

## Article

# Safety Assessment of Selenium-Biofortified Broccoli Seedlings: Insights from Repeated-Dose Toxicity and Genotoxicity Evaluation in Mice

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## Abstract

Selenium (Se) is an essential trace element; however, excessive exposure may cause adverse effects because of its narrow safety margin. This study evaluated the toxicological and genotoxic effects of Se-biofortified broccoli seedlings in male and female Swiss mice. Animals received control or Se-enriched broccoli seedlings orally for 40 days (15, 45, or 70 µg Se/kg body weight (BW)). General toxicity (body weight gain), hematological (complete blood count), biochemical (creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, and urea), histopathological, and micronucleus evaluations were performed. No significant changes in body weight or hematological parameters were detected. Biochemical alterations were restricted to males, with increased urea ( $51.90 \pm 2.14$  mg/dL) and decreased aspartate aminotransferase ( $128.4 \pm 14.45$  U/L) after 45 µg Se/kg BW. The highest Se dose increased spleen ( $0.60 \pm 0.04$  g/100 g BW) and ovary ( $0.29 \pm 0.03$  g/100 g BW) weights in females, decreased liver weight ( $5.82 \pm 0.10$  g/100 g BW), increased testis weight ( $0.76 \pm 0.04$  g/100 g BW) in males, induced renal and splenic histological alterations, and increased micronucleated polychromatic erythrocytes ( $0.46 \pm 0.04$ ). These findings indicate that 15 µg Se/kg BW was the safest dose evaluated. Future studies should investigate Se speciation and sex-dependent toxicological responses.



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**Keywords:** *Brassica oleracea* var. *italica*; food safety; micronucleus assay; sodium selenate

## 1. Introduction

Selenium (Se) is an essential trace element that exerts its biological functions through its incorporation into at least 25 human selenoproteins, including glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases. These selenoproteins play fundamental roles in antioxidant defense, maintenance of redox homeostasis, thyroid hormone metabolism, immune function, and regulation of inflammatory responses. Adequate Se intake is also associated with normal reproductive, neurological, and cardiovascular function and is essential for maintaining metabolic homeostasis. However, because Se has a narrow range between nutritional requirement and toxicity, both inadequate and excessive intake may adversely affect human health. This relationship has been described as a U-shaped dose–response between Se intake and disease risk, highlighting the importance of maintaining an adequate Se status to maximize health benefits while avoiding adverse effects [1–4].

Plants represent one of the main routes through which Se enters the human food chain, and its accumulation in edible tissues is strongly influenced by its availability in the soil [5,6]. In this context, Se biofortification of crops has emerged as a promising strategy to enhance dietary Se intake, particularly in regions where consumption does not meet nutritional requirements [7–10]. Among the crops suitable for Se biofortification, broccoli (*Brassica oleracea* L. var. *italica*) has emerged as one of the most promising vegetables because of its remarkable capacity to accumulate Se while maintaining high nutritional and functional value.

Broccoli, a member of the Brassicaceae family, is a cool-season vegetable crop widely cultivated in temperate and subtropical regions worldwide. It is characterized by an edible immature inflorescence composed of compact green flower buds and thick succulent stems and is recognized as one of the most nutritionally valuable vegetables [11–13]. Broccoli is an excellent source of dietary fiber; vitamins C, E and K; folates; carotenoids; and essential minerals, in addition to containing high concentrations of phytochemicals such as glucosinolates, isothiocyanates, and phenolic compounds, particularly flavonoids [14]. Experimental studies have consistently demonstrated that broccoli-derived phytochemicals modulate oxidative stress, inflammatory pathways, and detoxification enzymes, whereas clinical studies suggest that regular broccoli consumption may improve antioxidant status and reduce risk factors associated with cardiometabolic diseases and certain cancers [14,15].

Experimental evidence demonstrated that the interaction between Se and broccoli phytochemicals can enhance biological responses by increasing the expression of antioxidant-related enzymes, including thioredoxin reductase 1 and glutathione peroxidase [16–18]. Moreover, Se-enriched broccoli has been associated with chemoprotective mechanisms involving the regulation of oxidative stress, apoptosis induction, and modulation of cellular signaling pathways [19,20]. These findings highlight the potential of Se-biofortified broccoli as a functional food capable of combining the intrinsic health-promoting properties of broccoli with the physiological benefits of adequate Se intake.

Despite its essential physiological functions, Se exhibits a narrow margin between nutritional requirement and toxicity. Both inadequate and excessive Se intake can negatively affect human and animal health, with prolonged exposure to high levels resulting in selenosis, a condition associated with symptoms such as hair and nail alterations, gastrointestinal disorders, neurological effects, and tissue damage in severe cases [2,21,22]. The mechanisms involved in Se-induced toxicity remain only partially elucidated; however,

oxidative imbalance, reactive oxygen species generation, DNA damage, and oxidation of thiol-containing molecules have been proposed as important contributors to its toxic effects [2,23]. Furthermore, Se toxicity depends not only on exposure level but also on the chemical forms of Se. Organic Se compounds exhibit lower toxicity than inorganic forms [24,25].

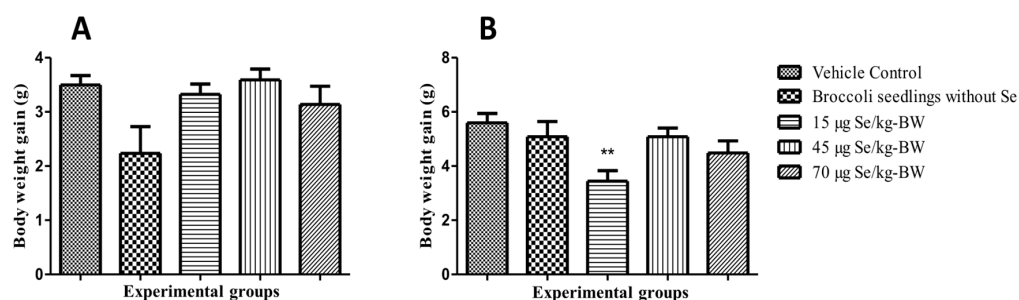
Although several studies have demonstrated the nutritional and functional benefits of Se-biofortified broccoli, most investigations have focused on Se accumulation, phytochemical composition, bioavailability, or biological activity, whereas comprehensive toxicological evaluations remain scarce [7,19,26,27]. Importantly, the toxicity profile of Se-enriched foods cannot be directly extrapolated from studies using isolated Se compounds because Se speciation, the food matrix, and interactions with endogenous phytochemicals influence Se absorption, metabolism, and biological responses [28–30]. Therefore, dedicated toxicological and genotoxic studies are essential to establish the safety of Se-biofortified foods intended for human consumption [24,25].

Therefore, despite the potential of Se biofortification as a nutritional strategy, evaluating the safety of Se-enriched foods is essential before recommending their consumption. To our knowledge, no previous study has simultaneously evaluated repeated-dose toxicity and *in vivo* genotoxicity of Se-biofortified broccoli intended for human consumption. Based on this premise, this study aimed to assess the effects of repeated oral exposure to Se-biofortified broccoli seedlings through toxicological and genotoxic evaluations in an experimental mice model.

