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# Effects of *Hibiscus sabdariffa* Linn. on Malondialdehyde and Glutathione Peroxidase in an Overtraining Rat Model characterized by Growth Hormone, Insulin-like Growth Factor-1, and Insulin-like Growth Factor Binding Protein-3

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## ABSTRACT

Overtraining is associated with impaired regeneration of body cells. One of the causes of overtraining syndrome is increased production of reactive oxygen species, causes decreased levels of antioxidants. *Hibiscus sabdariffa* Linn. contains antioxidants, especially anthocyanin. This study aims to investigate whether *H. sabdariffa* can prevent overtraining syndrome through decrease in MDA levels and increase in plasma GPx activity. The research subjects were 20 male rats divided randomly into 5 groups: C (control); C-Hib (400 mg/kg/day of *H. sabdariffa*); A-Ex (moderate aerobic exercise); OT (overtraining exercise); OT-Hib (overtraining exercise with *H. sabdariffa* 400 mg/kg/day). We examined GH, IGF-1, levels of free IGFBP-3, IGFBP-3 expression, MDA levels, and plasma GPx activity. We found that IGFBP-3 gene expression in OT and OT-Hib higher than in groups C, C-Hib and A-Ex. The differences between the groups were not statistically significant ( $p > 0.05$ ). MDA levels in the OT-Hib group were significantly lower than in group OT ( $p = 0.019$ ). The mean level of GPx in OT-Hib was higher than in group OT, but the difference was statistically not significant. There were strong negative correlations between GPx activities and both, MDA levels and IGFBP-3 gene expression. In conclusion, we found that *H. sabdariffa* decreases MDA levels and increases GPx activity in our overtraining model and can thus prevent overtraining syndrome.

**Keywords:** Physical exercise, overtraining syndrome, oxidative stress,  
*Hibiscus sabdariffa* Linn., IGFBP-3 gene expression.

## INTRODUCTION

Long-term training of athletes, especially in the preparation phase for competitions can lead to overtraining syndrome due to the volume, duration and intensity of exercise exceeding the athlete's capability. The overtraining syndrome is characterized by reduction of performance due to disturbances in physical condition, immunity, and emotion<sup>1-3</sup>. The occurring physical disturbance

settles for a certain period of time and cannot be overcome during this time despite being given adequate rest. This indicates an interruption in the recovery phase and longer time requirement to restore the body<sup>3-4</sup>. The recovery phase is needed to repair damaged tissues and to regenerate them through the processes of anabolic protein metabolism, replication, and differentiation of skeletal muscle cells. The mechanisms of the body response for tissue regeneration are mediated by

the insulin-like growth factor-1 (IGF-1). IGF-1 is produced in the liver through stimulation by growth hormone (GH) and in the plasma, it binds to its carrier protein, the insulin-like growth factor-binding protein-3 (IGFBP-3)<sup>2</sup>. As a response to adequate physical exercise the body should react by increasing the production of GH, IGF-1 and IGFBP-3<sup>3,5,6</sup>.

In a previous study unbound IGFBP-3 in the plasma (free plasma IGFBP-3) decreased in over-trained subjects<sup>6</sup>. The decrease of IGFBP-3 was considered an indicator or marker of overtraining syndrome<sup>7,8</sup>. However, the mechanism of the decrease of free IGFBP-3 in the plasma is not yet known. Interference between the actions of GH, IGF-1 and IGFBP-3 is estimated to occur in athletes with overtraining syndrome and the processes of anabolic protein metabolism and skeletal muscle cell replication and differentiation are considered to become impaired<sup>9</sup>.

Based on these considerations, questions arose how physical exercise and overtraining affect the levels of IGFBP-3 in the plasma and whether the levels of IGFBP-3 are influenced by the levels of IGF-1 and GH. Therefore, this study was conducted to determine the effect of aerobic exercise in the overtraining rat model on the levels of IGF-1, GH, IGFBP-3 and the interference with IGFBP-3 expression.

It was suggested that overtraining syndrome is associated with oxidative stress<sup>9</sup>. Excessive reactive oxygen species (ROS) can occur because of the increased oxygen consumption during severe aerobic exercise. Therefore, we investigated whether decreased IGFBP-3 in the overtraining syndrome is caused by impaired IGFBP-3 gene expression and how it is influenced by *Hibiscus sabdariffa*.

