Bangladesh Journal of Animal Science 52 (1): 29-37, March 2023 https://doi.org/10.3329/bjas.v52i1.65359

Bangladesh Journal of Animal Science
Journal homepage: http://www.banglajol.info/index.php/BJAS

Impact of black cumin and green tea on fertility, immunity and offspring morphology during reproductive aging in mice

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ARTICLE INFO

Article history:
Received: 13 February 2023
Revised: 20 March 2023
Accepted: 27 March 2023
Published: 31 March 2023

Keywords:
Black cumin, Green tea, Ovarian morphology, Pups per litter, Active reproductive life

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ISSN: 0003-3588

OPEN ACCESS

ABSTRACT

With the advancement of age in mammalian females, the reproductive ability is severely compromised, and infertility occurs. Black cumin seeds and green tea are believed to have pro-oxidant effects against various age-related disorders. Using mice model, this study was conducted to elucidate the impact of black cumin and green tea on fertility and offspring morphology of mammalian females during reproductive aging. For this, twenty aged female mice (≤180 days old) were served either with or without black cumin through feed, green tea through drinking water, or their combination. The mice were regularly checked for estrus cycle and mated with males. Similar reproductive patterns were observed in all the groups up to 285 days. After that, weak estrus or anestrus was observed in the control group; however, the active reproductive period was observed to extend up to 350, 375, and 432 days in mice treated with black cumin, green tea, and their combination, respectively. Accordingly, the treatment of black cumin and green tea significantly increased the average number of pups per litter, having comparatively higher birth weight and weaning weight. Moreover, the duration of hair development and eye-opening were significantly reduced in all treated mother. The serum immunoglobulin results revealed higher immunity in mice treated with black cumin and green tea treatment either separately or in combination. Similarly, healthy ovarian follicles with a compact layer of granulosa cells and expanded cumulus cell layer surrounded by oocytes in all the treated mice compared to those of untreated mice. These findings will be helpful for developing breeding strategies during the reproductive aging of mammalian females.

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Introduction

During follicular recruitment and selection in the mammalian ovary, the bulk of follicles in the developing cohort undergo atresia, and only one or a few, depending on animal species, reach the preovulatory stage (Hoque *et al*., 2021; Townson & Combelles, 2018). Follicular atresia is a universal periodic process that reduces the follicular reserves in the ovary (Matsuda *et al*., 2012). Apoptosis of ovarian cells and the alteration of endocrine functions are considered primary factors for follicular atresia (Turani *et al*., 2015). In mammalian females, after the peak fertility period, the follicular reserve is rigorously diminished; thereby, steroid synthesis becomes insufficient, and an unsatisfactory response to the gonadotropins occurs with the advancement of age (Findlay *et al*., 2009). Consequently, infertility occurs even with high nutritional management and advanced assisted reproductive techniques (Hellberg *et al*., 2004). Age-related infertility characterized by delayed estrus, anestrus, repeat

How to Cite

SAM Hoque, S Ahmed, MM Islam, MD Hossain and MU Habiba (2023). Impact of black cumin and green tea on fertility, immunity and offspring morphology during reproductive aging in mice. *Bangladesh Journal of Animal Science,* 52 (1): 29-37. https://doi.org/10.3329/bjas.v52i1.65359.

breeder, and conception failure is one of the major bottlenecks in dairy cattle production, especially in highly productive improved breeds (Amin *et al*., 2019; Hoque *et al*., 2017).

During aging, reactive oxygen species (ROS) accretion becomes higher as a by-product of mitochondrial oxidative phosphorylation (Zimnol *et al*., 2020). The ROS accumulation in excess amounts cause oxidative damage to mitochondria and lipids' peroxidation, leading to cellular apoptosis and death (Kwon *et al*., 2015). As a result, endogenous antioxidant enzymes are insufficient to scavenge the extreme ROS damage and the mature follicle undergoes atretic process (Agarwal *et al*., 2012). Consequently, anovulation, anestrus, and irregular estrus become prominent during aging. Therefore, antioxidant supplements from exogenous sources are necessary to mitigate excess ROS and to increase ovarian endocrine response in aged females. Our previous study reported that dietary supplementation of pyrroloquinoline quinone (PQQ) increases follicular development and ovulation and the number of offspring born without altering the estrus cycle and other fertility parameters (Hoque *et al*., 2021). Additionally, thymoquinone has been reported to prevent mitochondria-induced apoptosis in rat ovaries (Bouhlel *et al*., 2017).

