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Effects of Vitamin E and C on Placental Oxidative Stress: An *In Vitro* Evidence for the Potential Therapeutic or Prophylactic Treatment of Preeclampsia

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Abstract: Preeclampsia (PE) is a multisystem disorder that remains a major cause of maternal and foetal morbidity and death. To date, no treatment has been found that prevents the development of the disease. Endothelial dysfunction is considered to underlie its clinical manifestations, such as maternal hypertension, proteinuria and edema; and oxidative stress has been increasingly postulated as a major contributor to endothelial dysfunction in PE.

A large body of research has investigated the potential role of antioxidant nutrients in the prevention of PE in women at high increased risk of the disease. Therefore, the present study was primarily designed to assess the potential benefit of antioxidant supplementation on markers of placental oxidative stress in an *in vitro* model of PE, since we previously found that endothelin-1 (ET-1) is able to trigger the placental secretion of stress molecules. In this regard, we evaluated the effects of vitamin C, vitamin E and N-acetylcysteine (NAC), alone or in combination, in placental villi culture after exposure to ET-1. The effect of antioxidant nutrients on trophoblast cells proliferation and vitality was also evaluated.

The results obtained suggest that in a pathophysiological condition, such as PE, the deleterious effect of reactive oxygen species may be counteract by an antioxidant therapy, and there is the need to investigate the optimum dosing and timing of antioxidants administration, since an inappropriate antioxidant treatment in pregnant women may have deleterious consequences, reducing placental cells proliferation until to cell death.

INTRODUCTION

Preeclampsia (PE) is a human pregnancy-specific disorder that adversely affects maternal vascular function and fetal intrauterine growth, and the leading cause of maternal and perinatal mortality in developed countries [1]. A potential role for oxidative stress in the pathophysiology of PE emerged in the last years [2-4]. Synthesis of free radicals by a dysfunctional placenta and endothelium seems to play a central role in this association, since they, through the oxidative damage of biomolecules and lipid peroxidation, contribute to the systemic endothelial cell dysfunction and activation accounting for the clinical expression of the disease [5, 6].

On this basis, antioxidants such as vitamin C and vitamin E have been proposed as potential therapeutic or prophylactic treatment for PE [6]. Accordingly, Chappell *et al*. [7] in a randomised trial of 283 women at high risk for PE, showed a reduction in the incidence of the disease in women treated with vitamin C and E from the second trimester of pregnancy [7]. This effect was accompained by a diminution of the indices of oxidative stress towards values that were observed in a group of healthy women [8].

Vitamin C and E are two essential nutrients that can scavange free radicals and constitute a strong line of defence in retarding reactive oxygen species (ROS)-induced cellular damage. Indeed, vitamin E, mainly α-tocopherol, is the major peroxyl radical scavenger in biological lipid phases, such as membranes. Its antioxidant action has been ascribed to its ability to chemically act as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation [9]. With respect to vitamin C, it has been shown to scavange free radicals directly in the aqueous phases of cells and circulatory system, and also to protect membranes and other hydrophobic compartments from damage by rigenerating the antioxidant form of vitamin E [10]. A synergistic effect between the two vitamins *in vitro* and *in vivo* has been also demonstrated [9]. Indeed, the proposed mechanism of action was that when vitamin E intercepts a radical thus forming a complex α-tocopheroxyl-radical, wich can be reduced back to α-tocopherol by vitamin C or other reducing agents, thus attenuating the propagation of free radical reactions [11,12]. Thus vitamin C prevents the prooxidant activity of vitamin E by decreasing the activity of tocopheroxyl radical to α-tocopherol, thereby contributing to increased total antioxidant status and reducing oxidative stress [13].

With respect to oxidative stress and PE, in a previous study we found that endothelin-1 (ET-1), a potent endogenous vasoconstrictor peptide that is product in higher amount by endothelial and trophoblast cells during PE, affected the balance between prooxidant and antioxidant system in placental explants, and showed that ET-1 had a great relevance in triggering the secretion of stress molecules [14].

