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ORIGINAL ARTICLE

Evaluation role of Neem Leaf Extract on Mitotic Catastrophe in DMH-induced Colon Carcinogenesis in rats

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ABSTRACT
The present study attempted to explore the evaluation role of Neem leaf extract on Mitotic Catastrophe in DMH-induced colon carcinogenesis. Rats were segregated into four groups viz., normal control, DMH treated, Neem (Azadirachta indica) treated, DMH+ Azadirachta indica treated. Colon carcinogenesis was induced by weekly subcutaneous injections of DMH [30mg/Kg body weight] for two time durations of 10 weeks and 20 weeks. Azadirachta indica in the form of aqueous Azadirchta Indica leaf extract (AAILE) was administered orally at a dose rate of 100 mg /Kg/Body weight on alternate days three times a week for the entire duration of the study. DHM treatment resulted in a significant increase in the formation of micro nuclei (mitotic catastrophe) in the colons of DMH treated rats at both the time intervals. However, supplementation with Azadirachta indica significantly reduced the formation of micro nuclei in the DMH treated rats. Further, histological alterations were also observed following DMH treatment which however was appreciably prevented upon co-administration with Azadirachta indica. The study, therefore, concludes that Azadirachta indica proves as a useful agent in modulating DMH induced genotoxicity in rats.
Keywords: *Azadirachta indica, Dimethylhydrazine, Micronuclei, Mitotic, Neem, Catastrophe.*

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INTRODUCTION

Colon carcinogenesis ranks third among all cancers, the world over [1]. Experimental animals [2], the studies form our laboratory as well as earlier studies from various other laboratories; have proved the specificity of the DMH for induction of colorectal carcinogenesis [3-7]. The prime focus of the present study was to investigate modulatory potential of *Azadirachta indica*, if any, on mitotic catastrophe and colon histo-architecture of rats treated with DMH. Mitotic catastrophe is an important indicator of genotoxicity caused by carcinogens. It basically involves the formation of micronuclei in the cells due to faulty mitosis. It is a condition that is opted by a cell to counter rapid proliferation when apoptosis is withheld due to altered expressions of key apoptotic regulatory genes like bcl-2 and bax [8,9]. *Azadirachta indica* (neem) possess potent ability to remove cancerous phenotype as proved by various earlier studies, the world over. [10]. The chemopreventive effects of dietary doses of aqueous Neem leaf extract was studied on in-vivo murine system against 3H-B-á- P(Benz-á-pyrene)-induced initiation of cancer[11]. As colorectal carcinogenesis is primarily a result of improper dietary habits, the dietary chemopreventive agents are being explored extensively. Many dietary compounds are being investigated for their prominent roles in chemoprevention of colorectal cancer. *Azadirachta indica* is the chemopreventive of interest in the present study. Though *Azadirachta indica* has desirable biological activities against cancer but there is a paucity of information with regard to its modulatory role on mitotic catastrophe during colon carcinogenesis. So, the present study was focused to evaluate the efficacy of *Azadirachta indica* as a chemopreventive agent in modulating mitotic catastrophe during DMH induced colon carcinogenesis in rats.

MATERIALS AND METHODS

Animal care procedures followed in the current study were approved by the University Ethical Committee on Experimental Animals for Biomedical Research. Male Wistar rats weighing 120-150 g were procured from the Central Animal House of the Panjab University [Chandigarh, India]. All the animals were housed in polypropylene cages under hygienic conditions, and were provided with pellet diet and drinking water ad libitum. 40 Animals were segregated randomly and equally into four treatment groups. Animals in Group I served as normal controls and were given water and diet ad libitum. Rats in this group also received 1mM EDTA-saline subcutaneously per week, which was used as a vehicle for giving DMH treatment to group II animals.

Animals in Group II were given a weekly subcutaneous injection of DMH at a dose level of 30mg/Kg body weight dissolved in 1mM EDTA-normal saline [pH-6.5], for two time durations of 10 and 20 weeks [12]. Group III animals were given *Azadirachta indica* in the form of aqueous Azadirchta Indica leaf extract (AAILE) which was administered orally at a dose rate of 100 mg /Kg/Body weight on alternate days three times a week for the entire duration of the study. Animals in Group IV were given a combined treatment of DMH as well as *Azadirachta indica* in a similar manner as was given to Group II and Group IV animals, respectively.

