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RESEARCH ARTICLE

Amelioration of Methotrexate-Induced Testicular Toxicity in Rats by Two Varieties of Coconut Water: Effect on Sperm Characters, Oxidative Stress and Testosterone Levels

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Abstract

Methotrexate (MTX) is used as an anticancer drug in many types of cancer associated with organ toxicities. It generates free radicals responsible for tissue damage in organs like testis. Coconut water, a natural drink known for its nutritive values also possess components, which are rich in antioxidants. The present study was a novel study to evaluate the benefits of yellow and green varieties of coconut water in testicular damage caused by methotrexate in experimental animals. The study proved that, both the varieties of coconut water restored testicular structure and functions by recovering sperm function, testicular enzymes, antioxidant enzymes and improvement in tissue microscopy. We conclude that both green and yellow coconut water are beneficial and can be used as a natural remedy for methotrexate induced testicular toxicity.

Keywords: Antioxidant, Coconut Water, Methotrexate, Sperm Function

1. Introduction

Methotrexate (MTX) is used as anticancer drug in many types of cancers. It is indicated in acute lymphoblastic leukaemia, osteosarcoma, cancer of breast, lung, gestational choriocarcinoma, head and neck cancer. It is associated with many adverse effects. It produces nausea, vomiting, stomatitis, gingivitis, anorexia, diarrhoea, ulcers, and organ toxicities involving liver,bone marrow and testis[1]. Previous studies have reported damage (disorganization and vacuolization) in the seminiferous tubules of the testis, decrease in sperm count following administration of methotrexate (MTX)[2]. Oxidative stress has been reported to play an important role in the pathogenesis of MTX-induced testicular damage. High amounts of polyunsaturated fatty acids are found in the mammalian spermatozoa membranes, thereby making them susceptible to lipid peroxidation[3,4].

Coconut (*Cocos nucifera* L.) Water (CW), the liquid endosperm of coconut, is a nutritious sweet tasting drink, which contains several biologically active components

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comprising free amino acids like L-arginine, ascorbic acid, cysteine, auxins, minerals such as calcium, magnesium, and potassium[5,6]. Several studies have proven the bene ficial effect of these components on sperm count and sperm motility by its anti-peroxidative and free radical scavenging ability[7,8].

In southern part of India, coconut is available as two main varieties, green and yellow. Both the varieties are proven to have a difference in chemical composition and showed concentration-dependent free radical scavenging ability[7].Till date, there are no studies carried out to evaluate the benefits of the yellow variety on testicular toxicity caused due to MTX use. Since CW is nontoxic, cost-effective, and have above-mentioned benefits, we sought to conduct this study using Green Coconut Water (GCW) and Yellow Coconut Water (YCW) to evaluate its effect on MTX induced testicular toxicity using male Wistar albino rats.

2. Materials and Methods

The study commenced after approval by the Institutional Animal Ethics Committee [IAEC/KMC/91/2017]. Animals were procured from the Central Animal Facility of the institute in accordance with the Committee for the purpose of Control and supervision of experimentation on animal guidelines (CPCSEA). Male albino rats of Wistar strain weighing 100–150gm aged around 10 weeks were used for the study. The animals were housed under standard condition, 12:12 light-dark cycle, 50% humidity and 28°C temperature and provided with standard food granules and water *ad libitum*.

2.1 Chemicals/Drugs

GCW and YCW were procured from a local farm in Udupi which were authenticated by a taxonomist from the Department of Botany, Poornaprajna College, Udupi. MTX was obtained from the institutional pharmacy and all the other reagents used were purchased from Sigma Company and were of analytical grade.

2.2 Experimental Design

Twenty-four adult male rats were randomly divided into four groups of six rats each. MTX was administered intraperitoneally (IP) on day 1 at a dose of 20 mg/kg for all the groups except the normal control group[7]. Fresh coconuts of both varieties (yellow and green) of mature stage (10 months maturity) were harvested from the coconut tree (*Cocos nucifera*, a west coast tall variety) grown in the farm, de-husked, broken carefully and liquid endosperm was collected and administered orally to rats once daily for 15 days. The dose of GCW was administered at 4ml/100g and YCW at 2 ml/100g. The doses were chosen in accordance with previous study[4]. The rats were divided into four groups as follows:

Group 1: Control group received distilled water per orally for 15 days.

