

Study the Effect of an Antipyretic Drug, Thai Herbal Ha-Rak Formula on Platelet Aggregation in Healthy Thai Volunteers: A Randomized, Placebo - Controlled Trial

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ABSTRACT

Background: Fever may alter platelet homeostasis leading to bleeding tendency. Thai herbal Ha-rak formula (HRF), a traditional Thai remedy consisting of five medicinal plants, is indicated for relieving fever. However, its effect on platelet has not been evaluated. This study aims to investigate the effect of HRF on platelet aggregation.

Methods: A randomized, placebo, controlled trial was carried out in 46-healthy Thai volunteers, both male and female. The subjects either received the maximum recommended dose per day of HRF (1,500 mg) or placebo. Platelet aggregation, using aggregometer (AggRam), was assessed in platelet rich plasma (PRP) in response to each of three different agonists including epinephrine, adenosine diphosphate (ADP) and collagen at pre-dose and 8, 32 hours and 7-10 days after the first dose.

Results: All participants completed the study. Only few adverse events occurred which spontaneously improved without further treatment. Overall, analysis of platelet activity compared before and after HRF administration did not show significant difference of maximum percentage of platelet aggregation at any time point except the platelet response to collagen at 32 hours and 1 week after the first HRF dose. However, subgroup analysis characterized by sex, and platelet aggregation in response to all agonists did not reveal any significant change. The same results applied to subgroup analysis based on the different patterns of platelet aggregation.

Conclusion: HRF at a dose of 1,500 mg/day is well tolerated and has a significant effect on platelet aggregation only when induced by collagen.

Keywords: Thai herbal formula; platelet aggregation; epinephrine; ADP; collagen (Siriraj Med J 2017;69: 283-289)

INTRODUCTION

Fever is caused by various conditions. It is usually the first presentation of many conditions including infection. Certain infections such as dengue hemorrhagic fever can alter platelet homeostasis leading to risk of bleeding. In an endemic area, antipyretic acetaminophen is preferable to NSAIDs because it has no effect on platelet. Despite

commonly use of acetaminophen, some prefer alternative herbal remedies. Thai herbal Ha-rak formula (HRF) or Ben-Cha-Lo-Ka-Wi-Chian formula, one of the antipyretic recipes in the National List of Herbal Medicinal Products AD 2006, is commonly prescribed for relieving fever in Thai traditional folk medicine practice.¹ Despite the fact that HRF has long been used in humans, the mechanism

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of how HRF can relieve fever is still not known. HRF consists of 5 herbal plants including *Harrisonia perforate* Merr., *Capparis micracantha* DC., *Clerodendrum petasites* S.moore, *Ficus racemosa* Linn. and *Tiliacora triandra* (Colebr.) Diels. *Ficus racemosa* bark, which is the only herb in the HRF, that has a report that showed the antipyretic effect and inhibitory activity against cyclooxygenase (COX) enzyme.^{2,3} Blocking COX enzyme is known as a mechanism to alleviate fever and inflammation of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs). Moreover, inhibition of COX enzyme possibly alters platelet aggregation leading to bleeding tendency. The concept of reverse pharmacology has emerged in the study of herbal medicine. The reverse pharmacology relates to reversing the routine “laboratory to clinic” to “clinic to laboratory.”⁴ HRF have been use for many years by Thai traditional folk medicine practice, so one could say it has been tested for many years on lots of people, similar to the phase IV clinical trials. It is reasonable to study HRF in reverse pharmacology. This study aims to find the effect of HRF as a formula on platelet aggregation in healthy subjects.

MATERIALS AND METHODS

Study design and subjects

We conducted a randomized placebo controlled trial to compare the platelet function before and after administration of HRF or placebo in healthy volunteers. Subjects were male and female with age between 18 and 45 years, and considered healthy based on medical history, physical examination and laboratory test (hematology, blood biochemistry, lipid profiles, urine analysis and urine pregnancy test in female volunteers). Each subject was tested for baseline platelet aggregation. Subjects were excluded if they were using any drugs that are known to affect platelet aggregation for at least two weeks before the

study. Eligible volunteers were instructed to abstain from alcohol and smoking during the study. All participants provided verbal and written informed consent.

