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AMELIORATIVE EFFECTS OF NIGELLA SATIVA (KALVANJI) SEEDS ON SPERMATOGENESIS IN MICE WITH CISPLATIN-INDUCED TESTICULAR TOXICITY

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Abstract

Background: *Cisplatin-based chemotherapy is indicated for a variety of cancers such as tumors of testes, ovaries, lungs, urinary bladder, and lymphomas. However, its usefulness is marred by the adverse effect of the drug on spermatogenesis. Nowadays, herbs with claims of medicinal properties are being scientifically tested as a possible solution to drug-induced testicular injuries. Nigella sativa, commonly known as black cumin or kalvanji, is one such herb under investigation.*

Objectives: *To determine the Ameliorative effects of Nigella sativa (Kalvanji) seeds on spermatogenesis in mice with Cisplatin-induced testicular toxicity*

Methodology: *The study was conducted at the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar and Department of Histopathology, Rehman Medical Institute, Peshawar. Twenty-eight male Swiss albino mice were included in this study. They were randomized into four study groups i.e. Control, Cisplatin, Nigella Seeds and Cisplatin + Nigella Seeds groups. Control and Nigella Seeds groups had four mice each while Cisplatin and Cisplatin + Nigella Seeds groups had ten mice each. The mice were sacrificed on the 6th day and body weight was measured. The testes were dissected out and gross morphological features including weight, shape, color and texture of the testes were noted. This was followed by preparation of testicular tissue slides stained with Eosin & Hematoxylin. Histological examination was carried out under light microscope. Extent of spermatogenesis was recorded in terms of Johnsen Score whereby a number between 1 and 10 is assigned to testicular tissue depending on the generations of spermatogenic cells observed - 1 indicating no evidence of spermatogenic cells and 10 indicating intact spermatogenesis and presence of spermatozoa. Germinal epithelial height was measured using the image analyzer software ImageJ. Observations were also documented with regard to histological appearance of Sertoli and Leydig cells as well as the basement membrane. The numerical data was analyzed with SPSS version 23.*

Results: *Light microscopic study showed complete spermatogenesis in the testis of the animals in Control as well as Nigella Seeds group. Cisplatin treated animals suffered significant loss of spermatocytes, spermatids and spermatozoa. Statistically significant reduction (P <0.005) was observed for all the parameters (Mean Johnsen Score, Mean Germinal Epithelium Height, Mean weight loss and Mean Testicular Weight) when the Cisplatin-treated animals were compared to the other three experimental groups. These results signified that while Cisplatin treatment resulted in severe testicular toxicity, co-administration of an aqueous solution of Nigella seeds mitigated these changes to a statistically significant level.*
Conclusion: *On the basis of the results of this study it was concluded that Nigella seeds in the form of an aqueous solution have an ameliorative effect against the testicular toxicity of Cisplatin.*

INTRODUCTION

The diagnosis of cancer in males during adolescence and early reproductive years has increased globally in the recent past [1]. At the same time, new treatment protocols have been established that combine adjuvant chemotherapy with radiotherapy and surgical treatment for effective outcome of cancer treatment [2]. Due to prompt diagnosis and modern treatment regimens the chances of surviving malignant disease have increased dramatically [3,4]. Past two decades have seen an improvement in survival rates to 80% and above for most of the malignancies in adolescents and young adults [5–7]. Increase in long-term survival rates has made the prospect of starting a family possible for these young cancer survivors [1]. Unfortunately, anti-cancer chemotherapy has detrimental effects on male fertility [4]. Chemotherapeutic drugs target rapidly dividing cells [4,8], making the proliferative germ cells in testis susceptible to cytotoxic insult. The resulting azoospermia may be prolonged and temporary or completely irreversible [9]. For children, adolescents and young men in reproductive age who survive cancer, the post-cancer quality of life includes ability to father a normal child [9,10]. Chemotherapy-induced fertility impairment is therefore a serious concern for these patients [11].

Cisplatin is one of the most commonly prescribed anti-neoplastic drugs and forms the backbone of modern adjuvant chemotherapy [12,13]. However, its effectivity is marred by the fact that it can cause prolonged sterility [9] or even permanent infertility at higher cumulative doses [14]. In view of the young age of cancer survivors, the impact of Cisplatin-based chemotherapy on fertility is an important issue for patients and clinicians alike [15]. There is no safe and effective drug available as yet to preserve spermatogenesis in patients exposed to cytotoxic drugs[16]. In search of a fertility-preserving agent against Cisplatin, natural plant products and herb extracts have become the focus of research [17-19]. In this regard, Nigella sativa has also been a subject of interest.

