

# Proanthocyanidins produce significant attenuation of doxorubicin-induced mutagenicity via suppression of oxidative stress

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**Key words:** oxidative stress, chemoprotection, mutagenesis, doxorubicin, proanthocyanidins

**Abbreviations:** LMA, low melting agarose; PCE, polychromatic erythrocytes; MN, micronuclei; GSH, glutathione; TBARS, thiobarbituric acid reacting substance

This study has been initiated to determine whether proanthocyanidins can protect against doxorubicin-induced mutagenicity in mice and to elucidate the potential mechanism of this protection. Pretreatment of mice with proanthocyanidins (100 mg/kg/day, orally) for 7 days and simultaneously with doxorubicin (12 mg/kg, i.p.) for another day, significantly reduced the frequency of bone marrow DNA strand breaks and micronucleated polychromatic erythrocytes compared to doxorubicin-treated mice alone. Furthermore, proanthocyanidins caused a reduction in bone marrow suppression induced by doxorubicin treatment. In male germline, orally administration of proanthocyanidins (100 mg/kg/day, orally) for 7 consecutive days before and 7 consecutive days after treatment with doxorubicin (12 mg/kg, i.p.), significantly elevated the levels of sperm count and motility reduced by doxorubicin treatment. Furthermore, proanthocyanidins significantly decreased the elevated levels of spermatogonial and spermatocyte chromosomal aberrations and sperm head abnormality induced by doxorubicin. Prior administration of proanthocyanidins ahead of doxorubicin reduced the doxorubicin induced testicular lipid peroxidation and prevented the reduction in testicular non-protein sulfhydryl significantly. Conclusively, this study provides for the first time that proanthocyanidins have a protective role in the abatement of doxorubicin-induced mutagenesis and cell proliferation changes in germinal cells of mice that reside, at least in part, in their radical scavenger activity. Therefore, proanthocyanidins can be a promising chemopreventive agent to avert secondary malignancy and abnormal reproductive outcomes risks in cancer patients receiving doxorubicin-involved treatment.

## Introduction

An ideal chemotherapeutic treatment would selectively attack cancer cells without causing toxicity on normal tissues. Unfortunately, this ideal selectivity has not yet been reached by traditional chemotherapy, which is known to affect both neoplastic and proliferating normal cells. Modern therapies using multiple combinations of chemotherapeutic drugs reduce the cytotoxicity of these drugs to normal tissues, increasing the survival rates.<sup>1</sup> However, even after increasing the effectiveness of these treatments, secondary treatment-related malignancies have been reported after the use of combination chemotherapy.<sup>2-4</sup> Moreover, the adverse reproductive effects of anticancer agents have long been acknowledged: infertility is a common side effect of chemotherapy, which bears a substantial impairment on the quality of life for young survivors of cancer. There is growing concern over the effects of anticancer drugs on germ cells because treatments for cancer, including many of the more common pediatric cancers, have enjoyed rapidly expanding success rates,

often due in part to the aggressive use of high dose combination drugtherapies.<sup>5</sup> Although induction of non-transmissible lethal genetic damage could be expected to produce an adverse affect on fertility, the induction of transmissible damage is of greater concern because such genomic alterations might result in an increased risk of birth defects, genetic disease or cancer in the children of cancer survivors.<sup>6-8</sup>

Doxorubicin, also named adriamycin, is one of the most popular chemotherapeutic drugs used in the treatment of a variety of cancers. Doxorubicin has recognized effectiveness against solid and non-solid malignant tumors and is used in oncology protocols against malignancies such as Hodgkin disease, childhood leukemia and testicular cancer, which commonly affect young patients and children.<sup>9</sup> The preferential target of doxorubicin is the DNA of dividing cells; the drug intercalates within DNA strands causing cell cycle blockage in the G<sub>2</sub> phase, single-strand breaks<sup>10</sup> and inhibition of the activity of some nuclear proteins, such as DNA and RNA-polymerase and DNA-topoisomerase II.<sup>11</sup> It has been found that doxorubicin also interferes with an important

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molecule involved in chromosome stability and transcription, the DNA methyl-transferase 1-DNMT1,<sup>12</sup> inducing apoptosis.

The chemical structure of doxorubicin favors the generation of free radicals and the compound can bind to iron and form complexes with DNA, inducing DNA damage.<sup>13,14</sup> Some studies have demonstrated that oxidative damage is probably related to this formation of free radicals accompanied by a reduction in antioxidant capacity.<sup>15</sup> The clinical effectiveness of doxorubicin treatment is diminished due to the dose-limiting side effect of cardiotoxicity, which may occur in more than 20% of patients treated with anthracycline antibiotics.<sup>16</sup> Doxorubicin has also been reported to be a somatic cell mutagen in humans and animals.<sup>2,3,17</sup> Moreover, clinical and experimental studies have widely demonstrated the testicular toxicity caused by doxorubicin.<sup>18-20</sup> Lu and Meistrich<sup>21</sup> showed that even a low dose of doxorubicin (1 mg/kg) given to adult mice is able to target germ cells, mainly A1-A4 spermatogonia, leading to seminiferous epithelium depletion. In addition, doxorubicin can also harm type B spermatogonia<sup>22</sup> and primary spermatocytes depending on the treatment schedule.<sup>21</sup> The secondary malignancies and fertility preservation of young patients submitted to anticancer treatments are important aspect that must be considered, since the prognosis of 10 year survival after childhood leukemia has the projection to reach 90% until the end of 2010.<sup>7,23</sup> Thus, the chemotherapy schedules also need to be improved; on this scope, other supporting therapies must be investigated focusing on reducing undesirable effects and providing a better life quality to survivor patients.