The study protocol and related materials were reviewed and approved by Siriraj Institutional Review Board (Si 548/2009) of Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The study was conducted according to the International Conference on Harmonisation Good Clinical Practice (ICH-GCP) guidelines⁵ at the Siriraj Clinical Research Center (SICRC), Siriraj Hospital, Bangkok, Thailand.

According to the latest Thai National List of Herbal Medicinal products, the dose of 900-1,500 mg three times a day is recommended to relieve fever in adults. In this study, HRF was taken at this maximum dose, every 8 hours for a total of 3 doses. Subjects were randomly allocated into HRF or placebo groups (Fig 1) using a pre-printed randomization table. The volunteers were under medical observation by physicians and nurses starting from the first dose until 2 hours after the last dose. Physical examination and vital signs measurement were assessed prior to HRF administration as baseline and reassessed whenever subjects reported or experienced any adverse events. Blood samples were drawn at pre-dose for baseline, 8 hours after the first dose for the early onset of HRF on platelet aggregation, 32 hours after the first dose for the effect on platelet aggregation after multiple HRF doses, and 7-10 days after the first dose in the subjects whose previous platelet aggregation result was altered from that of baseline. Moreover, to avoid the diurnal variation of platelet aggregation, blood samples were collected at nearly the same time in the morning as shown in the protocol (Fig 2). Safety of HRF was monitored throughout the study period. All reported adverse events were evaluated and recorded by study physicians.

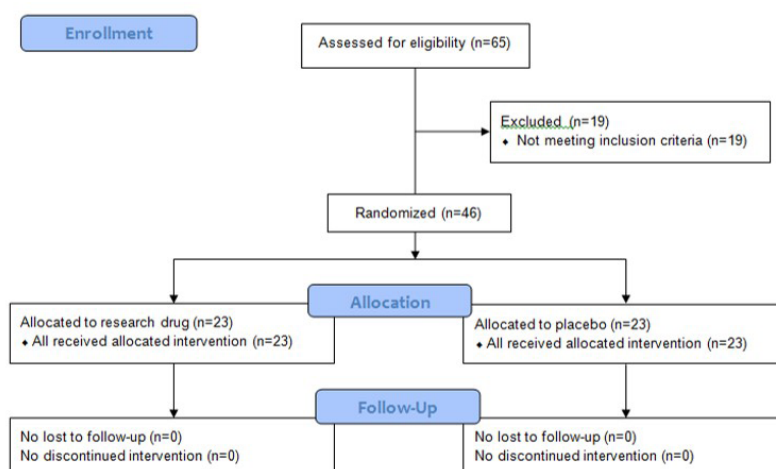


Fig 1. Subject recruitment, allocation and follow-up.

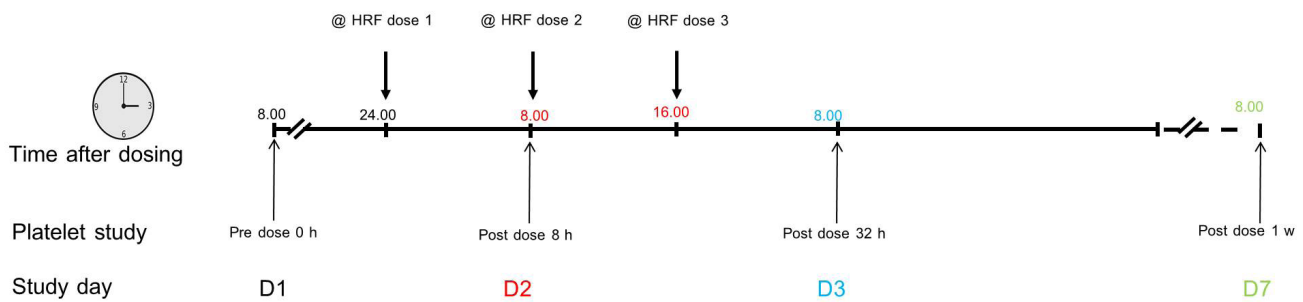


Fig 2. Protocol of blood sample collection that was performed nearly the same time in the morning.

Study drugs and placebo

HRF capsules (300 mg each) and placebo were manufactured under good manufacturing practice (GMP) by the Herbal Medicines and Products Manufacturing Unit, the Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Each batch had fingerprint as a quality assurance.

