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Salivary nitric oxide levels in inflammatory periodontal disease – a case-control and interventional study

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Parwani SR, Chitnis PJ, Parwani RN. Salivary nitric oxide levels in inflammatory periodontal disease – A case-control and interventional study.

Abstract: *Background:* Biochemical markers of inflammatory periodontal disease present in saliva can partially determine the extent of periodontal disease. Furthermore, collection of salivary constituents is a simple and non-invasive procedure. Nitric oxide (NO) has been linked to etiopathogenesis of inflammatory periodontal disease and is expressed in saliva. This study was conducted with the objective of estimating salivary NO levels in inflammatory periodontal diseases (gingivitis and periodontitis) and comparing these levels with control subjects. A re-assessment of these levels was also made after providing appropriate treatment with a view to ascertain its diagnostic and prognostic values. *Methods:* This was a case–control as well as an interventional study including a total of 90 (30 control, 30 gingivitis and 30 periodontitis) subjects. Saliva samples were collected from each subject, and NO levels were assayed by Griess reaction. *Results:* NO levels were increased significantly in gingivitis and periodontitis subjects as compared with controls. There was a statistically significant decrease in the NO levels in each study group after the healing period (corresponding to the reduced clinical signs of inflammation). Our study also correlated probing pocket depths with salivary NO levels in periodontitis group where we found a positive correlation between the two. *Conclusion:* Salivary NO levels can be utilized as a good indicator of the inflammatory status of the periodontium, and evaluating its levels in saliva by Griess reaction on a photoelectric colorimeter is a reliable, accurate and faster method to estimate the level of inflammation in periodontal tissues.

Key words: gingivitis; griess reaction; inflammatory marker; inflammatory periodontal disease; nitric oxide; nitric oxide synthase; periodontitis

Introduction

Gingivitis and periodontitis are chronic inflammatory diseases of the periodontium. Periodontitis is induced by gram-negative bacteria in subgingival pockets. These microorganisms induce cytokines from lymphocytes or fibroblasts (1). Macrophages, B cells and T cells also accumulate in periodontal lesions, and eventually bone resorption takes place (2).

Nitric oxide (NO), which is synthesized from L-arginine by nitric oxide synthase (NOS), plays a protective role in infectious diseases. NOS is classified into three distinct isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). iNOS is produced by immunocompetent cells such as macrophages infected with bacteria and is

involved in the regulation of inflammatory reactions (3). NO released by the iNOS is generated for long periods. Cytokines and other bacterial products stimulate the expression of iNOS and enhance the periodontal disease progression (2).

NO has been linked to the etiopathogenesis of periodontal disease (4) and is expressed in salivary glands as well as in their products (5). It is both a gas and a free radical (reactive oxygen species) that reacts with many biological molecules (6).

When NO is locally produced in high concentrations, it can act as a cytotoxic molecule, against cells infected by fungi, bacteria, protozoa, as well as tumoral cells and cells close to the production site resulting in tissue destruction (7). NO also has an important participation in bone metabolism regulation, directly acting over clastic cells (8).

This gas appears to play beneficial as well as detrimental role. Beneficial effects include antimicrobial activity and immune modulation (9). Detrimental effects include a cytotoxic action to the adjacent host tissues including alveolar bone (10). This NO-mediated cytotoxicity occurs in combination with the action of metalloproteinases and collagenases, liberated by activated macrophages, polymorphonuclear cells and resident fibroblasts (9).

Proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), interferon-gamma (IFN-γ) are produced by gingival fibroblasts, macrophages and lymphocytes of the periodontal system when challenged by the accumulation of bacteria, which may be responsible for the early reaction of gingivitis. Therefore, dental plaque formation increases NO production, by the upregulation of iNOS expression in gingival cells, which may be induced not only directly by bacterial proliferation but also indirectly through cytokine production stimulated by bacterial plaque (11).

NO has also been shown to directly activate both the constitutive and inducible forms of the cyclo-oxygenase (COX) enzyme, thus leading to an overproduction of prostaglandins (12). Prostaglandins are well-known inflammatory mediators, which by interaction with pro-inflammatory cytokines promote the progress of periodontal and other inflammatory diseases (13). NO is also a well-established endothelium-derived relaxing factor. So, when released after immunological stimulation, it can cause pathological vasodilation and tissue damage (11).

