Studies on the Cytological, Biochemical and Reproductive Toxicity of St. John’s Wort after Chronic Treatment in Swiss Albino Mice

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Abstract: St John’s Wort (SJW, Hypericum perforatum), a popular dietary supplement is immensely used in treating a broad range of psychiatric disorders. It is reported to possess both favorable and detrimental effects. Which are attributed to the active principles (hypericin and hyperforin) of SJW. Nevertheless, there is dearth of reports on toxicity of SJW. In view of the traditional significance of SJW, its immense use and a paucity of literature, it was found worthwhile to investigate its effect on (1) cytogenetic analysis of testicular chromosomes (2) morphology of sperms (3) dominant lethal assay (4) biochemical analysis of proteins, nucleic acids, MDA and NP-SH in the testes and (5) pituitary-gonadal hormones in plasma. The procedure included the oral treatment of mice with different doses (95, 190 and 380 mg/kg/day) of SJW for 90 days. The treatment caused significant changes in chromosomal aberrations, morphology of different types of sperms, rate of pregnancy and pre-implantation loss. However, the post-implantation losses were not affected significantly. Both prolactin and testosterone levels were found to increase at the highest dose, while the medium dose affected prolactin levels only. The nucleic acid concentration was depleted in the testicular cells. These changes might be attributed to oxidant activity as revealed by an increase in the concentrations of MDA and a decrease of NP-SH levels in testicular cells observed in the present study. The observed effects appears to be related to mutagenic and oxidant activity of terpenes, tannins, quercetin and flavonoids present in SJW.

Key words: St. John’s Wort • Cytology • Somatic Cells • Germ Cells • Nucleic Acids • Malondialdehyde • Nonprotein Sulphydryl Groups

INTRODUCTION

Hypericum perforatum L (St John’s Wort, SJW) is a widely distributed herbaceous herb. Traditionally, it is used in treatment of mild to moderately severe depression, insomnia, exhaustion, somatoform disorders, nervousity, convalescence; in addition to its application as a remedy for skin diseases, superficial injury, mucosal lesions and gastrointestinal illness [1, 2]. Samadi et al. [3] found topical application of SJW to facilitate cesarean wound healing and minimize formation of scar and its pain and pruritus. It is reported to protect against the apoptosis induced by H2O2 in human neuroblastoma cells and is shown to inhibit the free radical production in both cell-free and human vascular tissue [4]. Thomas et al. [5] reported hyperforin, a concentrated extract of SJW to treat premature ejaculation in rats. SJW is reported to improve quality of life in perimenopausal women [6].

Studies on toxicity of SJW revealed SJW to cause erythema, edema, alopecia, reduction in weight and changes in blood chemistry, in addition to skin reddening, itching, dizziness, constipation, fatigue, anxiety and tiredness in both humans and animals [7, 8]. The itching type of erythematous lesions were specific to light-exposed areas [9]. SJW is also known to cause a severe damage in both liver and kidney of wistar rats [10].

Capasso et al. [11] showed SJW to directly inhibit rat and human vas deferens contractility. Ondrizek et al. [12] found SJW to possess spermicidal effect, as revealed by inhibition of sperm motility and it had an adverse effect on oocytes and weight of newborn. The authors also demonstrated it to be mutagenic to sperm cells.
Thus a large number of papers are published on the pharmacology and toxicology effect of SJW. However, there is a paucity of literature on genotoxicity, biochemical and reproductive toxicity of SJW. The present study on the cytological and biochemical effects of SJW in germ cells of Swiss albino mice was undertaken in view of preliminary reports on reproductive toxicity of SJW [12], its folkloric importance and immense (prescribed or un-prescribed) use.

MATERIAL AND METHODS

Test Herbal Product: St John’s Wort marketed as dietary supplement by Nature’s way product (USA) in form of capsules was used as the test herbal product in the present study. Each capsule contained 300 mg of SJW.

Animal Stocks: Male Swiss albino mice (SWR) aged 6-8 weeks and weighing 25-28 g were obtained from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. The animals were fed on Purina chow diet and water ad libitum and were maintained under standard conditions of humidity, temperature and light (12 h, light/12 dark cycle). The conduct of experiments and the procedure of sacrifice (using ether) were approved by the Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Dose, Route and Duration of Treatment: The dose of SJW was determined by (i) Maximum Tolerated Dose (MTD) (ii) Human therapeutic dose with reference to the surface area rule and (iii) preliminary experiments conducted in our laboratory and reports in the literature [13, 14].