## 2. Results

### 2.1. *In Vivo* Safety Evaluation of Se-Biofortified Broccoli Seedlings

Throughout the 40-day experimental period, no clinical manifestations associated with toxicity were observed in either male or female mice. Signs such as hair loss, skin irritation, alterations in mucous membranes and eyes, fatigue, and soft or mucoid stools were absent, and all animals remained healthy until the end of the study. No mortality associated with the treatments was recorded. In female mice, body weight gain did not differ significantly between the vehicle control and treated groups (Figure 1). Conversely, males receiving 15  $\mu\text{g}$  Se/kg BW exhibited a significant decrease in body weight gain compared with the vehicle control group. Food and water consumption remained unchanged among all experimental groups in both sexes throughout the treatment.



**Figure 1.** Effects of Se-biofortified broccoli seedlings on body weight gain after repeated oral administration. Body weight gain of female (A) and male (B) Swiss mice following 40 consecutive days of treatment. Vehicle control: PBS (10 mL/kg body weight (BW)); broccoli control: non-biofortified broccoli seedlings; Se-enriched broccoli seedlings: 15, 45, and 70  $\mu\text{g}$  Se/kg BW. Data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test. \*\*  $p < 0.01$  versus the vehicle control group.

The hematological parameters evaluated after broccoli seedling administration are summarized in Table 1. Treatment with non-biofortified broccoli seedlings and all doses

of Se-enriched broccoli seedlings significantly reduced ( $p < 0.05$ ) mean corpuscular hemoglobin concentration (MCHC) compared with the vehicle control group in female mice. However, despite the statistical difference, these values remained within the reference range reported for this species (MCHC: 25.9–35.1 g/dL) [31]. In contrast, no significant alterations in hematological markers were observed in male mice from any treated group compared with the vehicle control.

**Table 1.** Hematological profile of female and male mice after 40 days of oral treatment with non-biofortified and Se-biofortified broccoli seedlings.

| Hematological Parameters          | Vehicle Control      | Non-Biofortified Broccoli Seedlings | Se-Enriched Broccoli Seedlings |                           |                           |
|-----------------------------------|----------------------|-------------------------------------|--------------------------------|---------------------------|---------------------------|
|                                   |                      |                                     | 15 $\mu\text{g Se/kg BW}$      | 45 $\mu\text{g Se/kg BW}$ | 70 $\mu\text{g Se/kg BW}$ |
| No. female                        | 8                    | 10                                  | 10                             | 10                        | 10                        |
| WBC ( $\times 10^3/\mu\text{L}$ ) | 8.51 $\pm$ 0.56      | 7.58 $\pm$ 0.94                     | 6.92 $\pm$ 0.99                | 7.73 $\pm$ 0.92           | 7.44 $\pm$ 1.00           |
| RBC ( $\times 10^6/\mu\text{L}$ ) | 10.84 $\pm$ 0.17     | 10.73 $\pm$ 0.21                    | 10.48 $\pm$ 0.20               | 10.46 $\pm$ 0.13          | 10.71 $\pm$ 0.18          |
| HGB (g/dL)                        | 14.98 $\pm$ 0.24     | 14.31 $\pm$ 0.23                    | 14.34 $\pm$ 0.26               | 14.17 $\pm$ 0.21          | 14.37 $\pm$ 0.25          |
| HCT (%)                           | 53.74 $\pm$ 0.59     | 53.89 $\pm$ 0.94                    | 53.26 $\pm$ 0.98               | 52.53 $\pm$ 0.66          | 53.91 $\pm$ 0.97          |
| MCV (fL)                          | 49.61 $\pm$ 0.50     | 50.27 $\pm$ 0.29                    | 50.86 $\pm$ 0.31               | 50.23 $\pm$ 0.27          | 50.36 $\pm$ 0.30          |
| MCH (pg)                          | 13.80 $\pm$ 0.16     | 13.42 $\pm$ 0.13                    | 13.71 $\pm$ 0.14               | 13.54 $\pm$ 0.09          | 13.42 $\pm$ 0.10          |
| MCHC (g/dL)                       | 27.80 $\pm$ 0.38     | 26.66 $\pm$ 0.14 *                  | 26.96 $\pm$ 0.18 *             | 26.98 $\pm$ 0.14 *        | 26.67 $\pm$ 0.13 *        |
| PLT ( $\times 10^3/\mu\text{L}$ ) | 1659.38 $\pm$ 159.10 | 1771.82 $\pm$ 67.94                 | 1702.22 $\pm$ 82.54            | 1610.80 $\pm$ 44.73       | 174.10 $\pm$ 65.03        |
| No. male                          | 8                    | 10                                  | 10                             | 10                        | 10                        |
| WBC ( $\times 10^3/\mu\text{L}$ ) | 9.24 $\pm$ 0.79      | 7.07 $\pm$ 0.55                     | 7.10 $\pm$ 0.88                | 7.06 $\pm$ 0.58           | 6.75 $\pm$ 0.36           |
| RBC ( $\times 10^6/\mu\text{L}$ ) | 10.38 $\pm$ 0.24     | 9.58 $\pm$ 0.51                     | 10.34 $\pm$ 0.26               | 10.62 $\pm$ 0.07          | 10.79 $\pm$ 0.18          |
| HGB (g/dL)                        | 13.65 $\pm$ 0.29     | 13.19 $\pm$ 0.50                    | 13.77 $\pm$ 0.30               | 13.77 $\pm$ 0.19          | 14.02 $\pm$ 0.12          |
| HCT (%)                           | 51.93 $\pm$ 1.13     | 49.12 $\pm$ 2.03                    | 52.42 $\pm$ 1.14               | 53.05 $\pm$ 0.38          | 53.64 $\pm$ 0.55          |
| MCV (fL)                          | 49.99 $\pm$ 0.29     | 51.62 $\pm$ 0.90                    | 50.74 $\pm$ 0.50               | 49.97 $\pm$ 0.22          | 49.76 $\pm$ 0.39          |
| MCH (pg)                          | 13.14 $\pm$ 0.06     | 14.09 $\pm$ 0.94                    | 13.36 $\pm$ 0.27               | 12.97 $\pm$ 0.15          | 13.01 $\pm$ 0.18          |
| MCHC (g/dL)                       | 26.29 $\pm$ 0.15     | 27.12 $\pm$ 1.27                    | 26.28 $\pm$ 0.30               | 25.95 $\pm$ 0.24          | 26.15 $\pm$ 0.19          |
| PLT ( $\times 10^3/\mu\text{L}$ ) | 1730.62 $\pm$ 121.20 | 1806.90 $\pm$ 166.30                | 1739.80 $\pm$ 98.36            | 1734.00 $\pm$ 74.90       | 1770.00 $\pm$ 109.10      |

Abbreviations: WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; BW, body weight. Data are expressed as mean  $\pm$  SEM. Experimental groups: vehicle control (PBS; phosphate-buffered saline containing 0.9% NaCl, 10 mL/kg BW); non-biofortified broccoli seedlings; and Se-biofortified broccoli seedlings administered at doses equivalent to 15, 45, and 70  $\mu\text{g Se/kg BW}$ . \* Data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test.  $p < 0.05$  versus the vehicle control group.

