

# The effects of acetazolamide on ischemia reperfused isolated hearts of 2- and 8-week-old rabbits

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## ABSTRACT

**الأهداف:** التحقق من تأثير الأستيازولاميد على عينات القلب المصابة بالافتقار بعد إعادة التروية لها والمعزولة من الأرانب التي تبلغ أعمارها 8 أسابيع و 8 أسابيع.

**الطريقة:** أُجريت هذه الدراسة في مركز كيرمنشاه لأبحاث العلوم الحيوية الطبية، كيرمنشاه، إيران وذلك خلال الفترة من مارس إلى سبتمبر 2011م. لقد قمنا بتقسيم الأرانب التي تبلغ أسبوعين من العمر (العدد: 17)، والأرانب التي تبلغ 8 أسابيع (العدد: 17) إلى مجموعتين وهما: مجموعة الشاهد (العدد: 9)، ومجموعة الدراسة (العدد: 8). بعدها قمنا بتعريض عينات القلب المعزولة إلى 35 دقيقة من الانقار الدموي، و30 دقيقة من إعادة التروية الدموية للقلب. وقمنا بإضافة الأستيازولاميد (100 ميكروغرام/لتر) إلى محلول التروية الدموية قبل حدوث الانقار بحوالي 10 دقائق في مجموعة الدراسة. وبعدها قمنا بتحليل مقاييس القلب التالية: الضغط البطيني، ومعدل نبضات القلب، ومنتج معدل الضغط، واستخدمنا اختبار تي من أجل تحليل هذه البيانات.

**النتائج:** أشارت نتائج الدراسة إلى أن إعطاء الأستيازولاميد لمجموعة الدراسة قد أدى إلى تغيير معدل نبضات القلب بشكل واضح من الناحية الإحصائية لدى الأرانب التي تبلغ من العمر أسبوعين (91±1.1%) مقارنةً بالأرانب التي تبلغ من العمر 8 أسابيع (96±0.8%) وكانت نسبة العودة إلى الوضع الطبيعي لمنتج معدل الضغط بعد إعادة التروية أقل لدى المجموعة التي تبلغ من العمر 8 أسابيع (28.9±3.4%) مقارنةً بالمجموعة التي تبلغ من العمر أسبوعين (45.2±3.5%) ( $p=0.005$ ).

**خاتمة:** لقد كانت استجابة عينات القلب للأنيدراز الكربونية لدى الأرانب التي تبلغ من العمر أسبوعين أفضل من استجابة الأرانب في المجموعة التي تبلغ من العمر 8 أسابيع، غير أن إعطاء الأستيازولاميد لم يؤدي إلى تفاقم تضرر القلب من الإفقار الدموي لدى الأرانب التي تبلغ من العمر أسبوعين. وهكذا أظهرت الدراسة بأنه على الرغم من الدور الرئيسي الذي تلعبه الأنيدراز الكربونية في وظائف عينات القلب الطبيعية فإنه لا يمكن اعتبارها عاملاً محددًا في تحديد مدى تضرر القلب من الافتقار الدموي لدى الأعمار المختلفة.

**Objectives:** To investigate the effects of acetazolamide on the ischemia-reperfused isolated hearts of 2- and 8-week-old rabbits.

**Methods:** This study was conducted at the Kermanshah Medical Biology Research Center, Kermanshah, Iran from March to September 2011. Two- (n=17) and 8-week old (n=17) rabbits were separately divided into 2 control (n=9), and test (n=8) groups. Isolated hearts were subjected to 35 minutes ischemia and 30 minutes reperfusion. Acetazolamide (100 microgr/l) was added to the perfusion solution for 10 minutes before ischemia in the test group. Cardiac parameters including ventricular pressure, heart rate (HR), and rate pressure product (RPP) were measured. Data sets were analyzed by t-test.

**Results:** Following acetazolamide administration the change percentage of HR was significantly different in the 2-week (91 ± 1.1%) compared with the 8-week (96 ± 0.8%) test groups ( $p=0.0016$ ). Recovery percentage of RPP in reperfusion was lower ( $p=0.005$ ) in the 8- (28.9 ± 3.4%) than the 2-week test groups (45.2 ± 3.5%).

