The Effects of Paulinia Cupana, on Genotoxic, Spermatogenic, Reproductive and Biochemical Changes In Sex Cells after Chronic Treatment in Male Swiss Albino Mice

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Abstract: Paulinia cupana (Guarana) is immensely used as a stimulant to increase physical activity and control obesity for a long term. The present study on the effects of Guarana on spermatogenesis, reproduction and biochemical changes in male Swiss albino mice was conducted, in view of a paucity of literature. Mice were orally treated with different doses (33, 66 and 133 mg/kg/day of the aqueous suspension of Guarana for 90 days by oral gavage. Animals in different control and treatment groups were used to conduct the following parameters: (1) cytological aberrations of the testis chromosomes; (2) spermatozoa abnormalities (3) study on reproduction and dominant lethal assay; (5) biochemical study on proteins, nucleic acids, malonaldehyde (MDA), non-protein sulfhydryl (NP-SH) and hormones. The treatment caused a significant increase in chromosomal aberrations and sperm abnormalities. The rate of pregnancy and pre- and post implantation losses were affected. The study on biochemical parameters showed increase of the concentrations of MDA and depletion of proteins, RNA, DNA and NP-SH in the testicular cells. The plasma levels of testosterone were decreased. The changes in MDA and NP-SH elucidated the role of free radical species in the induction of chromosomal aberrations and sperm abnormalities. The rate of pregnancy and pre- and post implantation losses were affected. The study on biochemical parameters showed increase of the concentrations of MDA and depletion of proteins, RNA, DNA and NP-SH in the testicular cells. The plasma levels of testosterone were decreased. The changes in MDA and NP-SH elucidated the role of free radical species in the induction of chromosomal aberrations, spermatozoa abnormalities and reproductive toxicity. The exact mechanism is not known, however, this might be due to the influence of the tannin contents of Guarana. In view of the observed effects, further evaluation of the toxicity of Guarana is suggested before it is further available for human use.

Key words: Guarana · Reproduction · Spermatogenesis · Nucleic Acids · Lipid Peroxides · Nonprotein-Sulfhydryl Groups · Mice

INTRODUCTION

Paulinia cupana (Guarana) belongs to the family ‘Sapindaceae’. It is a very popular plant in folk medicine, due to its weight reducing and stimulant properties, that is attributed to its caffeine content [1]. Dietary supplements that contain Guarana and Ma Huang are widely marketed and used in the U.S.A for treatment against obesity and enhancement of the athletic performance [2]. It is also used to control hot flashes in breast cancer survivors [3]. Herbal drug (Yerbe Mate) containing Guarana seeds and Damiana leaves is also reported to cause significant weight loss [4]. Nevertheless, as a drug, Guarana is sometimes trafficked as a natural stimulant or drug surrogate [5] and Guarana-based formulations are reported to be clinically toxic, like any other weight reducing herbal medicines (Ma huang, Gymnema sylvestre and Garcinia cambogia) which are known to cause rhabdomyolysis [6]. In addition, Ma huang and Guarana containing dietary supplements are found to cause serious cardiovascular toxicity [7]. In a woman, with a case history of mitral valve prolapse, use of Guarana is reported to cause intractable ventricular fibrillation [8]. Studies on mutagenicity showed extracts of Guarana to cause genotoxicity in Escherichia coli and Salmonella typhimurium [9].

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In toxicological studies, Adeyemo-Salami and Makinde, [10], showed Paullinia pinnata to cause elevation of alkaline phosphatase, aspartate aminotransferase, total cholesterol and triglycerides in mice and rats after sub-acute treatment. The present study on the effects of Guarana on spermatogenesis, reproduction and biochemical changes in male Swiss albino mice was conducted, in view of its significance as a folkloric medicine, long term use, reported acute toxicity and a paucity of literature.

MATERIALS AND METHODS

Test Herbal Product: Guarana (Paullinia cupana Kunth var. sorbilis) was used as the test herbal product in the present study. It is manufactured by Natural Balance, Inc., Castle Rock, CO, 80104, USA and marketed in form of capsules by General Nutrition Corporation (GNC) of USA in Saudi Arabia. Each capsule contains proprietary blend weighing 531 mg. The blend consists of Guarana (standardized seed extract yielding 72 mg of caffeine) ephedra (standardized plant body extract yielding 12 mg of ephedrine), passion flower (aerial portion extract), gotu kola (aerial portion extract), wood betoni (aerial portion). The other ingredients are magnesium stearate and gelatin.