The protective effects of an aqueous solution of nigella seeds have been studied against cimetidine-induced testicular toxicity [20]. However, the effects of have not been investigated against cisplatin induced testicular toxicity as yet. This study endeavors to study the amelioration of Cisplatin-induced testicular damage by Nigella seeds aqueous solution in a clinically relevant mouse model. As no proven products are available for protection against drug-induced reproductive toxicity [8], the protective role of Nigella seeds in this regard can revolutionize fertility and quality of life issues in cancer survivors. The current study was carried out to study the ameliorative effects of *Nigella sativa* seeds (aqueous solution) on spermatogenesis in Swiss albino mice with cisplatin-induced testicular toxicity.

Materials and methods

The study was conducted at the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar and Department of Histopathology, Rehman Medical Institute, Peshawar. In this experiment testicular toxicity was induced by intraperitoneal Cisplatin injection in the subjects, and the amelioration of the testicular damage by an aqueous solution of Nigella seeds was studied. A total of 28 male Swiss albino mice were employed in the

study. The inclusion criteria was male, sexually mature mice aged between 6 to 8 weeks were selected while the exclusion criteria was female mice, wice with visible signs of disease or injury such as skin lesions or lack of fur, male mice aged less than 6 weeks and mice older than 8 weeks. The mice were kept in standard cages in the animal house of IBMS and acclimatized for one week prior to the experiment. They were randomized into four study groups i.e. Control, Cisplatin, Nigella Seeds and Cisplatin + Nigella Seeds groups. Control and Nigella Seeds groups had four mice each while Cisplatin and Cisplatin + Nigella Seeds groups had ten mice each.

The Cisplatin group was injected intraperitoneally with Cisplatin at a dose of 2.5 mg/kg body weight. The Cisplatin + Nigella Seeds group received Cisplatin in the same dose as given to the Cisplatin only group, and this group also received aqueous solution of Nigella seeds at concentration of 1000 mg/kg body weight by gastric gavage. Animals in Control group were given intraperitoneal injection of 0.9% Normal Saline in a volume equivalent to Cisplatin group and served as vehicle control for Cisplatin. Animals in Nigella Seeds group served as control for aqueous solution of the seeds and were given the solution at concentration of 1000 mg/kg body weight by gavage. All the treatments were given once daily for five days. The mice were sacrificed on the 6th day and body weight was measured. The testes were dissected out and gross morphological features including weight, shape, color and texture of the testes were noted. This was followed by preparation of testicular tissue slides stained with Eosin & Hematoxylin. Histological examination was carried out under light microscope. Extent of spermatogenesis was recorded in terms of Johnsen Score whereby a number between 1 and 10 is assigned to testicular tissue depending on the generations of spermatogenic cells observed - 1 indicating no evidence of spermatogenic cells and 10 indicating intact spermatogenesis and presence of spermatozoa. Germinal epithelial height was measured using the image analyzer software ImageJ. Observations were also documented with regard to histological appearance of Sertoli and Leydig cells as well as the basement membrane. Data analysis was done using SPSS version 23 and Microsoft Excel. Results were subjected to appropriate statistical analysis and presented in tables. For this purpose, mean ± standard deviation values were calculated. Kruskall-Wallis Test was applied for comparison of the four studied groups amongst each other. If significant difference was found, the groups were further subjected to post-hoc Dunn's Test to determine which group differed from the others. For all the calculations, statistical significance was accepted at P value of less than 0.05.

