

Effect of *Maclura pomifera* Extract on Cisplatin-Induced Damages in Reproductive System of Male Rats ^[1]

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Abstract

In this study, we aimed to investigate the effect of *maclura pomifera* (MP) (known as cytotoxic, anti-tumoural, anti-inflammatory, antinociceptive, and anti-oxidative) extract upon the spermatological parameters in Cisplatin (anti-tumoural chemotherapeutic with Reactive Oxygen Species (ROS)- generating side-effects)-treated rats. A total of seventeen male Sprague Dawley rats, aged eight weeks and weighing 250-300 g, were used. Three experimental groups were assigned, as follows; Group 1 (n=5) as control (C): the animals received intraperitoneal (IP) injection of physiological saline and further saline via oral gavage during 5 days before and after the injection, Group 2 (n=6): single IP injection of 7 mg/kg Cisplatin (CP) and saline via oral gavage as above, and Group 3 (n=6): referred as MP + CP, oral administration of 500 mg/kg/day MP extract during 5 days before and after cisplatin (CP) injection, as above. At the end of the experiment, rats were decapitated and their testes and cauda epididymis weights as well as epididymal sperm parameters were analysed. The CP administration caused a significant (P<0.05) decline in sperm motility and density, with a significant increase in dead spermatozoa. The MP + CP combination also led to a significant decline (P<0.05) in the weights of total cauda epididymis and of testes, but significantly improved (P<0.05) sperm survival as compared to the CP alone. In conclusion; the findings suggested that the MP plus CP combination had an adverse effect upon the total weights of testes and cauda epididymis, however the dead sperm rate was markedly lower than the CP alone.

Keywords: Cisplatin, *Maclura pomifera* extract, Male rat, Sperm, Reproductive system

Erkek Ratlarda *Maclura pomifera* Ekstraktının Sisplatin ile Oluşturulmuş Üreme Sistemi Hasarı Üzerine Etkisi

Özet

Bu çalışmanın amacı, *maclura pomifera* (MP) (sitotoksik, anti-tümöral, yangı önleyici, analjezik/ağrı giderici, ve antioksidan olarak bilinen) ekstraktının sisplatin (ROS (Reaktif oksijen türevleri) -oluşturucu yan etkili anti-tümöral ajan) uygulanan erkek ratların spermatolojik parametreleri üzerine etkisini incelemektir. Araştırmada 8 haftalık yaşta, 250-300 g canlı ağırlığa sahip 17 adet erkek Sprague Dawley ırkı rat kullanıldı. Üç deney grubu oluşturuldu; Grup 1 (n=5): kontrol grubu, hayvanlara intraperitoneal (IP) serum fizyolojik enjeksiyonuyla birlikte, enjeksiyondan 5 gün öncesi ve sonrası boyunca oral gavaj yoluyla yine serum fizyolojik verildi. Grup 2 (n=6): Sisplatin (CP) grubu, hayvanlara bir kez IP 7 mg/kg CP enjeksiyonundan 5 gün öncesi ve sonrası boyunca oral yolla serum fizyolojik verildi. Grup 3 (n=6): MP + CP grubu, hayvanlara bir kez IP 7 mg/kg CP enjeksiyonuyla birlikte, enjeksiyondan 5 gün öncesi ve sonrası boyunca oral yolla günlük 500 mg/kg MP ekstraktı verildi. Deney sonunda ratlar dekapite edilerek testis ve kauda epididimis ağırlıkları ile epididimal sperm parametreleri incelendi. CP enjeksiyonu, sperm motilitesi ve yoğunluğunu önemli derecede (P<0.05) düşürdü ve ölü spermatozoa sayısını artırdı. MP + CP uygulaması, CP grubuna kıyasla toplam testis ağırlığı ve toplam cauda epididimis ağırlığını önemli derecede (P<0.05) azalttı, ancak canlı sperm sayısında önemli (P<0.05) bir artışa neden oldu. Sonuç olarak; MP + CP kombinasyonunun, CP grubuna kıyasla toplam testis ve cauda epididimis ağırlığı üzerine olumsuz bir etki göstermekle birlikte, ölü sperm sayısını önemli oranda azalttığı gözlemlendi.

