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Journal Articles

CONCENTRATION OF SERUM AMYLOID A IN CLINICALLY NORMAL ENDURANCE HORSES IN MALAYSIA

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SUMMARY

Endurance horses continuously undergoing training. This will cause inflammation which leads to acute phase reaction with the production of acute phase protein, especially serum amyloid A (SAA). The purpose of this study was to establish concentration of SAA in normal endurance horses in the blood serum using two-site enzyme linked immunoassay (ELISA) technique. Horse sera were aliquoted from blood taken from jugular venipuncture. The highest concentration of SAA was observed in horses rested between 12 months and 24 months. The lowest concentration of SAA was noticed in horses rested more than 24 months. All the horses between 6 and 11 years old have high SAA concentration. When resting intervals were compared against gender of the horses, it was noted that all mares have high SAA concentration compared to gelding and stallion. Whereas SAA concentration in Thoroughbred horses were high compared to Arabian horses in all rest intervals. The SAA concentration in horses rested more than 24 months was low most probably because the horses recovered well from the inflammatory process happened during the endurance race.

Keywords: Serum Amyloid A, endurance horse, training, age, rest interval, Malaysia

INTRODUCTION

Although long distance horse racing began in 1892, the first modern endurance ride was held in 1955. That race was a one-day event in which the horses compete in a 100-mile (160km) race from Nevada to California, United States of America (Nagy *et al*., 2012). In 1966, Australia held the 160km Tom Quilty Gold Cup and this event is considered the second oldest modern horse endurance race. However, there are records that show Spain and Portugal have held conducting endurance horse rides since the 1950s. The European Long Distance Rides Conference (ELDRIC) was formed was formed later in 1979 and under auspices of ELDRIC organised and regulated races were conducted in e North American, Australian and South Africa. The sports developed further when the endurance races became a Fédération Equestre Internationale (FEI) discipline in 1982 and subsequently most international rides are sanctioned and governed by FEI regulations (Nagy *et al*., 2012). The endurance races are categorised according to distance, which are 40, 80, 120 and 160km races. Since these endurance rides can be potentially hazardous to the health of the competing horses, the races are punctuated with compulsory stops, during which the horses are inspected by veterinarians at the veterinary gate to ensure that the horses are fit to continue to the next phase. Due to the nature of the sport, competing horses are prone to develop career-limiting or even worse, fatal metabolic and orthopaedic disorders. Thus in these races, horses are eliminated from the ride if the metabolic status or orthopaedic conditions show that they are not fit to continue.

The acute phase proteins (APPs) are known indicators of inflammation (Pepys *et al.,* 1983; Kent 1992; Gabay *et al.,* 1999; Cywińska *et al.,* 2012). The APPs are either positive indicators that increase or negative indicators that decrease in the presence of inflammation (Pepys *et al*., 1983; Aldred and Schreiber, 1993; Gabay *et al.,* 1999). Positive APPs further divided into major APPs, which can increase to 10 to 1000 folds and in the presence of inflammatory triggers and minor APPs, which increase minimally in the inflammations and infections (Pepys *et al*., 1983; Kent 1992; Kushner & Mackiewicz, 1993). In horses, the only major positive APPs is serum amyloid A (SAA) (Mozes *et al*., 1989).

SAA is synthesised in the liver and its production ceases immediately upon recovery from inflammatory diseases or conditions (Uhlar and Whitehead, 1999). The basal serum concentration is low and the reference range of SAA is narrow (Kent, 1992; Koj *et al.,* 1996).

Equine athletes, especially endurance horses, due to the nature of their activities are prone to the development of inflammatory conditions, either through injuries or diseases. Currently, there is no references values on SAA in endurance horses that could be used to determine the presence of inflammatory diseases and conditions in these horse. Thus, the aim of this research is to determine the level of SAA in competition endurances horses during their resting interval between races to develop a reference range and baseline for SAA in clinically healthy resting endurance horses.

MATERIALS AND METHODS

Horses

The study was conducted on 40 endurance horses from Selangor and Terengganu, Malaysia. All the horses were either Arabians or Thoroughbreds comprising of 20 geldings, 16 mares, and 4 stallions, with ages ranging from 6 to 22 years. All these horses had participated in endurances competitions within the period of 6 to 43

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months prior to this study. Horses included in the study were undergoing training. The horses were clinically normal based on history and physical examination. The age, breed, and gender of the horses were recorded.

