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Black Mulberry (*Morus nigra* L.) juice has positive effects on spermatological parameters in male New Zealand White rabbits

ÖZLEM YILDIZ-GULAY, MEHMET SUKRU GULAY

Department of Physiology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, 15300, Burdur, Turkey

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Yıldız-Gulay Ö., Gulay M. S.

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Summary

The current study aimed to investigate the possible effects of black mulberry juice (BMJ) consumption on spermatologic parameters, oxidant/antioxidant activity, and seminal plasma testosterone concentrations of male rabbits. Twenty-four healthy adult New Zealand White rabbits were randomly divided into 4 groups (n = 6). By oral gavage, 3.0 mL of distilled water, 0.75 mL BMJ + 2.25 distilled water, 1.50 mL BMJ + 1.5 mL distilled water, and 3 mL BMJ per 1 kg body weight were given daily for 9 weeks to rabbits in the C, LD, MD, and HD groups, respectively. Ejaculates were taken at the beginning and end of the experiments. Progressive motility, sperm concentration, and intact sperm rates were significantly increased especially in MD and HD groups. A significant decrease in malondialdehyde concentrations was apparent due to BMJ consumption. Moreover, there was a marked increase in catalase and glutathione peroxidase levels in the seminal plasma of the MD and HD groups. The current results indicated that, although seminal plasma protein levels and seminal plasma glutathione levels were not altered, overall oral BMJ treatments were able to improve sperm quality and antioxidant states of the male rabbits.

Keywords: antioxidants, reproduction, sperm concentration, sperm motility, testosterone

Black mulberry is a fruit species that can grow in temperate, tropical, and subtropical environments due to its high adaptability to different climatic and soil conditions. Mulberry (*Morus* spp.) belongs to the genus *Morus* of the family *Moraceae* of the order *Rosales* (39). Zhou and Gilbert (41) reported 16 species in Morus, whereas Koidzumi classified the genus Morus into 24 species in 1917 (19). Although as many as 68 species have been reported in Morus, nowadays only 10 to 16 are generally recognized (38). Among them, *M. alba* (white and purple mulberry), *M. nigra* (black mulberry), *M. rubra* (red mulberry), *M. australis*, *M. latifolia*, *M. multicaulis*, *M. ihou*, *M. Kagayamae*, *M. bombycis* are the mulberry species that their fruits are widely utilized and cultivated (35).

Black mulberry has a wide distribution in the world. Especially, Turkey, Northern Iran, Azerbaijan, Armenia, Syria, and China are some of the countries where black mulberry is grown (12). In the Anatolian region, the harvest period of black mulberry is between June and August (11). Black mulberry fruits are 2-3 cm long (11), juicy with a dark purple color, and have a unique slightly sour taste (24). The main components of the fruit are sugar (fructose 48%, glucose 52%) and organic acids such as citric (92%) and malic acid (8%). It is a high source of vitamins (e.g. vitamin A, ascorbic acid, and thiamin), minerals (e.g. Ca, P, K, and Fe), phenolic acids, and anthocyanins (11).

The interest in fruits with high antioxidant capacity and rich in anthocyanins and products produced from these fruits is increasing worldwide (12). Black mulberry also belongs to the preferred fruits in terms of its high anthocyanin and other phytochemical contents. Numerous studies have also proven the antioxidant properties of *M. nigra* by using *in vitro* methods (7, 10, 32). Black mulberry has exerted a broad range of biological and therapeutical activities including antidiabetic, anti-inflammatory, antimicrobial, and anticancer effects (22). It is also protective for several organs due to its antioxidant potential (22). These findings strongly suggest that *M. nigra* can be used as a promising natural source to prevent various health problems and improve male fertility.

Antioxidants are known to suppress reactive oxygen species (ROS) and lipid peroxidation (16) and many types of antioxidants have been used as ROS

scavengers to improve sperm characteristics (30). Anthocyanins constitute the main flavonoid group in black mulberry and there is a direct relationship between antioxidant activity and flavinoid content in this fruit (11). Flavonoids are very potent antioxidants that reduce oxidative damage in testicular tissue and improve semen quality (40). Due to its high flavonoid content, *M. nigra* could be a good candidate to ameliorate sperm quality; therefore, the current study examined the possible positive effects of *M. nigra* on some spermatological and oxidant/antioxidant parameters in male rabbits.

