Effect of Hyaluronidase and Methylprednisolone on Myocardial Function, Glucose Metabolism, and Coronary Flow in the Isolated Ischemic Rat Heart

MICHAEL J. ROVETTO

SUMMARY  Ischemia in the isolated perfused rat heart resulted in an increase in coronary vascular resistance. Studies were undertaken to determine the effect of hyaluronidase and methylprednisolone on this increase in resistance as well as on glycolytic rate and mechanical function of ischemic hearts. Neither hyaluronidase nor methylprednisolone affected the rate of glucose utilization in working perfused control or ischemic rat hearts. However, both agents prevented a reduction in coronary flow during a 2-hour ischemic period. Associated with the higher coronary flows were higher tissue concentrations of creatine phosphate and lower concentrations of lactate. These agents also prevented accumulation of tissue water in the ischemic hearts. Such changes would appear to be beneficial to the ischemic heart, although mechanical function of post-ischemic hearts was not enhanced by the presence of either hyaluronidase or methylprednisolone. The results, however, suggest that the reduction in myocardial infarct size noted with hyaluronidase and methylprednisolone may be due to their prevention of further reduction of coronary flow in marginally ischemic tissue.

GLUCOCORTICOIDS and hyaluronidase are reportedly beneficial in reducing infarct size and cellular damage during myocardial ischemia. In this regard, hyaluronidase was shown to be effective in dogs, rats, and man.

The mechanisms of action by which these agents protect the ischemic myocardium are not known. It has been suggested that glucocorticoids act through stabilization of membranes and reduce the release of lysosomal hydrolases. On the other hand, hyaluronidase was thought to reduce tissue fluid accumulation and therefore allow better perfusion of the ischemic tissue. However, Maroko et al. recently suggested that hyaluronidase might act to increase glucose entry in myocardial cells, thereby allowing more energy to be produced from glycolysis. This latter possibility was of particular interest since glucose availability is probably not the rate-limiting step in glycolysis during ischemia. Nevertheless, decreased flux of substrate through glycolysis could account for a lower than expected rate of energy production from this pathway. In this regard, hyaluronidase could stimulate glycolysis by reducing the high resistance for lactate efflux from ischemic cells. Lactate inhibits glycolysis at concentrations comparable to those observed during ischemia. The purposes of the present study were to determine the effect of methylprednisolone and hyaluronidase on: (1) the utilization of glucose by ischemic tissue, (2) the rate of coronary flow during ischemia, and (3) the recovery of mechanical function by reperfused ischemic hearts.

Methods

Hearts were removed from male Sprague-Dawley rats (300–350 g) anesthetized with pentobarbital (30 mg/kg, intraperitoneally and placed in cold 0.9% NaCl for about 30 seconds. The aorta was cannulated and the heart perfused retrograde for 10 minutes at a perfusion pressure of 60 mm Hg. During this perfusion period, the left atrial appendage was cannulated via the pulmonary vein.
hearts then were perfused at a left atrial pressure of 10 cm H2O for 10 minutes prior to starting a perfusate containing steroid or hyaluronidase or continuing perfusion with no drugs. Following an additional 10- to 30-minute perfusion period as a working heart (as indicated in the figures and tables), the hearts were either made ischemic or were perfused as working hearts (i.e., control group).

Ischemia was produced by a 60% reduction in coronary flow. This was achieved by the use of a one-way ball valve in the aortic outflow tract as previously described. Briefly, this procedure consists of reducing diastolic perfusion pressure which results in a reduction in coronary flow without an initial change in the cardiac work load. Due to insufficient oxygen delivery to the tissue, the left ventricle begins to fail, and peak systolic pressure declines from about 90 to 30 mm Hg within 6-10 minutes. Since coronary flow is dependent primarily on the systolic perfusion pressure, coronary flow decreases, and at 30 mm Hg peak systolic pressure, is only about 20% of that of control working hearts. If coronary flow continues to decline, ventricular function would not recover on restoration of coronary flow 25 minutes after the peak systolic pressure before aortic pressure and cardiac output were reached 30 mm Hg. 14 Therefore, to study the effects of ischemia over a longer time period, a minimum coronary perfusion pressure of 60 mm Hg and an additional 10 minutes were provided for stabilization at each filling pressure before aortic pressure and cardiac output were measured. No further changes in these parameters were found with longer times for equilibration.

