**In vivo Ipriflavone Mutagenicity and Cytotoxicity after Repeated Treatment Doses**

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**Abstract:** Ipriflavone is a semi-synthetic compound derived from soy and is used by humans as an alternative to hormone replacement, similar to phytohormones. Our study aimed to evaluate the mutagenic and cytotoxic effects of ipriflavone on bone marrow of rodents after repeated doses of treatment. In order to assess the mutagenicity and cytotoxicity of ipriflavone, male mice received different doses of ipriflavone (1.71, 8.57 and 42.85 mg.kg⁻¹) for five consecutive days. The rodent bone marrow micronucleus assay was used to verify mutagenicity and the ratio between polychromatic and normochromatic erythrocytes was used to verify cytotoxicity. Our findings suggest that ipriflavone does not induce mutagenic DNA damage, however, repeated use of ipriflavone at 1.71 or 42.85 mg.kg⁻¹ can trigger cytotoxic damage. For the conditions tested, the treatment clinic dose (8.57 mg.kg⁻¹) was the only one that did not induce mutagenic or cytotoxic damage. Our findings reinforce the safe use of ipriflavone and corroborate those found in the literature.

**Key words:** Ipriflavone, semi-synthetic phytohormon, mice bone marrow, rodent micronucleus assay.

**1. Introduction**

Approximately 200 million people worldwide are affected by the osteoporosis [1]. With increasing life expectancy throughout the 20th and 21st centuries, the projection for the number of osteoporotic fractures is steadily increasing, and fracture prevention remains a challenging clinical need.

On this hand, since ipriflavone increases bone mineral density, it has been extensively used as a nutraceutical supplement to treat degenerative bone disorders, such as osteoporosis [2]. Ipriflavone is a semi-synthetic compound derived from an isoflavone abundantly found in plants. Despite the use of this substance by humans, the efficacy and safety of ipriflavone uses in clinical trials still unclear, which trigger concerns regarding the adverse effects related to its prolonged use [3].

Studies on the toxic potential of ipriflavone present fundamental importance, since many substances used for humans may cause mutations in DNA and leads to the neoplasms development. Thus, among the bioassays used to evaluate DNA damage, it has been suggested the use of the *in vivo* micronucleus assay (MN), performed in rodents bone marrow erythrocyte, characterized as an important cytogenetic test used to evaluate potential genotoxic agents [4, 5].

Substances that show genotoxic action are known by its mutagenic and carcinogenic effects [6]. Thus, this study aimed to evaluate the mutagenic and cytotoxic potential of ipriflavone *in vivo* after repeated treatment doses.

**2. Methods**

**2.1 Treatment Doses**

The treatment concentrations were based on the clinical daily dose of ipriflavone [5], and the final
concentrations were 1.71, 8.57 and 42.85 mg.kg\(^{-1}\) b.w.. Ipriflavone was dissolved in DMSO and administered by gavage, simulating human clinical administration.

2.2 Animals and Treatments

The biotery of the Universidade Federal do Espírito Santo supplied 30 male Swiss albino mice (Mus musculus), aged 10-12 weeks and about 30 g b.w., were randomly selected. The animals were divided in six groups, containing five animals (n = 5), maintained into plastic cages under controlled conditions of light and temperature, with free access to water and food. Research Ethical Committee on Animal Use of the Universidade Federal do Espírito Santo (UFES) previously approved all protocols.

The ipriflavone-treated animals received a single dose of ipriflavone (1.71, 8.57 or 42.85 mg.kg\(^{-1}\) bw) per day via gavage for five consecutive test days. The group treated via gavage with NaCl 0.9% was adopted as the negative control group and the group that received DMSO (0.01 mL.g\(^{-1}\) bw) via gavage formed the solvent control group. The animals that received a single ip injection of cyclophosphamide (Asta Medica) at final concentration of 50.00 mg.kg\(^{-1}\) b.w. was assumed as the positive control group. Not all treatment doses exceeded 0.01 mL.g\(^{-1}\) b.w.