**Conclusion:** The 2-week hearts elicited more rapid response to carbonic anhydrase (CA) inhibition than the 8-week group. However, acetazolamide does not exacerbate ischemia-reperfusion (I/R) injury in the 2-week hearts. Therefore, it was revealed that after inhibition of CA, the age dependent pattern of I/R injury was similar to that of the normal hearts. In spite of the CA important role in the normal heart function, it is not a determining factor in I/R injury in different ages.

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Intracellular pH ( $\text{pH}_i$ ) is one of the most significant modulators of the cardiac function.<sup>1,2</sup> Steady-state  $\text{pH}_i$  is typically 7.1-7.3 in the cardiac cells.<sup>3</sup> It can be modestly reduced with an increase in heart rate and more dramatically during myocardial ischemia,<sup>1</sup> where it is responsible for much of the observed failure of contraction.<sup>4</sup> Myocardial ischemia is characterized by the inhibition of oxidative phosphorylation and the onset of anaerobic glycolysis with attendant lactic acid production, and intracellular acidification.<sup>5</sup> Acidosis decreases the electrical activity of the heart, and results in the rapid and reversible reduction of the heart rate.<sup>2</sup> Also, acidosis decreases the left ventricular developed pressure.<sup>6</sup> The  $\text{pH}_i$  is primarily regulated by buffers.<sup>1</sup> Carbon dioxide ( $\text{CO}_2$ )-hydrogen carbonate ( $\text{HCO}_3^-$ ) is one of the most important buffers in the heart,<sup>7,8</sup> and carbonic anhydrase (CA) enzyme plays a key role in this buffer<sup>1,2,9</sup>. The CA is functionally active in the heart, and is known to be expressed in the myocytes.<sup>1,10</sup> It can be blocked by acetazolamide, a membrane permeant CA inhibitor, which has been shown to inhibit fully intracellular CA activity in ventricular cardiomyocyte.<sup>9</sup> Inhibition of membrane-bound CA in rabbit's heart muscles might induce an intracellular acidosis, which decreases contractility of the heart.<sup>11</sup> The CA plays an important role in the recovery of intracellular pH and cardiac function following ischemia.<sup>7</sup> It has been reported that the control of intracellular pH and buffering capacity are age dependent in rabbit's myocytes. Furthermore, it has been suggested that increased tolerance of the neonatal heart to ischemic injury is related to myocardial pH regulation,<sup>12</sup> but the role of CA enzyme is not clear in this regard. Regarding the important role of CA enzyme, there are no adequate data regarding its effects on the ischemia reperfused hearts in different ages. Therefore, considering the significance of the subject, the current study conducted to investigate the role of acetazolamide on the ischemia reperfused isolated hearts of 2- and 8-week-old rabbits.

**Methods.** This study was conducted at the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran. The Ethics Committee of Kermanshah University of Medical Sciences approved all experiments, and all animals involved in the current study received humane care in compliance with institutional animal care guidelines. The New Zealand white rabbits (Razi Institute, Tehran, Iran) ( $n=34$ ) were used in this study. The 2- ( $n=17$ ) and 8-week old ( $n=17$ ) rabbits were randomly divided into 2 control ( $n=9$ ) and test ( $n=8$ ) groups, so that the 4 final