Biochemical analyses demonstrated that administration of both control and Se-enriched broccoli seedlings did not significantly modify biomarkers associated with hepatic function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin (BIL), or renal function, including creatinine (CREA) and urea (UR), in female mice (Table 2). However, male mice exposed to Se-enriched broccoli seedlings at 45  $\mu\text{g Se/kg BW}$  showed a significant increase (43%) in UR levels ( $p < 0.05$ ). Additionally, AST levels decreased by 45% and 46% in animals treated with control broccoli seedlings and Se-enriched broccoli seedlings at 45  $\mu\text{g Se/kg BW}$ , respectively. No significant differences were observed for the remaining biochemical parameters compared with the vehicle control group (Table 2).

The effects of broccoli seedling administration on relative organ weights in female and male mice are presented in Table 3. Overall, treatments induced changes in the relative weight of some organs in both sexes, except for the liver response, which differed between males and females. In females, Se-enriched broccoli seedlings significantly increased ( $p < 0.05$ ) the relative weight of the kidneys (15 and 45  $\mu\text{g Se/kg BW}$ ), ovaries (all tested doses), and the spleen and lungs (70  $\mu\text{g Se/kg BW}$ ). Furthermore, administration of non-biofortified broccoli seedlings also promoted a significant increase in the relative weight of the lungs and ovaries.

**Table 2.** Biochemical profile of female and male mice following repeated oral administration of non-biofortified and Se-biofortified broccoli seedlings for 40 days.

| Biochemical Parameters | Vehicle Control | Non-Biofortified Broccoli Seedlings | Se-Enriched Broccoli Seedlings |                  |                |
|------------------------|-----------------|-------------------------------------|--------------------------------|------------------|----------------|
|                        |                 |                                     | 15 µg Se/kg BW                 | 45 µg Se/kg BW   | 70 µg Se/kg BW |
| No. female             | 8               | 10                                  | 10                             | 10               | 10             |
| CREA (mg/dL)           | 0.50 ± 0.00     | 0.50 ± 0.00                         | 0.50 ± 0.00                    | 0.50 ± 0.00      | 0.50 ± 0.00    |
| BIL (mg/dL)            | 4.59 ± 1.38     | 2.52 ± 0.94                         | 1.69 ± 0.58                    | 1.73 ± 0.26      | 2.35 ± 0.46    |
| AST (U/L)              | 237.30 ± 35.87  | 228.90 ± 21.82                      | 199.90 ± 26.21                 | 244.10 ± 28.55   | 159.40 ± 23.41 |
| ALP (U/L)              | 65.55 ± 6.54    | 55.54 ± 6.54                        | 55.67 ± 4.62                   | 70.05 ± 5.40     | 53.87 ± 5.82   |
| UR (mg/dL)             | 39.56 ± 6.20    | 36.51 ± 4.72                        | 38.92 ± 3.74                   | 38.56 ± 2.52     | 34.09 ± 1.35   |
| No. male               | 8               | 10                                  | 10                             | 10               | 10             |
| CREA (mg/dL)           | 0.50 ± 0.00     | 0.52 ± 0.02                         | 0.51 ± 0.01                    | 0.50 ± 0.00      | 0.50 ± 0.00    |
| BIL (mg/dL)            | 1.14 ± 0.24     | 3.88 ± 1.52                         | 4.19 ± 1.35                    | 1.38 ± 0.32      | 2.22 ± 0.50    |
| AST (U/L)              | 239.90 ± 43.11  | 132.40 ± 29.57 *                    | 176.40 ± 24.72                 | 128.40 ± 14.45 * | 204.10 ± 48.34 |
| ALP (U/L)              | 78.79 ± 22.85   | 52.99 ± 9.10                        | 71.84 ± 17.01                  | 50.88 ± 5.51     | 143.00 ± 48.39 |
| UR (mg/dL)             | 36.35 ± 1.14    | 34.55 ± 2.40                        | 46.36 ± 3.59                   | 51.90 ± 2.14 *   | 46.80 ± 3.34   |

Abbreviations: CREA, creatinine; BIL, bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; UR, urea; BW, body weight. Data are expressed as mean ± SEM. Experimental groups: vehicle control (PBS; phosphate-buffered saline containing 0.9% NaCl, 10 mL/kg BW); non-biofortified broccoli seedlings; and Se-biofortified broccoli seedlings administered at doses equivalent to 15, 45, and 70 µg Se/kg BW. \* Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test.  $p < 0.05$  versus the vehicle control group.

**Table 3.** Relative organ weights of female and male mice after repeated oral administration of non-biofortified and Se-biofortified broccoli seedlings for 40 days.

