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Research Article

Protective Role Effect of Mannitol and Methylprednisolone on Testicular Injury Post Torsion-Detorsion in Rat

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Abstract

Introduction: Testicular torsion is one of the differential diagnoses of acute scrotal and is a urological emergency. When torsion occurs, the testicles experience ischemia, if blood flow is not restored, the damage will be irreversible starting from 4 hours and complete damage will occur at 8 hours to 12 hours after torsion.

Methods: The samples in this study were 30 male Wistar rats aged 14-18 weeks with an average weight of 180 gm-230 gm, involving 2 large groups consisting of one control group and four intervention groups.

Results: In the treatment group given both Mannitol and Methylprednisolone (group X1), there were significant decrease in MDA and Caspase-3 expression and significant increase in neutrophil adhesion. There was no significant different between group given Mannitol and/or Methylprednisolone and group underwent torsion-detorsion. Group X1 also showed the best results compared to other treatment groups regarding spermatogenesis and number of seminiferous tubular cell layer.

Discussion: The osmotic properties of Mannitol provide a protective effect against I/R injury by restoring microvascular circulation in the early phase of the I/R injury period. Methylprednisolone can reduce neutrophil migration which in turn can reduce ROS and ultimately reduce apoptosis in germ cells.

Conclusion: Antioxidant-antioxidant combinations are more effective than the use of a single antioxidant to reduce the occurrence of apoptosis and prevent reperfusion ischemic injury.

Keywords: Methylprednisolone; Mannitol; Torsion-detorsion; Diagnosis; Surgical intervention

Introduction

Testicular torsion is one of the differential diagnoses of acute scrotal and is a urological emergency [1,2]. It usually occurs in neonates or adolescent boys but can also be found in other groups. It is estimated that testicular torsion occurs annually in one in 4000 men up to the age of 25 years and has a role in testicular injury and infertility [2]. Although testicular torsion is most common in adolescent boys, it can occur in all age groups from neonates to adult [3]. Testicular torsion occurs when the arterial blood supply is interrupted due to torsion of the spermatic cord resulting in a loss of blood supply. When torsion occurs, the testicles experience ischemia, if blood flow is not restored, the damage will be irreversible starting from 4 hours and complete damage will occur at 8 hours to 12 hours after torsion [4].

Early diagnosis and prompt surgical intervention are both essential to prevent ischemic injury to the testis that can result in damage to the germ cells [2,4]. Surgical interventions to improve testicular torsion include detorsion of the testis and restoration of testicular blood flow. However, derotation or detorsion can result in testicular ischemic-reperfusion injury (I/R injury) [2]. In particular, IR injury causes anoxia, which results in the generation of large amounts of Reactive Oxygen Species (ROS), pro-inflammatory cytokines, cell adhesion molecules, and lipid peroxidation, followed by activation of the necrotic or apoptotic pathway leading to severe ischemic tissue damage [5,6]. ROS generation and proinflammatory neutrophils infiltration that occur in the early stages of I/R injury are essential in the pathogenesis of post ischemic injury and early control of the reperfusion phase to reduce I/R injury [7,8].

Mannitol given before partial nephrectomy can reduce the occurrence of ischemia that causes kidney damage. Mannitol has been used widely in treating or preventing medical conditions caused by an increase in the amount of interstitial fluid [9]. Apart from being a diuretic, Mannitol also acts as an intravascular free radical scavenger [10]. A number of *in vivo* studies have examined the effects of Mannitol as free radical therapy [11]. Research conducted by Kurt et al. [10] in a study of rats with testicular torsion, stated that the seminiferous tubule structure in the group given Mannitol had a better structure than the group that was not given Mannitol. In addition to the effect of reducing free radicals, through its osmotic properties, Mannitol also provides a protective effect against I/R injury. As a substance that has a hyperosmolar effect, Mannitol does not penetrate cellular membranes, causing hemodilution and tissue dehydration. Mannitol can reduce the level of free radicals significantly and can reduce the apoptotic index.

The anti-inflammatory effect of steroid drugs can reduce the formation of ROS through direct inhibition of phospholipase A2 and indirectly through improvement of leukocyte activity. Malondialdehyde (MDA) and Protein Carbonyl (PC), which are markers of free radical activity in tissue in the previous study, were shown to be significantly reduced in the intervention group compared to the control group. It can be concluded that therapy with the steroid

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Methylprednisolone protects testicular tissue in ischemia reperfusion injury caused by testicular torsion-detorsion [3].

