

# *Tribulus terrestris* improves metronidazole-induced impaired fertility in the male mice

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## Abstract

**Introduction:** Fruit extract of *Tribulus terrestris* (TT) bears aphrodisiac and antioxidative properties. Antimicrobial drug, metronidazole (MTZ) impairs the spermatogenic activity and fertility in males.

**Objective:** Validation of the use of fruit extract of TT as a supplement against MTZ-induced fertility impairment in males.

**Methods:** Adult Swiss strain male mice were administered with 500mg/kgBW/day of MTZ for 28 days. Low (100mg/kgBW/day) and high (200mg/kgBW/day) doses of TT were administered simultaneously with MTZ (500mg/kgBW/day) for same duration. All males were cohabited with virgin proestrus females. Vaginal plug formation was observed to calculate the libido index. Cohabited females were sacrificed on fifteenth day of gestation to dissect out the ovaries and uteri. Fertility index, quantal pregnancy, pre-implantation and post-implantation losses were calculated.

**Results:** MTZ-treated males showed unaltered mating ability, however, the females impregnated by such males exhibited marked alterations in the fertility index, quantal pregnancy and pre- and post-implantation losses. Supplementation with low dose of TT failed to restore such reproductive toxicities exhibited by administration of MTZ. However, the altered reproductive toxicities were reinstated to control values following supplementation with high dose of TT.

**Conclusion:** The fruit extract of TT may emerge as an effective herbal remedy, correcting the drug-induced fertility impairments in males.

**Keywords:** Female mice, fertility, male mice, metronidazole, *Tribulus terrestris*.

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## Introduction

Herbal extracts are well established for infertility therapy and fertility regulation. The role of certain herbs in improving the reproductive impairments is well documented.<sup>1-2</sup> *Tribulus terrestris* (TT) has emerged as a new source of antioxidant for infertility therapy.<sup>3</sup> It is a flowering

plant, belonging to family Zygophyllaceae<sup>4</sup> and bears several pharmacological properties.<sup>5-7</sup> This indigenous herb is also used in the Indian and Chinese system of medicine for the treatment of various reproductive disorders, especially in the males.<sup>8-10</sup> The fruit of TT contains a number of different chemical substances including saponins, glycosides, flavonoids, alkaloids, resins, tannins, sugar, sterols and essential oil.<sup>11-12</sup> Among the saponins isolated from the fruit extract of TT, protodioscin is the most popular active substance present in the extract,<sup>13-14</sup> bearing aphrodisiac property.<sup>10</sup> The existence of antioxidative flavonoid compound in the ethanolic fruit extract of TT has also been well documented.<sup>15-16</sup> Our earlier findings have reported safety evaluation of the fruit extract of TT on the male reproductive health of the laboratory mouse.<sup>17</sup>

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Further, ameliorating potentiality of TT has been proved against cadmium chloride<sup>18</sup>, cypermethrin<sup>19</sup> and diabetes<sup>20</sup> induced testicular injury in the rat.

Impairments in spermatogenic activity and fertility<sup>21</sup> and sperm morphology and fertility<sup>22</sup> of the male mice following administration of MTZ, in our earlier findings, raised the scope to carry out a supplementation study using the fruit extract of TT in order to ameliorate its side-effects. Metronidazole (MTZ) is the first nitroimidazole,<sup>23</sup> used for the treatment of *Helicobacter pylori* infections, amoebiasis, giardiasis, trichomoniasis, vaginal infections, antibiotic-associated pseudomembranous colitis, symptomatic amoebiasis<sup>24</sup> and Crohn's disease.<sup>24-25</sup> Earlier findings have suggested the intervention of metronidazole on the male reproductive health and fertility of laboratory rodents.<sup>26-34</sup> The drug at the dose of 500mg/kg BW/day, administered for 4 weeks, is reported to impair spermatogenic activity<sup>21</sup> and sperm morphology and fertility<sup>22</sup> in the mice.

Our earlier findings have reported the beneficial properties of TT in ameliorating the MTZ-induced spermatogenic inhibition, arising as a consequence of oxidative stress in the testis<sup>35</sup> and alterations in the histopathology of the epididymis<sup>36</sup> in the mice. These observations raised the possibility that the interrupted fertility in male mice<sup>21</sup> may also be corrected using the fruit extract of TT. Thus, the present study was designed with the aim to ascertain the use of the fruit extract of TT against MTZ-induced fertility impairment in the male mice.