Group 2: MTX at 20mg/kg/day, by IP route on day 1 and distilled water orally from day 1 to 15

Group 3: MTX at 20mg/kg on day1, by IP and GCW (4ml/100g) from day 1 to 15 per orally.

Group 4: MTX at 20mg/kg on day 1,by IP and YCW (2ml/100g) from day 1 to 15 per orally.

The body weight of rats before and after treatment was measured. All rats were sacrificed by cervical dislocation on day 16. Serum was assessed for testosterone levels. Reproductive organs were dissected out and the weight of testis and epididymis were measured. Estimation of sperm count, motility, and viability were done using a compound light microscope. The testicular tissue homogenate was prepared by mixing the tissue in 0.25 mol/l sucrose solution and 33% (w/v) and was homogenized using homogenizer. The supernatant was prepared by centrifugation at 10000 rpm for 10 min at 4°C. Testicular alkaline phosphatase level was estimated. Antioxidant enzymes and markers for lipid peroxidation were tested in the testicular tissue. The tissue was subjected to histopathological analysis by a pathologist.

2.3 Laboratory Analysis and Investigations

2.3.1 Reproductive Organ Weights

Testes and epididymis were dissected out at the end of the study and weighed. Testicular Index: The testicular index is used to assessthe proportionate change in weight of testis with respect to change in the body weight. It is also called as gonadosomatic index. It is a very useful parameter to assess testicular toxicity. It is calculated by dividing the left testis weight by the body weight and then multiplied by 100[9].

2.3.2 Sperm Count

The epididymis was minced in 1ml of Phosphate buffer saline (PBS) and the suspension was filtered through a mesh. An aliquot from the suspension (up to 0.5ml) was taken in a hemocytometer and was mixed with phosphate buffer saline in 1:10 dilutions. The suspension is then charged into Neubauer's counting chamber. The total sperm count in eight squares (except the central erythrocyte area) of 1 mm^2 each was determined and multiplied by $5x10^4$ to express the number of spermatozoa/epididymis[10].

2.3.3 Sperm Motility

It was done by the method as described by Sonmez *et al.*[11] Epididymis was macerated and mixed with 1 ml PBS. An aliquot of this solution was taken on the slide and the percentage of motility was evaluated microscopically at a magnification of 400 X.

2.3.4 Live and dead sperms (Viability)

A drop of the epidydimal content was mixed with an equal drop of 1% eosin stain as described by Metwally *et al.* Thin film slides were made, allowed to dry quickly and examined under a light microscope. Viable sperm remained colourless. Hundred sperms per rat were used for scoring and determining the viability percentage. Live to dead sperm ratio was also estimated[12].

2.3.5 Estimation of Lipid Peroxidation in Testicular Tissue

Malondialdehyde (MDA) is one of the most prevalent byproducts of lipid peroxidation during oxidative stress. Thiobarbituric acid (TBA) assay is the most commonly used method for determination of the MDA in biological tissues. The assay is based on a condensation reaction of two molecules of TBA with one molecule of MDA, in which the reaction rate depends on temperature, pH and concentration of TBA. The reaction is carried out in acidic solution at 100°C for time course of one hour[13].

2.3.6 Estimation of Superoxide Dismutase

Epinephrine can be auto-oxidized to adrenochrome by superoxide radicals. Maximum auto-oxidation of epinephrine takes place at pH-10.2. The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine to adrenochrome at pH 10.2 has been used as the basis for the assay of this enzyme. In this method epinephrine acts both as the source of superoxide radical and as the detecting system giving adrenochrome which can be monitored at 480 nm using spectrophotometer[14].

2.3.7 Estimation of Catalase

Catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen. The catalase activity in a sample is determined by measuring the decrease in hydrogen peroxide (H_2O_2) concentration observed following an incubation of the analyte sample with an H_2O_2 standard solution[15].