Active reproductive life is an essential parameter for farm animals because the farm economy depends on the number of parity and total milk production throughout the productive life (Islam *et al*., 2019). Moreover, the number of offspring born per litter is the most critical indicator of fertility and farm profitability in multiple-birth animals, including small ruminants and pets (Adu *et al*., 1979; Begum *et al*., 2022). Moreover, maternal immunity, the key factor for offspring survivability and growth performance (Cree *et al*., 2015) is rigorously compromised with the advancement of age. Offspring born from an aged mother have been reported to adversely affect offspring health, lifespan, and stress resistance in a range of taxa (Bock *et al*., 2019).

It is well known that black cumin (*Nigella sativa*) seed is rich in thymoquinone (Severina *et al*., 2013), and green tea (*Camellia sinensis*) is a vital source of PQQ (Kumazawa *et al*., 1995). Moreover, both black cumin seed and green tea are reported not only to have pro-oxidant effects against age-related skin and various organ disorders (Aljabre *et al*., 2015; Bouhlel *et al*., 2017) but also have a beneficial effect against female reproductive disorders (Kamal *et al*., 2021; Parhizkar *et al*., 2016). Although few studies have been conducted on the synthetic antioxidants and therapeutic treatments on reproductive aging (Sylwia *et al*., 2013; Zhou *et al*., 2018), little information are available on the use of natural sources against reproductive aging. Considering these realities, the present study was undertaken to elucidate the beneficial role of black cumin seed powder and green tea in mammalian age-related infertility and offspring physiology using a mouse model.

Materials and Methods

Materials, chemicals and reagents

All the chemicals and reagents were procured from Merck KGaA (Darmstadt, Germany). Black cumin seed powder and green tea were purchased from the local market.

Collection of mice and in vivo trial

Mature Swiss albino mice (≤180 days of age) were selected from the experimental animal facility of the Department of Animal Breeding and Genetics, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur-1706. Mice were reared, handled, and managed according to the Animal Research Ethics Committee (AREC) guideline of BSMRAU, Gazipur-1706, Bangladesh, under a 12 h light, 12 h dark schedule and provided with *ad libitum* feed and water. Twenty (20) female mice were separated and grouped into four (4) as control (no supplement), black cumin (20 mg/Kg body weight), green tea (20 mg/Kg body weight), and black cumin + green tea (20 mg+20 mg/Kg body weight). The acclimation period was 14 days. Black cumin was supplied through feed, and green tea through drinking water. The diet contain 4.0% fat, 20.0% protein, 65.0% carbohydrate, 5.0% fiber and 1% salt and vitamin-mineral premix.

Identification of the mouse estrous cycle

The mouse estrous cycle was identified by the visual observation method. Briefly, the mouse was grasped at the tail with the forepaws resting on the cage lid, and the vaginal opening was evaluated based on the conditions described by Champlin *et al*. (1973). Briefly, wide vaginal opening characterized by moist, swollen, pink tissue are considered as proestrus. The wide vaginal opening becomes less moist, less pink and less swollen in estrus condition. Narrow and non-swollen vaginal opening with white debris is the indications of metestrus. Small, closed vaginal opening without any tissue swelling is considered as diestrus.

Mating test

The natural mating experiment was designed and conducted using five females of each treated and an untreated group of mature mice, according to Hoque *et al*. (2021). After checking the estrous cycle stage, the male mice were placed in each case on the day of estrus and continued for eight months. In the meantime, the days of pregnancy and the number of pups in each litter were recorded. Chronologically, the length of active reproductive life was recorded.

