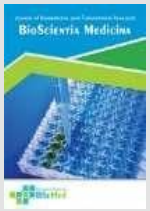
eISSN (Online): 2598-0580



Bioscientia Medicina: Journal of Biomedicine & Translational Research

Journal Homepage: www.bioscmed.com

The Effect of Combination of Green Tea and Roselle Extract on Morphology and Motility of Spermatozoa in Rats Induced by Monosodium Glutamate

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ARTICLE INFO

Keywords:
Morphology of spermatozoa
Motility of spermatozoa
Monosodium glutamate
Green tea extract
Roselle extract

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All authors have reviewed and approved the final version of the manuscript.

https://doi.org/10.37275/bsm.v6i9.576

A B S T R A C T

Background: Infertility is a reproductive system problem and has been increasing recently. One of the reasons is the poor quality of spermatozoa, which can be assessed through its morphology and motility. Excessive consumption of MSG can affect the quality of spermatozoa. The provision of a combination of green tea and roselle extract, which is an antioxidant and is expected to be able to counteract the effects of MSG's free radicals on spermatozoa. The objective of the study is to determine the morphology and motility of Wistar Rats' spermatozoa in the control group given MSG and in the experimental group given MSG and a combination of green tea and roselle extract at a dose of 200 mg/kg/day and 400 mg/kg/day for 14 days. **Methods:** Fifteen Wistar rats were randomly divided into three groups, a control group with MSG, group 1 with MSG and a combination of green tea and roselle extract at a dose of 200 mg/kg BW/day (P1), and group 2 with MSG and combination green tea and roselle extract at a dose of 400 mg/kg BW/day (P2). After 14 days of treatment, the morphology and motility of the spermatozoa of each mouse were studied and assessed microscopically. **Results:** In the morphological study, the control group had the lowest normal value and the highest abnormal value among all groups. Meanwhile, the P1 group had the highest normal and lowest abnormal values compared to the other two groups. **Conclusion:** The administration of a combination of green tea and roselle extract improved the morphology and motility of Wistar rats' spermatozoa. However, an increased dose of a combination of green tea and roselle extract was not proven to affect the morphology and motility of Wistar rats' spermatozoa.

1. Introduction

Green tea is one of the most popular drinks in the world and is widely consumed, especially in Asian countries. Green tea extract comes from the dried leaves of the tea plant (*Camellia sinensis*). The chemical composition of dried green tea extract is similar to fresh leaves extract, in contrast to black tea, which requires a fermentation process. Green tea consumption has been reported to reduce the risk of developing several types of tumors in Asian countries, especially gastrointestinal tumors. Current data suggest that the main mediator of this chemo-preventive effect is the presence of epigallocatechin-3-gallate in green tea.[1] Epigllocatechin-3-gallate (EGCG) is the most abundant polyphenol found in green tea, representing 67% of the total polyphenols in tea extract. These polyphenols have a role in a variety of chemical and biological activities in the body, including acting as an antioxidant, anti modulation of

carcinogen metabolism, inhibiting tumor growth, cell proliferation, and cell cycle arrest, induction of apoptosis, inhibiting invasion and metastasis, and inhibition of angiogenesis.[1]

Roselle flower plant (*Hibiscus sabdariffa*) is also believed to have antioxidant effects. This plant is a shrub that is usually used for making jellies, jams, and drinks. Its brilliant red color and unique taste make it a popular food product. The anthocyanin pigment is responsible for its color, so it is widely used as a food coloring. Recently, the biological activity of anthocyanins was believed to have antioxidant-like activity and provide protection against atherosclerosis. Anthocyanins were also found to be many times more potent than common antioxidants such as ascorbate.[2] Roselle flowers have the highest concentration of epigallocatechin-3-gallate, about 78%, whilst also containing a high amount of anthocyanins and type 3 collagen. This flower is used for making a combination of green tea and roselle extract products.[1,2] Combination of green tea and roselle extract is a herbal beverage product composed of natural ingredients. Hence, no side effects were found, and it is safer to consume and at an economical price range.