Dose Selection and Route of Administration: The doses selected to conduct different studies were based on the LD₅₀ (2.12 g/kg.) value. The different doses selected for Guarana were 33, 66 and 133 mg/kg., body weight/day, corresponding 1/64, 1/32 and 1/16, respectively of the LD₅₀ (2.12 g/kg.) value evaluated in our laboratory based on preliminary experiments [11]. The duration of treatment was 90 days (sub-chronic). The dosage form was aqueous suspension and the route of administration, gastric intubation (oral) in all the experiments.

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.

Experimental Groups: The experimental groups of mice consisted of the following: group 1, control (tap water); group 2, Guarana (33 mg/kg/day); group 3, Guarana (66 mg/kg/day); group 4, Guarana (133 mg/kg/day). In each group, a total of 30 male mice and 30 female mice were used. In studies on evaluation of reproductive performance 10 male mice and 30 female mice were used in each group. The allotment of mice for different experiments was as follows: (i) chromosomal aberrations (5 mice); (ii) studies on spermatozoa abnormalities (5 mice); (iii) evaluation of reproductive performance and dominant lethal assay (10 male mice and 30 female mice); (iv) biochemical evaluation (5 mice) and (v) endocrinology (5 mice);

Cytogenetic Analysis for Meiotic Chromosomes: In the analysis of the chromosomal aberrations, the mice were sacrificed after the last day of the sub-chronic treatment [12, 13]. The testis was removed in an isotonic sodium citrate solution. After peeling out the tunica albugenia, the seminiferous tubules were teased to form a cell suspension. The cell 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 fixative (methanol and acetic acid, 3:1). The chromosomal preparations were made by the air drying technique [12-14]. The coded slides were stained in Giemsa solution (10 per cent) and screened for the chromosomal aberrations including aneuploids, autosomal univalents, sex-univalents, polyploids and translocations.

Evaluation of Spermatozoa Abnormalities: The mice were sacrificed after the last day of sub-chronic treatment [12, 13, 15]. 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). After 10 minutes, the epididymis and vas deferens tubules were removed and the resultant sperm suspension evaluated for sperm content, motility percent. The motility percent of sperms was determined by their progressive and non-progressive movements observed under a compound microscope [16]. The sperm count was determined under a Neubauer haemocytometer [17]. The sperm suspension obtained was stained with 0.05% of eosin-Y, smears were made on slides, air-dried and made permanent. The spermatozoal morphology was examined by bright-field microscopy with an oil immersion lens. The different spermatozoal abnormalities (amorphous, banana shaped, swollen
achromosome, triangular head, macrocephali and rotated head) screened were those found in all the slides [15, 18, 19].

Reproductive Performance and Dominant Lethal Assay: The dominant lethal assay was carried to study the reproductive performance of male mice [20]. After the treatment, each male mouse in the treated and control groups was caged with three females for mating. 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 [13]. The pre-implantation loss was calculated by comparing the number of implantations per pregnant female in the treated and control groups. The post-implantation loss was determined by the number of dead implantations per pregnant female as a measure of dominant lethality [20].

Biochemical Evaluation
Estimation of Total Proteins: Total proteins were estimated by the modified Lowry method of Schacterle and Pollack [21]. Bovine serum albumin was used as standard.

Determination of Nucleic Acids: The method described by Bregman [22], 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. DNA was 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 colour was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

Malondialdehyde Estimation: The method described by Ohkawa et al. [23] was used. Malondialdehyde (MDA) was measured as an indicator of lipid peroxidation. Testes were homogenized in KCl solution and incubated with thiobarbituric acid. After centrifugation the pink clear layer was read at 532 nm. MDA bis (dimethyl acetal) was used as an external standard.

Effect of Guarana on Testis Chromosomes: The treatment caused a significant increase in the frequency of aneuploids (P<0.01), sex-univalents (P<0.05), polyploids (P<0.001) and the total percent chromosomal aberrations (P<0.001) at the high dose. The frequency of autosomal univalents showed an increasing trend (Table 1).

