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Hyrdoalcoholic Root Bark Extract of *Salacia oblonga* Prevented Mitomycin-C Induced Sperm Abnormality in Wistar Rats.

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ABSTRACT

The extract of root bark of *Salacia oblonga* (*SO*) belonging to the family Celastraceae was tested for the anti-mutagenic activity using sperm abnormality test in Wistar rats. The hydroalcoholic extract (0.5 and 1 gm/kg, b.w, p.o. daily for 7 days) was evaluated against Mitomycin-C (MMC-2 mg/kg, b.w, i.p.) induced testicular toxicity by estimating the sperm shape abnormality and sperm count. The sampling was done after 48 hours and 72 hours of the clastogen treatment. The antioxidant activity of the *SO* was evaluated by measuring the serum levels of superoxide dismutase(SOD) and catalase. The results indicated that prior treatment of *SO* had suppressed the changes produced by MMC. *SO* at a dose of 1.0 gm/kg bw had shown significant ($p<0.01$) inhibition in the sperm shape abnormality and sperm count in both the time intervals, while the lower dose (0.5 gm/kg, b.w) showed inhibitory effect mainly at 48 hr duration compared to the MMC group. The results also indicated that *SO* has improved ($p<0.01$) the status of serum antioxidant enzymes compared with the MMC group. The data from the study suggests that *SO* possess antimutagenic effect against MMC and the activity could be due its antioxidant potential.

Keywords: *Salacia oblonga,* Mitomycin-C*,* Sperm-shape abnormality*,* Sperm count*,* Antioxidant.

INTRODUCTION

Toxicological studies have undergone a significant evaluation during the past decade with much greater emphasis being placed on chronic toxicity, carcinogenicity, teratogenicity and mutagenicity. The environmental pollutants from various sources are known to cause the generation of reactive oxygen species (ROS) and results in the oxidative damage to tissues and mutation in somatic cells [1]. Several studies in the past have reported the important role of oxidative stress in the etiology of male infertility. However, at lower levels of oxidative damage, spermatozoa may retain the capacity to fertilize but still carrying significant levels of oxidative damage in their DNA. The subsequent repair of such damage in the zygote may result in the mutations associated with pre term pregnancy loss and variety of pathologies in the newborns including developmental defects and childhood cancer [2,3]. Many a time's hazards occur not only due to the presence of genotoxic agents but also due to lack of antimutagenic/anticarcinogenic agents in our diet. As we cannot avoid many of these agents, the best way to minimize the effect is by identifying the anti mutagens and desmutagens in our diet and increasing their use [4].

The sperm morphology test in rodents is commonly used for measuring the spermatogenic damage induced by the test agents. Studies have provided evidence that induced changes in sperm morphology reflect genetic damage in

male germ cells. Further, results have also demonstrated a high correlation between the mutagenicity of an agent and its ability to cause sperm shape abnormalities [5–7]. Epididymal sperm count is routinely used to assess the damage induced by disease/drug treatment to the male reproductive cells. Decrease in the sperm count could indicate reduced daily sperm production by the testis, obstruction of transport from the testis to the epididymis (e.g. obstruction of ductulus efferens) or an alteration in the epididymal transit time of sperm [8].

Now-a-days, natural products are driving the attention of researchers by showing comparably therapeutic activity with that of available drugs and with lesser side effects [9]. Several studies in the past indicated that the natural products can be used as chemopreventive agents against the mutagenic damages. These compounds exhibit the preventive effects against the mutagenesis by several pathways and important among them is by quenching the free radicals [4,10].

Salacia oblonga (Family: Celastraceae) commonly known as Saptrangi, is a subtropical under shrub found in the south Asian countries, known to have several medicinal properties such as hypoglycemic, hypolipidemic, anti-inflammatory, anti-oxidant etc [11–14]. The potential genotoxicity of *Salacia oblonga* root extract was evaluated using standard battery of tests recommended by US FDA and the results concluded that the extract is safe for human use and do not possess genotoxic potential [15,16]. However, the influence of *Salacia oblonga* root extracts against chemical induced cytogenetic damage in rodents with special reference to its anti-oxidant capabilities has not been found in the literature. Hence, we investigated the role of hydroalcoholic root extract of *Salacia oblonga* in mitomycin-C induced reproductive toxicity in male rats.

MATERIALS AND METHODS

Chemicals*:*

Ascorbic acid was purchased from Loba Chemie, Mumbai and Mitomycin-C was from Khandelwal Labs Pvt. Ltd, Mumbai. Other chemicals and stains used in this study were purchased from local supplier and are of analytical grade.

