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Article

# *Punica granatum* L. protects mice against hexavalent chromium-induced genotoxicity

**Renato Ivan de Ávila[1], Meirielle Teixeira Guerra[1], Kamilla de Almeida Souza Borges[1], Marcelo de Sousa Vieira[1], Luiz Marcos de Oliveira Júnior[1], Humberto Furtado[2], Mariana Flavia da Mota[1], Andréa Fernandes Arruda[2], Marize Campos Valadares[1,*]**

*[1]Celullar Toxicology and Pharmacology Laboratory - FarmaTec, Faculty of Pharmacy, Federal University of Goiás, Goiânia, GO, Brazil, [2]Analytical Spectroscopy Laboratory, Institute of Chemistry, Federal University of Goiás, Goiânia, GO, Brazil*

This study investigated the chemoprotective effects of *Punica granatum* L. (Punicaceae) fruits alcoholic extract (PGE) on mice exposed to hexavalent chromium [Cr(VI)]. Animals were pretreated with PGE (25, 50 or 75 mg/kg/day) for 10 days and subsequently exposed to a sub-lethal dose of Cr(VI) (30 mg/kg). The frequency of micronucleated polychromatic erythrocytes in the bone marrow was investigated and the Cr(VI) levels were measured in the kidneys, liver and plasm. For the survival analysis, mice were previously treated with PGE for 10 days and exposed to a single lethal dose of Cr(VI) (50 mg/kg). Exposure to a sub-lethal dose of Cr(VI) induced a significant increase in the frequency of micronucleated cells. However, the prophylactic treatment with PGE led to a reduction of 44.5% (25 mg/kg), 86.3% (50 mg/kg) and 64.2% (75 mg/kg) in the incidence of micronuclei. In addition, the 50 mg/kg dose of PGE produced a higher chemoprotective effect, since the survival rate was 90%, when compared to that of the non-treated group. In these animals, reduced amounts of chromium were detected in the biological materials, in comparison with the other groups. Taken together, the results demonstrated that PGE exerts a protective effect against Cr(VI)-induced genotoxicity.

**Uniterms:** Chemoprevention. Hexavalent chromium/induced genotoxicity. Oxidative damage. Pomegranate/chemoprotective effects. *Punica granatum*/chemoprotective effects. *Punica granatum*/pharmacognosy. *Punicaceae*/chemoprotective effects. *Punicaceae*/pharmacognosy.

Este estudo investigou os efeitos quimioprotetores do extrato alcoólico dos frutos da *Punica granatum* L. (Punicaceae) (EPG) em camundongos expostos ao cromo hexavalente [Cr(VI)]. Os animais foram pré-tratados com o EPG (25, 50 ou 75 mg/kg/dia) durante 10 dias e subsequentemente expostos a uma dose subletal de Cr(VI) (30 mg/kg). A frequência de eritrócitos policromáticos micronucleados na medula óssea foi investigada e os níveis de Cr(VI) foram quantificados nos rins, fígado e plasma. Para a análise de sobrevida, os camundongos foram previamente tratados com EPG durante 10 dias e expostos a única dose letal de Cr(VI) (50 mg/kg). A exposição à dose subletal de Cr(VI) induziu aumento significativo na frequência de células micronucleadas. Entretanto, o tratamento profilático com EPG levou à redução de 44,5% (25 mg/kg), 86,3% (50 mg/kg) e 64,2% (75 mg/kg) na incidência de micronúcleo. Além disso, a dose de 50 mg/kg de EPG produziu maior efeito quimioprotetor, uma vez que a taxa de sobrevivência foi de 90%, quando comparada àquela do grupo não tratado. Nesses animais, quantidades reduzidas de cromo foram detectadas nos materiais biológicos, em comparação com os outros grupos. Em conjunto, os resultados demonstram que o EPG exerce efeito protetor contra a genotoxicidade induzida pelo Cr(VI).

**Unitermos:** Quimioprevenção. Cromo hexavalente/genotoxicidade induzida. Dano oxidative. Romã/efeito quimioprotetor. *Punica granatum*/efeito quimioprotetor. *Punica granatum*/farmacognosia. *Punicaceae*/efeito quimioprotetor. *Punicaceae*/farmacognosia.

