RESPONSE OF NITRIC OXIDE AND HEAT SHOCK PROTEIN 70 IN KIDNEY TISSUE OF ALL OLD RATS RECEIVING DOXORUBICIN (FOLLOWING 8 WEEKS OF SWIMMING AEROBIC EXERCISE AND GARLIC EXTRACT DIET)

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ABSTRACT
This study is aimed to evaluate the response of Nitric oxide and Heat shock protein 70 in kidney tissue of all old rats receiving Doxorubicin following 8 weeks of swimming aerobic exercise and garlic extract diet in an empirical model of chronic renal diseases. To do this, 42 old male wistar rates 40-50 week with the weight mean 250-300 g were divided randomly into 6 groups: 1-Control, 2-Doxorubicin, 3-Doxorubicin-Saline, 4-Doxorubicin Doxorubicin-aerobic exercise, 5-Doxorubicin-garlic, 6-Doxorubicin-aerobic and garlic consumption. To evaluate the changes of groups, one-way variance analysis was used. To determine the difference between the groups, Tukey test was applied. The results showed that Doxorubicin treatment increased plasma level of Nitric oxide and Heat shock protein 70. Regular swimming exercises, garlic extract supplement and combined treatments for 8 weeks reduced the plasma level of Nitric oxide and Heat shock protein 70. In addition, there was no difference between the effective of three above treatments on reduction of Nitric oxide and Heat shock protein 70.

Keywords: Nitric Oxide, Heat Shock Protein 70, Doxorubicin, Swimming Aerobic Exercise, Garlic Extract

INTRODUCTION
Chronic renal failure is one of the chronic diseases as increased in the recent years. Failure is the dysfunction of kidneys leading to kidney disease (Al-Qattan et al., 2006). Now, the patients with chronic renal failure are increased and this disease involves a wide range of the elderly (Raisifar et al., 2009). The elderly patients with chronic renal failure is increased in the past two centuries in most countries namely Iran.

The annual growth of this disease is about 11% in Iran in accordance to the statistics of transplant management center and special diseases of ministry of health and has reached 40 thousand people in 2009 (Rashki et al., 2011). This disease is increased by 10 times in US in the past 20 years (Al-Qattan et al., 2006).

One of the complications of patients with kidney problem is hypertension as described as the increase of resistance against blood circulation (Tian et al., 2007). Nitric oxide is strong vasodilator for relaxation of smooth vascular muscles (Charles, 2007).

Also, it is an important mediator in different physiological activities of body as general regulation of vascular strength and blood pressure (Antoniades, 2005). Also, nitric oxide acts as an intra-cellular and inter-cellular messenger in physiological functions including cellular growth, apoptosis, vascular tonicity regulation, immune system, contraction power, heartbeat, Bronchus, Tonus , memory and learning (Bian, 2008; Harlan et al., 2001; Kawahara et al., 2006). Besides the useful physiological effects of nitric oxide on the body, if the concentration is increased, it affects the sport performance as activity inhibitor (Donati, 2009; Johnson et al., 2005). Sport activities can lead to reduced blood pressure or avoidance of medicine treatment in hypertension patients (Higashi & Yoshizumi, 2004; Kuru et al., 2009). De Moraes et al., (2004) applied endothelial-dependent [acetylcholine] and bradykinin] and -independent [sodium nitroprusside] vasodilators and assessed the effects of exercise training on renal vascular reactivity on isolated kidneys of white trained and non-trained rabbits.
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It was concluded that exercise training altered the rabbit kidney vascular reactivity, enhancing endothelium-dependent and-independent renal vasodilation. This effect seems to be related not only to an increased bioavailability of NO but also to the enhanced responsiveness of the renal vascular smooth muscle to nitric oxide.

