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Original article

The effect of systemic Isotretinoin on salivary tissue inhibitors of metalloproteinases 1 and 2 and salivary flow rate in periodontal disease

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ABSTRACT

Aims: To evaluate and compare changes in salivary flow rate and salivary levels of TIMP-1 and TIMP-2 in individuals taking oral Isotretinoin (INN) with those who do not take INN. To assess the variation in TIMP-1 and TIMP-2 as well as salivary flow rate observed at different stages of periodontal disease in comparison to those observed in the case of healthy periodontium.

Materials and methods: An examiner-blind case-control study involving 180 human adults divided into six groups based on their periodontal status. Clinical parameters, including pocket depth, clinical attachment level, and bleeding on probing were measured at six sites per tooth. Whole unstimulated saliva samples were collected from all subjects to evaluate salivary flow rate (SFR). Salivary TIMP-1 and TIMP-2 levels were detected using enzyme-linked immunosorbent assay (ELISA). Data were analyzed using IBM SPSS Software. The Kruskal Wallis test and Mann-Whitney U-tests were employed to verify any significant differences between the groups for all parameters. Multi-regression analysis was performed for each parameter tested in each group. All tests were compared at a significance level of 0.05.

Results: SFR was statistically significantly lower among all INN groups in comparison to the control groups ($P < 0.001$). TIMP-1 and TIMP-2 were significantly higher in all INN groups in comparison to the control groups, in both gingivitis cases ($P = 0.004$, $P < 0.0001$ respectively) and periodontitis cases ($P < 0.0001$).

Conclusion: Although INN reduces salivary flow rate, the findings of the present study revealed that it had an anti-inflammatory effect in periodontal biomarkers. Specifically, it was positively correlated with an elevation of salivary TIMP-1 and TIMP-2. Hence, INN might be a future additive medication to be further evaluated for the treatment of periodontal diseases.

1. Introduction

Isotretinoin (INN), which is also known as 13-*cis*-Retinoic acid (brand name; Roaccutane/Accutane-Roche, *Switzerland)*, is an oral administrative drug that belongs to a class of drugs known as retinoids (WHO Drug Information, 2017). It is predominantly used to treat severe nodular acne that has not responded to other treatment (Harms et al., 1986), and it has been approved for use as a treatment for acne in the United States since 1982 (Bauer et al., 2016). In terms of the anti-inflammatory properties, Isotretinoin has been found to inhibit the action of the matrix metalloproteinase-9 (MMP-9) in facial sebum without influencing the action of tissue inhibitors of metalloproteinases-1 and −2 (TIMP-1 and TIMP-2) (Papakonstantinou et al., 2005). It is well known that MMPs are a group of enzymes that are responsible for the degradation of most extracellular matrix proteins during organogenesis, growth, and normal tissue turnover (Uitto et al., 2000. 2003). They have been found to play a significant role in tissue destruction during the progression of periodontal diseases (Marcaccini et al., 2009). Although multiple studies have described

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the presence of various MMPs, such as MMP-2, MMP-7, and MMP-14 (Tervahartiala et al., 2000), in the gingival tissue, the most widely investigated MMPs in the GCF and saliva are MMP-8, MMP-9, and MMP-13 (Choi et al., 2004; Beklen et al., 2006; Kumar et al., 2006; Arias-Bujanda et al., 2020). Collagenases, especially MMP-8, and gelatinases, especially MMP-9, are used as markers of active periodontal destruction and disease activity in GCF, saliva and serum (Marcaccini et al., 2009; Rai et al., 2008; Ingman et al., 1996). It is believed that MMP-8 and −9 are primarily responsible for collagen degradation in the inflamed tissue observed in cases of gingivitis and adult periodontitis (14). Therefore, it is logical to consider these proteolytic enzymes to represent good indicators for periodontal inflammation. The destroying activity of MMPs can be controlled by inhibiting their action which can be mediated by the four members of the tissue inhibitor of metalloproteinase (TIMP) family (Brew et al., 2000). TIMPs proteins are responsible for regulating the extracellular activity of MMPs (Brew et al., 2000). Therefore, alterations in TIMP expression are known to occur in many disease processes (Uitto et al., 2000. 2003).