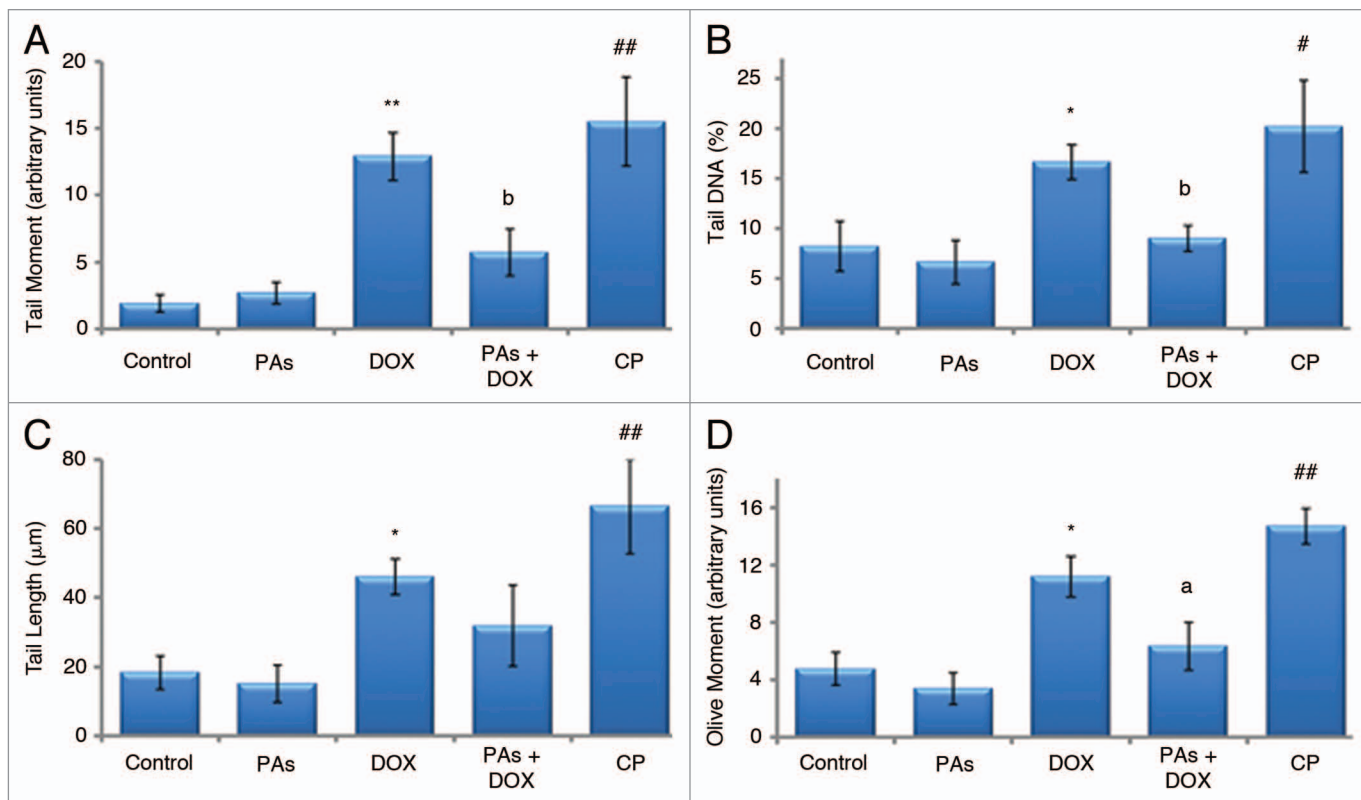
In this context, it was hypothesized that proanthocyanidins might be useful since proanthocyanidins have been shown to serve as free radical scavengers and antioxidants both in vitro and in vivomodels.<sup>24,25</sup> Proanthocyanidins, naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark, represent a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and other characteristics. Proanthocyanidins have been reported to exhibit a wide range of biological effects including anti-inflammatory, antiarthritic, antiallergic and prevent heart attack and skin aging.<sup>26,27</sup> Several in vitro studies have shown that treatment of epithelial cancer cells with proanthocyanidins results in a dose- and a time-dependent inhibition of cell growth.<sup>28-31</sup> In animal models, oral feeding of grape seed proanthocyanidin extract has been shown to inhibit ACP mutation-associated intestinal adenoma formation<sup>32</sup> and block skin cancer induction by UVB.<sup>33</sup> Other studies have also shown that topical application of proanthocyanidins significantly prevent tumor promotion in mouse skin.<sup>34,35</sup> Moreover, grape seed proanthocyanidin synergize the efficacy of doxorubicin against human breast carcinoma cells,<sup>28</sup> enhances doxorubicin-induced antitumor effect both in vitro and in vivo,<sup>29,30,36</sup> and reverses drug resistance indoxorubicin-resistant K562/DOX cells.<sup>30</sup> Additionally, proanthocyanidins strongly enhanced the anti-tumour effect of doxorubicin and completely eliminated myocardial oxidative stress and immunosuppression induced by doxorubicin in tumour-bearing mice.<sup>36</sup> Regarding epidemiology, a case-control study showed that increased consumption of proanthocyanidins is associated with reduced cancer risk.<sup>37</sup>

Considering the widespread use clinically of doxorubicin and the potential of proanthocyanidins to improve treatment efficacy and to reduce toxicity of doxorubicin in mice, we decided to study whether proanthocyanidins in combination with doxorubicin can ameliorate doxorubicin-induced mutagenicity towards normal mouse somatic and germinal cells. In addition, the possible mechanism underlying this modulation was assessed. The concept of providing protection against mutagenicity in non-tumour tissues will represent a promising approach of attacking the unwanted toxicity from conventional cytotoxic chemotherapy, and if successful, will allow the safe use of increased drug doses for the benefit of future cancer patients. The somatic cell mutagenicity was assessed by the bone marrow comet assay and micronucleus test. Spermatogonial and spermatocyte chromosomal analysis and spermiograms examination were undertaken in the current study as markers of germ cell mutagenicity. In addition, the biochemical alterations characteristic of oxidative stress activity induced by doxorubicin has been conducted by use of standard techniques.

## Results

**Bone marrow alkaline comet assay.** The results of the single cell gel electrophoresis analysis are shown in **Figure 1**. The level of the tail moment, tail length, tail DNA and olive moment recorded from the vehicle control group were 1.92, 8.22, 18.4 and 4.78, respectively. While, in the positive control group the average level of tail moment, tail length, tail DNA and olive moment were 15.5, 20.2, 66.4 and 14.7, respectively, which are significantly higher than that of the vehicle control mice. Level of bone marrow DNA damaged cells did not show any significant variation in proanthocyanidins-treated animals compared to the solvent control. On the other hand, tail moment, tail length, tail DNA and olive moment levels observed in doxorubicin-treated animals were 12.9, 16.6, 46.0 and 11.2, respectively, which are significantly higher than that of the solvent control group. Animals pretreated with proanthocyanidins showed significant decrease in the level of DNA damaged cells induced by doxorubicin to near to that in the control groups.