Animals:

Eight week-old healthy, laboratory bred, male Wistar rats weighing 180 ± 10 gm were maintained under standard laboratory conditions such as temperature 22-25° C, 12 hour light / dark cycle and provided water and pellet food *ad libitum*. The experiments were conducted in CPCSEA (Committee for the purpose of control and supervision of experiments on animals, Chennai, India) approved animal house after obtaining the prior approval from the Institutional Animal Ethics Committee.

Hydro-alcholic extract of root barks of *Salacia oblonga*:

The dried root bark of *Salacia Oblonga* (*SO*) was procured from local market and authenticated at Regional Research Institute (RRI), Bangalore (Ref No. RRI/BNG/SMP/ Drug Authentication/2007-08/214 d). The dried rook bark powder was extracted with the solvent system consisting of methanol and water in the ratio of 1:1. The extract was dried, weighed and suspended in 1% w/v CMC according to the dose and screened for the anti-mutagenic activity.

Dose and Treatment:

The animals are divided mainly into three groups' viz., control, challenge and treatment. The control group received saline (0.5 ml/kg) while the challenge group was treated with mitomycin-C (MMC-2 mg/kg, i.p.) [17]. In the treatment group, *SO* was tested in two doses (0.5 and 1.0 gm/kg, p.o) [18]. *SO* was administered daily for 7 consecutive days and on the 7th day MMC was administered. Sampling was done after 48 hr and 72 hr of MMC treatment. Ascorbic acid (100 mg/kg, p.o.) was used as an internal standard antioxidant agent [19].

Sperm shape abnormality assay [7]

The sperm shape abnormality was evaluated as per the procedure described by Wyrobek and Bruce (1975). The dissected cauda epididymis was minced in phosphate buffer (pH7.2) and the suspension free from the tissue fragment was stained for half an hour in 1% eosin yellow solution. A smear was prepared in the clean glass slide and about 700 spermatozoa cells were screened to identify the abnormality (Photo-1).

The caudal sperm count was done according to D'Souza (2004). The sperm cells were counted using the four chambers of naubauers slide [8].

Estimation of antioxidant enzymes
a. Superoxide dismutase (SOD) [20]:

The principle for measuring the SOD depends on the detecting the O_2generated during auto-oxidation of hydroxylamine. During the oxidation, nitro blue tetrazolium (NBT) is reduced and nitrite is produced in the presence of EDTA which can be detected colorimetrically at 560 nm. The concentration of SOD is expressed as mg protein/ml.

