Physical exercise and oxidative stress

Effect of intense physical exercise on the urinary chemiluminescence and plasmatic malondialdehyde

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ABSTRACT

Several studies have demonstrated that intense physical exercise causes oxidative stress in animals and humans, being possibly related, for instance, to fatigue and tissue lesions. However, the effects of high intensity exercise or training performed by athletes on the occurrence of oxidative stress are not fully clear, possibly due to methodological limitations. The objective of this study was to identify the occurrence of oxidative lesions in lipids due to physical training in athletes, through the quantification of the urinary chemiluminescence and plasmatic malondialdehyde (MDA). Post-exercise samples were collected after four training protocols: a) treadmill running (25-30 min); b) 20 km running performed by marathon runners; c) interval training accomplished by 400 m runners; d) soccer game with 50 min duration; and e) strength training with and without creatine supplementation. In the last four items, only the urinary chemiluminescence was evaluated. The conditions that presented elevation in urinary chemiluminescence after exercise completion were: a) 20 km running; b) soccer game; and c) strength training without creatine supplementation. The treadmill running increased plasmatic MDA concentration during and after its performance, and the plasmatic antioxidant capacity had an inverse behavior compared to the increase in MDA. The exercise used in this work promoted oxidative stress in a different way and this may be related to the duration and the intensity performed by athletes, and not only to intensity. In this work it was also observed that creatine ingestion associated with strength training might work as antioxidant.

Key words: Physical exercise. Oxidative stress. Metabolism. Reactive oxygen species.

INTRODUCTION

Studies on oxidative stress conducted with experimental animals and human beings have demonstrated that the increase on the metabolic activity furthers the occurrence of oxidative lesions in biomolecules[1-5]. As sportive training and competition elevate substantially the cellular metabolic activity, these lesions may present dimensions even larger in such conditions[6,7]. The oxidative stress involves increase on the formation of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), among others, generically called as reactive oxygen species (ROSs), in disadvantage of the available chemical and enzymatic antioxidant defenses[8]. There are evidences that the concentration of nitric oxide ($NO^{\bullet}$) and oxidant derivatives (reactive nitrogen species, RNSs) are high in the organism during physical exercise. However, once not many studies on the effect of physical exercise on the formation of RNSs are available, these derivatives will not be mentioned in this work any longer.

One may say, therefore, that intense physical activity may increase the ROSs intracellular formation, thus promoting oxidative stress. However, not many works on this problem involving human beings can be found because many procedures available to quantify oxidative lesions in biomolecules are invasive and of difficult application, especially those involving muscle biopsy. In this context, the objective of this work was to study the acute and chronic effect of the physical exercise on the urinary chemiluminescence in athletes involved in different intense physical activities (soccer game with 50 min duration, marathon runners, 400 m runners and strength training with and without creatine supplementation). Meanwhile, soccer players were submitted to treadmill running, where the urinary chemiluminescence, the total plasmatic antioxidant capacity and the malondialdehyde concentration (MDA) were quantified. Both the urinary chemiluminescence and the plasmatic MDA are considered as indicative of oxidative lesions in body lipids (lipid peroxidation).

MATERIAL AND METHODS

The studies were approved by the Ethics Research Committee from the Biomedicine Sciences Institute – University of São Paulo and all participants signed a consent form agreeing with the performance of the experimental procedures (physical effort, samples collecting, etc.). We emphasize that athletes who participate in this study do not smoke or use drugs forbidden by the International Olympic Committee. This fact was verified through personal interview.

Urinary chemiluminescence

The ROSs oxidative action on lipids, especially on lipids with biological membranes, promotes the formation of hydroperoxides that might be cyclized, generating heterocyclic compounds called as dioxietanes. These compounds may undergo thermal cleavage producing excited-state carbonyls (singlet or triplet, depending on the substituents on the dioxetane ring). The reduction of these carbonyls into the fundamental state with energy release as photons is the main factor responsible for the chemiluminescence observed in the urine. The luminescence intensity is, therefore, proportional to the concentration of excited carbonyls and reflects the extension of the tissue lipid peroxidation process.

