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Effect of Edible Bird Nest Supplementation against Busulfan-Induced Oligospermia in Adult Rats

(Kesan Suplemen Sarang Burung Walit terhadap Oligospermia Teraruh Busulfan pada Tikus Dewasa)

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ABSTRACT

WHO estimates that between 48 million couples and 186 million individuals are facing infertility globally. Male infertility contributes to one-third of the problem with oligospermia as a primary underlying cause. Edible bird nest (EBN), a nutritional natural product, has the potential to improve male fertility status. However, the scientific evidence on oligospermia is sparse. Therefore, this study aimed to evaluate the effectiveness of EBN supplementation in busulfan-induced oligospermia of adult rats. Thirty-six eight-week-old Sprague Dawley male rats (N=36) were randomly and equally divided into six groups (n=6): Control, Sham, Busulfan, EBN 250, 500, and 1000 mg/kg/daily. A single dose of 10 mg/kg busulfan was injected intraperitoneally to induce oligospermia. Daily EBN supplementation was administered for 28 consecutive days, started one day following the busulfan injection. Findings showed that sperm concentration, spermatogenesis index scoring, seminiferous tubule diameter, and germ cell height were reduced in busulfan-induced oligospermia. Meanwhile, the apoptosis status was significantly increased. EBN supplementation had significantly increased sperm concentration, spermatogenesis index scoring, germ cell height, and gene expression of p38 and MAPK14. EBN supplementation also reduced apoptosis in the oligospermia group in a dose-dependent manner. In conclusion, daily EBN supplementation has a protective effect in busulfan-induced oligospermia by increasing sperm production, preserving histological features of the testes, reducing apoptosis, and improving gene expression of p38 and MAPK14.

Keywords: Edible bird nest; oligospermia; p38; spermatogenesis; sperm concentration

ABSTRAK

WHO menganggarkan bahawa antara 48 juta pasangan dan 186 juta individu menghadapi ketidaksuburan di seluruh dunia. Kemandulan lelaki menyumbang kepada satu pertiga daripada masalah ketidaksuburan tersebut dengan oligospermia merupakan punca utama. Sarang burung walit (EBN), produk semula jadi yang mengandungi banyak khasiat, berpotensi untuk meningkatkan status kesuburan lelaki. Walau bagaimanapun, bukti saintifik mengenai oligospermia masih kurang. Oleh itu, kajian ini bertujuan untuk menilai keberkesanan suplementasi EBN terhadap oligospermia teraruh busulfan dalam tikus dewasa. Tiga puluh enam ekor tikus jantan Sprague Dawley (N=36) berumur lapan minggu dibahagikan secara rawak kepada enam kumpulan (n=6): Kawalan, Sham, Busulfan, EBN 250, 500 dan 1000 mg/kg/hari. Suntikan tunggal 10 mg/kg busulfan dilakukan secara intraperitoneal untuk mengaruh oligospermia. Suplementasi EBN diberikan selama 28 hari berturut-turut, bermula sehari selepas suntikan busulfan dilakukan. Hasil kajian menunjukkan kepekatan sperma, skor indeks spermatogenesis, diameter tubul seminiferus dan ketinggian sel germa menurun di dalam kumpulan oligospermia teraruh busulfan. Status apoptosis turut meningkat secara signifikan. Suplementasi EBN telah meningkatkan kepekatan sperma, skor indeks spermatogenesis, ketinggian sel germa serta pengekspresan gen p38 dan MAPK14 secara signifikan. Suplementasi EBN juga menurunkan status apoptosis secara kebergantungan dos. Kesimpulannya, suplementasi EBN harian mempunyai kesan perlindungan terhadap oligospermia teraruh busulfan dengan meningkatkan penghasilan sperma, memelihara ciri histologi testis, mengurangkan apoptosis dan meningkatkan pengekspresan gen p38 dan MAPK14.

Kata kunci: Kepekatan sperma; oligospermia; p38; sarang burung walit; spermatogenesis

Introduction

Globally, 15% of couples experience infertility, and male factors contribute to at least 50% of infertility cases (Cannarella et al. 2020). Male infertility is multivariate and complex, with a variety of factors contributing to a significant reduction in semen quality. Infertility include oligospermia, asthenospermia, necrospermia, and teratozoospermia (Sharma 2017). Of those aforementioned, oligospermia contributed between 40%-90% of male infertility cases (Maurya 2019).

Oligospermia condition in a study model, was achieved by inducing adult male Sprague Dawley rats by busulfan via intraperitoneal injection. According to Ogunlaja (2020), busulfan, also known as 1, 4-butanediol dimethanesulfonate, is a DNA alkylating agent, that interferes with mitosis during cell proliferation and causes apoptosis of cancer cells. However, as the testes contain proliferative spermatogonia cells, exposure to toxic agents such as busulfan may impair its proliferation. Disruption of proliferation would lead to a substantial loss of male germ, eventually disturbing spermatogenesis (Lai et al. 2015). Subsequently, it will lead to the clinical symptoms of oligospermia (Skurikhin et al. 2017). Besides interfering with cell proliferation, busulfan is also known to cause cytotoxicity through the generation of intracellular reactive oxygen species (ROS) (Li et al. 2018).

EBN is a nest made by specialised salivary glands secretion from the male swiftlet. It is produced mainly by *Aerodramus fuciphagus* during breeding and nesting season, and the bird species can be found primarily in Southeast Asia (Babji et al. 2015). EBN has been used in traditional Chinese medicines as a nutritional supplement for centuries. Previous study highlighted that the treatment improved sperm parameters significantly (Mendes et al. 2016; Sohrabipour et al. 2013). This can be associated with the presence of several reproductive hormones in EBN. The hormones are testosterone (T), follicle-stimulating hormone (FSH) (Albishtue et al. 2019a), estradiol (E2) and luteinising hormone (LH) (Ma & Liu 2012). These hormones regulate spermatogenesis (Albishtue et al. 2019b).

Spermatogenesis requires cell proliferation activity. In addition to the role of reproductive hormones in sperm generation and sperm quality, EBN has been proven to have proliferation properties in many conditions. It promoted the proliferation and migration of keratinocytes in human skin and helps to heal wounds (Terazawa & Shimoda 2020). It enhanced immune cells proliferation and antibody production (Dobutr et al. 2022) It is also reported to contain a high value of glycoprotein and amino acids, carbohydrates, calcium, sodium, and potassium (Zainab et al. 2013). Its carbohydrate components are composed of sialic acid which reported to be an effective antioxidant and able to improve fertility among couples (Hamzah et al. 2013). Several studies have been conducted in recent years to investigate the benefits of antioxidant treatments and showed that these compounds have a protective effect against oxidative stress damage. Antioxidant enzymes and free radical scavenger enhances spermatogenesis so that it can function normally and unaffected by oxidative stress (Kamaruzaman & Noor 2017).