On the other hand, one of the methods of cell defense against stress damage is a synthesis of a set of internal-cellular proteins protect as called Heat Shock Protein (HSP) (Campisi et al., 2003). The Researches showed that there was an inverse relationship between HSP 70 with tubule damage, apoptosis and kidney dysfunction. The increase of HSP 70 before and after ischemia damage led into the support of kidney performance against kidney damage (Wang et al., 2011). The expression of HSP70 in different tissues with sport activities can reduce damages and the previous preparation of sport activities can have supportive effects with the expression of HSP 70 in various tissues. Running on the uphill (7%) and downhill (-7%) can induce increases in HSP70 in the kidney and heart, and in the soleus and gastrocnemius muscles (Lollo et al., 2013). Long-term regular exercises by the increase of HSP 70 can increase antioxidant defense in heart (Soufi et al., 2008). Based on the effect of sport on the increase of HSP 70 as a stress factor, the effective mechanisms of sport exercises on the changes of stress protein level of HSP 70 in the renal tissue under pathological conditions including hypertension are not defined. Chang et al., (2006) showed the increased of the expression of HSP 70 in heart, lung, liver and kidney of all trained male wistar rats, compared to the non-trained groups ignoring their healthy or diabetics’ conditions. Thus, HSP 70 expression in different tissues with sport activities reduced the tissue damages and the sport preparation had supportive effect with extra expression of HSP70 in different tissues (Milne and Noble, 2002). The researches have shown that garlic extract reduced hypertension and renal damages and this was performed via improving anti-oxidant system and delay of renal damages (Štajner et al., 2008). Besides having antioxidant properties to protect heart can reduce blood pressure and with high level of different types of Flavonoids have strong antioxidant property and tissue protection (Al-Qattan et al., 2006). However, the chronic renal disease is associated with inflammation and oxidative stress (Vaziri, 2008). Based on the studies on inflammatory indices of NO and HSP 70 and the association of these factors with chronic renal disease on one hand and the lack of consideration of the researchers to the effect of regular swimming exercise and garlic on NO and HSP 70 among old rats, the following questions are responded as follows:

1- Is there any significant relationship between regular swimming activity and NO and HSP 70 in kidney tissue of old rats?
2- Is there any significant relationship between garlic extract diet and NO and HSP 70 in kidney tissue of old rats?
3- Is there any significant relationship between regular swimming activity with garlic diet and NO and HSP 70 in kidney tissue of old rats?

MATERIALS AND METHODS

The study population is 24 Wistar male rats aged 40-50 weeks with the mean weight 250-300g and after including in the study and after one week swimming, by random method, 6 following groups are used: 1- Control, 2- Doxorubicin, 3- Doxorubicin-saline, 4- Doxorubicin-exercise, 5- Doxorubicin-garlic, 6- Doxorubicin-aerobic exercise-garlic

Study Environment and Diet of Subjects

In the study, the subjects are housed in transparent poly carbonate cages, length 15*15*30cm, light to dark cycle 12:12 hours with the temperature 2±22°C and humidity 5±50% with suitable ventilation. The diet of subjects based on weighting based on every three days with special standard scale is 10g of natural diet for every 100g body weight per day. The required water of the animal is kept in the bottle 500mL special for laboratory animals.

Measures

1- Laboratory digital scale to weight the subjects with the sensitivity 0.001g
2- Swimming pool of activities of laboratory animals (rats) made in Iran
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3- Surgery tools (carpel blade, scissor, pence, etc.)
4- Timer (to regulate darkness and light of laboratory environment)
5- Temperature (to regulate the temperature of laboratory environment)
6- Laboratory tube, Erlenmeyer, acetone, alcohol, surgery glove (latex), syringe 2.5, 5cc, dissection tray, cotton, etc.

Exercise Protocol
Before the commencement of the main protocol, the trials of exercise group were trained for one week (five days per week and each time for five minutes) in order to learn swimming. The main exercise plan was as follows: swimming for 8 weeks (three days per week and 30 minutes for every session [13]) in water (temp=2±32°C). A five minutes interval was considered before and after the main exercise in order to let the trials warm and cool.