Therefore, the present study was primarily designed to assess the potential benefit of antioxidant supplementation on markers of placental oxidative stress [malondialdehyde (MDA), and glutathione (GSH)] induced by ET-1, as well as its effect on trophoblast cells proliferation and vitality. In this regard, we evaluated the effects of vitamin C and E

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alone or in combination, but also those of N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, that is known to stimulate GSH synthesis, to promote detoxification and to act directly as free radical scavenger.

MATERIALS AND METHODS

Placental Explants Culture

Placentae were obtained under sterile conditions from normal pregnancies undergoing elective caesarean section at term. Maternal consent was obtained according to the guidelines of Ethics Committee. Chunks of placental cotyledons weighing approximately 30 g were thoroughly rinsed in calcium- and magnesium-free Hanks' solution (HBSS), villous tissue was identified and isolated from membranes, large vessels, decidua and connective tissue under a dissection microscope. Small clusters of placental villi (50 mg/wet weight) were placed in a 24-well plastic plates and cultured in Ham's F10 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2-95% air. Culture medium was enriched with vitamin C or vitamin E or NAC, alone or in combination, at concentration of 5 pM. Culture medium was removed after two days of culture and the explants were treated with different concentrations of ET-1 (0-100 pg/ml), dissolved in fresh Ham's F10 medium, enriched with antioxidants, for 6 h. Indeed, we have shown previously that placental villi exposed to different ET-1 concentrations displayed a significant dose-dependent decrease of GSH and a significant dose dependent increase of MDA after 6 h of treatment [14]. Therefore, to investigate whether vitamin C, vitamin E and NAC blocks the oxidative stress condition triggered by ET-1, we measured their influence on GSH and MDA content in placental villi after 6 h of ET-1 treatment.

Experiments were performed at least six times, using a single placenta for each one.

Measurement of GSH and LPOs

For GSH measurement, placental tissues were homogenized using Mixer Mill MM 300 (QIAGEN, Milan, Italy) in EDTA-K^+ phosphate buffer (pH 7.4) and 10% (w/v) metaphosphoric acid (1:1) at 4°C, centrifuged at 2000 g for 10 min and the supernatants were stored at –80°C until the assay. GSH was measured by using a colorimetric assay kit (Cayman-Cayman Chemical Company, USA).

LPOs content was measured by the stable metabolite MDA with a HPLC assay as previously described [21]. Placental explants were homogenized in mixture (1:1) of 0,04 mol/L TRIS-HCl buffer (pH 7.4) and a solution of acetonitrile containing 0,1% butyl hydroxytoluene (4°C), to prevent the artificial oxidation of polyunsaturated free fatty acid during the assay, and centrifuged at 3000 g for 15 min at 4°C. Supernatants were stored at –80°C until the assay.

JEG-3 Choriocarcinoma Cells Culture

JEG-3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD; passage number 125), and were used within seven passages. JEG-3 cells were maintained routinely in RPMI medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2-95% air.

Effect of Antioxidants on JEG-3 Cells Proliferation After ET-1 Treatment

JEG-3 cells were plated at a density of 5 x 10^3 cells/well in 96-well plastic plates and were allowed to adhere to the wells overnight, then the cells were treated with different concentrations of the ET-1 (0-100 pg/ml) in RPMI medium enriched with vitamin C, vitamin E and NAC alone or in combination. After 24 h, JEG-3 vitality and proliferation rate were determined with (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromides (MTT) assay. MTT stock solution (5 mg/ml) was added to each well being assayed to equal one tenth the original culture volume and incubated for 4 h. At the end of the incubation period converted dye was solubilized with acidic isopropanol (0,04-0,1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Antiproliferative Activity of Vitamin C and E and NAC on JEG-3 Cells

JEG-3 cells were plated at a density of 5 x 10^3 cells/well in 96-well plastic plates and were allowed to adhere to the wells overnight, then the cells were treated with different concentrations ($5x10^{-12}$ – $5x10^{-8}$ M) of vitamin C, vitamin E and NAC for 24h. JEG-3 proliferation were determined by MTT assay, like described above.

