

*Full Length Research Paper*

# Effect of the plant growth regulator (4-Chlorophenoxy acetic acid) into the oxidative stress parameters in rat liver

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Accepted 24 November, 2011

4-Chlorophenoxy acetic acid (4-CPA) is one of the plant growth regulators that are widely used in greenhouses to increase yield and the quality of products. Plant growth regulators do not produce significant effects on organisms; however, their remnants on plants due to either high dose or inappropriate use may lead to certain adverse effects on humans. High doses of 4-CPA have been reported to cause hepatocellular necrosis. In our study, we investigated the oxidant and anti-oxidant parameters in the liver tissues of male and female rats exposed to 4-CPA before sexual maturity. The study was implemented on 20 day-old, 80 Wistar albino rats. Forty rats received 4-CPA/day until 50 days of age. The rats were randomized into five groups (a control group, a saline group and three 4-CPA groups that received 25 to 50 to 100 mg/kg/day, respectively); and each group were further divided according to gender, making a total of 10 groups. Comparison of each gender in control and saline groups showed no difference for MDA, GSH and NOx levels. However, exposure to 4-CPA caused a dose-dependent increase in MDA and a decrease in NOx levels. These findings may be the result of 4-CPA's effects on lipid peroxidation and inflammatory reactions in liver tissue.

**Key words:** Liver, plant growth regulator, endocrine disrupters, oxidative stress, 4-Chlorophenoxy acetic acid (4-CPA).

## INTRODUCTION

The synthesis, release, transport and excretion of hormones in humans, plants and animals may be affected by endocrine disruptors. Plant growth regulators (PGRs) are widely used in greenhouses to obtain maximum yield in every season (Aksglaede et al., 2006; Kaynak and Ersoy, 1997a; Kaynak and Memiş, 1997b). These substances induce high amount of pollen production per flower and therefore increase fruit production at temperatures either below 13°C or above 33°C. PGRs do not produce significant effects on organisms as they have very short half-lives and do not accumulate tissues because they are rapidly metabolized

and eliminated (Lee, 2007). However, their remnants on plants due to either high dose or inappropriate use may lead to certain adverse effects on humans. PGRs used in agriculture; such as majority of the pesticides and herbicides that are containing certain substances like endocrine disruptors, may cause detrimental effects as they are trapped in the fatty tissue and remain long-time in the body. PGRs have been reported to cause adverse effects particularly by contamination of industrial products (Lee, 2007; Solomon and Schetter, 2000).

Humans are exposed to plant growth regulators like 4-Chlorophenoxy acetic acid (4-CPA) all over the world. 4-CPA (developed by Dow Chemical in 1950) is an auxin belonging to phenoxy family which is declared as moderately hazardous-toxic by the World Health organization (United States Environmental Protection Agency (EPA, 1997). It is commonly employed in greenhouse vegetable

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production, particularly tomato production. It accelerates conversion of flowers to fruits when applied by spraying during the blossoming period (Kaynak and Ersoy, 1997a; Kaynak and Memiş, 1997b). It is shown that 4-CPA may exert its toxicity by inhibition of some antioxidant enzyme activities in human erythrocytes (Alicigüzel et al., 2001). PGRs and 4-CPA may also have detrimental effects on reproductive organs and endocrine system.

It has been reported that prepubertal exposure to 4-CPA may have a dose dependent apoptosis in reproductive organs for both male and female rats (Yeşilkaya et al., 2009; Massart et al., 2006; McLachan et al., 2006) and may cause hepatocellular necrosis at high doses through reactive oxygen species (ROS) produced by liver and neutrophils which may be responsible for hepatocyte damage (Kaynak and Memiş, 1997b; EPA, 1997; Giusti et al., 1995). To our knowledge, this is the first study that demonstrated the correlation between 4-CPA's effect on liver and the oxidant events. This study was to investigate the alterations in oxidant and antioxidant systems of male and female rats that were exposed to varying doses of 4-CPA before puberty.

