ビドロキシエチル・スターチを用いた犬赤血球の-80℃凍結保
存法の保存時間による影響

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<th>誌名</th>
<th>山口獣医学雑誌</th>
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<tr>
<td>ISSN</td>
<td>03889335</td>
</tr>
<tr>
<td>巻/号</td>
<td>34</td>
</tr>
<tr>
<td>掲載ページ</td>
<td>p. 35-48</td>
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<tr>
<td>発行年月</td>
<td>2007年12月</td>
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ORIGINAL ARTICLE

SOME EFFECTS OF THE STORAGE PERIODS OF CANINE ERYTHROCYTES AT -80°C CRYOPRESERVATION USING HYDROXYETHYL STARCH

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[Received for publication: December 25, 2007]

ABSTRACT

The objective of this study is to examine the relations between the storage periods of canine erythrocytes at -80°C cryopreservation and the quality of the erythrocytes.

The washed and concentrated blood, which was collected from six healthy dogs, was mixed with the equal volume of cryoprotective solution which contains 25%(w/v) hydroxyethyl starch (HES) and 60 mM NaCl. The mixture was put in plastic bags made from vinyl chloride and frozen in liquid nitrogen (-196°C). The bags containing the frozen mixture were immediately transferred to a -80°C deep-freezer and kept for 24 hours (one) month, 2 (two) months, and 3 (three) months. When the storage period of each bag was due in order to make separation possible by centrifugation, the thawed blood was mixed with the equal amount of physiological saline. The relations between the storage periods of erythrocytes at -80°C cryopreservation and the quality of the erythrocytes were evaluated by measuring the hemolysis and the osmotic fragility as well as by morphologically examining with scanning electron microscopy (SEM). The mean rates of hemoglobin recovery in 24 hours (one) month, 2 (two) months, and 3 (three) months groups were 97.8%, 95.6%, 96.3%, and 95.9%, respectively. With respect to the recovery rate, osmotic fragility, and morphology, there were no significant differences due to the different storage periods.

The result of the present study indicates that canine erythrocytes can be cryopreserved at -80°C for three months, longer than liquefied preservation. morphology, there were no significant differences due to the different storage periods.

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INTRODUCTION

In veterinary medicine, just as in human medicine, blood transfusion is indispensable as a treatment of big loss of blood, such as bleeding at the time of an operation, a traffic accident, declining of blood production, reduction of other blood components, and progressive erythrocytes destruction. In spite of such circumstances veterinary medicine is far behind human medicine regarding blood preservation. Therefore it is important to establish a cryopreservation method of canine erythrocytes in veterinary medicine. An advantage of cryopreservation over liquefied preservation is the extended preservation period.
While erythrocytes deteriorate rapidly and substantially in liquefied preservation\(^9\), they do not deteriorate much in \(-80^\circ\text{C}\) cryopreservation. This indicates that the cryopreservation of erythrocytes will be possible and useful in the practical field. It has been demonstrated that erythrocytes could be preserved as long as 20 to 30 years\(^{10}\). There are a few reports on the cryopreservation of canine erythrocytes using a cryoprotectant, such as glycerol (GL)\(^{14-20}\).

Cryopreservation is believed to induce the physical destruction of cell structure by the growth of crystals, the change of electrolyte solution and pH, the precipitation of protein, and the disappearance of structure water\(^{20}\). A cryoprotectant is used to avoid the destruction of cell structure during freezing. Intracellular protectants can pass through erythrocytes cell membrane freely because of their low molecular weight, while extracellular protectants cannot pass through the cell membrane because of the amount of high polymers. The former includes GL, dimethylsulfoxide (DMSO), and ethyleneglycol (EG), while the latter includes hydroxyethyl starch (HES), polyvinylpyrrolidone (PVP), polyglycol, and dextran.

Glycerol is widely used as an intracellular protectant. Due to the characteristic action and the toxic effect of GL, however, inadequate deglycerolization induces hemolysis after thawing\(^6\).

On the other hand, HES is degraded in the body by alpha-amylase and used as carbohydrate\(^6\). Because of its character, the exposure of erythrocytes preserved by HES to physiological saline is harmless. When HES is used as a cryoprotectant, therefore, it is expectable to eliminate the complicated operation of washing after thawing.

