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High-intensity exercise-induced oxidative stress in sedentary pre-pubertal & post-pubertal boys: A comparative study

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***Background & objectives*: High-intensity exercise results in oxidative stress in adult population. Impact of pubertal attainment on high-intensity exercise-induced oxidative stress in sedentary paediatric population has not been investigated in detail. The present study was conducted to investigate the extent of high-intensity exercise-induced oxidative stress in sedentary pre- and post-pubertal boys through estimation of serum thiobarbituric acid reactive substances (TBARS), total thiol content and activities of superoxide dismutase (SOD) and catalase (CAT).**

***Methods*: Sixty four sedentary pre-pubertal (n=32, age = 10.21±0.67 yr) and post-pubertal (n=32, age = 15.58±0.47 yr) boys performed incremental treadmill running exercise at 80 per cent of the age predicted maximum heart rate till volitional exhaustion. Blood sample (5 ml) was drawn from each individual before and after the exercise for estimation of oxidative stress markers.**

***Results*: Pre-exercise SOD activity and total thiol level showed significant positive relationship with age and were significantly higher in post-pubertal boys. Serum TBARS level, SOD and CAT activities increased while total thiol content decreased in both the groups following exercise. Post-exercise percentage change in TBARS, SOD activity and total thiol level was significantly higher in post-pubertal boys, and these variables had significant positive relationship with age. No significant intergroup variations were noted in CAT activity before or after exercise.**

***Interpretation & conclusions*: Extent of post-exercise oxidative stress increased significantly with attainment of puberty. However, baseline and post-exercise antioxidation status also increased significantly as a function of age with pubertal maturation allowing the post-pubertal boys to counter relatively higher oxidative stress more efficiently than their pre-pubertal counterparts. Post-exercise upregulation in CAT activity might not be influenced by age or pubertal maturation in this age group.**

Key words Catalase - oxidative stress - puberty - sedentary - superoxide dismutase - thiobarbituric acid reactive substance - total thiol

High-intensity endurance exercise increases oxygen demand in working muscle which in turn leads to generation of reactive oxygen species (ROS) due to leakage of electron from muscle mitochondria[1]. Such exercise-induced oxidative stress is accompanied by a parallel increase in activity of enzymatic antioxidants in the body[2,3]. The magnitude of such perturbation in redox status depends on intensity, duration and frequency of

exercise[1,3,4]. Chronic exposure to oxidative stress may lead to oxidation of protein, lipids and DNA, cardiovascular and metabolic disorders[2,5], peroxidative muscle damage[6], reduction in muscle force generation, muscular pain[2,4] *etc*.

Studies on exercise-induced oxidative stress response concentrated mostly on the adult individuals. The pattern of redox perturbation in children and adolescents following exercise is less known. It has been indicated that young individuals have less efficient antioxidant defence system[7], higher energy cost of exercise and more dependence on mitochondrial metabolism for energy supply[8,9] as compared to adults. These factors are likely to predispose younger population to higher degree of exercise-induced oxidative stress than adults. Significant post-exercise increases in thiobarbituric acid-reactive substances (TBARS) and catalase (CAT) activity has been reported both in pre-preubertal[10] and post-pubertal boys[6] after acute high-intensity exercise. It was also reported that running exercise led to a decrease in TBARS and superoxide dismutase (SOD) activity while reduced glutathione (GSH) level and CAT activity remained unchanged in adolescent runners[11]. Paltoglou *et al*[12] examined the effect of moderate aerobic exercise on normal and obese children belonging to the age group of 10-12 yr and indicated that antioxidation mechanisms might become more efficient with onset of puberty. However, in this study only pre-pubertal and early pubertal boys were included and the study focused mostly on the comparative analysis of post-exercise oxidative stress response with respect to status of obesity and not the effect of puberty on exercise-induced oxidative stress[12]. However, influence of puberty on exercise-induced oxidative stress can be assessed by comparing such response in pre-pubertal boys with those who have already attained puberty. Thus, the present study was aimed to assess and compare the impact of pubertal status (*i.e.* pre- and post-puberty) on high-intensity endurance exercise-induced oxidative stress response through estimation of serum TBARS level, total thiol content and activities of antioxidant enzymes like SOD and CAT in sedentary pre- and post-pubertal boys.