Blood Samples

Blood samples were taken from the jugular vein of horses while in stalls. The ambient temperature and environment at time of sampling were recorded. The sampling area of the jugular vein was occluded and swabbed with alcohol to sterilise. Blood samples were collected in two 3 mL plain tubes and allowed to clot for 20 minutes before centrifuging at 3400 × g for 10 minutes. Serum was separated and transferred to Eppendorf™ 1 mL tubes and stored at -20° C. The SAA levels were determined by the double sandwich ELISA.

Serum Amyloid A

Determination of the SAA was conducted using the ELISA kit (Cusabio, Immunology Consultants Laboratory, USA). The serum samples were first diluted to1/200 with by mixing 2 µL of serum sample with 398 µL of 1X diluent and the mixture mixed thoroughly. All reagents for the assay were brought into room temperature before use. 100 µL of blank and each standard solution of 2.25, 4.5, 9, 18, 36, and 72 ng/mL SAA were pipetted in duplicate into the respective well of the 96-well ELISA microtiter plate. One hundred microlitres of diluted samples were pipetted into the designated wells. The plate was covered placed on a level surface and incubated while shaking, on an orbital shaker at room temperature for 60 minutes. The contents of the wells were then aspirated and the plate washed with wash solution provided in the kit. One hundred microlitres of diluted antibody solution were pipetted into each well and the plate were covered, placed on a level surface, and incubated while shaking on an orbital shaker at room temperature for 20 minutes in a dark room. The contents of the wells were aspirated and the plate washed wash solution and the wells blotted to remove moisture. One hundred microlitres of diluted HRP-strepavidin was pipetted into each well and the plate similar incubated while shaking on an orbital shaker at room temperature for 20 minutes in a dark room. The contents of the wells were aspirated and the washed wash solution, and the plate blotted. One hundred microlitres of TMB substrate solution was pipetted into each well and the plate incubated while shaking on an orbital shaker at room temperature for 10 minutes in a dark room. The reaction was terminated by adding 100 µL of stop solution to each well. The absorbance (450nm) for the contents of the wells were spectrophometrically determined according to manufacturer's specifications.

Statistical Analysis

Statistical analysis, means and standard errors of mean were computed using SPSS® 20 for Windows® Microsoft. The results are expressed as the mean ± standard error of the mean (SEM). The SAA concentrations were compared among age group, gender, and breed of horses. Significance differences among means were determined at $\alpha = 0.05$.

RESULT

There were a total of 40 horses included in the study and the data from all these horses were analysed and presented here. Among the 40 endurance horses in this study 16 (40%) had participated in endurance race with the period ≤ 6 months, 14 (35%) 6 to 12 months, 4 (10%) 12 to 24 months, and 6 (15%) > 24 months prior to the study. The subjects comprised of 14 horses (35%) aged 6 to 11 years, 20 (50%) 12 to 18 years, and 6 (15%) > 19 years old. 36 were Arabian horses (90%) and 4 Thoroughbreds (10%) of which 20 (50%) were geldings, 16 (40%) mares and 4 stallions (10%).

Based on previous participation in endurance races, the mean serum amyloid A (SAA) concentrations were highest in horses that participated 12 to 24 months (7.22 ± 5.22 mg/L) followed in order by horses that participated ≤6 months (6.87 ± 1.74 mg/L), 6 to 12 months (3.71 ± 0.44 mg/L) and > 24 months (2.62 ± 0.30 mg/L) before the study (Figure 1).