Hence, with all these capabilities mentioned, it is postulated that EBN could likely reverse the oligospermia condition of study animals. During the last few decades, there has been increasing interest and growing evidence in oligospermia-related infertility cases and proper evaluation of them in clinical studies to recommend patients better solutions to their problems. Since EBN possesses positive proliferative effects and antioxidant activity, this may improve male reproductive fertility. Therefore, this study is imperative to introduce new insight into infertility associated with oligospermia and to enhance the market value of EBN by providing more evidence of its potential.

Materials and Methods

Busulfan treatment for oligospermia induction

Preparation of busulfan: busulfan (Sigma, USA) was initially dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) and further diluted with a sterile distilled water (1:1 v:v) to obtain a final concentration of 10 mg/kg busulfan in 50% DMSO. The busulfan solution was prepared immediately before use. The selection of this dose was based on a previous study that reported busulfan demonstrated a toxic effect in rat testis which subsequently lead to the development of oligospermia (Panahi et al. 2015).

Edible bird nest's supplementation

EBN supplementation was given to the rats one day after the busulfan injection was done. The EBN dose

of 250 mg/kg and 500 mg/kg were selected based on a previous pilot study (Careena et al. 2018; Jaffar et al. 2021). Meanwhile, EBN dose of 1000 mg/kg was selected based on maximum dosage recommendation by the Test Guideline OECD TG417 (2010).

A single batch of EBN extract in dried powder form was supplied by Glycofood Sdn. Bhd. The incorporation of EBN extract into the rat's food pellet were done and calculated based on Jaffar et al. (2021).

The animals were supplied with the EBN-enriched food pellets first, and once they had finished it all, the animal was supplied with the regular food pellets. This step ensured that EBN dosages were successfully supplied to the animal. The EBN supplementation was done daily for 28 consecutive days.

ETHICAL APPROVAL OF EXPERIMENTAL ANIMALS

All animal handling and procedures were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with approval code: (FP/2021/SITI FATIMAH/22-SEPT./1203-OCT.-2021-AUG.-2023).

ANIMAL CARE AND HANDLING

A total of 36 (N = 36) Sprague Dawley male rats at the age of 8 weeks, weighing 200-250 g, were supplied by Laboratory Animal Research Unit (LARU), Universiti Kebangsaan Malaysia (UKM). The rats were housed individually in a ventilated cage (IVC) at Animal Laboratory Level 8, Pre-clinical building, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). Acclimatization was done for 7 days before the experimental procedures were conducted. The facility was provided with 24 h air-conditioning, 12-h light and 12-h dark cycle. The ambient temperature was maintained at 22 ± 5 °C. The rats were fed with pellets, and clean water was provided *ad libitum*.

EXPERIMENTAL DESIGN

Following acclimatization, the rats were randomly and equally divided into six groups; with six rats in each group (n=6). The Control group was given a standard food pellet and the Sham group received 50% DMSO intraperitoneally. The Oligospermia group received a single intraperitoneal injection of 10 mg/kg busulfan. The other three groups received 10 mg/kg Busulfan along with EBN 250 mg/kg, 500 mg/kg, and 1000 mg/kg doses, respectively, for 28 days. The rats' body weights in each group were measured and recorded every week throughout the treatment. After 28 days of treatment, the rats were then euthanized. Blood is collected for serum preparation for biochemical analysis. Testis and epididymis were excised for sperm analysis and testicular histological changes.

EPIDIDYMAL SPERM COLLECTION AND SPERM ANALYSIS

The epididymis was first immersed in a pre-warmed PBS solution. Then cauda epididymis was separated from the epididymis and minced in a tube containing 2 mL of pre-warmed PBS (ThermoFisher Scientific, United States). The semen sample was then incubated in a water bath for 40 min at 37 °C to permit sperm to swim out of the epididymal tubules.

About 10 µL of pre-warmed sperm suspension was pipetted and placed on Makler Counting Chamber (Sefi Medical Instruments Ltd. Haifa, Israel). Sperm concentration was counted in a strip of ten grids under a 10× magnification bright field microscope (Olympus CH-2, Japan) (Jaffar et al. 2021).

According to WHO (2010) classification, sperm motility is categorized into progressive motility (PR), non-progressive motility (NP), and immotile (IM). About 10 µL of pre-warmed sperm suspension was pipetted, placed on a microscope slide, and covered with a coverslip. A total of 200 sperm cells were counted under a 40× magnification bright field microscope (Olympus CH-2, Japan). Each sample was measured in duplicate. The average percentage of sperm motility was expressed as a percentage of total motile sperm (PR + NP)/total counted sperm) × 100 (Jaffar et al. 2021).

Sperm viability was determined using a hypo-osmotic swelling test (HOST) solution. The HOST solution was mixed with the sperm suspension in a ratio of 1:10. Then, the mixture was incubated using a water bath for 30 min at 37 °C. About 10 µL of the mixture was pipetted, then smeared on a microscope slide, and left to dry at room temperature. The slides were then dipped in Diff-Quick Fix, Diff-Quick I, and eventually Diff-Quick II with 5 min for each dip. Excess stains from the slides were washed with distilled water. About 200 sperm cells were counted under a 40× magnification bright field microscope (Olympus CH-2, Japan). Each sample was duplicated. The viable sperm tails were stained and observed as either curved, thickened or shortened. Meanwhile, non-viable sperm showed straight and unstained tails (Jaffar et al. 2021).

TESTICULAR HISTOLOGICAL CHANGES

The excised testes were immediately fixed. Then, testes tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Testes tissues were sectioned with a microtome at a thickness of 3 µm. Mounted sectioned tissues on glass slides were stained with H&E. Histological changes of testes were examined under a light microscope (Olympus, Tokyo, Japan) at 200× magnification. Subsequently, 20 tubules of testis in the slides from each rat were selected randomly and analysed blindly for spermatogenesis indexing. The Spermatogenesis Index (SI), also known as Johnsen's scores involves the assessment of seminiferous tubules (STD) diameter, and germ cell height (Lestari et al. 2019; Panahi et al. 2015; Saygin et al. 2011).

APOPTOSIS DETERMINATION

Testis tissues were dried on a drying plate for 10 min at 60 °C. Next, the tissues were then dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Processed testes tissues were sectioned with a microtome at 3 µm thicknesses. Each testis tissues were then stained with 50 µL Annexin V-FITC (Elabscience, Elabscience Biotechnology Inc., USA) and cover slipped. Then, testis tissues were incubated for 1 h at room temperature in the dark. Testes tissues were washed three times for 2 min with PBS in a petri dish and placed on a shaker to dissolve the dye. After washing with PBS, the testes tissues were left to dry, then 1 µL of anti-fluorescence quenching agent (Elabscience, Elabscience Biotechnology Inc., USA) was added to each testes tissue and cover-slipped. Thereafter, the testis tissues were observed under a fluorescence microscope (Nikon Solutions Co., Ltd., Tokyo, Japan) at 20× magnification. In addition, 20 tubules of testis from each rat were selected randomly and analysed for apoptosis. The area of apoptosis on the testes tissues was detected by green fluorescence using a fluorescent microscope with a 530/30 nm band pass filter (Khorsandi et al. 2017).