Material & Methods

The study was conducted from August 2016 to November 2016 at Sports and Exercise Physiology Laboratory, department of Physiology, University of Calcutta, Kolkata, India.

The students belonging to two different age groups *i.e.*, 9-11 yr and 15-17 yr were selected from six State government-aided schools located in Kolkata, Howrah and Hooghly districts of West Bengal using simple random sampling method. Necessary permission was obtained from school authorities for conducting the study. The attainment of puberty was assessed by Tanner's staging criterion[13]. The individuals were divided into two groups *i.e.,* pre- (n= 32, 10.21±0.67 yr, range 9-11 yr) and post-pubertal (n=32, 15.58±0.47 yr, range 15-17 yr) boys based on their pubertal status. The pre-pubertal boys were in Tanner's Stage I while the post-pubertal boys belonged to Tanner's Stage III to IV.

For children and adolescents belonging to age group of 5-17 yr, any sedentary activity or recreational screen time of more than two hours per day including sedentary transport, extended sitting time, time spent indoors watching television and screen-based entertainments, sitting at school and reading was considered to be sedentary life style[14]. The children and adolescents of this age group are required to perform at least 60 min of moderate-to-vigorous-intensity physical activity daily along with regular breaks from sedentary behaviour[15]. The individuals who were finally selected for the study spent 8-9 h in sedentary pursuits as defined above. Moreover, the selected individuals did not perform the minimum one hour of moderate-to-vigorous physical activity. These individuals did not take part in any physical conditioning or training programme and were not associated with any professional sport. Individuals with history of any major disease(s) or undergoing any medication were excluded from the study.

The required sample size for the study was calculated by PS: Power and Sample Size Calculation software (version 3.1.2, 2014) that uses the approach suggested by Dupont and Plummer[16]. The expected mean difference between the pre- and post-exercise values (D) as well as the standard deviation (SD) of difference in the response of matched pairs (σ) for each parameter was ascertained from previous studies[10,11] as well as from pilot study conducted in this laboratory. The power of the study ($1-\beta$) was set at 0.8 and the level of significance was set at 0.05 for the purpose of sample size calculation. The highest sample size thus calculated was 16 for TBARS. The sample size estimates for the other parameters were found to be less than the estimates for TBARS. However, considering a 40 per cent dropout, 23 individuals were to be recruited in the study. Finally, 32 individuals were selected in each group.

Body mass index (BMI) of each individual was calculated by dividing the body weight in kilograms by the square of body height in metres (kg/m^2). The individuals did not participate in any physical exercise from two weeks prior to the experimental trials. Standard balanced diet was given to all the participants from one month before the experiment. The age-specific energy requirement and composition of balanced diet for the paediatric and adolescent individuals was determined based on the dietary guidelines issued by National Institute of Nutrition, Indian Council of Medical Research, India[17]. Written informed consent was obtained from all the individuals and their parents. The study was approved by the Human Ethics Committee of the department of Physiology, University of Calcutta.

Familiarization trial: The familiarization trial was conducted three weeks before the experimental trial not only to familiarize the individuals with the experimental protocol but also to select the speed and inclination of the treadmill at which the individuals attained their 80 per cent of age-predicted maximum heart rate (HR_{max}) as calculated from standard equation (220 − age in yr)[6]. Physical examination of the individuals was performed by recording their pre-exercise heart rate, blood pressure and electrocardiograph. The trial involved a progressively incremental treadmill (Viasys, Germany) running by increasing the speed (2 km/h) and inclination (1%) alternatively after each three minutes until 80 per cent of HR_{max} was reached[6].

Experimental trial protocol: Individuals reported at the laboratory at 0800 h after an overnight fast of 12 h. They were asked to take rest for 30 min on an easy chair. A heart rate monitor (Polar Electro Oy, Kempele, Finland) was secured on the individual's chest to monitor the resting, exercising and recovery heart rates. The pre-exercise heart rate and blood pressure were recorded after the resting period. Individuals performed warm-up exercise at a speed of 3 km/h at 0 per cent elevation for five minutes followed by progressive incremental treadmill running with change in speed (by 2 km/h) and elevation (by 1%) alternatively after each three minutes to reach the specific speed and grade that elicited 80 per cent of HR_{max} during the pre-experimental trial. The individuals continued to exercise at that specified speed and inclination until onset of fatigue as indicated by volitional exhaustion. No individuals reported any injury during the exercise trial.