## Materials and Methods

### Experimental animals

Thirty adult Swiss strain male mice (12 weeks old), and sixty adult Swiss strain female mice (10-12 weeks old), weighing 25-30g, were used in the study. All the mice were housed under standard husbandry conditions of temperature ( $24 \pm 20^\circ\text{C}$ ), light (photoperiod of 14 hours light and 10 hours dark) and relative humidity (60 to 70%), in polypropylene cages, with rice husk as the bedding material. Animals were maintained on pelleted food and water *ad libitum*. The use of the mice was approved by the Animal Ethical Committee, Banaras Hindu University, Varanasi, India (No. Dean/11-12/CAEC/263).

### Drug

MTZ (purchased from CDH, India) was dissolved in double distilled water and administered orally. The fruit of TT was purchased from the local market of Varanasi and

got identified from the Department of Botany, Banaras Hindu University (Voucher No. Zygo-2013-1). The extract of the fruit was prepared by adopting the method of Hussain and co-authors<sup>10</sup> and dissolved in distilled water for administration.

### Dose selection

The human therapeutic dose of MTZ was selected and translated to mice.<sup>37</sup> The doses of TT were standardized according to its ameliorating efficacy on MTZ-induced impairments in the fertility of the male mice.

### Experimental design

The animals were divided into six groups of five each in which Group I and II served as untreated and vehicle-treated (distilled water) controls, respectively. Mice of Group III were administered with MTZ (500mg/kg BW/day) while that of Group IV were administered with the fruit extract of TT (200mg/kg BW/day), for 28 consecutive days. Mice of Groups V and VI were co-administered with MTZ (500mg/kg BW/day) and both doses of the fruit extract of TT (100mg/kg BW/day and 200mg/kg BW/day), respectively, for 28 consecutive days.

### Mating ability and fertility test

Five males of each group were caged separately with two virgin proestrus females for overnight and according to the presence of vaginal plug and implantation sites in females, the mating ability and fertility of the males were assessed, respectively. The females impregnated with the treated males were weighed on the fifteenth day of gestation and sacrificed by cervical dislocation. The ovaries were removed to count the number of corpus luteum. To determine the total number of implantation sites, the uteri were dissected out and placed in 10% ammonium sulfide solution, which stained the hemosiderin pigment of resorbed implanted sites blue black.<sup>38</sup> The numbers of live implants, as well as the pre- and post-implantation losses were noted.

### Calculation

Libido index (%) = Total no. of males mated with females  $\div$  Total no. of males caged with females X 100

Fertility index (%) = Total no. of pregnant females  $\div$  Total no. of females exposed for mating X 100

Quantal pregnancy (%) = Total no. of pregnant females  $\div$  Total no. of females mated with the males X 100

Pre-implantation loss = Corpus luteum – (Total no. of resorbed implants + Total no. of live implants + Total no. of dead implants)

Post-implantation loss = Total no. of resorbed implants + Total no. of dead implants

### Statistical analysis

Percentage values of libido index, quantal pregnancy and fertility index were analyzed by using Chi-square test. The

values of the number of live implants as well as pre- and post-implantation losses were represented as mean  $\pm$  S.E. in each group and analyzed by ANOVA followed by Newman-Keul's multiple range. Values were considered significant at  $p < 0.05$ .

### Results

#### Libido index

The libido index of all the treated mice remained unimpaired, as compared with the controls (Table 1).

**Table 1. Effect of oral administration of MTZ and co-administration of MTZ and the fruit extract of TT for 28 consecutive days on the percentage of libido index, quantal pregnancy and fertility index**

Groups	Libido Index (%)	Quantal Pregnancy (%)	Fertility Index (%)
Untreated Control	100.00	100.00	100.00
Vehicle-treated Control	100.00	100.00	100.00
MTZ (500mg/kgBW/day)	83.33	37.50 <sup>a</sup>	25.00 <sup>a</sup>
TT (200mg/kgBW/day)	100.00	100.00	100.00
MTZ(500mg/kgBW/day) + TT (100mg/kgBW/day)	83.33	71.42	50.00
MTZ (500mg/kgBW/day) + TT (200mg/kgBW/day)	83.33	88.88 <sup>b</sup>	66.66 <sup>b</sup>

Values are expressed in percentage.