## METHODOLOGY

### Animals and study design

To determine liver effects of 4-CPA, malondialdehyde (MDA) and serum nitrite/nitrate (NOx) levels were measured for oxidant reactions indicators and glutathione (GSH) for antioxidant. Study was approved by the Ethical Committee of Gazi University involving the care and use of laboratory animals. The animals were obtained from the Experimental Research Center of Gazi University Medical School, Ankara, Turkey.

Study included 80 (40 male and 40 female) young Wistar albino rats. Rats were classified by gender 20 days after birth (the weaning period). All rats were kept in separate cages according to their group and gender with *ad libitum* standard food and tap water. Temperature of the laboratory was set to  $22 \pm 2^\circ\text{C}$ , with a relative humidity of  $50\% \pm 5$ . The animals were entrained to a 12 h light, 12 h dark cycle. 4-CPA was administered in three different doses (25 to 50 to 100 mg/kg/day). 20-days old male and female rats were divided into 5 groups according to the study design.

### Study groups of female and male rats

- Group A: Control group (n = 8); no application was done.
- Group B: Saline group (n = 8); received saline (0.5 ml/day)
- Group C: (n = 8); received 25 mg/kg/day 4-CPA dissolved in 0.5 ml saline.
- Group D: (n = 8); received 50 mg/kg/day 4-CPA dissolved in 0.5 ml saline.
- Group E: (n = 8); received 100 mg/kg/day 4-CPA dissolved in 0.5 ml saline.

All substances were given orally, once a day for 30 days. All rats were killed under intramuscular (IM) Rompun + Ketamin (50 mg/kg + 60 to 100 mg/kg) anesthesia 24 h after administration of the last dose; their livers were removed, freezeed by liquid nitrogen and stored at  $-86^\circ\text{C}$  until the analysis of study parameters. MDA level which is accepted as the indicator of lipid peroxidation in tissue was

measured by thiobarbituric acid reactive substances (TBARS) substance formation method; GSH level which indicates that the antioxidant capacity was measured by spectrophotometrically; and finally NOx (total nitrates and nitrites) was measured calorimetrically with Griess reaction.

## Biochemical determinations

### Determination of tissue MDA and GSH levels

Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Casini et al., 1986). Lipid peroxide levels are expressed in terms of MDA equivalents using an extinction coefficient of  $1.56 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$ . The GSH levels were determined by a modified Ellman method (Aykaç et al., 1986). The GSH levels were calculated using an extinction coefficient of  $13,600 \text{ mol}^{-1} \text{ cm}^{-1}$ . Measurements of MDA and GSH were carried out at room temperature using a spectrophotometer (UV 1208, Shimadzu, Japan).

### Determination of tissue NOx levels

NOx levels were measured with a Elisa reader by vanadium chloride ( $\text{VCl}_3$ )/Griess assay (Miranda et al., 2001). Prior to NOx determination, the tissues were homogenized in five volumes of phosphate buffer saline (pH = 7) and centrifuged at 2000 g for 5 min. 0.25 ml 0.3 M NaOH was added to 0.5 ml of the supernatant. After incubation for 5 min. at room temperature, 0.25 ml of 5% (w/v)  $\text{ZnSO}_4$  was added for deproteinization. This mixture was then centrifuged at 14000 rpm for 5 min and supernatants were used for the assays. Nitrate standard solution was serially diluted. After loading the plate with samples (100  $\mu\text{l}$ ), addition of vanadium III chloride ( $\text{VCl}_3$ ) (100  $\mu\text{l}$ ) to each well was rapidly followed by addition of Griess reagents, sulphanilamide (SULF) (50  $\mu\text{l}$ ) and N-(1-naphthyl) ethylenediamide dihydrochloride (NEDD) (50  $\mu\text{l}$ ). After the incubation (usually 30 to 45 min), samples were measured at 540 nm using an ELISA reader. The same procedure was applied to the plasma samples after deproteinization is carried out.

### Statistical analysis

Data are presented as means  $\pm$  SEM. Groups of data were compared with an analysis of variance (ANOVA) and by nonparametric Mann-Whitney U test using SPSS for Windows 7.0 pack. Values of  $P < 0.05$  were regarded as significant.