On the basis of evaluated MTD, (6.00 gm/kg.), the doses of SJW selected were 380, 760 and 1520 mg/kg/day corresponding to 1/16th, 1/8th and 1/4th, respectively [15]. The daily recommended dose of SJW (as inscribed on the commercially available bottle) for an adult human is 900 mg (3 capsules). According to the rule of surface area ratio [15] of mice (20 g) and man (60 kg), the calculated ratio is 0.0026 and the dose of SJW (per 50 mice weighing 1 kg) would be 117.0 mg/kg (0.0026 x 900 x 50 = 117.0 mg/kg.). The dose used experimentally is generally six times more than the calculated value (117 x 6 = 702.0 mg/kg/day). This is because the metabolic rate is more in mouse as compared to human being [16]. The medium dose (760 mg/kg/day) selected in the present study was based on the recommended human dose and the surface area rule and was around 1/8th of MTD. The low (380) and high (1520 mg/kg/day) doses were approximately half and double the dose selected, respectively. Aqueous suspension of SJW was administered by gastric intubation (oral) per kg/day for ninety days.

Experimental Groups: The experimental groups of mice consisted of the following: group 1, control (0.3 ml/mouse, tap water); group 2, SJW (380 mg/kg/day); group 3, SJW (760 mg/kg/day); group 4, SJW (1520 mg/kg/day). The different parameters of study were (i) fertility Index in male and female mice; (ii) evaluation of dominant lethal assay; (iii) analysis of spermatozoa abnormalities; (iv) study on chromosomal aberrations; (v) biochemical evaluation; (vi) endocrinology.

Reproductive Performance, Fertility Index and Dominant Lethal Assay: The methods described in male anti-fertility study [17] and dominant lethal assay [18, 19] were followed to evaluate the (i) rate of fertility in male mice (ii) induction of pregnancy (iii) total and pre-implantation loss and (iv) embryo-toxicity. After the treatment, each male mouse in the treated and control groups was caged with three females, which were allowed to stay with the male for one week. The female mice were necropsied 13 days following the mid-week of their caging and presumptive mating and the number of pregnant mice was recorded to determine fertility percent [20]. The pre-implantation loss was calculated by comparing the number of implantations per pregnant female in the treated and control groups. The dead foeti per pregnant female were determined to obtain the post-implantation embryonic loss [19].

Evaluation of Spermatozoa Abnormalities: The spermatozoa were obtained by making small cuts in caudae epididymis and vas deferens placed in 1 ml of modified Krebs Ringer-bicarbonate buffer (pH 7.4). To evaluate the spermatozoa abnormalities, the sperm suspension was stained with eosin, smears were made on slides, air-dried and made permanent. Coded slides were examined by bright-field microscope with an oil immersion lens. The different spermatozoa abnormalities screened were amorphous, banana shaped, swollen achrosome, triangular head, macrocephali and rotated head screened [19-22].

Cytological Analysis of Germ Cells: In analysis of the chromosomal aberrations, the mice were sacrificed after the last day of the treatment [19, 20]. The testes were removed in an isotonic sodium citrate solution and the seminiferous tubules were teased to form a cell suspension. The suspension was centrifuged and the
pellet re-suspended in the hypotonic citrate solution. After the second centrifugation the supernatant was discarded and the pellet suspended in a fixative (methanol and acetic acid, 3:1). The chromosomal preparations were made by the air drying technique [19, 20, 23]. The coded slides were stained in Giemsa solution and screened for the aberrations including aneuploids, autosomal univalents, sex-univalents and polyploids.

**Biochemical Evaluation:** The frozen samples of testes were used for estimation of proteins, ribose nucleic acid (RNA) and deoxyribose nucleic acid (DNA), MDA and NP-SH levels.

**Estimation of Total Proteins and Nucleic Acids:** Total proteins were estimated by the modified Lowry method of Schacterle and Pollack [24]. Bovine serum albumin was used as standard. The method described by Bregman [25] was used to determine the levels of nucleic acids. Testes were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted with ethanol. The levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

**Determination of MDA Concentrations:** The method described by Ohkawa et al. [26] was used. Testes were homogenized in TCA solution and the homogenate suspended in thiobarbituric acid. After centrifugation the optical density of the clear pink supernatant was read at 532 nm. Malondialdehyde bis (dimethyl acetal) was used as an external standard.