Most studies examined oxidative stress caused by physical exercise through lipid peroxidation, commonly determined by the production of its by-product, malondialdehyde (MDA)<sup>10-13</sup>. To overcome damages by free radicals, the body is equipped with endogenous antioxidants, both enzymatic, e.g., superoxide dismutase (SOD), catalase, and glutathione

peroxidase (GPx) and non-enzymatic, e.g., glutathione (GSH)<sup>14</sup>. Examples of exogenous non-enzymatic antioxidants are various vitamins and so-called secondary plant components like anthocyanin, flavonoids, and many others<sup>12</sup>. This study investigates the effect of exogenous antioxidants from *H. sabdariffa* to prevent the overtraining syndrome in rats, especially the impact on IGFBP-3 gene expression and on the plasma level of free IGFBP-3. The plant known as Red Tea or *Rosela* is a family member of *Malvaceae*, native to Asia or Africa<sup>15</sup>. In traditional medicine, calyx extract has been used for various indications; we were especially interested in its powerful antioxidant properties in the overtraining rat model<sup>16</sup>.

## MATERIALS AND METHODS

This research has been approved by the Ethical Committee of Medical Faculty, Universitas Indonesia / Cipto Mangunkusumo Hospital (No. 289 / H2.F1 / ETIK / 2013).

### Experimental Animals

This study was an experimental study using 30 male Wistar strain *Rattus norvegicus* (200 -250 g) provided by BPOM RI (Indonesian Food and Drug Administration). Before and during treatment, the rats were maintained healthy, fed a standard diet with free access to drinking water ad libitum. Cages were kept clean and air conditioned (ambient temperature 23±1°C) with a light-dark cycle of 12 hours. The animals were maintained properly according to the code of conduct of the animal handling commission in use of experimental animals. The animals were acclimatized for 3 weeks and to get them familiarized with the research conditions, two weeks for the environment and the third week for the training program. The size of the cages was 40cm x 50cm x 40cm and each cage contained five animals. Cages had a wooden base and were equipped with containers for food and water. Physical exercise devices used in this study were animal treadmills type L-6000 with six lanes (tracks) that can be used simultaneously. Duration, speed, and the slope of the tracks could be adjusted according to the desired training procedure. At the end of each track a metal plate was connected to electric current of 2 mA to deliver an electric shock

to the animal in case the animal stopped walking (or running) or did not follow the direction or velocity of the track. In the experiments the velocity of the track was set according to the aerobic exercise program or to the aerobic overtraining program established by Hohl<sup>17</sup>. To introduce the training program in the acclimatization phase and to minimize the stress in subsequent experiments the animal treadmill was set to low speed (~12m / min) without a slope. Duration of training was less than 10 minutes for each session in one day.

Animals were divided randomly into 5 groups: 1) control group (C), 2) control group with administration of *H.sabdariffa* at a dose of 400 mg/kg/ day (C-Hib), 3) group of rats with mild aerobic exercise (A-Ex), 4) group of rats with aerobic overtraining program (OT), 5) group of rats with aerobic overtraining program and administration of *H. sabdariffa* at a dose of 400 mg/kg/ day (OT-Hib). For the treatment given to the mild aerobic exercise group (A-Ex) the treadmill was running at a speed of 12m/min, for 10 min. The exercise was performed twice a week for 11 weeks<sup>17</sup>.

After completion of the 11 weeks training program, blood samples were taken 3 days later in order to eliminate the acute effect of aerobic physical exercise<sup>9</sup>. Rats were sacrificed with the application of ether and blood samples taken by cardiac puncture. The levels of serum GH, IGF-1 and IGFBP-3 were measured using ELISA Kits for rat growth hormone (GH ELISA Kit - Cusabio®), for rat insulin-like growth factor 1 (IGF-1 ELISA Kit - Cusabio®), and for rat insulin-like growth factor binding protein 3 (IGFBP-3 ELISA Kit - Cusabio®).