***Correspondence:** M. C. Valadares. Faculdade de Farmácia, Universidade Federal de Goiás. Praça Universitária esquina com a 1ª Avenida s/n - Setor Universitário, 74605-220 - Goiânia - GO, Brasil. E-mail: marizecv@farmacia.ufg.br

## INTRODUCTION

Chromium occurs naturally in the environment in different oxidation states, where Cr(III) and Cr(VI) are the most prevalent. The hexavalent form is usually linked with oxygen and is a strong oxidizing agent. Studies have shown that Cr(VI) is a mutagenic, carcinogenic and teratogenic agent, which easily penetrates the cell membrane in the form of chromate anion (Barceloux, 1999; Acharya *et al.*, 2006; Nickens *et al.*, 2010; Chang *et al.*, 2011; Okello *et al.*, 2012). Its molecular mechanisms of toxicity are not yet fully known, but it is believed that the intracellular increase in the production of reactive oxygen species (ROS) is probably a key factor in inducing cell damage (Ye *et al.*, 1999; Wu *et al.*, 2012). These reactive species can cause damage to macromolecules such as DNA, proteins and lipids, thereby inhibiting their functions (Valko *et al.*, 2007; Yan *et al.*, 2012) and may even trigger apoptotic cell death (Liu, Chen, 2006; Liu *et al.*, 2008).

Cr(VI) has been widely used in metallurgical, refractory and chemical industries. It has been considered a potent occupational carcinogen among workers involved in electrodeposition, metal finishes/welding, wood preservatives, organic synthesis procedures and in leather tanning (Barceloux, 1999; Shrivastava *et al.*, 2002; Acharya *et al.*, 2006; Nickens *et al.*, 2010). In such activities occupational exposure to Cr(VI)-contaminated dust and aerosols occurs predominantly through inhalation or skin contact (Zagrodzki *et al.*, 2007). Non-occupational exposure to Cr(VI) can also occur through cigarette smoke, automobile emissions and the inappropriate disposal of chromium-containing waste in the environment (O'Brien *et al.*, 2003; Russo *et al.*, 2005).

The literature has shown that the administration of antioxidant agents to animals exposed to Cr(VI) results in a reduction of bioavailability, nephrotoxicity and the mortality rate of these animals (Chow, 2002). Thus the use of antioxidant agents should be considered if the toxicological effects of this xenobiotic are reduced (Deb *et al.*, 2012).

Pomegranate [*Punica granatum* L. (Punicaceae)] has been reported to be a rich source of bioactive polyphenols, compounds with antioxidant properties, such as anthocyanins, hydrolysable and condensed tannins (Sudheesh, Vijayalakshmi, 2005; Lansky, Newman, 2007; Wang *et al.*, 2010). Various therapeutic properties are commonly attributed to this fruit. These include anti-inflammatory, antitumoral, diabetes prevention/treatment, antiulcer, antimicrobial, antiangiogenic, immunomodulating and antioxidant activities. The medicinal properties of this fruit have been widely investigated and numerous scientific papers have corroborated with its popular use (Prashanth *et al.*, 2001; Braga *et al.*, 2005; Menezes *et al.*, 2006; Vasconcelos *et al.*, 2006; Aquil, Ahmad, 2007; Oliveira *et al.*, 2010; Johanningsmeier, Harris, 2011).

Analyses of the antioxidant property of pomegranate have shown that this fruit is superior to other foods in terms of antioxidant effects, such as red wine, cranberries and green tea (Seeram *et al.*, 2008). Moreover certain reports have shown considerable antioxidant activities of *Punica granatum* in several *in vitro* and *in vivo* experimental models (Chidambara *et al.*, 2002; Cerda *et al.*, 2003; Ricci *et al.*, 2006; Bishayee *et al.*, 2011). Such effects have been attributed to its effective action against ROS (Ozgen *et al.*, 2008). As a consequence, *Punica granatum* is recognized as a potent chemoprotective agent and adjuvant in cancer treatment (Syed *et al.*, 2007). Our research group recently demonstrated that pomegranate alcoholic extract exerts antimutagenic activity against cyclophosphamide-induced DNA damage (Valadares *et al.*, 2010). Additionally this extract showed *in vitro* cytotoxic and *in vivo* antitumor activity due to its inhibition of Ehrlich ascites tumour growth, along with a decrease in the vascular pattern of the peritoneal wall and a consequent increase in survival time on mice (Oliveira *et al.*, 2010).