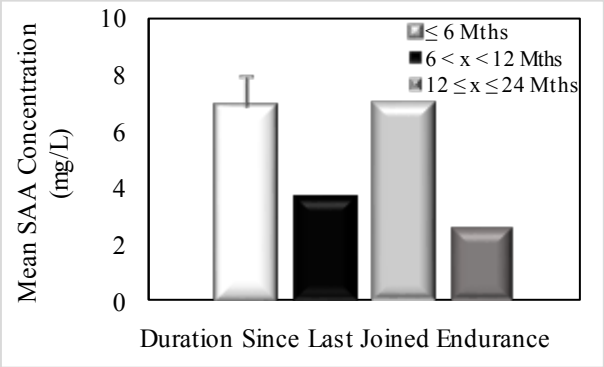

Figure 1. Comparative mean SAA concentration (mg/L) of clinically normal endurance horses (n = 40) at four periods from three stables

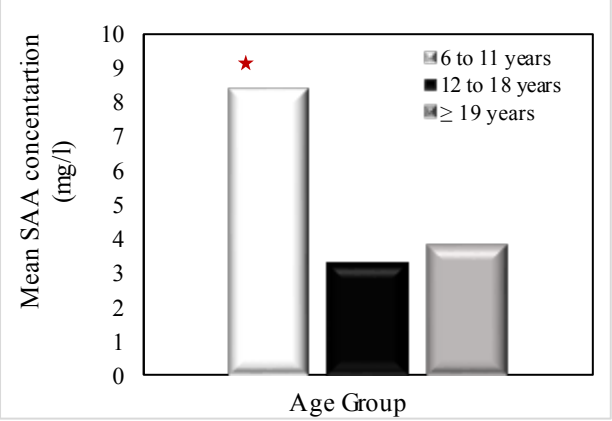

Figure 2. Comparative mean SAA concentration (mg/L) of clinically normal endurance horses (n = 40) at three age group from three stables. The '★' indicate significant mean difference

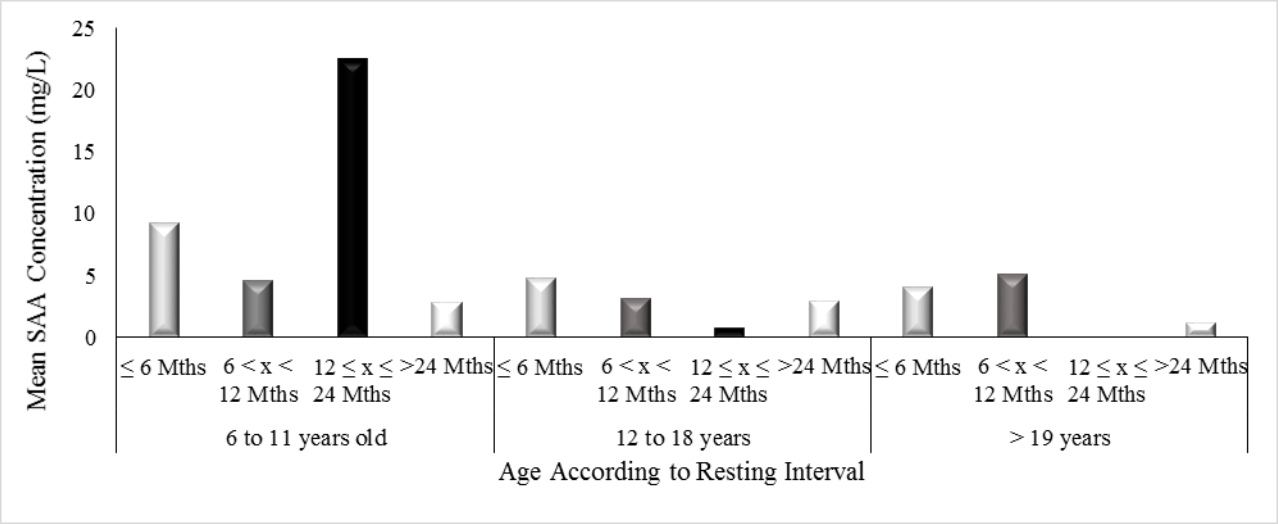

Figure 3. Comparative mean SAA concentration (mg/L) of clinically normal endurance horses (n = 40) at three age group according to rest interval from three stables