Micronucleus Assay

The micronucleus assay was carried out according to the method described by Schmid, 1975[13]. The tissues were washed with chilled homogenizing buffer (24mM Na2EDTA buffer pH 7.5 containing 75 mM of Nacl) and then homogenized at 500-800 rpm. The homogenates were then centrifuged at 7000 rpm for ten minutes. The supernatants were removed and fresh homogenizing buffer was poured to resuspend the pellets. The drop of the suspension was put at one end of the precleaned grease free microscopic slide and was spread using the coverslip held at 45 degrees into a smooth smear. The slides were air dried in dust free environment for at least 12 hrs before staining. The colon cells were then stained with May and Gurndwald stain for 1-2 min. followed by staining with Giemsa for 10 min. The slides were rinsed twice in distilled water dried and then rinsed in methanol. The slides were then cleared in xylene and mounted in DPX. Cells were counted for the presence of micronuclei using light microscope.

Histopathological studies

For the histopathological observations at light microscopic level, fresh tissue pieces of colon were immersion fixed formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5-7 ìm thick sections were double stained with hematoxylin and eosin and observed under light microscope.

Statistical analysis

The statistical significance of the data was determined by using one-way analysis of variance [ANOVA] followed by a multiple post-hoc test Least significant Difference [LSD] with 5% considered significant. The results are represented as Mean ± S.D. micronucleated cells. In the rats that received DMH treatment for both the durations of 10 and 20 weeks, a significant increase in the extent of micronucleus induction was observed when compared with normal rats (Table I). Moreover, in the rats that received *Azadirachta indica* along with DMH treatment, a significant reduction in the score of micronucleated cells was observed as compared to DMH treated animals.

RESULTS

The results obtained from various experiments conducted in this study are depicted in Tables I and II and Fig 1 -8. The data from various treatment groups have been compared with the normal control animals. However, results obtained from *Azadirachta indica* +DMH treated group were additionally compared with that of DMH group.

Micronucleus Assay

The frequency of micronucleus induction was expressed as percentage number of micronucleated cells. In the rats that received DMH treatment for both the durations of 10 and 20 weeks, a significant increase in the extent of micronucleus induction was observed when compared with normal rats (Table I). Moreover, in the rats that received *Azadirachta indica* along with DMH treatment, a significant reduction in the score of micronucleated cells was observed as compared to DMH treated animals.

HISTOLOGICAL CHANGES

The colons of normal control and *A. indica* treated rats showed normal histoarchitecture after hematoxylin and eosin staining (H&E) of the sections (Fig. 1, 3, 5, 7). The rats receiving 10 weeks of DMH treatment showed changes in the colon histoarchitecture (Fig. 2) and revealed its disorganization. There was a loss in nuclear polarity. Nuclear enlargement of epithelial cells with mild inflammation of lamina propria was evident. However, no signs of hyperplasia/dysplasia were observed. Moreover, *A. indica*

treated rats showed near normal structure of the colons as was seen in normal control animals (Fig. 3). Rats which received 10 weeks of combined *A. indica* and DMH treatments, the altered histoarchitecture showed improvement. The size and shape of the cells were uniform with mild nuclear enlargement (Fig. 4).

In the rats which received DMH treatment for 20 weeks, well differentiated signs of dysplasia were observed (Fig. 6). Nuclei were enlarged, thickening of epithelium was seen, cells were hyperchromatic and showed increased mitotic activity. Simultaneously, there was a loss in nuclear polarity However, following 20 weeks of *A. indica* treatment to DMH treated rats; histoarchitecture revealed no signs of dysplasia but indicated a little loss of nuclear polarity (Fig. 8).

DISCUSSION

The present study observed that coadministration of *Azadirachta indica* with DMH modulates appreciably the mitotic catastrophe induced by DMH. Further, the finding of the histological study clearly supports the Azadirachta indica, holds a promising anticancer potential with respect to colon carcinogenesis. The present study observed mitotic catastrophe in the DMH treated rats, which reflects a significant increase in the number of micronuclei in colon cells. The above assay revealed the damage to the chromosomes in the form of breaks and fragments which appeared as micronuclei in the proliferating cells. The results are in sync with the study by [14].