2.3.8 Serum Testosterone

The testosterone level in serum was estimated using ELISA kit. The testosterone ELISA is based on the principle of competitive binding between testosterone in the test specimen and testosterone-Horseradish Peroxidase (HRP) conjugate for a constant amount of rabbit anti-testosterone. Goat anti- rabbit IgG-coated wells were incubated with testosterone standards, controls, test samples, testosterone-HRP conjugate reagent and rabbit anti-testosterone reagent for 90 minutes. During the incubation, a fixed amount of HRP-labeled testosterone competes with the testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone-HRP immunologically bound to the well progressively decreases as the concentration of testosterone in the specimen increases. Unbound testosterone-peroxidase conjugate is then removed and the wells washed, followed by addition of Tetramethylbenzidine (TMB) reagent resulting in the development of blue color. The color development is stopped and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled testosterone in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve[16].

2.3.9 Testicular Alkaline Phosphatase (alp) Activities

Alkaline phosphatase (at an alkaline pH), hydrolysis the p-Nitrophenyl phosphateinto p-Nitrophenol and Phosphate. The rate of formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the Alkaline phosphatase activity in the sample[4].

2.3.10 Histopathological Examination

The autopsy samples as tissue bits were sectioned along the longest diameter for fixation in neutral buffered formalin solution for twelve hours at room temperature. The tissues sampled were placed in tissue cassettes, and then subjected to dehydration with graded dilutions of alcohol (70%, 95% & 100%). The tissue blocks were then cleared in xylene to allow for embedding media (paraplast) at 56 degrees Celsius. The tissue blocks were subjected to sectioning by a semi-automated microtome (Leica RM2235^{TM}), used to prepare sections (5µm thickness), in concordance with the control tissue used. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by

Hematoxylin & Eosin dyes for routine examination by light microscopy.

2.3.11 Statistical Analysis

Data was analyzed using SPSS V 20 software. Values were expressed as Mean ± S.D. Differences were considered significant if P≤0.05. Statistical analysis was done by using One- way ANOVA followed by post hoc Tukey test.

3. Results

3.1 Effect On Body Weight

Baseline weightswere comparable in all the groups. There was a significant increase in weight in all the groups except for MTX group, where there was weight loss recorded (Table 1).

3.2 Effect on Reproductive Organ Weight and Testicular Index

There was a significant loss of weight of reproductive organs (testis, epididymis) and decrease in the testicular index in MTX group compared to control group. The testicular weight, epidydimal weight and testicular index in GCW and YCW groups were increased compared to MTX group (Table 1).

Table 1. Alterations in Body weight, Reproductive organ weight and testicular index in different groups

Group	Body Wt(g)	Reproductive organ wt (g) and Testicular index (TI)			
	Pre-dose	Post-dose	Testis(g)	Epididymis(g)	T I (%)
Control	149±3.7	155.5±5.9	1.29±0.04	0.22±0.02	0.82±0.03
MTX	150±7.07	142.0±6.8**	0.84±0.01**	0.13±0.02**	0.58±0.03**
GCW+MTX	150.6±3.01	153.5±3.9##	1.35±0.04##	0.24±0.01##	0.88±0.04##
YCW+MTX	148.1±4.53	161.5±1.9##	1.48±0.03##††	0.31±0.02##††	0.91±0.03#

Data were expressed as mean ± SD. ANOVA and Tukey's post hoc tests were used for statistical analysis of data. ** significantly different from control group at p<0.01; # and # # significantly different from MTX group at p<0.05 and P<0.01 respectively;†† significantly different from GCW group at p<0.01.

3.3 Effect on Seminal Analysis

Sperm count, motility and sperm viability were significantly low in MTX group compared to control group (P<0.05). GCW group showed increased sperm count, motility and sperm viability compared to group 2. YCW showed increased: sperm count and motility compared to groups MTX and GCW; sperm viability compared to MTX group (Figure 1).

3.4 Effect on Testicular Lipid Peroxides measured as Malondialdehyde and Antioxidant Enzymes Superoxide Dismutase and Catalase

The MDA level was increased significantly while SOD, catalase levels decreased in MTX group compared to control group. The levels of antioxidants SOD and Catalase were significantly more and MDA was lesser in group GCW and YCW groups compared to group 2 (Table 2).