Considering that there is an ever growing worldwide risk of chromium poisoning in the aftermath of occupational exposure (Fen *et al.*, 2012), and with all the above-described date, as a background, this study set out to investigate the chemoprotective properties of *Punica granatum* L. fruits alcoholic extract (PGE) for mice exposed to Cr(VI).

## MATERIAL AND METHODS

### Botanical material

*Punica granatum* fruits were collected in the city of Goiânia, Goiás, Brazil (768 m altitude; 16° 40' 33.3'' South; 49° 14' 39.5'' West). The voucher specimens were deposited in the Federal University of the State of Goiás herbarium under the identification UFG-41497, Goiânia, GO. The fruits were dried in a ventilated oven at 36 °C, pulverized, macerated in 95% ethanol and rotaevaporated according to guidelines of the Brazilian Pharmacopoeia (1977).

### Animals and treatment

The experimental study was approved by the Ethics Committee for the Use of Animals of University of Goiás, in accordance with institutional protocols and

the guidelines of the Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care (Olfert *et al.*, 1993).

Swiss male mice (body weight: 30-38 g; age: 7-10 weeks) were obtained from Chemical Industry of the State of Goiás (IQUEGO). All animals were kept under constant environmental conditions with light-dark cycles and controlled temperature. Water and food were provided *ad libitum*.

*Punica granatum* alcoholic extract (PGE) was diluted in saline and Tween 80 (10%) for administration in animals. It was administered orally (gavage) in single daily doses of 25, 50 or 75 mg/kg for 10 days and subsequent exposure to Cr(VI), on the 11$^{th}$ day. For chemoprotection assay and survival evaluation, it was administered 30 mg/kg (sub-lethal dose) and 50 mg/kg (lethal dose) of Cr(VI) (potassium dichromate) intraperitoneally, respectively. Treatment groups were distributed as follows: Group I: PGE solvent; Group II: Cr(VI); Group III: 25 mg/kg PGE + Cr(VI); Group IV: 50 mg/kg PGE + Cr(VI); Group V: 75 mg/kg PGE + Cr(VI). These doses of PGE were chosen according previous studies from our group (Valadares *etal*., 2010).

### Investigation of chemoprotective effect of PGE

The animals (n=6 per group) were euthanized on the 12$^{th}$ day and bone marrow cells and kidneys, liver and plasma were obtained to micronuclei assay and determination of the Cr(VI) levels, respectively.

The micronucleus test was realized based on MacGregor *et al.* (1987) and Valadares *et al.* (2010). For each animal, three slides were made and stained with Giemsa dye. 1000 bone marrow cells for each animal were scored in a blind test using a light microscope (1000x magnification) and the number of micronucleated polychromatic erythrocytes (MNPCE) was determined. The frequency of MNPCE in each mouse was used as the experimental unit. The percentage of reduction in the frequency of micronuclei was calculated according to Manoharan and Banerjee (1985), as the following formula: % reduction = (mean frequency of damage in A - mean frequency of damage in B)/(mean frequency of damage in A - mean frequency of damage in C) x 100. Where, A = group exposed to Cr(VI) and without prophylactic treatment with PGE (Group II), B = group treated with PGE and exposed to Cr(VI) (Groups III, IV and V), C = group treated with the solvent from the PGE (Group I).

### Determination of the Cr levels

The Cr(VI) concentrations in plasma, kidneys and liver were measured using a AA 400 Flame Atomic Absorption Spectrometer (FAAS- Perkin Elmer, USA) with a chromium hollow cathode lamp (357.8 nm). Chromium determination procedure is based on sample incineration and solubilization in nitric acid, according to official methods of analysis (Association of official analytical Chemists – AOAC, 1990).