Free radicals and antioxidants have a positive correlation, in which an increased free radical concentration requires more antioxidants to neutralize.[3] The interest in antioxidant research and development has increased in recent years, along with research on free radicals. Free radicals or often called reactive oxygen species (ROS), are an atom or molecule that has an unpaired chain of electrons in their outer layer. Free radicals that enter the body are highly reactive and, in higher amounts, could be caused tissue damage. Free radicals can be divided into endogenous sources, such as autoxidation in the body, as well as exogenous sources, such as foods with high MSG content.[3] Monosodium glutamate (MSG) is a white crystalline powder that is widely used as a food enhancer and flavoring agent in various countries.[4] This component of MSG consists of 78% free glutamic acid, which binds to 12% sodium ions and 10% water, forming a sodium salt of L-glutamic acid. In general, the free form of glutamate is low in the daily diet, so it is necessary to add additional spices to enhance the taste.[4] The use of MSG throughout the world, including in Indonesia, is considered to be excessive, with a dose of 100-300 mg. This overuse of MSG has various toxic effects on various mammalian tissues, including male reproductive systems. Male infertility, testicular bleeding, changes in sperm production and morphology, reduced growth, obesity, and hypogonadism are the most frequent changes reported in cases of male infertility after MSG administration.[5,6]

Infertility or the inability to conceive in couples who are sexually active for a period of one year without using contraception is currently increasing. The infertility rate continued to increase from 4.2 million cases in 1990 to 48.5 million cases in 2010. In Asia, the prevalence of infertility has reached 8-12%, of which 40% are caused by male infertility. In Indonesia, the infertility rate has increased by around 15-20% from 50 million couples.[5,6] Low semen quality, azoospermia, and wrong intercourse are some of the causes of infertility.[5] These results indicate that sperm fluid plays an important role in the incidence of infertility. Seminal fluid can be assessed both macroscopically and microscopically. Macroscopically, the assessment of seminal fluid can be done by examining pH, coagulation, color, viscosity, odor, and semen volume. Meanwhile, microscopically, the assessment is based on spermatozoa concentration, motility, morphology, and agglutination.[7] Poor seminal fluid quality could increase the risk of infertility. This quality could be influenced by various factors, both internal and external. Internal factors include age, weight, hormones, genetics, congenital or acquired urogenital abnormalities, endocrine disorders, and immunological factors. Meanwhile, external factors include food, temperature, work, and lifestyle.[8] Unhealthy foods, such as foods that contain a lot of MSG and are consumed continuously, could increase free radical levels in the body and cause harmful effects on the quality of spermatozoa. Excessive consumption of MSG could increase levels of free radicals, which could cause oxidative stress to the

testes, resulting in spermatozoa cell damage. MSG can induce oxidative stress through the production of oxygen radicals and hydrogen peroxide, which causes oxidative DNA damage and cell membrane peroxidation, resulting in abnormal morphology to cell death. In addition, the reaction between MSG and saturated fatty acids in the spermatozoa cell membrane increases lipid peroxidase.[4] The continuous and excessive formation of lipid peroxidase could damage the mitochondrial DNA contributing to the change of spermatozoa motility.[9] An impairment in spermatozoa motility causes failure of penetration to the ovum cells, thus contributing to infertility.[10] The study would like to explore the effect of a combination of green tea and roselle extract on the morphology and motility of spermatozoa in Wistar rats exposed to MSG.

2. Methods

An experimental study with a post-test-only control group design was conducted by forming an experimental group and a control group, and the outcome data were recorded. The study was conducted on 15 Wistar rats (5 rats in each group), according to the sample amount calculations based on WHO guidelines and the Institutional Animal Care and Use Committee Guidebook. This study has been approved by the ethical committee of the Medical Faculty, Universitas Diponegoro, Semarang, Indonesia (No. 102/EC/H/FK-UNDIP/X/2020).

