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RESEARCH ARTICLE

Immunohistochemical expression of activated caspase-3 in the parotid salivary glands of rats after long administration of Myristica fragrans

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Abstract

Twenty male Sprague-Dawley rats (200 ± 15 g) were used in the present work, they were divided into two main groups, the study group comprises fourteen animals and the control group comprises six animals. The control group animals were received 1ml of distilled water orallyon a daily basis for the duration of the experiment (8 weeks). The study groupweregiven 1ml of the prepared (Myristicafragrans)nutmeg aqueous extract orally on a daily basis in the following doses (100 and 500 mg/kg b.w.) for 8 weeks. The study group was subdivided into 2 subgroups (7 animals each) according to the dose which are 100 mg/kg b.w. group (Sub gp A) and 500 mg/kg b.w. group (Sub gp B) respectively.The parotid salivary glands were dissected from each rat and prepared for paraffin section.Sections for histological studiesstained with H&E and Masson's trichrome stain, other sections were prepared for immunohistochemical study of activated caspase-3 expression. Histological results in Sub gp A showed intracellular cytoplasmic vacuoles of acinar cells, dilated ducts,lymphocytic infiltration, congested blood vessels and nuclear changes including hyperchromatism as well as pyknotic nuclei were observed. In addition, interacinar oedema was evident.In Sub gp B these changes were markedly severe. Immunohistochemical results revealed thatmost of the acinar and ductal epithelial cells of controlled group showed negative immunoexpression to caspase-3. Most of the nuclei and cytoplasm ofacinar and ductal cells of Sub gp A group revealed moderate positiveimmunoexpression to caspase-3.The reaction was more intense in Sub gpB .

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Introduction

The nutmeg tree (Myristica fragrans Houtt.) is a large, leafy evergreen plant originating from Moluccas (the Spice Islands) and now cultivated in the West Indies. It produces two spices – mace and nutmeg. Nutmeg is the seed kernel inside the fruit and mace is the lacy covering (aril) on the kernel **(Jukie, et al. 2006)**.Nutmeg, the seeds of Myristicafragrans(family Myristicaceae), is a well-known kitchen spice with a long-standing reputation as a psychoactive herb. Nutmeg at high doses is considered a cheap substitute to several drugs of abuse**(El-Alfy, et al. 2009)**.Nutmeg is one of the plants commonly found in Asian medical ingredients. It contains many bioactive compounds including camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, safrole, myristic acid, myristicin, elimicin and lignin compounds. M. fragrans extract has been shown to contain antibacterial activity against different genera of bacteria and antiviral activity against rotavirus **(Chirathaworn, et al. 2007)**.Nutmeg has aromatic, stimulant, narcotic, carminative, astringent, aphrodisiac, hypolipidemic, antithrombotic, anti-platelet aggregation, antifungal, antidysenteric, anti-inflammatory activities. It is used as a remedy for stomach ache, rheumatism and vomiting in pregnancy. The presence of two compounds, myristicin and

elemicin, is often related to intoxication and to the hallucinogenic action of nutmeg while safrole has been suspected to be carcinogenic. However, the mechanism by which these compounds excrete their activity is still a subject of extensive research. As a part of the growing consciousness of dietary habits, herbs and spices are becoming an important source of natural antioxidants. Since very little is known about the antioxidant activity of glycosidically bound volatile compounds, researches in this field are welcome **(Jukie, et al. 2006)**.Oil of nutmeg is useful in the treatment of inflammation of the bladder and urinary tract, halitosis, dyspepsia, flatulence, impotence, insomnia and skin diseases. It is also used externally as a stimulant and ointment as a counter-irritant. Most of the pharmacological properties of nutmeg are attributed to the compounds present in the essential oil. Mace oil possesses almost identical physiological and organoleptic properties to nutmeg oil. Nutmeg butter is a mild external stimulant used in the form of ointments, hair lotions and plaster, against rheumatism, paralysis and sprains **(Leela 2008)**.The effect of the aqueous extract of nutmeg seeds on the liver of mice was studied by **(Al-Hazmi, et al. 2004)**, they observed that Liver sections from nutmeg treated mice showed cellular changes in the form of hydropic degeneration (swelling and hypopigmentation), fatty degeneration, cellular vacuoles and some nuclear changes. The nuclear changes were in the form of redistribution of chromatin granules and its adherence to the nuclear membrane. These are considered as pre-necrotic changes. These findings were evident in all the nutmeg-treated mice regardless of the level of nutmeg.