Results

Light microscopic study showed complete spermatogenesis in the testis of the animals in Control as well as Nigella Seeds group. Cisplatin treated animals suffered significant loss of spermatocytes, spermatids and spermatozoa. In the Cisplatin + Nigella Seeds group the testis showed detrimental changes but they were less severe than the Cisplatin-treated group. Mean Johnsen Score was found to be a perfect 10 in Control group and Nigella Seeds group. The score had dropped to a low 6.000 ± 0.82 in Cisplatin treated group but less declined in Cisplatin + Nigella Seeds group at 8.700 ±0.48. (Figure 1) Mean Germinal Epithelial Height was highest in Control and Nigella Seeds groups (90.12 ± 1.10 and 90.22 ± 1.10 µm respectively), slightly reduced in Cisplatin + Nigella seeds group (84.94 ± 4.44 µm), and lowest in Cisplatin group (70.02 ± 2.26 µm). (Figure 2) Similarly, Mean Testicular Weight in Cisplatin group was the lowest of all four groups (0.17 ± 0.008 grams) as opposed to Control group (0.38 ± 0.008), Nigella seeds group (0.38 ± 0.002) and Cisplatin + Nigella Seeds Group (0.26 ± 0.002). (figure 3) There was no weight loss observed in animals of Control and Nigella Seeds groups, while Cisplatin-treated animals exhibited a great reduction in Mean Body Weight (13.30 ± 0.47 grams). This was a greater weight loss than that observed in Cisplatin + Nigella Seeds group (5.98 ± 0.54 grams). (Figure 4) Thus, statistically significant reduction ($P < 0.005$) was observed for all these parameters when the Cisplatin-treated animals were compared to the other three experimental groups. These results signified that while Cisplatin treatment resulted in severe testicular toxicity, co-administration of an aqueous solution of Nigella seeds mitigated these changes to a statistically significant level. (Table 2, 3, 4 and 5)

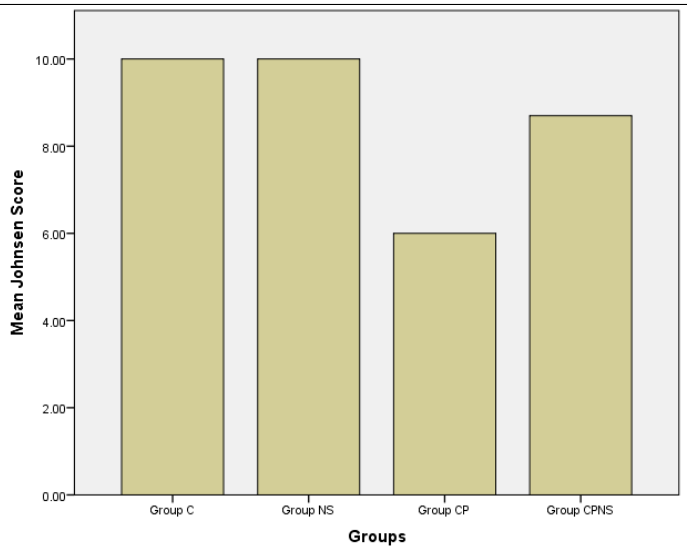

Figure 1. Mean Johnsen Score of all the groups.
C= Control, NS= Nigella Seeds, CP= Cisplatin, CPNS=Cisplatin + Nigella Seeds

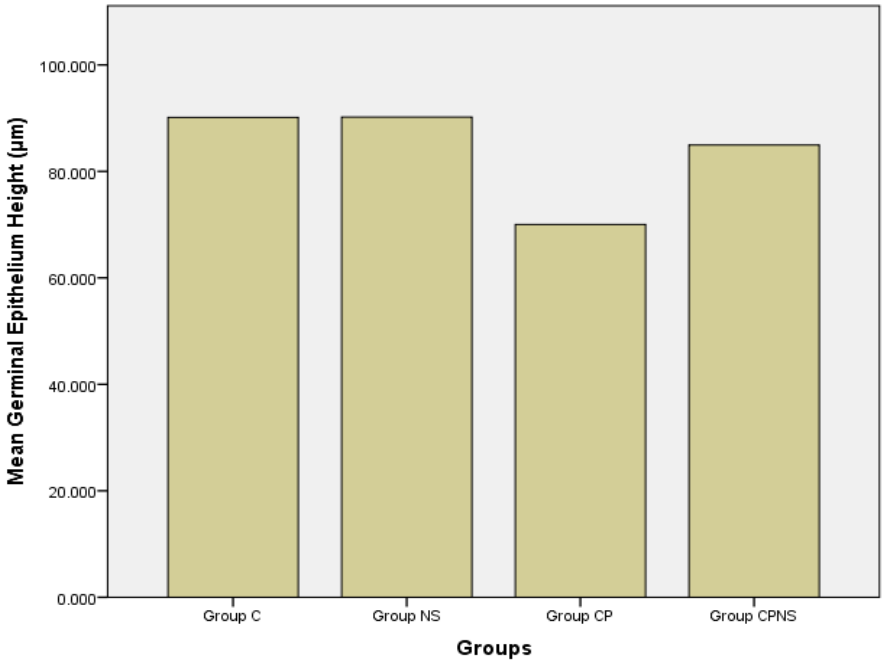

Figure 2. Mean Germinal Epithelium Height.