STATISTICAL ANALYSIS

Explants data were expressed as nmol/mg prot for GSH, and as nmol/gr tissue for MDA. JEG-3 data were expressed as per cent variations respect to control (each point represents six experiments, performed in triplicate) to better evidence the differences between the various treatments with ET-1.

The ANOVA for repeated measures test followed by Bonferroni post-hoc test was applied for statistical comparison. Differences were considered significant at a value of $P < 0.05$.

RESULTS

GSH levels in explants exposed to ET-1 (20-100 pg/ml) in presence of vitamin C 5 pM did not differ from those in control explants (Fig. **1A**). Same results we obtained in presence of NAC 5 pM, except for ET-1 100 pg/ml where it can possible to note a significant ($P < 0.05$) increase of GSH levels respect to control (Fig. **1A**). However, when placental villi were treated with ET-1 in presence of vitamin E 5 pM, we observed a significant ($P < 0.0001$) increase of GSH content that showed a dose-dependence respect to ET-1 concentration (Fig. **1A**). We also tested the effect of a combination of vitamin C and E, and NAC and vitamin E, and we found no change in GSH levels in the first case, but a significant ($P < 0.001$) increase of GSH content depending on ET-1 concentration in the second case (Fig. **1B**).

With respect to LPOs, the stable metabolite MDA was measured by using HPLC in placental villi exposed for 6 h to ET-1 (20-100 pg/ml) in presence of vitamin C, vitamin E and NAC, alone or in combination. When placental explants were treated with ET-1, HPLC chromatogram displayed a clear pick of MDA (Fig. **2A**), that were no more present in the chroma-

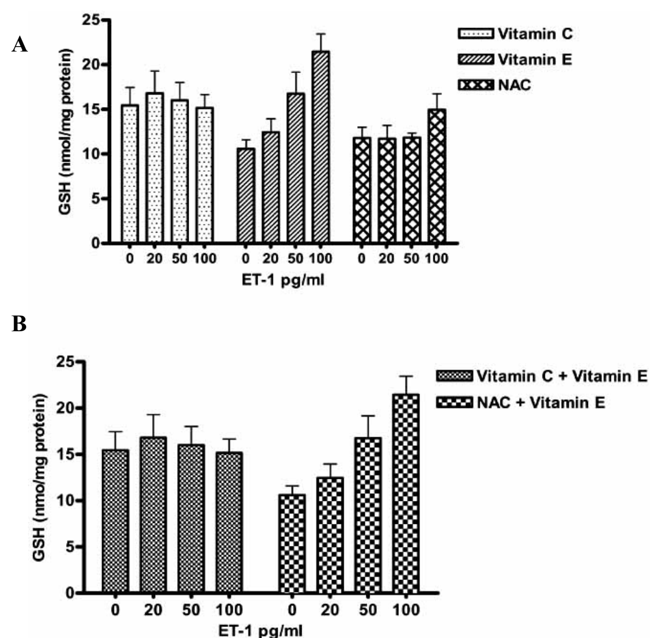

Fig. (1). Influence of vitamin C, vitamin E and NAC, alone (Fig. **1A**) or in combination (Fig. **1B**), on GSH levels in placental villi exposed for 6 h to different concentration of ET-1.

tograms of the placental villi treated with ET-1 in presence of the antioxidants alone or in combination. (Fig. **2B**).

Previously we also showed that ET-1 after 24 h of incubation [14] significantly decreased the vitality and proliferation of the JEG-3 cells, used as an *in vitro* model of the trophoblast cells. Therefore, we moved to evaluate the influence of the vitamin C, vitamin E and NAC on cell vitality and proliferation after 24 h of ET-1 treatment.

ET-1 significantly ($P<0{\cdot}001$) and dose-dependently decreased the proliferation rate of the JEG-3 cells (Fig. **3**). On the contrary, vitamin C not only blocked the inhibitory effect of ET-1, as also occurs when cells were incubated in presence of increasing amounts of NAC, but also stimulated JEG-3 cells proliferation. Use of the vitamin E alone displayed a moderate effect respect to antiproliferative action induced by ET-1 (Fig. **3A**).