The purpose of the present study was to evaluate changes in salivary flow and the salivary levels of TIMP-1, and TIMP-2 in patients who had been prescribed oral INN for the treatment of cutaneous acne before comparing the outcomes observed with those from patients suffering from different stages of periodontal disease.

2. Material and methods

2.1. Study sample

By using G Power software, a confidence level of 95% and a power level of 80% was set with a moderate effect size with a final sample size of 180 human subjects (Cohen, 1988). The study involved 6 groups with 30 subjects in each group. The study population consisted of 180 voluntary participants (>18 years old), consisting of 90 patients who were taking Isotretinoin and attending the Dermatology Clinic at King Khalid University Hospital and 90 control volunteers who were not taking the medication and were attending the Dental University Hospital at King Saud University. All subjects who were receiving 0.5 or 1.0 mg/kg/day dose of oral INN (Roaccutane®) for cutaneous lesions for a minimum of 3 months were included. Subjects were excluded from the study if they received any periodontal treatment or antibiotic therapy for medical or dental reasons three months prior to the investigation. In addition, any subject who was taking long-term medication that is known to affect the periodontal status or salivary flow or had a history of metabolic bone diseases, autoimmune diseases, diabetes, or postmenopausal osteoporosis was excluded. Pregnant women and smokers were also excluded from the study. The study protocol (E-19–3856) was approved by the Institutional Review Board (IRB) of King Saud University Medical City.

2.2. Clinical measures

After explaining the study protocol to the participants and obtaining their informed consents, two examiners (AZ and MK), who were blind to the patients' use of the medication (INN), assessed all teeth, except third molars, for periodontal clinical parameters. The clinical measurements that were performed included pocket depth, clinical attachment level, and bleeding on probing, which was measured at six sites per tooth using a William's probe. All measurements were read to the nearest 0.5 mm. Based on the collected clinical and radiographic data, each subject was diagnosed according to the latest classification scheme for periodontal diseases and conditions (2017) (Caton et al., 2018). After the INN-taking subjects underwent a periodontal examination they were divided into three groups. The first group was composed of subjects with healthy periodontium (HINN) (n = 30), the second group was composed of subjects with generalised plaque-induced gingivitis (GINN) (n = 30), and the third group was composed of subjects with generalised Stage I periodontitis (PINN) (n = 30). The negative control group, which included subjects who were not taking the medication (INN), were categorised in the same manner. The first group was composed of subjects with healthy periodontium (HC) (n = 30), the second group included subjects who were diagnosed with generalized plaque-induced gingivitis (GC) (n = 30), and the third group included subjects who were diagnosed with generalised Stage I periodontitis (PC) (n = 30) (AlJasser et al., 2021).

2.3. Intra-examiner and Inter-examiner reliability

The two examiners (AZ and MK) performed clinical measurements on two randomly selected participants. These measurements were repeated after 10 days, and the Cohen's Kappa Score was used to measure the reliability level.

2.4. Assessment of salivary flow rate

2.4.1. Subjective assessment

The subject's responses to a health questionnaire were measured by asking them four closed questions, as follow:

1- Does the amount of saliva in your mouth seem too little?
2- Does your mouth feel dry when eating a meal?
3- Do you have difficulty swallowing any food?
4- Do you sip liquids to aid in swallowing dry food?

"Yes" responses to these four questions were deemed to be significantly associated with a low salivary flow rate (Papakonstantinou et al., 2005).

2.4.2. Objective assessment

Subjects were instructed not to eat, smoke, or drink for at least three hours prior to saliva collection. To minimize fluctuations related to circadian rhythm in salivary secretion, all collections were performed at a fixed time of day. Subjects were instructed to relax and swallow all saliva present in their mouths five minutes before starting the saliva collection. While seated and leaning forward, they were asked to spit all the saliva they produced into a graduated test tube over a period of five minutes. The collected whole unstimulated saliva was then measured by volume and expressed as milliliters per minute (ml/min). SFR measurements were performed by one examiner (M.K.) (Navazesh and Kumar, 2008). The samples were then stored at −80 °C before being analyzed for TIMPs (Hu et al., 2007).