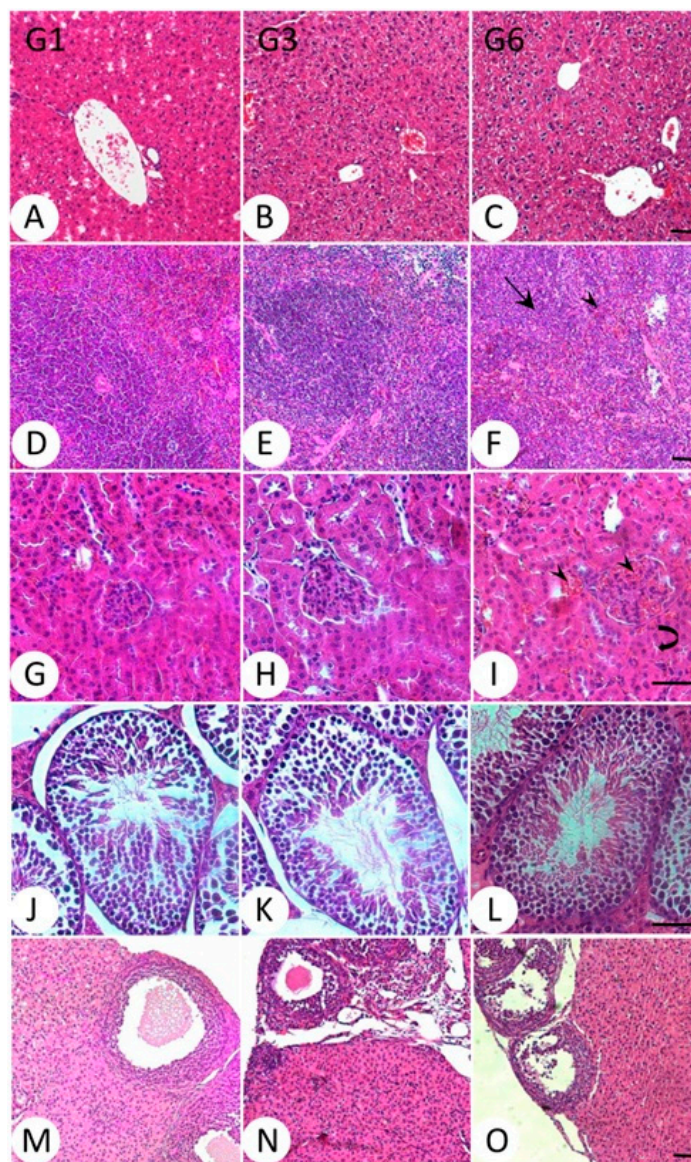
| Relative Organ Weights (g/100 g BW) | Vehicle Control | Non-Biofortified Broccoli Seedlings | Se-Enriched Broccoli Seedlings |                |                |
|-------------------------------------|-----------------|-------------------------------------|--------------------------------|----------------|----------------|
|                                     |                 |                                     | 15 µg Se/kg BW                 | 45 µg Se/kg BW | 70 µg Se/kg BW |
| No. female                          | 8               | 10                                  | 10                             | 10             | 10             |
| Liver                               | 6.40 ± 0.21     | 5.90 ± 0.15                         | 6.05 ± 0.23                    | 5.82 ± 0.12    | 5.99 ± 0.16    |
| Kidney                              | 1.32 ± 0.05     | 1.40 ± 0.04                         | 1.48 ± 0.03 *                  | 1.47 ± 0.04 *  | 1.46 ± 0.02    |
| Spleen                              | 0.46 ± 0.01     | 0.52 ± 0.04                         | 0.50 ± 0.02                    | 0.49 ± 0.02    | 0.60 ± 0.04 *  |
| Lung                                | 0.85 ± 0.03     | 1.02 ± 0.04 *                       | 0.94 ± 0.03                    | 0.90 ± 0.05    | 0.93 ± 0.04    |
| Heart                               | 0.54 ± 0.02     | 0.68 ± 0.05                         | 0.65 ± 0.04                    | 0.66 ± 0.03    | 0.66 ± 0.03    |
| Thymus                              | 0.50 ± 0.04     | 0.47 ± 0.03                         | 0.52 ± 0.05                    | 0.44 ± 0.03    | 0.43 ± 0.05    |
| Ovary                               | 0.14 ± 0.01     | 0.31 ± 0.04 *                       | 0.30 ± 0.04 *                  | 0.31 ± 0.04 *  | 0.29 ± 0.03 *  |
| Brain                               | 1.15 ± 0.03     | 1.24 ± 0.07                         | 1.24 ± 0.05                    | 1.21 ± 0.05    | 1.24 ± 0.06    |
| No. male                            | 8               | 10                                  | 10                             | 10             | 10             |
| Liver                               | 6.35 ± 0.21     | 5.81 ± 0.11 *                       | 5.96 ± 0.4                     | 5.66 ± 0.14 *  | 5.82 ± 0.10 *  |
| Kidney                              | 1.49 ± 0.07     | 1.53 ± 0.04                         | 1.70 ± 0.04 *                  | 1.61 ± 0.06    | 1.65 ± 0.03    |
| Spleen                              | 0.29 ± 0.02     | 0.35 ± 0.03                         | 0.29 ± 0.02                    | 0.29 ± 0.01    | 0.33 ± 0.01    |
| Lung                                | 0.72 ± 0.02     | 0.71 ± 0.03                         | 0.80 ± 0.03                    | 0.75 ± 0.03    | 0.82 ± 0.03    |
| Heart                               | 0.50 ± 0.01     | 0.56 ± 0.02                         | 0.68 ± 0.04 *                  | 0.61 ± 0.05    | 0.62 ± 0.03    |
| Thymus                              | 0.29 ± 0.03     | 0.27 ± 0.03                         | 0.33 ± 0.05                    | 0.37 ± 0.04    | 0.34 ± 0.05    |
| Testicle                            | 0.62 ± 0.02     | 0.68 ± 0.02                         | 0.67 ± 0.04                    | 0.70 ± 0.04    | 0.76 ± 0.04 *  |
| Brain                               | 0.94 ± 0.02     | 0.93 ± 0.03                         | 1.01 ± 0.03                    | 0.94 ± 0.04    | 0.99 ± 0.04    |

Abbreviation: BW, body weight. Data are expressed as mean ± SEM. Experimental groups: vehicle control (PBS; phosphate-buffered saline containing 0.9% NaCl, 10 mL/kg BW); non-biofortified broccoli seedlings; and Se-biofortified broccoli seedlings administered at doses equivalent to 15, 45, and 70 µg Se/kg BW. \* Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test.  $p < 0.05$  versus the vehicle control group.

In male mice (Table 3), the response differed from that observed in females. A significant reduction ( $p < 0.05$ ) in relative liver weight was observed after administration of non-biofortified broccoli seedlings and Se-enriched broccoli seedlings at 45 and 70 µg Se/kg BW. Moreover, Se-enriched broccoli seedlings significantly increased ( $p < 0.05$ ) the relative weight of kidneys (15 µg Se/kg BW), heart (15 µg Se/kg BW), and testes (70 µg Se/kg BW).

No significant changes were observed in the relative weights of spleen, lungs, thymus, or brain.

Histological evaluation of liver, kidney, spleen, testis, and ovary tissues after 40 days of oral administration of control or Se-enriched broccoli seedlings is shown in Figure 2. Animals from the vehicle control and non-biofortified broccoli seedling groups exhibited preserved tissue architecture in all evaluated organs. In contrast, treatment with Se-enriched broccoli seedlings at 70  $\mu\text{g Se/kg BW}$  induced morphological alterations characterized by disruption of the splenic white pulp and marked edema (Figure 2F), as well as renal edema associated with reduced renal tubules (Figure 2I).



**Figure 2.** Histopathological evaluation of liver (A–C), spleen (D–F), kidney (G–I), testis (J–L), and ovary (M–O) from mice after 40 days of repeated oral administration of non-biofortified and Se-biofortified broccoli seedlings. Panels in the first, second, and third columns correspond to G1 (vehicle control), G3 (non-biofortified broccoli seedlings), and G6 (Se-biofortified broccoli seedlings at 70  $\mu\text{g Se/kg body weight [BW]}$ ), respectively. No morphological alterations were observed in liver, kidney, testis, or ovary tissues. Treatment with Se-biofortified broccoli seedlings induced disruption of the splenic white pulp (arrow) and marked edema (arrowhead) in the spleen (F), as well as renal edema (arrowhead) and reduced renal tubule structures (curved arrow) in the kidney (I). Scale bar = 50  $\mu\text{m}$ .

## 2.2. In Vivo Genotoxicity Evaluation of Se-Biofortified Broccoli Seedlings

The frequency of micronucleated polychromatic erythrocytes (MNPCE) and the PCE/NCE ratio obtained from bone marrow analysis are presented in Table 4. No significant changes in the PCE/NCE ratio were observed in mice treated with non-biofortified or Se-biofortified broccoli seedlings compared with the vehicle control group, indicating the absence of cytotoxic effects on erythropoiesis. As expected, cyclophosphamide used as a positive control significantly increased ( $p < 0.05$ ) MNPCE frequency compared with the vehicle control group in both female and male mice, confirming the sensitivity and reliability of the assay.