Preparation of HRF solution for chromatogram fingerprinting

HRF recipe was accurately weighed and dissolved with 80% ethanol or 50% methanol, mixed and centrifuged at 15,000 rpm for 10 minutes at 4 °C. After precipitation, each supernatant was filtered through a 0.2 mm membrane filter and used for thin layer chromatography (TLC) or ultra-performance liquid chromatography (UPLC) analysis.

TLC chromatogram fingerprinting

Filtrated supernatant, mentioned above, was loaded to HPTLC plates coated with silica gel 60 F 254 on aluminium sheets (Merck, Germany) by sample applicator (Camag Linomat 5, Switzerland). Solvent system of hexane:ethyl acetate:acetic acid (31:14:5 v/v) was used as mobile phase for phenolic separation. The detection was examined under UV 254 nm, 366 nm and visible light after spraying with fast blue salt (FBS).

UPLC chromatogram fingerprinting

Another filtrate was injected into the UPLC with photodiode array detector (Water, Milford, A, USA). Separation was performed on an Acquity UPLC column, 100 mm × 2.1 mm I.D; particle size 1.7 mm (Water, Milford, MA, USA). The experiment was performed at a wavelength of 400 nm for 14 minutes.

Platelet aggregation study

The determination of platelet aggregation was

performed within 4 hours after blood drawn according to the recommendations for the standardization of light transmission aggregometry.⁶

Whole blood was collected in vacutainer tube mixed with 3.8% sodium citrate as an anticoagulant and centrifuged at 250 g for 10 minutes at room temperature. 300 microliters of platelet-rich plasma (PRP) was further centrifuged at 3,200 g for 2 minutes to obtain platelet free plasma (PFP). Platelet aggregation was determined by Born's technique⁷ using aggregometer (AggRam, Helena, USA). Platelet agonists, include epinephrine, adenosine diphosphate (ADP) and collagen, were purchased from Helena Chemical, USA.

Platelet aggregation was recorded as an increase in the light transmission after adding an agonist. The maximum amplitude of platelet aggregation was then measured and expressed as a percentage of difference between light transmission of PRP and of PFP. Percentage of aggregation was calculated from the below formula.

$$\% \text{ aggregation} = \frac{(A-B)}{(A-C)} \times 100$$

Note:

A = light transmission of PFP

B = light transmission of PRP with agonist_{maximum peak}

C = light transmission of PRP_{baseline}

Statistical analysis

Descriptive statistics were used to compare demographic data between subjects in HRF and placebo groups. The percent of platelet aggregation that was normally distributed were shown as means ± SD. Non normally distributed data was shown as median and range. The difference between each time point in each group was analyzed by one-way ANOVA and between groups by two-way ANOVA using statistical software GraphPad Prism 5 and SPSS respectively. Non parametric test was used when the data was not normally distributed. *P*-values less than 0.05 were accepted as statistically significant.

RESULTS

Subjects

Of the 65 subjects screened, 46 participants, with equal numbers of male and female, participated in this study. All participants completed the study. A total of 5 adverse events occurred including abdominal pain, loose stools in HRF group and vomiting, common cold and pancytopenia in placebo group (Table 1). All adverse events were spontaneously recovered. Three events,

abdominal pain, loose stools and vomiting were judged as related to HRF or placebo administration according to the study physician.

Demographic characteristics

Demographic and baseline characteristics were generally not different between the two groups, as shown in Table 2. The mean of height was higher in the placebo group than in the HRF group, corresponding to a greater number of male participants.

TABLE 1. Adverse events occurred in this study.

Adverse events	Number of adverse events	
	HRF	Placebo
Abdominal pain	1	-
Loose stools	1	-
Vomiting	-	1
Common cold	-	1
Pancytopenia	-	1

TABLE 2. Demographic data and baseline laboratory value.