We can find several reports regarding cryopreservation of human erythrocytes\(^{14,15,21}\), including a clinical test\(^{10}\). It is expected that HES can be used as a method of transfusing with no post-thaw washing. But we can find few reports\(^{17,20}\) about the possibility of cryopreservation of canine erythrocytes.

We found that by using HES erythrocytes could be cryopreserved at \(-196^\circ\text{C}\) and \(-80^\circ\text{C}\). We also learned that cryopreservation at \(-80^\circ\text{C}\) was safer than \(-196^\circ\text{C}\). Accordingly we conducted our experiment to establish the method of \(-80^\circ\text{C}\) cryopreservation with HES by examining four different storage periods of cryopreservation at \(-80^\circ\text{C}\).

**MATERIALS AND METHODS**

**Donor dogs:**

Blood was collected from six healthy dogs, which were bred at Yamaguchi University, Faculty of Agriculture, Veterinary Medicine. Prior to blood collection, physical and blood examinations of them were conducted. The weights of the dogs were from 7 to 15 kg, and their ages were from 2 to 7 years. Blood was steriley collected from a cervical vein. As anticoagulant, CPD (Blood bag CPDA, TERUMO) was added at the rate of 12.2 V/V%.

**Washing of blood:**

The collected blood samples were poured into 50 ml plastic test tubes. The plasma was separated by centrifugation at 3,000 rpm at \(4^\circ\text{C}\) for 5 minutes. The same volume of physiological saline was added to the separated blood. They were subsequently mixed well, and the supernatant fluid was removed after centrifugation at 3,000 rpm at \(4^\circ\text{C}\) for 5 minutes. After the centrifugation was performed three times, the value of hematocrit (Hct) was adjusted to approximately 80%.

**Cryopreservation with 25%(w/v) HES:**

HES(A-HYDROZETHYLSTARCH, Ajinomoto) solution consists of 25 wt% HES and 60 mM NaCl. The washed blood was mixed with the equal volume of HES solution. The mixture was poured into freezing bags (PHYSISALZ-FC 1000ml bag, Fuso), kept for 15 minutes at room temperature, and put between aluminum plates. The bags were subsequently submerged into the liquid nitrogen in the tank (CryoMed, Forma Scientific), and kept there for another 15 minutes. Then the bags were transferred into a deep-freezer (\(-80^\circ\text{C}\)) and stored for 24 hours (24-h group), 1 month (1-M group), 2 months (2-M group), or 3 months (3-M group).

**Thawing:**

In order to prevent erythrocytes from being destroyed by the recrystallization during warming, the freezing bags were quickly transferred to the warm water of \(43^\circ\text{C}\), shaken by hands, and thawed in less than 3 minutes.
The thawed blood was mixed with the equal volume of physiological saline, and separated by centrifugation at 3,000 rpm at 4°C for 10 minutes. After the supernatant solution was separated, the remaining cells were used as erythrocyte solution.

Experiment groups:

The experiment groups were divided into 5 (five), according to preservation periods, that is, 24-h, 1-M, 2-M, 3-M, and the control group with no cryopreservation. Both the osmotic fragility and the recovery rates of the control group were examined immediately after the blood was mixed with the equal volume of HES.

Measurement:

[1] Recovery rate:

The ratio of the quantity of hemoglobin of erythrocytes and the amount of hemoglobin lost in the supernatant fluid was calculated as the hemoglobin recovery rate. It was used as an index, which indicates the damage of erythrocytes caused by freezing and thawing. The recovery rate was calculated from the following formula using the hemoglobin value.

\[
\text{Recovery rate} = \frac{\text{Hemoglobin concentration of erythrocytes after centrifugation} \times \text{Amount of erythrocyte solution}}{\text{Hemoglobin concentration of supernatant fluid after centrifugation} \times \text{Amount of supernatant fluid}}
\]

To measure hemoglobin, the hemoglobin measure indicator (hemoglobin B-test wako) was used.

[2] Osmotic fragility test:

Osmotic fragility was determined by adding 0.02 ml of blood solution to 5.0 ml of buffered NaCl solution with concentration ranging from 0.1 to 0.9%, or to 5.0 ml of distilled water. The frozen-thawed erythrocytes were mixed with buffered NaCl solution or distilled water. The mixture was kept at 37°C for 30 minutes. It was centrifuged at 3,000 rpm for 5 minutes. The supernatant fluid was transferred into another set of tubes. The hemoglobin content of the supernatant fluid was determined spectrophotometrically at 546 nm. The percentage of hemolysis was calculated, assuming hemolysis in the biggest absorbance to be equal to 100%. The control value of the dog was also decided by using the mixture of washed erythrocytes and the equal volume of 25 wt% HES (control group).