groups were formed. The animals were anesthetized by injection of 60 mg/kg pentobarbital sodium (Sigma, Steinheim, Germany) intraperitoneally. The hearts were exposed through a midline thoracotomy and excised. Isolated hearts were arrested by immersion in ice-cold Krebs buffer (4°C). The aortic root was cannulated and the heart was retrogradely perfused in a non-circulating Langendorff apparatus with Krebs buffer (containing in mmol/l: sodium chloride 118; sodium hydrogen carbonate 25; potassium chloride 4.8; potassium dihydrogen phosphate 1.2; magnesium sulphate 1.2; glucose 11; and calcium chloride 1.2, at pH 7.4). The buffer was filtered through the Whatman paper catalog number 1002 125, and then bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at 37°C, and perfusion was performed under a constant hydrostatic pressure of 50 for the 2-, and 60 mm Hg for the 8-week old rabbit hearts. A small deflated balloon was inserted through the mitral valve into the left ventricle. The balloon was connected to a powerlab (model ML825, AD Instruments, New South Wales, Australia) through a pressure transducer (MLT844; AD Instruments, New South Wales, Australia), and linked to a computer. Then the balloon volume was adjusted to 5-10 mm Hg during the experiment. Therefore, different indices of myocardial function including left ventricular developed pressure (LVDP) in mm Hg, and heart rate as beat per minute (BPM) were assessed. The LVDP was defined as peak systolic minus end diastolic pressure (in mm Hg). Also, rate pressure product (RPP) was expressed as  $\text{RPP} = \text{LVDP} \times \text{HR}$ .<sup>13</sup> Coronary solution flow (CSF) was measured by regular timed collections of the coronary effluent. Hearts were subjected to 25 minutes stabilization period (baseline), 35 minutes global normothermic ischemia, and 30 minutes reperfusion period. Global normothermic ischemia was caused by clamping the aortic cannula, and immersing the hearts in perfusion solution at 37°C.<sup>14</sup> In addition to this protocol, the test groups' hearts were perfused for 10 minutes before ischemia with Krebs buffer containing 100 microgr/l acetazolamide (Iran Darou Pharmaceuticals Co. Ltd., Tehran, Iran). The change percentage of cardiac performance following drug administration was determined, and compared to the baseline. The level of ischemia-reperfusion (I/R) injury was assessed based on the cardiac functional recovery.

Results are reported as mean  $\pm$  standard error of mean (SEM), and statistical analysis between data sets was made by paired or unpaired t-test, and using the Graph Pad InStat, version 3.05, Graph Pad software. The confidence interval (CI) was 95% and  $p < 0.05$  was statistically considered the level of significant difference.

**Results.** The values of different cardiac parameters at baseline and thirtieth minute of reperfusion are shown in Table 1. Following drug administration, the cardiac functional parameters including HR, LVDP, CSF, and RPP were significantly decreased compared to the baseline. These parameters were reduced from baseline values to  $208 \pm 7.5$ ,  $67.8 \pm 2.5$ ,  $10.37 \pm 0.68$ , and  $14210 \pm 821$  in the 2-week old test, and to  $178 \pm 8.7$ ,  $79.2 \pm 3.4$ ,  $21.25 \pm 1.7$ , and  $14167 \pm 1067$  in the 8-week old test groups. As it is demonstrated in Figure 1, the reduction rate of HR in the 2-week old test group was significantly higher than that of 8-week-old test one. Compared with the baseline, the change percentage of the HR in the 2-week test group ( $91 \pm 1.1\%$ ) was significantly different ( $p=0.0016$ ) from that of the 8-week old test group ( $96 \pm 0.8\%$ ). On the other hand, following I/R, the recovery percentage of cardiac parameters in groups receiving acetazolamide was significantly lower than that of related controls. The main cardiac parameters, which significantly decreased in the 2- and 8-week old groups include HR and LVDP (Figure 2 & Figure 3). Also the different cardiac parameters of the 8-week-old test groups in reperfusion period were significantly lower than those of the related control (Table 1), which indicates the exacerbated I/R injury. The recovery percentages of cardiac parameters following I/R between the 2 test groups were compared in Figure 4. As shown in this figure, the LVDP, CSF, and

RPP recovery percentage in the 8-week old test group was significantly lower than that of the 2-week old test group. For instance, the recovery percentage of RPP in the 8-week old group ( $28.9 \pm 3.4\%$ ) was significantly different ( $p=0.005$ ) from that of the 2-week old group ( $45.2 \pm 3.5\%$ ), which reveals the exacerbated I/R injury in the 8-week old group.

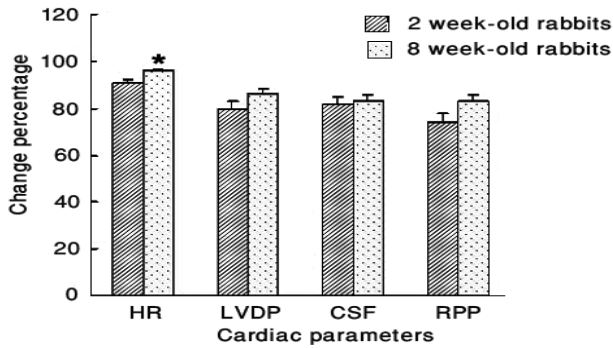
**Discussion.** The findings indicated different effects of acetazolamide on the function of the isolated rabbit hearts based on age. This is a new finding, which shows different roles of this agent at different ages. The inhibition of CA enzyme significantly decreases the cardiac performance of the 2 groups, but following acetazolamide administration the reduction of heart rate in the 2-week old group was significantly higher than that of the 8-week old group (as illustrated in Figure 1). This result indicates more enhanced reaction of the 2-week old hearts in response to CA inhibition than that of the 8-week old hearts.