Baseline characteristics	HRF (n=23)	Placebo (n=23)	P-values
Age (years)	29.2 ± 5.5	27 ± 5.1	0.18
Sex (number)			
Male	9 (39.1%)	14 (60.9%)	-
Female	14 (60.9%)	9 (39.1%)	-
Body weight (kg)	58 ± 10	62.9 ± 9	0.1
Height (cm)	164 ± 9	170 ± 7.8	0.02*
Body mass index (kg/m ²)	21.4 ± 2	21.6 ± 1.9	0.69
Hb (g/dL)	13 ± 1.3	13.8 ± 1.5	0.06
Hct (%)	39.2 ± 3.9	41 ± 5.5	0.06
WBC (x 10 ³ /uL)	6571 ± 1468	6616 ± 1142	0.9
Platelet (x10 ³ /uL)	279304 ± 65954	272782 ± 50988	0.89
FBS (mg/dL)	81.5 ± 7.9	84.2 ± 8	0.27
Total cholesterol (mg/dL)	179.7 ± 16.4	171.9 ± 25.1	0.22
Creatinine (mg/dL)	0.8 ± 0.2	0.8 ± 0.2	0.07
AST (U/L)	17.8 ± 3	19.2 ± 3.6	0.17
ALT (U/L)	11.9 ± 4.6	15.3 ± 7.8	0.12

Data are mean ± SD

* statistically significant at P < 0.05

Study drugs: Chromatogram fingerprinting

All of HRF and its extracted components were analyzed by TLC and UPLC to confirm the quality control of the recipe. The chromatogram fingerprints were shown in the Fig 3 and Fig 4, respectively.

Platelet aggregation

There was no significant change of average percentage of maximum platelet aggregation measured before and after dosing observed in both groups, except for the collagen-induced platelet aggregation in HRF group (Fig 5). Subgroup analysis by sex also showed no significant change in percentage of maximum aggregation.

Moreover, subgroup analysis of participants to different patterns of platelet aggregation was performed. According to the study of Ketsa-ard et al; Yee et al and Hayes et al⁸⁻¹⁰ the pattern of aggregation was classified to hypo-normal if platelets respond to 25 μ M epinephrine only in a primary phase of aggregation, while hyper-aggregation occurred if platelets respond to 1 μ M epinephrine in a secondary phase of aggregation. Normal aggregation represents platelets respond to 1 and 25 μ M epinephrine in a concentration-dependent manner in both primary and secondary phases of aggregation. Neither of these patterns had significant effects on the percentage of aggregation at any time during HRF administration.

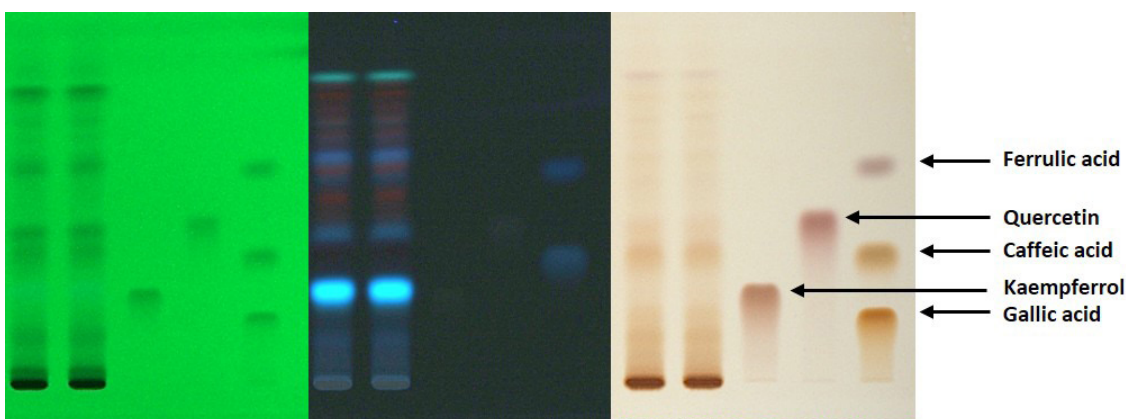
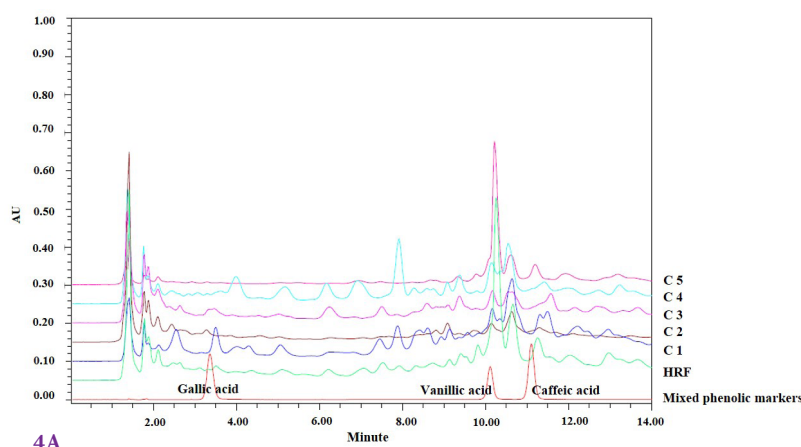
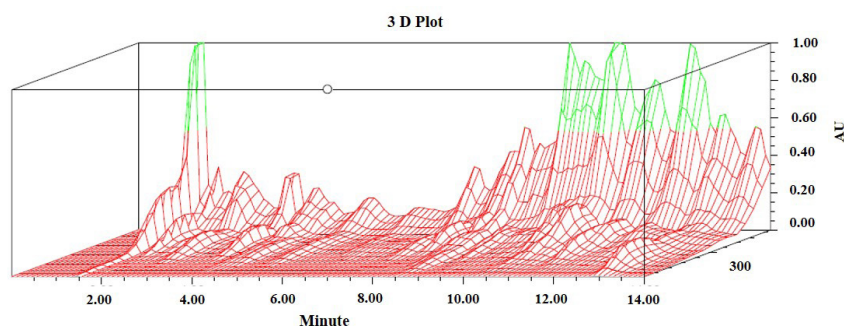