Blood sample (5 ml) was drawn from antecubital vein of each individual before the commencement of the exercise trial (T1) and immediately after cessation of exercise (T2) for biochemical estimation of oxidative stress markers. Blood was collected in Vacutainer tubes having no additive and was allowed to clot at room temperature for 30 min. The tubes were centrifuged for 15 min at 4°C, and the supernatant (serum) was collected. Biochemical analyses were performed immediately after serum collection. The blood collection and handling was done following the standard guidelines to avoid the impact of any pre-analytic variable on accuracy of the assay[18].

Biochemical analysis: The extent of lipid peroxidation in serum was assayed by the method of Wright *et al*[19]. The amount of TBARS in the sample was measured spectrophotometrically at 535 nm using ultraviolet (UV) spectrophotometer (Bio-Rad Laboratories, Berkeley, USA) and was calculated and expressed as MDA equivalent using molar extinction coefficient of 1.56×10^5/M/cm for MDA. The results were expressed as μmol/l.

Serum CAT activity was measured following the method of Claiborne[20] using H_2O_2 (Merck, Darmstadt, Germany) as substrate. The decrease in absorbance due to decomposition of H_2O_2 was determined spectrophotometrically at 240 nm using UV spectrophotometer (Bio-Rad Laboratories) at 30 sec interval for two minutes. CAT activity was expressed as U/mg of protein [one unit represents the amount of protein needed for degradation of 1 μmol of H_2O_2 per minute (*i.e.,* μmol of H_2O_2 consumed or decomposed/min/mg protein)]. Molar extinction coefficient for H_2O_2 was taken as 0.0394/mM/cm. Total protein of the serum sample was estimated by the method of Lowry *et al*[21].

SOD enzyme activity was assayed by the method of Marklund and Marklund[22]. The rate of inhibition of auto-oxidation of pyrogallol by SOD was estimated by recording changes in optical density at 420 nm over 120 sec using UV spectrophotometer. The enzyme activity was expressed in terms of units/mg protein in which one unit of enzyme activity corresponded to the amount of enzyme that inhibited the auto-oxidation reaction by 50 per cent.

Reduced serum total thiol (-SH) groups were assayed according to the method of Ellman[23] as modified by Hu[24]. Absorbance values recorded at 412 nm using UV spectrophotometer were used to calculate serum total thiols using the molar extinction coefficient 13,600/M/cm, and values were expressed as μM/l.

Statistical analysis: Statistical package for social sciences (SPSS, v20.0, Chicago, IL, USA) was used for the statistical analysis of the data. Normality of the data was tested by Shapiro-Wilk test, and the data were found to be normally distributed. Paired *t* tests were performed separately in each group to locate significant difference between the mean values of TBARS, SOD, CAT and total thiol content obtained before and after the exercise trial. Independent Sample *t* tests were performed to locate any significant difference in mean values of oxidative stress markers between the two groups and also to find significant intergroup difference in the mean value of percentage change recorded in each parameter following exercise. Multiple regression analysis was performed to assess the influence of different independent variables such as age, BMI and heart rate in explaining the proportion of variability observed in different oxidative stress parameters.

Results

Physical characteristics, BMI, pre-exercise heart rate, blood pressure, running time to exhaustion and distance covered by individuals during exercise trial are presented in Table I. Post-pubertal boys had significantly higher BMI ($P<0.001$), body height ($P<0.001$), body weight ($P<0.001$), pre-exercise heart rate ($P<0.05$), systolic and diastolic blood pressure ($P<0.001$) as compared to the pre-pubertal group. However, the mean running time to exhaustion and average distance covered during treadmill running did not differ between the groups.

The pre-exercise and the post-exercise values as well as percentage change in markers of oxidative stress are presented in Table II. Pre-exercise TBARS level showed no significant inter-group difference (Table II). TBARS level increased significantly in both the groups ($P<0.001$) following treadmill running. The post-exercise values of TBARS as well as percentage increase following exercise were significantly higher in post-pubertal boys ($P<0.001$) (Table II).