Preparation and Consumption of Garlic Extract
To start extraction process, maceration of garlic is used. A total of 50 gr ground garlic was poured in a 1 liter balloon and methanol was added up with a ratio of 1:3 and was placed on a shaker for 24 hours. The solution, then, was filtered by Buchner funnel and methanol was added up to the remained slag. The slag was filtered again after 24 hours and was added to the first extract. The obtained solution, then, was distilled in a vacuum distillation device at a temperature of 50°C and at a speed of 70 rpm until the solution volume was decreased to 1/5 of its initial volume. At this point, the extract tank was separated from the device. When the solution was cooled it was decanted for three times with 50 ml chloroform. The remained volume was poured in a Petri dish with a given weight and was opened within device at 50°C. When the extract was dried, it was weighted and every ¼ gram of the extract powder was mixed with 56 ml distilled water.

The supplement group and supplement-exercise group were received 1 ml garlic extract for every kilogram of their body weight on daily basis for 8 weeks through oral gavage. Saline group, however, was received the same volume of saline supplement in the same manner (Alkreathy et al., 2010).

Preparation Method and Injection of Doxorubicin and Saline
Doxorubicin is diluted using distilled water to provide the required dosage (8.5mg/kg weight of body). Based on the results of study of Chen et al., (2013), Doxorubicin is injected by an insulin syringe as peritoneal injection once at the first week. Also, based on the probable effects of injection in Doxorubicin treatment group, for equalization of conditions for all subjects, other groups received saline for the same value (chloride sodium 0.9%). To avoid the influence of circadian heart rhythm rate, the injections were homogenous at 10 a.m.

Blood Sample Collection and Biopsy Operations
Blood sample collection and biopsy operations were conducted 48 hours after the last exercise session and after 10-12 hours of fasting. The rats were anesthetized by intra peritoneal injection of Ketamin (30-50 mg/kg) and Xylazine (3-5 mg/kg).

Then, kidney tissue was immediately removed and placed in a freezer at -70°C for measuring HSP and NO levels purposes. Blood sampling was performed directly from heart using heparin syringe and to extract plasma by centrifuge, was kept for 150min at the speed 3000 rpm at temperature -80°C. To avoid the influence of circadian heart rhythm rate, sampling operation was started at 8:00 a.m. and was completed at 11:30 a.m.

Providing Tissue Homogenized Samples
A total of the frozen collected tissue was placed in liquid nitrogen after being powdered. The tissue, then, was homogenized in protease buffer (PBS, pH 4.7) with 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol, 1mM PMSF, leupeptin (1 μg/ml), sodium vanadate (0.5 mM, AEBSF (100 mg/ml). The obtained solution was centrifuged for 20 minutes at speed= 12000 rpm at the temperature 4°C. The solution was frozen at the temperature -20°C for chemical analysis.

Biochemical Analysis of Variables
Plasma level of NO is measured by converting NO to its stable metabolites (reduction of nitrate ions to nitrite via Nitrate reductase) and measurement of concentration of nitrate /nitrite using colorimetric
method based on Griess reagent. To evaluate HSP70, at first the samples were removed from the freezer and placed for 30 at room temperature. Then, they were up and down for 5 times to eliminate the gradient of concentration of freezing and melting and the concentration of the samples was uniform. All measures were performed by quantitative method using Enzyme-Linked-Immuno-sorbent Assay (ELISA) using Elisa kits (Rat Eliza kit, Assaypro co, USA) based on the instruction of the manufacturing company with the change coefficient (CV%:7.3) and sensitivity (15.6pg/ml).

**The Statistical Analysis**

The statistical computations are performed using SPSS software, version 20 and plotting charts is performed by Excel software. For classification of dispersion indices, descriptive statistics is used. To evaluate the homogeneity of variance, Levin test is used and to evaluate the changes among groups, ANOVA is used and to determine the difference between groups, Tukey test is used.