Anahtar sözcükler: Sisplatin, *Maclura Pomifera* ekstraktı, Erkek rat, Sperm, Üreme sistemi

INTRODUCTION

Maclura pomifera (MP), a member of Moraceae family, is a dioecious tree that inhabits in the Southwestern United

States. It is also a widely cultivated hardwood tree in Turkey. The plant is known as osage orange, horse apple, mock orange or hedge apple ^[1]. Numerous articles have indicated that the MP and its components have several



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biological activities including cytotoxic [2], anti-tumoural [3], anti-inflammatory and antinociceptive activities [1].

The MP contains lectins [2], xanthenes [4] and flavone-type compounds [1]. *Maclura pomifera* has antioxidant capacity, attributable to flavonoid type components especially iso-flavones, osajin and pomiferin [5]. The antioxidant activities showed marked difference between osajin and pomiferin. Vesela et al. [5] showed that the antioxidant profile of highly active pomiferin was comparable to the reference compounds used, while osajin showed only low activities. Pomiferin also has inhibitory effects for anti-cholinesterase [6] and histone deacetylase (HDAC) [7]. Chromatin is a dynamic macromolecular complex that consists of DNA, histones, and non-histone proteins. Histones are small basic proteins consisting of a globular domain and a more flexible and charged NH₂-terminus protruding from the nucleosome [8].

The HDAC inhibitors act in dual way, as having inhibitory effect on the proliferation and inducing differentiation and/or apoptosis of tumour cells in culture and in animal models. Some HDAC inhibitors have been shown to have a potent anti-tumoural effect with a little toxicity *in vivo* in animal models [8]. The HDAC inhibitors exhibit an anti-proliferative activity on tumour cells [7]. Yang et al. [9] suggested that pomiferin has an anti-proliferative effect not only on transformed breast epithelial cells but also on the normal cells. Robert and Rasool [10] emphasised that the HDAC inhibitors are triggered in cancer and leukemia cells by widespread histone acetylation and actual increases in reactive oxygen species (ROS). Also, the HDAC inhibitors may increase DNA damage following treatment with DNA-damaging chemotherapies by inhibition of DNA repair mechanism.

Cisplatin (CP), known as one of the most effective anti-tumoural chemotherapeutic drugs, is widely used in the treatment of various cancer types [11-13]. Despite the fact that CP has a powerful effect on the destruction of neoplastic cells; it has also some dose-dependent side effects, including; cytotoxicity, nephrotoxicity [14,15], hepatotoxicity [16] and reproductive toxicity [17]. The CP exposure can break the redox balance of tissues, suggesting that biochemical and physiological disturbances may result from the oxidative stress [18]. The ROS, like singlet molecular oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide are normally produced in the subcellular compartments of testis, particularly within the mitochondria. They are then scavenged by the antioxidant defence system like the enzymes of the related subcellular compartments such as plasma membrane, cytosol, acrosome, nucleus, equatorial segment, midpiece and flagellum [19]. However, this balance can readily be broken by the chemicals like the CP, that disrupts the prooxidant-antioxidant balance, leading to cellular dysfunction [20]. Also, the CP can induce free radical toxic stress and spermatid DNA damage [21,22].

To protect the live cells within the body against the

CP's deleterious effects, numerous studies have been conducted. Various studies suggested that some antioxidant substances like lycopene, ellagic acid [18], royal jelly [23], melatonin [24], roselle and ginger [25] and selenium [26] have all ameliorative effects against the CP-induced damages in the male reproductive system. However, in the literature, there appears no study available about the effect of *maclura pomifera* extract on the spermatological parameters in CP-exposed rats. Hence, the combined effects of *maclura pomifera* and CP administration upon the reproductive parameters of male rats were investigated in this study.

MATERIAL and METHODS

Chemicals

Cisplatin (50 mg/100 ml) was purchased from Ebewe Pharma® (Unterach-Austria). To obtain the MP extract, fruits of *maclura pomifera* were collected from Ankara province in September and then were washed and removed from the shell. After the slicing into 100 g portions, they were wrapped with a filter paper. The extraction was performed routinely in 250 ml distilled water on a magnetic mixer at room temperature during one day. By removing the water in the lyophilisator, the extract of raw fruit juice was obtained. This process was repeated twice with two-day interval and both extracts were pooled and used as *maclura pomifera* extract. The extract was kept at +4°C during the study.