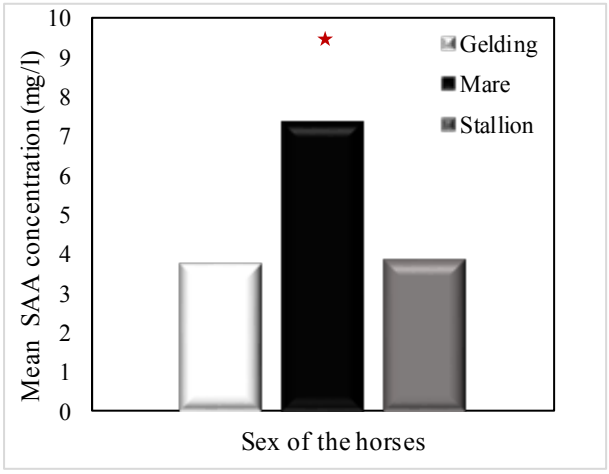

Figure 4. Comparative mean SAA concentration (mg/l) between clinically normal endurance geldings (n = 20), mares (n = 16) & stallion (n = 4)

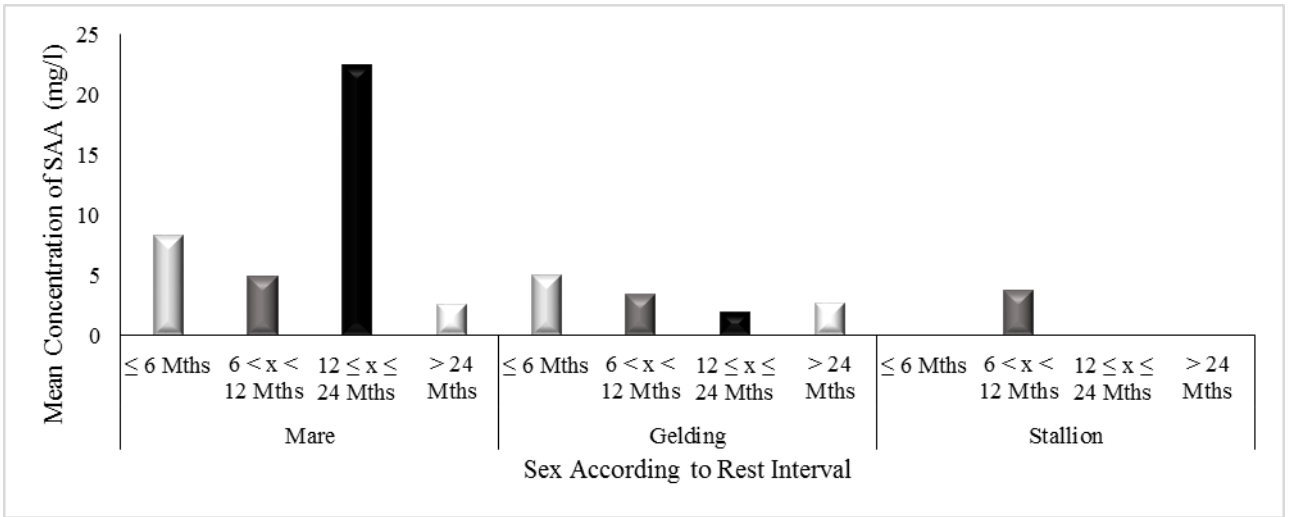

Figure 5. Comparative mean SAA concentration (mg/L) between clinically normal endurance geldings (n = 20), mares (n = 16) & stallions (n = 4) according to rest interval

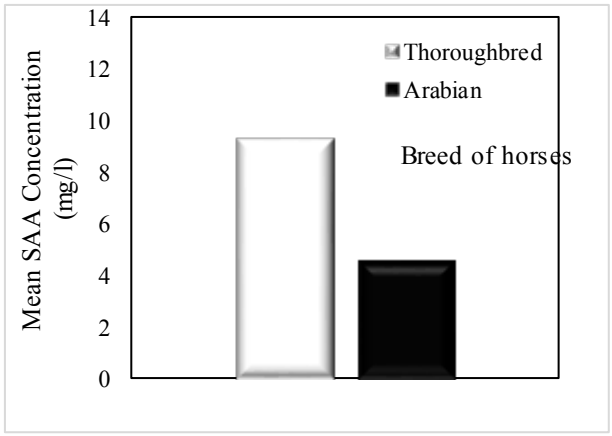

Figure 6. Comparative mean SAA concentration (mg/L) between Thoroughbred (n = 4) and Arabian (n = 36) endurance horses. The '★' indicate significant mean difference