REVERSE TRANSCRIPTION-QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION PCR (RT-qPCR) for GENE EXPRESSION OF p38 and MAPK14

In this experiment, 30 mg testis tissues were used for RNA extraction. Total RNA was isolated according to manufacturer's instructions using GF-1 Total RNA Extraction Kit (Vivantis, Malaysia). Gene expressions of p38 and MAPK14 were analyzed using appropriate primer sequences (Table 1). Approximately 2 µL of cDNA was amplified in 20 µl final volume of each qPCR reaction containing SYBR Green Master Mix (Vivantis, Malaysia), 0.2 µL of 10 pM forward and reverse primers (Vivantis, Malaysia), and 8 µl RNAse-free water. After 10 min initial incubation at 95 °C, qPCR amplification was performed in 40 cycles using the following program: 95 °C for 3 min, 95 °C for 30 s and 60 °C for 40 s. Expression levels were normalized to B-actin (IDT, lowa, USA) and cyclin A2 (IDT, lowa, USA) housekeeping gene (Khorsandi et al. 2017).

STATISTICAL ANALYSIS

All data were analysed using the IBM® SPSS® Statistics version 27 (IBM Corp., New York, NY, USA). Data were subjected to a one-way ANOVA with the post-hoc Tukey HSD test to evaluate the independent variables. The values were expressed as a mean ± standard error of mean (SEM) and were considered significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

Our previous study reported that EBN supplementation gradually improved the male reproductive system in normal adult male Sprague Dawley rats (Jaffar et al. 2021). To further understand the EBN potential in improving male fertility, this study evaluated the effect of EBN supplementation on the oligospermia rat model

TABLE 1. Gene sequences

	Forward	Reverse
p38	CGA GCG ATA CCA GAA CCTG	GCG TGA ATG ATG GAC TGA AA
MAPK 14	TCG GCA CAC TGA TGA CGA AA	TCA TGG CTT GGC ATC CTG TT
β-actin	TAC AAC CTT CTT GCA GCT CCT	CCT TCT GAC CCA TAC CCA CC
Cyclin A2	TGG ATG GTA GTT TTG AAT CAC CC	TGG CCC GCA TAC TGT TAG TG

with higher dose of EBN. On top of oligospermic model compared to normal model, our current study assessed four parameters, including sperm quality, histological changes, apoptosis, and gene expressions of p38 and MAPK14. Previous study has shown that 250 mg/kg/daily EBN supplementation in normal adult Sprague Dawley rats resulted in substantial changes in most of the mentioned parameters. As an extension of the study, this study applied an additional dose of 500 mg/kg EBN supplementation to evaluate further EBN's capability to improve male infertility status. The limit dose of 1000 mg/kg EBN supplementation was selected based on the OECD Test Guideline 407 (OECD 2008).

SPERM ANALYSIS

Figure 1 illustrates the effects of EBN on sperm parameters consist of sperm concentration, sperm motility and sperm viability. A significant increase was noted in sperm concentration ($p < 0.05$) following different doses of EBN (Figure 1(A)). However, there were no significant differences in sperm motility (Figure 1(B)) and sperm viability (Figure 1(C)) following 250 mg/kg, 500 mg/kg, and 1000 mg/kg EBN supplementation. The microscopic examination is illustrated in Figure 2.

A previous study have reported a possible relationship between testes and sperm quality that play a crucial role in male fertility (Budin et al. 2017). The current results indicate that EBN supplementation has increased sperm concentration significantly compared to the Control and Oligospermia group. These findings align with the previous study in which the results demonstrated a positive effect on sperm concentration following the 250 mg/kg EBN supplementation (Jaffar et al. 2021). In general, the increase in sperm count corresponds to an increase in epididymal weight. Henceforth, increased in epididymal weight indicates an increase in sperm production or spermatogenesis (Jaffar et al. 2021; Mao et al. 2018; Teves & Roldan 2022).

It is also noted that the increased sperm concentration is dose-dependent on EBN supplementation. Mechanism related to sperm concentration improvement in oligospermia model rats are yet to be determined. Busulfan is known to cause cytotoxicity through damaged DNA structure and prevents the proliferation or differentiation of spermatogonia stem cells (Chen, Liang & Wang 2018). We postulated that an increase in sperm concentration may be due to the increased spermatogonia proliferation which was influenced by sialic acid (Aswir & Wan Nazaimoon 2011). Sialic acid is a major component of EBN. Presence of sialic acid led to sialylation in sperm which is vital for providing a negatively charged glycocalyx, enriching the molecular structure of the sperm surface and impactful for spermatogenic cells (Yi et al. 2023).

Despite the evident increase in sperm concentration observed in the oligospermia model as a result of EBN supplementation, there were no statistically significant changes noted in sperm motility and viability when compared to the Control group. It is noteworthy that sperm motility is dependent on energy and calcium ions (Barbagallo et al. 2020). Oligospermia has been linked to low levels of calcium ions (Chyra-Jach et al. 2020) or the impairment of crucial pathways regulating sperm motility, such as the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and the Ca^{2+} signaling pathway (Jaffar et al. 2019). Busulfan-induced oligospermia exhibited a decline in sperm motility due to injury to the blood–testis barrier, leading to an up-regulation of matrix metalloproteinase 9 activity and non-collagenous 1 (Jiang et al. 2022). Despite the similarity in busulfan dosage between that study and ours, EBN was unable to bring about changes in sperm motility.

Supplementation with EBN did not affect the viability of sperm. The sperm viability method is based on membrane permeability and the osmosis process. Our study induced oligospermia, which caused changes in membrane ultrastructure, including disruption of the lipid and protein components, leading to a loss of selective permeability capabilities and cell death (Ibrahim et al. 2008). One of the mechanisms that explains cell death is DNA damage. Previous *in vitro* studies have confirmed that the severity of DNA damage varies and depends on sperm preparation techniques, the incubation time of the sperm suspension, and storage temperature (Nabi et al. 2014; Zhang et al. 2011). In our study, sperm was harvested mainly from the cauda epididymis, which stores mature sperm. During harvesting, there is a chance that the sperm population came from the caput epididymis because the structures are close together. A population mixture produced various susceptibilities to the viability test (HOST). This phenomenon led to a false negative result of sperm viability, which is a limitation of this study.

HISTOLOGICAL EXAMINATION OF TESTIS

Histological examination of testicular sections showed a

significant difference in the spermatogenesis index was found among the groups (Chi-square = 289.138, p = 0.001, df = 20) (Figure 3(A)). On the other hand, there was a significant difference in seminiferous tubules diameter (Figure 3(B)) and germ cell height diameter (Figure 3(C)) ($p < 0.05$) in the Oligospermic group compared to the Control and Sham groups. Overall, EBN supplementation caused a significant effect on all histological examinations following 250 mg/kg, 500 mg/kg, and 1000 mg/kg EBN supplementation. The microscopic examination is illustrated in Figure 4.