The term apoptosis is given to a morphologically distinct mode of cell death. In terms of tissue kinetics,apoptosis might be considered a mechanism that counterbalance the effect of cell proliferation by mitotic division.On the other hand, excessive apoptosis might cause organ atrophy and failure **(Majno and Joris.,1995)**. Caspase-3 had been found to be necessary for normal brain development as well as its typical role in apoptosis ,where it is responsible for chromatin condensation and DNA fragmentation **(Peter andJanicke,1999)**.Caspase-3 is one of the key executioners of apoptosis**(Cohen,1997)**.Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspase 3, in particular, has many cellular targets and when it is activated, produces morphologic features of apoptosis **(Jang et al., 2002)**. To date, 14 caspases have been implicated in the apoptotic pathway cascade. Among these, caspase 3 is considered to be a major execution protease **(Nicholson, 1999)**.

The cleavage of caspase 3 from its pro-form to its active form has been shown to be critical for its role in apoptosis. Once activated it cleaves cytoplasmic structural proteins such as actin and cytokeratins or nuclear proteins such as poly (ADP- ribose) polymerase (PARP) and lamins. The active caspase 3 induced activation of caspase-activated deoxyribonuclease (CAD), also called DNA fragmentation factor-40 (DFF), that is integrally involved in degrading DNA and apoptosis in different cells **(Nicholson, 1999; Kim et al., 2001)**.

Very few studies were done on oral tissues but some researches were done to study the various effects of nutmeg on different organs such as the kidney, liver, spleen, heart, testes, brain, etc. Despite these studies over the last few decades, the pathogenesis of nutmeg on the oral tissues is still unclear. There has been considerable controversy regarding the pathogenesis of nutmeg with conicting evidences as to whether it represents true pathology. Accordingly, The aim of the present work is to examine the changes occurred in the parotid gland by nutmeg, compared with that of the control tissues, to determine its pathogenesis using:

- Histological study using H&E and Masson's trichrome stains.
- Immunohistochemical study of activated caspase-3 expression.

2-Material and methods

Animals:

Twenty male Sprague-Dawley rats (200 ± 15 g) were used in the present study. The animals were housed in cages at the Faculty of Dentistry, Damanhour University, under the optimal experimental conditions.

They were divided into two main groups:

- The Control group: comprised six animals. These animals received 1ml of distilled water orally (using an oropharyngeal tube) on a daily basis for the duration of the experiment (8 weeks).

- The study group were given 1ml of the prepared (Myristicafragrans) nutmeg aqueous extract orally (using an oropharyngeal tube) on a daily basis in the following doses (100 and 500 mg/kg b.w.) for 8 weeks. The study group was subdivided into 2 subgroups (7 animals each) according to the dose which are 100 mg/kg b.w. group (Sub gp A) and 500 mg/kg b.w. group (Sub gp B) respectively.

Preparation of the nutmeg seed's extract

Nutmeg seeds were obtained from local markets. The dry seeds were washed thoroughly to remove dust, fungal spores and/or other undesired particles, then left to dry at room temperature overnight. The seeds were then macerated into a fine flour-like paste using a mortar and pestle to pass through 0.2 mm mesh. Aliquot weight of nutmeg powder (125g) was soaked in 500 ml hotdistilled water and left to stand for 72 hrs.Then filtered the extract.The extract was kept frozen until used.**(Olaleyeet al.2006)**.

Histological procedures: Drury &Wallington (1980)

At the end of the study, the animals were humanely sacrificed under anaesthesia.Then the parotid salivary glands were dissected out, and fixed immediately in 10% neutral buffered formalin solution. Then the specimens were washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Sections of 4-5 µ thick were obtained and mounted on clean glass slides and stained with: Haematoxylin and Eosin (H&E) to verify histological details and Masson' s trichrome stain for demonstration of collagen fibers.