The urine was collected in this work before and after the exercise and two other collections were done after the end of the treadmill running: one shortly after and another three hours later. The urine was kept frozen until analysis, being centrifuged at 10,000 g for 10 minutes at room temperature after melting. The measures

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were performed using 3 mL of urine supplemented with 1 mL of sodium phosphate 0.1 M, pH 6.2. Creatine was measured through procedure described by Heinegard and Tinderstrom[9] and its value was used to correct the samples dilution. A previous absorbance measurement of the urine sample is required in order to avoid excessive luminous absorption that could influence the luminescence quantification. In this work, whenever the absorbance of the centrifuged sample presented value above 0.3, it was diluted with Milli Q water up to this limit. The method was originally described by Lissi *et al.*[10] and the luminescence intensity measured in liquid scintillator.

Malondialdehyde (MDA)

Aldehydes are frequently produced when lipoperoxides are metabolized by aerobic organisms. Their identification provides an indirect index of oxidative lesions as result of the lipid peroxidation. MDA is one of the most abundant aldehydes resulting from tissue lipid peroxidation, especially from arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). The Yagi[11] procedure was adopted for MDA quantification by means of fluorescence, in which the plasmatic MDA concentration is measured through sample excitement in 515 nm and quantification in 553 nm. The MDA quantity was expressed in nmol per mL of plasma. The procedure's sensitiveness allows the utilization of the volume of 50 μL of blood samples collected from the earlobe. As these measurements concurred with the determination of the blood lactate obtained from the same site, it was decided for collecting of samples in blood vessel at the forearm cubital region, in which approximately 5.0 mL of blood was obtained from each collection, with a total of 1.0 mL of plasma after cooled centrifugation. 0.5 mL of plasma were used in this experiment and 0.5 mL at the dosage of the total plasmatic antioxidant capacity.

Total antioxidant capacity

The plasmatic antioxidant capacity was determined through the Whitehead[12] method, using the chemiluminescence technique to measure the antioxidant capacity of biological fluids. The light issue emission occurs when the chemiluminescent substrate (luminol) is oxidized by H_2O_2 in reaction catalyzed by "horseradish peroxidase". The stabilities and intensities of the light emitted are high due to the addition of *p*-iodine phenol and its emission depends on the constant production of intermediate free radicals derivative of *p*-iodine phenol, luminol and O_2. For this reason, the light emission is sensible to antioxidants but reestablished when these antioxidants are consumed in the reaction. As the generation of intermediate free radicals is constant, the light suppression period is directly related to the amount of antioxidant in the sample. The assay is sensible to measure the antioxidant capacity of biological samples, being expressed relatively to the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), soluble analogous of the vitamin E. The result is expressed as equivalent of trolox per liter of sample.

Simulated soccer game and strength training – The soccer training involved two groups of 12 players enrolled in the Physical Education and Sports Graduation School-USP, using the entire field, but in only 10 students the samples were collected. The duration was of 50 minutes and the strength training was composed of pulling and throwing; to elevate the value of the maximal strength in the respective exercises was the objective during the entire training period (three times a week during three months). Two weeks later, the strength training always used load close to maximal (80-90% of the 1RM) with only a few repetitions with no interval (6-8 series with 3-6 repetitions with 3-5 minutes of interval between series).

Marathon and 400 m runners training – Athletes specialized in marathon ran 20 km (heart rate of 180-185 bpm) as part of their daily training that represents the general physical preparation period in which the main objective is to work with large volumes of weekly training. The group of 400 m runners performed one daily training session with specific exercises for the anaerobic power development (training with intervals), also corresponding to the general physical preparation period. The main part of the training was composed of a session of 3 x 4 series of 300 m with intervals of 3 minutes between each exercise and of 5 minutes between each series. The rhythm required was of 36-38 seconds in each exercise, and the exercise with intervals was performed in 400-m athletics track and the 20 km running, at the University of São Paulo campus.

Strength training and creatine supplementation – 18 students from the Santos School of Physical Education, who already presented practical experience for about one year with exercises typically performed in gymnastics academies, were divided into two groups of nine students, namely: groups A and B. These students were previously submitted to muscular hypertrophy training in tests battery and entered the first and second preparation weeks. After this period, they entered in the hypertrophy training weeks. The supplementation with monohydrated creatine (CrH_2O) was used according to Volek *et al.*[13] protocol modified by Souza Jr.[14].