Presently, if available literature is compiled, we know that in inflammatory periodontal disease, salivary NO levels increase. But the question that remains unanswered is whether resolution of inflammation by periodontal therapy can bring down salivary NO levels (and its detrimental effects to oral tissues). So, this study was conducted with an aim to estimate and correlate the salivary NO levels in inflammatory periodontal diseases and re-assess these levels after providing appropriate periodontal therapy (to resolve inflammatory changes) with a view to ascertain its diagnostic and prognostic values.

Materials and method

This study was a case–control trial (to compare salivary NO levels of gingivitis and periodontitis group patients as against those of controls) as well as an interventional study (where salivary NO levels were re-estimated after treating gingivitis and periodontitis patients and also permitting a healing period) carried out in the Department of Periodontics, Modern Dental College, Indore. Subjects in the age range of 20–60 years with sex proportion at random were selected. Study group included 60 patients categorized into gingivitis and periodontitis groups of 30 patients each. Control group included 30 subjects without any inflammatory disease of the periodontium. Ethical clearance for the study was obtained from ethical committee of the institution.

Patient selection and study and control group allocation were based on the inclusion and exclusion criteria, which were as follows:

Inclusion criteria

Individuals with a minimum of 15 teeth were enrolled in the study.

Control group

Individuals exhibiting no bleeding on probing and Ramfjord's Periodontal Disease Index (PDI) (14) score ≤1.

Gingivitis group

Individuals with more than 30% of sites with the presence of bleeding on probing and Ramfjord's PDI score ranging from 2 to 3.

Periodontitis group

Individuals with more than 30% of sites with the presence of mobility, recession, furcation and/or periodontal pockets (clinical attachment loss) and Ramfjord's PDI score ranging from 4 to 6. (Patients from the periodontitis group were merged from mild, moderate and severe subgroups as the aim of our study was not to compare the treatment modalities but to reduce the component of inflammation by any pocket reduction therapy).

Exclusion criteria

Pregnant women, subjects treated for gingivitis or periodontitis in last 6 months, subjects on antibiotics or anti-inflammatory therapy, subjects using antimicrobial mouthwashes, tobacco consumers, subjects with systemic diseases such as diabetes mellitus/hypertension, periapical infection with any tooth or any other form of systemic inflammatory involvement.

Informed consent was obtained from all the participants of the study.

Clinical parameters evaluated were as follows:

For the control and gingivitis groups

Greene and Vermillion's simplified oral hygiene index (OHI-S) (which was recorded to assess the overall status of patient's

oral hygiene) (14), gingival bleeding on probing (present/absent) and Ramfjord's PDI (14).

For the periodontitis group

In addition to the above-mentioned parameters, probing pocket depths, clinical attachment levels (recording the deepest reading), furcation involvement (Hamp *et al*., 1975) (15), gingival recession (recorded as present/absent) and mobility (Muhlemann's method) (16) were also estimated.

Ramfjord's PDI was taken as a common index for all the three groups because this index has both gingivitis and periodontitis components.

Orthopantamographs were also a necessary adjunct for diagnosis in periodontitis group patients.

These radiographs were taken on Planmeca model no. PM 2002 CC Proline panoramic machine, which was standardized at 65-70 kVp as per the age and built of the patient. Exposure time was kept constant, i.e. 18 s.

Ramfjord's PDI was modified in three aspects:

Instead of University of Michigan Number 0 probe, a graduated manual pressure-sensitive periodontal probe (B/Braun DB764R™ Aesculap, Tuttlingen, Germany) calibrated at a constant probing force of 0.2 N (Fig. 1) was used; crevicular measurements were recorded on buccal, mesial, distal and lingual/palatal surfaces instead of only on mesial and buccal surfaces; if a particular tooth was absent, the tooth distal to the missing tooth was used for scoring (in posterior teeth) and the contralateral tooth was used for scoring (in anterior teeth). In case, if the contralateral tooth was also missing, the tooth distal to the missing tooth was considered for scoring in anteriors.