Busulfan induces cytotoxicity as well as testicular injury. These two factors may have contributed significantly to the pathological changes (Hosseini et al. 2014; Nasimi, Tabandeh, & Roohi 2018; Panahi et al. 2015; Vahdati et al. 2015). This study found that oligospermia model presented with histopathological changes in testicular tissues. This abnormality was represented by the abnormal results of the spermatogenesis index, seminiferous tubules diameter, and germ cell height. Testicular injury causes neutrophils to migrate into the affected area and release proteases, cytokines, myeloperoxidase and other inflammatory mediators, causing pathological changes that damage tissue (Wang 2018). A previous study has shown that green tea that is consumed naturally and has antioxidant properties was able to repair histopathologic damage in the testis and increased antioxidant enzymes following inflammatory injury (Martins et al. 2021). Thus, we hypothesized that EBN supplementation was responsible for improving spermatogenesis scoring and germ cell height in our study.

PERCENTAGE OF APOPTOSIS

The current results demonstrated the Oligospermic model significantly increases the percentage of apoptosis compared to the Control, Sham and all EBN groups (Figure 5). Meanwhile, all EBN supplementation significantly decreases the percentage of apoptosis when compared to the Oligospermic model. However, no significant differences when compared to the Control and Sham groups. The percentage of apoptosis was measured by Image J software and evaluated by the ANOVA test. A significant difference in the percentage of apoptosis was found among the groups (ANOVA = 50.512, p = 0.001, df = 5). The microscopic examination is illustrated in Figure 6.

Spermatogenesis is a continuous cell generation process that involves mitosis and meiosis (Zeng et al. 2019). On the other hand, spermatogenic cell apoptosis reduces cell numbers by removing abnormally dividing cells. This action prevents abnormal spermatozoa formation (Kim et al. 2009). p38 MAPK pathway has been reported to play an essential role in regulating cell growth, differentiation, proliferation and, importantly, apoptosis (Sui et al. 2014). Notably, p38 MAPK was also found in mouse testis spermatogenic cells (Luo et al. 2022). Thus, a well-regulated apoptosis mechanism in spermatogenic cells is crucial for spermatogenesis. An imbalance can result in abnormal spermatogenesis leading to oligozoospermia, asthenozoospermia, and azoospermia (Pergialiotis et al. 2016).

In the present investigation, instead of focusing on the cell surface death receptor pathway, we explored an early marker for apoptosis. This involved the use of annexin V-FITC, which targets the externalization of phosphatidylserine in apoptotic cells. Our research demonstrates that EBN has the ability to decrease the count of apoptosis in the oligospermia model. The reduction is dose-dependent, with 1000 mg/kg of EBN supplementation showing the lowest percentage of apoptosis. We hypothesized that higher doses increase the likelihood of preserving and enhancing spermatogonia proliferation in oligospermic rats. These findings align with a previous study indicating that EBN reduces early apoptotic membrane phosphatidylserine externalization and inhibits caspase-3 cleavage (Yew et al. 2014) as a neuroprotective substance. Notably, our study is the first to document the anti-apoptotic effect of EBN in an oligospermia model.

GENE EXPRESSION OF p38 AND MAPK14

Gene expression of p38 and MAPK14 were determined by qPCR analysis after normalization with β-actin and cyclin A2 housekeeping gene. There was a significant difference among the groups in gene expression of p38 (Figure 7(A)) and MAPK14 (Figure 7(B)) ($p < 0.05$). Overall, EBN supplementation caused a significant effect on gene expression of p38 and MAPK14 following 250 mg/kg, 500 mg/kg, and 1000 mg/kg EBN supplementation. The amplification curve and melt curve of gene expressions of p38 and MAPK14 are illustrated in Figure 8.

Numerous preceding studies have suggested that the modulation of male reproductive function involves MAPK cascades. Within the testis, p38 and MAPK14 play pivotal roles in governing gene expression, cell cycle proliferation, differentiation of germ cells, germ cell apoptosis, cell migration, and survival

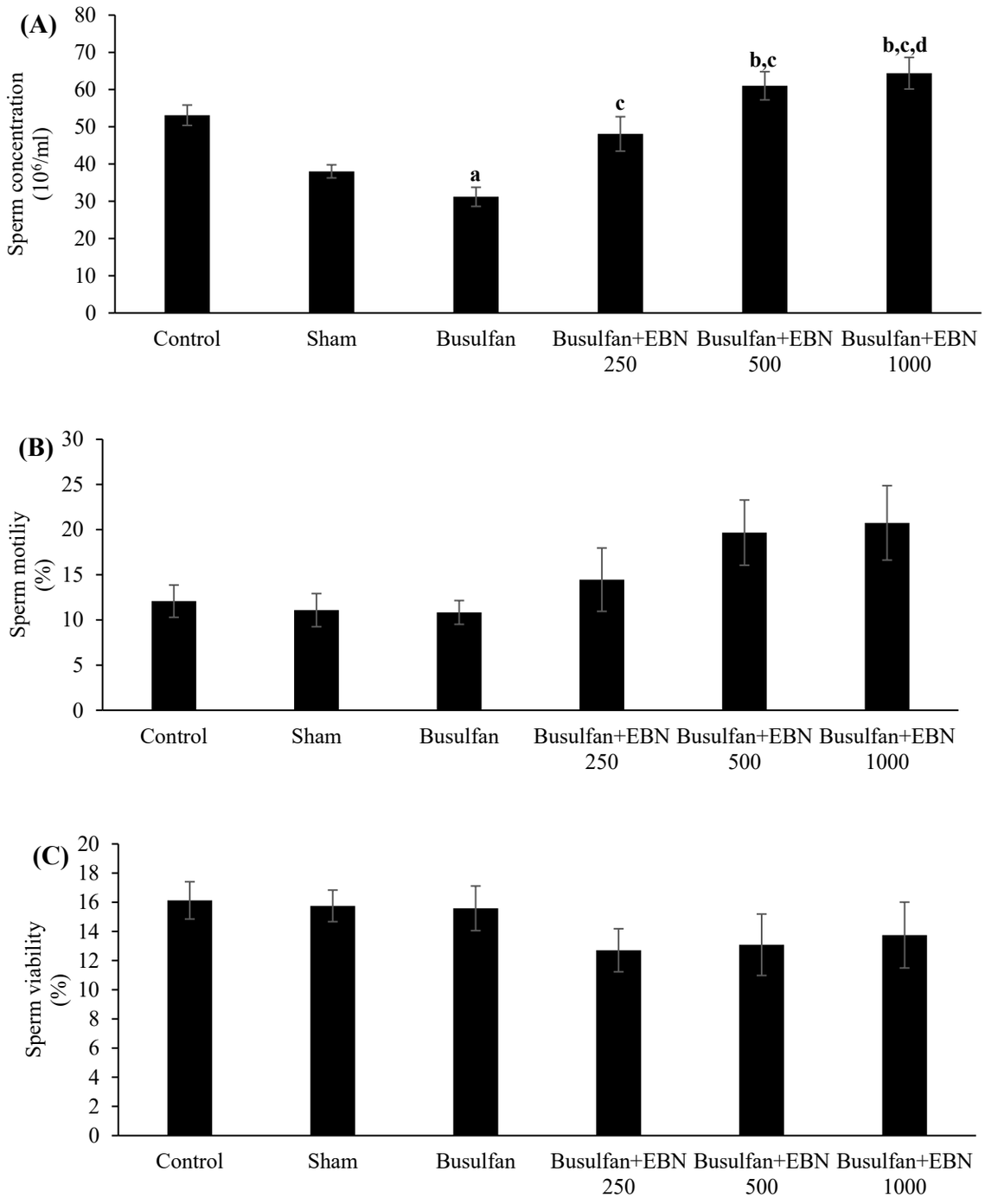

FIGURE 1. Effects of EBN supplementation on (A) sperm concentration, (B) sperm motility, and (C) sperm viability in each group. The values are expressed as mean ± SEM. * $p < 0.05$ when compared to the control. [a]significant difference compared with the control group, [b]significant difference compared with the sham group, [c]significant difference compared with the busulfan group, [d]significant difference compared with the Busulfan+EBN 250 group