[3] Scanning electron microscopy

The frozen-thawed and washed erythrocytes were fixed with 1.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 (one) hour, washed with 0.1 M phosphate buffer three times, and dehydrated with an ascending ethanol series 50% to 100%. The specimens were dried by a critical point dryer and coated with gold in an ion-coater apparatus. They were observed under a scanning electron microscope (JSM 6100, Japan Electron Optics Laboratory) at accelerating voltage of 15 kv. Erythrocytes were classified into 3 groups on the basis of shape of erythrocytes (Discocyte, Echinocyte-1, and Echinocyte-2) (Fig. 4). They were counted from the scanning electron photomicrographs taken at the magnification of x1000 on two randomly selected areas in each sample, and the ratio of each erythrocyte was calculated. Discocyte was defined as the erythrocyte with the smooth surface of membrane. Echinocyte-1 was defined as the erythrocyte with protrusions on the surface of membrane, while Echinocyte-2 was defined as the erythrocyte with sharper protrusions than those of Echinocyte-1.

Statistical analysis:

The results present the mean ± standard deviation (SD). Statistically significant difference was analyzed by t-test. Differences at p<0.05 were considered significant.
RESULTS

1. Recovery rate:

The recovery rates of 24-h, 1-M, 2-M, and 3-M groups were 97.8±1.6%, 95.6±3.6%, 96.3±3.3%, and 95.9±3.3%, respectively. The recovery rate of the control group was 98.9±0.9% (Table 1). With respect to the recovery rate (p>0.05), there were no significant differences among the groups.

2. Osmotic fragility test:

There are relations between the osmotic fragility of erythrocytes and the geometrical structure. Osmotic pressure causes expansion, hemolysis, the change of membrane permeability, and damage, which results in the termination of structure maintenance. In order to find out the damage of erythrocytes during cryopreservation, the osmotic fragility of the HES groups and the control group was tested. The results of the test are shown in Fig. 1 to Fig. 3. Compared with the mean value of the control group, the graph curves of the cryopreserved groups move to the right, indicating that the osmotic fragility of erythrocytes increases. In addition, the concentration of NaCl producing 50% hemolysis is one of the evaluation methods which determine the degree of the damage of erythrocytes membrane. As the graph curves show, the concentration of NaCl producing 50% hemolysis in the control group, 24-h, 1-M, 2-M, and 3-M groups were 0.47±0.029%, 0.50±0.030%, 0.54±0.029%, 0.53±0.024%, and 0.54±0.031%, respectively. Regarding the osmotic fragility, there were no significant differences among the groups.

3. Scanning electron microscopy:

The percentages of discocytes in the 24-h, 1-M, 2-M, and 3-M groups were 82.3±8.1%, 82.3±8.1%, 79.3±7.2%, and 80.5±7.0%, respectively. The percentages of echinocyte-1 were 12.93±5.0%, 13.3±5.8%, 15.7±4.7%, and 14.8±5.4%. The percentages of echinocyte-2 were 4.9±3.8%, 4.3±2.9%, 5.0±3.3%, and 4.6±2.5%. In the control group, the percentages of discocytes, echinocyte-1, echinocyte-2 were 98.5±1.7%, 1.7±2.0%, and 0% (Fig. 5, Fig. 6, Table 2). With respect to the morphological analysis by using scanning electron microscopy, there were no significant differences among the groups.

DISCUSSION

The preservation of blood in the liquid state is useful and simple. Blood can be preserved for 21 days. Many attempts have been made to extend the preservation period of blood in the liquid state. However, the preservation of blood in the liquid state for a prolonged time has not materialized yet. At present, the cryopreservation is the sole method which enables the preservation of blood for a longer period.

Blood transfusion is performed in the field of veterinary medicine. However, unlike human medicine, neither the blood donation system nor the blood bank is established. It is difficult to prepare the needed amount of blood all the time, that is, the matching type of the fresh blood or the liquefied preservation blood. At the time when a transfusion is performed, some crucial issues must be considered, which include how to avoid infection or immunity antibody, and how to prevent side effects or the isoimmunization in case of a rare blood type. Unfortunately, the present condition is far from being satisfactory to deal with the cases which need an autotransfusion.