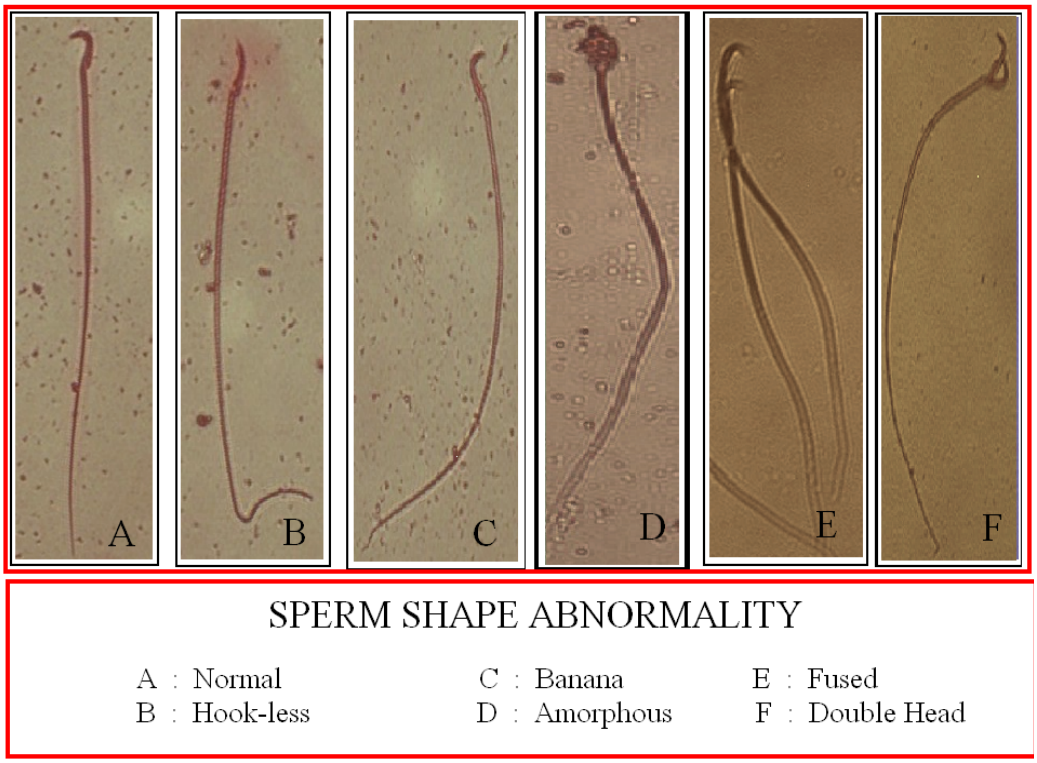

Photo-1: Abnormalities in sperm morphology

b. Catalase [21]:

The estimation of catalase activity was done by determining the decomposition of H_2O_2 at 240 nm in an assay mixture containing the phosphate buffer (0.25 M, pH 7). One international unit of catalase utilized is that amount which catalyzes the decomposition of 1 mM H_2O_2 per min at 37°C and expressed in terms of mg protein /ml.

c. Total proteins [22]:

The estimation of total protein was done as per the procedure of Lawry et al (1951). In the alkaline medium, peptide bonds of proteins react with cupric ions in biuret reagent to form violet colored complex with an absorption maximum at 546 nm. Intensity of the color formed is directly proportional to the concentration (mg/dl) of total protein in the sample.

Statistics

The statistical significance of the results was carried out using one-way Anova followed by multiple comparisons by Bonferroni test [23]. $p<0.05$ was considered to indicate significance.

RESULTS

Effects of the hydro-alcoholic extract of SO on sperm morphology and sperm count after the administration of MMC:

The antimutagenic study indicated that the hydroalcoholic extract of *SO* prevented dose dependently the reproductive toxicity induced by MMC. Administration of MMC (2 mg/kg) had increased significantly ($p<0.001$) the sperm shape abnormality and it was also found to decrease the number of caudal sperm count in both 48 hr and 72 hr time intervals. The treatment of *SO* was observed to reduce the changes induced by MMC. At lower dose, *SO* (0.5 gm/kg) had reduced the number of abnormal shaped sperms ($p<0.05$) and had increased the germination of sperm cells only in 48 hr interval, while in 72 hr, it did not produced any change compared to the MMC. *SO* at higher dose (1 gm/kg) had exhibited significant ($p<0.01$) suppression of sperm shape abnormality and elevation in the sperm count after 48 hrs, but in the longer duration (72hrs) the effect was found to be less significant ($p<0.05$) compared to the MMC group. The action of *SO* was found to be more prominent at higher dose compared to the lower dose. Further, administration of *SO* (1 gm/kg) to the control group did

not exert any effect on sperm count and sperm shape morphology in both the tested time intervals. Ascorbic acid (100 mg/kg) as a standard antioxidant had showed a significant ($p<0.001$) suppression in the total sperm shape abnormality and minimized the effect of MMC on the caudal sperm count (Table-1 and Table-2)

Effect of the hydro-alcoholic extract of *SO* on serum antioxidant status after the administration of MMC.

The comparison of MMC treated animals with the control indicated a significant ($p<0.001$) reduction in the level of SOD, catalase and total proteins. The pretreatment of *SO* in the MMC administered animals produced a significant elevation in the levels of SOD and catalase. *SO* at lower dose (0.5 gm/kg) had increased ($p<0.05$) the antioxidant status only in the 48 hr group compared to MMC group. On the other hand, when *SO* was tested at 1gm/kg, a significant increase in the level of SOD, catalase and total proteins was observed in both 48 hr ($p<0.01$) and 72 hr ($p<0.05$) intervals in comparison with MMC group. However, the administration of *SO* (1 gm/kg) to the normal animals did not produced any change in the levels of the antioxidant enzymes. Administration of ascorbic acid to the MMC challenged animals indicates that the treatment had significantly ($p<0.001$) improved the antioxidant status, without affecting the antioxidant enzyme levels in the control animals (Table-3).