The results show that the highest value of NO of plasma is dedicated to Doxorubicin group and the minimum value is dedicated to control group.

The results of Shapiro Wilk (Tables 1, 2) and Levin test (P=0.636 for plasma level of NO, P=0.535 on HSP 70 of kidney tissue) refer to the normal distribution of data and homogeneity of variances. To evaluate the changes of plasma level of NO and HSP70 of kidney tissue of old rats being treated with Doxorubicin after a regular aerobic exercise with garlic extract, ANOVA is used and in case of significance of the difference between the groups, to clarify the difference, Tukey test at significance level P<0.05 is used.

- **H1: There is No Significant Difference between the Changes of Plasma Level of NO in Exercise Groups, Garlic Extract and Combination of Exercise-Garlic of Old Rats Receiving Doxorubicin.**

Based on the computed F value (14.285) and its significance at the level P=0.000, H0 regarding the non-significance between NO levels in different groups with confidence interval 95% is rejected.

![Figure 1: The Comparison of the Mean of NO Levels in Different Groups](image)

*: The difference significance to control group
#

: The difference significance to Doxorubicin and Doxorubicin+ saline groups
## Table 1: The Mean and Standard Deviation of Plasma Level of NO (µmoles/l) in Different Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>Doxorubicin+ Saline</th>
<th>Doxorubicin+ Exercise</th>
<th>Doxorubicin+ Garlic</th>
<th>Doxorubicin+ Exercise + Garlic</th>
</tr>
</thead>
<tbody>
<tr>
<td>P Value</td>
<td>0.838</td>
<td>0.322</td>
<td>0.143</td>
<td>0.556</td>
<td>0.423</td>
<td>0.636</td>
</tr>
<tr>
<td>Mean</td>
<td>42.77</td>
<td>38.34</td>
<td>36.10</td>
<td>22.20</td>
<td>20.77</td>
<td>20.77</td>
</tr>
<tr>
<td>SD</td>
<td>6.41</td>
<td>7.92</td>
<td>5.61</td>
<td>6.07</td>
<td>4.72</td>
<td>8.72</td>
</tr>
</tbody>
</table>

P value: Schapiro-Wilk test

## Table 2: The Mean & Standard Deviation Indices of HSP70 of Kidney Tissue (Pictogram/ml/g/Protein) of Different Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>Doxorubicin+ Saline</th>
<th>Doxorubicin+ Exercise</th>
<th>Doxorubicin+ Garlic</th>
<th>Doxorubicin+ Exercise + Garlic</th>
</tr>
</thead>
<tbody>
<tr>
<td>P Value</td>
<td>0.591</td>
<td>0.878</td>
<td>0.846</td>
<td>0.555</td>
<td>0.783</td>
<td>0.535</td>
</tr>
<tr>
<td>Mean</td>
<td>60.77</td>
<td>61.20</td>
<td>62.84</td>
<td>82.12</td>
<td>83.62</td>
<td>43.77</td>
</tr>
<tr>
<td>SD</td>
<td>14.51</td>
<td>14.63</td>
<td>11.86</td>
<td>15.55</td>
<td>10.94</td>
<td>9.56</td>
</tr>
</tbody>
</table>