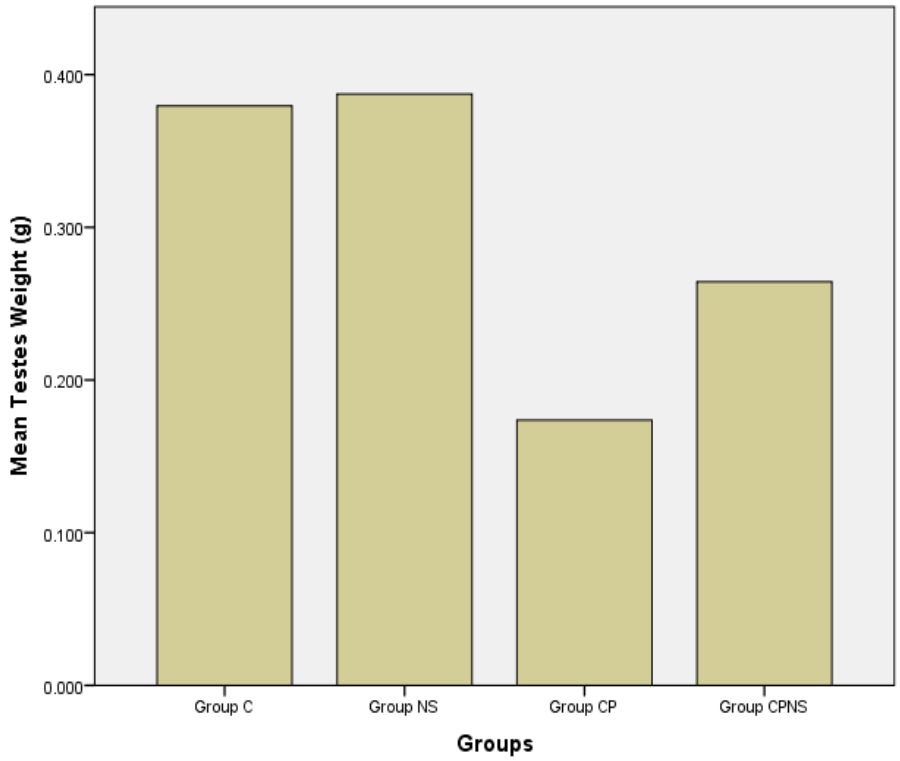

Figure** Error! No text of specified style in document.. **Mean Testicular Weight of all four groups.