**Table 4.** Genotoxicity assessment of Se-biofortified broccoli seedlings based on micronucleus formation in mouse bone marrow cells.

| Sex    | Treatment                  | MNPCE/4000 PCEs<br>(Mean $\pm$ SEM) | PCE/NCE<br>(Mean $\pm$ SEM) |
|--------|----------------------------|-------------------------------------|-----------------------------|
| Female | Vehicle Control            | 0.32 $\pm$ 0.03                     | 2.37 $\pm$ 0.21             |
|        | Cyclophosphamide           | 0.80 $\pm$ 0.09 *                   | 0.98 $\pm$ 0.14             |
|        | Broccoli seedlings control | 0.48 $\pm$ 0.04                     | 1.89 $\pm$ 0.11             |
|        | 15 $\mu$ g Se/kg BW        | 0.40 $\pm$ 0.04                     | 2.05 $\pm$ 0.13             |
|        | 45 $\mu$ g Se/kg BW        | 0.45 $\pm$ 0.05                     | 2.23 $\pm$ 0.21             |
|        | 70 $\mu$ g Se/kg BW        | 0.49 $\pm$ 0.05                     | 2.30 $\pm$ 0.21             |
| Male   | Vehicle Control            | 0.26 $\pm$ 0.02                     | 4.19 $\pm$ 0.65             |
|        | Cyclophosphamide           | 0.78 $\pm$ 0.05 *                   | 1.49 $\pm$ 0.18             |
|        | Broccoli seedlings control | 0.32 $\pm$ 0.03                     | 3.66 $\pm$ 0.52             |
|        | 15 $\mu$ g Se/kg BW        | 0.38 $\pm$ 0.02                     | 4.01 $\pm$ 0.78             |
|        | 45 $\mu$ g Se/kg BW        | 0.43 $\pm$ 0.04                     | 2.85 $\pm$ 0.24             |
|        | 70 $\mu$ g Se/kg BW        | 0.46 $\pm$ 0.04 *                   | 4.86 $\pm$ 0.90             |

Abbreviations: MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes. Experimental groups: vehicle control (PBS; phosphate-buffered saline solution containing 0.9% NaCl, 10 mL/kg BW); positive control (cyclophosphamide, 50 mg/kg); non-biofortified broccoli seedlings; and Se-biofortified broccoli seedlings administered at doses equivalent to 15, 45, and 70  $\mu$ g Se/kg BW. \* Data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison test.  $p < 0.05$  versus the vehicle control group.

Regarding the broccoli seedling-treated groups, no significant differences in MNPCE frequency were observed in female mice treated with either non-biofortified or Se-biofortified broccoli seedlings compared with the vehicle control group. In contrast, male mice exposed to Se-biofortified broccoli seedlings at the highest dose (70  $\mu$ g Se/kg BW) exhibited a significant increase ( $p < 0.05$ ) in MNPCE frequency, suggesting a potential genotoxic response (Table 4).

## 3. Discussion

The present study provides an integrated safety evaluation of Se-biofortified broccoli seedlings using toxicological, biochemical, histological, and genotoxic endpoints. Since Se exhibits a narrow range between nutritional requirements and excessive exposure, safety assessment represents an essential step for validating Se-enriched crops intended for human consumption [1,2].

Body weight monitoring represents one of the primary endpoints in repeated-dose toxicity studies, reflecting possible systemic effects caused by prolonged exposure to test substances [1]. Male mice receiving Se-enriched broccoli seedlings at 15  $\mu$ g Se/kg BW showed a significant reduction in body weight gain compared with the vehicle control group. However, this effect was not reproduced in animals treated with the higher doses (45 and 70  $\mu$ g Se/kg BW), indicating the absence of a monotonic dose–response relationship. Because the treatment groups consisted of independent animals, the values observed at

the higher doses should not be interpreted as a recovery from the response detected at 15 µg Se/kg BW.

Non-monotonic dose–response relationships have been described in toxicological studies and may result from adaptive physiological responses, threshold effects, or complex biological regulatory mechanisms, although such responses should be interpreted cautiously and ideally confirmed by reproducible evidence and mechanistic investigations before being considered biologically meaningful [32,33]. In addition, the reduction in body weight gain was not accompanied by changes in food or water consumption, clinical signs of toxicity, or a consistent pattern of adverse effects in the remaining toxicological endpoints evaluated at the same dose. Therefore, this isolated finding should be interpreted with caution, and additional studies are required to determine whether it represents a true biological effect or reflects normal biological variability.

No clinically relevant hematological alterations were detected in male mice after administration of either non-biofortified or Se-biofortified broccoli seedlings. In females, although a significant reduction in mean corpuscular hemoglobin concentration (MCHC) was observed in all treated groups compared with the vehicle control, this alteration was not accompanied by changes in other erythrocyte-related parameters, including mean corpuscular volume (MCV), red blood cell count (RBC), and mean corpuscular hemoglobin (MCH) [34]. Furthermore, the MCHC values observed in broccoli-treated female mice remained within the physiological reference range reported for this species [31], suggesting that this variation was not associated with a clinically relevant hematological effect.

Exposure to excessive Se levels has been associated with toxic effects in different organs, with the liver being considered one of the main targets due to its central role in Se metabolism, detoxification, and the synthesis of selenoprotein P (SELENOP), the major Se transport protein in plasma [24,25,35,36]. Oral administration of broccoli seedlings, regardless of Se biofortification, resulted in a tendency toward reduced relative liver weight. This effect reached statistical significance only in male mice treated with non-biofortified broccoli seedlings and Se-biofortified broccoli seedlings at 45 and 70 µg Se/kg BW. The reduction in relative liver weight was accompanied by decreased AST levels in males receiving non-biofortified broccoli seedlings and Se-biofortified broccoli seedlings at 45 µg Se/kg BW. Available evidence indicates that excessive Se exposure may induce hepatic alterations [37–39]. However, the similar reductions in relative liver weight and AST observed in mice receiving non-biofortified broccoli seedlings indicate that these responses cannot be attributed exclusively to Se exposure. In addition to Se, broccoli contains glucosinolates, isothiocyanates (particularly sulforaphane), and phenolic compounds, which have been shown to modulate hepatic antioxidant defenses, phase II detoxification enzymes, and redox-sensitive signaling pathways [40]. Accordingly, the contribution of these phytochemicals to the observed hepatic responses cannot be excluded. Nevertheless, these hepatic responses were restricted to male mice. Sex-dependent differences in hepatic Se metabolism and selenoproteins regulation may have contributed to this pattern, although the mechanisms underlying these responses were not investigated in the present study [36,41].

Experimental studies have shown that excessive Se exposure may induce structural and functional renal alterations, including nephritis, renal papillary degeneration, and increased kidney weight, indicating that the kidneys represent an important target organ in Se toxicity [25,42–44]. Both female and male mice treated with Se-biofortified broccoli seedlings exhibited a significant increase in relative kidney weight. Moreover, male mice receiving Se-biofortified broccoli seedlings at 45 µg Se/kg BW showed significantly increased UR levels, suggesting possible changes in renal function. Histopathological evaluation further supported these findings, which revealed renal morphological alterations, including

edema and reduction in renal tubule structures, in both female and male mice exposed to the highest Se dose (70 µg Se/kg BW). These results suggest that renal effects induced by Se-biofortified broccoli seedlings occurred mainly at higher Se exposure levels.