Animals and Experimental Protocols

Seventeen male Sprague Dawley rats (Specific Pathogen Free) aged eight weeks old and weighing 250-300 g, were used in this study. The animals were obtained from Atatürk University Medical & Experimental Research & Application Centre (Erzurum, Turkey) and were housed therein under standard laboratory conditions (24±2°C, 40-60% humidity, a 12 h light: 12 h dark cycle). A commercial pellet chow (in the sack of 50 kg, Bayramoglu Food Co./Erzurum-TR) and fresh drinking water were available *ad libitum*. Rats were divided into 3 groups, as follows; In Group 1 (n=5), served as control (C), animals were exposed to intraperitoneal (IP) physiological saline injection along with further saline via oral gavage during 5 days before and after the injection. In Group 2 (n=6), referred to as Cisplatin (CP), rats were exposed to single dose (7 mg/kg) of IP injection of the CP along with saline via oral gavage for 5 days before and after the CP injection. In Group 3 (n=6), named as cisplatin + *maclura pomifera* (MP + CP), animals received single dose of IP injection of the CP (7 mg/kg) and oral administration of MP extract (500 mg/kg/day) during 5 days before and after the cisplatin injection.

Before the study started, the approval of Committee for Institutional Animal Care and Use was provided from

Atatürk University Local Board of Ethics (the approval number: 2013/141, dated on 22nd Oct., 2013).

Collection of Samples

Rats were sacrificed under ether anaesthesia. Both testes were then removed. Cauda epididymides were separated from both testicles and the connective tissues were cleansed by using anatomic scissors. Cauda epididymides and testicles both were then weighed. To obtain semen samples from both cauda epididymises, a modified method, previously described by Sonmez et al.^[27] was used. Briefly, the epididymides were minced by anatomical scissors in 1 ml of physiological saline (0.9% w/v NaCl) in a petri dish. Afterwards, the pieces of epididymides were allowed to incubate at room temperature for 15 min to provide the migration of all spermatozoa from the epididymal tissue into the fluid. Epididymal tissues were removed from dishes by tweezers. The fluid obtained was used as semen sample.

Sperm Evaluation

To evaluate the percentage of sperm motility, light microscope equipped with heated stage was used. Briefly, a slide was placed on a light microscope with a heated stage warmed up to 37°C. A droplet of semen sample was dropped on the slide and the percentage of sperm motility was determined routinely by visual observation. The motility estimations were performed in three different fields for each sample. Then, the mean of the three consecutive estimations was used as the final motility score ^[27].

The epididymal sperm concentration was determined by using a slight modification of the method (using eppendorf tubes instead of routine haemocytometer), as described by Sonmez et al.^[27]. The semen sample was drawn into the eppendorf tube at 10 µl volume with an automatic pipette and 990 µl eosin solution was added. The solution contained 5 g sodium bicarbonate, 1 mL formalin (35%, v/v) and 25 mg eosin per 100 mL distilled water. After vortexing the eppendorf tubes for 15 sec, approximately 10 µl of diluted sperm suspension was transferred into the counting chambers of Thoma chamber and allowed to settle down for 5 min. The sperm cells in both chambers were counted by using phase contrast microscope at the magnification of 400x.

To determine the percentage of morphologically abnormal spermatozoa, the methods described by Sonmez et al.^[27] and Turk et al.^[20] were used. Briefly, the slides were stained with eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate). The slides were then examined under a phase contrast microscope at 400x magnification. A total of 200 sperm cells were examined on each slide and the head, mid-piece, tail and total abnormality rates of spermatozoa were all expressed as percentage ^[20].

To determine the percentage of dead sperm, the stained

slides prepared originally for determination of abnormal spermatozoa were used. The slides were examined under a phase contrast microscope at 400x magnification and 200 sperm cells were counted on each sample. According to the staining status (eosin uptake) of the head of sperm cells, they were classified as dead (stained head) or alive (unstained head).

Pathological Examinations

Tissue samples of testis were fixed in Bouine's solution. Tissues were exposed to alcohol-xylol series, embedded in paraffin blocks, sectioned 5 µm in thickness and stained with Hematoxylin-Eosin dye. The evaluation was performed according to Johnsen classification criteria with examination of 100 seminiferous tubules selected randomly in each sample under the light microscope ^[28].

Statistical Analyses

All values of sperm parameters and testicular traits studied were presented as mean ± standard error of means (S.E.M.). The differences were considered significant when $P < 0.05$. Statistical analyses were performed using analysis of variance (One-way ANOVA) and post hoc Tukey test by using the SPSS/PC (Version 20) software programme. Also, data from spermatological parameters and testis traits were analysed further to find possible correlations exist between them with Pearson-correlation test.