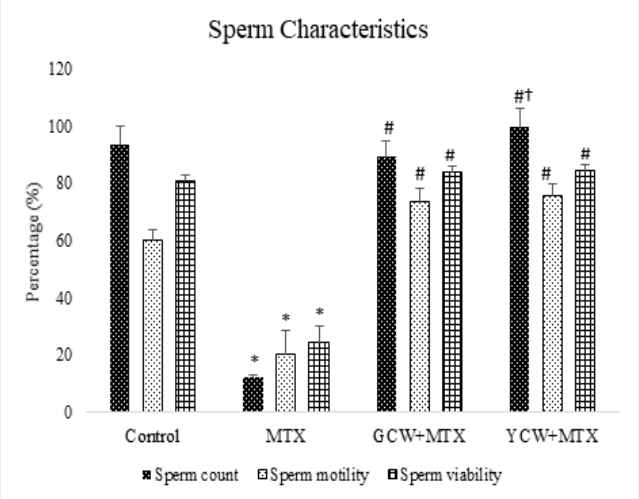

Figure 1. Effect of methotrexate and methotrexate with coconut water on sperm characteristics. Data were expressed as mean ± SD. ANOVA and Tukey's post hoc tests were used for statistical analysis of data. * significantly different from control group at p<0.01. # significantly different from MTX group at P<0.01.† significantly different from GCW at P<0.05.

Table 2. Effect of methotrexate and simultaneous administration of green coconut water and yellow coconut water on testicular melondealdehyde, superoxide dismutase and catalase activity

Groups	MDA(nMol/mg)	SOD (U/mg)	CAT (U/mg)
Control	0.23±0.01	0.14±0.01	0.41±0.03
MTX	0.90±0.05*	0.06±0.02*	0.06±0.01*
GCW+MTX	0.30±0.03#	0.14±0.01#	0.23±0.03#
YCW+MTX	0.24±0.01#†	0.21±0.02#†	0.38±0.02#†

Data were expressed as mean ± SD. ANOVA and Tukey's post hoc tests were used for statistical analysis of data. * significantly different from control group at p<0.01. # Significantly different from MTX group at P<0.01.† significantly different from GCW at P<0.01.

3.5 Estimation of Serum Testosterone

The testosterone levelwas reduced in MTX group compared to control group. On the other hand, hormone levels significantly increased in GCW and YCW groups compared to MTX group (Figure 2).

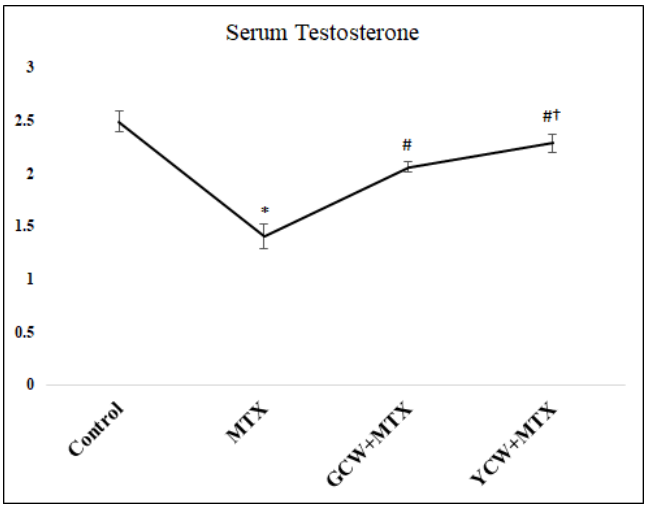

Figure 2. Effect of methotrexate and methotrexate with coconut water on serum testosterone levels. Data were expressed as mean ± SD. ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *significantly different from control group at p<0.01. # significantly different from MTX group at p<0.01.† significantly different from group GCW at P<0.01.

3.6 Effect on Testicular Alkaline Phosphatase

Alkaline phosphatase enzyme was significantly more in the MTX group compared to control group. The testicular ALP levels reduced significantly in rats treated with green and yellow coconut water compared to MTX group (Figure 3).

