prepared fresh and sterilized through a 0.22 µm filter (Acrodisc; Pall Gelman Laboratory, Ann Arbor, MI).

Table1. Chemical composition of concentrate mixture and *Nigella sativa* seed.

Chemical analysis %	Concentrate mixture	*N. sativa*
DM	93.32	96.20
OM	94.13	81.60
CP	18.0	24.60
CF	7.32	8.64
EE	2.9	23.66
NFE	65.91	24.90
Ash	5.87	4.70

DM, dry matter; OM, organic matter; CP, crude protein; CF, Crude fiber; EE, ether extract; NFE, Nitrogen free extract

Collection of germinal vesicle oocytes:

Five females of each group were injected with 7.5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet). The injected females were sacrificed 44-48 h after PMSG injection through cervical dislocation. Ovaries were removed and GV-stage oocytes were released by puncturing of ovarian follicles with 30-G sterile needles under a stereomicroscope. The GV-stage oocytes were released into HEPES tissue cell culture medium (TCM) 199 supplemented with 5% fetal bovine serum (FBS). Oocytes were immediately collected using glass pipette with tip diameter larger than the diameter of cumulus-enclosed oocytes and graded into cumulus enclosed GV oocytes (Grade I), partial cumulus GV oocytes (Grade II) and denuded oocytes (Grade III) (Figs 1 A, B, C) (Mohammed and Attaai, 2011).

Timing of germinal vesicle breakdown and polar body extrusion:

Cumulus cells were stripped from oocytes through glass pipette having tip diameter smaller than the diameter of cumulus-enclosed oocytes. The denuded oocytes were cultured in TCM199 supplemented with 10.0% FCS. The oocytes were investigated for timing of germinal vesicle breakdown and timing of 1st polar body extrusion during *in vitro* maturation (Fig 1 D, E) (Mohammed *et al*., 2008; Mohammed *et al*., 2010; Mohammed, 2014).

Collection of cleaving embryos:

Five females of each group (N. sativa and control) were superovulated by injection of 7.5 IU of PMSG (Folligon, Intervet) followed by 7.5 IU of hCG (Chorulon, Intervet) 48 h later and mated with the same males of proven fertility. The same males were used to inseminate females of both control and N. Sativa group to exclude the paternal effect on embryo cleavages. Late 1-cell stage embryos were collected in Hank's balanced salts supplemented with 10% FCS from the oviducts 29-30 h after hCG injection. The collected late 1-cell stage embryos of N. sativa (66 embryos) and control (59 embryos) group were washed and cultured in KSOM+AA medium in vitro and followed for cleavage every 15 min (Fig 1 F).

Quality of collected embryos

Five females of each group (N. sativa and control) were used for embryo collection. 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 of *N. sativa*

(61 embryos) and control (57 embryos) group were graded into three categories; good, fair and bad embryos (Fig1 G, H, I).