**Bone marrow micronucleus assay.** As shown in **Table 1** the frequency of MNPCE and % PCE observed in untreated mice (0.3 and 48.6, respectively) and treatment with proanthocyanidins (0.28 and 48.2, respectively) were found to be in the same ranges. In the positive control group the frequency of MNPCE and % PCE were 1.48 and 42.2, respectively, which are significantly higher than that of the vehicle control mice ( $p < 0.01$ ). Doxorubicin treatment was found to induce a significant increase in the frequency of MNPCE (2.14;  $p < 0.01$ ). Moreover, the mitotic activity was suppressed after treatment with doxorubicin as compared to control (35.0;  $p < 0.01$ ). The frequency of MNPCE induced by doxorubicin was significantly inhibited in mice pretreated with proanthocyanidins when compared to MNPCE induced by doxorubicin alone (0.86;  $p < 0.01$ ). Moreover, the mitodepression induced by doxorubicin was also restored in the pre-treatment group; however, this amelioration was not significant when compared to the values observed in the group treated with doxorubicin alone.



**Figure 1.** Level of DNA damaged (A) tail moment, (B) tail DNA, (C) tail length and (D) olive moment induced by proanthocyanidins (PAs) and/or doxorubicin (DOX) or cyclophosphamide (CP) (mean  $\pm$  SD). \* $p < 0.01$  and \*\* $p < 0.01$  versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test); <sup>#</sup> $p < 0.05$  and <sup>##</sup> $p < 0.01$  versus control; <sup>a</sup> $p < 0.01$  and <sup>b</sup> $p < 0.01$  versus doxorubicin alone (Mann-Whitney U test).

**Spermatogonial chromosomal analysis.** The results of the spermatogonial metaphase chromosomal analysis are shown in Table 2. The average percentage of aberrant spermatogonial metaphases recorded from the vehicle control group of mice was 4.8 with average chromosomal aberrations (excluding gaps) per hundred metaphases in them was 3.2. Whereas, in the positive control group the average percentage of aberrant spermatogonial metaphases was 42.0 and the average chromosomal aberrations (excluding gaps) per hundred metaphases was 38.4, which are significantly higher than that of the vehicle control mice ( $p < 0.01$ ). Proanthocyanidins alone failed to induce spermatogonial chromosomal aberrations confirming its non-clastogenicity. On the other hand, doxorubicin treatment induced aberrant spermatogonial metaphases was 11.2 and the average chromosomal aberrations (excluding gaps) per hundred spermatogonial metaphases in them was 18.0 ( $p < 0.05$ ). However, when pre-treatment of proanthocyanidins was given prior to doxorubicin treatment, significant decreased rates of mutagenic changes were observed when compared to clastogenesis induced by doxorubicin alone ( $p < 0.01$ ) and all types of chromosomal aberrations induced by doxorubicin were found to be reduced by proanthocyanidins.

**Spermatocyte chromosomal analysis.** The results of the spermatocyte diakinesis-metaphase I analysis are shown in Table 3. At week 4 post-treatment, the average percentage of aberrant primary spermatocytes recorded from the negative control group of mice was 2.8. In the positive control group of mice the induced

**Table 1.** Frequency of MNPCE and mitotic activity (% PCE) in bone marrow of mice treated with proanthocyanidins (PAs) and/or doxorubicin (DOX) or cyclophosphamide (CP) (mean  $\pm$  SD)

| Treatment groups | % MNPCE (mean $\pm$ SD)      | % PCE (mean $\pm$ SD)       |
|------------------|------------------------------|-----------------------------|
| Control          | 0.30 $\pm$ 0.10              | 48.6 $\pm$ 2.6              |
| PAs              | 0.28 $\pm$ 0.08              | 48.2 $\pm$ 2.1              |
| DOX              | 2.14 $\pm$ 0.22**            | 35.0 $\pm$ 3.3**            |
| PAs + DOX        | 0.86 $\pm$ 0.24 <sup>b</sup> | 40.6 $\pm$ 4.2              |
| CP               | 1.48 $\pm$ 0.35 <sup>#</sup> | 42.2 $\pm$ 3.4 <sup>#</sup> |

\*\* $p < 0.01$  versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test); <sup>#</sup> $p < 0.01$  versus control; <sup>b</sup> $p < 0.01$  versus doxorubicin alone (Mann-Whitney U test).

percentage of aberrant primary spermatocytes was 18.2, which is statistically significant ( $p < 0.01$ ) compared with that of the negative control group of mice. Proanthocyanidins treatment did not exhibit a significant difference in the frequency of numerical and structural chromosome aberrations compared to the solvent control at the tested dose. Doxorubicin treatment induced 13.2 average percentages of aberrant primary spermatocytes, which is statistically significant ( $p < 0.01$ ) compared with that of the negative control group of mice. However, proanthocyanidins treated doxorubicin-induced mice group encountered with a statistically significant decline in aberrant primary spermatocytes compared to doxorubicin-treated mice alone ( $p < 0.01$ ).