Post-pubertal boys had significantly higher pre-exercise serum total thiol (-SH) level ($P<0.001$) compared to pre-pubertal boys (Table II). Serum total thiol content reduced significantly in both the groups ($P<0.001$) after high-intensity exercise with post-exercise value being significantly lower in post-pubertal boys ($P<0.001$) compared to pre-pubertal. The magnitude of percentage decrease in total thiol level following exercise was also significantly higher in post-pubertal boys ($P<0.001$) (Table II).

Table I. Physical characteristics, pre-exercise heart rate, blood pressure, running time to exhaustion and distance covered by the pre- and post-pubertal boys

Groups	Body height (cm)	Body weight (kg)	BMI (kg/m^2)	Pre-exercise HR (beats/min)	Blood pressure (mm of Hg)		Running time (min)	Distance covered (km)
					Systolic	Diastolic		
Pre-pubertal boys (n=32)	130.77±4.29	22.11±2.02	12.94±1.16	68.66±3.24	95.84±4.82	59.56±4.36	51.58±3.25	7.18±0.57
Post-pubertal boys (n=32)	158.50±3.43***	50.81±2.95***	20.25±1.26***	70.69±3.60*	111.56±4.15***	70.63±2.32***	52.79±7.57	7.61±1.38

Values were expressed as mean±SD. *$P<0.05$; ***<0.001compared to pre-pubertal boys. HR, heart rate; SD, standard deviation; BMI, body mass index

CAT activity increased significantly in both pre-pubertal (P<0.001) and post-pubertal boys (P<0.001) from the basal level following exercise. No significant differences were noted between the groups in pre-exercise and post-exercise values as well as percentage increase in CAT activity (Table II). SOD activity also increased significantly in both pre-pubertal (P<0.001) and post-pubertal boys (P<0.001) following exercise. Post-pubertal boys showed significantly higher pre- and post-exercise value as well as higher post-exercise percentage increase in SOD activity (P<0.001) (Table II).

The result of the multiple regression analysis suggested that the models assessing impact of age, BMI and heart rate on baseline antioxidant status showed good level of prediction as indicated by higher values of multiple correlation coefficient (R) (R=0.738 for baseline total thiol and R=0.694 for baseline SOD activity). Similarly, the models examining the impact of age, BMI and heart rate on exercise-induced percentage change in oxidative stress parameters also showed good level of prediction as evident from higher R values (R=0.731 for TBARS, R=0.820 for total thiol and R=0.857 for SOD activity) (Table III). The baseline value as well as percentage change in total thiol and SOD activity showed higher coefficient of determination (R^2) indicating that the models captured substantial proportion of variability explained by the independent variables (Table III). Percentage change in TBARS also had higher value of R^2 (R^2=0.534). Significant F ratios for all the parameters except for CAT indicated that models were good fit for the data (Table III). Age showed significant beta coefficient (β) for baseline total thiol level (β=0.745, P<0.01), post-exercise percentage change in thiol level (β=0.622, P<0.01), percentage increase in TBARS (β=0.576, P<0.05), baseline SOD activity (β=0.501, P<0.05) and percentage change in SOD activity (β=0.449, P<0.05). BMI also showed significant beta coefficient with post-exercise percentage increase in

Table II. Changes in indices of oxidative stress following exercise

Variable	Group	Before exercise (T1)	After exercise (T2)	Per cent change (between T1 and T2)
LPO (TBARS)	Pre-pubertal boys	2.03±0.71	3.85±1.30***	93.50±39.15
	Post-pubertal boys	1.98±0.45	5.89±0.78$^{***,\#\#\#}$	208.93±60.80$^{\#\#\#}$
Total thiol (-SH)	Pre-pubertal boys	309.21±34.72	246.64±35.81***	20.24±8.47
	Post-pubertal boys	367.80±21.45$^{\#\#\#}$	215.93±33.87$^{***,\#\#\#}$	41.24±9.13$^{\#\#\#}$
SOD	Pre-pubertal boys	2.04±0.32	2.80±0.41***	38.16±12.56
	Post-pubertal boys	2.81±0.51$^{\#\#\#}$	4.60±0.71$^{***,\#\#\#}$	66.46±28.75$^{\#\#\#}$
Catalase	Pre-pubertal boys	0.67±0.16	1.09±0.18***	66.10±31.49
	Post-pubertal boys	0.71±0.37	1.12±0.55***	70.77±70.18