**Quantification of the NP-SH Levels:** The method of Sedlak and Lindsay [27] was used to determine the levels of NP-SH. The testes were homogenized in ice cold 0.02 M ethylene-o-amine tetra acetic acid disodium (EDTA) before mixing with TCA. The homogenate was centrifuged at 3000g. The supernatant was suspended in tris buffer, 5-5'-dithiobis-(2 nitrobenzoic acid) (DTNB) and read at 412 nm against reagent blank with no homogenate.

**Estimation of Hormones:** The plasma samples were analyzed to estimate the concentrations of human Chorionic Gonadotropin, Luteinizing hormone, Follicle stimulating hormone, Estradiol, Prolactin and Testosterone. The analysis was done by direct immune-enzymatic colorimetric method based on ELISA. The procedure used for each hormone was according to the method described for the particular kit [28].

**Statistical Analysis:** The different studies undertaken were statistically analyzed by Analysis of Variance. Some parameters in the study on reproductive performance were analyzed using a Chi-square test.

**RESULTS**

**Effect of SJW on Fertility Index in Male and Female Mice:** The fertility in male and female mice was 90 percent in the control group. The prolonged treatment with SJW at the high dose was found to reduce the fertility to 60 percent in male mice and 70 percent in the female mice (Table 1).

**Effect of SJW on the Induction of Dominant Lethal Mutations after Sub-Chronic Treatment in Male Swiss Albino Mice:** In mating week 1, the sub-chronic treatment of male mice with SJW was found to significantly ($P<0.05$) decrease the percent of pregnant female mice at the high dose. There were no significant changes in the total, live and dead foeti per pregnant female mice and the percent dead embryos at any of the doses of SJW. In mating week 2, the sub-chronic treatment of male mice with SJW failed to induce a significant reduction in the percent pregnant mice. There was a significant decrease in the total implants ($P<0.01$) and live implants ($P<0.05$) per pregnant female mice at the high dose. There was an increasing trend of the dead implants per pregnant female and the percent dead embryos (Table 2).

**Effect of SJW on Spermatozoa Morphology:** The sub-chronic treatment with SJW was found to significantly ($P<0.05$) increase the amorphous type and total abnormalities at the medium and high doses (190 and 380 mg/kg., body weight/day). The other sperm abnormalities (banana shaped, swollen achromosome and macrocephali) were increased significantly ($P<0.05$) at the high dose of SJW. There was a trend increase of rotated and triangular head spermatozoa abnormalities at different doses of SJW (Table 3).

**Effect of SJW on Testes Chromosomes:** The sub-chronic treatment with SJW induced a statistically significant ($P<0.05$) increase in the frequency of aneuploids, polyploids and total chromosomal aberrations at the medium and high doses (190 and 380 mg/kg., body weight/day) (Table 4).
Table 1: Effect of St. John’s wort on fertility index in male and female mice after sub-chronic treatment in male Swiss albino mice

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment and dose (mg/kg. Body weight/day)</th>
<th>No. of male mice mated</th>
<th>No. of fertile male mice</th>
<th>Fertility percent</th>
<th>No. of female mice mated</th>
<th>No. of fertile female mice</th>
<th>Fertility percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (0.3 ml tap water/mouse)</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>10</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>St. John’s wort (95)</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>10</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>St. John’s wort (190)</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>10</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>St. John’s wort (380)</td>
<td>10</td>
<td>6</td>
<td>60</td>
<td>10</td>
<td>7</td>
<td>70</td>
</tr>
</tbody>
</table>

10 mice (male or female) were used in each group

*P > 0.05 (Chi square test)

Table 2: Effect of St. John’s wort on the induction of dominant lethal mutations after sub-chronic treatment in male Swiss