Fig 3. HPTLC fingerprints of HRF developed in mobile phase of hexane: ethyl acetate : acetic acid (31:14:5 v/v) visualized under UV 254, 366 nm and visible light (after spraying with fast blue salt; FBS). Lane 1-2: HRF, Lane 3-5: phenolic markers (from bottom to top); gallic acid, kaempferol, caffeic acid, quercetin and ferulic acid.



4A



4B

Fig 4. UPLC-PDA chromatogram, two dimension (A) and three dimension (B), of HRF and 5 components: C1=*Harrisonia perforate* Merr (Kon-ta), C2=*Capparis micracantha* DC (Ching-chee), C3=*Clerodendrum petasites* S.Moore (Thao-Yai-Mom), C4=*Ficus racemosa* Linn. (Madua-Chumporn), C5=*Tiliacora triandra* (Colebr.) Diels. (Ya-nang).

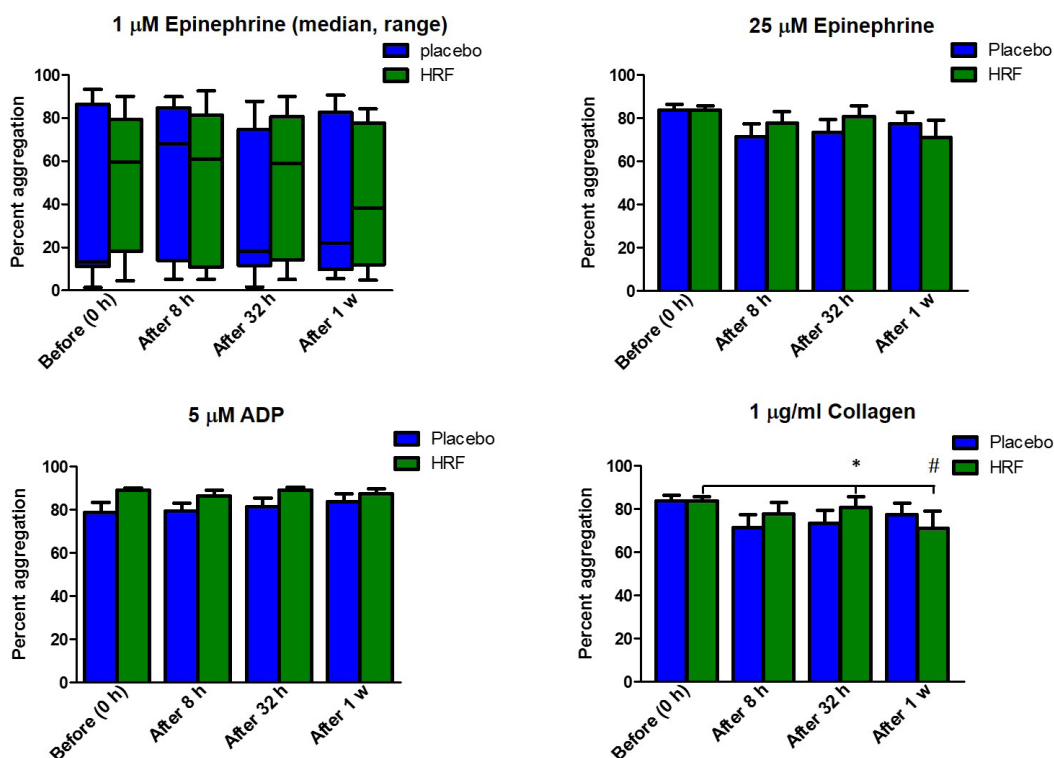


Fig 5. Percentage of platelet aggregation in response to each agonists at various times. (* p= 0.04, # p=0.05 using Repeated ANOVA)

DISCUSSION

To our knowledge, this is the first study that examined, *ex vivo*, the effect of HRF on platelet aggregation in humans. Platelet aggregation is a highly complex sequence of events involving multiple receptors and signaling pathways, therefore, many agonists could theoretically be used in testing platelet aggregation. Three agonists including collagen ADP and epinephrine, were used in this study. Collagen itself does not induce platelets to aggregate but subjects platelet to change shape and substances release. ADP is an agonist-induced platelet aggregation. Epinephrine potentiates the aggregation response to ADP and some other mediators. Induction of platelet by collagen, ADP or epinephrine possibly involves at least 3 different pathways of platelet aggregation. We further classified subjects into hyper aggregation normal aggregation and hypo or disaggregation for subgroup analysis. Generally, no significant difference in percentage of platelet aggregation was observed between HRF and placebo groups. Moreover, subgroup analysis according to platelet status including gender revealed no significant change of percent aggregation between before and after treatment. However, a significant effect on platelet aggregation when induced by collagen was observed in HRF group at 32 hours and around 1 week after the first dose. Collagen-induced platelet aggregation