In the light of all these data, present study was designed to investigate the testicular effects of Mannitol and Methylprednisolone in rats post torsion-detorsion.

Materials and Methods

The samples in this study were 30 male Wistar rats aged 14-18 weeks with an average weight of 180 gm-230 gm. Samples were maintained at room temperature of 28.0°C ± 2.0°C with lighting from 09.00-21.00 and given adequate food and drink. The inclusion criteria were rats that were healthy (active movement), having 2 testes (right and left) and no abnormalities, especially in the abdominal and genital regions. Male Wistar rats showing behavioural changes (activity appearing weak) were excluded. Wistar rats that died during the initial maintenance and treatment before decapitation were dropped out.

Animal experiments

This research was an experimental study with a "randomized parallel study with control group design". This study involved 2 large groups consisting of one control group and four intervention groups. The control group was a group of research subjects who were not applied torsion.

The first treatment group (group X1) was a group of research subjects who were subjected to a torsion on the left testicular funiculus of 720° and a detorsion was carried out 2 hours later without being given intravenous medical therapy prior to detorsion. In the second treatment group (group X2), Mannitol was given 2 hours after the testicular detorsion carried out. In the third treatment group (group X3), Methylprednisolone was given 2 hours after the testicular detorsion was carried out. In the fourth treatment group (group X4), a combination of Mannitol and Methylprednisolone was given 2 hours after testicular torsion was performed.

After 2 hours of testicular detorsion, the research subjects were performed orchidectomy to assess the expression of MDA, Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α), Caspase-3, grade of neutrophil adhesion, assessment of the number of seminiferous tubular cell layers, histopathological grading of spermatogenesis, and assessing the number of necrosis cells in the seminiferous tubules.

Histological evaluation

Testicular tissues were fixed in 10% paraformaldehyde and then embedded in paraffin blocks. Sections cut at 4 μm thickness were stained with Hematoxylin and Eosin (H&E) and Indirect Immunoperoxidase Staining using monoclonal antibody specific for MDA, TNF-α, IL-6, and Caspase-3. The sections were then viewed under light microscope to determine the histological changes.

In H&E stained sections, grading of spermatogenesis and the number of necrosis cells in the seminiferous tubules was measured and recorded. Johnsen scoring system was used for the grading of testicular histopathology.

Statistical analysis

Data from research was recorded, collected and processed with a computer program. Data was presented in tables and graphs (Tables 1-10) (Figures 1-8). Before the analysis was carried out, a normality test was carried out using the Saphiro-Wilk test because the number of samples was less than 50. The analysis between the independent variable and the dependent variable with the ratio variable (numerical) using One way Anova because the normality test was obtained with the normal distribution of data. The test was then continued with Post Hoc. The analysis between the independent variable and the dependent variable of used Kruskal Wallis and continued with the Mann Whitney test because it was obtained the abnormal distribution of data. After obtaining the necessary data, the results of the data are processed in the research discussion.

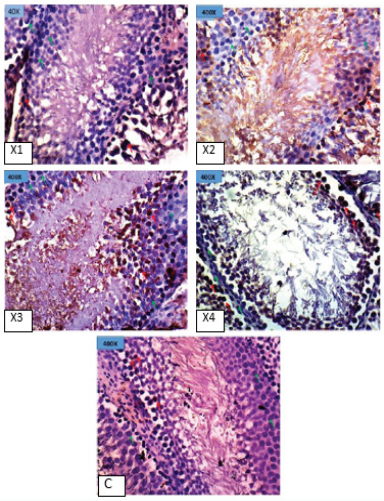

Figure 1: Group X1: About 10% of the cytoplasm stained brown (red arrow), and not stained (green arrow); Group X2: About 50% of the cytoplasm stained brown (red arrow), and not stained (green arrow); Group X3: About 40% of the cytoplasm stained brown (red arrow), and not stained (green arrow); Group X4: About 95% of the cytoplasm stained brown (red arrow), and not stained (green arrow); Control Group: About 1% of the cytoplasm stained brown (red arrow), and not stained (green arrow).