Material and methods

The current study was approved by Hatay Mustafa Kemal University, Local Ethics Committee for Animal Experiments (2022/04-12). In the first phase of the study, 24 adult male New Zealand rabbits aged 10-12 months with an average weight of 2680-3045 g were used. Male rabbits were obtained from the same place and were not used in any previous study prior to the current experiment. They were kept in the Experimental Research Application and Research Center in laboratory conditions throughout the experiment.

Animals were kept in galvanized cages with 1 rabbit in each cage. Rabbits were fed with standard pellet feed containing 9% crude ash, 20% crude protein, 14% crude cellulose, 0.5% calcium, 0.5% phosphorus, and 0.2% sodium (Mirisan Feed and Oil Industry, Hatay, Turkiye). Rabbits had free access to feed and water. The environment of the rabbits was 50-55% humidity, 22 ± 2°C temperature, and 14 : 10 hours light : dark cycle throughout the study. The general behavior, alertness, appetite, and presence of nasal discharge were controlled throughout the experiment. Moreover, the body temperature and respiratory rate of the rabbits were checked by a veterinarian when necessary.

Experimental protocol. Prior to the experiment, rabbits were allowed to acclimatize to the laboratory setting for 10 days. The rabbits were also trained to ejaculate in the artificial vagina during this acclimatization period.

The experimental phase of the study lasted 9 weeks (49 days for one spermatogenesis period + 14 days for sperm storage and transfer period in the epididymis). At the start of the second phase, New Zealand rabbits were divided into 4 treatment groups (n = 6/group); group 1 (control group – C; 3.0 mL distilled water per 1 kg body weight), group 2 (low dose black mulberry group – LD; 2.25 mL water per 1 kg body weight containing 0.75 mL black mulberry juice – BMJ), group 3 (medium dose black mulberry group – MD; 1.50 mL of water containing 1.50 mL of BMJ for 1 kg body weight), group 4 (high dose black mulberry group – HD; 3.0 mL/kg BMJ for 1 kg body weight. According to the protocol, all rabbits in the groups were given a total of 3 mL of liquid (water and/or BMJ water mixture) per kg body weight orally daily throughout the study. The treatment doses were chosen based on the doses used in a previous study in the literature on the spermatologic effects of pomegranate juice in rats (36). Oral gavages were given daily between 8:00 and 9:00 hr. All rabbits were weighed weekly, changes in weight levels were noted and doses were adjusted accordingly.

Black mulberry samples were collected from Eğirdir, Isparta/Turkiye region from a single tree. All fruit samples were selected for shape and color uniformity and picked at the commercially ripe stage. The 100 grams of samples were individually packaged in polyethylene bags, rapidly frozen at –20°C, and kept at this temperature until their use. 15 minutes before each oral gavage administration, the frozen black mulberries were minced in cheesecloth to extract the juice and immediately used for oral gavage administration.

Spermatological evaluation. The ejaculate samples were collected directly through the artificial vagina into graduated and warmed glass tubes. After the female was brought to the male rabbit, the time taken by the male rabbit to ejaculate was recorded as response time. Volume and weight were measured after removing the gel portion of the ejaculate. Ejaculate volume, ejaculate weight, sperm pH, sperm concentration, abnormal sperm percent, and motility were measured from ejaculates collected at the beginning (at day 1) and end of the experiment (at day 63) according to the previously used protocol (5).

After the measuring spermatological parameters, the remaining ejaculates were centrifuged at 2000 rpm for 20 minutes to separate the seminal plasma and 10 µL of the seminal plasma was dropped into a refractometer (Atago, SPR-N, Japan) and total protein in seminal plasma were recorded. The remaining seminal plasma samples were kept at –5°C for testosterone analysis.

Oxidant and antioxidant parameters. At the end of the experimental period, rabbits were euthanized under isoflurane anesthesia. Following euthanasia, the left testis and epididymis were removed, washed with saline at 5°C, and weighed. The left testis was stored at –80°C for the determination of some oxidant-antioxidant parameters.