**Table 2.** Frequency of spermatogonial chromosomal aberrations induced by proanthocyanidins (PAs) and/or doxorubicin (DOX) or cyclophosphamide (CP) (mean  $\pm$  SD)

| Treatment groups | Average % of aberrant spermatogonial metaphases | Types and number of chromosomal aberrations |    |                 |   |     | Total aberrations (excluding gaps) | Average aberrations/100 spermatogonial metaphases |
|------------------|---|---|----|-----------------|---|-----|------------------------------------|---|
|                  |   | Chromatid type                              |    | Chromosome type |   | F/M |                                    |   |
|                  |   | G   | B  | G               | B |     |                                    |   |
| Control          | 4.80 $\pm$ 1.09                                 | 6   | 2  | 0               | 1 | 5   | 8                                  | 3.20 $\pm$ 1.09                                   |
| PAs              | 3.60 $\pm$ 1.60                                 | 5   | 2  | 0               | 0 | 4   | 6                                  | 2.49 $\pm$ 0.84                                   |
| DOX              | 11.2 $\pm$ 3.60                                 | 24  | 18 | 7               | 4 | 18  | 40                                 | 18.0 $\pm$ 3.93*                                  |
| PAs + DOX        | 6.40 $\pm$ 1.60                                 | 14  | 9  | 3               | 3 | 7   | 19                                 | 8.35 $\pm$ 2.31 <sup>b</sup>                      |
| CP               | 42.0 $\pm$ 7.07                                 | 48  | 47 | 6               | 7 | 28  | 82                                 | 38.4 $\pm$ 7.77 <sup>#</sup>                      |

Fifty metaphases were scored for chromosomal aberrations per mouse, for a total of 250 metaphases per treatment. \* $p < 0.05$  versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test); # $p < 0.01$  versus control; <sup>b</sup> $p < 0.01$  versus doxorubicin alone (Mann-Whitney *U* test). G, gaps; B, breaks; F, fragments; M, minutes.

**Table 3.** Frequency of spermatocyte chromosomal aberrations induced by proanthocyanidins (PAs) and/or doxorubicin (DOX) or cyclophosphamide (CP) (mean  $\pm$  SD)

| Treatment groups | Different structural chromosomal aberrations screened |                      |     |            |    | Total structural aberrations (%) |
|------------------|---|----------------------|-----|------------|----|----------------------------------|
|                  | X-Y univalents  | Autosomal univalents | F/B | Polyploidy | MV |                                  |
| Control          | 7   | 5                    | 1   | 1          | 0  | 2.80 $\pm$ 1.09                  |
| PAs              | 4   | 6                    | 1   | 0          | 0  | 2.20 $\pm$ 0.44                  |
| DOX              | 41  | 24                   | 4   | 3          | 4  | 13.2 $\pm$ 1.4**                 |
| PAs + DOX        | 15  | 16                   | 2   | 1          | 2  | 7.20 $\pm$ 1.0 <sup>b</sup>      |
| CP               | 32  | 25                   | 6   | 2          | 5  | 14.0 $\pm$ 2.2 <sup>#</sup>      |

\*\* $p < 0.01$  versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test); # $p < 0.01$  versus control; <sup>b</sup> $p < 0.01$  versus doxorubicin alone (Mann-Whitney *U* test). F, fragments; B, breaks; MV, multivalents having a chain of four chromosomes.

**Table 4.** Frequency of spermatozoa count, motility and abnormalities of mice after treatment with proanthocyanidins (PAs) and/or doxorubicin (DOX) or cyclophosphamide (CP) (mean  $\pm$  SD)

| Treatment groups | Sperm count ( $\times 10^6$ /couda epididymis) | Sperm motility (%)          | Sperm abnormalities (%)     |
|------------------|--|-----------------------------|-----------------------------|
| Control          | 50.2 $\pm$ 6.7                                 | 78.4 $\pm$ 7.4              | 3.2 $\pm$ 1.0               |
| PAs              | 53.0 $\pm$ 7.3                                 | 81.0 $\pm$ 9.4              | 2.3 $\pm$ 0.8               |
| DOX              | 34.8 $\pm$ 3.3*                                | 55.6 $\pm$ 11.8*            | 9.1 $\pm$ 2.3*              |
| PAs + DOX        | 38.0 $\pm$ 5.7                                 | 64.6 $\pm$ 8.5              | 5.0 $\pm$ 1.3 <sup>a</sup>  |
| CP               | 41.0 $\pm$ 3.6 <sup>#</sup>                    | 47.0 $\pm$ 5.8 <sup>#</sup> | 12.4 $\pm$ 2.9 <sup>#</sup> |

\* $p < 0.05$ , \*\* $p < 0.01$  versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test); # $p < 0.01$  versus control; <sup>a</sup> $p < 0.01$  versus doxorubicin alone (Mann-Whitney *U* test).

**Spermiograms analysis.** The results of the spermiograms analysis are shown in Table 4. Treatment of mice with proanthocyanidins did not affect the parameters studied as compared to the control value. At week 8 post-treatment, the average percentages of sperm count, sperm motility and sperm with abnormal morphology were 50.2, 78.4 and 3.2, respectively, in the negative control group of mice and 41.0, 47.0 and 12.4, respectively, in the positive control group, which are statistically significant in comparison with that of the negative control group ( $p < 0.01$ ). The percentage sperm count and sperm motility were significantly decreased after treatment with doxorubicin compared to the value observed in the control group ( $p < 0.05$ ). In animals treated with

doxorubicin the percentages of sperm count and sperm motility were 34.8% and 55.6%, respectively. Moreover, doxorubicin significantly increased the percentage abnormal sperm to 9.1% compared with the negative control group ( $p < 0.05$ ). Pre-treatment of mice with proanthocyanidins for 7 consecutive days before and 7 consecutive days after doxorubicin treatment significantly decreased the percentage of abnormal sperm relative to the values obtained after treatment with doxorubicin alone ( $p < 0.05$ ). The percentage sperm count and sperm motility were also restored with proanthocyanidins pretreatment; however, these ameliorations were not significant when compared to the values observed in the group treated with doxorubicin alone.

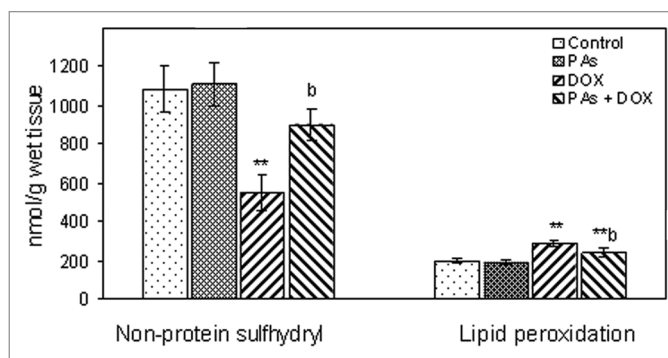
**Testicular oxidative stress markers.** The effect of proanthocyanidins on doxorubicin-induced oxidative stress in mice was assessed by measuring testicular non-protein sulfhydryl (GSH) and lipidperoxidation levels and the results are shown in Figure 2. The testicular level of GSH and lipid peroxidation in the solvent control were 1,084 and 198.6 nmol/gwet tissue, respectively. Testicular GSH and lipid peroxidation levels did not show significant variation in proanthocyanidins-treated animals compared to the solvent control. The GSH level observed in doxorubicin-treated animals was significantly decreased compared to the solvent control (546;  $p < 0.01$ ). Animals pre-treated with proanthocyanidins showed a significant increase in GSH level over the doxorubicin-treated group ( $p < 0.01$ ). A significant rise in testicular lipid peroxidation was observed in the doxorubicin group compared to the solvent control (288;  $p < 0.01$ ). Pre-treatment of