The combination of two antioxidants (vitamin C plus vitamin E or NAC plus vitamin E) were used in combination also blocked the inhibitory effect of ET-1 on cells proliferation (Fig. **3B**).

We also tested the effect of the increasing concentrations ($5x10^{-12}$ – $5x10^{-8}$ M) of vitamin C, vitamin E and NAC on JEG-3 cells proliferation. Vitamin C showed a marked antiproliferative effect, achieving an inhibition of cell growth until to 70% at higher doses. The effect of the NAC and the vitamin E was more moderate respect to the vitamin C and the maximum inhibition was of the 25% (Fig. **4**).

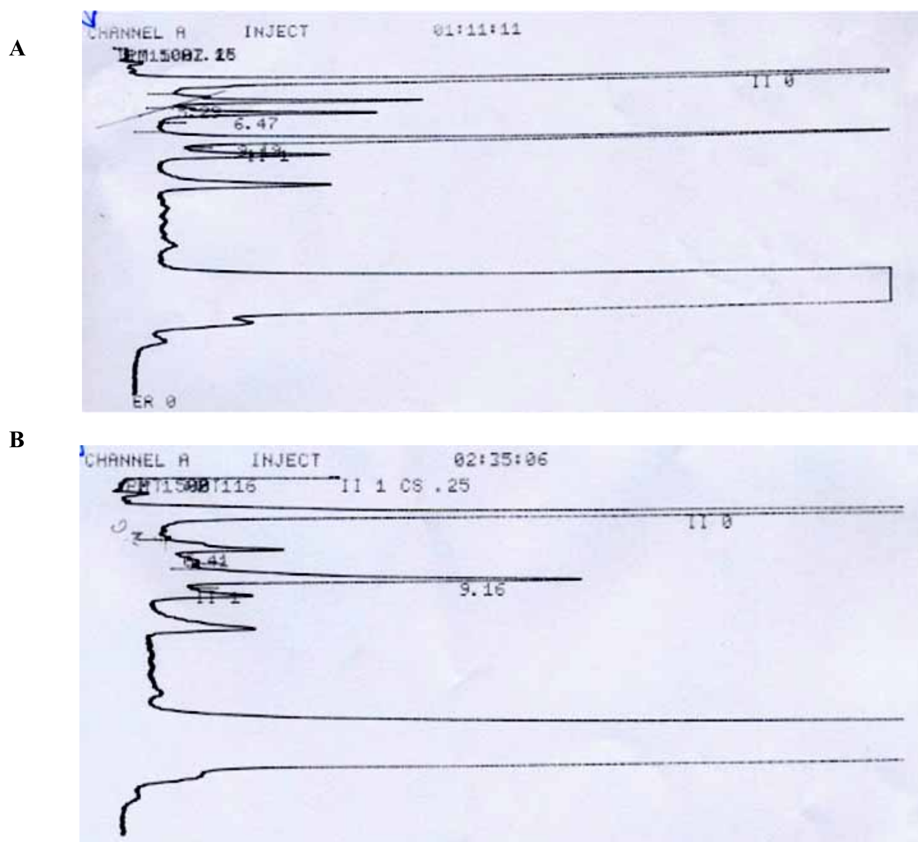

Fig. (2). HPLC chromatograms of MDA obtained in placental explants exposed to ET-1. Arrow indicates the pick of MDA in placental explants exposed to ET-1 (Fig. **2A**) and in placental explants exposed to ET-1 in presence of antioxidants, where no relevant pick is observed (Fig. **2B**).