Testicular tissue removed from the deep freezer was weighed and transferred to glass tubes while maintaining their cold temperatures. A buffer containing 1.15% potassium chloride was added to the samples as a 1/10 dilution. Next, the tissues were homogenized in a homogenizer at 16,000 rpm for 3 minutes, again maintaining their cold temperatures. Malondialdehyde (MDA) was determined in a portion of these homogenates. MDA levels in tissues were calculated based on the reaction of MDA, one of the aldehyde products of lipid peroxidation, with thiobarbituric acid (25). The glutathione peroxidase (GSH-Px) activity was measured spectrophotometrically at 340 nm by reading the difference in absorbance during the oxidation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP) (21). The tissue activity of the catalase (CAT) was determined by calculating the amount of substrate at a wavelength of 240 nm (1). Glutathione (GSH) level was calculated by the method of Sedlak and Linsay (29) and was measured spectrophotometrically at 412 nm.

Seminal Plasma Testosterone. Rabbit-specific ELISA kits were used for the determination of seminal plasma testosterone levels (BT-LAB E0039Rb Rabbit Testosterone ELISA Kit, Intra-Assay: CV < 8%, Inter-Assay: CV < 10%). Hormone analysis was performed according to the manufacturer's instructions (Bioassay Technology Laboratory, China).

Statistical analysis. All values are given as mean ± SD. PROC ANOVA procedure of SAS statistical program was used for statistical evaluation. Results with statistical differences were compared with the Tukey test. In all statistical applications, the difference between the groups was considered significant when $P < 0.05$.

Results and discussion

The general health status of the rabbits was controlled throughout the study and no clinical problems were seen in the rabbits during the experiment. In addition, no problems were encountered such as infections, loss of appetite, weakness, alertness to the surroundings, and dehydration markers. Overall, there were no notable changes among the groups for the parameters tested from samples taken at the beginning of the experiment; spermatological parameters, seminal plasma testosterone levels, and response time were similar for rabbits in the different treatment groups (Tab. 1). Likewise, none of the BMJ doses had significant effects on the testis or epididymis weights of rabbits during the 9-week period when compared to controls. In addition, ejaculate volume, ejaculate weight, ejaculate pH, seminal plasma proteins, and response time were not altered due to treatment (Tab. 2).

Effects of BMJ on progressive motility, sperm concentration, and normal-abnormal sperm percent are presented in Table 2. Both medium and high doses of the treatment resulted in a significant increase in progressive motility when compared to the rabbits in the C group ($P < 0.01$), whereas the change in sperm motility was not significant between C and LD groups. Although LD and MD did not cause significant changes in sperm concentration when compared to C, HD treatment was able to increase sperm concentration over the C group ($P < 0.05$). Moreover, sperm quality was positively changed due to BMJ treatment; intact spermatozoa rates were higher for rabbits in MD and HD groups compared to the C group ($P < 0.02$). Although the frequency of tail abnormalities was not affected by any of the treatments, the frequency of head abnormalities was lower in MD and HD groups ($P < 0.01$).

Testicular MDA, CAT, GSH-Px, and GSH levels tested in the current experiment were in Table 3. High and medium BMJ doses resulted in decreased MDA levels compared to C ($P < 0.04$). While none of the BMJ doses resulted in a significant alteration in the tissue GSH-Px levels, both medium and high-dose BMJ elevated the tissue GSH and CAT activities when compared to controls ($P < 0.02$).

Recently, our knowledge of mammalian infertility has been increasing, and our knowledge of

Tab. 1. Some initial reproductive parameters (mean ± SD) of male New Zealand White rabbits prior to the experiment