(Bonney 2017; Cuenda & Sanz-Ezquerro 2017; Li, Mruk & Cheng 2009). Our study showed a significant increase in the gene expressions of p38 and MAPK14 with EBN supplementation compared to the Control group. Various earlier studies have demonstrated that EBN supplementation promotes cell proliferation *in vitro*, including the proliferation of rabbit corneal keratocytes (Zainal Abidin et al. 2011), human adipose tissue (hADSCs), and normal human fibroblast cells (NHFs) (Roh et al. 2012). In an *in vivo* study, EBN supplementation also stimulated the proliferation and activation of B cell antibodies (Zhao et al. 2016) facilitated the reproduction of uterine structures (Albishtue et al. 2019a). Nevertheless, the precise mechanism by which EBN supplementation regulates the p38 and MAPK14 pathways remains unclear.

Hence, it has been demonstrated that EBN exhibits antioxidant and anti-apoptotic properties (Falk et al. 2011; Mehraein et al. 2011), which contribute to the activation of cell proliferation via the MAPK pathway (Roh et al. 2012). Based on this, we hypothesize that EBN supplementation could potentially diminish apoptosis, boost the gene expressions of p38 and MAPK14, and ultimately foster increased spermatogonia proliferation.

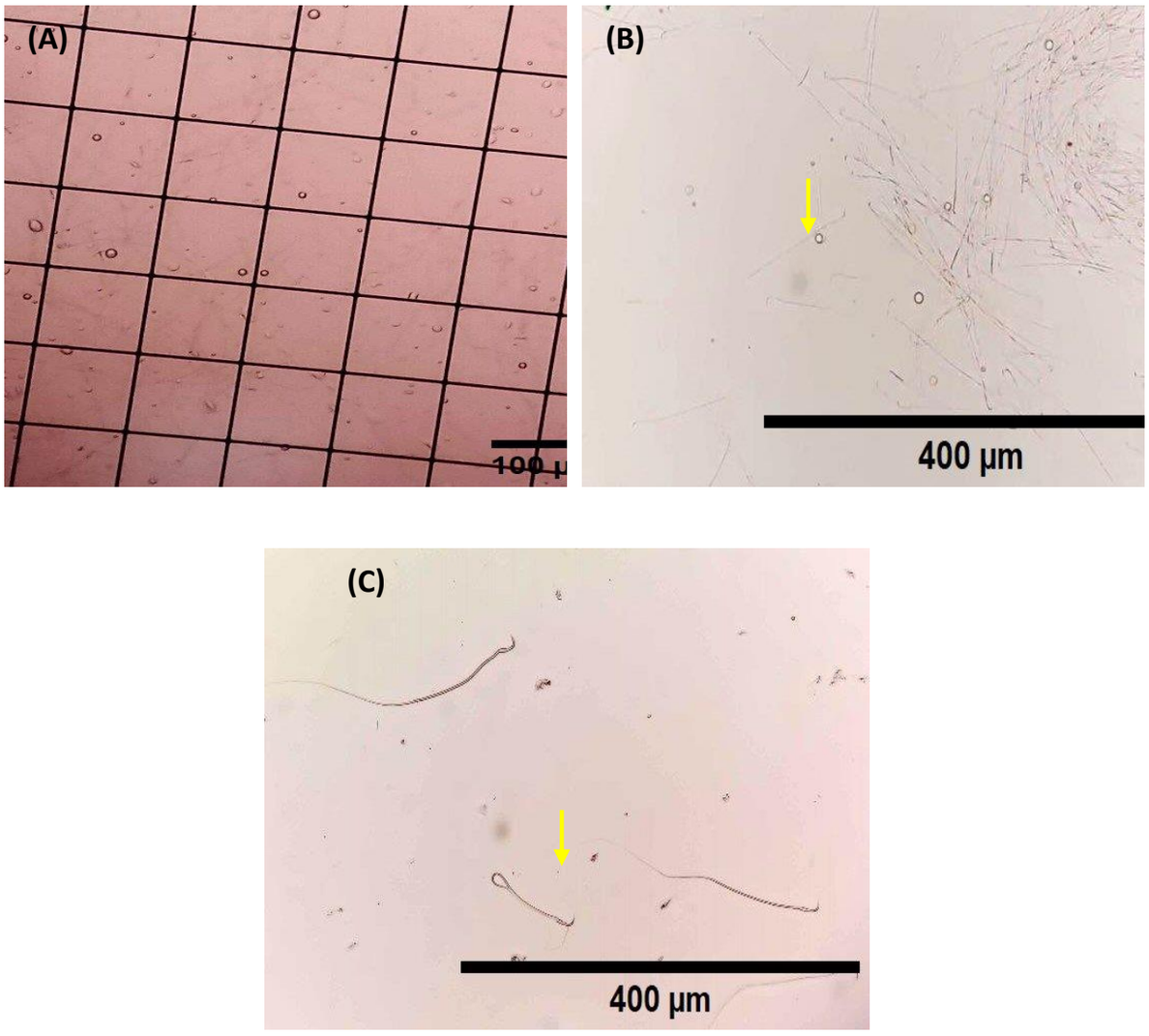

FIGURE 2. Sperm analysis of rats from (A) sperm count on a Makler chamber; (B) sperm motility; (C) sperm viability (Scale bar = 400 µm)