RESULTS

Reproductive Organ Weights

Total testes weights (TTW) of rats in all groups were presented in *Table 1*. Although the TTW of MP + CP group was significantly ($P < 0.05$) lower than in the control group, but there was no significant difference ($P > 0.05$) either between the MP + CP and CP groups or between the control and CP groups.

Total cauda epididymis weights (TCEW) were presented in *Table 1*. The mean TCEW of MP + CP group was statistically ($P < 0.05$) lower than those in the control group. But, no significant difference ($P > 0.05$) was found either between the MP + CP and CP groups or between the control and CP groups.

Table 1. Total testes and cauda epididymis weights

Tablo 1. Toplam testis ve cauda epididymis ağırlıkları

Groups	N	Total Testes Weights (g)	Total Cauda Epididymis Weights (g)
Control	5	2.660±0.082 ^a	0.414±0.0264 ^a
CP	6	2.605±0.038 ^{ab}	0.348±0.0187 ^{ab}
MP + CP	6	2.403±0.070 ^b	0.296±0.0165 ^b

^{a,b} Different superscript letters show significant differences between groups ($P < 0.05$)

Epididymal Sperm Parameters

Sperm motility percentages were presented in *Table 2*. In terms of sperm motility rate, both the CP and MP + CP group had significantly ($P < 0.05$) lower values than those in the control group. However, no significant difference ($P > 0.05$) was found between the CP and MP + CP groups.

Sperm cell densities in all the groups studied were presented in *Table 2*. It was significantly ($P < 0.05$) higher in the controls than both in the CP and in MP + CP groups. However, there were no differences ($P > 0.05$) between the CP and MP + CP groups.

Epididymal sperm abnormalities were classified as head, mid-piece, tail and total sperm abnormality. There was no significant difference ($P > 0.05$) among all the groups in terms of all abnormalities concerned. The values of all the sperm abnormalities of groups were presented in *Table 2*.

Dead sperm rates were presented in *Table 2*. The rate of CP group was significantly ($P < 0.05$) higher than in the control and in the MP + CP groups. However, there was no difference ($P > 0.05$) between the control and MP + CP groups.

Correlations between all the reproductive parameters studied were presented in *Table 3*.

Pathological Findings

The structures of seminiferous tubules were normal in the control group. In this group, the germ cells in the seminiferous tubules were arranged regularly and the normal course of spermatogenesis was evident (*Fig. 1*). However, both in the CP and MP + CP groups although the borders of tubules appeared normal, the sequence of germ cells were disordered and there were significant ($P < 0.05$) evidence of degeneration. Furthermore, oedema in the interstitial fields in these groups was evident (*Fig. 2* and *Fig. 3*). Based on the Johnsen's Score, there was a significant ($P < 0.05$) difference between the control and CP groups, between the control and MP + CP groups, but no differences ($P > 0.05$) were found between treatment groups (*Table 4*).

DISCUSSION

Despite the fact that the CP exhibited deleterious side effects, it is still one of the most effective chemotherapeutic agents that is widely used against various cancer types [11-13]. Cisplatin-based chemotherapy results in toxicological changes in other tissues such as liver, kidney and testes [17]. Chemotherapy-induced gonadal toxicity and the recovery of spermatogenesis are interrelated with the type of drugs, their doses and duration of therapy used [29]. Treatment with chemotherapeutic agents causes germinal

Table 2. Semen and reproductive parameters of all the experimental groups

Table 2. Bütün grupların sperma ve reproduktif parametreleri

Groups	N	Motility (%)	Density (10 ⁶ /ml)	Head Abnormality (%)	Mid-piece Abnormality (%)	Tail Abnormality (%)	Total Abnormality (%)	Dead Sperm Rate (%)
Control	5	55.59±1.20 ^a	135.000±27.9 ^a	14.9±1.6	5±1.4	10.5±0.6	30.5±2	34.6±1.1 ^b
Cisplatin	6	42.12±1.29 ^b	55.338±6.26 ^b	11.9±1.3	4.2±1.9	12.7±0.7	28.9±1.2	42.3±1.2 ^a
MP + CP	6	44.04±0.96 ^b	52.343±7.59 ^b	13.2±1.1	3±1.4	9.3±1.7	25.4±2.6	38.3±0.8 ^b
Significance		P<0.05	P<0.05	NS	NS	NS	NS	P<0.05