Follow-up examinations

After completion of active periodontal therapy, examination of the periodontium was repeated, recording the same clinical parameters again after 3 and 6 weeks in gingivitis and periodontitis groups, respectively. For gingivitis, scaling and polishing was the treatment administered, and for periodontitis, either scaling and root planing (SRP) alone or SRP + flap surgery (as per the need in individual case) was carried out. To be specific, all patients in periodontitis group were treated initially with SRP. After 1 month, re-evaluation of the periodontal condition was made by reprobing the entire mouth, with rechecking for the presence of calculus and signs of persistent inflammation. Patients with persistent inflammation in areas of moderate-to-deep pockets were treated with flap surgery for pocket elimination i.e. sulcular incision flap, papilla preservation flap or modified Widman flap depending on anatomical and pocket morphology. Seven of thirty patients with periodontitis showed healing with SRP alone and the rest were treated with flap surgery.

Saliva collection and NO quantification

Approximately 2 ml of whole unstimulated saliva was collected in sterile plastic bulbs from the subjects by spitting method (17). Salivary estimation of NO was carried out using the Griess colorimetric reaction (18). The Griess reagent (1:1 mixture of 1% sulphanilamide and 0.1% N-naphthyl ethylene diamine dihydrochloride in 5% orthophosphoric acid) reacted with nitrite present in biological fluid (saliva) to produce a purple azo dye end-product, which was measured on photo-electric colorimeter (Digital Photo Colorimeter E1™; Fig. 2) with a maximum absorbance at 570 nm. The intensity of this colour is directly proportional to the concentration of NO present in the sample (18).

A standard curve was obtained using different concentrations of the standard solution, i.e. sodium nitrite ($NaNO_2$; Fig. 3), which showed linear relationship of optical density (salivary NO level) with concentration of standard sodium nitrite.

Equal volumes of ethylene diamine dihydrochloride solution and sulphanilamide were mixed to obtain Griess reagent (0.1 and 1 g %) 10 min before of its use.

Three test tubes were then taken on a test tube stand, which were labelled as blank (B), standard (S) and test (T). One millilitre of distilled water was taken in a test tube labelled as 'B'. One millilitre working standard solution of

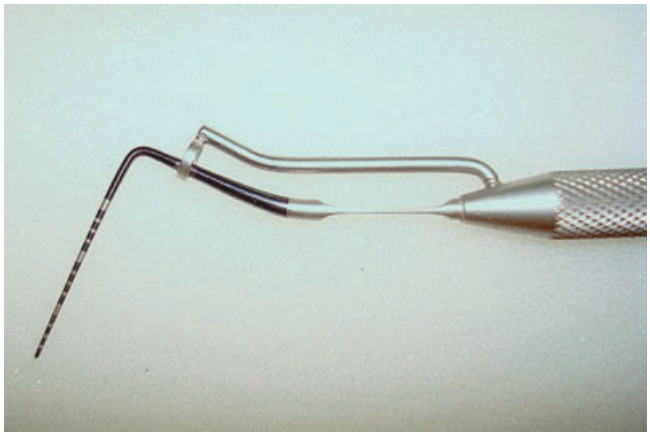

Fig. 1. Graduated manual pressure sensitive probe.

Fig. 2. Digital Photo Colorimeter E1™.

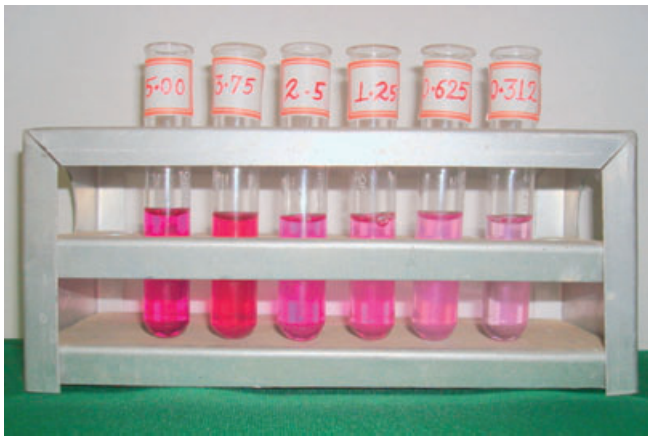

Fig. 3. Griess reaction: standard solution at varying concentrations.

sodium nitrite was taken in a test tube 'S'. One millilitre whole saliva was taken in a test tube 'T'. Then, 1 ml of reconstituted Griess reagent was added to all the tubes 'B', 'S' and 'T'. All these tubes were kept for 10 min at room temperature.