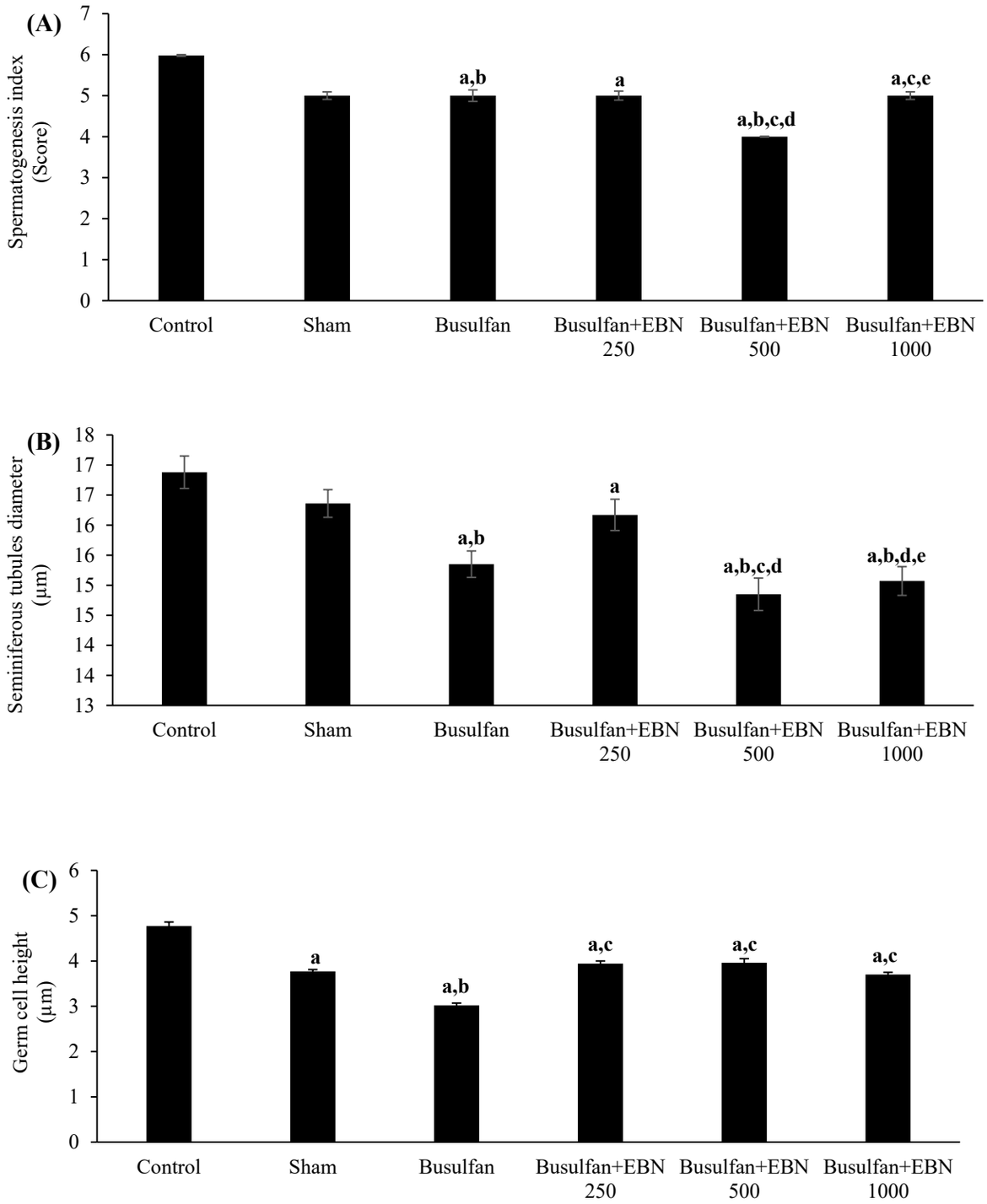

FIGURE 3. Effects of EBN supplementation on (A) spermatogenesis index, (B) seminiferous tubules diameter, and (C) germ cell height in each group. The values are expressed as mean ± SEM. * $p < 0.05$ when compared to the control. [a]significant difference compared with the control group, [b]significant difference compared with the sham group, [c]significant difference compared with the busulfan group, [d]significant difference compared with the Busulfan+EBN 250 group, and [e]significant difference compared with the Busulfan+EBN 500 group

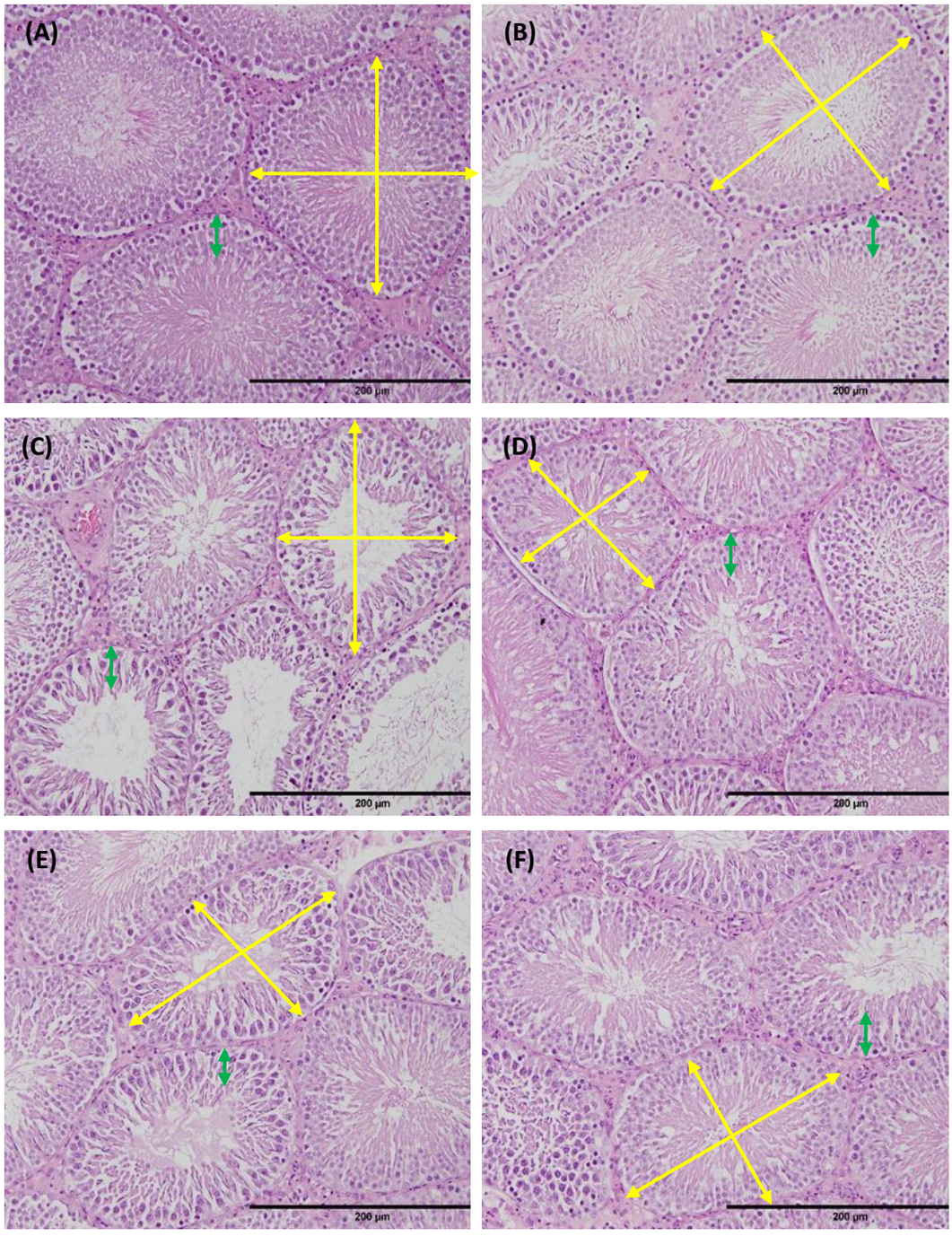

FIGURE 4. Histopathological section of testis tissues stained by hematoxylin and eosin (H and E) such as seminiferous tubules diameter and germ cell height from (A) control group; (B) sham group; (C) busulfan group; (D) Busulfan+EBN 250 group; (E) Busulfan+EBN 500 group; (F) Busulfan+EBN 1000 group (Yellow arrow: seminiferous tubules diameter, Green arrow: germ cell height, Scale bar = 200 µm)