2.5. Enzyme-linked immuno-sorbent assays

Enzyme-linked immunosorbent assay (ELISA) (Elabsciences ®, Houston, Texas, USA) was used to study the levels of TIMP-1 and TIMP-2 within the saliva samples. Twenty-four hours before analysis, samples were transferred to storage at 4 °C for gradual thawing. After thawing, the saliva samples were centrifuged for 20 min at 1000 rcf in a bench top-refrigerated centrifuge before the supernatant was collected to carry out the assay. A duplicate of each saliva sample was used in the micro-ELISA plates to verify the accuracy. The Sandwich-ELISA ELISA kit was employed in accordance with the manufacturer's instructions. The micro-ELISA plate was pre-coated with an antibody specific to Human TIMP-1 and

TIMP-2. Standards and samples were added to appropriate micro-ELISA plate wells and combined with the specific antibody. Wells containing Human TIMP-1 and TIMP-2 biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by adding a Stop Solution, which appeared yellow in color. The optical density (OD) was measured at a wavelength of 450 nm ± 2 nm using a Biotek Synergy HT microplate reader (Synergy HT, Biotek, Vermont, USA). The OD value was proportional to the concentration of Human TIMP-1 and TIMP-2.

2.6. Data Analysis:

Data were analyzed using IBM SPSS statistical software, Version 21.0 (IBM Inc., Chicago, USA). Non-parametric statistical tests (Kruskal Wallis test and Mann-Whitney *U* test) were used as the outcome variables were skewed, to compare the mean ranks of the outcome variables in relation to the six study sub-groups within each group and between the two groups. Pearson Chi-square test was used to compare the distribution of categorical responses across the study groups. Kappa statistics were used to quantify the agreement and report the intra and inter-examiner reliability. A *P*-value of $\leq$ 0.05 was deemed to be of statistical significance.

3. Results:

A total of 180 subjects with a mean age of 24.8 years were included in this study. Of the 180 subjects, 101 were females (56%) and 79 were males (44%). Table 1 shows the descriptive statistics (mean, standard deviation, and frequencies) of the study variables across the six study groups.

3.1. Subjective assessment of salivary flow rate

The differences in the subjects' responses to the four questions were highly statistically significant between the INN groups and control groups ($P < 0.0001$). A higher proportion of the subjects in the INN groups responded positively to all four questions (i.e., 'Yes'), in comparison to the subjects in the control groups, as shown in Table 2 and Table 3.

3.2. Objective assessment of salivary flow rate

The comparison of mean saliva rate across the six study groups revealed that there was a highly statistically significant difference ($P < 0.001$) in the amount of saliva collected, as shown in Fig. 1. Overall, the mean saliva rate of the three control groups was statistically significantly higher than the rate observed among the other three INN groups. Within the three INN groups, the mean saliva rate values of PINN group were statistically significantly lower than the other two study groups, HINN and GINN. Among the three control groups, the mean saliva rate values were higher in the PC group in comparison to the other two control groups, HC, and GC. The comparison of the mean saliva rate between the GINN and GC groups revealed that the saliva rate production of the participants in the GINN group was statistically significantly lower ($P = 0.019$) than that of the GC group. The comparison of the mean saliva rate between the PINN and PC groups revealed that the saliva rate production of the participants in the PINN group was statistically significantly lower ($P < 0.0001$) than that of the PC group.

3.3. Enzyme-linked immunosorbent assay (ELISA)

There was no statistically significant difference in the mean ranks of the MMP-9/TIMP-2 between the GINN and GC groups ($P = 0.712$). However, a comparison of the mean ranks of TIMP-1 and TIMP-2 levels revealed that there was a highly statistically significant difference between the GINN and GC groups ($P = 0.004$, and $P < 0.0001$, respectively). The values were significantly higher in the GINN group than the GC group, as shown in Fig. 2. The comparison of the mean ranks of TIMP-1, TIMP-2, and MMP-9/TIMP-2 between PINN and PC groups showed highly statistically significant difference ($P < 0.0001$). The mean ranks of the TIMP-1 and TIMP-2 were significantly higher in the PINN group than the PC group, as shown in Fig. 3.