The research and data collection was done for 4 weeks, from August to September 2020. Experiments and tissue sample collections were conducted at the Animal Laboratory of Universitas Setya Budi Solo, while morphology and motility tests were done by analysts at the Laboratory of the Universitas Setya Budi Solo. MSG was prepared at a dose of 400mg / 200gBW / day diluted with distilled water to a volume of 1 ml, while a combination of green tea and roselle extract products was prepared at a dose of 200 mg/kg BW / day dissolved with distilled water up to 1 ml volume for experimental group 1, and Combination green tea and roselle extract dosage 400 mg/kg BW / day dissolved with distilled water up to 1 ml volume for experimental group 2. Then, the 2 white Wistar rats were divided into 3 groups randomly as follows: the control group (K) was given MSG solution at a dose of 400mg / 200gBW / day dissolved in 1 ml of distilled water for 14 days; the experimental group 1 (P1) was given MSG solution at a dose of 400mg / 200gBW / day dissolved in 1 ml of distilled water and Combination green tea and roselle extract at a dose of 200 mg/kg BW / day dissolved in 1 ml of distilled water for 14 days; experimental group 2 (P2) was given MSG solution at a dose of 400mg / 200gBB / day dissolved in 1 ml of distilled water and Combination green tea and roselle extract at a dose of 400 mg/kg BW / day dissolved in 1 ml of distilled water for 14 days.

The data was collected once a day in the morning for 14 days. On day 15 the rats were terminated with chloroform, then a cut was made from the cauda epididymis until the ampulla of the vas deferens. Furthermore, sequencing and dilution were conducted in order to obtain the mouse sperm. The epididymis was placed in a petri dish that contained 0.9% NaCl solution. Then the epididymis was scrubbed with a scalpel to get the spermatozoa. Then the spermatozoa samples were examined for morphology and motility. To examine the morphology, the Rats' sperm was put into a petri dish containing a solution of 0.9% NaCl. NaCl solution was used because it is an isotonic solution that keeps cells from being damaged. Two drops of seminal fluid were used and then flattened with another glass object. Furthermore, the object-glass containing the specimen was dried and then added methanol and dried again. Then the slide is dripped with eosin Y and dried. Eosin Y is used to dye the cytoplasm pink. If there is excess color, rinse with distilled water. Distilled water was used to rehydrate specimens and facilitate staining that requires water. Then the slide was dyed with methylene blue and waited to dry. Methylene blue is used to dye the nucleus blue. Furthermore, the sperm morphology was observed under a microscope with a magnification of 400 times, and the percentage of normal and abnormal morphology was calculated.

To examine the motility, sperm are collected in a petri dish containing 0.5 µL of 0.9% NaCl solution. Sperm were taken from a petri dish using a pipette. Sperm are placed on a glass object and then covered with a deck glass. Then the object-glass is placed under a microscope and examined at 40x magnification. Observations were made in five fields of view with the following motility criteria: Criteria a (spermatozoa is moving fast and forward), criteria b (spermatozoa is moving slowly forward), criteria c (spermatozoa is only moving on the spot), criteria d (spermatozoa is not moving). Spermatozoa motility was divided into two groups, progressive (if the spermatozoa meet criteria a and b) and not progressive (if the spermatozoa meet criteria c and d).

Data analysis was performed using computer software. The data were tested for normality by using the Shapiro-Wilk test, then continued with statistical testing. If the results of the Shapiro-Wilk test show normal distribution data, the chosen statistical test is ANOVA, whereas if the data is not normally distributed, the chosen statistical test is the non-parametric Kruskal-Wallis test. Data is considered significant if the p-value is ≤ 0.05. If the test results show that H0 fails to be rejected (there is no difference between groups), then a post hoc test is not carried out, whereas if a difference is found, a post hoc test will be carried out. The post hoc test performed depends on the results of the Test of Homogeneity of Variances; If the same variance is obtained (significance value> 0.05), then a post-hoc test will be carried out, whereas if a different variance is obtained (significance value 0.05) then a post-hoc test with Mann-Whitney is obtained.

3. Results

Figure 1 shows the histopathological description of spermatozoa morphology. Figure 1a shows the histopathological picture of spermatozoa morphology in the control group that received MSG. Figure 1a shows visually that spermatozoa with normal morphology are found in quite a few, less than 30%. Figures 1b and c show visually that spermatozoa with normal morphology in groups P1 and P2 were found quite a lot, around 80-90%.