mice with proanthocyanidins was found to significantly decrease the lipid peroxidation relative to the values obtained after treatment with doxorubicin alone (242;  $p < 0.01$ ). However, this amelioration was still significant when compared to the values observed in the control group ( $p < 0.01$ ).

## Discussion

The impact of proanthocyanidins on doxorubicin-induced mutagenesis and cell proliferation changes in germinal cells of mice has not been reported yet. Proanthocyanidins have been reported to exhibit a wide range of biological effects including antioxidant, anti-inflammatory and anticancer effects.<sup>26,27</sup> In spite of their potent efficacies, proanthocyanidins shows little toxicity or mitogenicity in tests using mice. In fact, though 2 g/kg of grape seed proanthocyanidin extract, which was 240 times of the estimated daily ingestion of proanthocyanidins by human, was administered orally, to mice, no evidence of mutagenicity was found.<sup>38</sup> The LD<sub>50</sub> value of the grape seed proanthocyanidin extract was found to be greater than 4 g/kg in the male and female rats combined in the oral acute toxicity study. Bombardelli and Morazzoni<sup>39</sup> reported that acute oral LD<sub>50</sub> value of proanthocyanidin oligomers was approximately 4 g/kg. Therefore, grape seed proanthocyanidin extract was not toxic, as well as the proanthocyanidin oligomers. Since, proanthocyanidins are safe and readily available antioxidant, so one needs to examine its interaction with drugs. The current study demonstrates that proanthocyanidins were neither mutagenic nor cytotoxic with the dose tested. Moreover, they are able to protect mouse somatic and germinal cells against the doxorubicin-induced mutagenesis as observed by reduction in the bone marrow DNA damage and MN frequencies, testicular chromosomal aberrations and sperm head abnormality. Although, in some examined parameters the values decreased were still higher than that of the control and proanthocyanidins alone-treated groups. Moreover, the mitodepression, sperm motility and sperm count were also restored with proanthocyanidins pre-treatment. The positive control mutagen cyclophosphamide was used and this compound produced the expected responses and the results of this compound were in the same range as those of earlier studies.<sup>40-42</sup> These data confirmed the sensitivity of the experimental protocol followed in the detection of mutagenic effects.

An *in vivo* cyto-mutagenicity studies in mice has shown that doxorubicin is a somatic and germ-cell mutagen capable of inducing DNA damage, both numerical and structural chromosome aberrations.<sup>20,43-46</sup> In agreement with the above-cited report, the present experiment showed that exposure to doxorubicin caused significant increase in the bone marrow DNA damage and MN frequencies, spermatogonial and spermatocyte chromosomal aberrations and abnormal sperms as compared to the values obtained after treatment with the solvent control. Prior administration of proanthocyanidins ahead of doxorubicin challenge ameliorated the semutagenic markers. A similar effect has been also reported, for chemical-induced mutagenesis. Cisplatin injected animals pre-treated with grape seed proanthocyanidins have significantly fewer chromosomal damage in mouse cells



**Figure 2.** Effect of proanthocyanidins (Pas) and/or doxorubicin (DOX) on testicular lipid peroxidation and non-protein sulfhydryl (GSH) concentrations in mice (mean  $\pm$  SD). \*\* $p < 0.01$  versus control; <sup>b</sup> $p < 0.01$  versus doxorubicin alone (One way ANOVA and post hoc Tukey-Kramer multiple comparison test).

than those pre-treated with the vehicle.<sup>47</sup> More recently, Yalçin et al. found that treatment of mice with grape seed proanthocyanidins for 28 days greatly attenuated the doxorubicin-elicited changes in the levels of bone marrow chromosome aberrations and erythrocytes MN frequencies. Furthermore, Sugisawa et al. found that grape seed proanthocyanidins showed dose-dependent prevention of H<sub>2</sub>O<sub>2</sub> chromosomal damage evaluated by cytokinesis block micronucleus assay *in vitro*. Grape seed proanthocyanidins also prevented DNA oxidative damage in various tissues and DNA fragmentation induced by different agents.<sup>49-51</sup>

The exact mechanism by which proanthocyanidins protected against doxorubicin-induced mutagenesis in somatic and germinal cells is not well known. One possible explanation for the protection against mutagenesis is that simultaneous treatment with proanthocyanidins would allow interception of free radicals generated by doxorubicin before they reach DNA and induce mutagenesis. In the present work, in order to evaluate whether the observed anti-mutagenic effect was due to an enhancement of the scavenger of free radicals generated by doxorubicin, oxidative stress markers such as lipid peroxidation and GSH were done after the animals were treated with doxorubicin, compared with the prior treatment with proanthocyanidins and the solvent control animals. The present study demonstrates that proanthocyanidins pre-treatment reduced the doxorubicin induced testicular lipid peroxidation and prevented the reduction in testicular GSH significantly. The increased GSH level suggests that protection by proanthocyanidins may be mediated through the modulation of cellular antioxidant levels. These observations confirm earlier studies in which proanthocyanidins were reported to scavenge free radicals and lipid peroxides.<sup>52,53</sup> Moreover, Ray et al.<sup>54</sup> suggested that *in vivo* protection of DNA damage by proanthocyanidins might be due to detoxification of cytotoxic radicals and presumed contribution to DNA repair.