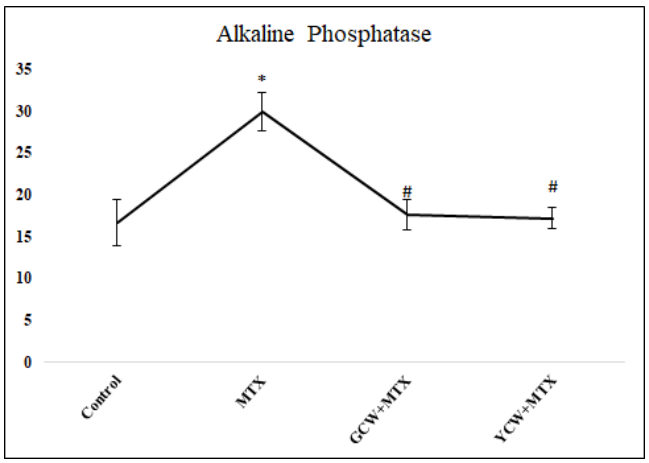

Figure 3. Effect of methotrexate and methotrexate with coconut water on Alkaline phosphatase enzyme levels in testis. Data were expressed as mean ± SD. ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *significantly different from control group at p<0.01. # significantly different from MTX group at p<0.01.

3.7 Histopathology

The testicular architecture was normal in control rats. The seminiferous tubules, germ cells, Sertoli cells were normal in number and morphology. Stroma and Leydig cells were unremarkable (Figure 4A). In MTX group, seminiferous tubule showed a reduction in diameter, intertubular edema along with tubular disruption, with luminal necrosis. Maturation sequence of germ cells was disrupted with more than 60% necrosis of spermatozoa and spermatids along with a severe decrease in Sertoli cells. Minimal stromal edema was seen, with a mild decrease in number of Leydig cells (Figure 4B). In GCW group, seminiferous tubules showed a mild reduction in diameter, minimal intertubular edema and minimal tubular disruption with focal necrosis. Germ cell maturation sequence was normal with patchy necrosis of spermatozoa and spermatids.

Sertoli cells were normal in number with a mild reduction in Leydig cells (Figure 4C). In YCW, seminiferous tubules were normal in diameter without any intertubular edema or necrosis. There was very minimal tubular disruption, but germ cell maturation sequence was normal without necrosis of spermatozoa and spermatids. Sertoli cells and Leydig cells were normal in number (Figure 4D).

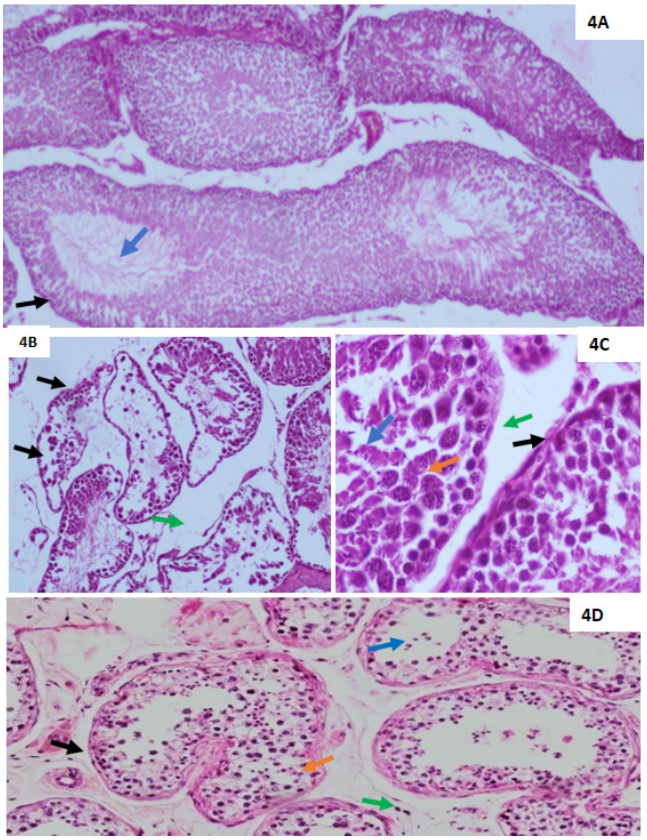

Figure 4. Effect of methotrexate and methotrexate with coconut water on histopathological changes in rat testis. 4A: Control, 4B: MTX, 4C: MTX with Green coconut, 4D: MTX with Yellow coconut, BLUE ARROW: Spermatozoa, ORANGE ARROW: Spermatogonia, GREEN ARROW: Edema, BLACK ARROW: Seminiferous tubule architecture