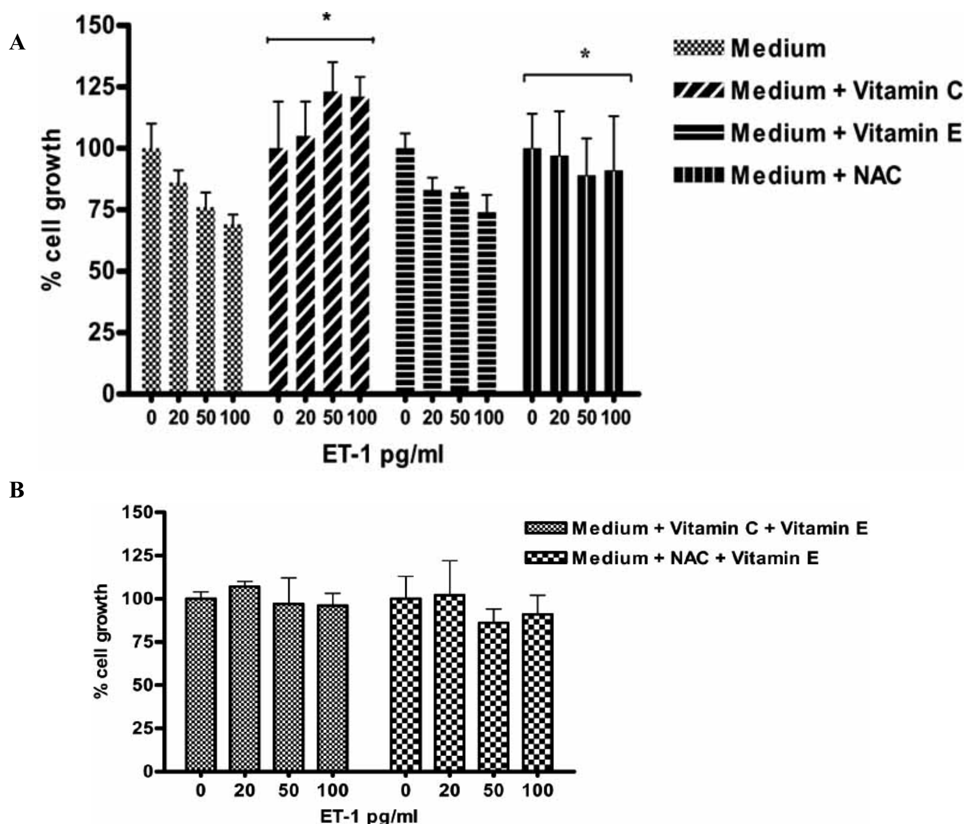

Fig. (3). Proliferation in JEG-3 cells. Placental cells were exposed to ET-1 in absence of antioxidant nutrient and in presence of vitamin C, vitamin E and NAC, alone (Fig. **3A**) or in combination (Fig. **3B**). Cell growh was estimated after 24 h by MTT method. Results are expressed as mean ± SD of six experiments, each one performed in triplicate. * P< 0,05.

DISCUSSION

The present study was designed to value the hypothesis that well known antioxidants, such as vitamin C, vitamin E and NAC, may lead to an improvement in the oxidative stress, since endothelial and placental dysfunction in PE results from oxidative stress [5,6]. As consequence, antioxidants have been proposed as a potentially advantageous prophylactic measure for PE [2-4], since treatment with vitamin E and C have been reported to reduce the incidence of PE in women and to decrease the biochemical indices of oxidative stress [8]. Moreover, in patients with severe PE plasma α-tocopherol was significantly decreased compare with controls, which is thought to be caused by the fact that antioxidants may be utilized to a greater extent to counteract free radical-mediated cell disturbances, resulting in a reduction in their plasma levels [11,12].

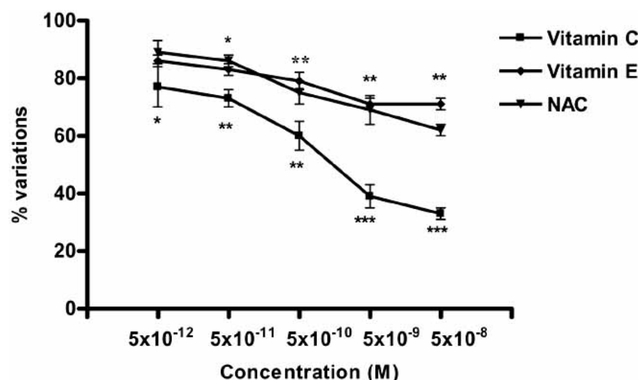

Fig. (4). Antiproliferative effect in JEG-3 cells exposed to increasing concentrations of vitamin C, vitamin E and NAC. Cell proliferation was estimated after 24 h by MTT method. Results are expressed as mean ± SD of six experiments, each one performed in triplicate. * P < 0,05; ** P < 0,01; *** P < 0,001.