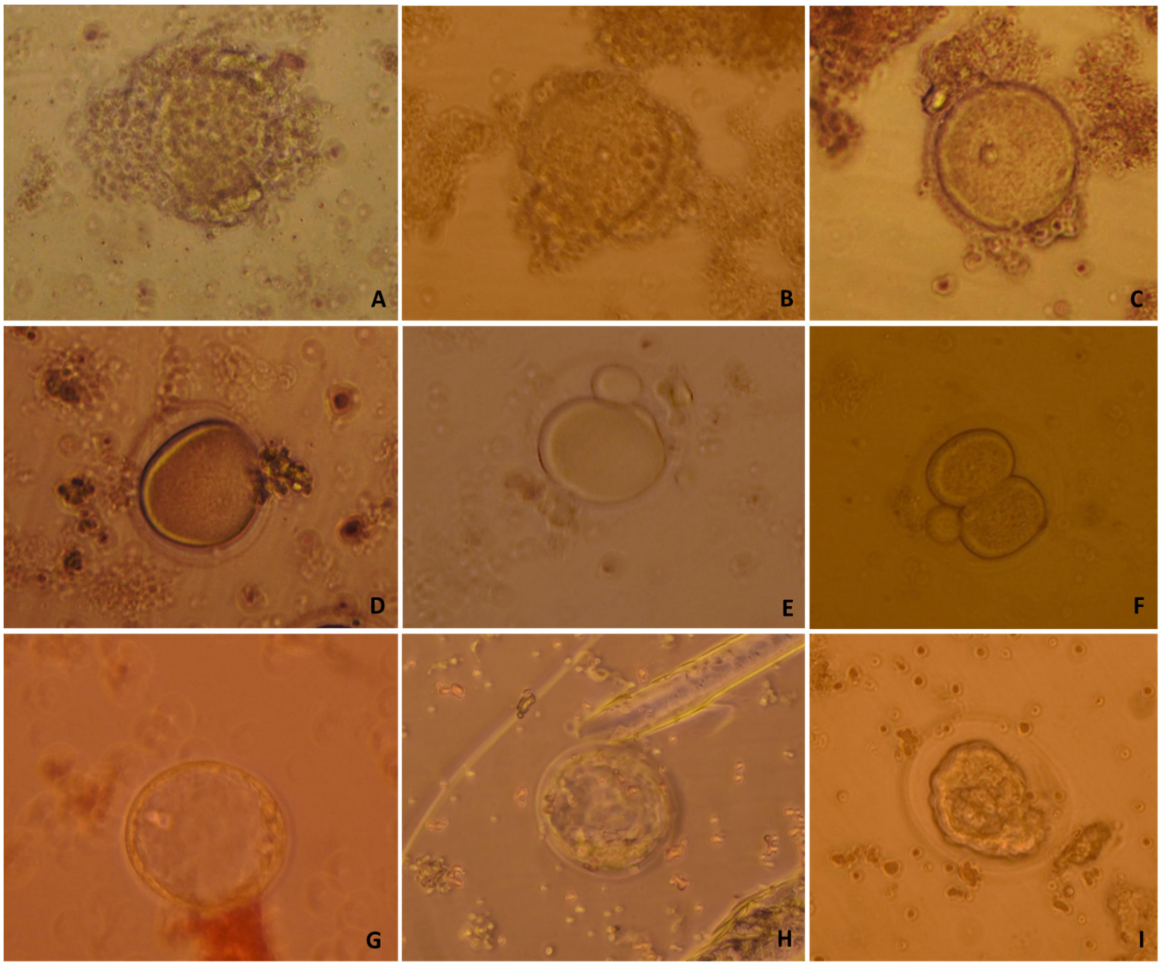

Fig. 1. Categorizing of developmental competence of oocytes and embryos upon *N. sariva seed* supplementation (5.0%) in mice; A) cumulus-enclosed (good) germinal vesicle oocytes; B) partial cumulus-enclosed fair germinal vesicle oocytes; C) denuded (bad) germinal vesicle oocytes; D) germinal vesicle breakdown; E) extruded polar body; F) two-cell stage embryo; G) Good quality embryo; H) fair quality embryo; I) bad quality embryo

Reproductive performance:

Ten females of each group (*N. sativa* and control) were used for checking reproductive performance. Females with vaginal plug after insemination were considered pregnant and checked for parturition 4 times/day since day 18 of hCG injection. The numbers and weight of pops per female were counted and weighted for each group.

Statistical analysis:

Statistical analysis was done according to general linear model (G.L.M) of S.A.S program (2001), version 8.2. Differences between groups in all data were evaluated by one-way ANOVA. Duncan Multiple Range Test (Steel and Torrie, 1980) was used to test the effect of treatments. The data were presented as mean ± S.E.M. Level of significance was set at $P<0.05$. Statistical model was as follows:

$Y_{ij}= \mu + T_i + E_{ij}$

Where: Y_{ij}= the observation ij, μ =the overall mean, T_i = the effect due to treatment i., E_{ij} = the experimental error.

RESULTS AND DISCUSSION

Oocyte quality

Sixty-eight GV oocytes collected from five females of *N. sativa* group and fifty-eight GV oocytes collected from five females of control one. The GV-stage oocytes collected from ovarian follicles 44-48 h after PMSG injection were graded into good, fair and bad quality. Quality of oocytes was improved by *N. sativa* supplementation. Percentage of cumulus enclosed GV (good) oocytes were significantly ($P < 0.05$) increased whereas denuded (bad) oocyteswere significantly decreased in *N. sativa* group compared to control one. The percentage of good, fair and denuded oocytes in *N. sativa* group was 61.99 ±0.77%, 21.60 ± 2.49% and 16.38 ± 1.71%, respectively versus 55.76 ± 1.29%,

17.16 ± 2.14 and 27.06 ± 2.00% in control one (Figure 2).