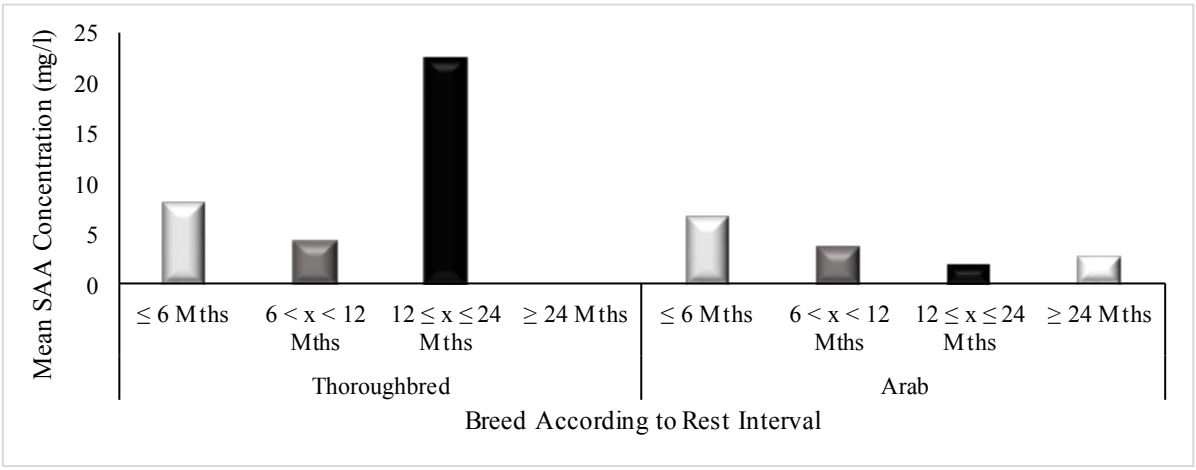

Figure 5. Comparative mean SAA concentration (mg/L) between Thoroughbred (n = 4) and Arabian (n = 36) endurance horse according to rest interval

According to age groups, the mean SAA concentrations were highest in horses aged 6 to 11 years (8.38 ± 2,25 mg/L) followed in order by horses aged >19 years (3.80 ± 0.55 mg/L) and between 12 to 18 years (3.28 ± 0.42 mg/L) (Figure 2). There was no difference in SAA concentrations between horses aged 6 to 11 years and 12 and 18 years (Figure 2).

The SAA concentrations among horses of various age groups and during the resting interval were compared. The SAA concentrations were highest in young horses age 6 to 11 years old (22.53 ± 0.00 mg/L) that participated in endurance races12 to 24 months followed by those that raced < 6 months prior to the study (Figure 3). The SAA concentration among horses of other age and prior race participation groups differ significantly.

Mares appeared to show highest mean SAA concentration (7.33 ± 1.96 mg/L) followed by stallions (3.78 ± 0.79 mg/L) and geldings (3.67 ± 0.66 mg/L) (Figure 4). Among the mares, the group that participated in endurance races 12 s to 24 months prior to the study had highest SAA concentration (22.53 ± 0.00 mg/L), followed by those that participated ≤ 6 months (8.30 ± 2.84 mg/L), 6 to 12 months (4.88 ± 0.12 mg/L), and finally > 24 months before the study (Figure 5). In geldings, the mean SAA concentrations (22.53 ± 0.00 mg/L) were highest in those that participated in endurance ≤6 months before the study and decreased with duration of rest period (Figure 5).

The SAA concentrations in Thoroughbred horses (9.25 ± 3.95 mg/L) were twice as high compared to the Arabian horses (4.57 ± 0.82 mg/L) (Figure 6). Among the Thoroughbred horses, those horses that last participated in endurance races 12 to 24 months before the study showed the highest SAA concentrations (23.53 ± 0.00 mg/L), followed in order by horses that participated ≤ 6 months (8.3 ± 2.84 mg/L), 6 to 12 months before the study (Figure 7). In Arabian horses, the SAA concentrations were highest for the horses participated ≤ 6 months (6.11 ± 6.99 mg/L), followed in order 6 to 12 months (3.67 ± 1.73 mg/L), > 24 months, and 12 to 24 months before the study (Figure 7).

DISCUSSION

This study was undertaken to determine the level of SAA in clinically normal endurance horses during the rest period in-between races. Several factors has been taken into considerations are the effect of resting interval, age, gender, and breed on the mean SAA concentrations.