From a cellular perspective, one of the most advantageous features of proanthocyanidins oligomers free radical scavenging activity is that, because of its chemical structure, it is incorporated within cell membranes. This physical characteristic along with its ability to protect against both water and fat-soluble

free radicals provides incredible protection to the cells against free radical changes on chromosomes.<sup>55</sup> Experimental studies verify the antioxidant capacities of proanthocyanidins. These polyphenols are scavengers of superoxide, hydroxyl, peroxy and peroxynitrite radicals,<sup>56,57</sup> they chelate redox-active metals<sup>56,58</sup> and they can protect cell membranes against oxidative attack.<sup>57</sup> In RBCs subjected to oxidative stress, proanthocyanidins markedly decreased membrane lipid peroxidation, recycled oxidized tocopherol in the membrane and delayed hemolysis.<sup>59</sup> Doxorubicin has been shown to be metabolically activated to a free radical state and interacts with molecular oxygen to generate superoxide radicals.<sup>13,14</sup> Superoxide radicals can deplete antioxidant cellular sulfhydryl compounds and react with hydrogen peroxide to form highly reactive hydroxyl radicals through the iron-catalyzed Haber-Weiss reaction.<sup>60,61</sup> Secondarily derived hydroxyl radicals can cause protein, DNA damage and initiate lipid peroxidation, ultimately inducing tissue damage, mutagenicity and leukaemia.<sup>62-64</sup> The use of certain antioxidants such as  $\beta$ -glucan, selenium, *N*-acetylcysteine, lycopene and vitamin C may help to decrease of the cardiotoxicity and genotoxicity created by doxorubicin and may inhibit mutagenesis and carcinogenesis.<sup>44,65,66</sup> Importantly, these effects were associated with a concomitant alteration of the antioxidant potential. Proanthocyanidins were found to elevate reduced glutathione, glutathione peroxidase, superoxide dismutase and to reduce the oxidation of polyunsaturated fatty acids and provide protection against lipid peroxidation.<sup>52,53,67</sup> Thus, the antioxidant activity of proanthocyanidins might be responsible for the protection against mutagenicity induced by doxorubicin.

A crucial consideration of coadministration of doxorubicin and proanthocyanidins is how it will possibly affect the anticancer treatment efficacy; there are, however, important differences between these two processes, which suggest that a reduction in side effects does not necessarily go hand-in-hand with a reduction in the antitumor effects. Grape seed proanthocyanidins reduced cardiotoxicity<sup>54</sup> and ameliorated growth inhibitory effects and apoptosis in non-malignant human liver cells<sup>68</sup> from several different anthracyclines: The mechanism of action on these preventions is unclear, whereas the current understanding is that proanthocyanidins protect the heart from doxorubicin-induced oxidative damage via suppression of oxidative stress.<sup>36,69,70</sup> In conclusion, a critical point of this study is the possibility that there may be a therapeutic window for the use of doxorubicin in combination with proanthocyanidins, so that its mutagenic side effects in normal cells are minimized. The mutagenic effects of doxorubicin might be, at least in part, mediated by an oxidative stress mechanism that may be prevented or reduced by radical scavengers. doxorubicin has a direct inhibitory effect on topoisomerase II, an important component of its antitumor activity, and this will be unchanged by any manipulations that alter the redox reaction. Apart from the regulatory role of proanthocyanidins on MDA production observed in the current work, the anti-mutagenic effects of proanthocyanidins could be possibly ascribed to the irradical scavenger activity that modulated the mutagenic responses and cell proliferation changes induced by doxorubicin. Therefore, we can conclude that proanthocyanidins can be

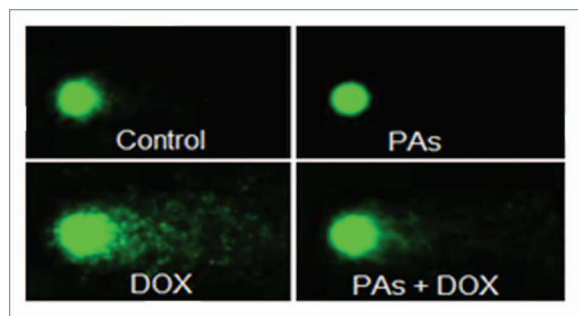
promising chemoprotective agent and might be useful to avert secondary malignancy and abnormal reproductive outcomes risks in cancer patients receiving doxorubicin-involved treatment.

## Materials and Methods

**Chemicals.** Doxorubicin was supplied as vials (Adriablastina, Farmitalia Carlo Erba, Italy). The vial contents were thoroughly dissolved in sterile dH<sub>2</sub>O in darkness 10–15 min before use and administered by intraperitoneal injection within 1 h following preparation. It was administered at a dose level of 12 mg/kg.<sup>43,44</sup> Grape seed proanthocyanidin extract was provided by Spectrum Chemical Mfg. Corp., (CA USA, CAS-No 84929-27-1) and stored at 4°C in light-tight containers until used. The composition of this preparation was to consist of 95% (w/w) proanthocyanidins (75–80% oligomeric proanthocyanidins and 3–5% monomeric proanthocyanidins). Proanthocyanidins thoroughly dissolved in distilled water and administered through oral intubations at the dose levels of 100 mg/kg/day for 7–14 consecutive days. The antimutagenic dose for the proanthocyanidins was chosen by reference to our earlier study in reference 47. Cyclophosphamide (Sigma-Aldrich St. Louis, MO) was dissolved in sterile dH<sub>2</sub>O and used at a concentration of 25 mg/kg as a positive control mutagen. All other chemicals and reagents used were of analytical grade.

**Animals.** Adult male white Swiss albino mice, weighing 20–25 g (10–12 weeks old), were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were maintained under standard conditions of humidity, temperature (25  $\pm$  2°C) and light (12 h light/12 h dark). They were fed with a standard mice pellet diet and had free access to water. All animal experimentations described in the manuscript were conducted in accord with accepted standards of humane animal care in accordance with the NIH guidelines and the legal requirements in Kingdom of Saudi Arabia.