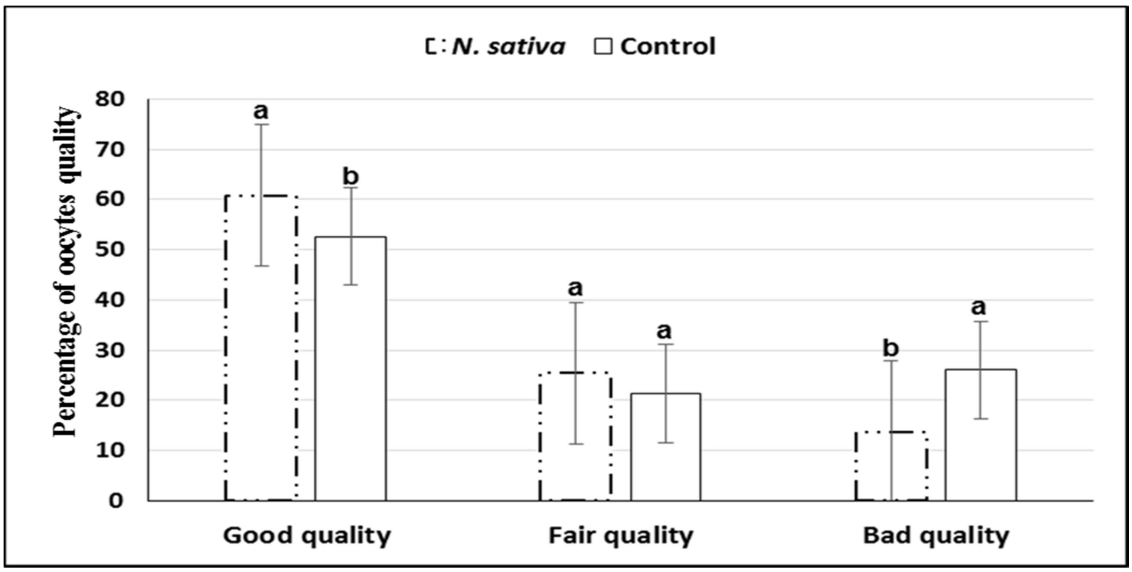

Fig. 2. Quality of germinal vesicle oocytes of *N. sativa* and control groups.
a,b; Values with different superscripts between groups significantly differed at P < 0.05.

The *N. sativa* seeds has high levels of protein, fat, carbohydrates in addition to various vitamins, minerals and beta-carotene (Ahmad *et al*., 2013; Gharby *et al*., 2015; Mohammed and Al-Suwaiegh, 2016), which might improve the quality of oocyte and support further embryonic development. The improvement of oocyte quality in *N. sativa* seeds group compared to control one might be related to high levels of *β*-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. In a study, Ahmad *et al*. (2013) found estrogen-like activity of *N. sativa* extract in immature female rats, which was assumed to stimulate follicular development and corpora lutea formation. The females treated with *N. sativa* extract exhibited an increase in the concentration of serum total protein and progesterone hormone.

Timing of germinal vesicle breakdown and polar body extrusion

The sixty-eight GV oocytes of *N. sativa* group and the fifty-eight GV oocytes of control one were cultured for *in vitro* maturation. They were investigated for timing of germinal vesicle breakdown and polar body extrusion. No differences wereobserved in timing of germinal vesicle breakdown of *N. sativa* and control oocytes, which occurred 2-3 hr. of starting maturation. Extrusion of 1st polar bodies of both *N. sativa* and control oocytes occurred 9-12 hr (Figure 3). *N. sativa* supplementation did not significantly change percentage of oocyte maturation (95.48% ± 1.30 vs. 93.56% ± 1.15). Timing of germinal vesicle breakdown (GVBD) and polar body extrusion affects arrangements of chromosomes during maturation and further embryonic development (Mohammed *et al*., 2008; Mohammed *et al*., 2010 and Mohammed, 2014). It seems that the timing occurrence of germinal vesicle breakdown and polar body extrusion in such study supported further embryonic development and reproductive performance.