The samples were prepared by wet-digestion using $HNO_3$ (65%) and $H_2O_2$ (30%), and diluted to a final volume of 50 mL with ultrapure water for the chromium determination. The optimum operation conditions were 20 nm band width, 25 mA lamp current and 2.5 mL.min$^{-1}$ acetylene flow rate in an air/acetylene flame. The chromium quantification was carried out using multiple standard additions in the prepared solutions e the results were expressed in the amount of Cr presents in each organ or plasm pool. A minimum of three replicate analyses was performed for each sample. Five solutions, each containing 3.0 mL of organ sample solution , were prepared by additions of 0, 50, 200, 350, and 500 µL of 200 mg/L chromium stock solution (prepared using $CrO_3$ in $HNO_3$ 5%) for a 5 mL final volume. The results of mice group exposed to Cr(VI) only were used as a comparison with the groups pretreated with PGE and exposed to this toxic metal.

### Survival curve

For the survival test, three groups of animals (n = 10 per group) were previously treated during 10 days by gavage with different PGE doses (25, 50 or 75 mg/kg) and Cr(VI) group received only saline and Tween 80 (10%) solution. On the 11$^{th}$ day, all groups were exposed to the Cr(VI). From 12$^{th}$ day, the rate of survival of the animals was monitored. At the end of the study, the surviving animals were euthanized (day 30).

### Statistical analysis

Survival was evaluated by the Kaplan-Meier test and Log-Rank test *a posteriori*. Micronuclei frequency and Cr levels in biological tissues were analyzed by the one-way Analysis of Variance (ANOVA) followed by Tukey's test. Statistical significance was established as $p<0.05$.

## RESULTS

### Investigation of the chemoprotective effect

Cr(VI) exposure induced a significant increase in the frequency of MNPCE, as compared to that of the control group ($p<0.05$). On the other hand, prophylactic treatment

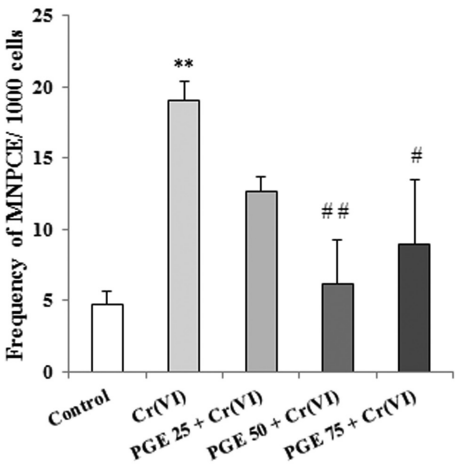


**FIGURE 1** – Effects of prophylactic oral treatment with PGE (25, 50, and 75 mg/kg/day) for 10 days on the frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice exposed to a sub-lethal dose of Cr(VI) (30 mg/kg). Each bar presents mean±SD. (**p<0.01 vs. control; #p<0.05 and ##p<0.01 vs. Cr(VI). One way ANOVA and Tukey's test, p<0.05).

with PGE followed by exposure to Cr(VI) produced a significant reduction in the frequency of micronuclei (p<0.05) (Figure 1). The study found that animals pretreated with 25, 50, or 75 mg/kg of PGE showed reductions of 44.5, 86.3 and 64.2% in the incidence of micronuclei, respectively. In a preliminary study, similar results were observed when animals exposed to Cr(VI) were therapeutically treated with PGE, i.e., the treatment was given after exposure (data not shown).

### Quantification of Cr levels

As shown in Figure 2, only those mice exposed to a sub-lethal dose of Cr(VI) showed an increase in Cr levels, in contrast with the animals pretreated with 25 and 50 mg/kg doses of PGE. Treatment with these doses significantly reduced the Cr levels in plasm, kidneys and liver (p<0.001), while mice pretreated with 75 mg/kg of PGE showed Cr levels similar to the Cr(VI) group.

### Survival analysis

All the mice exposed to a single Cr(VI) lethal dose (50 mg/kg) died between 12$^{th}$ and 14$^{th}$ day. On the other hand, treatment with PGE doses increased the survival rate of the animals exposed to this toxic metal. The 50 mg/kg dose of PGE presented the best survival rate, since 90% of the animals survived until the end of the assay, while 25 mg/kg and 75 mg/Kg of PGE gave rates of 60 and 30%, respectively (p = 0.0096) (Figure 3).