In the studies of recovery of ventricular function, the hearts were reperfused for 10 minutes at an aortic hydrostatic perfusion pressure of 60 mm Hg and an additional 10 minutes as working hearts prior to determining cardiac performance. Cardiac performance was assessed prior to and following ischemia by generating function curves with left coronary flow 25 minutes after the peak systolic pressure. Methylprednisolone (MP) (Solu-Medrol, Upjohn) was dissolved directly in the perfusate containing 11 min glucose. The control perfusate was Krebs-Henseleit bicarbonate buffer without vehicle at a final concentration of 0.25 mg/ml. This concentration of MP would be equivalent to a circulating blood concentration at a dose of 30 mg/kg provided the steroid were distributed only in the blood. Hyaluronidase (Sigma) was prepared as a stock solution in 0.9% NaCl-5% bovine serum albumin and added to the perfusate to give a final concentration of 4 U/ml and 0.01% bovine serum albumin. The control hearts were perfused with buffer containing 0.01% albumin. The albumin appeared to give more reproducible results with longer perfusion times and may have acted to stabilize the hyaluronidase. This concentration of hyaluronidase was approximately equal to that which would have obtained in the whole animal studies of Maroko et al., 5 provided that the dose they used was distributed only in the plasma.

Glucose utilization was determined by the release of 3H2O from 2-3H-D-glucose. 15 The 3H released from glucose completely exchanges with H2O in the glycolytic pathway and was separated from labeled glucose on Dowex resin. 16 Tissue adenine nucleotides, creatine phosphate, and lactate concentrations were determined in 6% perchloric acid extracts of quickly frozen hearts using standard enzymatic techniques. 17 The hearts were frozen during perfusion by clamping them between blocks of metal precooled in liquid nitrogen.

Results

EFFECT OF HYALURONIDASE

The effects of hyaluronidase on glucose utilization in control hearts and ischemic hearts with constant coronary flow are shown in Table 1. Hyaluronidase was without effect on glycolytic rate. Tissue concentrations of ATP and creatine phosphate (CP) decreased and lactate concentrations increased in hearts ischemic for 30 min compared to the values for aerobic control working hearts. However, there were no differences between these concentrations in the ischemic hearts perfused with and without hyaluronidase in the perfusate. These data indicate that hyaluronidase was without significant effect on the glycolytic rate of, and lactate efflux from, ischemic tissue.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (min)</th>
<th>Coronary flow (ml/min)</th>
<th>Glucose utilization (µmol/g min)</th>
<th>Concentration (µmol/g dry tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Creatine phosphate</td>
</tr>
<tr>
<td>Perfusate without</td>
<td>0</td>
<td>15.7 ± 0.4</td>
<td>4.8 ± 0.2</td>
<td>23.2 ± 2.5</td>
</tr>
<tr>
<td>drug</td>
<td>15</td>
<td>2.4 ± 0.4</td>
<td>3.4 ± 0.2</td>
<td>13.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.5 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>13.7 ± 2.3</td>
</tr>
<tr>
<td>Perfusate plus</td>
<td>0</td>
<td>13.7 ± 0.8</td>
<td>4.8 ± 0.3</td>
<td>22.1 ± 2.0</td>
</tr>
<tr>
<td>hyaluronidase</td>
<td>15</td>
<td>2.4 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>14.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.4 ± 0.1</td>
<td>3.2 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Prior to making the hearts ischemic, they were perfused for 10 minutes at aortic perfusion pressures of 60 mm Hg followed by an additional 10-minute perfusion period as working hearts at left atrial pressures of 10 cm H2O. The end of this second perfusion period is referred to as 0 time; 15 and 30 minutes refer to the duration of the perfusion period following ventricular failure (i.e., peak systolic pressure of 30 mm Hg). Coronary flow and glucose utilization data for 0 min are from these hearts subsequently made ischemic. Tissue high energy phosphate and lactate concentrations for this time are means ± SEM for two additional groups of four hearts each. These values are not changed during 30 min of perfusion as working hearts. 18 The values for the ischemic hearts are means ± SEM for eight hearts.
Hearts were next made ischemic and, following ventricular failure, coronary flow was provided by a constant hydrostatic perfusion pressure of 30 cm H$_2$O for 2 hours. The data for hearts perfused with and without hyaluronidase in the perfusate are shown in Figure 1. Under these conditions, hyaluronidase did not alter the glycolytic rate of the ischemic hearts. The coronary flow of hearts perfused with regular Krebs-Henseleit buffer was decreased by about 30% compared with the flow of hearts perfused with buffer containing hyaluronidase ($P < 0.01$). Despite the lack of effect on the control working heart coronary flow, hyaluronidase prevented a decrease in the coronary flow during the 2-hour ischemic period. Tissue lactate concentration was lower ($P < 0.005$) and the creatine phosphate concentration was higher ($P < 0.01$) in ischemic hearts perfused with hyaluronidase in the perfusate, compared with hearts which were perfused with regular buffer (Fig. 2). That these differences were probably due to the higher rate of coronary flow in the hyaluronidase-treated hearts which provided more oxygen and better washout of lactate from the tissue was shown in two ways. First, six hearts were perfused without hyaluronidase for 1 hour at a coronary flow of 3 ml/min, and the lactate and creatine phosphate concentrations were 14 ± 3 and 20 ± 2 μmol/g dry tissue, respectively, and were not different from the values for hyaluronidase-treated hearts perfused for 2 hours at comparable rates of coronary flow (creatine phosphate data in Figure 2). Second, coronary flow was provided with regular buffer with a hydrostatic perfusion pressure of 40 cm H$_2$O. At the end of 2 hours, coronary flow had declined from 3.8 ± 0.2 to 3.2 ± 0.1 ml/min and the tissue concentrations of lactate and creatine phosphate were 16 ± 4 and 19 ± 3 μmol/g dry tissue, respectively, and again were not different from those in hyaluronidase-treated hearts with a coronary flow of 3 ml/min. The data in Table 1 lend additional support to this contention.