Collection of blood and assessment of serum immunoglobulin level

At the end of the trial period, the blood was collected using 5 ml syringe with 18 G needle from the heart of the anesthetized mice. After centrifuging at 2000 g for 10 minutes, the serum was collected, and immunoglobulin levels as IgG, IgM and IgA were measured using specific kits (Crescent Diagnostics, Arkanlabs) in a spectrophotometer (BMG Labtech, Germany) according to the manufacturer's instructions.

Collection of ovaries and histological examination

Ovaries were collected at the end of the experiment from euthanized mice, according to Hoque *et al*. (2019). Ovaries were then trimmed and fixed overnight at 4°C in 4% (w/v) paraformaldehyde in phosphate buffer saline, dehydrated, and embedded in paraffin. Then, five µm thick sections were cut from the paraffin block, mounted on slides, deparaffinized, and rehydrated using ethanol, followed by incubation in xylene and staining with hematoxylin-eosin to visualize the nuclei and cytoplasm. Morphological study was performed according to Myers *et al*. (2004) under a phase contrast microscope (Zeiss, Germany).

Statistical analysis

All data from five replicates in each group, either treated or control, were analyzed by one-way analysis of variance (ANOVA) followed by LSD posthoc test using IBM SPSS Statistics for Windows, Version 20.0 (IBM, 2011).

Results

Effect of black cumin and green tea on the reproductive cycle

The cyclic mature mice were regularly checked for the estrus cycle stages. Similar estrus patterns along with pregnancy and postpartum intervals were observed in all the groups irrespective of treated or untreated condition up to a particular time (285 days) period (Figure 1).

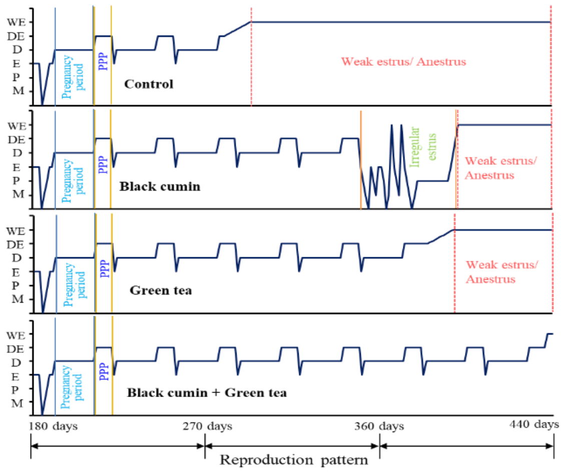

Figure 1: Reproductive pattern of mice treated with or without black cumin, green tea, or black cumin+green tea. The individual graph shows different reproductive cycle stages: WE-Weak estrus, DE- Delivery of pups (parturition), D- Diestrus, E- Estrus, P- Proestrus, M-Metestrus, and PPP-Postpartum period.

After that, weak estrus or anestrus was observed in the control group, but the normal reproductive period was extended for additional five cycles in both the black cumin and green tea treated group (Figure 1). However, the reproductive cycle was extended for three cycles (a total of eight additional cycles compared to the control). In comparison between the green tea and black cumin group, a three cycle period of irregular estrus was observed before showing weak estrus or anestrus in black cumin, whereas the green group was observed to be directly shifted from regular estrus to the anestrus period (Figure 1).

Effect of black cumin and green tea on active reproductive life and the number of pups per litter

The number of pups per litter and the active reproductive life were recorded throughout the experimental period, and the results were compiled in Figure 2. The length of active reproductive life was observed significantly ($p<0.05$) increased by both black cumin (350.5±8.24 days) and green tea (375.5±3.38

days) treatment, which was further significantly ($p<0.05$) increased by treating with a mixture of black cumin and green tea (432.5±6.58 days) compared to the control (285.0±2.21 days) mice group (Figure 2A). The average number of pups per litter was observed significantly ($p<0.05$) increased by both black cumin (8.25±0.37) and green tea (8.75±0.45) compared to that of control (5.75±0.35). However, a further significant ($p<0.05$) increase in the number of pups per litter was obtained by the combination of black cumin and green tea treatment (10.25±0.36) compared to those of black cumin treatment separately (Figure 2B).