The treatment given to the aerobic exercise overtraining (OT and OT-Hib) group was treadmill running with a gradual increase of duration and speed over the 11 weeks of the program. *H.sabdariffa* extract was obtained from the Herbal Research Centre in Bogor, Indonesia and administered through a syringe, every day for 11 weeks, except on Saturdays and Sundays. Our dosage of 400 mg/kg/day is based on an investigation of the influence of *H. sabdariffa* in rats with diabetes mellitus referring to the optimal dose of 400mg/kg/day<sup>18</sup>.

### Measurement of parameters

Blood sampling performed on day 3 post-treatment was done for measuring, GH, IGF-1, IGFBP-3, gene expression of IGFBP-3, MDA, and GPx. Rats were sacrificed with ether, then blood samples were taken by cardiac puncture. Measurement of the levels of GH, and IGF-1, was performed by ELISA technique as mentioned above. MDA content was measured by means of a TBARS (TCA Method) Assay Kit No. 100870 (Cayman Chem, Ann Arbor, MI, USA) based on the method of Yagi<sup>19</sup>. GPx content was measured using RS 505 Backpack kit from Randox Laboratories Limited<sup>20</sup>. The measurements were done in the Department of Biochemistry of Medical Faculty, Universitas Indonesia. In addition, the liver tissue was taken to examine the gene expression of IGFBP-3, was done in the Research Laboratory of Department of Oral Biology, Faculty of Dentist, Universitas Indonesia. Liver tissue was soaked with Ringer's solution and then stored at -80°C. Qualitative RT PCR (qPCR) method was used to examine the gene expression. Measurement of gene expression was performed in the following stages: extraction of RNA, preparation of cDNA, RT-PCR.

### RNA extraction

Liver homogenate was produced by adding 1 mL of Trizol® Reagent to 50 mg of liver tissue. Then the samples were incubated at room temperature and continued to be centrifuged at a speed of 12,000 x g for 15 minutes at 4°C. In the separation phase, the liquid part was taken from the sample. In the RNA precipitation stage, the sample was incorporated with 0.5 mL of isopropanol 100% for each 1 mL of the Trizol® Reagent, then incubated for 10 minutes and centrifuged with a speed of 12,000 x g for 10 min at 4°C. Furthermore, the RNA was washed by removing the supernatant. Subsequently, the RNA pellet was washed with 1 mL of ethanol 75% for each 1 mL of Trizol® Reagent. Then it was briefly vortexed before being centrifuged at a speed of 7500 x g for 5 min at 4°C. For RNA resuspension about 20-50 mL RNase-free water was used to be mixed into the tube and then the tube was incubated in a water bath for 10-15 minutes.

### DNA Preparation

Mixing iScript 5x reaction mix, iScript reverse transcriptase, nuclease-free water and RNA template (100 fg to 1 pg total RNA) were mixed to reach a total volume of 20  $\mu$ L. The mixture was incubated at 25°C for 5 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes.

### Primer design

Primers used in the quantitative RT PCR can be seen in Table 1.

### RT-PCR

Entered into each well of cDNA were 1  $\mu$ L of Fp (B-actin or IGFBP 3 depending on the well), 1  $\mu$ L of Rp (B-actin or IGFBP 3 depending on the well), 1  $\mu$ L of Syber Green Reagent 5  $\mu$ L, and 2  $\mu$ L of dH<sub>2</sub>O, so each well contained a total volume of 10  $\mu$ L. Gene amplification was accomplished as follows: polymerase activation stage, 95°C for 190 seconds; the stage of denaturation, 95°C for 15

seconds; annealing (AT 10 seconds), elongation (72°C, 20 seconds), quantification (QT 5 seconds).

### Statistical analysis

Data were analyzed using SPSS 17; analysis was performed with one way ANOVA followed by post-hoc LSD. The level of statistical significance was set at  $p < 0.05$ . Correlations were calculated using Pearson's analysis.