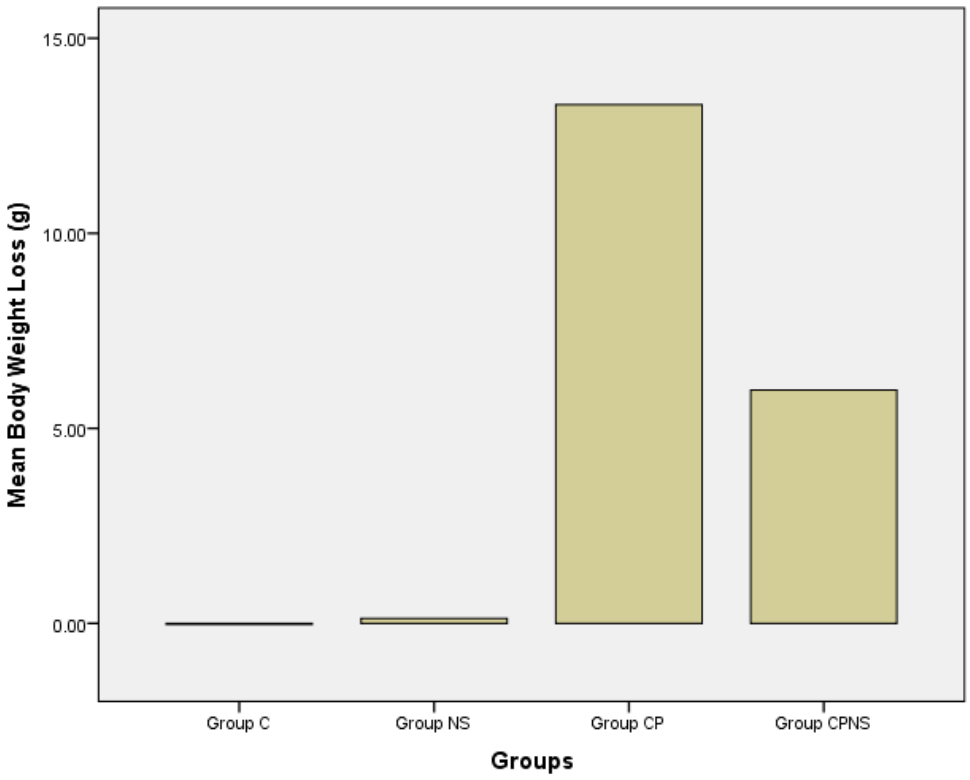

Figure 4: Mean Weight loss in the four experimental groups.

Table 2. Pair-wise Comparison of Changes in Body Weight

Groups Compared	P Value
Cisplatin vs. Control	0.000*
Cisplatin vs. Cisplatin +Nigella Seeds	0.039*
Cisplatin vs. Nigella Seeds	0.001*
Cisplatin + Nigella Seeds vs. Nigella Seeds	0.483
Cisplatin + Nigella Seeds vs. Control	0.305
Control vs. Nigella seeds	1.000

Table 2: Pair-wise Comparison of Mean Testicular Weight

Groups Compared	P Value
Cisplatin vs. Control	0.002*
Cisplatin vs. Cisplatin + Nigella Seeds	0.039*
Cisplatin vs. Nigella Seeds	0.000*
Cisplatin + Nigella Seeds vs. Nigella Seeds	0.163
Cisplatin + Nigella Seeds vs. Control	0.816
Control vs. Nigella Seeds	1.000

Table 3. Pair-wise Comparison of Mean Johnsen Score

Groups Compared	P Value
Cisplatin vs. Control	0.000*
Cisplatin vs. Cisplatin + Nigella Seeds	0.033*
Cisplatin vs. Nigella Seeds	0.000*
Cisplatin + Nigella Seeds vs. Nigella Seeds	0.351
Cisplatin + Nigella Seeds vs. Control	0.351
Control vs. Nigella Seeds	1.000

Table 4: Pair-wise Comparison of Germinal Epithelial Height

Groups Compared	P Value
Cisplatin vs. Control	0.001*
Cisplatin vs. Cisplatin + Nigella Seeds	0.028*
Cisplatin vs. Nigella Seeds	0.001*
Cisplatin + Nigella Seeds vs. Nigella Seeds	0.576
Cisplatin + Nigella Seeds vs. Control	0.576
Control vs. Nigella Seeds	1.000

Discussion

Cisplatin treatment can cause prolonged or permanent azoospermia in cancer patients [21]. The protective effects of an aqueous solution of nigella seeds have been studied against cimetidine-induced testicular toxicity[22]. The current study was undertaken to determine if an aqueous solution of Nigella seeds can alleviate testicular toxicity effects of Cisplatin in Swiss albino mice. Various parameters were evaluated for this purpose including body weight, testicular weight, and histological features of testis. The results of this study show partial but significant prevention of

toxic effects of Cisplatin with regard to the studied parameters.

Cisplatin administration can cause reduced body weight in experimental animals [23, 24]. This reduction in body weight is attributed to Cisplatin-induced anorexia, emesis and gastrointestinal toxicity [25, 26] and the resultant decrease in food intake [27, 28]. In this study, Cisplatin-treated animals suffered significant weight loss as compared to the controls, corroborating the evidence from previous studies. On the other hand, administration of aqueous solution of Nigella sativa attenuated Cisplatin-induced weight loss in animals of Cisplatin + Nigella seeds group. Previously, use of antioxidants has shown efficacy against Cisplatin-induced emesis and weight loss [29], and Nigella seed extracts possess high level of antioxidant activity [30]. The seeds also act as appetite stimulant [31] which can counter the anorexia caused by Cisplatin. Due to these properties, Nigella seeds appear to play important role in preventing excessive loss of body weight caused by Cisplatin as suggested by the findings of this study.