4. Discussion

In the present study, MTX reduced the weight of the testis, epididymis and adversely affected sperm characteristics. Reduction in reproductive organs weight and testicular index indicates testicular toxicity and disturbed androgenesis[17]. Treating with GCW and YCW restored the organ weight (Table 1).

Sperm count is the most sensitive indicator of spermatogenesis while sperm motility indicates sperm fertilizing capacity[18,19]. All the sperm characteristics like count, motility and viability were reduced with MTX, indicating its toxic effects on testis whereas supplementing CW improved all the above sperm parameters (Figure 1). The reduction in sperm count could be due to: the direct toxic effect due to excess MTX induced lipid peroxidation in testis and reduced biosynthesis[20].

The raised free radical decreases the levels of testosterone by damaging Leydig cells. MTX is also known to reduce testosterone biosynthesis secondary to its suppressive effects on LH levels[20]. Testosterone is required for the normal spermatogenesis as it plays a vital role for maintaining the structural morphology and normal physiology of seminiferous tubules[21]. Its reduction results in decreased sperm count and testicular atrophy[22] (Figure 2).

Sperm motility is affected due to lipid peroxidation as previous study has demonstrated the linear relationship of decline in sperm motility with lipid peroxidation[23]. Disruption of germ cell membrane by MTX leads to high levels of MDA. We found that both the varieties of coconut water showed significant improvement in sperm count and motility (Figure 1). Exact reason as to this beneficial effects of CW is not known but apart from itsantioxidant activity, previous studies have demonstrated the role of L-Arginine (L-Arg), a component of CW in improving sperm count and viability in men[24]. Nitric oxide formed from L-arginine by the enzyme NOS and ascorbic acid, stimulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland and increased the level of androgens[25]. L-arginine is required for the synthesis of polyamines like spermine, spermidine in seminal fluids and synthesis of various basic proteins. YCW contains five times higher L-Arg levels compared to GCW and might have contributed to the marked improvement in sperm functions with YCW compared to all other groups[26].

CW is rich in ascorbic acid. Studies have demonstrated its antioxidant effects and its ability to reverse infertility in animal models[27]. We found a reduction in MDA levels and increase in antioxidant enzymes SOD and catalase levels with both the varieties of CW. The antioxidant activity was more pronounced in YCW compared to GCW (Table 2)

The ALP is a known marker for inflammation in testicular tissue. Its levels was raised in MTX group which indicates intense inflammation and cellular degeneration owing to its direct toxic effects on testicular parenchyma (Figure 3)[28]. Histopathological data in our study confirms the disturbance of tubular structure with edema, disruption of germ cell maturation and decrease inSertoli

and Leydig cells in MTX group. These histological changes along with the abnormal biochemical parameters like raised oxidative stress, confirmed the MTX toxic effects on testis. The results are consistent with the previous study[2]. However, in GCW there was improvement in histological changes as shown by mild reduction in seminiferous tubules diameter, minimal edema and necrosis. Germ cell maturation was normal with full recovery of Sertoli cells but mild reduction in Leydig cells was observed. In YCW treated group, there was complete restoration of structure and morphology of the seminiferous tubule, germ cell maturation sequence, recovery of Sertoli and Leydig cells to normal levels without any evidence of necrosis and it was supported by reduction of oxidant stress induced by MTX (Figure 4). This confirms the protection offered by CW against the MTX toxicity in testis.

5. Conclusion

The study confirms that both the green and yellow coconut water have a protective effect in MTX-induced testicular damage. Both types of CW were able to effectively restore disturbed sperm characteristics by their natural antioxidant effects and ability to normalize elevated testosterone levels. Among both the varieties, yellow coconut water restores the sperm structure and functions to a greater extent.

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