These data also suggest that during minimum flow ischemia with constant coronary flow, a steady state develops with regard to the concentration of the high energy phosphates, ATP and creatine phosphate. When peak systolic pressure reached 30 mm Hg, the concentrations of creatine phosphate and ATP were 7 and 15 μmol/g, respectively. During the ischemic period, ATP remained at this level whereas creatine phosphate increased to 18 and 25 μmol/g dry tissue in hearts perfused with regular and hyaluronidase containing buffers, respectively (Fig. 2).

Since hyaluronidase prevented an increase in coronary vascular resistance, it was of interest to determine whether recovery of mechanical function following ischemia was facilitated by the presence of this enzyme. Cardiac function was assessed by ventricular function curves prior to and following 2 hours of ischemic minimum flow perfusion provided by a hydrostatic perfusion pressure of 30 cm H$_2$O (Table 2). The mechanical function of control aerobic working hearts perfused for an equivalent time as the ischemic hearts did not change significantly following a 2-hour perfusion period (group 7 in Table 2). Moreover, the values for this group were not different from the preischemic values for hearts made ischemic. Following ischemia, however, ventricular function was significantly depressed in both groups of hearts, and hyaluronidase did not alter the degree of recovery (groups 1 and 2 in Table 2). During the recovery period when the hearts were perfused retrograde at 60 mm Hg aortic pressure (Langen-
TABLE 2  Effects of Hyaluronidase and Methylprednisolone on Ventricular Function