4. Discussion

This study was concerned with the oral side effects of INN, which is a significant reduction in SFR, as reported subjectively by the medication takers and measured objectively by the clinicians by measurement of the whole unstimulated saliva (Erdemir et al., 2017; Lupi-Pégurier et al., 2007; Oikarinen et al., 1995). In the current study, a significantly higher reduction in SFR was observed in the group with generalized periodontitis Stage I in comparison to other groups. Although a lower SFR was found in subjects with generalized periodontitis Stage I who were taking INN, the anti-inflammatory biomarkers TIMP-1 and TIMP-2 were significantly higher in these subjects than in those who were not taking the medication, with the same periodontal diagnosis and a much higher SFR. This finding is contrary to the hypothesis that there is a correlation between SFR and periodontal disease. Furthermore, in addition to the current study, multiple studies have proved the weakness of this relation (Crow and Ship, 1995; Shaila et al., 2013; Syrjälä et al., 2011).

To the best of our knowledge, the present study is the first of its kind to illustrate the relation between isotretinoin use and salivary levels of TIMP-1 and TIMP-2. Previous speculations can be confirmed by changes of tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2), which are considered to represent anti-inflammatory biomarkers that increase according to states of peri-

Table 1
Descriptive statistics of outcome variables across the six study groups.

Variables	HC	HINN	GC	GINN	PC	PINN
Age (years) mean(SD)	24.9(5.4)	24.7(3.5)	25.2(7.8)	23.6(3.8)	26.2(2.4)	24.1(3)
Gender (Males:Females)	11:19	16:14	16:14	13:17	15:15	8:22
Objective assessment of salivary flow rate (ml/min)						
Saliva Rate Mean(SD)	0.34(0.20)	0.28(0.17)	0.40(0.22)	0.28(0.15)	0.72(0.16)	0.22(0.11)
Enzyme-linked immunosorbent assay ELISA (ng/mL)						
TIMP-1 Mean(SD)	1.3(1.8)	4.14(4.7)	0.78(1.4)	3.24(3.5)	4.2(3.2)	147.7(171.2)
TIMP-2 Mean(SD)	1.6(3.8)	26.7(28.9)	2.2(2.9)	9.4(10.5)	3.2(2.8)	23.7(12.0)
MMP-9/TIMP-2Mean(SD)	2164.6(7068.9)	145.9(730.6)	178.4(330.4)	33.3(100.5)	250.8(196.9)	4.5(2.9)

*Statistically significant; BOP, bleeding on probing; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of matrix metalloproteinase; HC, Control subject with healthy periodontium; HINN, INN using subject with Healthy periodontium.; GC, Control subject with Gingivitis; GINN, INN using subject with Gingivitis; PC, Control subject with Generalized Periodontitis Stage I; PINN, INN using subject with Generalized Periodontitis Stage I.

Table 2
Subjective assessment of salivary flow rate, comparison between the Gingivitis groups.

	GINN		GC			
	Number (Yes:No)	% (Yes:No)	Number (Yes:No)	% (Yes:No)	X^2-value	*P*-value
1- Does the amount of saliva in your mouth seem too little?	24:6	80:20	8:22	26.7:73.3	17.14	<0.0001*
2- Does your mouth feel dry when eating a meal?	18:12	60:40	1:29	3.3:96.7	22.23	<0.0001*
3- Do you have difficulty swallowing any food?	15:15	50:50	2:28	6.7:93.3	13.87	<0.0001*
4- Do you sip liquids to aid in swallowing dry food?	27:3	90:10	10:20	33.3:66.7	20.38	<0.0001*

* Statistically significant; GC, Control subject with Gingivitis; GINN, INN using subject with Gingivitis.

Table 3
Subjective assessment of salivary flow rate, comparison between the periodontitis groups.

		PC		PINN		
P-value	X^2-value	% (Yes:No)	Number (Yes:No)	% (Yes:No)	Number (Yes:No)	
<0.0001*	60.0	0:100	0:30	100:0	30:0	**1- Does the amount of saliva in your mouth seem too little?**
<0.0001*	56.13	0:100	0:30	96.7:3.3	29:1	**2- Does your mouth feel dry when eating a meal?**
<0.0001*	56.13	0:100	0:30	96.7:3.3	29:1	**3- Do you have difficulty swallowing any food?**
<0.0001*	56.13	0:100	0:30	96.7:3.3	29:1	**4- Do you sip liquids to aid in swallowing dry food?**

* Statistically significant; GC, Control subject with Gingivitis; GINN, INN using subject with Gingivitis.