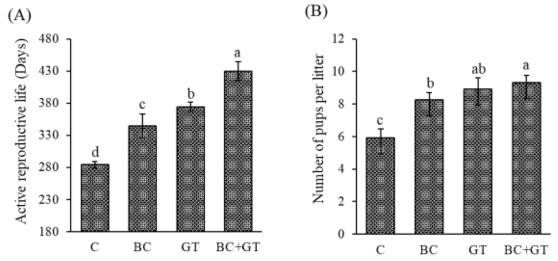

Figure 2: Active reproductive life (A) and the number of pups per litter (B) of mice treated with black cumin, green tea or black cumin+green tea. C, Control; BC, Black cumin; GT, Green tea; BC+GT, Black cumin + Green tea. Values are specified as mean ± SD (n=5). Values with different lowercase letters (a, b, c or d) symbolize significant variation ($p<0.05$) among the treatments [LSD =16.906 for (A) and 0.710 for (B)].

Effect of black cumin and green tea on ovarian morphology and follicular health

The ovaries were collected and checked for ovarian morphology after hematoxylin-eosin staining to evaluate the effect of black cumin and green tea on ovarian health at the end of the experimental period. A compact layer of granulosa cells was observed in the antral follicles, with an expanded cumulus cell layer surrounded by oocytes in all the treated group compared to the control (Figure 3). However, the mixture of black cumin and green tea showed a noticable improvement in the frequency of both granulosa cells and the cumulus cell layer within the antral follicles (Figure 3).

Effect of black cumin and green tea on the serum immunoglobulin level

Serum immunoglobulin levels were measured to evaluate the immunity status of aged mice, and results were compiled in Table 1. It was observed that the IgG level was significantly ($p<0.05$) decreased by both the black cumin and green tea treatment, which was further significantly ($p<0.05$) decreased by the black cumin and green tea combination treatment compared to those of control.

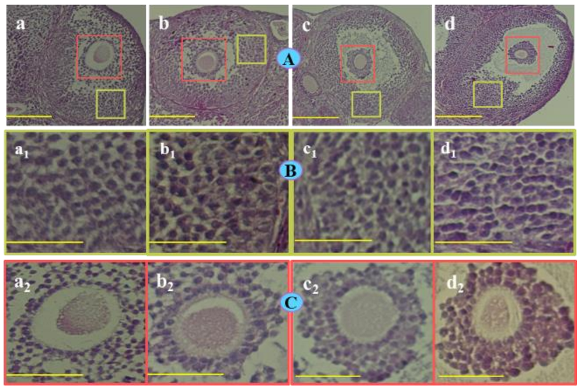

Figure 3: Histological sections of ovaries after Hematoxylin and Eosin (HE) stain**,** showing (A) Morphology of the preovulatory follicles, (B) Distribution and concentration of granulosa cells, and (C) distribution and concentration of cumulus cells within the follicles for different treatments. Where, (a, a_1, a_2) represent the control, (b, b_1, b_2) Black cumin, (c, c_1, c_2) Green tea and (d, d_1, d_2) Black cumin + Green tea. Scale bar showing 100 μm for A, and 200 μm for B and C.

The IgM levels were similar ($p>0.05$) in all treated and untreated mice. The IgA level was significantly ($p<0.05$) higher in treated mice than in control. Among the treatment groups, green tea and the combination of black cumin and green tea group showed significantly higher ($p<0.05$) IgA levels than that of the black cumin treatment.