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment and dose (mg/kg. Body weight/day)</th>
<th>Pregnant females Total</th>
<th>Live</th>
<th>Dead</th>
<th>Percent dead embryos</th>
<th>Pregnant females Total</th>
<th>Live</th>
<th>Dead</th>
<th>Percent dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (0.3 ml tap water/mouse)</td>
<td>27/30 (90.00)</td>
<td>10.86±0.57</td>
<td>10.33±0.54</td>
<td>0.52±0.19</td>
<td>14/29(4.78)</td>
<td>11.87±0.43</td>
<td>11.35±0.49</td>
<td>0.52±0.22</td>
</tr>
<tr>
<td>2</td>
<td>St. John’s wort (95)</td>
<td>21/30 (70.00)</td>
<td>11.10±0.30</td>
<td>10.52±0.36</td>
<td>0.52±0.16</td>
<td>11/23(4.74)</td>
<td>11.70±0.42</td>
<td>11.20±0.47</td>
<td>0.55±0.21</td>
</tr>
<tr>
<td>3</td>
<td>St. John’s wort (190)</td>
<td>20/30 (66.67)</td>
<td>11.55±0.34</td>
<td>10.85±0.31</td>
<td>0.65±0.22</td>
<td>13/29(4.74)</td>
<td>10.65±0.71</td>
<td>10.08±0.67</td>
<td>0.65±0.15</td>
</tr>
<tr>
<td>4</td>
<td>St. John’s wort (380)</td>
<td>17/30* (56.70)</td>
<td>11.12±0.42</td>
<td>10.65±0.41</td>
<td>0.47±0.15</td>
<td>8/198(4.23)</td>
<td>9.67±0.48**</td>
<td>8.86±0.80*</td>
<td>0.81±0.24</td>
</tr>
</tbody>
</table>

Figures between parentheses denote percent.

*P <0.05; ** P <0.01 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test)

Table 3: Effect of St. John’s wort on sperm abnormality in Swiss albino mice after sub-chronic treatment

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Different Spermatozoal abnormalities screened/Total</th>
<th>Control (tap water, 0.3 ml/mouse/day)</th>
<th>St. John’s wort (95)</th>
<th>St. John’s wort (190)</th>
<th>St. John’s wort (380)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amorphous</td>
<td>0.14±0.02</td>
<td>0.21±0.03</td>
<td>0.66±0.12*</td>
<td>0.66±0.23*</td>
</tr>
<tr>
<td>2</td>
<td>Banana shaped</td>
<td>0.22±0.05</td>
<td>0.30±0.06</td>
<td>1.61±1.00</td>
<td>0.46±0.09*</td>
</tr>
<tr>
<td>3</td>
<td>Swollen acrosome</td>
<td>0.21±0.08</td>
<td>0.30±0.04</td>
<td>0.42±0.06</td>
<td>0.52±0.11*</td>
</tr>
<tr>
<td>4</td>
<td>Triangular head</td>
<td>0.45±0.07</td>
<td>0.41±0.03</td>
<td>0.74±0.15</td>
<td>1.04±0.38</td>
</tr>
<tr>
<td>5</td>
<td>Macrocephali</td>
<td>0.31±0.07</td>
<td>0.34±0.06</td>
<td>0.50±0.16</td>
<td>0.54±0.05*</td>
</tr>
<tr>
<td>6</td>
<td>Rotated head</td>
<td>0.09±0.03</td>
<td>0.16±0.07</td>
<td>0.12±0.02</td>
<td>0.20±0.08</td>
</tr>
<tr>
<td>7</td>
<td>Total abnormalities</td>
<td>1.44±0.24</td>
<td>2.05±0.21</td>
<td>3.19±0.57*</td>
<td>6.24±1.90*</td>
</tr>
<tr>
<td>8</td>
<td>Total sperms screened</td>
<td>5020</td>
<td>6000</td>
<td>5060</td>
<td>4990</td>
</tr>
</tbody>
</table>

Five mice were used in each group

* P <0.05 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters)

Table 4: Effect of St. John’s wort on testis chromosomes in Swiss albino mice after sub-chronic treatment

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Different chromosomal abnormalities screened/Total</th>
<th>Control (tap water, 0.3 ml/mouse/day)</th>
<th>St. John’s wort (95)</th>
<th>St. John’s wort (190)</th>
<th>St. John’s wort (380)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aneuploids</td>
<td>3.61±0.48</td>
<td>4.45±0.65</td>
<td>5.58±0.59*</td>
<td>5.50±0.37*</td>
</tr>
<tr>
<td>2</td>
<td>Autosomal univalents</td>
<td>3.15±0.41</td>
<td>3.25±0.37</td>
<td>2.53±0.42</td>
<td>2.99±0.26</td>
</tr>
<tr>
<td>3</td>
<td>Sex-univalents</td>
<td>2.79±0.27</td>
<td>3.18±0.58</td>
<td>3.20±0.59</td>
<td>2.45±0.56</td>
</tr>
<tr>
<td>4</td>
<td>Polyploids</td>
<td>3.12±0.56</td>
<td>3.75±0.73</td>
<td>5.34±0.48*</td>
<td>4.99±0.52*</td>
</tr>
<tr>
<td>5</td>
<td>Translocations</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Total-percent aberrations</td>
<td>12.62±1.05</td>
<td>14.08±1.72</td>
<td>16.65±1.38*</td>
<td>15.94±0.78*</td>
</tr>
<tr>
<td>7</td>
<td>Total stages screened</td>
<td>600</td>
<td>547</td>
<td>530</td>
<td>500</td>
</tr>
</tbody>
</table>