is a complex process and involves synergistic action of many G-protein-coupled receptors and their ligands. The precise role of these receptors in the processes of activation and aggregation is still poorly defined.¹¹ Increased platelet aggregation at 32 hours while decreased platelet aggregation at 1 week after the first dose of HRF might be due to the complexity of these receptors. The metabolites during HRF metabolism might interact with the different receptors in the process of platelet activation and aggregation which alter platelet aggregation. However, this finding remains unclear. Moreover, at 1 week after the first dose of HRF, an affected platelet should be replaced by the new platelet because the average life span of a platelet is about 8-10 days.¹² Nevertheless, this preliminary data suggests HRF consumers to be cautioned about to bleeding risk. Another explanation for HRF effect on platelet aggregation is phenolic compounds which are well known for being particularly attributed to the antiplatelet activity of plant extracts.¹³ *Ficus racemosa* Linn., one of HRF's components, is an excellent source of phenolic compounds exhibiting significant antioxidant activity in terms of radical scavenging and anti-lipid peroxidation activity.¹⁴ Therefore, *Ficus racemosa* Linn. may exert some inhibition effects on platelet activity. On the other hand, *Ficus racemosa* stem bark extract, despite being a rich source of phenolics and flavonoids,

was reported to induce aggregation of human platelets.¹¹ These contradictory evidences indicated the complicated effects of HRF on platelets. Moreover, not only phenolic compounds, but also other phytochemical constituents exist in HRF which produce a variety of activities. These activities possibly overshadow antiplatelet activity of phenolic compounds. Furthermore, the interaction of the five components in HRF can result in unpredictable platelet effect.

CONCLUSION

This study was the first *ex vivo* investigation of the effects of HRF on human platelet aggregation. The significant change in the percentage of platelet aggregation after HRF administration was observed when collagen is an agonist. Further study is needed to confirm and elucidate. Although no serious adverse events were reported, an awareness of bleeding risk when taking HRF is recommended.

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