P value: Schapiro Wilk test

## Table 3: The Results of ANOVA of NO Levels

<table>
<thead>
<tr>
<th>P Value</th>
<th>F Value</th>
<th>Mean of Squares</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>The Variance of NO Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000*</td>
<td>14.285</td>
<td>644.804</td>
<td>5</td>
<td>3224.019</td>
<td>Intergroup</td>
</tr>
<tr>
<td></td>
<td>45.140</td>
<td>1625.026</td>
<td>36</td>
<td></td>
<td>Intragroup</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>4849.045</td>
<td>41</td>
<td></td>
<td>Total</td>
</tr>
</tbody>
</table>
Table 4: The Results of Tukey Test and Inter-Group Changes of NO Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>Doxorubicin + saline</th>
<th>Doxorubicin + exercise</th>
<th>Doxorubicin + garlic</th>
<th>Doxorubicin + exercise + garlic</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>2.000</td>
<td>4.428</td>
<td>6.671</td>
<td>20.571</td>
<td>22.071</td>
<td>---</td>
</tr>
<tr>
<td>p</td>
<td>0.993</td>
<td>0.818</td>
<td>0.444</td>
<td>0.000*</td>
<td>0.000*</td>
<td>p = 0.000*</td>
</tr>
<tr>
<td>M</td>
<td>20.071</td>
<td>17.642</td>
<td>15.400</td>
<td>1.500</td>
<td>0.998</td>
<td>---</td>
</tr>
<tr>
<td>p</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.002*</td>
<td>p = 0.002*</td>
<td>p = 0.998</td>
<td>p = 0.998</td>
</tr>
<tr>
<td>p</td>
<td>0.783</td>
<td>0.001*</td>
<td>0.005*</td>
<td>0.005*</td>
<td>0.000*</td>
<td>---</td>
</tr>
<tr>
<td>M</td>
<td>2.428</td>
<td>0.988</td>
<td>0.988</td>
<td>2.242</td>
<td>2.428</td>
<td>---</td>
</tr>
<tr>
<td>p</td>
<td>0.983</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

*There is a significant difference

The above results show that Doxorubicin treatment leads to the increase of plasma level of NO (P=0.000, 51.60%) in old rats. 8 weeks of aerobic exercise (P=0.002, 74.51%), garlic extract (P=0.000, 85.21%) and combined intervention (P=0.000, 96.95%) with the reduction of NO in old rats. There is no difference between the effects of three above interventions to reduce NO levels. Finally, there is a significant difference between the changes of plasma level of NO in exercise groups, garlic extract consumption and combination of exercise-garlic of old rats in empirical model of chronic renal disease and H0 is rejected.

- H2: There is No Significant Difference between the Changes of HSP70 of Kidney Tissue in Exercise Groups, Garlic Extract and Combination of Exercise-Garlic of Old Rats in Empirical Model of Chronic Renal Disease.
Table 5: The Results of ANOVA of HSP70 of Kidney Tissue

<table>
<thead>
<tr>
<th>P Value</th>
<th>F Value</th>
<th>Mean of Squares</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>The Variance HSP70 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000*</td>
<td>9.273</td>
<td>1574.852</td>
<td>5</td>
<td>7874.262</td>
<td>Intergroup</td>
</tr>
<tr>
<td></td>
<td></td>
<td>169.832</td>
<td>36</td>
<td>6113.934</td>
<td>Intragroup</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td>13988.196</td>
<td>Total</td>
</tr>
</tbody>
</table>

Based on the computed F value (9.273) and its significance at the level P=0.000, H0 regarding the non-significance between HSP70 levels of kidney tissue in different groups with confidence interval 95% is rejected.

Table 6: The Results of Tukey Test and Inter-Group Changes of HSP70 Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>Doxorubicin+ saline</th>
<th>Doxorubicin+ garlic</th>
<th>Doxorubicin+ exercise+ garlic</th>
</tr>
</thead>
<tbody>
<tr>
<td>M=17.000</td>
<td>M=-17.428</td>
<td>M=-19.071</td>
<td>M=-38.357</td>
<td>M=-39.857</td>
<td>***</td>
</tr>
<tr>
<td>p=0.170</td>
<td>p=0.150</td>
<td>p=0.092</td>
<td>p=0.000*</td>
<td>*p=0.000</td>
<td></td>
</tr>
<tr>
<td>M=22.857</td>
<td>M=22.428</td>
<td>M=20.785</td>
<td>M=1.500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=0.026*</td>
<td>p=0.030*</td>
<td>p=0.053</td>
<td>p=1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=0.044*</td>
<td>p=0.050</td>
<td>P=0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M=2.071</td>
<td>M=1.642</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=1.000</td>
<td>p=1.000</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M=0.428</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=1.000</td>
<td></td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*There is a significant difference