Values are expressed as means±SD. $^{***}P$<0.01 compared to pre-exercise value within group; $^{\#\#\#}P$<0.01 compared to pre-pubertal boys. TBARS, thiobarbituric acid reactive substance; LPO, lipid peroxidation; SOD, super oxide dismutase; SD, standard deviation

Table III. Multiple regression of different oxidative stress parameters with age, body mass index (BMI) and heart rate as predictor variables

Variables	Coefficient (β)			R	R^2	Adjusted R^2	F ratio
	Age	BMI	HR				
Baseline TBARS level	−0.014	0.024	−0.063	0.060	0.004	−0.049	0.068
Per cent change in TBARS level	0.576*	0.153	0.023	0.731	0.534	0.509	21.752***
Baseline total thiol level	0.745**	0.017	−0.108	0.738	0.545	0.521	22.729**
Per cent decrease in total thiol level	0.622**	0.226	−0.053	0.820	0.673	0.656	38.424***
Baseline SOD activity	0.501*	0.197	0.011	0.694	0.482	0.454	17.648***
Per cent change in SOD activity	0.449*	0.370*	0.115	0.857	0.734	0.720	52.510***
Baseline catalase activity	0.563	−0.756	−0.090	0.324	0.105	0.054	2.075
Per cent change in catalase activity	−0.160	0.059	0.069	0.108	0.012	−0.043	0.211

P^{*}<0.05; **<0.01;***<0.001. TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; HR, heart rate

SOD activity. Pre-exercise heart rate did not show any significant beta value with any of the oxidative stress parameter studied. Results also indicated that baseline activity as well as percentage change in CAT activity had lower R and R^2 value, insignificant F ratio and did not show significant β coefficient with any of the independent variable studied (Table III).

Discussion

The result of the present study indicated that the extent of lipid peroxidation increased significantly following high-intensity exercise in both pre- and post-pubertal boys. This suggested that exercise stress led to free radical-induced peroxidation of membrane lipids resulting in elevated blood level of TBARS[6]. In contrast to the present study, Tong *et al*[11] reported insignificant increase in serum TBARS level following 21 km run in adolescent runners. They recruited trained individuals who performed moderate-intensity running exercise at a speed selected by the individuals themselves. The extent of lipid peroxidation is influenced by the intensity of exercise performed, and high-intensity exercise has been shown to induce higher degree of lipid peroxidation as compared to low and moderate exercise[25]. Unlike the exercise protocol used by Tong *et al*[11], an experimentally controlled high-intensity exercise was used in the present study which led to significant oxidative stress leading to lipid peroxidation among sedentary boys. The level of exercise-induced lipid peroxidation was much higher in post-pubertal boys in the present study as indicated by significantly higher post-exercise elevation in TBARS level compared to pre-pubertal group. Post-exercise percentage increase in TBARS was >two times higher in post-pubertal boys (208.93% increase) as compared to their pre-pubertal counterparts (93.50% post-exercise elevation). Result of the multiple regression analysis suggested that age and BMI accounted for majority of the variability in percentage increase in TBARS following exercise, and there was significant positive relationship of post-exercise percentage increase in TBARS with age. Therefore, observations like significantly higher post-exercise percentage increase in TBARS level among post-pubertal boys together with significant linear relationship of this variable with age indicated that magnitude of exercise-induced lipid peroxidation might have increased with advancement of age and physical maturity. Growth and maturation during puberty has induced an increase in muscle mass with proliferation of mitochondria[26], and hence, the post-pubertal boys are likely to have higher relative number and proportion of mitochondria in muscle fibres due to increase in muscle mass during puberty. Greater oxygen demand during exercise by substantially higher muscle mass coupled with higher mitochondrial respiration in post-pubertal boys might have resulted in higher ROS generation and consequent lipid peroxidation. However, further studies are needed to confirm this finding.