Five mice were used in each group

* P <0.05 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters).
Effect of SJW on Proteins, Nucleic Acids, MDA and NP-SH: The sub-chronic treatment with SJW was found to inhibit significantly (P<0.05) the testicular levels of RNA and DNA at the high dose. Prolonged treatment with SJW significantly (P<0.05) increased and decreased the testicular concentrations of MDA and NP-SH, respectively, at the medium and high doses (Table 5).

Effect of SJW on Certain Pituitary-Gonad Hormones: The levels of prolactin were increased (P<0.01) at the medium and (P<0.001) high dose. The concentrations of testosterone were also increased significantly (P<0.001) at the high dose. In female mice, a trend increase and decrease was observed in the plasma levels of progesterone and leutenizing hormone, respectively (Table 6).

**DISCUSSION**

The sub-chronic treatment with SJW was found to decrease the percent fertility in male mice which is found to influence reduction of fertility in female mice and pre-implantation losses. However, the treatment failed to significantly increase dead foeti per pregnant female and percent dead embryos. The embryonic loss, before implantation, observed in the present study cannot be distinguished between the fertilized and unfertilized eggs.

The data on fertility index and the dominant lethal assay are in agreement with reports in the literature on the sexual dysfunction caused by SJW [29]. The exact mechanism of the reduction of fertility index and the reduction of pregnant female mice in the dominant lethal assay is not known, however, it appears that the biochemical changes observed in the present study might be responsible for reduced fertility. SJW is well known for its cytotoxic [30, 31] and oxidant [32] activity. The oxidant and cytotoxic activity observed in the present study on the biochemical changes, the reduction of fertility index and the fertility of females mated to treated male mice are confirmed by reports on the spermatotoxic and mutagenic effects of SJW [12]. The study on the plasma levels of hormone showed increase of prolactin levels in the male mice. The observed changes in the plasma hormones might have also contributed to the low fertility rate observed [33].
significant increase in the proportion of aneuploids, polyploids and the frequency of total chromosomal aberrations. The long term treatment of SJW showed a significant increase observed in the proportion of amorphous, banana shaped, swollen achrosome type and the marocephalous type. Thus the induced pre-implantation loss might be related with the observed sperm abnormalities and the testicular chromosome aberrations. The data is ascribed to the cytotoxic [30, 31] and oxidant [32] activity of SJW. Nevertheless, the results obtained in the dominant lethal assay clearly indicate that there is late onset of the induced damage in form of pre-implantation loss, while such a change is not observed in the first week of mating. The discrepancy might be related to the dual role (oxidant and antioxidant) role of SJW [32].

The data on induction of chromosomal aberrations in the testis and the pre-implantation loss observed in the dominant lethal assay are supported by the reports in the literature on spermatotoxic and mutagenic [12] and sexual dysfunction causing potentials of SJW [29]. These results are ascribed to the cytotoxic [30, 31] and oxidant [32] activity of SJW.

The biochemical analysis of the testicular levels of MDA and NP-SH showed increase and decrease respectively. There was significant depletion of DNA and RNA in the testis. The analysis of plasma hormones revealed the increase of the plasma levels of prolactin and testosterone in male mice. Furthermore, these changes might be responsible for the sperm abnormality, chromosomal aberrations and pre-implantation loss caused by SJW. These changes might be related to the oxidant status of the testicular cells, observed after treatment with SJW. Our results are confirmed by the spermatotoxic and mutagenic [12], DNA damaging and genotoxic [34] potentials of SJW. The constituent(s) responsible for the observed activity are not exactly known, however, these changes might be related to the hypericin, hyperforin, quercetin and flavonoids present in SJW.

Further experiments are suggested on clinical and experimental toxicity of SJW under exposure to light to determine the exact mechanism of action and the role of active principles of SJW.

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