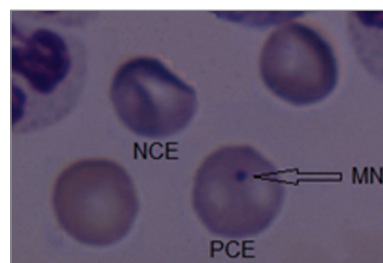
**Experimental groups.** Hundred healthy male mice were selected from the acclimatized stock, divided randomly into five groups of 20 mice each and caged separately as follows: Group 1: mice served as a control group and treated daily with sterile dH<sub>2</sub>O for 7–14 consecutive days; Group 2: mice were treated with proanthocyanidins at a dose of 100 mg/kg/day, once a day, for 7–14 consecutive days; Groups 3: mice were treated with proanthocyanidins at a dose of 100 mg/kg/day for 7–14 consecutive days and a single dose of 12 mg/kg doxorubicin was administered on the day 7, one hour after regular proanthocyanidins exposure. Groups 4: mice were injected with doxorubicin in a single dose of 12 mg/kg; Group 5: mice were injected with cyclophosphamide in a single dose of 25 mg/kg as a positive control mutagen. In cyto-mutagenicity tests a single dose treatment for one cell cycle duration is adequate for both qualitative and quantitative assessments, so the alkaline comet assay, micronucleus assay and spermatogonial metaphase chromosome aberration study were undertaken at 24 h post-treatment with doxorubicin. Oxidative stress markers such as testes lipid peroxidation and non-protein sulfhydryl were assessed as a possible mechanism underlying this amelioration 24 h post-treatment with doxorubicin. To assess the



**Figure 3.** Representative fluorescence comet images of bone marrow cells showing undamaged cells [(Control) or proanthocyanidins (PAs)], highly damaged cell of animal treated with doxorubicin (DOX) and low damaged cell of animal treated with PAs and DOX (PAs + DOX).

potential transmission of such effects from spermatogonia in the male germline and amelioration of these effects by proanthocyanidins, primary spermatocytes and sperm were examined 4 and 8 weeks after exposure to doxorubicin, respectively. In the primary spermatocytes and spermograms analysis the animals were treated as described above but mice were treated with proanthocyanidins for 7 consecutive days before and 7 consecutive days after doxorubicin injection (i.e., 14 days).

**Bone marrow alkaline comet assay.** After 24 h of doxorubicin treatment i.e., on 8<sup>th</sup> day of the experiment, five mice from each of the five groups were sacrificed by cervical dislocation. The single cell gel electrophoresis comet assay was performed essentially by the guidelines of Tice, et al. with slight modifications. In brief, the bone marrow cells from one femora were collected in tubes containing foetal calf serum then centrifuged and resuspended in ice-cold PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free, pH 7.4). 10  $\mu\text{l}$  of cell suspension (~10,000 cells) were mixed with 85  $\mu\text{l}$  of 0.5% low melting agarose (LMA), distributed onto the end frosted conventional slides precoated with 1.5% normal melting agarose in PBS. After the agarose solidifies, other 85  $\mu\text{l}$  of LMA was layered and kept over ice for 10 min. The slides were prepared in duplicate. Finally, the slides were immersed in freshly prepared chilled lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) containing freshly added 10% DMSO and 1% Triton X-100]. These slides were kept overnight at 4°C and placed in a horizontal gel electrophoresis tank filled with freshly made electrophoresis buffer (1 mM  $\text{Na}_2\text{EDTA}$  and 300 mM NaOH, pH > 13). The slides were left in the buffer for 30 min to allow DNA unwinding and expression of alkali labile sites as DNA breaks. Subsequently, electrophoresis was performed at 24 V, 300 mA for 30 min at 4°C. All these steps were performed under dimmed light. After electrophoresis, the slides were washed three times with cold neutralization buffer [0.4 M Tris (pH 7.5)]. The slides were stained with ethidium bromide (20  $\mu\text{g}/\text{ml}$ ) for 5 min, dipped in chilled distilled water and then a cover slip was placed. Slides were placed in a dark humidified chamber to prevent drying of the gel and analyzed within 3–4 h. The slides were studied using a fluorescent microscope (Nikon, Japan) equipped with appropriate filters. The microscope was connected to a computer through a charge coupled device camera (Fig. 3). Fifty individual cells were



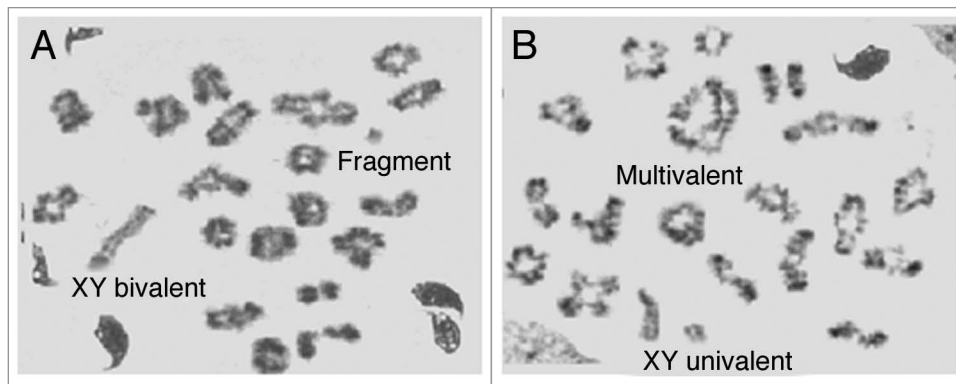
**Figure 4.** Microscopic image of bone marrow cells showing enucleated normochromatic erythrocyte (NCE) as well as nucleated polychromatic erythrocyte (PCE) cells. One PCE contains a micronucleus (MN).

selected for calculations for each analysis; all experiments were carried out at least three times, each with two parallel slides per data point. Single cells were analyzed with TriTek CometScore version 1.5 software. The parameters studied to assess the DNA damage were the tail moment (arbitrary units), tail DNA (%), tail length ( $\mu\text{m}$ ) and olive moment (arbitrary units).

**Bone marrow micronucleus assay.** The remaining femora from the same animals used for the alkaline comet assay was used for estimation of micronuclei (MN) frequencies and mitotic activity. Bone marrow smears were done and the slides were stained with May-Gruenwald/Giemsa solutions as described earlier in reference 72. Per animal, 1,000 polychromatic erythrocytes (PCE) were blindly scored microscopically for the presence of MN (Fig. 4). In addition the number of PCEs among 1,000 normochromatic erythrocytes (NCE) per animal was recorded to evaluate bone marrow suppression, mitotic activity was calculated as  $\% \text{ PCE} = [\text{PCE}/(\text{PCE} + \text{NCE})] \times 100$ .