	C	LD	MD	HD	P <
Ejaculate volume (ml)	0.72 ± 0.06	0.71 ± 0.07	0.77 ± 0.08	0.70 ± 0.06	NS
Ejaculate weight (mg)	0.74 ± 0.05	0.75 ± 0.05	0.79 ± 0.07	0.72 ± 0.07	NS
Progressive motility (%)	57.7 ± 3.86	59.4 ± 2.89	57.5 ± 4.27	59.2 ± 3.37	NS
Ejaculate pH	7.25 ± 0.18	7.18 ± 0.15	7.25 ± 0.16	7.27 ± 0.17	NS
Sperm concentration (× 10^6/ml)	263.2 ± 38.5	243.0 ± 19.2	252.5 ± 37.4	241.5 ± 34.1	NS
SP Proteins (g/dl)	3.20 ± 0.17	3.18 ± 0.11	3.13 ± 0.15	3.15 ± 0.15	NS
Intact spermatozoa (%)	79.0 ± 1.67	77.8 ± 2.22	78.3 ± 2.02	80.0 ± 1.96	NS
Head defect (%)	11.5 ± 1.37	11.3 ± 1.50	11.5 ± 1.76	10.2 ± 1.17	NS
Tail defect (%)	9.33 ± 1.22	10.8 ± 1.47	10.0 ± 1.49	9.33 ± 1.41	NS
SP Testosterone (ng/ml)	24.56 ± 6.97	24.00 ± 4.70	22.71 ± 8.20	23.85 ± 8.43	NS
Response time (sec)	13.83 ± 1.47	12.66 ± 2.73	12.3 ± 1.63	11.5 ± 2.07	NS

Explanations: C – control; LD – low dose mulberry dose group; MD – medium dose mulberry group; HD – high dose mulberry group, SP – seminal plasma, NS – not significant

Tab. 2. Some reproductive parameters (mean ± SD) of male New Zealand White rabbits at the end of the experiment

	C	LD	MD	HD	P <
Ejaculate volume (ml)	0.73 ± 0.34	0.85 ± 0.09	0.88 ± 0.07	0.84 ± 0.06	NS
Ejaculate weight (mg)	0.79 ± 0.12	0.87 ± 0.08	0.91 ± 0.09	0.90 ± 0.08	NS
Progressive motility (%)	63.3^{a} ± 3.21	66.0^{ab} ± 3.84	70.0^{bc} ± 3.16	71.3^{c} ± 3.01	0.01
Ejaculate pH	7.32 ± 0.19	7.13 ± 0.16	7.20 ± 0.14	7.30 ± 0.28	NS
Sperm concentration (× 10^6/ml)	276.3^{a} ± 24.3	293.8^{ab} ± 17.6	308.1^{ab} ± 28.9	330.2^{b} ± 44.8	0.05
SP proteins (g/dl)	3.26 ± 0.32	3.31 ± 0.21	3.26 ± 0.12	3.33 ± 0.27	NS
Intact spermatozoa (%)	83.0^{a} ± 2.60	83.0^{a} ± 2.76	87.0^{b} ± 2.75	86.8^{b} ± 3.12	0.02
Head defect (%)	9.83^{a} ± 1.16	8.33^{ab} ± 1.20	6.50^{b} ± 1.37	7.00^{b} ± 2.28	0.01
Tail defect (%)	7.16 ± 1.83	8.66 ± 1.96	6.50 ± 1.64	6.16 ± 1.16	NS
Left testis weight (g)	2.80 ± 0.40	2.96 ± 0.17	2.98 ± 0.14	3.08 ± 0.16	NS
Left epididymis weight (g)	0.86 ± 0.11	0.86 ± 0.07	0.88 ± 0.11	0.89 ± 0.12	NS
SP Testosterone (ng/ml)	35.09 ± 8.13	34.91 ± 4.53	35.8 ± 4.69	34.5 ± 4.03	NS
Response time (sec)	8.83 ± 2.78	9.33 ± 1.21	8.50 ± 1.04	7.83 ± 1.72	NS

Explanations: C – control; LD – low dose mulberry dose group; MD – medium dose mulberry group; HD – high dose mulberry group, SP – seminal plasma, NS – not significant. Values in the same line with different letters are significantly different

Tab. 3. The effects of black mulberry administration on some oxidant-antioxidant parameters (mean ± SD) in testicular tissues of New Zealand White Rabbits

	C	LD	MD	HD	P <
MDA (nmol/g protein)	$4.60^{a} \pm 1.09$	$4.27^{ab} \pm 0.97$	$3.31^{b} \pm 0.64$	$3.33^{b} \pm 0.72$	0.04
CAT (IU/g protein)	$85.8^{a} \pm 6.41$	$91.1^{ab} \pm 3.90$	$92.2^{b} \pm 5.16$	$95.9^{bc} \pm 3.02$	0.02
GSH-Px (IU/g protein)	22.2 ± 3.54	23.4 ± 2.81	26.3 ± 4.08	27.1 ± 4.85	NS
GSH (nmol/g protein)	$1.47^{a} \pm 0.43$	$1.50^{a} \pm 0.32$	$1.90^{b} \pm 0.27$	$2.02^{b} \pm 0.21$	0.02