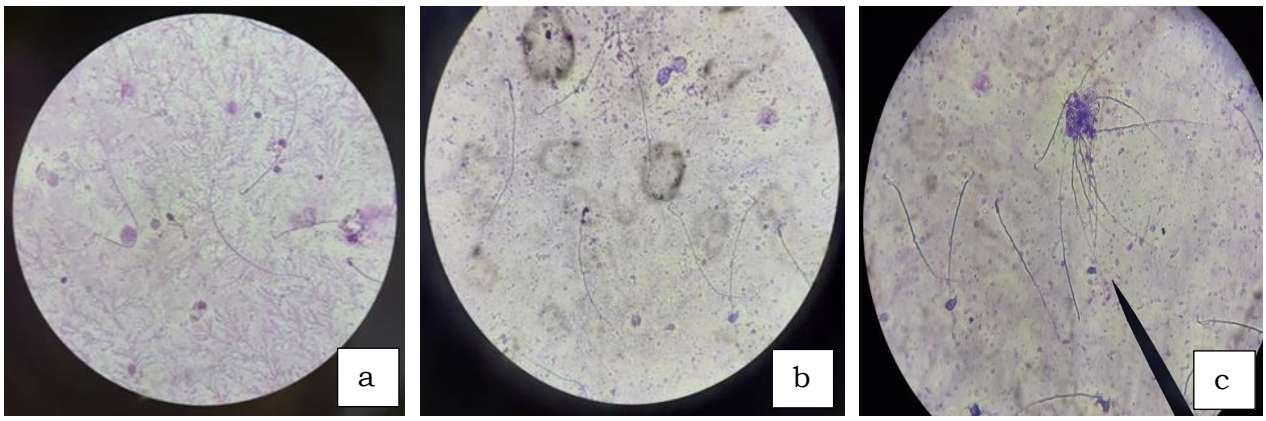

Figure 1. Histopathological picture of spermatozoa morphology. (a) The control group, MSG at a dose of 400 mg/200 g BW/day, (b) Group P1, MSG, and combination green tea and roselle extract 200 mg/kg BW, (c) Group P2, MSG and Combination green tea and roselle extract 400 mg/kg BW.

Table 1 shows the percentage of spermatozoa with normal morphology. Groups P1 and P2 showed that the percentage of spermatozoa with normal morphology was 3 times higher than the control group. Table 2 also further confirms statistically that the percentage of spermatozoa with normal morphology between groups P1 and P2 is much higher than in the control group. However, there was no significant difference in the percentage of spermatozoa with normal morphology between groups P1 and P2.

Table 1. Percentage of spermatozoa with normal morphology

Group	Mean ± SD	p
Control	27.70 ± 20.37	0.000*
P1	90.50 ± 2.92	
P2	87.50 ± 2.18	

*p<0.05, ANOVA test

Table 2. Post-hoc analysis of spermatozoa with normal morphology

Group	Control	P1	P2
Control		0.005*	0.006*
P1	0.005*		0.221
P2	0.007*	0.221	

*p<0,05, post-hoc analysis – Games Howell

Table 3 shows the percentage of spermatozoa with normal motility. Groups P1 and P2 showed that the percentage of spermatozoa with normal motility was 3 times higher than the control group. Table 4 also further confirms statistically that the percentage of spermatozoa with normal motility between groups P1 and P2 was much higher than in the control group. Table 4 also shows the significant difference in the percentage of spermatozoa with normal morphology between groups P1 and P2.

Table 3. Percentage of spermatozoa with normal motility

Group	Mean ± SD	p
Control	21.30 ± 9.91	0.006*
P1	81.80 ± 2.08	
P2	89.60 ± 0.96	

*p<0.05, ANOVA test

Table 4. Post-hoc analysis of spermatozoa with normal motility

Group	Normal	P1	P2
Control		0.000*	0.000*
P1	0.000*		0.001*
P2	0.000*	0.001*	

*p<0.05, post-hoc analysis – Games Howell

4. Discussion

Consuming a combination of green tea and roselle extract as an antioxidant can increase the normal value and reduce the abnormal value in the morphology of spermatozoa which has been given free radicals in the form of MSG. So, it can be concluded