## RESULTS

### Levels of Growth Hormone, IGF-1, and IGFBP-3 and IGFBP-3 Gene Expression

In Table 2, GH levels were not significantly different between all groups ( $p > 0.05$ ), although they tended to be lower in C-Hib and OT. On the other hand, GH level in OT-Hib was highest in all groups. Levels of IGF-1 were not significantly different between all groups ( $p > 0.05$ ), although IGF-1 was higher in the OT-Hib, OT and C-Hib groups

**Table 1: Primer pairs used in Real Time PCR quantification**

Gene Bank ACC No	Gene Name size	PS Forward 5' to 3'	PS Reverse 3' to 5'	Amplificon	AT	QT
NM 012588	IGFBP 3	CGC TAC AAA GTT GAC TAT GAG	CGT CTT TCC CCT TGG T	292	60	79
NM 001101	B Actin	CTA CGT CGC CCT GGA CTT CGA GC	GAT GGA GCC GCC GAT CCA CAC GG	385	60	79

Abbreviations: GenBank Accession numbers, PS=Primer sequence, AT=annealing temperature, QT=quantification temperature, bp=base pairs, IGFBP-3=insulin like-growth factor binding protein-3.

**Table 2. The levels of GH, IGF-1, IGFBP3 and IGFBP3 gene expression**

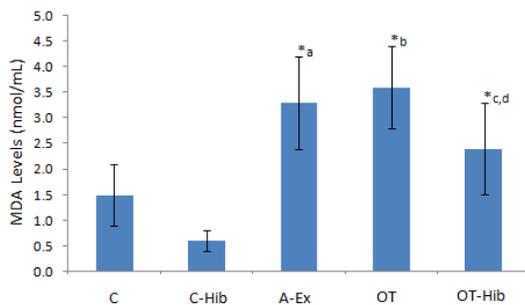
Group	GH [mIU/L]	IGF-1 [mIU/L]	IGFBP-3 [mIU/L]	IGFBP-3 Gene expr. [arb. units]
C	198.4±26.2	4.1±0.9	17.4±10.2* vs. OT-Hib	2.5±0.3
C-Hib	182.4±17.4	5.1±0.8	19.1±11.5	2.6±0.4
A-Ex	211.7±11.3	4.2±0.6	22.4±9.7	2.7±0.3
OT	176.8±29.2	5.2±1.7	18.2±9.9	3.2±1.2
OT-Hib	215.5±27.3	5.3±1.3	39.7±6.2* vs. OT	3.1±0.4
Significance, $p < 0.05$	n.s.	n.s.	* significant differences	n.s.

Abbreviations: C, control group; C-Hib, control-*H.sabdariffa*; A-Ex, mild aerobic exercise; OT, overtraining exercise; OT-Hib, overtraining exercise-*H.sabdariffa*; n.s., not significant.

than in C and A-Ex. Levels of IGFBP-3 were significantly different ( $p < 0.05$ ) between OT-Hib and both, groups C and OT. IGFBP-3 gene expression was not significantly different between all groups ( $p > 0.05$ ); it was higher in OT and OT-Hib groups than in the other groups (C, C-Hib, and A-Ex), where their values were almost alike.

**MDA Levels**

MDA levels differ significantly between groups A-Ex ( $3.3 \pm 0.9$  nmol/mL), C ( $1.5 \pm 0.6$  nmol/mL), and C-Hib ( $0.6 \pm 0.2$  nmol/mL; Fig.1, \*a), between OT ( $3.6 \pm 0.8$  nmol/mL) and C, C-Hib, OT-Hib ( $2.4 \pm 0.9$  nmol/mL; Fig.1, \*b) and between OT-Hib and C, C-Hib (\*c,d), all differences with  $p < 0.05$ . Highest values were found in group OT, followed by A-Ex. In group OT-Hib MDA levels were lower than in the two former groups (Fig. 1).



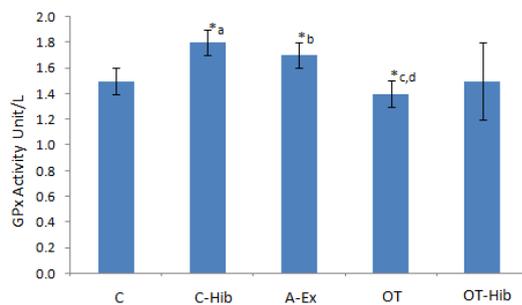
**Fig. 1:** MDA levels in control (C), control-*H.sabdariffa* (C-Hib), mild aerobic exercise (A-Ex), overtraining exercise (OT) and overtraining exercise-*H.sabdariffa* (OT-Hib) groups. Mean values, error bars indicate standard deviations. (Note: test results performed with  $\alpha = 0.05$ )

**GPx Activities**

Lowest GPx activity was found in the OT group ( $1.4 \pm 0.1$  U/L), which was significantly different from C-Hib ( $1.8 \pm 0.1$  U/L) and A-Ex ( $1.7 \pm 0.1$  U/L; Fig.2,\*c,d). OT-Hib group ( $1.5 \pm 0.3$  U/L) shows the same value as controls and is significantly lower than group C-Hib ( $1.8 \pm 0.1$  U/L; Fig. 2,\*a), whereas A-Ex significantly differs from controls ( $1.5 \pm 0.1$  U/L; Fig.2,\*b); all these differences with  $p < 0.05$  (Fig. 2).