Immunohistochemical examination: Ramos-Vara (2005)

Immunohistochemistry for detection of caspase-3 was performed on paraffin sections mounted on coated glass slides. Antigen was retrieved in citrate buffer (PH 6.0) microwave digestion (2 cycles of 12 minute each). Endogenous peroxidase was blocked with 0.05% hydrogen peroxide for 30 min. After incubation with a 1:20 dilution of normal horse serum, the slides were incubated overnight at 4˚C with primary antibodies (Rabbit anti-active caspase-3,Millipore,AB3623). Secondary antibodies associatedwith a streptavidin-biotin-peroxidase method were applied. Diaminobenzidine was used as chromogen. All sections were counter-stained with haematoxylin. The sections were washed with phosphate buffered saline after each step. Negative controls were used using non-immune serum instead of the primary or secondary antibodies.

3-Results:

Examination of H&E stained control group sections of the parotid salivary glands revealed that the gland consisted of secretory acini and ducts. These serous acini appeared round and had a narrow lumen. The acini were lined by pyramidal cells with apical acidophilic cytoplasm. Their nuclei were prominent, deeply stained and spherical in shape and basally situated. The duct system presented intercalated, striated and excretory ducts. The intercalated ducts were compressed between the acini. The striated ducts were lined by a single layer of columnar cells which showed well-defined outlines and central, rounded, darkly stained nuclei**(Fig.1)**.H&E stained Sub gpA sectionsshowed vacuoles, dilated ducts, congested blood vessels and nuclear changes including hyperchromatism well as pyknotic nuclei were observed. In addition, interacinar oedema was evident**(Fig.2)**.H&E stained Sub gp B sections showedmarked increase in vacuoles, appearance of wide inter-acinar spaces, dilatation in ducts, congested blood vessels and the connective tissue septa surrounding the lobes and lobules showed increase in the thickness and was infiltrated by inflammatory cells. The excretory ducts were dilated. The intralobular& interlobular blood vessels showed wide dilatation and were engorged with blood more nuclear changes due to severe effect ofnutmegon the parotid gland tissue**(Fig.3)**.

Masson's trichrome stain results of the control group showed very fine collagen fibers**(Fig.4)**.while inSub gpA sections showedthick collagen fibers**(Fig.5).** InSub gp B sections showedvery thick collagen fibers,dilatation of the ducts and congested blood vessels**(Fig.6).**

Immunohistochemistry detection of caspase-3 in the control group revealed thatthe secretory acini and ducts gave negative immunoexpression for caspase-3**(Fig.7).**while in Sub gp A sections the nuclei of both secretory acini and

ducts revealed moderate positive immunoexpression for caspase-3**(Fig.8).**In Sub gp B sections the nuclei and cytoplasm of acinar cells showed more intense positive immunoexpression for caspase-3 **(Fig.9).**

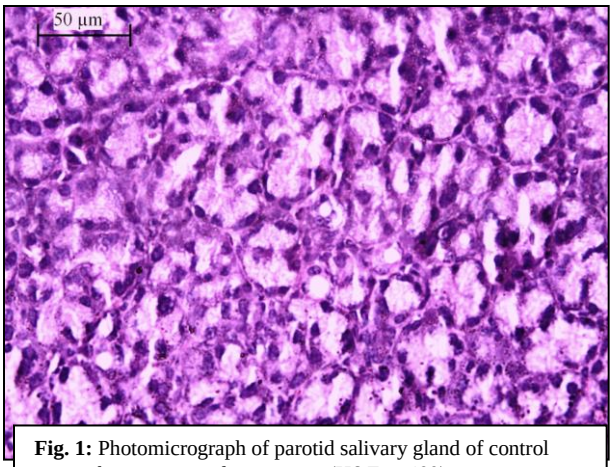


Fig. 1: Photomicrograph of parotid salivary gland of control group showing normal structure. (H&E x400).