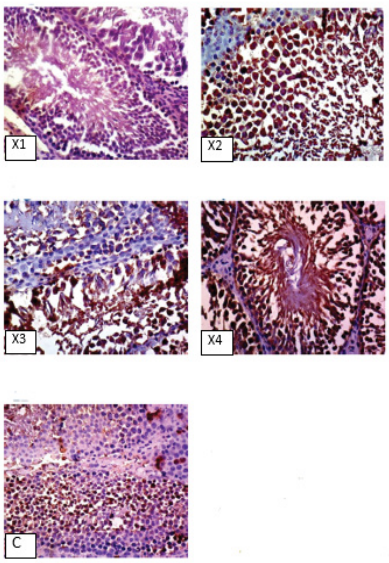

Figure 2: Immunohistochemistry Test of TNF-α of each group. Group X1: Moderate intensity, 80%; Group X2: Strong intensity, 90%; Group X3: Strong intensity, 80%; Group X4: Strong intensity, 100%; Control Group: Moderate intensity, 50%.

Results

In this study, there was no necrotic cell found in all groups.

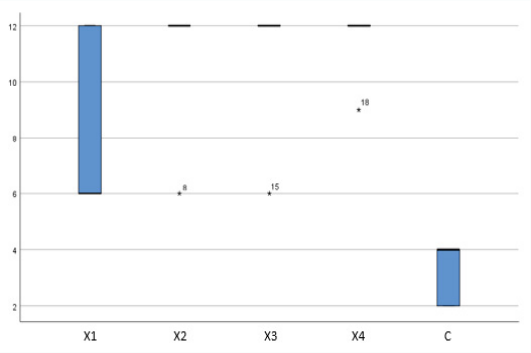

Figure 3: Characteristics of IL-6 Expression.

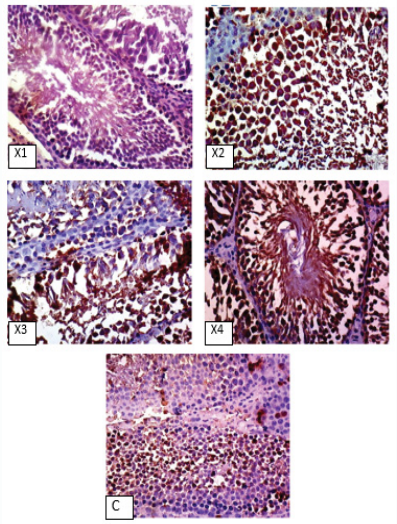

Figure 4: Immunohistochemistry Test of IL-6 of each group. Group X1: Moderate intensity, 80%; Group X2: Strong intensity, 90%; Group X3: Strong intensity, 90%; Group X4: Strong intensity, 100%; Control Group: Moderate intensity, 60%.

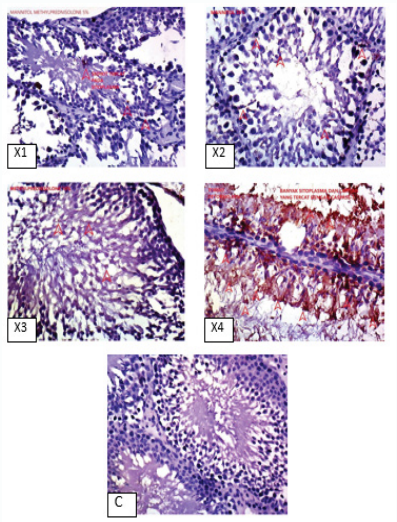

Figure 5: Immunohistochemistry Test of Caspase-3 with 400× magnification. Group X1: Weak intensity, 5%; Group X2: Weak intensity, 30%; Group X3: Weak intensity, 30%; Group X4: Strong intensity, 80%; Control Group: Negative result.