that the combination of green tea and roselle extract affects the morphology of spermatozoa that have been exposed to MSG. However, the insignificant difference between the P1 and P2 groups indicated that whether or not spermatozoa morphology was normal or not was influenced by the dose of Combination of green tea and roselle extract given. The conclusion of this study, according to the other research, concluded that MSG administration could increase the abnormal morphology of spermatozoa.[11-13] MSG is a free radical in the body externally, which can affect spermatogenesis. Consuming MSG as much as 4mg/kg/day for 15 days was proven to cause spermatogenesis problems. If converted to humans, this dose corresponds to the provision of MSG of 22.165 mg/kg/day.[14-16] The mechanism of MSG as an oxidative stress inducer can be explained by the oxygen radicals and hydrogen peroxide produced. The production of these oxidizing agents, in turn, causes oxidative DNA damage and peroxidation of cell membranes. DNA and cell membrane damage is what affects the abnormal morphology leading to cell death. Excess MSG can result in hypothalamic damage, which results in decreased GnRH (gonadotropin-releasing hormone) secretion. Thus, the stimulation of LH (luteinizing hormone) and FSH (follicle-stimulating hormone) also decreased. The LH hormone acts on the Leydig cells to regulate the secretion of testosterone, while the FSH hormone affects the Sertoli cells, which synthesize androgen-binding protein (ABP) that binds to testosterone. Testosterone is used in the process of cleavage and maturation of spermatogonia into spermatozoa (spermiogenesis). This spermiogenesis disorder can result in a decrease in the morphology of normal spermatozoa. Apart from morphology, this study also examined the effect of a combination of green tea and roselle extract administration on the motility of spermatozoa following the consumption of MSG.

Antioxidants can increase the motility of spermatozoa that have been exposed to oxidants.[17-19] Consuming MSG continuously at excessive doses can increase levels of free radicals that can cause oxidative stress. Physiologically, ROS is produced during the spermatogenesis process, but its presence must be neutralized with antioxidants. ROS and antioxidant imbalance will cause cell damage, including spermatozoa cells. Oxidative stress on the testes can result in damage to spermatozoa cells. MSG reacts with saturated fatty acids on the spermatozoa cell membrane and increases lipid peroxidase.[4] The continuous and excessive formation of lipid peroxidase can damage mitochondrial DNA, which will interfere with sperm motility.[9] In addition, damage to cells in the spermatozoa's neck will result in damage to the mitochondria, causing insufficient energy supply to the tail and resulting in impaired sperm motility. Impaired spermatozoa motility cause failure of spermatozoa penetrations to the ovum cells, thus affecting the occurrence of infertility.[10] Research on the provision of Combination green tea and roselle extract on the morphology and motility of spermatozoa has never been done before. A combination of green tea and roselle extract is a herbal drink that is rich in antioxidants and is widely consumed by patients who have abnormal cell growth disorders, such as cysts or cancer. A combination of green tea and roselle extract content includes 78% green tea (*Camellia sinensis*), bovine collagen, and purple roselle (*Hibiscus sabdariffa*).

Green tea has high levels of polyphenols (catechins), about 30-40%. Catechins are water-soluble, colorless, and give a bitter taste. These catechin compounds consist of epicatechin (EC) 1-3%, epicatechin-3-gallate (ECG) 3-6%, epigallocatechin (EGC) 3-6%, and epigallocatechin gallate (EGCG) 7-13%. The four EGCG compounds are the most abundant antioxidants and have the strongest antioxidant effects. Polyphenols can capture hydroxyl group free radicals without oxidizing fat, protein, and DNA in cells. The ability of polyphenols to scavenge these free radicals is 100 times more effective than vitamin C and 25 times more effective than vitamin E.[4] Roselle flower petals contain vitamin C, vitamin A, and amino acids. A total of 18 amino acids among all the amino acids the body needs are contained in roselle

flower petals, including legnine and arginine, which are important in the body's cell rejuvenation process. In addition, roselle flower petal extract is rich in organic acids, polysaccharides, and flavonoids. Roselle flower petals also contain 211.2 mg of vitamin C, which can increase endurance. [20] The various contents above indicate that a combination of green tea and roselle extract is a drink with high antioxidants.

5. Conclusion

The administration of a combination of green tea and roselle extract improved the morphology and motility of Wistar rats' spermatozoa. However, an increased dose of a combination of green tea and roselle extract was not proven to affect the morphology and motility of Wistar rats' spermatozoa.

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