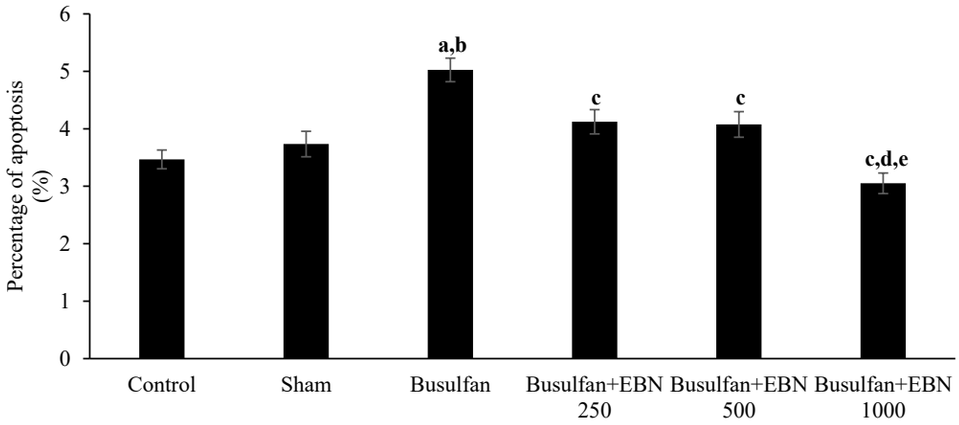

FIGURE 5. Effects of EBN supplementation on the percentage of apoptosis in each group. The values are expressed as mean ± SEM. * $p < 0.05$ when compared to the control. [a]significant difference compared with the control group, [b]significant difference compared with the sham group, [c]significant difference compared with the busulfan group, [d]significant difference compared with the Busulfan+EBN 250 group, and [e]significant difference compared with the Busulfan+EBN 500 group

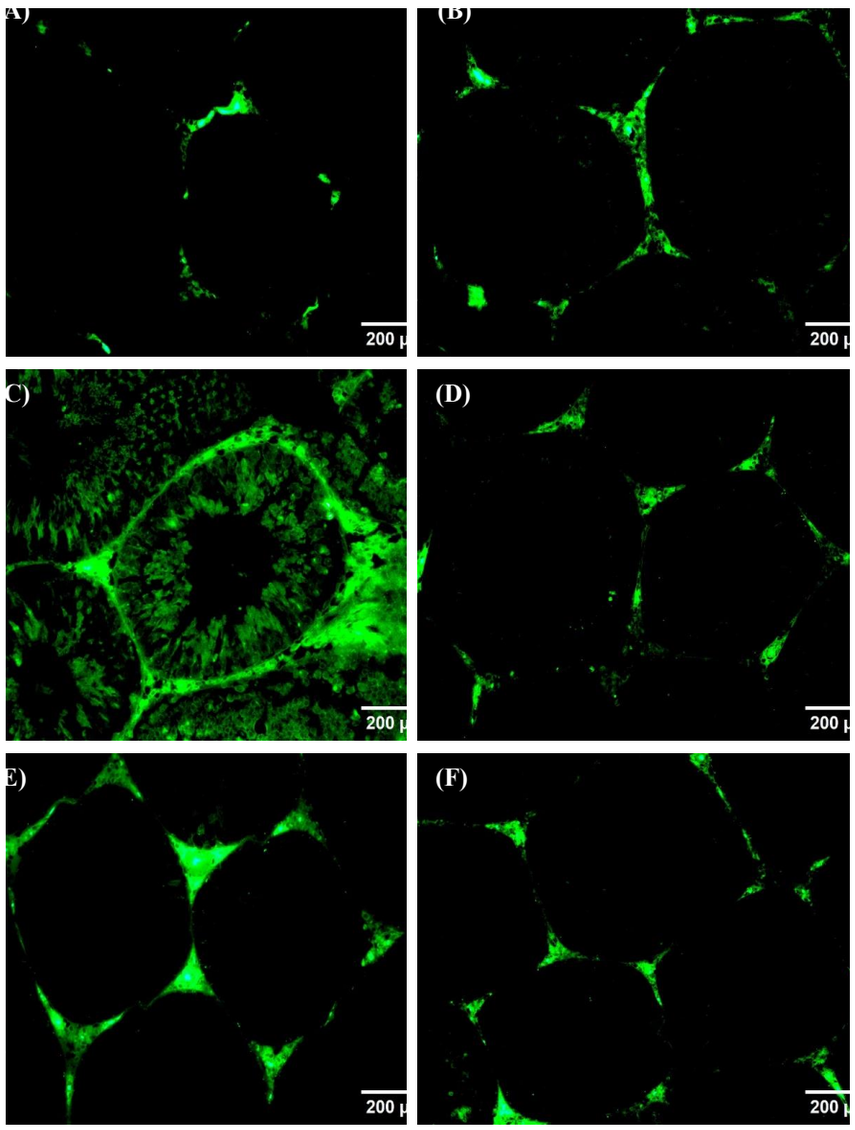

FIGURE 6. Immunofluorescence staining in rat testes by using Annexin V-FITC with excitation and emission wavelengths of 490 and 525 nm from (A) control group; (B) sham group; (C) Busulfan group; (D) Busulfan+EBN 250 group; (E) Busulfan+EBN 500 group; (F) Busulfan+EBN 1000 group (Scale bar = 200 µm)