Effect of Guarana on Reproduction and Dominant Lethal Assay in Male Mice: The long-term treatment with Guarana reduced the Percentage of pregnant female mice significantly (P<0.05) at medium and (P<0.01) high dose. At this dose the total and live implants per pregnant female mice were also significantly (P<0.01) decreased. There was no effect on dead implants/pregnant female mice. The dead embryos percent Revealed fan increasing trend (Table 3).
Table 1: Effect of Guarana on testis chromosomes in Swiss albino mice after sub-chronic treatment

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Different chromosomal abnormalities</th>
<th>Control (tap water, 0.3 ml/mouse/day)</th>
<th>Guarana (33)</th>
<th>Guarana (66)</th>
<th>Guarana (133)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aneuploids</td>
<td>2.61±0.52</td>
<td>3.53±0.56</td>
<td>4.39±1.13</td>
<td>7.34±0.37**</td>
</tr>
<tr>
<td>2.</td>
<td>Autosomal univalents</td>
<td>2.98±0.53</td>
<td>3.91±0.73</td>
<td>3.73±0.40</td>
<td>4.13±0.64</td>
</tr>
<tr>
<td>3.</td>
<td>Sex-univalents</td>
<td>2.96±0.68</td>
<td>3.19±0.32</td>
<td>4.36±0.85</td>
<td>6.87±1.10*</td>
</tr>
<tr>
<td>4.</td>
<td>Polyploids</td>
<td>3.23±0.79</td>
<td>4.14±0.81</td>
<td>5.64±0.52</td>
<td>11.79±0.96***</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>11.71±1.20</td>
<td>14.78±0.59</td>
<td>18.45±2.19</td>
<td>30.13±2.39***</td>
</tr>
<tr>
<td>7.</td>
<td>Total stages screened</td>
<td>500</td>
<td>525</td>
<td>500</td>
<td>480</td>
</tr>
</tbody>
</table>

Five mice were used in each group

*P < 0.05, **P < 0.001 (One-way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters).

Table 2: Effect of Guarana on epididymal spermatozoa in Swiss albino mice after sub-chronic treatment

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Different Spermatozoal abnormalities</th>
<th>Control (tap water, 0.3 ml/mouse/day)</th>
<th>Guarana (33)</th>
<th>Guarana (66)</th>
<th>Guarana (133)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amorphous</td>
<td>0.42±0.06</td>
<td>0.41±0.07</td>
<td>0.57±0.08</td>
<td>0.86±0.24</td>
</tr>
<tr>
<td>2.</td>
<td>Banana shaped</td>
<td>0.29±0.05</td>
<td>0.31±0.05</td>
<td>0.41±0.07</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td>3.</td>
<td>Swollen acrosome</td>
<td>0.42±0.05</td>
<td>0.36±0.05</td>
<td>0.43±0.05</td>
<td>0.73±0.27</td>
</tr>
<tr>
<td>4.</td>
<td>Triangular head</td>
<td>0.41±0.11</td>
<td>0.37±0.07</td>
<td>0.42±0.09</td>
<td>0.88±0.18</td>
</tr>
<tr>
<td>5.</td>
<td>Macrocephali</td>
<td>0.27±0.04</td>
<td>0.22±0.04</td>
<td>0.27±0.06</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>6.</td>
<td>Rotated head</td>
<td>0.12±0.02</td>
<td>0.11±0.01</td>
<td>0.14±0.02</td>
<td>0.41±0.13*</td>
</tr>
<tr>
<td>7.</td>
<td>Total abnormalities</td>
<td>1.93±0.19</td>
<td>1.79±0.21</td>
<td>2.24±0.18</td>
<td>3.86±0.87*</td>
</tr>
<tr>
<td>8.</td>
<td>Total sperms screened</td>
<td>5176</td>
<td>5441</td>
<td>5560</td>
<td>5200</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 3: Effect of Guarana on the induction of dominant lethal mutations 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>Pregnant females/ Fertility percent</th>
<th>Mean Implants/pregnant female ± S.E.</th>
<th>Mean Implants/pregnant female ± S.E.</th>
<th>Percent female fertility</th>
<th>Percent dead embryos</th>
<th>Percent male fertility</th>
<th>Percent dead embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (0.3 ml tap water/mouse)</td>
<td>30/30(100)</td>
<td>11.17±0.43</td>
<td>10.57±0.47</td>
<td>18/335(5.37)</td>
<td>11.17±0.43</td>
<td>10.57±0.47</td>
<td>18/335(5.37)</td>
</tr>
<tr>
<td>2</td>
<td>Guarana (33)</td>
<td>25/30(83.33)</td>
<td>9.60±0.95</td>
<td>9.13±0.92</td>
<td>14/288(4.86)</td>
<td>9.60±0.95</td>
<td>9.13±0.92</td>
<td>14/288(4.86)</td>
</tr>
<tr>
<td>3</td>
<td>Guarana (66)</td>
<td>23/30(76.67)*</td>
<td>9.17±1.01</td>
<td>8.63±0.98</td>
<td>16/275(5.82)</td>
<td>9.17±1.01</td>
<td>8.63±0.98</td>
<td>16/275(5.82)</td>
</tr>
<tr>
<td>4</td>
<td>Guarana (133)</td>
<td>16/30(53.33)**</td>
<td>6.83±1.22**</td>
<td>6.27±1.13**</td>
<td>17/205(8.29)</td>
<td>6.83±1.22**</td>
<td>6.27±1.13**</td>
<td>17/205(8.29)</td>
</tr>
</tbody>
</table>