**Spermatogonial chromosomal analysis.** For spermatogonial metaphase chromosomal aberration study, at 20 h post-treatment with doxorubicin, five mice from each of the five groups were colchicized intraperitoneally at the rate of 4 mg/kg. After an interval of 4 h of colchicine treatment, the mice were sacrificed by cervical dislocation. The testes were dissected out and washed thoroughly in 2.2% tri-sodium citrate at 37°C to remove the adhered fat, seminiferous tubules were teased out in 1% tri-sodium citrate at room temperature for 20 min. The tubules were suspended in Carnoy's fixative. Two to three changes of fixative were required and the tubules were then suspended in 50% of acetic acid. Finally, the tubules were suspended in 1 ml of fresh fixative after centrifugation from the acetic acid and slides for spermatogonial metaphase chromosome preparations were made following the flame-drying giemsa technique of Adler.<sup>73</sup> More than 250 well-spread spermatogonial metaphases from each group of mice were examined randomly and the chromosomal aberrations in them were recorded. Aberrant spermatogonial metaphases with chromatid/chromosome gaps, chromatid/chromosome breaks, fragments and minutes, were recorded. Percentages of aberrant metaphases (inclusive of gaps) and aberrations excluding gaps per 100 metaphases were calculated for each animal.

**Spermatocyte chromosomal analysis.** For primary spermatocytic chromosome analysis, mice were treated with proanthocyanidins for other 7 consecutive days after a single treatment with



**Figure 5.** Microscopic images of aberrant spermatocytes at diakinesis-metaphase I with fragments (A), multivalents and XY univalents (B).

doxorubicin i.e., for 14 days treatment with proanthocyanidins. At week 4 post-doxorubicin treatment, five mice from each of the five groups were sacrificed cervical dislocation. The first meiotic chromosome preparations were made according to the air-drying technique detailed earlier in reference 73. The testes were removed and placed in 2.2% isotonic tri-sodium citrate solution. The tunica albuginea was peeled out and seminiferous tubules were teased to form a cell suspension. The suspension was centrifuged for 5 min at 1,000 rpm and the pellet was re-suspended in 1.1% hypotonic tri-sodium citrate solution for 20 min at room temperature. After centrifugation the supernatant was discarded and the pellets were re-suspended in Carnoy's fixative. Two to three changes of fixative were required before the preparation of slides. Finally, the cells were suspended in 1 ml of fixative and burst open on a clean slide to release chromosomes. At least four slides were made for each animal and allowed to dry overnight. The coded slides were stained with giemsa and scored as detailed earlier in reference 73. Hundred well spread diakinesis-metaphase I per animal were analyzed under bright field microscope for chromosomal aberrations. The types of aberrations recorded in diakinesis-metaphase I cells included univalents, fragments, breaks, polyploids and multivalents having a chain of four chromosomes (Fig. 5). The percentage of total structural aberrations for each group was calculated.

**Spermograms analysis.** The remaining animals (five from each group) were sacrificed at week 8 post-doxorubicin treatment for spermograms analysis i.e., on 49<sup>th</sup> day of the last 14 days treatment with proanthocyanidins. Immediately after cervical dislocation, both *caudae epididymes* of each animal were dissected and incisions were made. Then they were placed individually into tubs filled with 3 ml of fetal calf serum. The tubs were placed on an Eppendorf incubator at 32°C for 30 min to allow the sperm to actively leave the epididymes. The tissue residuals were removed from by filtration through 80  $\mu$ m pore-size nylon mesh. The filtrate was used for evaluation of epididymal sperm parameters as follow: Sperm count and motility were determined under the light microscope using a Neubauer hemacytometer according to the World Health Organization manual for the examination of human semen,<sup>74</sup> and two counts per animal were averaged. For sperm-shape

abnormality, aliquots of sperm suspensions were stained with 1% eosin-Y and the smears were made on clean glass slides, air-dried and made permanent. The stained slides were examined by bright field microscope and the abnormalities were categorized as close as to those described by Wyrobek and Bruce.<sup>75</sup> At least 500 sperms per animal were assessed for morphological abnormalities which included triangular, without hook, banana shape, amorphous and tail abnormality.

**Determination of oxidative stress markers in testicular cells.** To study the effect of proanthocyanidins on the oxidative stress induced by doxorubicin, the testes from the same animals used for the comet and micronucleus assays were used for estimation of lipid peroxidation and non-protein sulfhydryl (GSH). The testes were weighed, rinsed with ice-cold deionized water and dried with filter paper. The testes were homogenized in buffer [Tris 10 mM, EDTA 1 mM, PMSF 1 mM, pH 7.5]. GSH was assayed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the protocol described by Ellman.<sup>76</sup> The homogenate was mixed with trichloroacetic acid and centrifuged at 3,000 g. The supernatant was suspended in tris buffer, DTNB and read at 412 nm against reagent blank with no homogenate. The concentration of GSH was calculated from a standard curve that was obtained from freshly prepared standard solution of reduced glutathione. Lipid peroxidation was measured in the testes using thiobarbituric acid reacting substance (TBARS) and was expressed in terms of malondialdehyde (MDA) content.<sup>77</sup> Sample aliquots were incubated with trichloroacetic acid and thiobarbituric acid. The samples assayed for MDA contained 1 mM butylated hydroxytoluene in order to prevent artificial lipid peroxidation during the boiling step. The mixture was heated in a boiling water bath for 30 min, an equal volume of n-butanol was added and the final mixture was centrifuged. After centrifugation the optical density of the clear pink supernatant was read at 532 nm. As 99% TBARS is MDA so lipid peroxidation levels of the samples were calculated from the standard curve using the 1,1,3,3-tetramethoxypropane.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard deviation (SD) of the means. The analyzed parameters were tested for homogeneity of variance and normality and were found to be normally distributed. The data were, therefore, analyzed by employing non-parametric tests, Mann-Whitney *U* test or Kruskal-Wallis



test followed by Dunn's multiple comparisons test. Data on oxidative stress parameters were analyzed using analysis of variance, followed by Tukey-Kramer for multiple comparisons. Results were considered significantly different if the p-value was <0.05.

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