Previous studies revealed that the inhibition of membrane bound CA in rabbit's cardiomyocytes results in intracellular acidosis and reduction of cardiac function.<sup>11</sup> However, the findings of the current study showed that the reduction of HR in the 2-week-old test group is greater than that of the 8-week-olds. It could be the result of more severe susceptibility of the 2-week

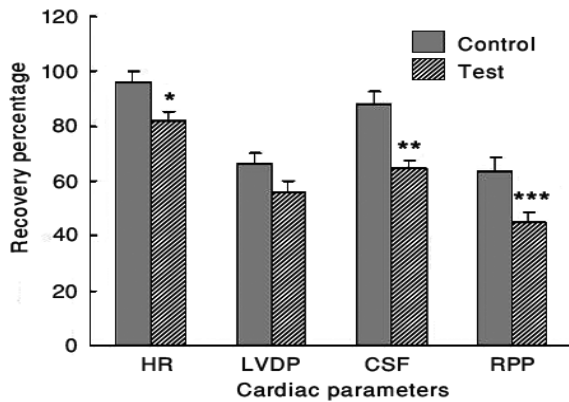
**Table 1** - Cardiac parameters before ischemia (baseline) and at the thirtieth minutes of reperfusion in 2- and 8-weeks old isolated rabbit's heart.

Groups	Parameters							
	Baseline				Reperfusion at the thirtieth minute			
	HR	LVDP	CSF	RPP	HR	LVDP	CSF	RPP
Control, weeks old, (n=9)	$220 \pm 18.02$	$76 \pm 2.49$	$12.12 \pm 1.04$	$16787 \pm 727$	$212 \pm 10$	$49.8 \pm 3.5$	$10.63 \pm 1.06$	$10512 \pm 770$
Test, 2 weeks old, (n=8)	$228 \pm 7.98$	$84 \pm 2.9$	$12.45 \pm 0.51$	$19308 \pm 1101$	$187 \pm 8.8$	$46.9 \pm 2.9$	$8.08 \pm 0.44$	$8649 \pm 668$
2 weeks old p-value	0.419	0.055	0.795	0.07	0.085	0.552	0.051	0.091
Control, 8 weeks old, (n=9)	$195.6 \pm 9.8$	$93.2 \pm 2.59$	$27.55 \pm 1.6$	$18205 \pm 912$	$173 \pm 6.83$	$53.4 \pm 5.3$	$21.27 \pm 1.39$	$9268 \pm 975$
Test, 8 weeks old, (n=8)	$184 \pm 8.1$	$91.7 \pm 3.4$	$25.32 \pm 1.4$	$16875 \pm 971$	$160 \pm 6.1$	$29.8 \pm 2.86^*$	$13.11 \pm 0.76^\dagger$	$4689 \pm 292^\ddagger$
8 weeks old p-value	0.392	0.726	0.333	0.334	0.189	0.002	0.0002	0.0007

HR = heart rate (beat/minute), LVDP = left ventricular developed pressure (mm Hg), CSF = coronary solution flow (milliliter/minute), RPP= rate pressure product (LVDP×HR). \* $p=0.002$ , 95% confidence interval (CI): -36.9 - -10.1; † $p=0.0002$ , 95% CI: -11.67 - -4.65, ‡ $p=0.0007$ , 95% CI: -6869.2 - -2288.8 versus 8-week-old controls