## DISCUSSION

Cr(VI) has been shown to induce chromosomal aberrations and mutations (Balansky *et al.*, 2000; O'Brien *et al.*, 2003; Rodriguez *et al.*, 2011). In this study, as expected, it was detected that Cr(VI) led to an increase in the frequency of micronucleated polychromatic erythrocytes. In contrast, PGE reduced micronuclei frequency, especially at 50 mg/kg dose, thus showing its chemoprotective property. The antigenotoxic

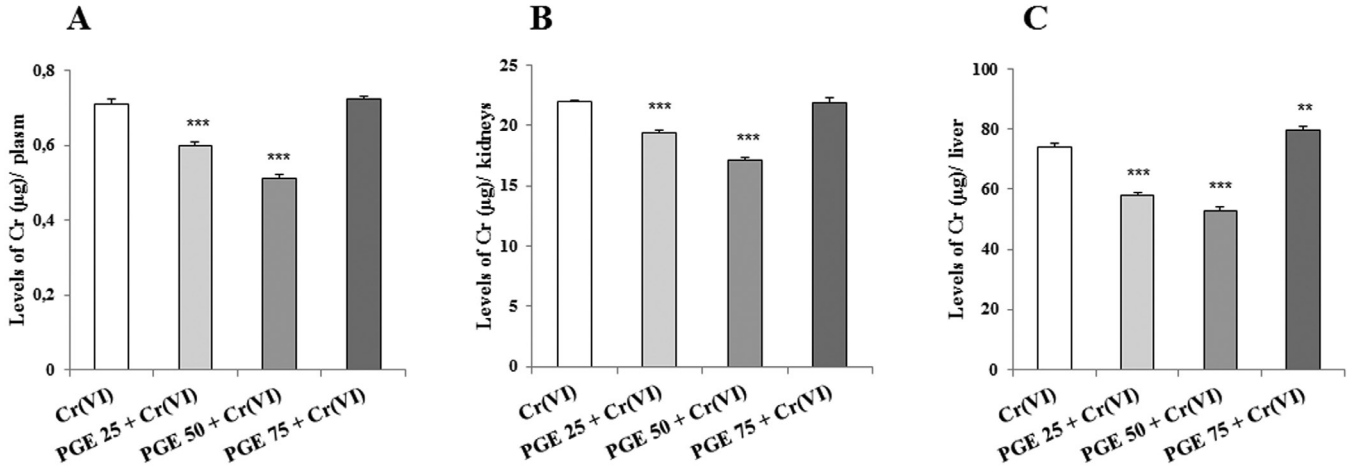


**FIGURE 2** – Chromium levels present in each plasm (A), kidneys (B) and liver (C) pool of mice pretreated with PGE (25, 50 and 75 mg/kg) and exposed to a sub-lethal dose of Cr(VI) (30 mg/kg). The chromium quantification was carried out using multiple standard additions in the prepared solutions and the results were expressed in the amount of Cr present in each organ or plasm pool. Each bar presents mean±SD of three replicate analyses for each sample. (**p<0.01 and ***p<0.001 vs. Cr(VI). One way ANOVA and Tukey's test, p<0.05).

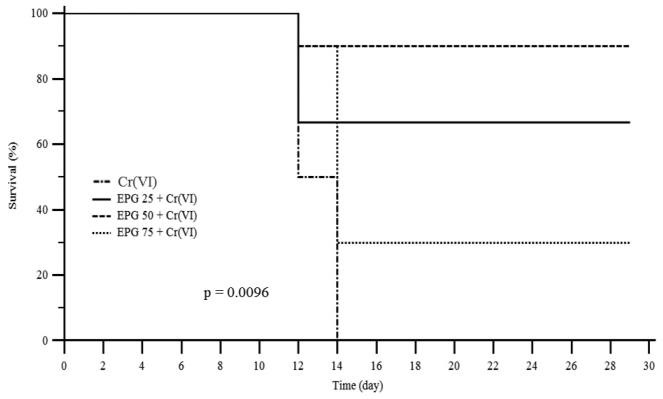


**FIGURE 3 -** Survival analysis of animals prophylactically treated with different PGE doses (25, 50 and 75 mg/kg/day) for 10 days and exposed to a lethal dose of Cr(VI) (50 mg/kg) on the 11th day. The survival rate was monitored until the 30nd day. (p = 0.0096. Kaplan-Meier test and Log-Rank test, $p<0.05$).