^{a,b} Different superscript letters show significant differences between groups ($P < 0.05$); NS: Non-significant

Table 3. Correlations between all the reproductive parameters studied

Table 3. Çalışılan bütün reproduktif parametrelerin arasındaki korelasyonlar

Reproductive Parameters	Motility	Density	Dead sperm Rate	Head Abnormality	Mid-Piece Abnormality	Total Sperm Abnormality	Total Testis Weight	Total Cauda Epididymis Weight
Motility		(+) **	(-) **					(+)
Density	(+) **		(-) *					(+) **
Dead sperm rate	(-) **	(-) *						
Head abnormality						(+) **		
Mid-piece abnormality						(+) *		
Total sperm abnormality				(+) **	(+) *			
Total testis weight								(+) **
Total cauda epididymis weight	(+) *	(+) **					(+) **	

(+): Positive correlation, (-): Negative correlation; ** Correlation is significant at 0.01 level; * Correlation is significant at 0.05 level



Fig 1. Control group. The structure appears normal (HE staining)

Şekil 1. Kontrol grubu. Yapı normal görünümde (HE boyama)

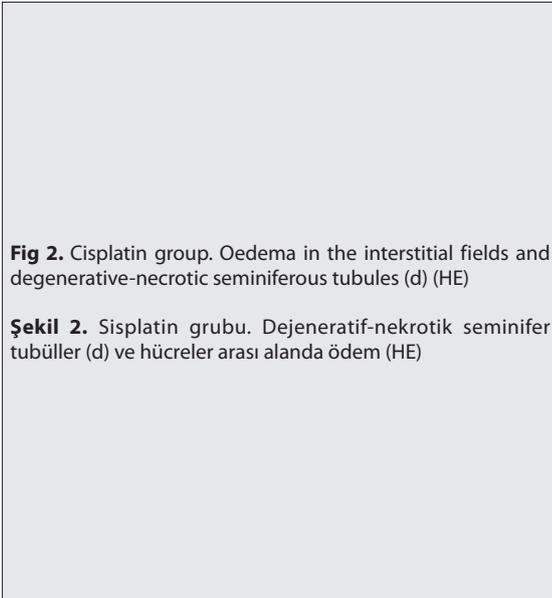


Fig 2. Cisplatin group. Oedema in the interstitial fields and degenerative-necrotic seminiferous tubules (d) (HE)

Şekil 2. Sisplatin grubu. Dejeneratif-nekrotik seminifer tubüller (d) ve hücreler arası alanda ödem (HE)



epithelial damage leading to oligo- or azoospermia [17]. Atessahin et al. [17] reported that the CP administration (7 mg/kg) resulted in a marked decrease in the concentration and motility together with an increase in the rates of all types of sperm abnormalities in rats. Similar findings with the epididymal sperm were reported by Salem et al. [26], using the 10 mg/kg dose. It was presumed that declines both in the motility and density of sperm cells observed herein might stem from lipid peroxidation induced by CP [17,18,26]. The latter workers also reported that the administration of CP further caused a marked decline in the TTW and TCEW. Present results showed that the administration of CP (7 mg/kg) caused a marked ($P < 0.05$) decline in sperm motility and density, together with an increase in the number of dead sperm. These results were similar to other studies [17,26]. On the other hand, the CP treatment did not affect the TTW, TCEW and sperm cell

abnormalities such as the head, mid piece, tail and total abnormalities. This effect might be dependent on the management, sensitivity or health conditions of rats.

Pomiferin, one of the components of *Maclura pomifera* used in this study has inhibitory effects for anti-cholinesterase [6] and histone deacetylase (HDAC) [7]. The HDAC inhibitors function as an anti-proliferatives on tumour cells [7]. Indeed, Yang et al. [9] suggested that pomiferin has anti-proliferative effect not only on transformed cells but also on the normal cells. Herein, the combination of *maclura pomifera* extract with the CP (MP + CP) caused a marked decline in the TTW and TCEW which might depend on the inhibitory effect of pomiferin. As known, the HDAC inhibitors act as anti-proliferative agent for tumour cells, but with a mild toxicity [8]. Normal body cells could also be affected by this undesirable effect [9]. Presumably, this