Based on Table 6, HSP 70 of kidney tissue of old rats was increased after Doxorubicin treatment (P=0.000, 87.63%). 8 weeks of garlic consumption (P=0.030, 25.48%), combined intervention of exercise and garlic (P=0.026, 26.01%) led into the reduction of HSP 70 in kidney tissue of old rats. The heat shock protein levels 70 of kidney tissue in exercise groups (P=0.053, 23.49%) was reduced non-significantly but it was higher than its levels in healthy old rats and there was no difference between the effect of three above interventions to reduce HSP 70 of kidney tissue. Thus, there is a significant difference between the changes of heat shock protein level 70 of kidney tissue in exercise, garlic extract and combination of exercise of old rats in empirical model of chronic renal disease.

Discussion and Conclusion

The final purpose of this study is the evaluation of NO and HSP70 response of kidney tissue of old rats receiving Doxorubicin following 8 weeks of regular exercises and garlic extract consumption in empirical model of renal chronic disease. Based on the study findings, Doxorubicin treatment leads to the increase of plasma level of NO in old rats and 8 weeks of aerobic exercise is with the reduction of NO in old rats.
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It is consistent with the results of study of Husain (2003) as sport activities can increase NO-dependent vasodilation without the increase of endothelial nitric oxide synthase and it is justified via the increase of anti-oxidant as the increase of Super oxide dismutase and increase of Glutathione peroxidase. Also, the effect of sport activities on the increase of response to acetylcholine and NO-dependent vasodilation with reduction of blood pressure in empirical models of hypertension with the dysfunction of NO is supported by Kuru et al., (2009); Ribeiro et al., (2010); De Moraes et al., (2004). Other results of study show that HSP 70 levels of kidney tissue of old rats was increased significantly after Doxorubicin treatment and 8 weeks of exercise had no significant effect. Speaker et al., (2014) showed that six weeks of exercise via the increase of heat shock protein 72 enhanced the factor of Monocyte chemo attractant protein-1, interleukin 10 and interleukin -6 of white fat tissue of thin rats. Lewis et al., (2013) showed that mild sport activities could increase HSP70 levels of skeletal muscles of middle age rats. Also, Parra et al., (2008) reported over expression of renal HSP70 in three empirical models of salt-sensitive hypertension in Sprague-Dawley rats with inhibition of nitric oxide (NO3 weeks of drinking water with L-NAME), short-term angiotensin II (10ng injection/min, 2 weeks), protein overload in urine with protein load (2 weeks of Intraperitoneal injections of bovine serum albumin, 2g in 6mL saline 0.9%). In a study, Rodríguez-Iturbe et al., (2004) referred to the supportive role of heat shock protein in vascular damage in hemodynamic stress. The results of new study by Wang et al., (2011) showed that there was an inverse relationship between HSP70 with tubule damage, apoptosis and kidney dysfunction. The present study showed that garlic extract (P=0.030, 25.48%, p=0.000, 85.21%) and combined intervention (p=0.026, 26.01%, P=0.000, 96.95%) was with the reduction of NO level of plasma and HSP70 of kidney tissue in old rats. In addition, there is no difference between the effects of three above interventions to reduce the above variables. Also, Doxorubicin treatment increased the plasma level of NO and HSP70. Also, regular swimming exercises, garlic extract supplement and combined intervention for 8 weeks led into the renal support against oxidative stress and inflammation of treatment with Doxorubicin. Thus, sport exercises, antioxidant supplements and combined methods were used as non-medicine therapy methods to reduce renal damage against the damages of oxidative stress and inflammation of age increase.

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