In the present study we used an *in vitro* model to assess the effect vitamin C, vitamin E and NAC on oxidative stress, since we previously found that ET-1 is able to trigger the placental secretion of stress molecules [14]. Briefly, we found that supplementation with vitamin C, vitamin E and NAC, alone or in combination, prevented the ET-1-induced decrease of GSH, an antioxidant molecule that protects cells from reactive oxygen species [15], as well as the ET-1-induced secretion of MDA, an oxidative stress molecole that plays a central role in oxidative damage, since it has toxic effect on cells by disorganizing oxidative phosphorilation in mitochondria and by reacting with proteins and DNA bars [16-20].

Vitamin C is a potent scavenger of superoxide radicals and may thus have helped to maintain intracellular GSH concentration, in fact no changes were observed in total content of tripeptide GSH in placental villi exposed to ET-1 in

presence of vitamin C. On the contrary, we observed an increasing of GSH content when placental villi were exposed to ET-1 in presence of vitamin E and NAC. We can explain this as an adaptive response to elevated levels of oxidizing species that elicit a specific compensatory increase in antioxidant molecule activities. Vitamin E, in fact, is the major peroxyl radical scavenger in biological lipid phases such as membranes. Its antioxidant action has been ascribed to its ability to chemically act as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation and protecting the organism against oxidative damage. Indeed, the interaction between vitamins C and E in the antioxidant defense of biochemical systems is well established because vitamin C can reduce tocopheroxyl radicals directly or indirectly and thus support the antioxidant activity of vitamin E. Upon oxidation these micronutrients need to be regenerated in the biological setting, hence the need for further coupling to nonradical reducing systems such as GSH. Because the metabolism of the reactive species should be regulated selectively at or near the sites of their generation, increases in the expression of these reducing systems in preeclamptic placentas would act as a protective mechanism by which placental cells minimize tissutal oxidative damage. Same conclusions we can hypothesize for NAC, used both as a precursor in the synthesis of GSH and as a direct radical scavenger.

Indeed, our results shows an inhibition of the MDA production, one of the most markers of secondary end products of lipid peroxidation, supporting the evidence of the protective effect of vitamin supplementation in placental villi exposed to ET-1 since the process of lipid peroxidation of membranes has been implicated as one of the primary events in oxidative cellular damage and has been shown to be associated with fine structure disturbance and subsequent function loss of biological membranes.

The second important result of this study is the opposite effect of the antioxidant substances on basic cell physiological process. Indeed, vitamin C, vitamin E and NAC improved cell survival when JEG-3 cells were exposed to ET-1, but increasing concentrations of these nutrients were able to induce cell death and to block cell proliferation when JEG-3 were maintained in culture without ET-1.

The first approach to this study has been to verify the antiradicalic activity of the vitamins and NAC by DPPH test. We found IC_{50} values to concentrations higher than 1 μM; but when we exposed JEG-3 cells to vitamins and NAC at this concentration we observed an antiproliferative effect respect to control cells. Therefore, we have exposed JEG-3 cells to increasing concentrations of vitamins and NAC and we observed a progressive change of cell morphology, a detachment of the cells from the bottom of the culture plate and a consequent cell death (data not shown).

These results suggest at least two considerations. First of all, in a pathophysiological condition such as PE where oxidative stress is an important component of the disease, the deleterious effect of reactive oxygen species may be counteract by an antioxidant therapy. On the other hand, there is the need to investigate the optimum dosing and timing of antioxidants administration, since an inappropriate antioxidant treatment in pregnant women may have deleterious consequences

In conclusion, vitamin C, vitamin E and NAC, alone or in combination, protect the human placenta against the deleterious effects of reactive oxygen species, and further support the linkage between ET-1 and reactive oxygen species, and a likely explanation of their effect is through this mechanism. These data would support the hypothesis that ET-1 would induce an initial free radicals overgeneration, promptly scavenged by supplementation with vitamin C and E, therefore reinforcing placental resistance to oxidative injury.

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