The kidneys play a central role in Se homeostasis and excretion and are therefore particularly susceptible to excessive Se exposure. Under supranutritional conditions, Se may shift from its physiological antioxidant function to a pro-oxidant behavior, promoting oxidative stress through thiol oxidation and increased generation of reactive oxygen species, which may contribute to tubular injury and renal dysfunction [45]. The increase in UR only in males, despite structural alterations in both sexes at the highest dose, suggests a sex-dependent functional renal response. Differences in renal Se handling and selenoproteins expression may contribute to this pattern [36,41,46], although tissue Se and oxidative stress markers were not measured. Together, these observations suggest that the kidney represents one of the primary target organs of Se toxicity under the present experimental conditions.

At 70 µg Se/kg BW, female mice showed increased relative spleen weight, white pulp disruption, and marked edema, in contrast to findings reported after exposure to isolated Se compounds [24]. Because the spleen is sensitive to redox and inflammatory disturbances, excessive Se exposure may have contributed to the observed architectural changes [45]. The female-specific response may also reflect sex-dependent regulation of immune and redox pathways, although these mechanisms were not directly assessed. Differences from previous studies may additionally be related to Se speciation and its incorporation into the broccoli matrix.

Se is required for normal reproductive physiology, but excessive exposure may impair reproductive function through oxidative and redox-sensitive mechanisms [35,36,39,41,46]. Relative testis weight increased in males receiving 70 µg Se/kg BW, whereas relative ovary weight increased in females at all tested doses. However, no histopathological alterations were detected. Thus, these weight changes were not accompanied by overt structural injury and may represent adaptive tissue responses; reproductive performance, hormonal status, and oxidative stress markers would be required to determine their functional significance.

Finally, the micronucleus assay is widely applied in both in vitro and in vivo studies to detect clastogenic and aneugenic effects, since micronucleus formation reflects chromosomal damage resulting from chromosome fragments or whole chromosome loss [47]. Cyclophosphamide was included as a positive control due to its well-established genotoxic activity, which occurs mainly through DNA alkylation and oxidative stress generation after metabolic activation [48]. Based on MNPCE frequency and the PCE/NCE ratio, no genotoxic response was detected in female mice treated with either non-biofortified or Se-biofortified broccoli seedlings. However, in male mice receiving Se-biofortified broccoli seedlings at the highest dose (70 µg Se/kg BW), a significant increase (43%) in micronucleus frequency was observed compared with the vehicle control group, indicating chromosomal damage under this exposure condition. Nevertheless, the absence of changes in the PCE/NCE ratio suggests that this effect was not accompanied by detectable bone marrow cytotoxicity. These findings indicate that chromosomal damage occurred without detectable impairment of bone marrow erythropoiesis.

Evidence from micronucleus assays indicates that the genotoxic effects of Se are highly dependent on its chemical species, concentration, biological model, and food matrix. Isolated Se compounds have demonstrated both genotoxic and antigenotoxic activity in micronucleus assays, depending on the experimental conditions [49]. In contrast, Se-enriched yeast showed no genotoxicity in an in vivo mouse micronucleus assay [50], whereas Se-enriched rice and green tea exhibited antigenotoxic or anticlastogenic effects against chemically induced genetic damage [48,51]. Moreover, non-biofortified broccoli seed extract did not induce micronuclei in mice [52]. These apparently divergent findings

reinforce that the increase in MNPCE observed in the present study at the highest dose should be interpreted in the context of Se dose, speciation, duration of exposure, biological sex, and the broccoli matrix. Collectively, these findings reinforce that Se-related genotoxicity is highly context-dependent and influenced by Se dose, chemical speciation, biological sex, and food matrix.

The variability in toxicological responses reported in previous studies also highlights the importance of Se speciation and food matrix in determining its biological effects. Se toxicity depends not only on the total amount consumed but also on its chemical species, bioavailability, and metabolic fate. Broccoli predominantly accumulates Se as organic species, including Se-methylselenocysteine, methylselenocysteine, selenomethionine, and selenocystine, which differ substantially from the inorganic forms commonly evaluated in toxicological studies [28,53]. In addition, the plant matrix may further modulate Se bioavailability. Zeng et al. [54] demonstrated that methylselenocysteine exhibited lower bioavailability when consumed as part of broccoli than as an isolated compound, highlighting the influence of the food matrix on Se absorption. Therefore, the toxicological profile observed likely reflects the combined influence of Se dose, chemical speciation, and the broccoli matrix rather than Se concentration alone.

Overall, the present findings indicate sex-dependent toxicological responses. Although both sexes were exposed to identical Se doses, females predominantly exhibited hematological and splenic alterations, whereas males showed hepatic, renal functional, and genotoxic changes. Experimental and clinical studies have demonstrated that Se metabolism exhibits marked sexual dimorphism, including differences in tissue distribution, urinary excretion, Se retention, and the expression of several selenoproteins involved in antioxidant defense and Se transport, such as SELENOP, Glutathione peroxidase 3 (GPX3), and Iodothyronine deiodinase 1 (DIO1) [35,36,41]. Sex hormones have also been proposed to modulate Se metabolism and selenoproteins hierarchy. Although the mechanisms responsible for the sex-specific responses observed remain to be elucidated, these findings reinforce the importance of considering biological sex as a relevant variable in toxicological evaluations of Se-biofortified foods.

Furthermore, Se intake levels associated with potential health benefits have been reported to range around 50–100 µg/day, although the biological effects depend on several factors, including baseline Se status and exposure conditions [55]. Considering the findings of the present study, the human equivalent dose (HED) of Se-biofortified broccoli seedlings was estimated using body surface area (BSA) normalization according to the dose translation approach proposed for animal-to-human extrapolation [56]. The HED was calculated using the equation  $HED = \text{animal dose} \times (\text{Km animal} / \text{Km human})$ , considering Km values of 3 for mice (body weight: 0.02 kg; BSA: 0.007 m<sup>2</sup>) and 37 for humans (body weight: 60 kg; BSA: 1.6 m<sup>2</sup>). Based on this approach, the highest dose evaluated in mice (70 µg Se/kg BW), which was associated with toxicological alterations, corresponds to an estimated human exposure of approximately 5.67 µg Se/kg BW, equivalent to 340 µg Se/day for a 60 kg adult. Collectively, these findings indicate that the toxicological alterations observed in mice occurred at an estimated human exposure substantially higher than Se intakes generally associated with nutritional benefits. Nevertheless, extrapolation of safety thresholds to humans should be made cautiously because species differences, Se status, and long-term dietary exposure were not evaluated in the present study.

This study has some limitations. Although male and female mice were included, the findings are restricted to a single animal strain and a 40-day exposure period, which may limit extrapolation to long-term human consumption. Se speciation, tissue Se accumulation, reproductive function, and additional molecular markers of oxidative stress were not evaluated. Moreover, despite the use of blinded microscopic assessments, the treatment

administration and data collection stages were not fully blinded, which may represent a potential source of bias. Therefore, the findings should be interpreted within the context of the experimental model and exposure conditions used.