**Correlations**

Correlations were calculated between all parameters measured, between physiological parameters (GH, IGF-1, IGFBP-3) correlations were expected, because they are not considered independent (e.g., strong correlation of 0.784 between IGF-1 and IGFBP-3 expression). More



**Fig. 2:** GPx activity in control (C), control-*H.sabdariffa* (C-Hib), mild aerobic exercise (A-Ex), overtraining exercise (OT), and overtraining exercise-*H.sabdariffa* (OT-Hib) groups. Mean values, error bars indicate standard deviations. (Note: test results performed with  $\alpha = 0.05$ )

important were correlations of MDA levels and GPx activities, e.g., between levels of MDA and IGFBP3, and between IGFBP3 expression and GPx activities. There were strong negative (inverse) correlations between GPx activities and both, MDA levels ( $r = -0.527$ ) and IGFBP3 expression ( $r = -0.625$ ,  $p = 0.006$ ). No further correlations could be found; after our preliminary experiments, we had suspected strong negative correlation between the levels of MDA and IGFBP3; however, this result could not be confirmed in our main experiments.

**DISCUSSION**

Overtraining is experienced as a decrease in performance and physiological functions within weeks up to years caused by psychological and physiological factors, although the exact mechanisms are not yet fully understood<sup>1-3,21-26</sup>. In humans, conditions are more common in populations who have high levels of physical activity such as athletes in training programs or during training camps to achieve maximum performance. Research on overtraining syndrome using human

subjects has obstacles, not only because of ethical concerns, but also because of differing designs and incomparable protocols. Therefore, researchers use male Wistar rats as test subjects in an overtraining animal model<sup>17</sup>. This model applies standard physical overtraining exercise protocols and allows for analysis of biomarkers from body tissues and comparison of results between studies.

At the time of starting the treatment phase in our experiments, the age of the rats was eleven weeks and at the end of the treatment phase 22 weeks. The age of 11-22 weeks in rats is equivalent to 17-28 years of age in man<sup>27</sup>.

Growth hormone (GH) is secreted from the pituitary glands (hypophysis) under regulation of the hypothalamic GHRH<sup>4,28</sup>. Physical exercise is one of the factors that increase the secretion of GHRH and thus, GH level<sup>4,29,30</sup>.

The significance of these results strongly depends on the time of measurements after physical exercise<sup>29</sup>. GH increased immediately post-training as an acute response and returned to normal within 6 hours after exercise because of the negative feedback mechanism of the axis GH/IGF<sup>14</sup>. We conducted the GH plasma assay in response to chronic stimulus so that there were no significant differences; however, there was a tendency to decreased GH in the OT group<sup>29</sup>.

The release of GH by anterior pituitary gland into circulation stimulates the liver to produce IGF-1<sup>5,6,29,30</sup>. Apart from liver, GH also goes into other tissues such as muscles and bones stimulating the formation of IGF-1 via these networks<sup>5,6</sup>, thus constituting the main factor that increases IGF-1 secretion after aerobic exercise<sup>29-31</sup>. Two weeks of aerobic exercise increased IGF-1 levels in circulation<sup>31</sup>. However, the increase was temporary only in the acute phase and returned to the original levels within 10-15 minutes after training<sup>30</sup>.

The secretion of IGFBP-3 is also mediated by the activation of the GH/IGF axis, i.e., the levels of IGF-1 and IGFBP-3 exert positive correlations with GH secretion<sup>4</sup>. Thus, elevated levels of IGF-1 and IGFBP-3 found in physical exercise are due to increased secretion of GH from pituitary gland,

which then stimulates IGF-I release from the liver and other tissues<sup>26-31</sup>.