Similar observation was also observed during benzo(a)pyrene lung carcinogenesis in our earlier study [15]. Mitotic catastrophe is a compensatory mechanism which involves faulty mitosis and is adopted by a cell when the programmed cell death (apoptosis) fails to control rapid proliferation of the cells during cancer. Further, the histological changes revealing well differentiated signs of dysplasia and hyper proliferation of colonic cells support the above observation and signify inactivation of apoptosis. The observation is in corroboration with the study by Slatter et al [16]. *Azadirachta indica* co-administration significantly decreased the formation of micronuclei in the DMH treated rats. This observation confirms the anti-clastogenic effects of *A. indica* against DMH induced genotoxic damage to the colon cells. The earlier studies supported our observations, whereas extracts of *A. indica* were documented to exert anti-clastogenic effects though several mechanisms. Subapriya *et al*. [17] evaluated the effects of pretreatment with *Azadirachta indica* on DMBA-induced genotoxicity and oxidative stress in male Swiss albino mice. In another study, Subapriya *et al* [18]. evaluated the effects of pretreatment with *Azadirachta indica* against n-methyl-n'-nitro-n-nitrosoguanidine (MNNG)-induced genotoxicity in male Swiss albino mice. Interestingly, *Azadirachta indica* supplementation to DMH treated rats appreciably modulated the histopathological changes. These histological modulations by *Azadirachta indica* could be ascribed to the ability of *Azadirachta indica* in maintaining the membrane integrity by scavenging the free radicals. Secondly, *Azadirachta indica* could have moderated the hyper-proliferation of the colonic cells by stimulating apoptotic genes which then contributed towards observed decrease in micronuclei formation. The study therefore concludes that *Azadirachta indica* acts as an effective modulator of mitotic catastrophe and histopathological changes during colorectal carcinogenesis.

Table I. Effect of *A. indica* on % incidence of micronucleus formation in colons of rats subjected to 10 and 20 weeks of DMH treatment

Groups	Percentage of micronucleated cells
Normal control	0.33 ± 0.40
DMH treated	1.83 ± 0.60 b
***A. indica* treated**	0.50 ± 0.35
DMH+ *A. indica* treated	1.10 ± 0.41 a,x

Values are expressed as means ± S.D; aP<0.01 and bP<0.001, when compared with normal control group; xP<0.01, when values of Group IV are compared with Group

Table II. Effect of *A. indica* on % incidence of micronucleus formation in colons of rats subjected to 20 weeks of DMH treatment

Groups	Percentage of micronucleated cells
Normal control	0.16 ± 0.25
DMH treated	1.91 ± 0.64 b
***A. indica* treated**	0.60 ± 0.41
DMH+ *A. indica* treated	1.20 ± 0.41 b,x

Values are expressed as means ± S.D.
aP<0.01 and bP<0.001, when values are compared with normal control group.
xP<0.01, when values of Group IV are compared with Group

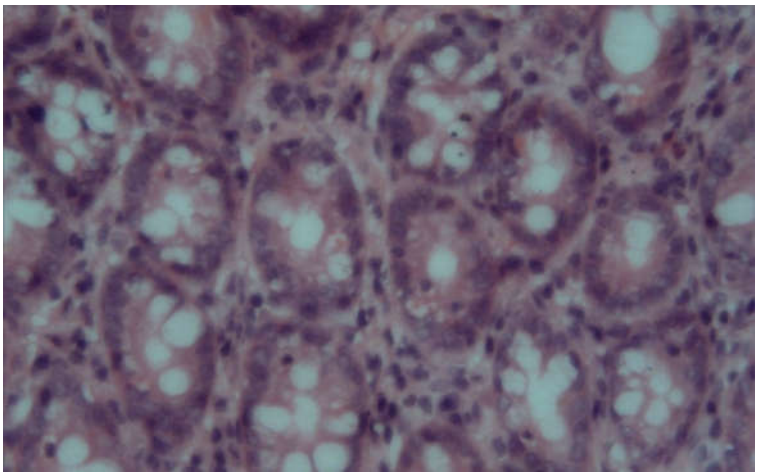

Fig 1. Showing the normal histoarchitecture of rat colon (10 week).