Explanations: C – control; LD – low dose mulberry dose group; MD – medium dose mulberry group; HD – high dose mulberry group, NS – not significant. Values in the same line with different letters are significantly different. MDA – malondialdehyde; CAT – catalase; GSH-Px – glutathione peroxidase; GSH – glutathione

oxidative stress has led to the development of many new therapeutic approaches (37). Although there are many antioxidants that have the potential to reduce oxidative stress and improve sperm quality (30), there is not enough scientific evidence for their efficacy. The results of the current study showed for the first time that daily BMJ intake for 63 days caused an increase in progressive motility, sperm concentration, intact sperm percent, and a decrease in the proportion of abnormal sperm due possibly to decreased oxidative stress in male rabbits.

All cellular components such as lipids, proteins, nucleic acids, and sugars are targets for oxidative stress (20). The degree of damage due to oxidative stress strongly depends on the number and the duration of exposure to free oxygen radicals (16). Unsaturated fatty acids are the most vulnerable molecules to oxidation in the sperm plasma membrane. Reactive oxygen radicals can attack unsaturated fatty acids in the cell membrane and initiate a chemical chain called lipid peroxidation (4).

Increased levels of ROS have been associated with decreased sperm motility (2). According to one theory, H_2O_2 can cross the sperm cell membrane and interferes with the function of some vital enzymes such as glucose-6-phosphate dehydrogenase via the hexose monophosphate shunt (3). This allows the intracellular production of NADPH, which is then used by the enzyme system known as NADPH oxidase as an electron source to enhance free oxygen radicals production by spermatozoa (3). Another hypothesis is based on a series of events that result in the phosphorylation of axonemal proteins and a decrease in sperm motility, both of which cause a decrease in membrane fluidity that is essential for sperm-oocyte fusion (20). The decrease in spermatozoa motility in samples kept overnight is closely related to lipid peroxidation (14) and studies with antioxidants such as vitamin E provide evidence that antioxidants can protect the sperm membrane from lipid peroxidation and preserve sperm motility for a longer time (17).

Oxidative stress often causes DNA damage in the sperm cell, and ROS can also cause mutations that reduce semen quality (18, 31). Increased reactive oxygen levels are associated with increased DNA fragmentation and decreased DNA methylation (34). Infertile men with high levels of free oxygen radicals have been shown to carry more chromosomal fragmentations than fertile men (9).

Black mulberry fruit may have important effects on human health and nutrition due to its antioxidant capacity (15). The fruit is rich in vitamin A, ascorbic acid, phenolic compounds, and anthocyanin pigments. Phenolic compounds attract attention due to their high antioxidant properties in the prevention of degenerative diseases (32). Anthocyanins are an important group of water-soluble phenolic pigments that prevent free radicals (18). Anthocyanins are compounds that can be used against various diseases (such as oral and dental diseases, hypertension, diabetes, anaemia, cardiovascular diseases, and cancer) because of their antioxidant capacity (23, 32). It has been stated that cyanidin prevents oxidative damage caused by hydroxyl radicals and thus protects DNA from oxidative damage (26). In the present study, significant decreases in MDA levels, a by-product of lipid peroxidation, and significant increases in GSH, GSH-Px, and CAT activities were observed in testicular tissue samples of rabbits administered different doses of BMJ. These findings indicate that BMJ has a strong anti-oxidative effect on the male reproductive system and preserve sperm cell from possible oxidative damage.

In recent studies, some antioxidants or natural products with strong antioxidant properties had beneficial spermatological effects in male Zealand White rabbits (5, 13). In the current study, the significant improvements observed in sperm count, progressive motility, and ejaculate quality may be attributed to the prevention of the overproduction of free radicals due to the antioxidant properties of BMJ. Thus, it can be concluded that there is a positive correlation between BMJ intake and sperm parameters, and oral BMJ could be a good candidate for preventing oxidative stress in the male reproductive system. The possible effects of BMJ in other tissues and organs are worth investigating.

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Corresponding author: Prof. Dr. Mehmet Sukru Gulay, Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Physiology, Burdur, Turkey