Five mice were used in each group

*A total of 10 male and 30 females were used in each group

*P < 0.01 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test) Percent female fertility *P < 0.05; **P < 0.001 (Chi square test)
Percent dead embryos : P>0.05 (Chi square test)

Table 4: Effect of Guarana on Protein, RNA, DNA, MDA and NP-SH concentrations in testicular cells of Swiss albino mice after Sub-chronic treatment

<table>
<thead>
<tr>
<th>SlNo</th>
<th>Treatment Dose (mg/kg. Body weight)</th>
<th>Proteins (mg/100 mg tissue)</th>
<th>RNA (µg/100mg tissue)</th>
<th>DNA (µg/100mg tissue)</th>
<th>Malondialdehyde concentrations (mmol/g wet tissue)</th>
<th>NP-SH concentrations (mmol/100 mg wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (tap water, 0.3 ml/mouse)</td>
<td>14.14±0.54</td>
<td>509.51±17.28</td>
<td>222.62±10.42</td>
<td>202.64±10.42</td>
<td>135.36±9.38</td>
</tr>
<tr>
<td>2</td>
<td>Guarana (33)</td>
<td>13.75±0.44</td>
<td>472.71±19.96</td>
<td>231.42±9.89</td>
<td>200.61±15.84</td>
<td>119.25±5.68</td>
</tr>
<tr>
<td>3</td>
<td>Guarana (66)</td>
<td>12.55±0.23</td>
<td>427.71±28.98</td>
<td>180.68±15.63</td>
<td>220.81±19.26</td>
<td>116.78±4.51</td>
</tr>
<tr>
<td>4</td>
<td>Guarana (133)</td>
<td>12.04±0.68*</td>
<td>398.28±17.29**</td>
<td>188.13±9.76*</td>
<td>246.61±15.84*</td>
<td>104.14±8.05*</td>
</tr>
</tbody>
</table>

Five mice were used in each group

*P < 0.05; ** P < 0.01 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters)
Table 5: Effect of Guarana on certain pituitary-gonadal hormones in plasma of male Swiss albino mice after Sub-chronic treatment

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Determination of pituitary-gonadal hormones in plasma</th>
<th>Control (tap water, 0.3 ml/mouse/day)</th>
<th>Guarana (33)</th>
<th>Guarana (66)</th>
<th>Guarana (133)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Human-Chorionic Gonadotropin</td>
<td>1.07±0.33</td>
<td>0.80±0.20</td>
<td>0.70±0.08</td>
<td>0.60±0.06</td>
</tr>
<tr>
<td>2.</td>
<td>Leutening hormone</td>
<td>1.40±0.62</td>
<td>1.30±0.52</td>
<td>1.26±0.46</td>
<td>1.58±0.65</td>
</tr>
<tr>
<td>3.</td>
<td>Follicle-Stimulating Hormone</td>
<td>1.20±0.43</td>
<td>1.40±0.32</td>
<td>1.30±0.26</td>
<td>1.26±0.32</td>
</tr>
<tr>
<td>4.</td>
<td>Estradiol</td>
<td>0.24±0.09</td>
<td>0.20±0.08</td>
<td>0.10±0.03</td>
<td>0.37±0.04</td>
</tr>
<tr>
<td>5.</td>
<td>Prolactin</td>
<td>2.20±1.00</td>
<td>1.62±0.85</td>
<td>1.86±0.73</td>
<td>1.96±0.66</td>
</tr>
<tr>
<td>6.</td>
<td>Testosterone</td>
<td>5.00±0.58</td>
<td>4.00±1.50</td>
<td>3.92±1.20</td>
<td>1.00±0.30</td>
</tr>
</tbody>
</table>

Five mice were used in each group

*P<0.01(One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters)

Effect of Guarana on Proteins, RNA and DNA concentrations in testicular cells: The sub-chronic treatment with Guarana decreased significantly the testicular levels of proteins and DNA (P<0.05) and RNA (P<0.01) (Table 4).