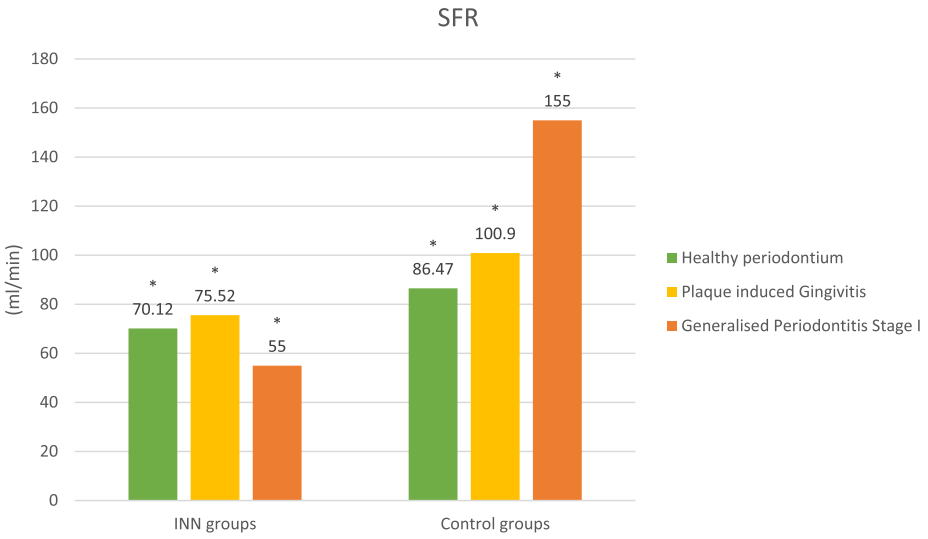

Fig. 1. Comparison between the six groups in the mean ranks of their SFR.

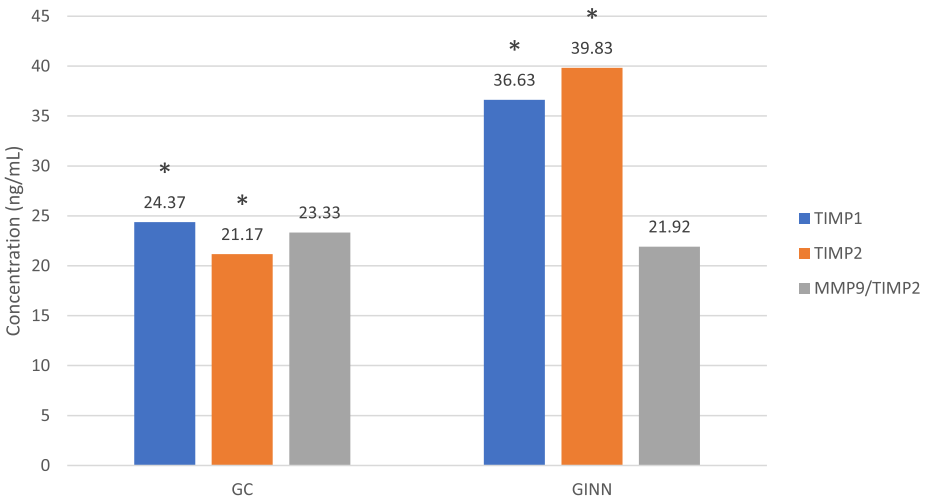

Fig. 2. Comparison between Control subjects with Gingivitis (GC) and INN taking subjects with Gingivitis (GINN) in the levels of TIMPs.