## 4. Materials and Methods

### 4.1. Plant Material

Broccoli seedlings were produced and Se-biofortified under greenhouse conditions as previously described by Bachiega et al. [26] and Ávila et al. [7]. Briefly, seeds of *Brassica oleracea* var. *italica* (cv. Avenger, Sakata Seed Sudamerica, Bragança Paulista, SP, Brazil) were cultivated in 200-cell black polyethylene trays containing coconut fiber substrate (Amafibra<sup>®</sup>, Holambra, SP, Brazil). After 15 days of germination, seedlings were randomly divided into two experimental groups and treated with either distilled water (2 mL; non-biofortified group) or sodium selenate solution (50 µM, 2 mL; Sigma-Aldrich, St. Louis, MO, USA; Se-biofortified group). Following treatment application, seedlings were maintained under greenhouse conditions for an additional 15 days, completing a 30-day cultivation period. No visual symptoms of toxicity were observed after Se biofortification.

Subsequently, the edible aerial parts were harvested, washed with distilled water, frozen at −20 °C for 48 h, and freeze-dried using a L101 freeze dryer (Liobras, São Carlos, SP, Brazil) at a condenser temperature of −55 °C and a chamber pressure of 271 µHg for 72 h. The freeze-dried samples were stored at −20 °C until further analyses. Se accumulation was quantified by inductively coupled plasma mass spectrometry (ICP-MS; ELAN<sup>®</sup> DRC-e, PerkinElmer, Waltham, MA, USA) (Appendix A), resulting in concentrations of  $0.25 \pm 0.04$  and  $61.03 \pm 3.15$  µg Se/g dry weight in non-biofortified and Se-biofortified broccoli seedlings, respectively. The data generated from the repeated-dose oral toxicity study, including hematological, biochemical, relative organ weight, histopathological, and micronucleus evaluations, were derived from the doctoral thesis of Bachiega [57].

### 4.2. Animals

The animal experiments were conducted between January and July 2018 under the ethical approval granted by the Ethics Committee on Animal Use of the University of Campinas (CEUA-UNICAMP; approval numbers 4319-1 and 4319-1(A)/2017). The experimental procedures were conducted following internationally accepted principles for laboratory animal care, including the International Guiding Principles for Biomedical Research Involving Animals (CIOMS-ICLAS, December 2012), and the Brazilian Guidelines for the Care and Use of Animals for Scientific and Educational Purposes [58].

Male and female Swiss mice (18–25 g) were supplied by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB), University of Campinas (UNICAMP, São Paulo, Brazil). Before the beginning of the experimental procedures, mice underwent an acclimatization period of at least seven days. Animals were maintained in groups of five per polypropylene cage (49 × 34 × 16 cm) containing sterile wood shavings bedding (*Pinus* sp.). Environmental enrichment was provided using sanitized polyvinyl chloride (PVC) tubes and two to three sheets of white paper per cage.

Throughout the experimental period, animals were kept under controlled environmental conditions, including temperature maintained at  $22 \pm 2$  °C and a 12 h light/dark cycle (lights off at 18:00 h). Standard commercial rodent diet (Biobase, Biotécnicas Indústria e Comércio Ltda, Pouso Alegre, MG, Brazil) and drinking water were provided *ad libitum*.

For the repeated-dose toxicity study, the number of animals per group was defined according to OECD Guidelines 407 and 474 recommendations [58–60]. As recommended for guideline-based toxicity studies, multiple toxicity endpoints were assessed, and no single primary outcome measure was used for sample size determination.

#### 4.3. Experimental Design

Male and female mice were randomly allocated to six experimental groups before treatment initiation: a vehicle control group (PBS, pH 7.0;  $n = 8/\text{sex}$ ), a positive control group for the mammalian erythrocyte micronucleus assay ( $n = 8/\text{sex}$ ), a group receiving non-biofortified broccoli seedlings ( $n = 10/\text{sex}$ ), and three groups treated with Se-biofortified broccoli seedlings at doses corresponding to 15, 45, or 70  $\mu\text{g Se/kg BW}$  (0.25, 0.74, and 1.15 g broccoli seedlings/kg BW, respectively;  $n = 10/\text{sex/group}$ ). The individual animal was considered the experimental unit for all analyses. Animals from different experimental groups were housed under identical environmental conditions, and treatments and measurements were performed using standardized procedures to minimize potential confounding factors. No animals or data points were excluded from the analyses. Histopathological and micronucleus slides were coded by an independent technician, and investigators responsible for microscopic evaluations were blinded to the experimental group allocation until all analyses were completed.

Cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA) was administered to the positive control group at a single intraperitoneal dose of 50 mg/kg 24 h before euthanasia to validate the mammalian erythrocyte micronucleus assay. Broccoli seedling preparations were suspended in phosphate-buffered saline (PBS, pH 7.0), whereas cyclophosphamide was diluted in the same vehicle. The administration volume was standardized at 10 mL/kg body weight (BW) for both oral and intraperitoneal administration. The Se doses were selected based on previously published toxicological studies [24,25].

Freeze-dried broccoli seedlings were ground into a fine powder, and the amount required for each treatment group was calculated based on the Se concentration previously determined by ICP-MS and the combined body weight of the animals in each group. Daily portions of the lyophilized powder were weighed in advance, individually stored in separate containers for up to one week, and maintained dry until use. Immediately before administration, each daily portion was suspended in phosphate-buffered saline (PBS, pH 7.0), homogenized, and administered by oral gavage. Individual administration volumes were recalculated every three days according to the body weight recorded throughout the 40-day treatment period, maintaining a constant dosing volume of 10 mL/kg BW and the intended Se doses.

#### 4.4. Repeated-Dose Oral Toxicity Study and Mammalian Erythrocyte Micronucleus Test

The experimental procedures were adapted from OECD Guidelines 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) and 474 (Mammalian Erythrocyte Micronucleus Test) [59,60]. Animals received the respective treatments once daily by oral gavage for 40 consecutive days. Throughout the experimental period, mice were monitored daily for clinical signs of toxicity and mortality. Humane endpoints included severe clinical signs of distress, marked deterioration of general health, or any condition requiring early euthanasia according to animal welfare criteria. Individual body weight was recorded before treatment initiation and every three days thereafter. Food and water intake was measured at the same intervals for each experimental group. At the end of the treatment period, animals were anesthetized with ketamine (Dopalen<sup>®</sup>, Ceva Saúde Animal, Paulínia, SP, Brazil) and xylazine (Anasedan<sup>®</sup>, Ceva Saúde Animal, Paulínia, SP, Brazil) (200 and 20 mg/kg, respectively), and blood samples were collected from the retro-orbital plexus. Subsequently, mice were euthanized by cervical dislocation, followed by necropsy and bone marrow collection.