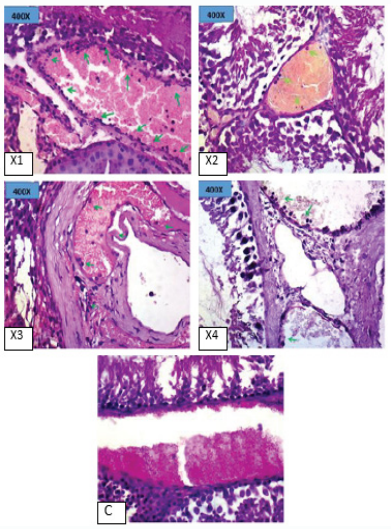

Figure 6: Group X1: Neutrophil margination (green arrow) in most of the blood vessel (Score 3); Group X2: Neutrophil margination (green arrow) in most of the blood vessel (Score 2); Group X3: Neutrophil margination (green arrow) in most of the blood vessel (Score 2); Group X4: Neutrophil margination (green arrow) in most of the vascular circularly arranged (Score 2); Control Group: No neutrophil margination in blood vessel.

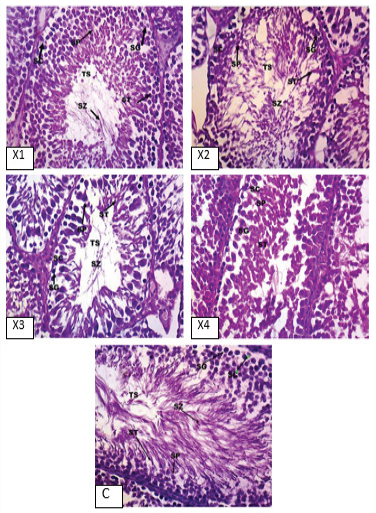

Figure 7: Image of Seminiferous Tubule of All Groups with 40X Magnification.

Discussion

In this study, it was found that the testes of Wistar rats subjected to a torsion of 720° for 2 hours had begun damaging the cells. This was indicated by an increase in the expression of MDA in the testes with torsion for 2 hours compared to the control group (p= 0.005). These results are consistent with research conducted by Turner et al. [12] which shows that at 720° testicular torsion for 2 hours a severe ischemic process occurred. The process of increasing MDA expression occurs due to the lipid peroxidation process which is a reaction that occurs during oxidative stress [13,14]. Oxidative stress is a state of imbalance between reactive oxygen species (ROS) and the ability of the biological system to detoxify the active intermediate. Tissue injury is the most common cause of oxidative stress wherein tissue injury can occur in the ischemic reperfusion process which is often referred to as I/R injury.

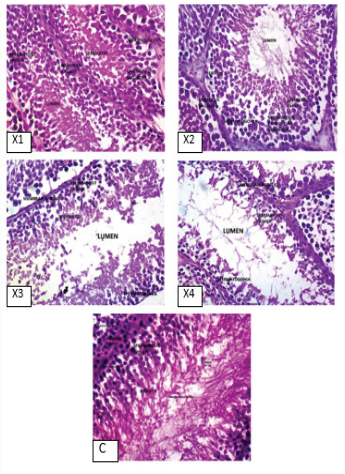

Figure 8: Histopathology of the Number of Seminiferous Tubular Cell Layer with HE Staining.

Table 1: Mann Whitney Test of Expression of MDA.

Group	X2	X3	X1
X4	0.015	0.011*	0.004*
X2	-	0.221	0.014*
X3	-	-	0.134

Table 2: Mann Whitney Test of The Expression of TNF-α.

Group	X2	X3	X4	C
X1	0.238	0.126	0.054	0.017*
X2	-	0.881	0.317	0.006*
X3	-	-	0.317	0.006*
X4	-	-	-	0.005*

Table 3: Mann Whitney Test of The Expression of IL-6.

Group	X2	X3	X4	C
X1	0.221	0.221	0.12	0.007*
X2	-	1	0.881	0.006*
X3	-	-	0.881	0.006*
X4	-	-	-	0.006*

Table 4: Descriptive Statistics of The Expression of Caspase-3.

Group	Mean	Std. Deviation	Min	Max
X1	1.6	1.517	0	4
X2	3.2	1.789	2	6
X3	3.6	1.673	2	6
X4	6.4	2.881	2	9
C	1.2	1.789	0	4

Table 5: Mann Whitney Immunoreactive Score (IRS) of The Expression of Caspase-3.

Group	X2	X3	X4
X1	0.104	0.068	0.020*
X2	-	1.65	0.08
X3	-	-	0.106
X4	-	-	-

Table 6: Mann Whitney Test of Neutrophil Adhesion.