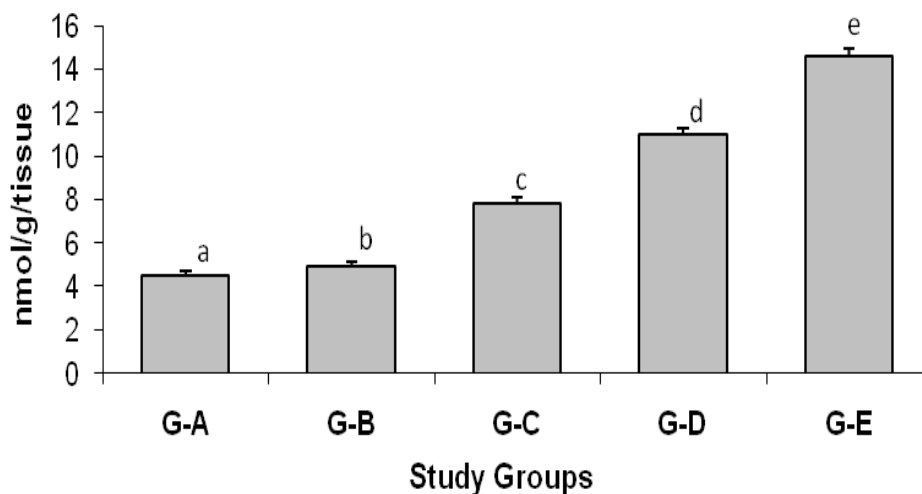
## RESULTS

### TBARS levels in tissues

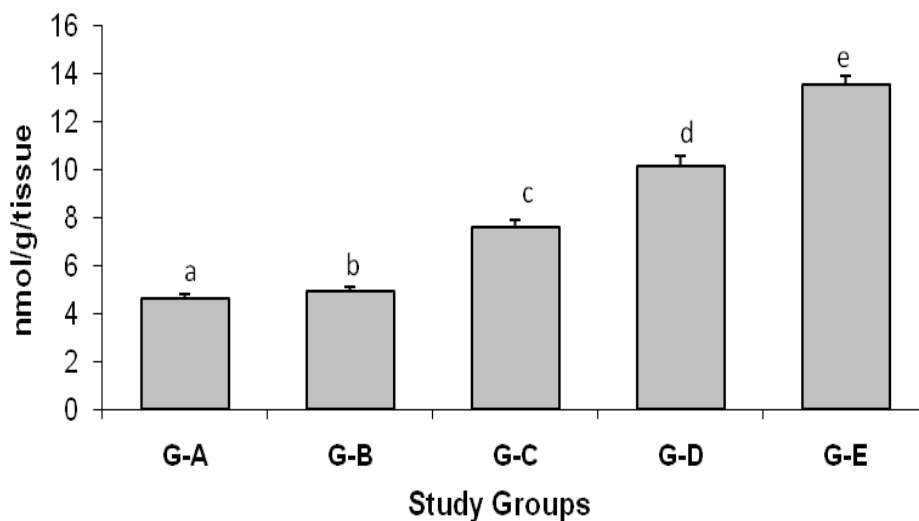
There was no statistically significant difference between female or male rats of the control group (Group A) which received no substance and of the saline group (Group B). There was dose dependent changes for tissue MDA levels between 4-CPA groups and the control group (Figures 1 and 2).

### GSH levels in tissues

There was no statistically significant difference between



**Figure 1.** Tissue TBARS levels in female rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D, G-E: Group E. The data were analyzed by performing one - way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.001$ : a-c, a-d, a-e, b-c, b-d, b-e, c-d, c-e and d-e