DISCUSSION

The administration of hydroalcoholic extract of *Salacia oblonga* (*SO*) had prevented the sperm abnormality and enhanced the sperm count in the MMC treated Wistar rats. The inhibitory effect was observed when *SO* was tested at 1.0 gm/kg in both the 48 hr and 72 hr durations. The antioxidant study indicated that SO elevated the serum levels of SOD and catalase in MMC administered animals. Ascorbic acid used as an standard antioxidant

Table-1: Effect of the hydro-alcoholic extract of SO on sperm count and sperm morphology 48 hrs after administration of MMC

TREATMENT (Dose)	SPERM SHAPE MORPHOLOGY					% Total abnormality	SPERM COUNT (10^6)
	Hookless	Banana	Amorphous	Fused	Double head		
Control(Saline 0.5 ml/kg)	8	12	9	13	1	1.02 ± 0.04	35.33 ± 0.20
S0 (1gm/kg)	12	11	9	12	2	1.09 ± 0.04	35.13 ± 0.27
Ascorbic Acid(100 mg/kg)	10	13	7	12	3	1.09 ± 0.03	35.18 ± 0.15
MMC (2 mg/kg)	47	37	28	58	19	4.52 ± 0.09[a]	24.34 ± 0.30[a]
MMC + *S0* (0.5gm/kg)	28	45	36	46	25	4.26 ± 0.04*	25.20 ± 0.24*
MMC + *S0* (1gm/kg)	32	40	36	44	23	4.11 ± 0.04**	26.01 ± 0.21**
MMC + Ascorbic Acid(100 mg/kg)	39	37	29	43	13	3.85 ± 0.09***	26.60 ±0.38***

Values are expressed as Mean ± SEM, SO-Salacia oblonga, MMC-Mitomycin-C, n=8
Statistics: One way Anova followed by multiple comparison by Bonferroni test.
a p<0.001 compared with control
** p<0.05,*
*** p<0.01,*
**** p<0.001 compared with mitomycin-C*

Table-2:Effect of the hydro-alcoholic extract of SO on sperm count after 72 hrs of administration of MMC.

TREATMENT (Dose)	SPERM SHAPE MORPHOLOGY					% Total abnormality	SPERM COUNT (10^6)
	Hookless	Banana	Amorphous	Fused	Double head		
Control(Saline 0.5 ml/kg)	8	12	9	13	1	1.02 ± 0.04	35.33 ± 0.20
S0 (1gm/kg)	11	11	9	12	2	1.07 ± 0.04	35.27 ± 0.25
Ascorbic Acid(100 mg/kg)	9	10	11	12	2	1.04 ± 0.03	35.22 ± 0.24
MMC(2 mg/kg)	46	37	28	58	19	4.50 ± 0.10[a]	24.27 ± 0.34[a]
MMC + *S0* (0.5gm/kg)	54	43	20	51	18	4.43 ± 0.10	24.39 ± 0.34
MMC + *S0* (1gm/kg)	50	41	14	51	20	4.24 ± 0.05*	26.10 ± 0.51*
MMC + Ascorbic Acid(100 mg/kg)	42	48	28	27	14	3.78 ± 0.10***	27.42 ±0.52***

Values are expressed as Mean ± SEM, SO-Salacia oblonga, MMC-Mitomycin-C, n=8
Statistics: One way Anova followed by multiple comparison by Bonferroni test.
a p< 0.001 compared with control
** p<0.05,*
**** p<0.001 compared with Mitomycin-C*

Table-3: Effect of the hydro-alcoholic extract of SO on serum antioxidant status in MMC treated rats.

Treatment (dose)	After 48 hrs SOD (mg protein/ml)	After 48 hrs Catalase (mg protein/ml)	After 48 hrs Total Protein (mg/dl)	After 72 hrs SOD (mg protein/ml)	After 72 hrs Catalase (mg protein/ml)	After 72 hrs Total Protein (mg/dl)
Control(Saline 0.5 ml/kg)	0.74 ± 0.05	9.61 ± 0.48	7.73 ± 0.32	0.74 ± 0.05	9.61 ± 0.48	7.73 ± 0.32
S0 (1gm/kg)	0.71 ± 0.03	9.13 ± 0.32	7.36 ± 0.28	0.71 ± 0.03	9.32 ± 0.53	7.21 ± 0.34
Ascorbic Acid (100mg/kg)	0.70 ± 0.04	9.08 ± 0.35	7.18 ± 0.37	0.69 ± 0.04	9.18 ± 0.531	7.11 ± 0.37
MMC (2mg/kg)	0.21± 0.03[a]	3.932 ± 0.44[a]	4.77 ± 0.40[a]	0.20 ± 0.04[a]	3.82 ± 0.48[a]	4.65 ± 0.36[a]
MMC + S0 (0.5gm/kg)	0.34 ± 0.042*	5.27 ± 0.37*	6.04 ± 0.38*	0.21 ± 0.03	4.06 ± 0.50	4.87 ± 0.34
MMC + S0 (1gm/kg)	0.39 ± 0.03**	6.31 ± 0.34**	6.74 ± 0.36**	0.34 ± 0.04*	5.31 ± 0.39*	5.97 ± 0.37*
MMC + Ascorbic Acid (100mg/kg)	0.43 ± 0.02***	6.92 ± 0.45***	6.88 ± 0.22***	0.41 ± 0.02***	6.84 ± 0.27***	6.87 ± 0.25***