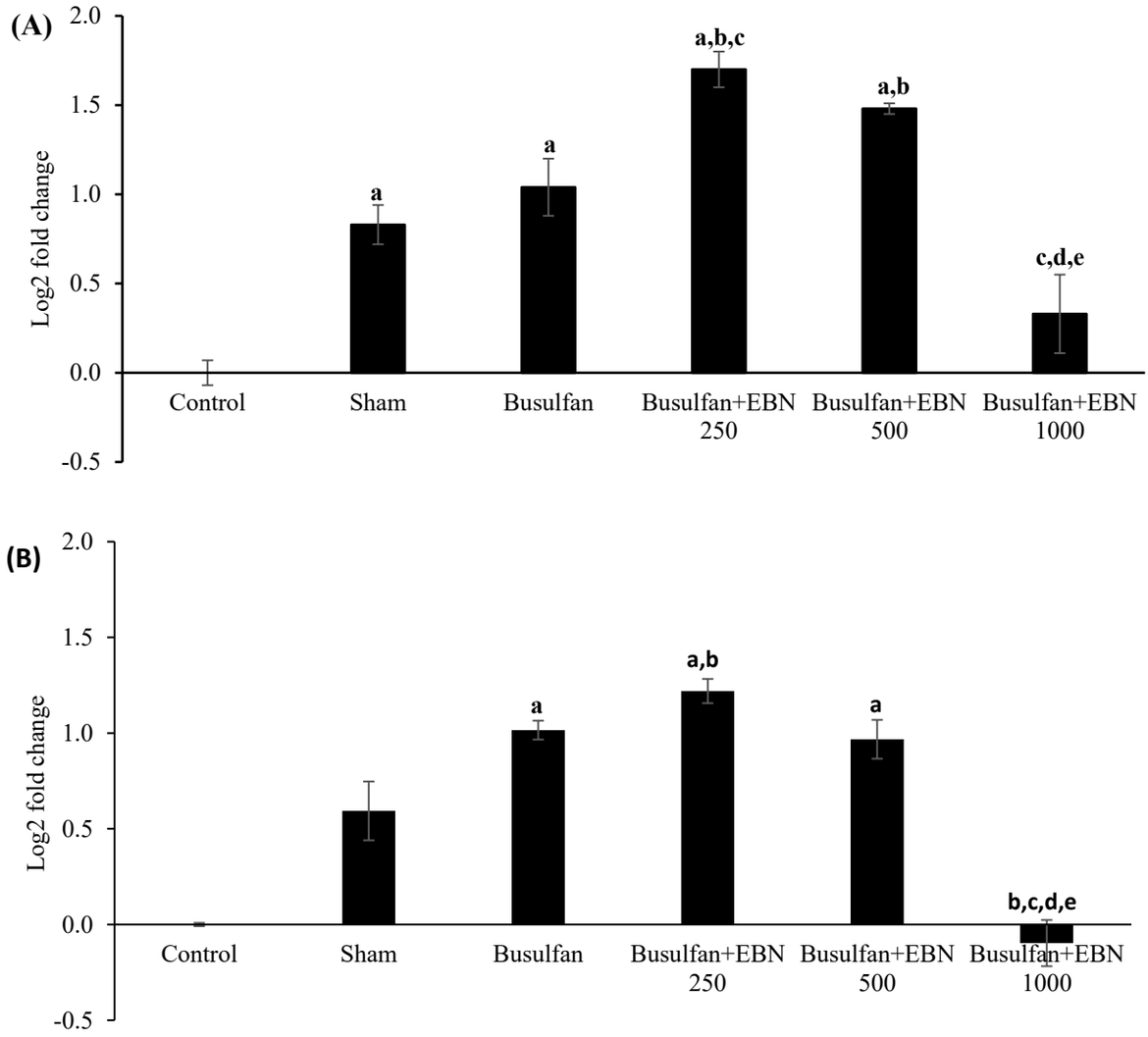

FIGURE 7. Effects of EBN supplementation on (A) $\log_2$ fold change of p38 and (B) $\log_2$ fold change of MAPK14 gene expression in each group. The values are expressed as fold change in relative to the control (0 baseline) and mean ± SEM. * $p < 0.05$ compared to the control. [a]significant difference compared with the control group, [b]significant difference compared with the sham group, [c]significant difference compared with the Busulfan group, [d]significant difference compared with the Busulfan+EBN 250 group, and [c]significant difference compared with the Busulfan+EBN 500 group

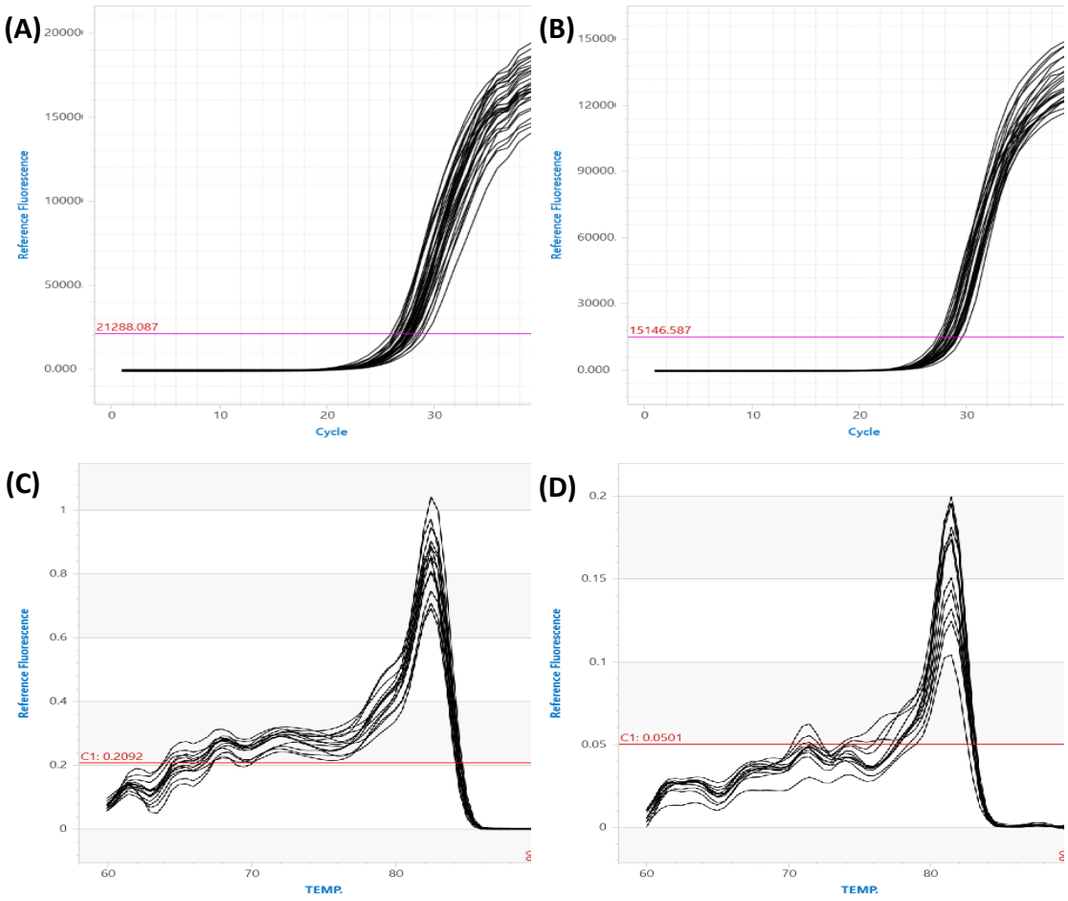

FIGURE 8. Amplification curve and melt curve of gene expressions of p38 and MAPK14 from (A) Amplification curve of p38; (B) Amplification curve of MAPK14; (C) Melt curve of p3; (D) Melt curve of MAPK14

CONCLUSIONS

The present study contributes substantially to EBN's potential to improve oligospermia in men. The selected doses of EBN studies were found safe to apply as they preserve sperm quality, protects testicular tissue structure, and reduces apoptosis activity.

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