<sup>a</sup>: significantly different from controls by Chi-square test at  $p < 0.05$ , <sup>b</sup>: significantly different from MTZ (500mg/kgBW/day) by Chi-square test at  $p < 0.05$

#### Fertility index

The females exhibited marked reduction in the fertility index to 25% when impregnated with the male mice, administered with 500mg/kg BW/day of MTZ (Table 1). This marked reduction was reinstated to 50% in the females, impregnated with the male mice, co-administered with 500mg/kg BW/day of MTZ and 100mg/kg BW/

day of the fruit extract of TT and to 66.66% in the females impregnated with the male mice co-administered with 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT (Table 1).

#### Quantal pregnancy

The females exhibited marked reduction in the quantal

pregnancy to 37.50% when impregnated with the male mice, administered with 500mg/kg BW/day of MTZ (Table 1). This marked reduction was reinstated to approximately 71.42% in the females impregnated with the male mice co-administered with 500mg/kg BW/day of MTZ and 100mg/kg BW/day of the fruit extract of TT and to 88.88% in the females impregnated with the male mice co-administered with 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT (Table 1).

### Number of live implants

The number of live implants declined significantly in the

females cohabited with the male mice, administered with 500mg/kg BW/day of MTZ, as compared with the controls (Table 2; Fig. 1B). The females impregnated with the male mice, co-administered with 500mg/kg BW/day of MTZ and 100mg/kg BW/day of the fruit extract of TT, did not show significant increase in the number of live implants (Table 2; Fig. 1D). However, significant increase was noticed in the same in the females impregnated with the male mice, co-administered 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT, as compared to those administered with 500mg/kg BW/day of MTZ (Table 2; Fig. 1E), thus attained the values similar to the controls.

**Table 2. Effect of oral administration of MTZ and co-administration of MTZ and the fruit extract of TT for 28 consecutive days on the number of live implants, pre-implantation loss and post-implantation loss**

Groups	No. of live implants	Pre-implantation Loss	Post-implantation Loss
Untreated Control	7.62 ± 0.80	3.98 ± 0.94	0.75 ± 0.25
Vehicle-treated Control	7.25 ± 0.53	4.25 ± 1.13	0.41 ± 0.14
MTZ(500mg/kgBW/day)	2.75 ± 1.1 <sup>a</sup>	6.25 ± 1.1	1.25 ± 0.52
TT (200mg/kgBW/day)	8.33 ± 1.07	3.50 ± 0.76	0.33 ± 0.36
MTZ(500mg/kgBW/day) + TT (100mg/kgBW/day)	3.33 ± 1.13	6.08 ± 1.12	0.83 ± 0.22
MTZ(500mg/kgBW/day) + TT (200mg/kgBW/day)	5.41 ± 1.25 <sup>b</sup>	3.16 ± 0.73	0.16 ± 0.11

Values are mean ± SE of 10 females.

Values are considered significant by ANOVA followed by Newman-Keul's multiple range test at  $p < 0.05$ .

a: significantly different from controls, b: significantly different MTZ(500mg/kgBW/day).



**Fig. 1** Implantation sites in the uterus of the female mice impregnated with (A.) the control male mice. Note the normal range of the numbers of implanted embryos in the left and right uteri. (B.) the male mice administered with 500mg/kgBW/day of MTZ for 28 days. Note the post-implantation loss indicated by the least number of implanted embryos in the uterus, the dead implant (red arrow) and the resorption sites indicated by scars (black arrow) observed in the uterus devoid of implanted embryos. (C.) the male mice administered with 200mg/kgBW/day of the fruit extract of TT for 28 days. Note the normal range of the numbers of implanted embryos in the left and right uteri. (D.) the male mice co-administered with MTZ (500mg/kgBW/day) and the fruit extract of TT (100mg/kgBW/day) for 28 days. Note the post-implantation loss indicated by the dead implant (red arrow), the partial recovery in the number of implanted embryos as the left and right uteri of one female was still devoid of implanted embryos. (E.) the male mice in Gr. VIII, co-administered with MTZ (500mg/kgBW/day) and the fruit extract of TT (200mg/kgBW/day) for 28 days. Note the recovery in the number of implanted embryos in the uterus. Also note a dead implant (red arrow) in the left uteri of two females.