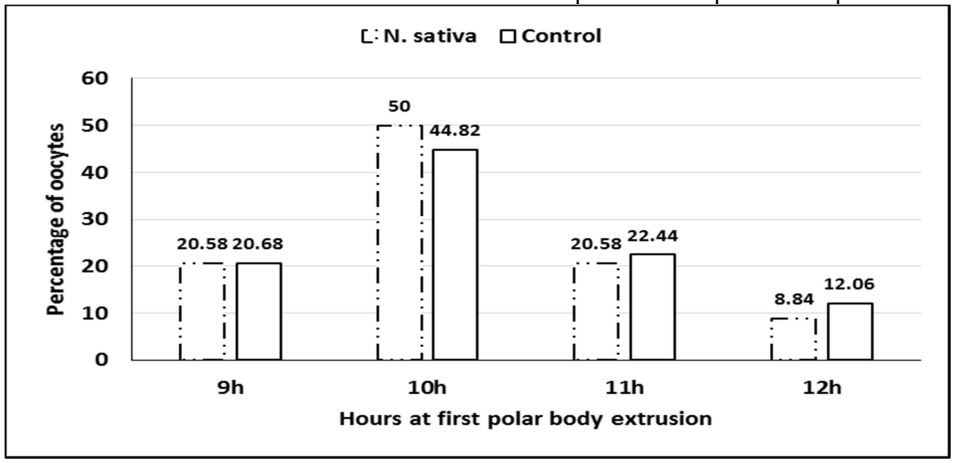

Fig.3. Percentage of oocytes at timing extrusion of first polar body of *N. sativa* and control groups.

Timing of embryo cleavage and quality and reproductive performance

Fifty late 1-cell stage embryos were collected at 29-30 hr. of hCG injection of both *N. sativa* and control were explored for first cleavages. Timing explored for first cleavage of late 1-cell stage embryos to two-cell stage embryos did not differ between *N. sativa* and control group where all cleavages occurred within one hour and half. Sixty-five embryos were collected from five females of *N. sativa* group whereas fifty-six were collected from five females of control group at day 4.0 of hCG injection were graded for quality. Percentages of embryos of good, fair and bad quality were significantly different between *N. sativa* and control groups. Percentage of embryos of good quality (60.8% ± 0.79 vs. 52.58% ± 0.75; $P < 0.05$) and fair quality (25.43% ± 2.54 vs. 21.34% ± 1.89; $P > 0.05$) increased in *N. sativa* group compared to control one. On the other hand, percentage of embryos of bad quality decreased in *N. sativa* group compared to control one (13.76% ± 1.75 vs. 26.06% ± 1.25; $P < 0.05$, Figure 4).