It was also evident that the pre-exercise serum total thiol content was significantly higher in post-pubertal boys than the pre-pubertal boys. The baseline total thiol level in serum showed significant positive relationship with age. This indicated that the systemic antioxidation status improved with attainment of puberty and maturation. Total thiol content of serum decreased significantly in both the groups following exercise with post-exercise level being significantly lower in post-pubertal boys. Data on impact of exercise on serum total thiol content for this age group are not available in the literature. Previous studies investigated the effect of exercise on GSH level, and indicated that GSH level declined in both pre-[10,27] and post-pubertal boys following exercise[28]. Significant positive relationship of age with post-exercise percentage decrease in total thiol level indicated that the magnitude of exercise-induced oxidation of serum thiol increased as a function of age. This suggested that post-pubertal boys encountered increased generation of ROS following exercise which was neutralized by the thiol resulting in lower post-exercise value and higher percentage decrease as compared to the pre-pubertal boys. This finding was in agreement with significantly higher TBARS level in post-pubertal boys. The data suggested that attainment of puberty not only upregulated the antioxidation status but also endowed the post-pubertal boys with the ability to counter higher exercise-induced oxidative stress as compared to the pre-pubertal boys.

CAT activity increased significantly in both the groups following exercise suggesting that the exercise stress upregulated the activity of this antioxidant enzyme that countered the hydrogen peroxides produced during high-intensity exercise. Our finding was in agreement with the previous studies which suggested that CAT activity was responsive to exercise stress and increased significantly in both pre- and post-pubertal boys[6,10,12]. However, no significant differences were noted between the groups in terms of pre- and post-exercise values as well as percentage increase in CAT activity following exercise. The results of multiple regression analysis indicated that

predictors of physical maturation such as age and BMI did not significantly explain the variability of baseline as well as exercise-induced percentage change in CAT activity. These findings indicated that both pre- and post-pubertal boys responded almost similarly in terms of elevation in CAT activity when challenged with high-intensity exercise, and pubertal transition might not have any significant influence on the baseline as well as exercise-induced upregulation in CAT activity.

Baseline activity of SOD was also found to be significantly higher in post-pubertal boys, and it showed significant positive relationship with age. These findings suggested that attainment of puberty upregulated the expression of this enzyme in post-pubertal boys. Exercise stress increased the activity of serum SOD in both the groups. Majority of the SOD activity in vasculature and plasma is accounted by the copper/zinc-containing extracellular SOD (ecSOD) which is responsible for combating vascular oxidative stress[29]. The expression of ecSOD from vascular muscle is increased by exercise stress, and this increase in ecSOD expression in response to exercise stress in both the groups represented an important physiological adaptation that counterbalanced the increase in vascular superoxide production following exercise[30]. The post-exercise percentage increase in SOD activity had significant positive relationship with age and BMI suggesting that exercise-induced SOD response increased linearly with increase in age and physical maturation. Therefore, it may be argued that attainment of puberty might improve the SOD-mediated antioxidant response to exercise and such response increased as a function of age.

The present study had certain limitations as it examined one specific aspect of exercise-induced oxidative stress namely lipid peroxidation which reflected the extent of peroxidation of biological membranes in response to high-intensity exercise. Evaluation of exercise-induced oxidation of other biomolecules such as protein and DNA as well as post-exercise response of other antioxidant markers such as GSH peroxidase and total antioxidant capacity were not studied. Adult age groups were not considered in the present study.

In conclusion, the present investigation suggested that high-intensity incremental running exercise resulted in significant oxidative stress among both pre- and post-pubertal sedentary boys. However, post-pubertal boys might be more susceptible to exercise-induced lipid peroxidation as compared to their pre-pubertal counterparts in response to similar exercise stress. The baseline antioxidant status as measured by resting SOD activity and total thiol content also appeared to improve significantly with attainment of puberty. Pubertal maturation also endowed the post-pubertal boys with superior ability to counter higher post-exercise oxidative stress as evident from significantly higher upregulation in post-exercise serum SOD activity and higher relative reduction in total thiol content as compared to pre-pubertal boys. Therefore, performance of any such high intensity exercise or training at this age should be accompanied by adequate recovery time and dietary antioxidant supplementation to avoid any deleterious effect of oxidative stress on health. Since pre-pubertal children are likely to have lower baseline antioxidant level, special care must be taken while engaging this age group in professional sports demanding novel acute high-intensity exercise.

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***Conflicts of Interest*:** None.

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