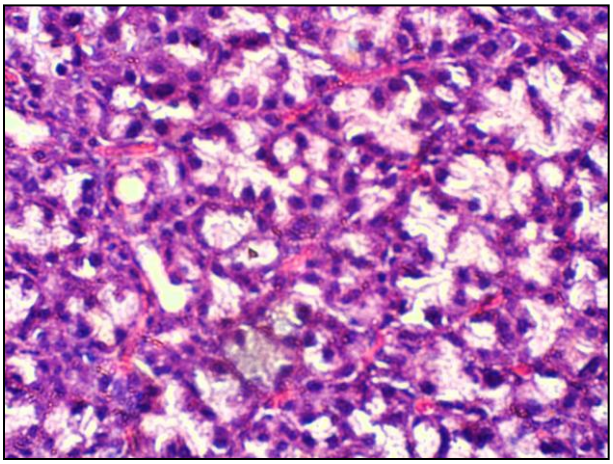
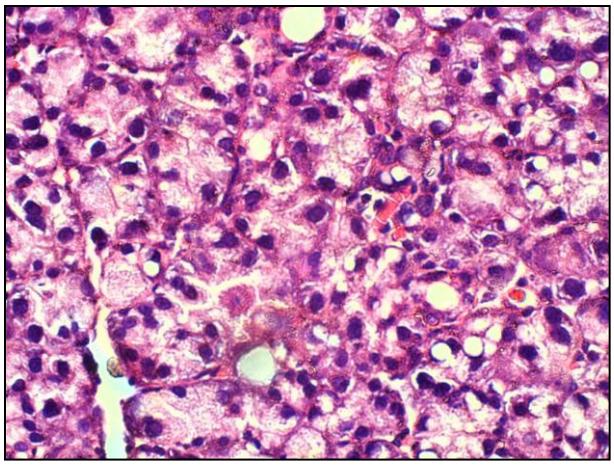


Fig. 2: Photomicrograph of parotid salivary gland of Sub gpA showing vacuoles, interacinar oedema, dilated ducts, congested blood vessels and hyperchromatism as well as pyknotic nuclei. (H&E x400).



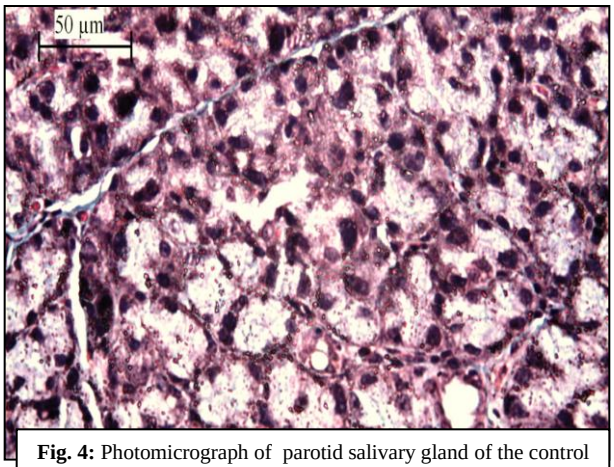


Fig. 4: Photomicrograph of parotid salivary gland of the control group showed very fine collagen fibers. (Masson' s tricrome x400).

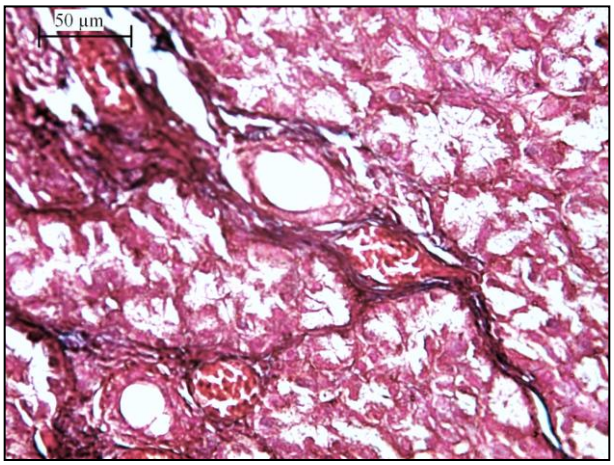


Fig. 5: Photomicrograph of parotid salivary gland of Sub gpA sections showed thick collagen fibers. (Masson' s tricrome x400).