The readings of blank, standard and test samples were recorded on the colorimeter against green filter (480–570 nm). These readings were in the form of optical density (O.D./% transmission), which were then converted to NO levels by the standard conversion formula.

Calculations (19, 20)

$$\text{NO concentration in } \mu g\ \% = \frac{\text{O.D. of 'T'} - \text{O.D. of 'B'}}{\text{O.D. of 'S'} - \text{O.D. of 'B'}} \times \text{concentration of standard}$$

NO values were then subjected to statistical evaluation.

NO levels were evaluated from saliva of individuals from all groups on day 1. In the study group, these were also evaluated after providing appropriate treatment, i.e. after 3 weeks after the last day of treatment in gingivitis group and after 6 weeks in periodontitis group.

Observations and results

Observations were obtained for different groups at pre- and post-treatment stages.

See Tables 1, 2, 3 and 4 and Figs 4 and 5. Table 1 shows the mean, standard deviation and range of O.D., NO levels and probing pocket depth in controls, gingivitis and periodontitis groups at pre- and post-treatment stages. Figure 4 shows mean values of O.D. in controls, gingivitis group and periodontitis group at pre- and post-treatment stages. Figure 5 shows values of NO levels in controls, gingivitis group and periodontitis group at pre- and post-treatment stages.

The three groups, i.e. control, gingivitis and periodontitis (at pre-treatment stage), were analysed by one-way analysis of variance, and it was found that highly significant differences were present between the groups for O.D. as well as NO levels (Table 2).

Student's independent *t*-test was conducted to know the differences in O.D. and NO levels between the groups at pre-treatment stage (Table 3).

Paired *t*-test was performed to evaluate the differences between the same group at pre- and post-treatment stage in study groups and also to know the differences in probing pocket depths in periodontitis group at pre- and post-treatment stages (Table 4).

The relationship of probing pocket depth with salivary NO levels was analysed by correlation analysis, and it was found that a highly significant positive relationship existed between these two parameters, i.e. as the probing pocket depth increased, NO levels also increased and *vice versa*. Correlation coefficient '*r*' at pre-treatment stage was 0.871 with a *t*-value 9.364, and at post-treatment stage, '*r*' was 0.915 with a *t*-value of 11.197, which was statistically significant at 1% level of significance.

Discussion

In this study, salivary NO production was measured indirectly using the level of nitrite in saliva of 30 control, 30 gingivitis subjects and 30 periodontitis subjects. It was observed that NO levels were increased in gingivitis and periodontitis sub-

Table 1. **Mean, standard deviation and range of O.D., NO levels and probing pocket depth in controls, gingivitis and periodontitis groups at pre- and post-treatment stages**

	Pre-treatment		Post-treatment	
Group	Mean ± SD	Range	Mean ± SD	Range
Control subjects				
Optical density	0.36 ± 0.12	0.20–0.64	–	–
NO levels	241.10 ± 83.72	132.0–421.0	–	–
Patients with gingivitis				
Optical density	0.65 ± 0.10	0.41–0.80	0.40 ± 0.08	0.24–0.54
NO levels	430.60 ± 67.97	270.0–528.0	269.07 ± 53.08	159.0–368.0
Patients with periodontitis				
Optical density	0.81 ± 0.12	0.68–1.22	0.49 ± 0.06	0.44–0.77
NO levels	537.67 ± 80.06	447.0–803.0	326.73 ± 41.03	289.0–507.0
Probing depth	3.99 ± 0.66	2.99–5.20	1.19 ± 0.33	1.00–2.59

Table 2. **Analysis of variance for O.D. and NO levels in control, gingivitis and periodontitis groups at pre-treatment stage**

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F* value	*P*
Optical density					
Between groups	2	3.17	1.585	116.35	0.000**
Within groups	87	1.185	0.014		
Total	89	4.356			
NO levels					
Between groups	2	1353253	676626	112.5	0.000**
Within groups	87	523124	6012.9		
Total	89	1876378			