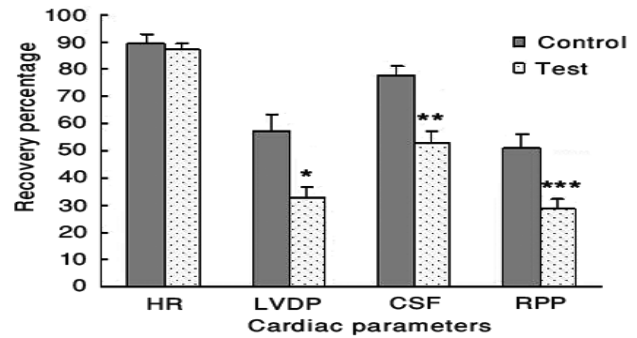


**Figure 1** - Change percentage (in comparison to baseline) of the different cardiac parameters following acetazolamide administration in 2- (n=8) and 8-weeks (n=8) old groups. HR = heart rate (beat/minute), LVDP = left ventricular developed pressure (mm Hg), CSF = coronary solution flow (milliliter/minute), RPP= rate pressure product (LVDP x HR). Data are expressed as the mean  $\pm$  standard error of mean. Following is a representation of *p*-values and confidence intervals of the pair columns, which have been compared: HR - \**p*=0.0016, 95% confidence interval (CI): 0.0249 - 0.0855; LVDP - *p*=0.19, 95% CI: - 0.030 - 0.1399; CSF - *p*=0.94, 95% CI: -0.074 - 0.079; RPP - *p*=0.059, 95% CI: -0.191 - 0.004.

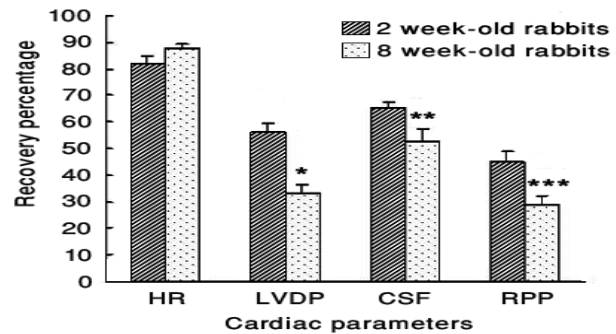


**Figure 2** - Recovery percentage of the different cardiac parameters following ischemia reperfusion in 2 controls (n=9) and test (acetazolamide) (n=8) groups in 2-week-old isolated rabbit's hearts. HR = heart rate (beat/minute), LVDP = left ventricular developed pressure (mm Hg), CSF = coronary solution flow (milliliter/minute), RPP= rate pressure product (LVDP x HR). Data are expressed as the mean  $\pm$  standard error of mean. The following is a representation of the *p*-values and confidence intervals of the pair columns, which have been compared: HR - \**p*=0.014, 95% confidence interval (CI): -0.2528 - - 0.03325; LVDP - *p*=0.137, 95% CI: -0.2285 - 0.03471; CSF - \*\**p*=0.0009, 95% CI: -0.3527 - -0.1121; RPP - \*\*\**p*=0.012, 95% CI: -0.324 - -0.0454.

old hearts to acidosis. Higher glycolytic capacity in the younger hearts results in more lactate production, which in turn causes more reduction of intracellular pH.<sup>15</sup> In this case, acidosis enhances intracellular calcium, and thereby, it can activate calcium-activated potassium ( $K_{Ca}$ ) channels, and giving rise to cellular hyperpolarization.<sup>16</sup> The density of these channels is



**Figure 3** - Recovery percentage of the different cardiac parameters following ischemia reperfusion in 2 controls (n=9) and test (acetazolamide) (n=8) groups in 8-week-old isolated rabbit's hearts. HR = heart rate (beat/minute), LVDP = left ventricular developed pressure (mm Hg), CSF = coronary solution flow (milliliter/minute), RPP= rate pressure product (LVDP x HR). Data are expressed as the mean  $\pm$  standard error of mean. Following is a representation of *p*-values and confidence intervals of the pair columns, which have been compared: HR - *p*=0.619, 95% confidence interval (CI): - 0.1043 - 0.06425; LVDP - \**p*=0.003, 95% CI: -0.3969 - - 0.09453; CSF - \*\**p*=0.0005, 95% CI: -0.3657 - -0.1264; RPP - \*\*\**p*=0.003, 95% CI: -0.3586 - -0.08643.