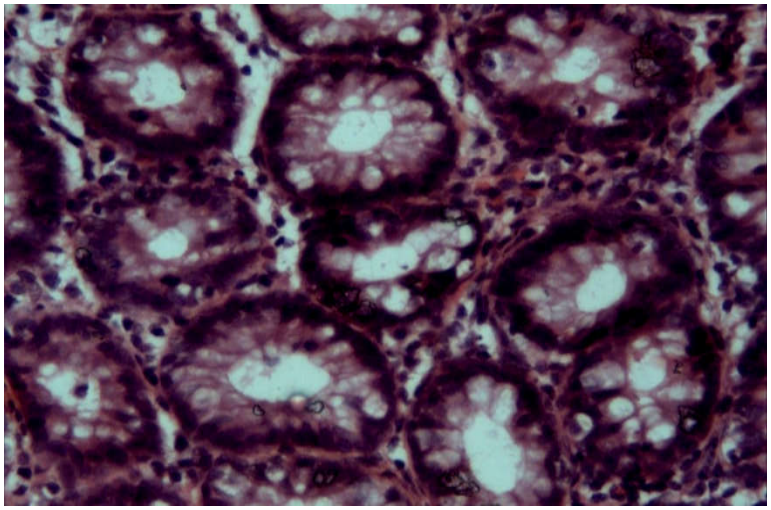

Fig 2. Showing the altered colonic histoarchitecture of rats which received 10 weeks of DMH treatment

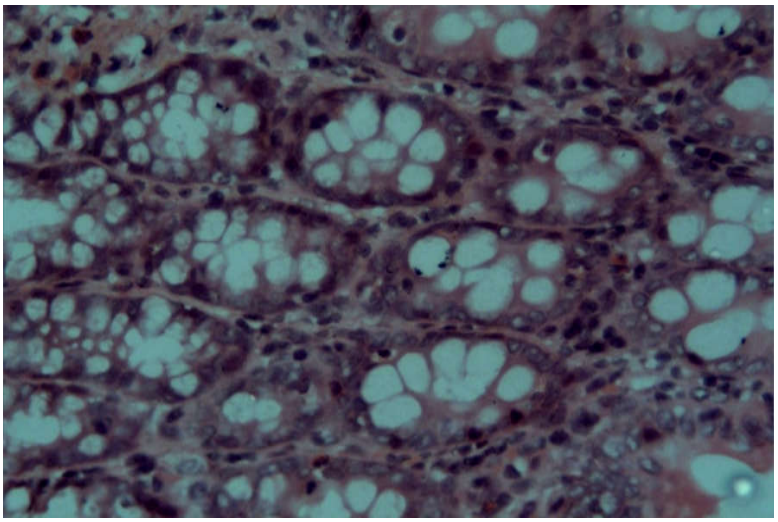

Fig3. Showing the colonic histo-architecture of rats which received 10 weeks of A. indica treatment

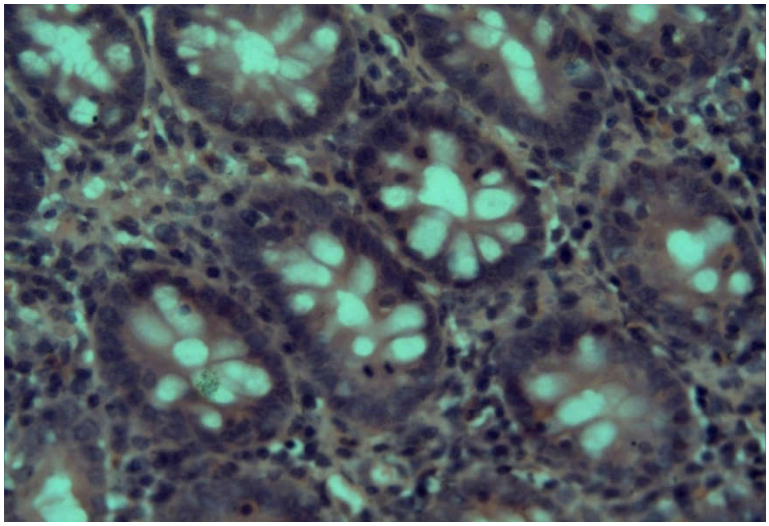

Fig.4. Showing the colonic histo-architecture of rats which received 10 weeks of DMH + *A. indica* treatment