<table>
<thead>
<tr>
<th>Group</th>
<th>Left atrial pressure</th>
<th>Power (g x cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preischimic</td>
<td>Postischimic</td>
</tr>
<tr>
<td></td>
<td>5 10 15 20</td>
<td>5 10 15 20</td>
</tr>
<tr>
<td>1 Regular perfusate 2 hours ischemia</td>
<td>14 10.5±1.7</td>
<td>2 14.4±3.4</td>
</tr>
<tr>
<td>2 Hyaluronidase perfusate, 2 hours ischemia</td>
<td>14 12.2±2.0</td>
<td>0.25 mg per ml perfusate</td>
</tr>
<tr>
<td>3 Regular perfusate, 2 hours ischemia</td>
<td>6 13.8±2.2</td>
<td>25.6±9.4</td>
</tr>
<tr>
<td>4 Methylprednisolone perfusate, 2 hours ischemia</td>
<td>6 14.0±1.1</td>
<td>25.6±9.4</td>
</tr>
<tr>
<td>5 Regular perfusate 20 min ischemia</td>
<td>9 16.7±2.7</td>
<td>25.6±9.4</td>
</tr>
<tr>
<td>6 Methylprednisolone perfusate, 20 min ischemia</td>
<td>7 17.6±2.2</td>
<td>25.6±9.4</td>
</tr>
<tr>
<td>7 Regular perfusate aerobic</td>
<td>6 11.8±2.7</td>
<td>25.6±9.4</td>
</tr>
</tbody>
</table>

The values are means ± SEM for the number of hearts indicated by the values in parentheses for each group. The appropriate control groups are given immediately before the experimental groups. Ventricular power was calculated as mean arterial pressure minus left atrial pressure (cm H₂O) multiplied by aortic flow (ml/sec). The 20 minutes in the two ischemic groups refers to the ischemic period following ventricular failure. Coronary flows of hearts in the 20-minute ischemic groups were not maintained following ventricular failure and averaged 0.5 ml/min prior to reperfusion.

dorff perfusion), the coronary flows were 6.9 ± 0.4 for the control ischemic hearts and 8.2 ± 0.5 for the hyaluronidase-treated hearts. Comparable values for the preischimic hearts were 9.2 ± 0.6 and 9.4 ± 0.4 for the control and hyaluronidase-treated hearts, respectively. Thus, the flow was significantly decreased in the nontreated hearts, compared with preischimic values, whereas it was not changed in the treated hearts. Although the differences were small, the effect of hyaluronidase on coronary flow was manifest in the postischemic Langendorff hearts. In contrast, the coronary flows during the postischemic ventricular function curves were not different between the control and hyaluronidase hearts, as both groups exhibited an average 37% decrease in coronary flow compared with the preischimic values. It was not possible to estimate how much of the flow reduction was due to the decreased mechanical function of these hearts as opposed to some other factor restricting coronary flow.

EFFECT OF METHYLPRÉDNIOSOLONE

Figure 3 illustrates the glycolytic rate prior to and during 2 hours of ischemia in hearts in which coronary flow was provided by a constant aortic perfusion pressure of 30 cm H₂O. As with hyaluronidase, glycolytic rate was not affected by the presence of 0.25 mg of methylprednisolone per ml of perfusate. However, methylprednisolone also prevented the increase in coronary vascular resistance during the ischemic period (Fig. 4). Due to the higher rate of coronary flow in methylprednisolone-treated hearts, the amount of tissue lactate was reduced and creatine phosphate was maintained at a higher concentration at the end of the ischemic period (Table 3). The lower lactate concentration was not due to a direct effect of the steroid, since it was not different in methylprednisolone, compared with nontreated control hearts perfused at constant coronary flows (data not shown but comparable to those in Table 1).

Methylprednisolone induced a consistent significant increase in coronary flow when the perfusate of aerobic working hearts was switched from regular buffer to buffer containing methylprednisolone. This increase in flow was only about 1 ml/min in each heart and, due to variations in the coronary flow rate between hearts, does not appear as a significant increase (Fig. 4). The vasodilation was not evident following ventricular failure when coronary flow...
was provided by a constant aortic perfusion pressure so that the initial rates of minimum coronary flow was equal in the two groups of hearts.

Methylprednisolone, like hyaluronidase, did not prevent the reduction in ventricular function following ischemia (groups 3 and 4 in Table 2).

Similar results also were obtained for hearts in which coronary flow was allowed to continue to decline following ventricular failure, and ventricular function was assessed in reperfused hearts 20 min after a decline in peak systolic pressure to 30 mm Hg.