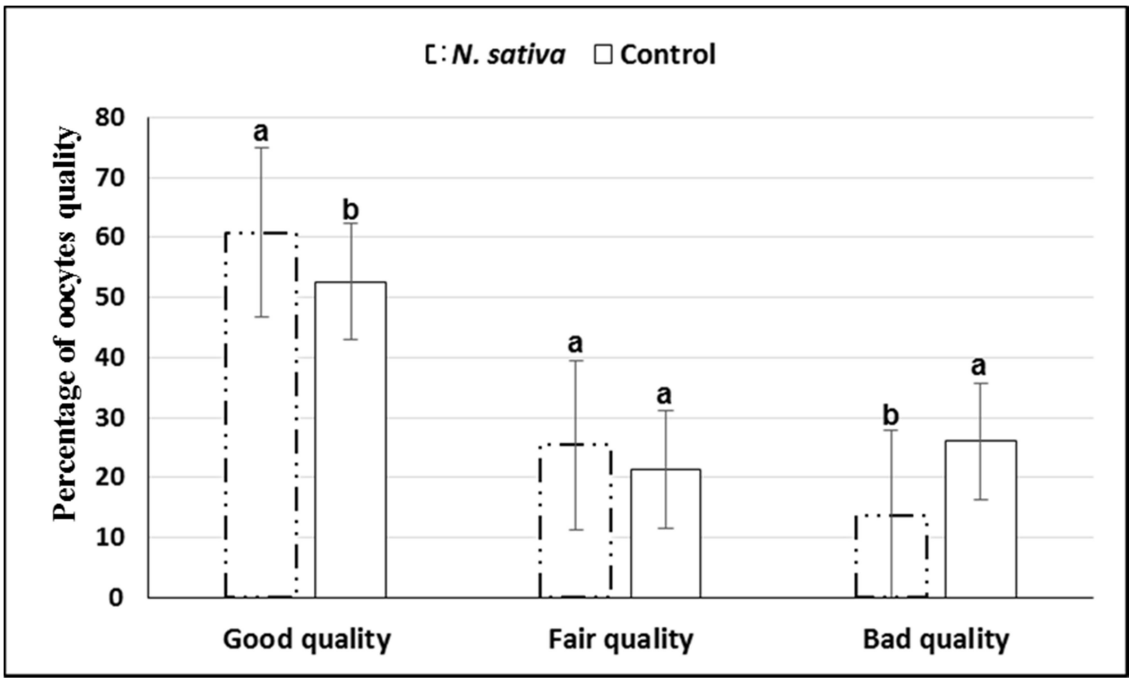

Fig. 4. Quality of embryos of *N. sativa* and control groups.
a,b; Values with different superscripts between groups significantly differed at $P < 0.05$.

Furthermore, average of offspring number (9.2 ± 0.34 & 8.1 ± 0.29; $P < 0.05$) and weight (11.71 ± 0.41g& 9.72 ± 0.36 g; $P < 0.05$) at birth of ten deliveries were significantly ($P<0.05$) increased in the *N. sativa* group compared to control one (Figure 5). The improvement of number of litter size and weight might be attributed also to the beneficial effects of *N. sativa seed* on body health and ovarian function. The recorded body weight during the experimental period indicates increasing of body weight gain with *N. sativa* group compared to control one as recorded in other studies. It is recorded in several studies that *N. sativa seed* significantly increased feed intake and feed conversion in addition to the significant increase of body growth (Mohamed, 2007 and Abd El-Rahman*et al.,* 2011). It has been indicated that *N. sativa seeds* stimulate appetite and increase peristaltic action of the stomach and bowels (Inamul, 2004 and DerMarderosian *et al*., 2005). The *N. sativa* oil contains 57.9% concentrations of essential fatty acids (EFAs) including linoleic acid (C18:2) (Omega-6) whereas the concentration of linolenic acid (18:3n-3) (Omega-3) is 0.2%. Therefore, *N. sativa* omega 6:3 ratio is 56:1. These lipids are important for supporting normal reproductive function and absorption of carotenoids and other fat-soluble nutrients. *N. sativa* oil contains β-carotene in concentration 569–593 μg g^{-1} oil (Ramadan and Mörsel, 2002). There are beneficial effects of β-carotene on reproduction reported of various species. β-carotene supplementation in rats (Chew and Arche, 1983) and pigs (Brief and Chew, 1985 and Kostoglou *et al*., 2000) increased litter size and decreased embryonic mortality. Haliloglu *et al*. (2002) found a positive correlation between beta-carotene level and corpus luteum weight and function of pregnant cattle. Schweigert *et al*. (2002) studied the effect of dietary β-carotene of gilts on uterine fluid composition and early embryonic development. They found that β-carotene resulted in a higher number of early developing embryos. Injections of β-carotene and tocopherol to superovulated Holstein cows improved embryo quality (Sales *et al*., 2008). Furthermore, *N. sativa* has uterine stimulant (Inamul, 2004; DerMarderosian *et al*., 2005).

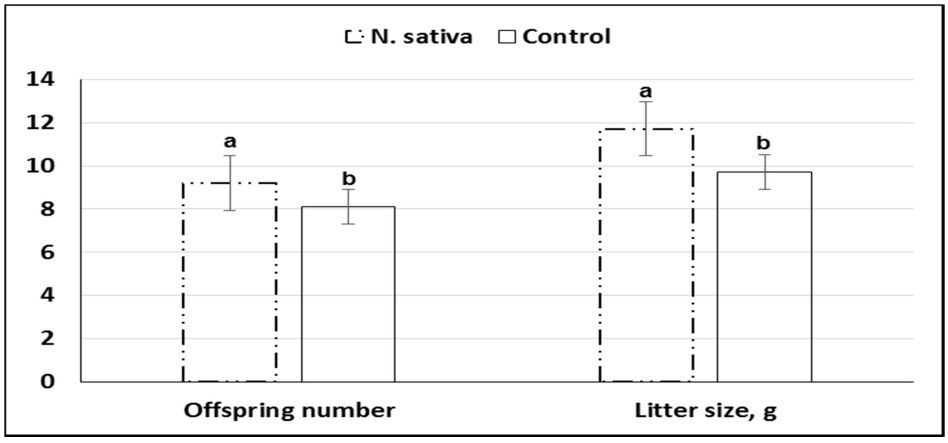

Fig. 5. Offspring number and weight (g) of litter size of *N. sativa* and control groups.
a,b; Values with different superscripts between groups significantly differed at P < 0.05.