**Highly significant.

Table 3. ***t*-values for O.D. and NO levels at pre-treatment stage of the different groups**

Character	Mean	Mean	*t*-value	*P*	Sig.
Optical density					
Control & gingivitis	0.36	0.65	9.82	0.000	Highly sig.
Control & periodontitis	0.36	0.81	9.62	0.000	Highly sig.
Gingivitis & periodontitis	0.65	0.81	5.71	0.000	Highly sig.
NO levels					
Control & gingivitis	241.10	430.60	14.24	0.000	Highly sig.
Control & periodontitis	241.10	537.67	14.02	0.000	Highly sig.
Gingivitis & periodontitis	430.60	537.67	5.58	0.000	Highly sig.

Table 4. ***t*-values for O.D. and NO levels at pre- and post-treatment stages of the different groups**

Character	Mean	Mean	*t*-value	*P*	Sig.
Pre- and post-treatment gingivitis					
Optical density	0.65	0.40	17.64	0.000	Highly sig.
NO levels	430.60	269.07	17.72	0.000	Highly sig.
Pre- and post-treatment periodontitis					
Optical density	0.81	0.49	17.04	0.000	Highly sig.
NO levels	537.67	326.73	17.36	0.000	Highly sig.
Probing pocket depth	3.99	1.19	21.91	0.000	Highly sig.

jects as compared with controls. This was in accordance with the following studies:

Batista *et al.* (4) showed a significant increase in the number of iNOS$^+$ PMNLs (polymorphonuclear leucocytes) in the biopsy samples of gingivitis and periodontitis subjects as compared with those of control subjects. Sharma *et al.* (21) conducted a study that also showed an increase in NO levels in chronic gingivitis and chronic periodontitis subjects as compared with the control group. Matejka *et al.* (22) in a study showed increased levels of amino acids related to NO in

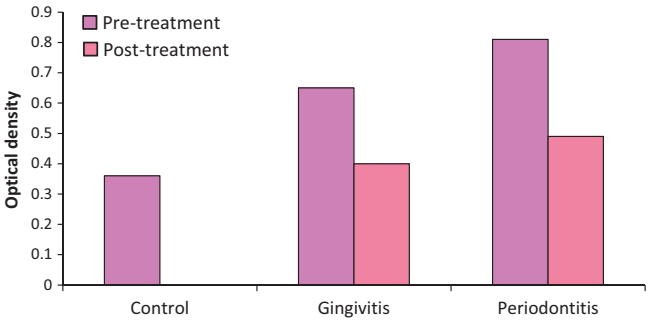

Fig. 4. Mean values of optical density in controls, gingivitis and periodontitis subjects at pre-treatment and post-treatment stages.

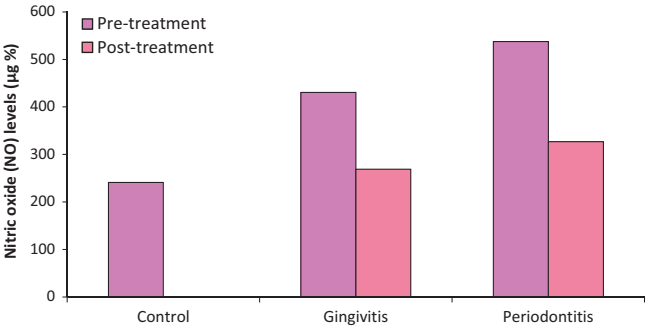

Fig. 5. Mean values of nitric oxide (NO) levels in controls, gingivitis and periodontitis subjects at pre-treatment and post-treatment stages.

inflammatory periodontal disease. Lappin *et al.*, Kendall *et al.* and Hirose *et al.* (23–25) also observed an increase in NO levels in periodontitis subjects as compared with control group. They demonstrated that periodontitis diseased tissue exhibited greater levels of iNOS expression than the healthy tissue. Yet another study showing an increase in salivary NO levels in periodontitis cases as compared with control group was conducted by Koshti *et al.* (19). Reher *et al.* (18) conducted another similar study that correlated the salivary NO levels in control and periodontitis groups. NO levels in chronic generalized periodontitis (moderate and advanced) groups were higher than in the control group. Also, it was observed that in the periodontitis group, NO levels increased as the severity of the disease increased. They also found a positive correlation between NO levels and probing pocket depth.