**Figure 4** - Comparison of the recovery percentage of the different cardiac parameters following ischemia reperfusion in 2- (n=8) and 8-week-old (n=8) isolated rabbit's hearts of the test (acetazolamide) groups. HR = heart rate (beat/minute), LVDP = left ventricular developed pressure (mm Hg), CSF = coronary solution flow (milliliter/minute), RPP= rate pressure product (LVDP x HR). Data are expressed as the mean  $\pm$  standard error of mean. The following is a representation of the *p*-values and confidence intervals of the pair columns, which have been compared: HR - *p*=0.15, 95% confidence interval (CI): -0.02384 - 0.1361; LVDP - \**p*=0.0005, 95% CI: -0.3441 - -0.1206; CSF - \*\**p*=0.036, 95% CI: -0.2290 - - 0.008921; RPP - \*\*\**p*=0.005, 95% CI: -0.2677 - -0.05662.

age dependent and more abundant in lower ages,<sup>17</sup> and the role of the acetazolamide in the activation of these potassium channels has been revealed in a previous study<sup>16</sup>.

As a limitation of this study we could not determine the level of myocyte hyperpolarization, however, the result of the current study including significant

decrease of HR in the 2-week old hearts can be explained by the reduction of the myocytes' excitability following hyperpolarization, which has been reported in other studies.<sup>15-17</sup> As demonstrated in Figures 2 & 3, the 2 acetazolamide recipient groups showed more exacerbated I/R injury, and less recovery of the cardiac performance than that of the control. This result is obviously expected in the presence of CA inhibition, since the reduction of the buffering capacity could naturally lead to the exacerbated I/R injury. On the other hand, the reduction of the cardiac parameters in the 8-week old hearts at this stage is related to LVDP, as demonstrated in Figure 3. Previous studies have shown that intracellular acidosis immediately reduces the cardiac contractility, which is largely due to a decrease in myofilament  $\text{Ca}^{2+}$  responsiveness.<sup>18</sup> Therefore, consistent with other reports, the present results could be explained by this mechanism.

As presented in Figure 4, I/R injury significantly exacerbated in the 8-week old hearts compared with that of the 2-week olds group. Several reports revealed that the intensity of the I/R injury is age-dependent, and also the recovery of myocardial function following ischemia decreases by aging.<sup>19-23</sup> The tolerance of the younger hearts against ischemia possibly is due to the adaptation to an environment of low oxygen tension in the embryonic period.<sup>24</sup> In fact, the restricted oxygen access before birth of an animal could activate the cellular and metabolic protective mechanisms that protect the heart from low oxygen supply. Following birth and living at an atmosphere which is full of oxygen, these protective mechanisms gradually diminish with aging, and heart resistance is higher in the post-natal period due to activity of this protective mechanism.<sup>24,25</sup>

Therefore, the better myocardial recovery in the 2-week old groups could be explained by these mechanisms, which is consistent with other studies. However, the current study showed the rapid and more significant reduction of the 2-week old group's heart rate in the presence of the acetazolamide. In spite of this intensive reaction of the 2-week old hearts to CA inhibitor, the severity of I/R injury is lower than that of the 8-week old group. On one hand, it shows the important role of the cellular buffering capacity, especially CA in cardiac function, and on the other hand, it can be concluded that CA is not solely a determinant factor in the myocardial recovery and heart's tolerance against I/R injury. The role of the other age-dependent protective mechanisms should be emphasized based on the current study. In the previous study, it has been suggested that the higher age-dependent cardiac tolerance against I/R

is probably due to the higher cellular buffering capacity of the younger rabbits.<sup>12</sup> However, based on the present findings it can be concluded that the CA buffering system is not a determining factor in the age-dependent tolerance of the heart in I/R.

In conclusion, the current study showed that the 2-week-old hearts react to CA inhibition more rapidly than the 8-week-old hearts. However, CA inhibition does not exacerbate I/R injury in the 2-week old hearts compared with the 8-week old groups. In fact, the results showed that after blocking of CA, the age dependent pattern of I/R injury was similar to that of the normal hearts. Therefore, in spite of the essential role of buffering capacity in the preservation of normal heart function, it is not a determinant factor in I/R injury of the hearts at different ages. The important effect of the other age-dependent protective mechanisms in I/R should be emphasized, and remained to be elucidated in future studies.

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