GENERAL DISCUSSION

As a part of our series of studies involved in improvement of reproduction through feed additives, the current study was designed to investigate the effects of 5.0% *N. sativa* seeds supplementation on oocytes maturation and embryos development in addition to reproductive performance of mice. For that purpose, female albino mice were given 5.0%*N. sativa* seeds in diets for 4-weeks. The obtained results indicated the significant improvement of oocytes and embryos quality in addition to numbers and weights of litter size after parturition are presented in (figures 2-5). Nutritional supply of *N. sativa* seeds may be used as an alternative to hormonal treatments to enhance reproductive performance. *N. sativa* seeds has high levels of protein, fat, carbohydrates in addition to various vitamins, minerals and beta-carotene (Ahmad *et al*., 2013; Gharby *et al*., 2015; Mohammed and Al-Suwaiegh, 2016). European Food Safety Authority accepted alfalfa leaf extract of *N. sativa* as a safe dietary supplement rich in proteins and vitamins. High biomass production of *N. sativa* together with its high content of phenolic compounds and saponins make it a good source of bioactive compounds.

CONCLUSION

Dietary supplementation of *N. sativa seeds* could enhance reproductive performance of mice through the improvement of oocyte quality and preimplantation embryo development. Further studies are still required to investigate the developmental competence of *in vitro* matured oocytes and the resulting embryos upon *N. sativa* seeds supplementation in mammals followed by embryo transfer.

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تـأثير اضافه حبة البركة الى الغذاء على إنضاج البويضات وتطور الأجنة في الفئران

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يهدف البحث إلى دراسة قدرة البويضات والأجنة على التطور تحت تأثير حبة البركة. تم دراسة قدرة البويضات والأجنة على التطور للفئران (عدد ٢٥ أنثى) المغذاة على حبة البركة (٥%) مقارنة بمجموعه المقارنه (عدد ٢٥ أنثى) غير المغذاة على حبة البركة. تم حقن إناث الفئران بمعدل ٧.٥ وحدة دولية من هرمون جونادوتروبين مصل الفرس الحامل(PMSG) متبوعة بالحقن مرة اخرى بعد ٤٨ ساعة بهرمون جونادوتروبين المشيمة الآدمى، (hCG) ثم التلقيح بذكور خصبة. جمعت البويضات غير الناضجة من المبايض بعد ٤٨ ساعة من الحقن بهرمون جونادوتروبين مصل الفرس الحامل لفحص جودة البويضات، توقيت انهيار تركيب النواة وخروج الأجسام القطبية، ومعدل الإنضاج (%). تم جمع الأجنة في مرحلة الخلية الواحدة من قناة المبيض بعد ٢٩-٣٠ ساعة من الحقن بهرمون hCG ثم فحصت لتوقيت أول إنقسام أما أجنة البلاستوسيست فجمعت من قرني الرحم بعد ٩٦ ساعة من الحقن بهرمون hCG وتقييمها. وقد أوضحت النتائج تحسن جودة انضاج البويضات في حين لم يختلف توقيت إنهيار تركيب النواة ومعدل الإنضاج بين مجموعة حبة البركة ومجموعة المقارنه. وبالرغم من أن حبة البركة لم تغير توقيت الإنقسام الأول للأجنة، إلا أن جودة الأجنة زادت معنوياً ($P<0.05$) في مجموعة حبة البركة. كما أن عدد المواليد (٩,٢ ± ٠,٣٤ & ٨,١ ± ٠,٢٩) ووزنها (١١,٧١ ± ٠,٤١ & ٩,٧٢ ± ٠.٣٦) عند الولادة زاد معنوياً ($P<0.05$) في مجموعة حبة البركة مقارنة بمجموعة المقارنه على الترتيب. ونستنتج من هذة الدراسه أن إضافة حبة البركة بنسبة ٥% زاد من الكفاءة التناسلية للفئران من خلال تحسينهالجودة البويضات وتطور الأجنة قبل الإنغراس فى الرحم.