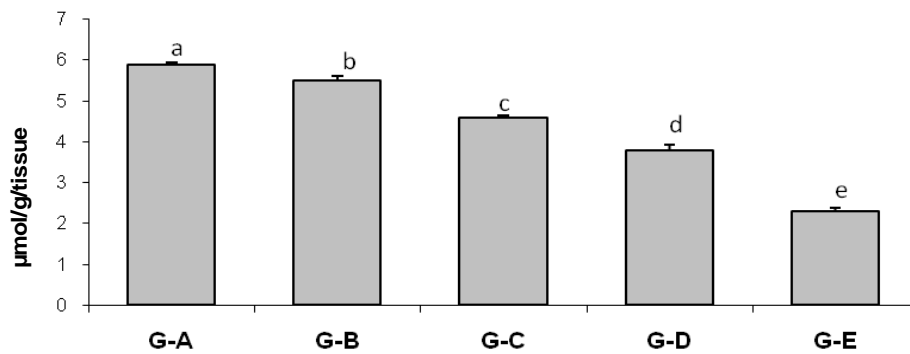
**Figure 2.** Tissue TBARS levels in male rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D, G-E: Group E. The data were analyzed by performing one-way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.01$ : c-d and d-e.  $P < 0.001$ : a-c, a-d, a-e, b-c, b-d, b-e and c-e.

either gender of the control group (Group A) that received no substance and the saline group (Group B). Comparison of 4-CPA groups with the control group revealed significant dose-dependent decreases in tissue GSH levels (Figures 3 and 4).

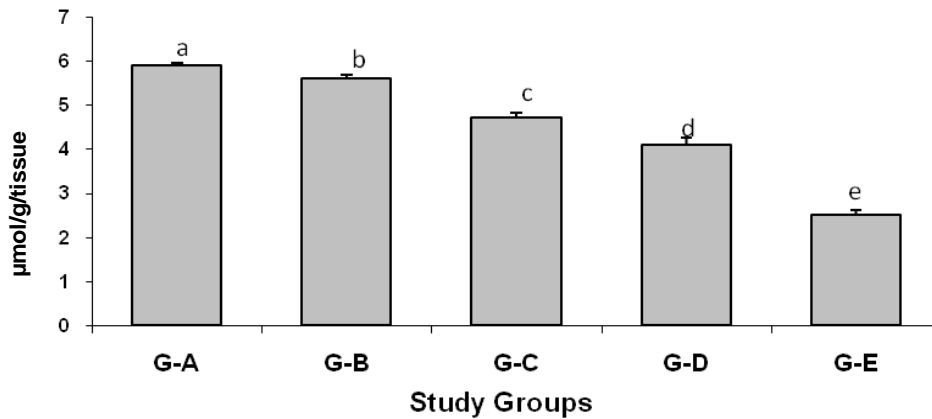
#### NOx levels in tissues

There was no significant differences between the female

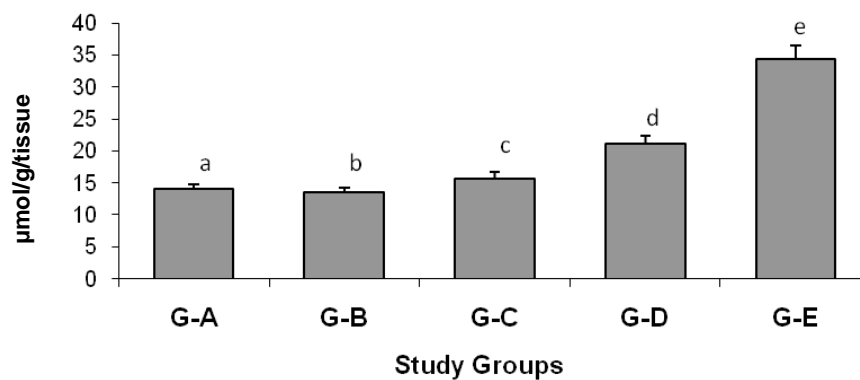
rats of the control group (Group A), saline group (Group B) and 25 mg/kg/day 4-CPA group (Group C). However, comparison of 4-CPA groups that received 50 mg/kg/day (Group D) and 100 mg/kg/day (Group E) with the control group showed a dose dependent increase in tissue NOx levels (Figure 5). Male rats of the control group (Group A) and saline group (Group B) showed no significant difference. 4-CPA groups showed a dose-dependent increase in tissue NOx levels as compared to control group (Figure 6).