Similarly, our study also correlated probing pocket depths with salivary NO levels in periodontitis group where we found a positive correlation between the two.

In contrast to these above studies, one report [by Aurer *et al.* (26)] has described a reduction in salivary NO levels in individuals with adult periodontitis and with aggressive periodontitis. They explained that according to Kankanian *et al.* (27), saliva from healthy subjects stimulates NO synthesis in PMNLs, while the saliva from patients with gingivitis or periodontitis suppresses it. Alternate explanation that the authors gave for this decrease was a reduction in local parasympathetic

neural activity (in inflammatory conditions), which would result in reduced NO produced by salivary glands (28).

In our study, as observed from results, NO levels at pre-treatment stage in patients with gingivitis were statistically higher than NO levels in control group subjects. In the periodontitis group, the pre-treatment NO levels were significantly higher statistically than NO levels in control group and gingivitis group subjects at pre-treatment stage. This could be explained that as compared with the control group, gingivitis group patients exhibit more inflammation and signs of matrix degradation. This was also correlated with the presence of bleeding on probing where biological activity of NO plays a role in vasodilatation and inhibition of platelet adhesion and aggregation. Secondly, the higher NO levels in periodontitis pre-treatment group as compared with gingivitis pre-treatment group can be correlated with the greater amount of matrix degradation and alveolar bone loss seen in patients with periodontitis.

Our study was an interventional study in which NO levels in the gingivitis and periodontitis groups were re-estimated after treatment. Our results showed that there was a statistically significant decrease in the NO levels in each study group after the healing period (corresponding to the reduced clinical signs of inflammation) e.g. the NO levels during the post-treatment evaluation in the gingivitis group were statistically significantly decreased as compared with those at the pre-treatment evaluation stage. Similarly, the NO levels at the post-treatment evaluation stage in the periodontitis group were also statistically significantly decreased as compared with the pre-treatment NO levels in the same group.

However, the post-treatment NO levels in the study groups did not come down to the control group NO levels. Probably, the treatment that was rendered, i.e. scaling, root planing and antibiotic and anti-inflammatory prescription after flap surgery, was not sufficient to bring down the NO levels to the control group NO levels, or alternatively there could be some unforeseen systemic factor that had an overplay e.g. stress (29).

In future, studies can be taken up with the administration of chemical inhibitors of NOS or antioxidants (30) (as NO also belongs to reactive oxygen species group), which will probably reduce post-treatment NO levels in gingivitis and periodontitis groups to control group NO levels, signifying complete health of periodontal tissues.

Conclusion

In this study, we aimed to estimate the salivary levels of NO in controls, chronic generalized gingivitis subjects and chronic generalized periodontitis subjects in order to evaluate the correlation of salivary NO levels with the inflammatory status of the periodontium.

From the data collected from the study, following compilation could be made:

1 The increase in salivary NO levels was statistically highly significant in gingivitis and periodontitis subjects as compared with controls.

2 The difference between salivary NO levels of gingivitis and periodontitis was also statistically highly significant, suggesting that as the inflammatory process faces the shift to a more destructive stage, salivary NO levels also increase.

3 As the probing pocket depths in periodontitis subjects increased, the salivary NO levels also increased – suggesting a linear relationship between the severity of periodontitis and NO levels in saliva.

4 Post-treatment salivary NO levels decreased in gingivitis and periodontitis groups. However, these levels persistently remained at a higher level in comparison with the control group.

So, to conclude, salivary NO levels can be utilized as a good indicator of the inflammatory status of the periodontium, and evaluating these levels in saliva by Griess reaction on a photoelectric colorimeter is a reliable, accurate and faster method to estimate the level of inflammation in periodontal tissues.

However, further studies need to be conducted as to whether the administration of some chemical inhibitors of NOS or antioxidants in addition to mechanical therapy can be of some help to modulate the host response in inflammatory periodontal diseases.

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Conflicts of interest

This is to state that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could have inappropriately influenced the results of the study.

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