The mean SAA concentrations for horses that participated in endurance races for 12 to 24 months were high. There was one outlier in this group of horses, showing very high mean SAA concentration of 22.50 ± 0.00 mg/L, while the range of SAA concentrations for the rest of horses in this group was 0.00 to 4.00 mg/L. In general, the mean SAA concentration of these horses was similar to that of horses that participated in endurance races ≤ 6 months but higher than those that participated for 6 to 12 months and > 24 months. The results suggest that the SAA concentration in endurance horses is related

to the period of participation in races. Based on the SAA concentrations levels, the group of horses in this study that participated in endurance races for durations of < 6 months and > 24 months had developed some inflammatory reactions (Cywińska *et al.,* 2013). These findings may be incidental and could also be due to orthopaedic injuries. Orthopaedic injuries in active endurance horses are common, and could be the result of tears in forelimb suspensory ligament and superficial digital flexor tendons (Gomide *et al*., 2006) and metabolic disorders (Allen *et al.,* 1988). At this juncture, the correlation between SAA concentration and duration of participation in races before resting is evident; but, there are other factors that contributes to SAA concentrations in these horses that include fitness, intensity and method of training, race conditions and environment (Jacobsen *et al.*, 2006; 2007).

Young horses aged 6 to 11 years old horses tended to have higher mean SAA concentration than their older counterparts. Young horses are to yet to adapt to the rigors of training and competition. Thus, it is presumed that they are more prone to inflammatory reactions and injuries during the on-going process of adaptation. Older horses aged 12 to 18 years in this group are adapted to the training and fit for endurance races, thus, they showed less tendency to show inflammatory responses as indicated by the low SAA concentrations (Giori *et al*., 2011). However, musculature loses elasticity with age, which could contribute to muscle injuries and damage during intense training and rigorous races. However, even in fit horses, the SAA concentrations also vary with age; that is middle aged horses with greater muscle elasticity are expected to show lower SAA than old horses, as suggest by the current study (Kenyon *et al.*, 2007).

The SAA concentrations in endurance horses vary with gender. For example, in this study of resting horses, geldings and stallions showed much lower mean SAA concentrations than mares. Mares are more affected by hormonal cycles than either geldings or stallions. It is expected that the oestrus cycle in mares could have contributed to the development of inflammatory responses in these horses. It was also previous shown that during proestrus and metestrus, mares tend to exhibit very aggressive and stressful behaviour, which may result in inflammatory response and subsequent increase in SAA concentration (Hultén *et al.*, 1997).

There is variability in mean SAA concentration among horse breeds. Thoroughbred horses had high SAA concentration than Arabian horses, due to composition of their fast twitch muscle fibres (Thiruvenkadan et al., 2009). Fast twitch muscle fibres are highly anaerobic, although provides the Thoroughbred with speed but also accumulate a lot more muscle-damaging lactate than the Arabians. Since lactate accumulation in muscles lead to exhaustion, these horses are more prone to injuries and inflammation, thus, increase in SAA concentration. Arabian horses on the other hand have slow twitch aerobic muscle fibres. These muscles generate high energy. These muscles are "fatigue-resistant" and capable of reducing the toxic end-products of metabolism. Since endurance rides are long duration submaximal intensity aerobic events, horses with slower twitch fibers are ideal for these races (Revold *et al.,* 2010). Arabian horses with these attributes have low tendency to be affected by exertions during endurance rides. This is clearly reflected by the low SAA concentrations in the Arabian horses than the Thoroughbreds as indicated by their low SAA concentrations (Adamu *et al.*, 2014).

One of the objectives of the this study was to develop the reference range for SAA in endurance horses, The study proposed the reference range for endurance race horses to be from 2.09 to 8.01 mg/L. In fact, 77.5% of the horses in this study showed SAA concentration within this range.

CONCLUSION

It is suggested that the reference range for SAA concentration of endurance race horses in Malaysia is from 2.09 to 8.01mg/L. It is proposed that SAA concentrations can be used as an indicator of fitness and ability of horses to complete endurance races. The owners should allow for longer rest periods for horses that show SAA concentrations exceeding the reference range.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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