Previous studies have demonstrated loss of germinal epithelial cells including spermatogonia, spermatocytes and spermatids due to acute Cisplatin toxicity [32, 33]. The depletion of germ cells has consequences on the histologic appearance of seminiferous epithelium. The epithelial height is reduced and large vacuoles are seen in the thickness of the epithelium due to cellular losses [34, 35]. The Johnsen Score, based on presence or absence of particular cell types in the seminiferous tubules, is also reduced [36]. The germ cell loss also affects weight of testis considering that testicular weight is largely dependent on presence of abundant spermatogenic cells in the seminiferous tubules [34]. Previously, reduced testicular weight consistent with loss of germ cells has been observed with Cisplatin administration [21]. In present research, a significant declination of testis weight was noted in the Cisplatin-treated group compared to the vehicle control group. In histological findings of this study, Cisplatin produced a marked depletion of germ cells. Spermatozoa were completely absent from all the tubules in the entire Cisplatin group. Spermatocyte and spermatid populations were grossly eliminated in a vast majority of tubules. Parameters pertaining to normal spermatogenesis such as epithelial height and Johnsen Score were markedly low in Cisplatin-treated animals than in the Control group animals. The observations of previous studies regarding Cisplatin toxicity effects on testicular weight and cells of seminiferous epithelium are thus augmented by this current study.

In the present study, supplementation with an aqueous solution of Nigella seeds to Cisplatin-treated animals prevented the exaggerated loss of testicular weight as opposed to animals that received Cisplatin only. Also, animals in Cisplatin + Nigella seeds group showed significantly less susceptibility to cytotoxic effect of Cisplatin against germ cells as compared to Cisplatin group animals. Both the epithelial height and Johnsen Score, although less than the Control group, were significantly better than the Cisplatin-treated animals. The histological picture showed evidence of preserved spermatogenesis in most, if not all, of the tubules. With a Johnsen Score of either 8 or 9, the spermatocytes, spermatids and in some tubules even spermatozoa had escaped cytotoxic effects of Cisplatin. In past researches, depletion of germ cells and concurrent reduction of testis weight has been attenuated by agents that reduce oxidative stress in testicular tissue [94]. Moreover, improvement of Johnsen Score as well as epithelial thickness is documented with use of phytomedicinal products that reduced markers of oxidative injury in testis [37]. In previous studies, Nigella sativa has shown preservation of spermatogenesis in experimental animals exposed to other gonadotoxic agents that act by inducing oxidative injury in testis [38]. According to the findings of this study, co-administration of Nigella sativa as an aqueous solution is highly effective against damage to testicular germ cells caused by Cisplatin and this amelioration is possibly due to the antioxidant active substances in the seeds.

In some previous experiments employing same Cisplatin dose regimen as the current study, vacuole formation in Sertoli cells was found in Cisplatin-treated mice [39]. In yet another investigation involving same dose of Cisplatin in a 5-day cycle, Sertoli cells appeared unharmed [44]. The findings of this study agree with the latter study showing normal cell morphology and population of Sertoli cells in all the study groups.

In a study with same Cisplatin exposure protocol as the current work which employed electron microscope, the basement membrane had wavy and

irregular appearance [35]. In the current study, appearance of basement membrane was normal across all the study groups. This difference may because the present study was done using a light microscope while the previous work studied the structure of the basement membrane by means of electron microscope. There was also no observable detachment of the germinal epithelium from the basal lamina in the present work which is contrary to previous reports [40]. Similarly, abundance of Leydig cells was preserved across all the study groups. Due to lack of histological findings for Sertoli cell, basement membrane, and Leydig cells damage the role of Nigella seeds could not be demonstrated in present study as far as these features were concerned.

Conclusion

Based on the results of this study, it is concluded that Cisplatin administered to mice in a therapeutic equivalent dose for five days as practiced in a single treatment cycle for humans causes loss of spermatocytes, spermatids and spermatozoa and a decline in Johnsen Score to as low as 5. At the given dose, Sertoli and Leydig cells appear undamaged as far as histological appearance of these cells is concerned. Nigella sativa in the form of aqueous solution significantly attenuates the impaired spermatogenesis, testicular weight reduction and body weight loss caused by Cisplatin. The aqueous solution has shown potential of maintaining the Johnsen score at 8 or 9.

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