potential of the *Punica granatum* leaf for protecting against cyclophosphamide-induced DNA damage in mice has also been reported (Dassprakash *et al.*, 2012). Chidambara *et al.* (2002) showed the chemoprotective effect on rats pretreated with 50 mg/kg of pomegranate peel and then exposed to carbon tetrachloride ($CCl_4$). In this study, hepatic parameters such as catalase, peroxidase, superoxide dismutase and lipid peroxidation were restored to normal values and histopathological damage was prevented. Similar findings were shown in the hepatic (Pirinccioglu *et al.*, 2012) and renal (Moneim, El-Khadragy, 2012) tissue of rats pretreated with pomegranate juice and exposed to $CCl_4$. Moreover, pomegranate seeds attenuated cisplatin-induced acute nephrotoxicity and hepatotoxicity in rats probably due to their antioxidant, radical-scavenging and antiapoptotic effects (Cayır *et al.*, 2011).

These chemoprotective properties assigned to *Punica granatum* are quite interesting, since kidneys and liver are essential organs for maintaining body homeostasis and are susceptible to drug and chemical effects which may cause renal and liver dysfunction (Cayır *et al.*, 2011). Because of that, attention has been focused on the toxic action of Cr(VI), since it is highly hepatotoxic (Rafael *et al.*, 2007) and nephrotoxic (Khan *et al.*, 2010; Molina-Jijón *et al.*, 2011). Moreover, certain studies have indicated that this metal accumulates in the liver and kidneys (Sutherland *et al.*, 2000; O'Brien *et al.*, 2003; Collins *et al.*, 2010; Chang *et al.*, 2011), corroborating the results found here. However, the mice pretreated with the 25 and 50 mg/kg doses of PGE showed an interesting change in the bioavailability of chromium, since its levels in the kidney, liver and plasma were reduced. The best results were observed with the 50mg/kg/day dose. In this respect, Bishayee *et al.* (2011) found that pomegranate may act through the induction of Nrf2-regulated cytoprotective enzymes, which results in enhancing the excretion of the electrophilic carcinogen and reducing the formation of free radicals inductors of tumorigenesis. In contrast, the 75 mg/kg dose of PGE did not affect the bioavailability of this xenobiotic. Sánchez-Lamar *et al.* (2008) observed that pomegranate is toxic, i.e. genotoxic to mice at doses greater than or equal to 70 mg/kg.

Published reports have shown that the use of natural products rich in antioxidants, such as polyphenols, can reduce the toxic effects of Cr(VI) (Arreola-Mendoza *et al.*, 2006; Kalayarasan *et al.*, 2008; Pedraza-Chaverri *et al.*, 2008; Guha *et al.*, 2010; Khan *et al.*, 2010; Molina-Jijón *et al.*, 2011; Deb *et al.*, 2012). Thus, the chemoprotective effect demonstrated here may be related, at least in part, to the reduction of Cr(VI)-induced oxidative stress, since pomegranate contains polyphenols with antioxidant properties, such as punicalin and punicalagin, its main metabolites. Moreover, one cannot exclude the possibility of this fruit exerting a chelating effect, probably due to the presence of tannins, which are widely studied as chelating agents (Urquiaga, Leighton, 2000; Chin *et al.*, 2009; Karamac, 2009a). In particular, these bioactive phytoconstituents exhibit strong antioxidant properties when compared to phenolic compounds of low molecular weight(Amarowicz *et al.*, 2005). In addition, its antioxidant properties can result from free radical scavenging activities but ability to chelate metal ions also play an important role (Karamac, 2009b). It has been considered that the binding of metal ions with tannins can stabilize the oxidative damage produced by these ions (Andrade *et al.*, 2005). Moreover, pomegranate bark converted into activated carbon has been identified as an effective adsorbent for removing Cr(VI) ions from wastewater (Nemr, 2009). Okello *et al.* (2012) also showed Cr(VI) being removed from environmental samples using quercetin, a naturally occurring flavonoid, and its two synthetic derivates, quercetin pentaphosphate and quercetin sulfonic acid.

The results of this study indicate that PGE protected against the genotoxicity induced by Cr(VI). However further studies are needed to elucidate the exact mechanisms involved in the protective effects of PGE against the damage induced by chromium. Moreover studies with individual phenolic compounds of this fruit could be undertaken to elucidate the different protective mechanisms and possible associated effects.

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