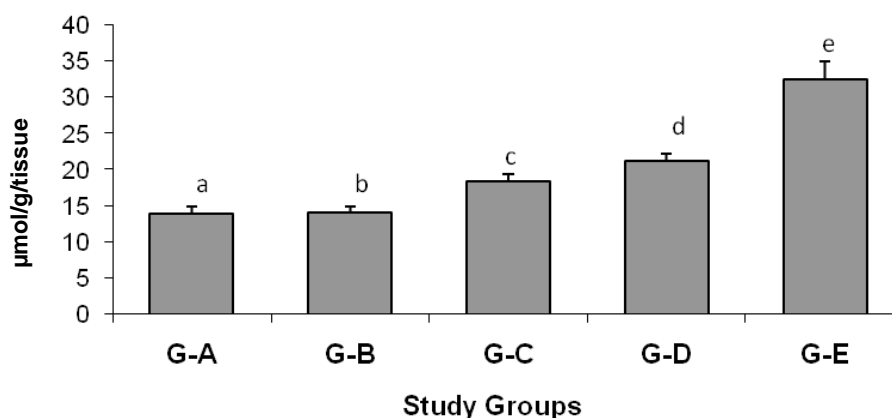
**Figure 3.** Tissue GSH levels in female rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D, G-E: Group E. The data were analyzed by performing one-way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.01$ : a-d, b-d, c-d and d-e.  $P < 0.001$ : a-c, a-e, b-c, b-e and c-d.



**Figure 4.** Tissue GSH levels in male rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D, G-E: Group E. The data were analyzed by performing one-way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.01$ : b-d, c-d and d-e.  $P < 0.001$ : a-c, a-d, a-e, b-c, b-e and c-e.



**Figure 5.** Tissue NOx levels in female rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D, G-E: Group E. The data were analyzed by performing one-way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.01$ : b-d, c-d, c-e and d-e.  $P < 0.001$ : a-d, a-e and b-e.



**Figure 6.** Tissue NOx Levels in male rats. G-A: Group A, G-B: Group B, G-C: Group C, G-D: Group D and G-E: Group E. The data were analyzed by performing one-way Mann-Whitney U tests. Values are given as arithmetic mean  $\pm$  standard error.  $P < 0.01$ : a-c, b-c and d-e.  $P < 0.001$ : a-d, a-e, b-d, b-e and c-e.

## DISCUSSION

Plant growth regulators like 4-CPA is widely used in the world. The aim of the present study was to show the alterations in oxidant and antioxidant systems in rat liver due to 4 CPA which demonstrated that exposure to 4-CPA caused a dose-dependent increase in MDA and a decrease in NOx levels.

In today's world, food demand is continuously expanding, so that endocrine disruptors are being increasingly used in agricultural practice. These conditions bring about some issues in the use of PGRs. These substances are widely used in greenhouses and are usually accepted as non-toxic if used in proper amounts and at proper time. There is no sufficient data about the effect of these substances on human health, which evidently have the potential for incongruous use at high doses with the expectation to increase the yield (Aksglaede et al., 2006; Kaynak and Ersoy, 1997a; Kaynak and Memiş, 1997b). 4-CPA was used in the present study as one of the most commonly used PGRs in greenhouses (Kaynak and Ersoy, 1997a; Kaynak and Memiş, 1997b). Previous studies in the literature that examined the toxic effects of PGRs and 4-CPA have reported certain impacts on reproductive organs and endocrine system. Particularly, prepubertal exposures to these substances have been noted to produce negative effects (Yeşilkaya et al., 2009; Massart et al., 2006; McLachan et al., 2006).

Present literature indicates that the adverse effects of this substance may arise particularly during the prepubertal period where tissue and organ development is the highest. For this reason, administration of 4-CPA was started when the rats were 20 days old and continued until the 50th day when rats reach sexual maturity. Drug administration period of 30 days corresponds to the

first 2 years in human life in our study. Alicığüzel et al. (2001) have observed that 4-CPA selectively reduced catalase and G6PDH enzymes with no influence on other antioxidant enzymes of human erythrocytes *in vitro*.