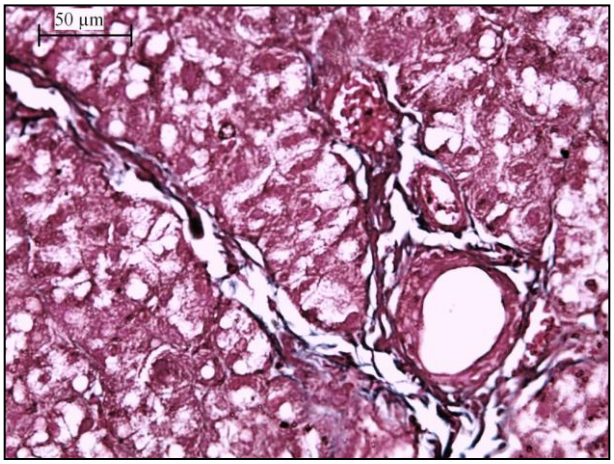
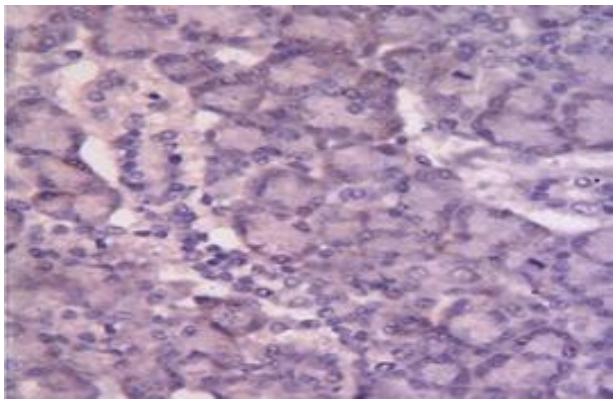


Fig.3: Photomicrograph of Sub gp B showing marked increase in vacuoles, appearance of wide inter-acinar spaces, dilatation in ducts, congested blood vessels. The excretory ducts were dilated. The intralobular& interlobular blood vessels showed wide dilatation and were engorged with blood more nuclear changes (H&E x400).

Fig. 6: Photomicrograph of parotid salivary gland of Sub gp B showing marked increase in thickening of collagen fibers, dilatation of the ducts and congested blood vessels (Masson' s tricrome x400).



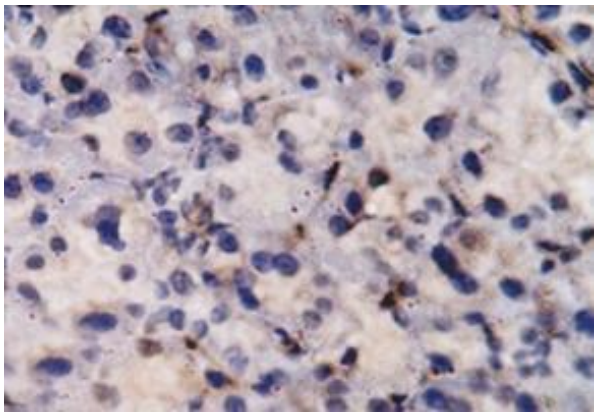


Fig. 7: Photomicrograph of parotid salivary gland of control group showing negative immune reaction in the acinar and ductal cells. (Caspase-3 x400)

Fig. 8: Photomicrograph of parotid salivary gland of Sub gpAshowing moderate positive immunoexpression of caspase-3 in some nuclei and cytoplasm of acinar& ductal cells.(Caspase-3 x400).

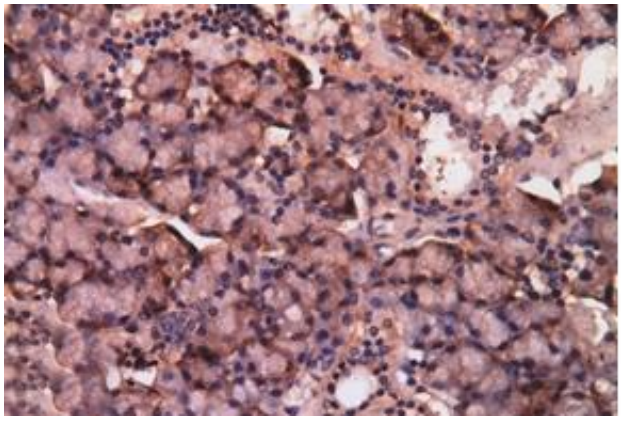


Fig.9 : Photomicrograph of parotid salivary gland of Sub gp B showing intense positive immunoexpression of caspase-3 in most nuclei of acinar and ductal cells (Caspase-3 x400).