Group	X2	X3	X1
X4	0.166	0.65	0.606
X2	-	0.419	0.042
X3	-	-	0.343

Table 7: Analysis of Histopathological Grading of Spermatogenesis.

Group	N	Mean ± SD	Median	Min	Max
X1	5	10.00 ± 0.00	10	10	10
X2	5	9.00 ± 0.00	9	9	9
X3	5	9.00 ± 0.00	9	9	9
X4	5	7.00 ± 0.00	7	7	7
C	5	10.00 ± 0.00	10	10	10

Table 8: Comparative Test of Spermatogenesis Between Groups.

Group	X2	X3	X4	C
X1	0.003*	0.003*	0.003*	1
X2	-	1	0.003*	0.003*
X3	-	-	0.003*	0.003*
X4	-	-	-	0.003*

Table 9: Descriptive Statistics of The Number of Seminiferous Tubular Cell Layers.

Group	Mean	Std. Deviation	Min	Max
X1	51.6	4.099	48	58
X2	44.8	2.28	42	48
X3	45.2	3.899	36	50
X4	37.2	1.789	40	40
C	52	1.414	50	54

Table 10: Post Hoc Bonfferoni Test of The Number of Seminiferous Tubular Cell Layer.

Group	X2	X3	X4
X1	0.014	0.024	0.000002*
X2	-	1	0.005*
X3	-	-	0.003*
X4	-	-	-

In the group receiving Mannitol (group X2) and Methylprednisolone only (group X3), there were significant differences in MDA expression compared to the torsion-detorsion group (group X4). This was consistent with a study conducted by Omer Kurt et al. [10], where it was stated that Mannitol had a protective effect on testicular torsion. Mannitol was previously used to reduce I/R injury in partial nephrectomy operations by attracting free radicals. In addition to these functions, the osmotic properties of Mannitol also provide a protective effect against I/R injury by restoring microvascular circulation in the early phase of the I/R injury period [15,16]. This is because Methylprednisolone can reduce neutrophil migration which in turn can reduce ROS and ultimately reduce apoptosis in germ cells [3,17].

In the treatment group given both Mannitol and Methylprednisolone (group X1), there was a significant difference in MDA expression compared to the torsion-detorsion group (p=0.004). This result had the most significant difference when compared with single administration of Mannitol or Methylprednisolone. This is something new in this field.

The effectiveness of Mannitol and Methylprednisolone as a protective effect of testicular cell damage by torsion-detorsion showed that there was no difference between groups X1, X2, X3 and X4 with p values >0.05, both seen from the expression of TNF-α and IL-6. This result was different from the research conducted by Kurt et al. where Mannitol had a protective effect against testicular damage due to torsion-detorsion [10].

The onset of action of a drug begins when the drug enters the plasma and ends until it reaches the Minimum Effective Concentration (MEC). The administration of Methylprednisolone in this study used an intravenous line, so that the drug directly enters the blood vessels and has a fast onset of action. Hazra et al. [18] and Haughey et al. [19] mentioned the rapid onset of action in intravenous administration of Methylprednisolone in mice. The highest drug levels occur shortly after intravenous administration of Methylprednisolone. The drug will be in the blood of the mice for more than 6 hours. This study is a reference that the intravenous administration of Methylprednisolone in this study which was given shortly after detorsion and with a duration of 2 hours until the testicular tissue collection indicated that the drug

would react according to the onset of action and had not reached MEC when the rat testicular tissue was taken. Likewise, Mannitol was administrated intravenously 2 hours before orchidectomy treatment in this study. Jeffries et al. [20] stated that intravenous administration of Mannitol had an effect on increasing intracranial pressure within 15-30 minutes. Mannitol, as well as Methylprednisolone, initiate action when the drugs get into the bloodstream. Mannitol has a half-life of about 100 minutes.

In this study, it was proved that there was an effect between giving Mannitol and Methylprednisolone therapy on Caspase-3 expression (p=0.032). The X1 group (Mannitol and Methylprednisolone) was the group with the lowest Caspase-3 expression in the treatment group compared to the group given single therapy of Mannitol or Methylprednisolone (p = 0.020).