It has been reported that oxidative stress is the unwanted effect of endocrine disruptors on organism. Oxidative stress has been suggested as one of the major causes of apoptotic cell death. In normal situation, antioxidant enzymes detoxify the reactive oxygen radicals formed by the organism. However, endocrine disruptors are considered to inhibit antioxidant enzymes by several mechanisms and cause apoptotic cell death by increasing reactive oxygen radicals (Pasqualotto et al., 1984; Wang et al., 2004; Bhat et al., 2006; Giwercman et al., 2007; Sirotkin and Grossmann, 2007; Yeşilkaya et al., 2009). A study on an organochlorine pesticide, methoxychlor, has shown that it has increased reactive oxygen radicals through oxidative stress which lead to growth retardation of antral follicles in the ovary and ovarian atresia (Markiewicz et al., 2003).

In another study, no change was determined in the biochemical hormone levels of female rats, but dose dependent cell death in the ovarian tissue, epithelium and stroma of the uterus was observed that suggests the cause of apoptosis is oxidative stress (Yeşilkaya et al., 2009).

In our study, liver was examined as it is a major target organ for elimination of toxic substances. Previous studies that used phenoxy acid derivatives have shown that cytochrome P-450 levels in the liver were increased and certain metabolic enzymes were also affected (Bağcı et al., 2005). In addition, toxicity studies on rats have reported reduced appetite, weight loss and findings of hepatocellular necrosis (EPA, 1997). On the other side, one study that examined the cytogenetics of human lymphocytes reported that 4-CPA had no genotoxic effect

(Noritaka et al., 1991).

It has been shown that 4-CPA lead liver necrosis; therefore, we aimed to demonstrate whether this effect is through oxidative stress in liver.

Indole acetic acid (IAA), another member of auxin group like 4-CPA, is synthesized by plants, microorganisms and mammals (Qureshi and Baig, 1993). Oliveria et al. (2007) determined that this auxin reduces lipid peroxidation. However, *in vitro* IAA was converted to reactive oxygen species in the presence of peroxidases and increased lipid peroxidation (Folkes and Wardman, 2001). Lins et al. (2006) have found that IAA increased liver and plasma levels of TBARS that supported this finding. Additionally, Kim et al. (2006) have shown that IAA has increased apoptosis by peroxidation in human melanoma cells. Our results showed that 4-CPA, which is a member of auxin group, exerted similar effects on liver tissues of both male and female rats and resulted in a dose-dependent increase in MDA levels and subsequent reduction in GSH levels. These changes in MDA and GSH levels proved that PGRs increase lipid peroxidation.

NOx contribution to oxidant damage is variable. NOx may react with and eliminate the superoxide anion from the medium or act as an active oxidant by peroxynitrite formation.

As it is well known, NOx level increases by stimulation of nitric oxide synthetase (NOS) when an inflammatory response commences. During normal physiologic conditions, eNOS dependent NO production provides paracrine control of hepatic satellite cells, however, variable NO levels during pathologic conditions may lead to inflammation and uncontrolled apoptosis (Langer et al. 2006; Smith and Smith, 2006). Increased liver NOx levels in our study may be possibly related with inflammation induced by 4-CPA.

Wong et al. (2001) have demonstrated that glutathione S transferase in liver cells may help detoxification of NO-mediated reactive nitrogen species, such as peroxynitrite, myeloperoxidase, H<sub>2</sub>O<sub>2</sub> and nitrite. Same study has also demonstrated that increased amount of NO-mediated reactive nitrogen species inhibited the synthesis of glutathione-S-transferase (GST) (Wong et al., 2001).

In conclusion, our results suggest that prepubertal exposure to plant growth regulators like 4-chlorophenoxy acetic acid which is widely used in agriculture, increases lipid peroxidation independent of gender and possibly cause inflammatory reactions in liver tissues. Finally, the maximum daily doses, the adverse effect and maximum amount of remnants allowed for these substances should be determined and controlled.

## ACKNOWLEDGEMENTS

The Institutional Animal Care and Use Committee of Gazi University approved all the animal procedures and the Project was supported by the Scientific Research Fund of

Gazi University (Project No: GÜ-ET-07-002).

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