Effect of black cumin and green tea on the morphology of pups

The morphology of pups was assessed by periodic body weight from birth to weaning (21 days) and duration of hair growth and eye-opening and the results are compiled in Table 2. It was observed that the birth weight and the body weight at 7-day, 14-day, and weaning weight were significantly ($p<0.05$) increased by black cumin, green tea and black cumin+green tea treatment compared to the control. However, no significant ($p>0.05$) difference was observed among the treatments. As shown in the Table 2, the pups born from the mother treated with either black cumin, green tea, or black cumin+green tea have been observed to grow hair at a significantly ($p<0.05$) earlier period (4.40 to 5.40 days) with significantly ($p<0.05$) shorter duration of eye-opening (9.20 to 9.80 days) compared to those of control (6.80 days and 13.40 days, respectively).

Table-1: Serum immunoglobulin level of mice treated with or without black cumin, green tea or black cumin + green tea (n=5).

Treatments	IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)
Control	9.98±0.37[a]	0.14±0.02[a]	0.42±0.06[c]
Black cumin	8.32±0.39[b]	0.11±0.06[a]	0.61±0.04[b]
Green tea	8.38±0.28[b]	0.10±0.02[a]	0.69±0.02[a]
Black cumin + Green tea	7.32±0.58[c]	0.12±0.05[a]	0.71±0.03[a]
LSD	0.564	0.0571	0.056

Values were shown as Mean±SD. The superscripts with different lowercase letters in each column differ significantly (p<0.05).

Table-2: Morphology of pups born from mice treated with or without black cumin, green tea or black cumin + green tea (n=5).

Parameters	Treatments				LSD
	Control	Black cumin	Green tea	Black cumin + Green tea	
Birth weight (g)	1.62±0.13[b]	2.04±0.13[a]	2.06±0.21[a]	2.16±0.11[a]	0.20
Day-7 weight (g)	3.32±0.28[b]	4.20±0.26[a]	4.42±0.16[a]	4.26±0.21[a]	0.31
Day-14 weight (g)	7.28±0.26[b]	8.32±0.28[a]	8.48±0.13[a]	8.52±0.08[a]	0.27
Day-21 weight (g)	9.04±0.21[b]	12.28±0.43[a]	12.42±0.61[a]	12.54±0.39[a]	0.58
Days required for hair appearance	6.80±0.84[a]	5.40±0.89[b]	4.80±0.84[b]	4.40±0.55[b]	1.06
Days required for eye opening	13.40±0.89[a]	9.80±0.84[b]	9.20±0.84[bc]	9.40±0.84[c]	1.14

Values were shown as Mean±SD. The superscripts with different lowercase letters in each column differ significantly (p<0.05).

Discussion

Reproductive aging involves a combination of factors affecting the reproductive organs and the quality of gonads and gametes. The declining quality and quantity of follicular reserves in females during aging leads to infertility and endocrine abnormality (Comizzoli and Ottinger, 2021). Naturally, reproductive aging can come about several months or years before the physiological aging of the organism (Nikolaou and Templeton, 2003). Besides the natural aging of the organism, environmental factors, including free radical accumulation, endocrine disruptors, poor nutrition, or physiological stress, can accelerate reproductive aging (Iwata, 2017). However, follicular disappearance in the mammalian ovary is the primary cause of reproductive aging (Babayev *et al*., 2016). The follicle disappearance by atresia primarily occurs due to the apoptosis of ovarian cells (Glamoc and Saraga-babic, 2005). In addition, steroidogenic alteration may also involve in atretic processes during the follicular development process (Hoque *et al*., 2016; Yu *et al*., 2004). During the typical estrus cycle of the adult female, the spontaneous changes in the gonadotropin level alter the cyclic follicle recruitment and the development of preovulatory follicles. From the diestrus to the proestrus stage, the increased FSH and LH levels induce follicular development from the secondary to the antral stage (Baerwald *et al*., 2012; Hoque *et al*., 2011). FSH acts on the dominant follicles to further develop to the preovulatory stage from the proestrus to the estrus stage (Khandoker *et al*., 2011; McGee and Hsueh, 2000). However, FSH-induced proliferation and differentiation of granulosa and cumulus cells are regulated by mitochondrial oxidative phosphorylation to produce ATP (Hoque *et al*., 2019), leading to excessive ROS generation.