Several previous studies showed that the expression level of Caspase-3 decreased with the administration of Mannitol and Methylprednisolone alone. By combining Mannitol and Methylprednisolone, it was obtained better results both qualitatively and statistically. This could occur with the possibility of a synergistic combination of the effects of Mannitol and Methylprednisolone which differ slightly in their function of reducing the rate of apoptosis.

Activated neutrophils that migrate to the walls of blood vessels block capillaries and prevent tissue reperfusion which leads to tissue necrosis. Meanwhile, the treatment group that received Mannitol compared with the torsion treatment group did not get significant results (p = 0.166) and the treatment group that received Methylprednisolone compared to the torsion treatment group did not get any significant results (p = 0.650). This was not in accordance with the research conducted by Yazawa et al. [17] which states that when giving glucocorticoids in this case dexamethasone reduces the adhesion degree of Neutrophils performed at 12 hours after reperfusion. The same thing also happened to the treatment group that received Mannitol+Methylprednisolone compared to the torsion-detorsion treatment group which did not get significant results (p=0.606). An interesting result was obtained in the X1 group (Torsion-detorsion + Mannitol + Methylprednisolone) which had a greater value of the grade of neutrophil adhesion than the X4 group (Torsion-detorsion). This might be due to one of the side effects of Mannitol and Methylprednisolone which can increase glucose levels. Where according to research by Morigi et al. [21] and Omi et al. [22] an increase in blood glucose levels can induce adhesion of leukocytes to endothelial cells.

Regarding the number of seminiferous tubule cell layers, group X1 showed the best results compared to other treatment groups. The average number of seminiferous tubule cell layers in the Mannitol + Methylprednisolone group had the number of seminiferous tubular cell layers that was closer to the control group. This study also proved that there was an effect between giving Mannitol Methylprednisolone therapy on the number of seminiferous tubule cell layers (p = 0.000496). Group X1 (Mannitol+Methylprednisolone) was the group that had the greatest number of seminiferous tubular cell layers in the treatment group that was treated with torsion-detorsion compared to the group with single therapy of Mannitol or Methylprednisolone (p=0.000002).

The HE staining results in the Mannitol and Methylprednisolone combination therapy group also showed the most complete picture of spermatogenesis compared to the Mannitol or Methylprednisolone group alone. Group X1 showed complete spermatogenesis in the seminiferous tubules, namely there were all stages of spermatogenic cells including a large number of spermatozoa, while the group experiencing torsion and detorsion had peeling and disorganization of the tissue possibly due to the initiation of a degenerative process as a result of the increase of lipid peroxidation and depletion of cellular ATP. Testicular torsion induces release of apoptosis-activating cytokines, which in turn causes widespread apoptosis in the testicular germ epithelium. Therefore, the high incidence of infertility in patients with testicular torsion is likely due to repeated episodes of apoptosis [23]. Antioxidant-antioxidant combinations are more effective than the use of a single antioxidant to reduce the occurrence of apoptosis and prevent reperfusion ischemic injury [24]. In this study, a combination administration of Mannitol and Methylprednisolone was performed and showed significant results (p <0.05).

The basic process of necrosis involves the loss of the integrity of the cell membrane leading to the entry of extracellular ions and fluids into the intracellular resulting in swelling of the cells and organelles. Proteolytic enzymes leave the lysosome and enter the cytosol which causes the digestion process of cellular components that does not change and cell destruction. Necrotic cells in the electron microscope can be seen as cells whose cytoplasm are increasingly transparent, there is swelling of organelles, dilatation of the nuclear membrane, and condensation of chromatin. This increased cell volume can ultimately result in disruption of the plasma membrane by uncontrolled leakage of cellular components into the cytosol and interstitial space [25]. Our results showed that ischemic injury resulting from 720^0 torsion of spermatic cord did not reveal macroscopic necrosis of testicular cells (histopathological) significance after 2 hours of torsion suggests there may be persistent blood flow to the testes and testicular viability despite the degree of testicular torsion.

The incidence of testicular necrosis or atrophy increases with the duration of torsion. Necrosis and atrophy are known as the outcomes of ischemia-related injury and as indicators of late and irreversible testicular injury [26]. This study suggests that the necrosis process may have occurred at the molecular level but has not been shown microscopically, proven by the absence of necrotic cells.

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