After 1RM tests, anthropometrical measurements were performed and during the two preparation weeks, the daily CrH_2O intake of individuals was of 30 g divided into six equal doses of 5 g with intervals of 3 and 4 hours with a daily total of 30 g in the first supplementation week (30 g/day for 7 days, total of 210 g) or placebo (maltodextrin), corresponding to the third training week. After the initial intake period, the groups received a maintenance quantity of 5 g/day for 42 days, total of 210 g, corresponding to the last 5 training weeks.

The substance CrH_2O was supplied by Protech Systems and the placebo substance (maltodextrin) was purchased from a store of nutritional supplements. The substances were conditioned into tablets by Pedrosa & Delsin – Farmácia de Manipulação Ltda., CGC 00243338/0001-74. Both substances CrH_2O and maltodextrin were equally conditioned into tablets containing 0.5 g in order to avoid participants of knowing who would be taking creatine tablets. For this experiment, the training protocol used was the protocol proposed by Souza Jr.[14], applied during eight weeks. The first two weeks (phase A) were used for the neuromuscular adjustments of the individuals and the next six weeks (phase B) were aimed at increasing the maximal muscular strength and the muscular mass (hypertrophy).

The preparation week (phase A) was composed of exercises performed with 50% of the 1RM with intervals of 120 seconds between them. The hypertrophy training (phase B) was composed of the use of 80% of the maximal load using the 1RM test as reference, 4 series of 8-10 repetitions with 120 seconds of intervals between series and of 120 seconds between exercises for another muscular group. The creatine and placebo supplementation began in the third week along with the beginning of the hypertrophy training.

The training protocol consisted of the division of the muscular groups and exercises for the respective groups as well as the specific training days. In the two first weeks, the volunteers performed 3 series with 12 repetitions in all exercises proposed, only changing the abdominal exercises, which were performed twice a week with 5 series of 20 repetitions each series and with 50% of the 1RM. For further details on the training procedure adopted in this experiment, see Souza Jr.[14].

Treadmill exercise – The training exercise in motorized treadmill was performed according to protocol developed especially to assess the athlete's maximal anaerobic capacity (Mart test = maximal anaerobic running test)[15]. The intensity was progressively increased according to the athlete's capacity to support the physical effort imposed until close to exhaustion, which remained between 25 and 30 minutes.

The athletes were submitted to treadmill test with concurrent determination of O_2 intake, heart rate, respiratory quotient and blood lactate. The blood samples collecting frequencies for lactate and other parameters dosage are found in tables 1 and 2. Blood for lactate dosage was collected from the left earlobe with the aid of heparinized capillaries with capacity of 25 µL of blood. The plasmatic MDA and antioxidant capacity determinations were performed in plasma obtained as described above, in other words, blood collected from forearm cubital region at the same time intervals. Heparinized disposable syringes were used for these blood collections and hemolyzed samples were rejected. The individual results were compared with results in rest. In other words, the athletes have been their own control group for the parameters analyzed.

TABLE 1
Blood and respiratory parameters of athletes (n = 10) indicative of treadmill exercise intensity

Plasmatic lactate (mmol/L)	Respiratory quotient	Time (min) of exercise (samples collection frequency)
0.89 ± 0.07	0.86 ± 0.06	(rest)
2.24 ± 0.11	1.23 ± 0.09	3
2.54 ± 0.10	1.34 ± 0.09	6
3.02 ± 0.12	1.40 ± 0.08	9
5.45 ± 0.17	1.47 ± 0.07	12
9.79 ± 0.20	1.52 ± 0.08	23
9.90 ± 0.18	1.57 ± 0.09	26

All results compared with rest situation are significantly higher ($p < 0.01$).
Results are expressed as average ± standard deviation.