EFFECT OF VASODILATORS AND LACTATE ON CORONARY VASCULAR RESISTANCE

Table 4 shows data obtained from hearts perfused at 35 cm H<sub>2</sub>O perfusion pressure following failure with buffer containing 40 mM lactate, 5 μg of papavarine per ml, or 0.5 mM adenosine. These data indicate that perfusates containing these agents did not alter the magnitude of the increase in coronary vascular resistance during ischemia.

EFFECT OF ISCHEMIA ON TISSUE WATER CONTENT

One of the possible effects of hyaluronidase is to reduce extravascular fluid accumulation. To determine the effect of hyaluronidase and methylprednisolone on tissue water content, ischemic hearts were perfused for 2 hours in the presence and absence of these agents and were cut open, carefully blotted on filter paper, weighed, and dried to constant weight. During the ischemic period, there was an increase in tissue water content of about 20% from 3.95 ± 0.04 (20.3% dry) to 5.05 ± 0.25 ml/g in the nontreated hearts. However, in both treated groups of hearts, there was very little change in the tissue water content (3.89 ± 0.09 and 4.07 ± 0.22 ml/g for hyaluronidase and methylprednisolone hearts, respectively). Therefore, an increase in tissue water was associated with the increase in coronary vascular resistance in the nontreated ischemic hearts.

Discussion

The effects of two reportedly beneficial agents for reducing myocardial infarct size were studied in isolated ischemic rat hearts. The advantages of this model over an in situ regional ischemic model are (1) the entire heart receives reduced coronary flow, which reduces the sampling error when tissues are frozen for analysis; (2) the metabolic status of the tissue can be determined following various degrees of ischemia, since substrate delivery and product removal can be measured at known rates of coronary flow; and (3) postischemic mechanical function of tissue subjected to low flow can be measured without interference by contractile activity of nonischemic tissue.

The disadvantages of this model, compared with in situ regional ischemic models, appear to be that (1) the absence of blood prevents reactions such as platelet aggregation from occurring and also gives high rates of coronary flow, (2) the hearts are not subjected to the neural and hormonal influence that they would be in vivo, and (3) whatever influences adjacent nonischemic tissue may have on ischemic tissue are eliminated. Aside from these con-
siderations, the ischemic model used in the present studies appears to be well suited for evaluating the efficacy of agents which may protect the ischemic myocardium and for determining the mechanisms of action of beneficial agents.

The mechanism of action of hyaluronidase and glucocorticoids in reducing myocardial infarct size is not known. It has been suggested that hyaluronidase reduces ischemic damage by decreasing tissue fluid accumulation\(^{6,9}\) or by increasing substrate entry into the ischemic tissue.\(^{4}\) Release of lysosomal hydrolases appears to be an early consequence of ischemia.\(^{16,19}\) and glucocorticoids may prevent this release and protect ischemic tissue.\(^{5,8}\) Neither of these agents modified the rate of glycolytic energy production in ischemic perfused rat hearts in the present studies. However, the fact that both reduced the magnitude of the increase in coronary vascular resistance during ischemia suggest that this may be a primary mode of action of these agents. The mechanism(s) whereby hyaluronidase and methylprednisolone exert this effect is not known.

The finding that the coronary vascular resistance progressively increased in the ischemic model employed in this study suggests that the enlargement of myocardial infarct size in regional ischemia may have as its basis a similar increase in resistance to coronary flow. Such a possibility has not been previously demonstrated although, in a preliminary report, Askenazi et al.\(^{39}\) found that hyaluronidase prevented a reduction in coronary flow in ischemic dog myocardium which was attributed to an effect of hyaluronidase on collateral blood flow. Oliveira and Levy\(^{4,8}\) also considered edema development during ischemia an important contributory factor to ischemic cellular damage, and the beneficial effect of hyaluronidase in both experimental animals and patients was thought to be due to reduction of tissue edema.\(^{6,9}\) The use of hyperosmotic agents to protect the ischemic myocardium may also act to reduce the amount of tissue water which accumulates during ischemia. Willerson et al.\(^{21}\) have shown that hyperosmotic mannitol increases coronary flow in ischemic tissue, presumably through a reduction in cellular water. Also, the "no-reflow" phenomenon observed with reperfusion of ischemic tissue\(^{22}\) might be an extension of changes in tissue water content initiated during the ischemic period. In this manner, either small changes in coronary vascular resistance occur during ischemia and become larger on reperfusion, or the increased resistance in regional ischemic models becomes apparent only upon reperfusion. The present studies would suggest the latter possibility, since coronary flow was reduced by a smaller percentage on reperfusion than during the ischemic period. In this regard, in blood-perfused tissues, part of the "no-reflow" may be due to red cells plugging coronary vessels which have a reduced caliber. Krug et al.\(^{23}\) found no evidence of thrombosis following 30 to 120 min of ischemia, whereas, with reperfusion from 1 to 6 hours after temporary coronary artery occlusion, the capillaries were dilated, partly ruptured, and filled with erythrocytes. Regardless of whether or not the presence of red cells magnifies the resistance changes, several lines of evidence suggest that tissue edema is an important contributory component to tissue damage during myocardial ischemia.