Effect of Guarana on MDA and NP-SH concentrations in testicular cells: The treatment with Guarana (high dose) caused a significant (P<0.05) increase and decrease in the testicular levels of MDA and NP-SH, respectively (Table 4).

Effect Of Guarana On Certain Pituitary-Gonadal hormones: The levels of testosterone were decreased significantly (P<0.05) in male mice at the high dose (Table 5).

DISCUSSION

The sub-chronic treatment with Guarana for a period of 90 days was found to cause reproductive toxicity as revealed by reduction in fertility percent and pre-implantation losses and increase of dead implants per pregnant female and dead embryos percent. The embryonic loss, before implantation, observed in the present study is difficult to distinguish between the fertilized and unfertilized eggs. The reduction of fertility after long term treatment with Guarana is supported by significant depletion of the testosterone levels in the plasma of male mice. There are no parallel studies on reproductive toxicity of Guarana, however; the treatment is shown to debilitate the sperms to pierce the oocytes [26]. The causal factors for these events are described as chromosomal anomalies [12]. Our study is supported by Enciso et al. [27] who found chromosomal abnormalities are related with elevated sperm DNA fragmentation. The changes in the rate of pregnancy and pre-implantation loss are in agreement with the data on chromosomal aberrations and spermatozoa abnormalities in the male germ cells observed in the same study, which showed a significant increase in the frequency of aneuploids, sex-univalents, polyplioids, total chromosomal aberrations, rotated head shaped and the total spermatozoa abnormalities after sub-chronic treatment with Guarana. There is no parallel study on spermatozoa. However, earlier studies demonstrated dependence of sperm abnormalities on chromosomal aberrations [17, 27, 28]. These results are in agreement with data on depletion of testicular nucleic acids, observed in the same study. This might be attributed to the generation of lipid peroxidation as revealed by increase and depletion of MDA and NP-SH respectively in the target cells. This confirms a previous report [29]. On the sensitization of target cells by the oxidant status. Lipid peroxidation is reported to cause destruction of cell membranes, resulting in toxic injury to cells and tissues [30, 31]. Furthermore, the depletion of endogenous antioxidants are known to interfere with meiosis and cause chromosomal aberrations and disrupts the development of spermatozoa [13, 32, 33]. Nevertheless, the results obtained in the present study are not in accordance with the reports of Mattei et al., [34] which revealed the antioxidant potentials of Guarana in vitro. The discordance in these results is the difference in the plant products in the present study. Furthermore, in vitro protocol was used by Mattei et al.[34]; whereas, the method used in this study involved the conduct of in vivo experiments to measure the MDA and NP-SH concentrations in both the testes.

The exact mechanism of Guarana-related reproductive toxicity, adverse effects on chromosomes and spermatozoa and the biochemical changes is not known. Nevertheless, the observed effects may be related to the tannin content. Tannins are described dietary carcinogens and as DNA damaging compounds which interfere with...
full use of proteins by biological system [35, 36]. Besides
the role of tannins, the adverse effects of Guarana may
also be related with its caffeine content. Caffeine is
known to increase catecholamines and norepinephrine
[37], which proven to be oxidative mutagens [38] and
cause accumulation of free radicals, cytotoxicity and
apoptosis [39]. Furthermore, the observed toxicity may
also be due to the oxidant and cytotoxic potentials of
xanthine constituents of Guarana [40].

Taken together, the results obtained in this study
apparently show the reproductive, genotoxic and
biochemical toxicity of the sub-chronic treatment of
Guarana in the germ cells of mice. These effects might
have serious consequences that might cause malignancy
and/or induce sterility and teratogenicity, in addition to
increasing the frequency of mutations in the exposed
population. In view of the observed effects of
Guarana, it is suggested to evaluate its clinical toxicity.

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