Numerous studies reported that the excessive ROS in mitochondria directly activates the caspase pathway and induces cellular apoptosis (Orrenius *et al*., 2015) and consequently, follicular atresia and anovulation occurs. Irregular estrus or cessation of estrus caused by anovulation is the most prominent sign of reproductive aging (Khatun *et al*., 2022; Richards, 1980). It is well known that thymoquinone and PQQ are potent mitochondria-targeted antioxidants naturally rich in black cumin and green tea, which are believed to improve reproductive performance and stimulate cellular growth (Nakano *et al*., 2014) by reducing oxidative stress. PQQ content of green tea is 29.6 ng/ml (Kumazawa *et al*., 1995), whereas thymoquinone content of black cumin is 21% (Yimer *et al*., 2019). In our study, black cumin and green tea treatment might neutralize the excess mitochondrial ROS in ovarian cells, thereby increasing the preovulatory follicular health, including granulosa and cumulus cells integrity, which might ultimately increase the levels of endocrine hormones and also induce the down-regulated pathways and increased ovulation rate. Therefore, a regularity of estrus, increased number of pups per delivery, and overall active reproductive life were significantly increased compared to control. Black cumin and green tea are not only sources of antioxidants but also have immune-boosting capacities that improve the immune status and slower physiological aging by protecting against different infections (Yimer *et al*., 2019). In our study, the increased IgA and decreased IgG levels revealed the immunity-boosting feature of black cumin and green tea that might enhance the body's defense mechanisms and lengthen healthy living. Consequently, the active reproductive life became longer.

A mother's age during birth directly correlates with the offspring's physiological development. Offspring born from aged mothers showed a decline in health, lifespan, and stress resistance in mammals, including mice and humans (Cree *et al*., 2015). Moreover, offspring physiology might be altered according to the maternal environment, including food, climate, or stressors exposure (Jones and Boelaert, 2015). Furthermore, maternal immunity is reflected in the growth and development of offspring (Asad *et al*., 2018). In this study, the higher birth weight and weaning weight, earlier hair development, and shorter eye-opening duration might result from maternal health and immunity improved by black cumin and green tea treatment.

Although few studies have been conducted using purified antioxidants, there is no study using natural crude sources of mitochondria-targeted antioxidants in mammalian age-related infertility. In this study, it is revealed that black cumin and green tea significantly improved reproductive life and fertility during aging. However, for a concrete understanding of the mechanisms of how black cumin and green tea improve age-related infertility, further study is recommended considering mitochondrial integrity and cellular apoptosis. Age-related infertility is the most common problem in livestock production, especially in dairy farming, with improved cattle breeds in Bangladesh. These findings will be helpful in developing strategies for treating age-related infertility in cattle. Consequently, the reproductive life of dairy animals will be increased, and farmers will be directly benefited in terms of total lifetime milk yield and the number of progeny.

Therefore, it can be concluded that both black cumin and green tea alone or in a combination mixture significantly improve immunity, fertility and reproductive life during the aging of mammalian females. These findings will be helpful in designing reproductive strategies in mammalian females during aging.

Author's contribution

The study was conceptualized and designed by SAM Hoque. Data collection and analysis were carried out by SAM Hoque, S Ahmed, MM Islam, MD Hossain and MU Habiba. SAM Hoque drafted the manuscript. The manuscript was reviewed, revised, edited and then approved by SAM Hoque, S Ahmed, MM Islam, MD Hossain and MU Habiba. Fund and research allocation were provided by SAM Hoque and S Ahmed.

Conflicts of Interest statement

There are no competing interests, as stated by the authors.

Funding

The Government of the People's Republic of Bangladesh funded this study through the special allocation project by Ministry of Science and Technology [Project number: 2020/BS-137].

Data availability

All the data generated during the experiment were included in the manuscript. The research data are available from the corresponding author upon logical request.

Ethical Approval

This experiment was conducted according to the guideline of Animal Research Ethics Committee (AREC) guideline of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh.

Consent for publication

The authors approved this manuscript for publication in the Bangladesh Journal of Animal Science.

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