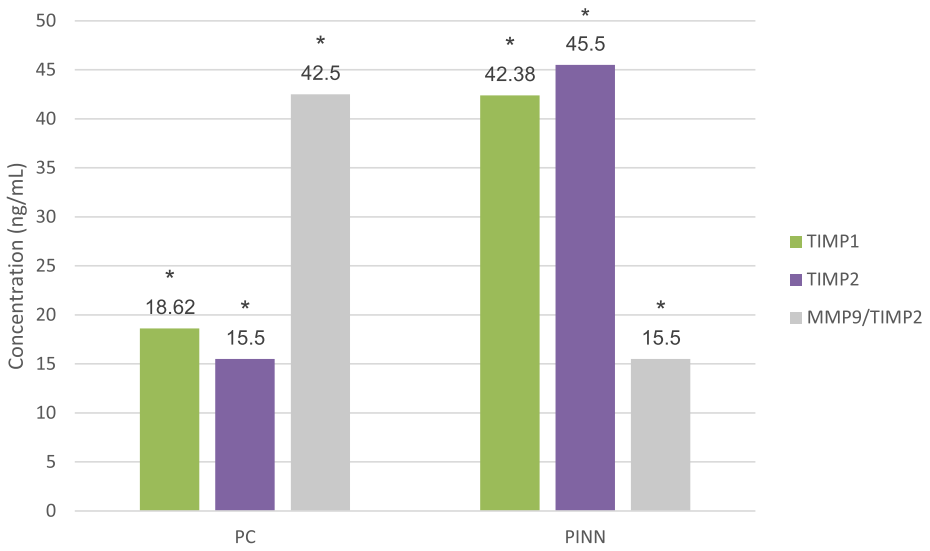

Fig. 3. Comparison between Control subjects with Generalized Periodontitis Stage I (PC) and INN taking subjects with Generalized Periodontitis Stage I (PINN) in the levels of TIMPs.

odontal health (Nizam et al., 2014). In the present study, statistically significant higher levels of TIMP-1 and TIMP-2 were found in patients taking INN compared to those who were not taking the medication. These results can indicate the strong anti-inflammatory effect of INN, which has already been proven and used to treat different systemic inflammatory diseases in various medical fields; for example, inflammatory acne vulgaris, psoriasis (Arechalde and Saurat, 2000), and rosacea (Rebora, 2002). Finally, no previous studies have measured salivary TIMP-1 and TIMP-2 in similar participants.

An in-vitro experiment investigated the effect of different forms of vitamin A on human gingival fibroblast. The author concluded that Vitamin A helped to increase gingival fibroblast population, growth, collagen production, and connective tissue strength (Ozick, 1993). Moreover, this investigation led to a patent award on the local use of Vitamin A safe forms, including INN for the treatment of severe signs of periodontitis, which strongly supports the findings of the present clinical study. Another study, however, found that systemic retinoids have no positive effect on periodontal disease when observed in Papillon Lefevre Syndrome patients (Systemic retinoid medication and periodontal health in patients with Papillon-Lefèvre syndrome., 1996). This difference in the findings can be attributed to variations in the nature of the periodontal diseases that were examined in this study. Specifically, the present study focused only on plaque-induced gingivitis and chronic periodontitis, which differ from more severe forms of periodontal diseases as aggressive periodontitis in the pathogenicity and rate of progression as well as the host immune response (Heller et al., 2012; Baer, 1971; Ford et al., 2000. 2010 Jun). One additional study reported a case diagnosed with non-plaque induced gingivitis which INN was believed to be associated with (Mahajan et al., 2011).

The current study had several limitations. First, the collection of data for each subject was performed one single time, and no further follow-up was pursued. This might be of importance since the patients usually require a long course of therapy with INN, and thus, INN response might differ from one individual to another. In addition, performing follow-up studies with patients can lead to the detection of the potential side effects associated with long-term use and, thus, generate a better understanding of the condition. Another limitation concerns the fact that the evaluation of the subjects' TIMPs levels was based purely on their saliva. This approach represents a less accurate method than gingival tissue biopsy or gingival crevicular fluid. Finally, the present study evaluated SFR depending only on the amount of un-stimulated saliva.

5. Conclusion:

Although it is well known that Isotretinoin causes oral dryness, the findings of the present study demonstrated that it had an anti-inflammatory effect on periodontal parameters. The elevation in salivary TIMP-1 and TIMP-2 was detected in patients with plaque-induced gingivitis and chronic periodontitis. This can promote its possible future use as an adjunct treatment modality for periodontal diseases. These findings need to be confirmed by future well-designed longitudinal studies and clinical trials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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