TABLE 2
Blood and urinary parameters of athletes (n = 10) indicative of oxidative lesions in body lipids during and after the treadmill exercise

Urinary chemiluminescence (cpm/mg of creatine)	MDA (nmol/mL of plasma)	Plasmatic antioxidant capacity (µmol of Trolox – Eq/L)	Time (min) of exercise
910.0 ± 10.0	0.21 ± 0.012	483 ± 88	(rest)
	0.23 ± 0.013	470 ± 65	3
	0.28 ± 0.011* (↑ 33%)	465 ± 50	6
	0.19 ± 0.011	650 ± 90* (↑ 34%)	9
	0.16 ± 0.012* (↓ 24%)	655 ± 88* (↑ 36%)	12
	1.5 ± 0.009* (↑ 714%)	233 ± 12* (↓ 52%)	23
895.8 ± 9	1.6 ± 0.013* (↑ 761%)	200 ± 14* (↓ 59%)	26
657.8 ± 8* (↓ 28%)			3 h after

* $p < 0.01$ compared with rest situation.
MDA = malondialdehyde; cpm = counting per minute.
Results are expressed as average ± standard deviation. ↑ increase; ↓ drop.

The motorized treadmill especially developed to assess human beings used is the *Quinton 65* model, Quinton Instruments, Seatle-WA, and the blood lactate analysis occurred using automated analyzer (model 2300 stat plus, Yellow Springs Instruments, Yellow Springs, OH). The metabolic analysis system used in the respiratory quotient study was the *Teen-100* – Aeroesport Inc. USA, all equipments from Ladesp (Sportive Performance Laboratory – Department of Sports-USP). The fluorimeter employed in the MDA and the total plasmatic oxidant capacity analyses was the Yellow Springs model YSI 53 (Biochemistry Laboratory – Department of Biochemistry – IQ-USP) and the scintillation counter used was the Packard *Tri-carb* system (Department of Histology – ICB-USP). All reagents were obtained from Sigma and Merck. The statistical analysis used the Student's *t* test for non-paired means after the two-way analysis of variance.

RESULTS

Data with regard to the effects of the treadmill exercise on the parameters used with the objective of monitoring its intensity are described in table 1. One observes from the analyses that the physical exercise was effective in promoting great physical requirement in function of the blood lactate concentration obtained at the end of the test and the respiratory quotient value. All results compared to rest values in table 1 are significantly higher ($p < 0.01$). Data with regard to the effect of the exercise on the urinary chemiluminescence are described in table 2. One observes in this table that the only significant effect obtained for this parameter was the reduction (28%) three hours after the end of the exercise.

Table 2 shows significant increase on the plasmatic MDA, especially at 6 min (33%), 23 min (714%) and 26 min (761%) of physical exercise. It is also observed in this experiment that the plasmatic MDA was reduced between 9 and 12 min times, being significantly lower (24%) at 12 min of running. The total plasmatic antioxidant capacity (table 2) increased significantly at 9 min (34%) and 12 min (36%) of exercise, while decreased significantly at 23 min (52%) and 26 min (59%) of exercise.

The results obtained with marathon runners group are described in table 3. Both pre-training (31.5%) and post-training (41.5%) results were obtained for this group, being significantly higher when compared to the inactive individuals group. In the 400 m runners group (table 3), no significant difference was found in the pre- and post-training urinary chemiluminescence when compared to the inactive individuals group. In fact, some reduction tendency is verified in this parameter for this group relatively to the inactive individuals.

TABLE 3
Urinary chemiluminescence (cpm/mg of creatine) of athletes before and after physical exercise and intense training

Groups	Before	After
Inactive individuals	926 ± 39	–
Marathon runners	1,352 ± 23 (↑ 31.5%)#	1,582.0 ± 69 (↑ 41.5%)#
400 m runners	900.0 ± 97	890.0 ± 97
Soccer (1)	965.5 ± 34	1,465.0 ± 37 (↑ 58%)# (↑ 52%)*
Soccer (2)	955.5 ± 30	1,640.4 ± 26 (↑ 77%)# (↑ 71%)*
Strength training (1)	955.0 ± 29	1,630.0 ± 23 (76%)# (71%)*
Strength training (2)	956.5 ± 28	1,135.4 ± 20 (↓ 43%)**

Compared with inactive individuals; $p < 0.01$.
* After compared with before; $p < 0.01$.
** Strength training 2 (after) compared with strength training 1 (after); $p < 0.01$.
Results are expressed as average ± standard deviation. Marathon runners: 284 samples in 10 athletes were analyzed after 20 km running at heart rate of 180-185 bpm. 400 m runners: 10 athletes and 10 inactive individuals, all male (average of 24 analyses each group). Soccer (1): simulated soccer game with 50 min (average of 24 analyses of 10 players); Soccer (2): strength training of soccer players (average of 24 analyses of 10 players). Strength training (1): before and after with placebo; strength training (2) before and after with creatine with 9 students each group. ↓ drop; ↑ increase.