4-Discussion:

The current study revealed varying changes in the glandular architecture including changes in the acini, ducts, connective tissue stroma and blood vessels. These changes increased from Sub gpA to Sub gpB according to the increase in the dose of nutmeg extract administered . Histological studies showed dilatation of the ducts and this finding was attributed to accumulation of the salivary secretion and failure of exocytosis due to glandular injury and dysfunctioncaused by nutmeg administration.Vacuolization is one of the apparent results in this study. Vacuoles increased in Sub gp B rats giventhe highest dose of nutmeg extract (500 mg/kg b.w.) than those given the lower dose (100 mg/kg b.w.)(Subgp A). Several studies of **Eweka et al.,(2010), Al-Hazmi et al (2004) and (Adjene, 2010)**

performed on rat or mice kidneys, liver and brain showed cytoplasmic vacuolizations on the administration of nutmeg and thus is agreed with our findings.**(Al-Hazmi, et al. 2004)**also studied the effect of the aqueous extract of nutmeg seeds on the kidney and the liver of mice and observed similar effects. Kidney sections from nutmeg treated mice, showed some pathological changes in the form of hydropic degeneration. Moreover, liver sections showed cellular changes in the form of hydropic degeneration (swelling and hypopigmentation), fatty degeneration, cellular vacuoles and some nuclear changes. The nuclear changes were in the form of redistribution of chromatin granules and its adherence to the nuclear membrane. These were considered as pre-necrotic changes. These findings were evident in the nutmeg-treated mice and increased with the increase of the dose of nutmeg. **(Henics and Wheatley 1999)**explained that in mammalian cells, intense vacuolation leaded to cell death or apoptosis (shrinkage necrosis, the cell cytoplasm becoming intensely crowded and dense) and this is compatible with our finding of hyperchromatism well as pyknoticnuclei.The cell might therefore be expected to compensate by swelling and vacuolating. Since this does not occur in the vast majority of cases, the cell membrane must become more permeable during apoptosis. Vacuoles reported in apoptotic cells tend to be either lipid filled or autophagic vacuoles. Moreover, presence of congested blood vessels was a very prominent feature in nutmeg treated parotid glands in the present investigation and increased in Sub gp B than in Sub gpA.This agreed with **Olaleye et al. (2006)** who studied the effect of nutmeg on several organs. He noticed congestion of blood vessels in the liver and kidney.The dilatation and congestion of the blood vessels might be a part of inflammatory response to bring more blood to the areas of fibrosis or degeneration as was explained by **Moubarak (2008).**In addition to the previous findings, various signs of degeneration wereapparent in our present study and the nuclei of acinar cells showed all signs of nuclear changes. This was confirmed by the results reported by **(Alalwani, 2013)**in the kidneys ofmale animals.

Our imunohistochemical staining confirm our histological findings of parotid salivary glands.This is clear in Sub gp B experimental group using caspase 3 antibodies which revealed intense +ve reaction to activated caspase 3 in most nuclei and cytoplasm of the acini. the reaction was intense and in the ducts cells.. In contrast, Sub gpA experimental group groups showed moderate +ve immunoreactions in most acini and duct cells. While the control group showed –ve immunoreactions. Thus, this is the first study to demonstrate that nutmeg-induced salivary gland apoptosis which mediated by caspas-3 activation.Caspase-3 had many cellular targets and when activated, produces morphologic features of apoptosis **(Jang et al., 2002).Majno and Joris.,(1995)** stated that excessive apoptosis might cause organ atrophy and failure. The active caspase-3 induced activation of caspase-activated deoxyribonuclease,also called DNA fragmentation factor-40 (DFF),that is involved in degrading DNA and apoptosis in different cells **(Kim et al.,2001).**The negative immunoreaction observed in acini and duct cells in all control groups, might be attributed to the physiological cell death. These findings also came in accordance with those of **Olney et al.(2002)** and**Tenkova et al(2003).**

5-Conclusion:-

Administration of nutmeg had damaging effects and induce apoptosis of the parotid salivary glands particularly when taken at high doses.

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