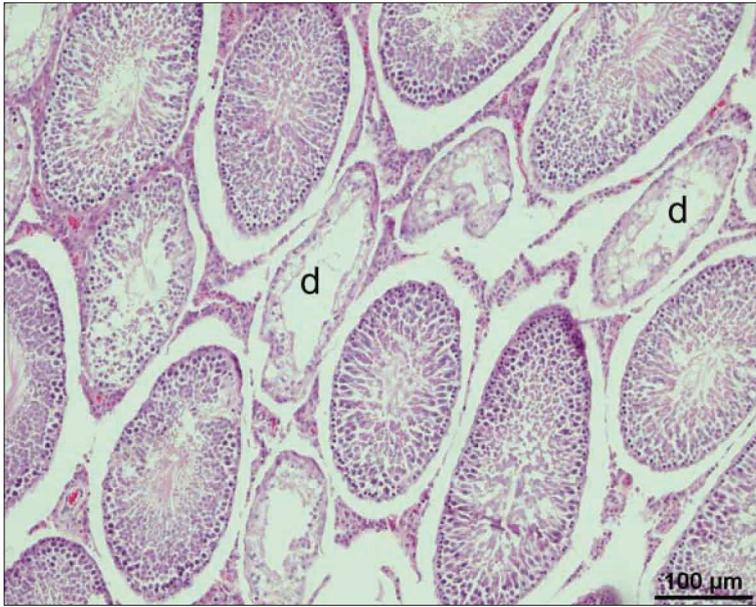


Fig 3. MP + Cisplatin group. Oedema in the interstitial fields and degenerative-necrotic seminiferous tubules (d) (HE)

Şekil 3. MP + Sıslatin grubu. Dejeneratif-nekrotik seminifer tubüller (d) ve hücreler arası alanda ödem (HE)

Table 4. Jonhsen Score table of all the experimental groups
Tablo 4. Bütün deney gruplarının Jonhsen Skor tablosu

Jonhsen Scores	Groups		
	Control	Cisplatin	MP + CP
Mean Score	9.6±0.24 ^a	8.4±0.24 ^b	8.6±0.24 ^b

^{a-b} Different superscript letters show significant differences between the groups (P<0.05)

phenomenon may be explained by a dual mechanism, as the inhibition of testicular tissue proliferation by the pomiferin's inhibitory effect for the HDAC and degenerative side effect of the CP administration simultaneously. While degenerative effects of chemotherapeutic agent might have been compensated for by the natural proliferation in the CP group, the anti-proliferative property of pomiferin might have blocked the cell proliferation in the MP + CP group. The positive correlation observed between the TTW and TCEW strongly indicates that the weights of these tissues are closely interrelated with each other.

Treatments of *Maclura pomifera* extract for 5 days pre- and post-treatment together with the CP had no effect on decreased sperm motility and density. These side effects could be the result of CP's side effects on the male reproduction system. Degeneration and oedema in testicular tissues both in the CP and in MP + CP could have led to the low sperm cell density and motility rate. The oedema in testicular tissue may have been caused an obstruction in the testicular tubules or epididymal lumens. In the one hand, a possible obstruction in this field could be the result of decreased sperm transport from the seminiferous tubules into the cauda epididymis. On the other hand, *maclura pomifera* extract might have had a protective effect on dead sperm rate in the MP + CP group. In this group, the rate of dead sperm was markedly lower than in the CP group but similar to that in the control group. This might

have resulted from the protective effect of antioxidant component of *maclura pomifera* extract.

As expected, a positive correlation was observed between the abnormalities of head and mid-piece and the total sperm abnormality. Furthermore, a negative correlation was found between the sperm motility and dead sperm rate. Decreases in cauda epididymal sperm density had resulted neither from the atrophy in the seminiferous tubules nor from the oedema in the interstitial field. Instead, decreased production might have resulted from the acute necrosis in seminiferous tubules originating from the cisplatin administration. It could be speculated that, as this situation might decrease the sperm density, the TCEW decreased. Indeed, this hypothesis is supported by positive correlation observed between sperm density and the TCEW.

In conclusion, it appeared that pre- and post-treatment with *maclura pomifera* extract (500 mg/kg/day) for 5 days had a limited beneficial effect on the reproductive parameters in the CP-exposed male rats. According to our results, the usage of *maclura pomifera* extract together with the CP; i) had an unfavourable effect on the TTW and TCEW, ii) it was ineffective for protecting the motility rate and sperm density rates, while iii) being protective against the death of the sperm. However, for achieving more reliable results, it is recommended

that future studies should be planned using longer treatment days, larger numbers of rats in the different groups and different concentrations of *maclura pomifera* extract to be given.

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