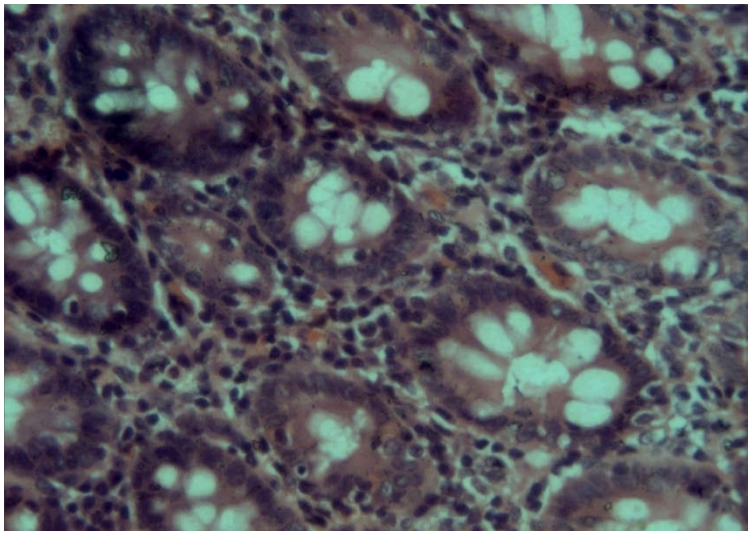

Fig5. Showing the normal histo-architecture of rat colon (20 week)

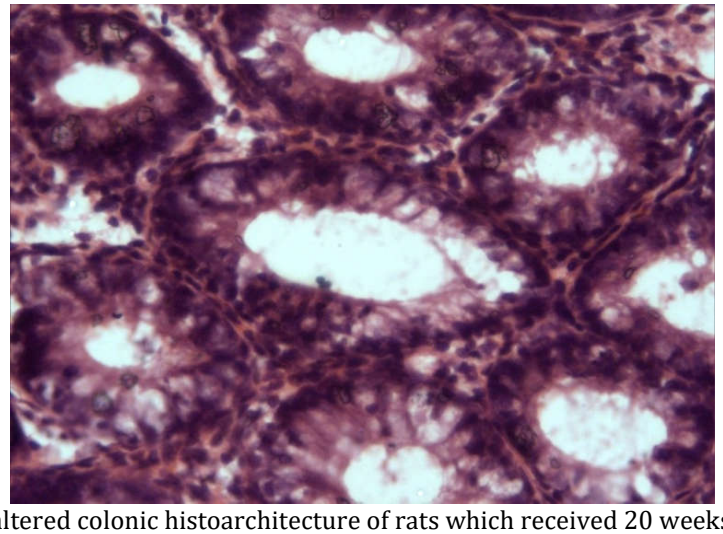

Fig.6. Showing the altered colonic histoarchitecture of rats which received 20 weeks of DMH treatment

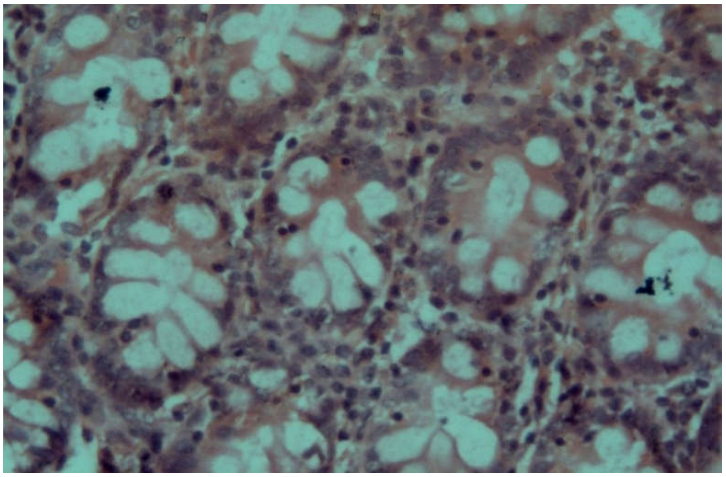

Fig.7. Showing the colonic histoarchitecture of rats which received 20 weeks of A. indica.