Values are expressed as Mean ± SEM, SO-Salacia oblonga, MMC-Mitomycin-C, n=8 Statistics: One way Anova followed by multiple comparison by Bonferroni test.
a p<0.001 compared with control;
** p<0.05,*
*** p<0.01,*
**** p<0.001 compared with MMC*

agent produced significant antimutagenic effect against the MMC induced nuclear damages besides, suppressing the oxidative stress.

MMC is one of the clinically used toxic anticancer drugs. It produces the cytotoxicity by an electrophilic attack on the nucleophilic site in the DNA. MMC is commonly used as a clastogen in pre-clinical studies as it produces chromosomal damage in variety of cells [17]. Further, the administration of MMC has been reported to suppress the levels of antioxidant enzymes which contribute in the oxidative stress [24]. In our study too, administration of MMC had enhanced the percentage of sperm abnormality besides reducing the sperm count and antioxidant enzyme levels (Table-1, 2 and 3). As reported, oxidative stress plays major role in the cytonuclear damages of the cells. Free radicals in the oxidative stress initiate a chain of reaction in the body which ultimately damages the cellular component including DNA which finally results in serious complications like mutations [25].

The sperm morphology assay in rodents is a well established method to study the role of clastogens on the reproductive cells. The formation of normal sperm heads involves a series of intricate, synchronous morphological and biochemical steps, viz., machette formation, the replacement of histones with protamine, etc[26,27]. Disturbances in these sequences results in the formation of abnormal sperms. The defect in the sperm morphology can be studied by evaluating the number of abnormalities like hook less, banana shaped, amorphous, fused, double head etc. The changes in the head portion and tail of the sperm have been reported to affect the nuclear composition and motility of the spermatozoa respectively [5–8].

SO in this study have shown protective effect against the damages caused by MMC. Administration of *SO* had produce dose dependent inhibition in the MMC mediated alterations in the sperm shape abnormality, sperm count and antioxidant enzyme status (Table 1, 2 and 3). The effect was found to be dose dependant, only in 48 hrs of MMC treatment group. The inability to prevent the alteration at lower dose (0.5 gm/kg) after 72 hrs indicates that as the duration of exposure of MMC is increased, the generation of free radicals will be more, which could not be inhibited by lower dose of *SO* (0.5 gm/kg). As mentioned, *SO* have exhibited significant antioxidant effect both *in vivo* and *in vitro* [14]. The potential to enhance the level of SOD and catalase could have contributed in the anti-clastogenic effect of *SO* against MMC.

The research conducted on certain plants like *Cistanchis deserticolae, Hippophae rhamnosides, Angelicae sinesis* etc revealed that these herbs inhibited the sperm shape abnormality produced by the clastogenic agents due to the antioxidant potential [28,29]. These studies suggests that enhancement in the level of antioxidant enzymes like SOD, Catalase, glutathione etc is responsible for the detoxification and reduction in the oxidative stress mediated by several carcinogens and mutagens [24].

Ascorbic acid tested as a standard antioxidant agent had produced significant inhibition in the sperm abnormality and oxidative stress induced by MMC [30]. In the earlier study too, administration of ascorbic acid has prevented the mutagenic changes caused by MMC due to its ability to protect the electrophilic attack on the nucleophilic sites of the nucleus, primarily by reducing the concentration of the free radicals [31]. This information suggests that compounds possessing the antioxidant property could prevent the mutagenic changes caused by the clastogens. Since *SO* is already reported to be an antioxidant [14], we suggests that in this study too *SO* has protected the germinal cells against the damage caused by the oxidative stress due to its antioxidant property.

CONCLUSION

The present study indicate that the hydroalcoholic root bark extract of *Salacia oblonga* (*SO*) possess antimutagenic effect against the testicular toxicity caused by mitomycin-C (MMC). The extract prevented the incidence of abnormal shaped sperms and minimized the changes on sperm count induced by the clastogen. The antioxidant enzyme estimation in serum indicated that *SO* has got the potential to enhance the level of SOD and catalase. The ability of the extract of *SO* to prevent the testicular damage caused by the known mutagens could play an important role in overcoming several mutations related defects caused due to the oxidative stress.

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