The results obtained for soccer players (table 3) in simulated game with 50 min duration revealed that in this condition the physical exercise may elevate significantly the urinary chemiluminescence, when compared with the rest situation (52%) and with inactive individuals (58%). In other words, despite the fact that the urinary chemiluminescence does not increase significantly after treadmill test at maximal intensity, it is possible that it would occur after the game. The same situation is observed in the strength training period for the same athletes (table 3) in which a significant increase on the urinary chemiluminescence was observed when compared with inactive individuals (77%) and with rest situation (71%).

In studies conducted with strength/hypertrophy training associated to creatine intake (table 3) as energetic supplementation, the

results suggest the existence of protective effects of the creatine on the urinary chemiluminescence. Indeed, it was verified, comparatively to the group with placebo supplementation, that the urinary chemiluminescence is significantly lower (43%) in group treated with creatine.

DISCUSSION

The exercise's intensity and duration are factors that determine the type of energetic substrate used. In the case of treadmill exercise (table 1) performed until exhaustion, the main energetic substrate used was the muscular glycogen. This conclusion is based on the fact that both the plasmatic lactate and the respiratory quotient increased significantly when compared to the pre-exercise values. Therefore, this type of exercise is characterized as of high intensity. In this condition, a decrease on the plasmatic MDA concentration was verified (table 2) after the beginning of the exercise and a drop on the urinary chemiluminescence (table 2) during and after exercise; these effects may reflect the presence of antioxidant capacity sufficient to protect the organism at the initial exercise moments but not after this phase.

Indeed, a decrease on the plasmatic MDA is verified in the beginning of the exercise in agreement with the presence of high plasmatic antioxidant content (table 2). On the other hand, the plasmatic MDA elevation after 12 min of exercise occurs concurrently to the drop on its total antioxidant capacity. It is important to emphasize that the high lactate plasmatic concentration verified in the blood of individuals who performed this type of exercise is propitious to the plasmatic antioxidant capacity, once it was already verified that it presents antioxidant properties[1,7]. Still, one may say that the plasmatic elevation of lipid peroxidation products during extended intense physical exercise may reveal the lack of capacity of the organism to support the long-term continuous oxidative stress.

The reduced urinary chemiluminescence values detected after the end of the exercise support the previous statement that the organism presents antioxidant capacity sufficient to be protected at the beginning of the exercise, but it is not in agreement with the high MDA content found after 12 min of exercise. In fact, despite the drop on the urinary chemiluminescence (table 2) shortly after the exercise has not been significant, this becomes more evident when it is verified that this parameter presents drop even higher three hours after the end of the exercise. Therefore, it is possible that the organs present at the splenic region may have sufficient antioxidant capacity to protect themselves against the exercise effects on their ROSs production systems, what may reflect in lower chemiluminescence index detected in the urine[15].

Lipoperoxides may be transferred from one organ or tissue to another and metabolized by those with high antioxidant capacity[16-18]. This is particularly important during intense exercise because the splenic region presents blood flow strongly diminished in such condition, while it is increased in the skeletal muscles. Thus, besides the possibility of lipoperoxides being metabolized by organs at the splenic region, hemodynamic factors make the transportation of these lipoperoxides towards this region difficult, elevating their concentration in plasma and in other tissues where lipoperoxides are produced during intense physical exercises. These statements are based on data obtained by Maugham *et al.*[19], who demonstrated that lipoperoxides present their concentrations increased in plasma until six hours after intense exercise like the exercise performed in this work. Therefore, due to the recovery of the renal blood flow in this time interval, the evaluation of the urinary chemiluminescence of athletes submitted to intense exercise in a time interval longer than that used in this work becomes necessary.