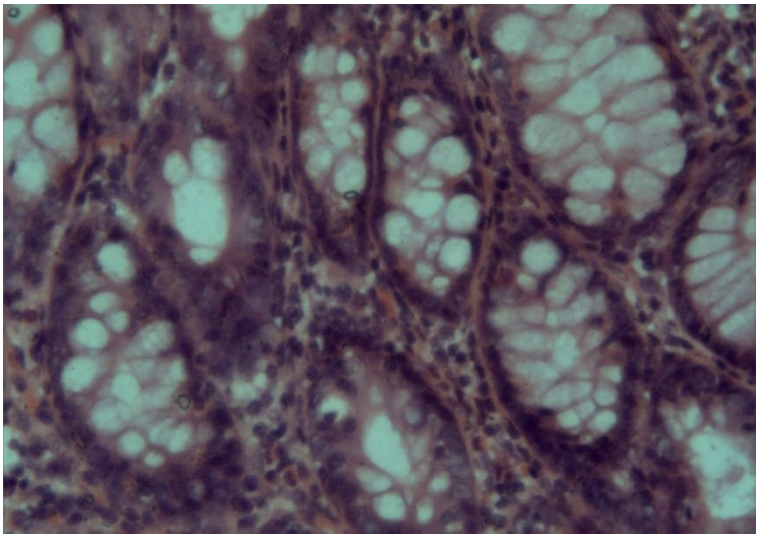

Fig.8. Showing the colonic histoarchitecture of rats which received 20 weeks of DMH + A. indica treatment.

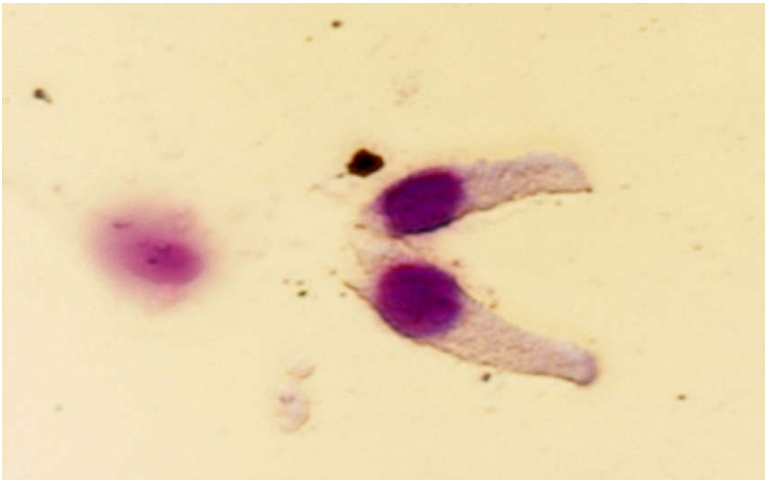

Fig. A. Showing the normal colon cells.

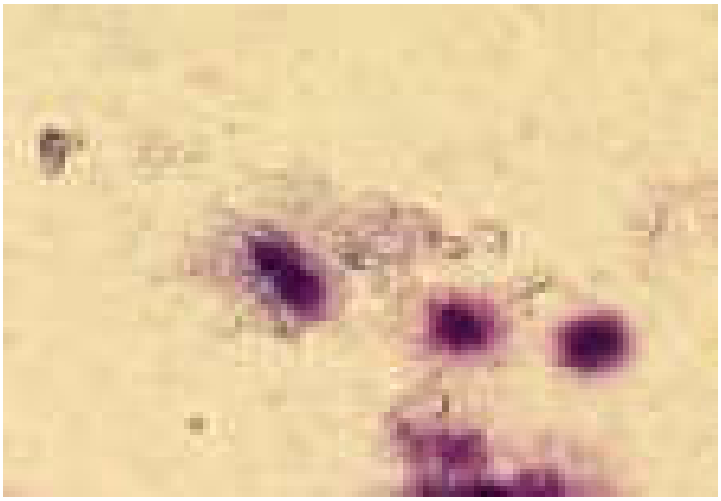

Fig. B. Showing the micronucleated colon cells

COMPETING INTERESTS

The authors have declared that no competing interest exists

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