The possibility that increased vascular resistance during ischemia may be an active process rather than a passive constriction should be considered. Agents such as adenosine and papavarine which inhibit an active contraction of the vascular smooth muscle cells do not prevent the increase in vascular resistance during ischemia. Coronary vascular resistance also can be increased by decreasing the perfusate pH below about 6.7.\(^{24}\) However, the pH of the coronary effluent did not decrease below 7.1 under the conditions used in the present study. In addition, maintenance of the coronary effluent pH at 7.4 with additional buffer in the perfusate failed to prevent the increase in resistance during ischemia. An additional argument against the hypotheses that H\(^{+}\) is responsible for these changes is that an increase in resistance occurred when the initial coronary flow following failure was 4 ml/min rather than 3 ml/min and, at this higher coronary flow, coronary effluent pH was higher than at the lower coronary flow.

Another factor that might have influenced coronary resistance during ischemia was the lactate produced during ischemia. However, addition of 40 mM lactate to the perfusate failed either to increase the magnitude of the resistance change or to induce it earlier.

The tissue distribution of the increased water is unknown. Whalen et al.\(^{25}\) attributed the increase in tissue H\(_2\)O, found after reperfusion of myocardium which had been ischemic 40 min, to an increase in cell H\(_2\)O content. However, in their studies, the ischemic tissue was receiving virtually no flow. It is possible that ischemic perfusion of the tissue during ischemia would lead to interstitial edema prior to the cellular edema which occurs when the cells are irreversibly damaged.

If the fluid accumulation is interstitial rather than cellular, the action of hyaluronidase in preventing an increase in coronary vascular resistance during ischemia might be due to clearing of the ground substance from interstitium and subsequent washout of oncotic substances including hyaluronic acid and proteins. Methylprednisolone would not be expected to act in a similar manner. However, the glucocorticoids are well known membrane-stabilizing agents, and the beneficial action of methylprednisolone in reducing tissue water content may be through stabilization of capillary membranes or through a reduction of cellular protein loss into the interstitial space. In the first case, membrane characteristics of the capillaries would have to be changed during ischemia to increase net fluid filtration. Kloner et al.\(^{27}\) have reported that, following 90 minutes of regional ischemia in the in situ dog heart, there is extensive capillary damage. Also, Meeney\(^{28}\) has pointed out the importance of the capillary factor in myocardial ischemia, and Feola and Glick\(^{27}\) demonstrated by measuring cardiac lymph that capillary permeability is increased during 2 hours of regional ischemia. The permeability of the capillary membranes apparently does increase during ischemia, and glucocorticoids may prevent it. The factors responsible for increased capillary membrane permeability are not known.

Glucocorticoids reportedly\(^{5,6}\) reduce the loss of cellular enzymes during ischemia. It is not known, however, to
what extent enzyme leakage into the interstitial space would alter capillary filtration.

Regardless of the factors responsible for the increase in tissue water during ischemia, the increased coronary vascular resistance was associated with increased tissue water. However, other alterations leading to simultaneous increases in both resistance and tissue water have not been ruled out. Nevertheless, the increased coronary vascular resistance appears to warrant special consideration among factors which increase the magnitude of tissue damage during ischemia.

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References

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