Although the statements previously made on treadmill exercise performed by the athletes, an elevation on the urinary chemiluminescence is verified in table 3 for soccer players after the performance of a single training session in simulated game (50 min) as well as the urinary chemiluminescence after strength training sessions in their general physical preparation phase (table 3). The urinary chemiluminescence measurement occurred before and after single training session and the training did not present suppressive effect on the urinary chemiluminescence, which remained high during all this period, probably because the objective was to increase the players' maximal strength. In other words, this type of physical training must not have provided significant effect on stimulating antioxidant defenses in order to protect the athlete's organism during the training period.

The average-duration intense physical exercise used in this experiment as well as marathon and ultramarathon[20,21] promote increases on the plasmatic MDA, but the effect of the extended exercise on the urinary chemiluminescence remains unclear. Indeed, besides the high plasmatic MDA detected by Kanter *et al.*[20,21] group, in studies involving ultramarathon runners, athletes specialized in marathon events present increased urinary chemiluminescence after training with intense physical exercise (table 3). Data presented in table 3 suggest that long-duration intense physical activities performed by human beings promote more oxidative stress than short or average-duration physical activities performed at high intensities. Furthermore, data show that the daily physical training does not develop the antioxidant capacity of marathon runners in such way to protect them from oxidative lesions due to ROSs, especially when their pre-training urinary chemiluminescence results are compared with results from inactive individuals and runners in general (table 3).

Studies involving marathon and ultramarathon runners just like the studies conducted by Kanter *et al.*[20,21] found significant correlation between the lipoperoxide plasmatic concentrations and the creatina kinase enzyme and the muscular lactate dehydrogenase (LDH-M). This group observed that the lipoperoxide concentrations and the activity of these enzymes increase in plasma after 50 miles of running. The researchers concluded that oxidative lesions occurred in the muscular fibers of these individuals during exercise in function of the increased plasmatic concentration of these enzymes. Thus, both the increased plasmatic MDA, as suggested by Kanter *et al.* studies, and the elevated urinary chemiluminescence in the present study may indicate the occurrence of oxidative tissue injuries in individuals who perform extended intense physical activities, showing the need of dietary supplementation with chemical antioxidants for these individuals.

With regard to the strength training and oxidative stress, only a few studies are available. One of them has verified that when isometric contraction prevails in the strength training, oxidative lesions may occur in biomolecules as demonstrated through lipoperoxides blood measurements[22]. Thus, it is possible that the strength training would also induce the oxidative stress. Besides, it was verified in studies involving strength training associated to creatine intake as energetic supplement (table 3) that the lower urinary chemiluminescence detected in these individuals suggests the existence of protective antioxidant effects from this substance. This probably occurs because the creatine ingested along the strength training may maintain the intramuscular ATP values longer without favoring high activation of the purine degradation cycle, main O_2-independent catabolic process responsible for the ROSs intramuscular production[23].

Not many data on the quantification of metabolic markers of the purine degradation cycle activation in intense physical exercise associated to the creatine ingestion are available. Therefore, further experiments are required in order to evaluate whether this hypothesis is plausible. However, it has been verified that the physical performance is elevated during repeated sessions of maximal exercise associated to the ingestion of 20 g/day for 5-7 days of creatine[23]. Furthermore, a drop was verified both in the ammonia and in the hypoxanthine concentrations during intense exercise

preceded by creatine ingestion[24]. Although the effect of creatine supplementation and oxidative stress during intense exercise has not yet been systematically studied, it is possible speculating that the low hypoxanthine production as result of this procedure may reduce its catabolism into xanthine and urate with lower concurrent production of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$. Therefore, it is possible that the additional creatine ingestion before intense physical exercise performed in short period of time involving strong muscular contractions may act as antioxidant.

CONCLUSION

Treadmill running performed at high intensity by human beings shows that the average-duration physical activity (20-30 min), rather than the short (strength training) and the long duration physical activities (20 km running and 50 min soccer game) reduces the urinary chemiluminescence. The first type of exercise, on the other hand, increases the plasmatic MDA concentration. Therefore, the intense exercise stimulates the oxidative stress in human beings in a different way, depending on the duration. Although the strength/hypertrophy training elevates the urinary chemiluminescence values, the creatine ingestion associated to this type of training may be beneficial as antioxidant protection.

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