### **Pre-implantation loss**

Females impregnated with the male mice administered with 500mg/kg BW/day of MTZ showed a marked increase in the pre-implantation loss, as compared to the controls (Table 2; Fig. 1B). However, this loss was restored, comparable to the controls, only in the females impregnated with the males co-administered with 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT (Table 2; Fig. 1E).

### **Post-implantation loss**

Females impregnated with the male mice administered with 500mg/kg BW/day of MTZ showed a marked increase in the post-implantation loss, as compared to the controls (Table 2; Fig. 1B). However, a noticeable restoration was noted by showing a decrease in such losses, comparable to the controls, only in the females impregnated with the males co-administered with 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT (Table 2; Fig. 1E).

### **Discussion**

The present study centres around the ameliorating potentiality of the fruit extract of TT against MTZ-induced fertility impairment. The ameliorating fertilizing potentiality was evidenced by evaluating the libido index, quantal pregnancy, number of live implants, pre-implantation loss and post-implantation loss.

In the present study, MTZ administration did not affect the libido index of the mice. The libido index ranging between 80-90% suggests the unaltered mating ability of the mice, administered with 500mg/kg BW/day of MTZ. Since, libido index is an androgen-dependent parameter<sup>38</sup> therefore, its unaltered range noticed in the present study reflects the optimal level of serum testosterone in the mice administered with MTZ (500mg/kg BW/day). This reflection is supported by the unaltered level of serum testosterone as reported in our earlier finding<sup>21</sup>.

In spite of the unaltered mating ability of the treated males, significantly reduced number of live implants, with profound elevation in the pre-implantation and post-implantation losses were noted in the females, impregnated with the males, administered with 500mg/kg BW/day of MTZ. These findings suggest the impaired fertilizing potential of the spermatozoa, which may be due to alterations in the sperm quality. A high percentage of sperm

with progressive motility is related to high fertilization index.<sup>39</sup> Therefore, the decline in the fertility index of the females with reduced number of live implants in the uteri might be due to severely depressed motility of epididymal spermatozoa in the mice administered with 500mg/kg BW/day of MTZ as reported in our earlier findings,<sup>36</sup> thus, suggesting an impaired sperm transport in the reproductive tract.

Further, the decline in quantal pregnancy indicates the anti-implantation effect of MTZ. Pre-implantation losses might have arisen due to disruption of events which are prerequisite for fertilization or an impairment in the production of cytokines, growth factor and various types of adhesion molecules either by the developing blastocyst or by the uterine epithelium around the site of implantation.<sup>40-42</sup> The increase in the post-implantation loss following 500mg/kg BW/day of MTZ administration may be attributed to the direct damage to the sperm DNA, caused by the free radicals or reactive oxygen species released by the inflammatory cells.<sup>42</sup> The post-implantation loss may also be attributed to the oxidative stress, produced in the testis of MTZ-treated mice, as reported in our earlier study.<sup>35</sup>

In the present study, fertility of the MTZ-treated males was improved following co-administrations of MTZ and 200mg/kg BW/day of the fruit extract of TT, as evidenced by recovery in the fertility index, quantal pregnancy, the number of live implants and pre- and post-implantation losses in the females impregnated with such males. Our earlier findings reporting the reappearance of several spermatozoa in the epididymal lumen, as evidenced through histopathological study, following co-administrations of 500mg/kg BW/day of MTZ and 200mg/kg BW/day of the fruit extract of TT<sup>36</sup>, also support the present study. Therefore, improvements in the number of live implants and pre- and post-implantation losses might be due to the beneficial properties exhibited by the fruit extract of TT on the quality and quantity of spermatozoa,<sup>43-47</sup> thus increasing the fertility potential of the MTZ-treated mice.

### **Conclusion**

MTZ, administered at the dose of 500mg/kg BW/day for 28 days impairs the fertility in the males. The fruit extract of TT bears the potentiality to restore the impaired

fertility and hence, may emerge as an effective herbal remedy in improving the drug-induced fertility impairments in the males.

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### Conflict of interest

None.

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