As shown in Table 2, GH in the OT group was lower than in the C, A-Ex and OT-Hib groups. In the A-Ex group GH was slightly increased, consistent with reports that physical exercise can increase the secretion of GH indirectly through increased secretion of GHRH. This response is closely related to the physical exercise intensity and the higher the intensity, the greater the GH increase. The increase was found immediately after exercise as an acute response and returned to normal after 6 hours, post workout because of the negative feedback mechanism of the axis GH/IGF1. This study measured the levels of GH in response to chronic stimulus so that there are no significant differences, although there is a tendency of decrease in baseline GH in OT group. Decrease in baseline GH in OT group will influence the secretion of IGF-1 and IGFBP-3. As we know, GH stimulates the liver to produce IGF 1. Decrease in GH will correlate with a decrease in IGF-1, but in this study, we found a tendency of IGF-1 increase in the OT group. This increase is suspected to occur because of the initial significant decrease in IGFBP-3 reported in our previous study<sup>8</sup>. Approximately, 98% of IGF-1 in plasma binds to IGFBP-3. Thus, a decrease in IGFBP-3 will cause the amount of free IGF-1 to increase. Although the reduction mechanism is still unclear, the decrease in IGFBP 3 is suspected to be caused by interference on the axis of GH/IGF1 or interruption in the biosynthesis of IGFBP3 in liver cells. Hibiscus seems to interfere with the GH/IGF-1 axis, as we can assume from the results of C-Hib group with lower GH but higher IGF-1 levels compared to controls.

IGFBP-3 gene expression (Table 2) is highest in the two overtraining groups, without and with Hib, higher than in control, C-Hib, and A-Ex groups. Obviously, overtraining up-regulates IGFBP-3 gene expression in terms of physical adaptation. However, increased IGFBP-3 gene expression does not match the low IGFBP-3 levels in blood (Table 2), although they were not as rapidly decreased as in our previous study<sup>8</sup>.

On the first glance, the results of this study seem to show that physical overtraining exercise

does not interfere with the process of regulation of IGFBP-3 gene expression, because differences are not significant. However, the strong negative correlation between GPx activity and IGFBP-3 gene expression suggests interference with the regulation of the GH/IGF-1 axis. On the other hand, there is also a strong negative correlation between GPx activity and MDA levels suggesting that antioxidant Hibiscus extract interferes with the oxidative stress in overtraining syndrome as indicated by the highest levels of MDA (Fig. 1). This assumption is supported by the lower MDA levels of the over-trained groups with *H. sabdariffa* extract (OT-Hib) as compared to groups OT and A-Ex; IGFBP-3 gene expression is slightly (although not significantly) lower and circulating levels of IGFBP-3 are significantly higher than in the over-trained group (OT) and the highest level of all groups.

More research is needed to clarify the exact mechanism of the decrease of IGFBP-3 levels in overtraining syndrome which is associated with oxidative stress. High levels of IGFBP-3 in the OT-Hib group indicate the role of *H. sabdariffa* as an antioxidant in the counteraction towards overtraining syndrome. On the other hand, it must still be clarified whether (and/or how) *H. sabdariffa* interferes with the GH/IGF-1 axis down to the cellular level to express IGFBP-3 protein.

Antioxidants can prevent or inhibit the oxidative damage and degradation of proteins, lipids (e.g., in cell membranes), and DNA/RNA. *H. sabdariffa* contains – among other substances - flavonoids, ascorbic acid, protocatechuic acid (PCA), and anthocyanin<sup>32</sup>. The latter was found to be the most potent antioxidant compound in *H. sabdariffa* extract raising total antioxidant capacity and especially increasing GPx activity<sup>33,34</sup>. This was the major reason why we investigated the GPx activity under our experimental conditions. In our study *H. sabdariffa* increased GPx activity in groups C-Hib versus control and attenuated it to normal control values in the OT group.

In conclusion, *H. sabdariffa* extract appears to prevent symptoms of overtraining syndrome in rats via antioxidant mechanisms, it decreases MDA levels, normalizes GPx activity, and strongly increases levels of circulating IGFBP-3. The strong negative correlation between GPx activity and IGFBP-3 gene expression needs further clarification with respect to the interference with the GH/IGF-1 axis down to the cellular level.

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