2.3 Micronucleus Test

At the end of the treatment, the mice were euthanized and, to perform the micronucleus test, erythrocytes from the bone marrow of the rodents were obtained [7]. Slides containing bone marrow erythrocytes were prepared and the cells were stained with Leishman, in order of differentiation between normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE). Ipriflavone mutagenicity was established by the frequency of micronucleated polychromatic erythrocytes (MNPCE) in a total of 2000 PCE per animal, and cytotoxicity was established by the ratio of PCE to NCE in a total of 2000 cells. In both analyses, the cells were analyzed under an optical microscope, at 1000x magnification.

2.4 Statistical Analysis

Normality was accessed by the Shapiro Wilk test and data are presented as mean ± standard deviation. The mutagenicity and cytotoxicity of ipriflavone was established by comparing the results obtained with the negative control group, ANOVA post hoc Dunnett’s test (p < 0.05).

3. Results and Discussion

Data regarding mutagenicity and cytotoxicity of ipriflavone after repeated treatment doses (5 days) are summarized in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MNPCE/1000 PCE ± SD</th>
<th>p</th>
<th>PCE/NCE ± SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (0.9%) 0.01 mL.g(^{-1}) bw</td>
<td>11.60 ± 3.17</td>
<td>-</td>
<td>0.503 ± 0.068</td>
<td>-</td>
</tr>
<tr>
<td>DMSO 0.01 mL.g(^{-1}) bw</td>
<td>5.10 ± 2.92***</td>
<td>0.0001</td>
<td>0.463 ± 0.036</td>
<td>0.6122</td>
</tr>
<tr>
<td>Ipriflavone 1.71 mg.kg(^{-1}) b.w.</td>
<td>10.30 ± 3.37</td>
<td>0.8182</td>
<td>0.414 ± 0.110</td>
<td>0.0327</td>
</tr>
<tr>
<td>Ipriflavone 8.57 mg.kg(^{-1}) b.w.</td>
<td>4.20 ± 2.57***</td>
<td>&lt; 0.0001</td>
<td>0.510 ± 0.073</td>
<td>0.9997</td>
</tr>
<tr>
<td>Ipriflavone 42.85 mg.kg(^{-1}) b.w.</td>
<td>4.50 ± 1.08***</td>
<td>&lt; 0.0001</td>
<td>0.396 ± 0.061</td>
<td>0.0071</td>
</tr>
<tr>
<td>Cyclophosphamide 50.00 mg.kg(^{-1}) b.w.</td>
<td>33.00 ± 4.57***</td>
<td>&lt; 0.0001</td>
<td>0.407 ± 0.054</td>
<td>0.0185</td>
</tr>
</tbody>
</table>

Following the mutagenicity analysis, when compared to the negative control group (NaCl 0.9%), the solvent control group (DMSO) showed a reduction in the frequency of micronuclei. DMSO, the substance used to dilute ipriflavone, has non-enzymatic antioxidant activity [8–10] and may have acted in the
fight against oxidant radicals, reducing the frequency of mutagenic damage, as reported by Delarmelina et al. [6] when investigating the antimutagenicity of ipriflavone. Similarly, doses of 8.57 and 42.85 mg.kg⁻¹ also reduced the frequency of micronuclei. In our study, none of the treatment doses induced mutagenic damage (increased micronucleus frequency).

The PCE/NCE ratio provides information on cytotoxicity. Thus, when compared to the control group, the clinical dose (8.57 mg.kg⁻¹) did not induce cytotoxic damage, while doses of 1.71 and 42.85 mg.kg⁻¹ were able to induce, achieving a cytotoxic effect similar to the positive control group (cyclophosphamide 50.00 mg.kg⁻¹). Belcavello et al. [5] when evaluating the cytotoxicity of ipriflavone in rodents in an acute form (single treatment dose) did not observe the induction of cytotoxic damage.

Thus, our findings suggest that the use of repeated doses of ipriflavone, below or above the clinical dose, can trigger cytotoxic damage.

4. Conclusions

For the conditions evaluated in our study, ipriflavone was not able to induce mutagenic DNA damage, however, the repeated use of this substance can lead to the development of cytotoxic damage. For all doses tested, the clinical treatment dose (used for humans) was the only one that did not trigger mutagenic or cytotoxic damage. Thus, our findings reinforce the safe use of ipriflavone. However, we consider that further investigations are needed on the biological activities of ipriflavone.

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Conflict of Interest

The authors state that there is no conflict of interest.

References