For the micronucleus assay, bone marrow samples were obtained from both femurs of each animal by flushing with fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cell suspension was centrifuged ( $120 \times g$  for 5 min), and the

resulting pellet was resuspended and used to prepare at least two smears per animal. Slides were fixed, stained with Leishman's stain (Sigma-Aldrich, St. Louis, MO, USA), and analyzed using light microscopy (100× magnification; Leica Microsystems, SME model, Wetzlar, Germany). Cytotoxicity was assessed by determining the polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) ratio through the evaluation of 500 erythrocytes per animal. Genotoxicity was determined based on the frequency of micronucleated polychromatic erythrocytes (MNPCE), obtained after scoring 4000 PCE per animal.

#### 4.5. Hematological and Biochemical Analyses

Blood samples obtained from each animal were divided according to the required analyses. For hematological assessment, samples were transferred into tubes containing EDTA as anticoagulant and processed using an automated hematology analyzer Sysmex pocH-100iV (Sysmex Corporation, Kobe, Japan). The evaluated hematological parameters included white blood cells (WBC), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT).

For biochemical analysis, blood samples were processed according to the requirements of each parameter. Serum was obtained after centrifugation and analyzed using an automated clinical chemistry analyzer Reflotron® Plus analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with specific reagent strips (Reflotron® tests, Roche Diagnostics GmbH, Mannheim, Germany). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and urea (UR) levels were determined using serum obtained from non-heparinized blood samples. Bilirubin (BIL) and creatinine (CREA) were measured using the same blood samples collected for hematological evaluation.

#### 4.6. Necropsy and Histopathological Analysis

Following euthanasia, all animals were subjected to a complete macroscopic examination to identify possible gross pathological alterations. The heart, liver, spleen, lungs, kidneys, brain, thymus, and reproductive organs (testes or ovaries) were collected and relative organ weight was calculated as organ weight/body weight × 100.

For histopathological evaluation, liver, spleen, kidney, and reproductive tissue samples (testis or ovary) were fixed in 10% buffered formalin for 24 h and subsequently maintained in 70% ethanol until processing. After dehydration, tissue fragments were embedded in paraffin, sectioned at 5 µm thickness using a microtome Leica RM2235 (Leica Microsystems, Wetzlar, Germany), stained with hematoxylin and eosin (H&E), and evaluated by light microscopy.

#### 4.7. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed separately for female and male mice. Statistical analyses were conducted using SAS software version 9.0 (SAS Institute Inc., Cary, NC, USA). Data distribution and variance homogeneity were assessed using the Shapiro–Wilk and Box–Cox tests, respectively. When the assumptions of normality and homogeneity of variance were satisfied, comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. For datasets that did not meet these assumptions, the Kruskal–Wallis test followed by Dunn's multiple comparison test was applied. Differences were considered statistically significant at  $p < 0.05$ .

## 5. Conclusions

The present study provides new evidence regarding the safety of Se-biofortified broccoli seedlings using an integrated *in vivo* approach based on repeated-dose toxicity and genotoxicity assessments. Se-biofortified broccoli seedlings did not induce relevant changes in body weight gain or hematological parameters in male and female mice. However, exposure to higher Se levels, especially at 70 µg Se/kg BW, promoted organ-specific alterations, including changes in relative organ weight and histopathological findings in the kidney and spleen. In addition, male mice exposed to the highest Se dose showed increased micronucleus frequency, indicating a genotoxic response under this experimental condition. These findings demonstrate that the biological effects of Se-biofortified broccoli seedlings are dose-dependent and support their potential use as a dietary Se source at adequate intake levels. Future studies should investigate the long-term safety and bioavailability of Se-biofortified broccoli, as well as the molecular mechanisms underlying the toxicological and genotoxic effects observed at higher Se doses, to better define safe consumption limits and optimize biofortification strategies.

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**Data Availability Statement:** The data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|        |                                                                                    |
|--------|------------------------------------------------------------------------------------|
| ALT    | Alanine aminotransferase                                                           |
| ALP    | Alkaline phosphatase                                                               |
| ANOVA  | Analysis of variance                                                               |
| AST    | Aspartate aminotransferase                                                         |
| BIL    | Bilirubin                                                                          |
| BSA    | Body surface area                                                                  |
| BW     | Body weight                                                                        |
| CEMIB  | Multidisciplinary Center for Biological Investigation on Laboratory Animal Science |
| CEUA   | Committee for Ethics in Animal Use                                                 |
| CIOMS  | Council for International Organizations of Medical Sciences                        |
| CONCEA | National Council for the Control of Animal Experimentation                         |
| Cyp    | Cyclophosphamide                                                                   |
| CREA   | Creatinine                                                                         |
| DIO1   | Iodothyronine deiodinase 1                                                         |
| EDTA   | Ethylenediaminetetraacetic acid                                                    |

|         |                                                        |
|---------|--------------------------------------------------------|
| GPX3    | Glutathione peroxidase 3                               |
| H&E     | Hematoxylin and eosin                                  |
| HCT     | Hematocrit                                             |
| HED     | Human equivalent dose                                  |
| HGB     | Hemoglobin                                             |
| ICP-MS  | Inductively coupled plasma mass spectrometry           |
| MCH     | Mean corpuscular hemoglobin                            |
| MCHC    | Mean corpuscular hemoglobin concentration              |
| MCV     | Mean corpuscular volume                                |
| MN      | Micronucleus                                           |
| MNPCE   | Micronucleated polychromatic erythrocytes              |
| NaCl    | Sodium chloride                                        |
| NCE     | Normochromatic erythrocytes                            |
| OECD    | Organisation for Economic Co-operation and Development |
| PBS     | Phosphate-buffered saline                              |
| PCE     | Polychromatic erythrocytes                             |
| PLT     | Platelet count                                         |
| PVC     | Polyvinyl chloride                                     |
| RBC     | Red blood cell count                                   |
| SAS     | Statistical Analysis System                            |
| Se      | Selenium                                               |
| SELENOP | Selenoprotein P                                        |
| SEM     | Standard error of the mean                             |
| UNICAMP | University of Campinas                                 |
| UR      | Urea                                                   |
| USP     | University of São Paulo                                |
| UFMT    | Federal University of Mato Grosso                      |
| WBC     | White blood cell count                                 |

## Appendix A

The total Se contents were determined using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) quadrupole (ELAN<sup>®</sup> DRC-e, PerkinElmer, Waltham, MA, USA). Freeze-dried samples (100 mg) were decomposed in a microwave oven (DGT 100 Plus, Provecto, Analítica, Brasil), using a mixture of 4 mL HNO<sub>3</sub> and 200 µL H<sub>2</sub>O<sub>2</sub> for 7 min at 400 W. The conditions were as follows: 1200 W ICP RF power, 15 L min<sup>−1</sup> plasma gas flow rate, 1.1 L min<sup>−1</sup> auxiliary gas flow rate, 0.75 L min<sup>−1</sup> nebulizer flow, 7.0 V lens voltage, 1000 V pulse state voltage, <sup>78</sup>Se isotope monitored, and 0.5–25 µg L<sup>−1</sup> concentration range of the analytical curve. The results were expressed as µg Se g<sup>−1</sup> fresh weight (FW).

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