Even when the blood transfusion to animals is necessary due to their chronic anemia and some other long-term medical treatment, an autotransfusion may not be applied clinically, because the period of the conventional refrigeration preservation of blood is only 21 days, and the needed blood is not always available. It is supposed that cryopreservation has the advantage of the period of storage and the state of cells. It is reported that the preservation as long as 37 years is not impossible. When erythrocytes are kept under −80°C (especially at −196°C), the normal structure is retained. They are suited to prolonged preservation.

The protection mechanism of hydroxyethyl starch (HES) used in human medicine is as follows. HES is classified into an extracellular (non-penetrating) cryoprotective additive. In contrast to glycerol, as a plasma substitute, HES offers the advantage of being a nontoxic, biodegradable compound. There are various opinions about the process of freeze protection. It is suggested that the holes on the surface of the cell membrane are closed by HES, so that the cells are protected from the ice crystals in the cytoplasm which will be otherwise formed.
Dehydration prevents the growth of the ice crystals, which will cause cytoplasmic damage.

It is reported that quick freezing is effective for the cryopreservation of human erythrocytes, as well as for the quality of the blood platelet. It is necessary to freeze quickly and control the molecular movement, because the extracellular (non-penetrating) cryoprotective effect of HES is not strong.

HES is the polysaccharide of molecular weight $10^4$ to $10^5$. It is biodegrades in the body by alpha-amylase, and used as carbohydrate. Moreover, it has less antigen, so its clinical application is carried out with PVP or dextran as a plasma expander at the time of bleeding. The freezing erythrocytes by HES may be transfused without washing after thawing, because they are not hemolized by direct exposure to physiological salt solution. The licensed drug HES offers the advantage of being a nontoxic, biodegradable cryoprotectant and plasma substitute.

When GL is used as a cryoprotectant, the cryopreserved blood cannot be transfused immediately after thawing. GL must be washed steriley and removed first. If deglycerolization is inadequate, hemolysis will occur. And GL toxicity will be suspected. On the other hand, erythrocytes frozen with HES can normalize oxygen transport and blood volume loss in case of extended hemorrhage. For this reason HES cryopreserved erythrocytes can be transfused by no washing after thawing. And HES may be suitably used for cryopreservation of canine erythrocytes. Attempts have been made to use HES for the cryoprotectant as an alternative to glycerol. It is reported that the possibility of RBC cryopreservation at $-196^\circ C$ was examined by using a small quantity of canine erythrocytes with HES. The demerit of the cryopreservation in liquid nitrogen is the possible damage of the freezing bag at the time of thawing. It seems that the cryopreservation by $-80^\circ C$ deep-freezer is safer, as well as more suitable for a large amount of blood. It is reported that the cryopreservation of canine erythrocytes at $-80^\circ C$ could result in a slight injury. The erythrocytes cryopreserved at $-80^\circ C$ using HES could be used for autologous transfusion. In this study, therefore, we limited the periods of cryopreservation of erythrocytes no more than three months (3-M). As a result, we found out that at $-80^\circ C$ erythrocytes could be preserved longer than at liquefied preservation.

The recovery rate is an important guideline which determines the usefulness of the cryopreservation of erythrocytes. When fresh blood is used for cryopreservation, the recovery rate after thawing is high. In this study no significant differences were noticed among the storage periods (p > 0.05). It is conceivable that the destruction of erythrocytes was much lower during the preservation. The existence rate within the vital body is another important guideline that shows the usefulness of erythrocytes. The existence rate within the living body is measured with $^{51}$Cr method. The blood transfusion effect index is decided by the product of the collection rate and the erythrocytes existence rate measured 24 hours after the blood transfusion.

The cell membrane of erythrocytes is tender, but it lacks elasticity. It will explode, therefore, if the amount of water taken in erythrocytes exceeds the critical capacity. The osmotic fragility of the erythrocytes in cryopreservation rises by the presence of ice crystals, which are formed inside and outside the freezing cells. A hole on erythrocytes membrane bigger than the diameter of 64Å of a hemoglobin molecule may bring about some sort of damage on erythrocyte membrane, such as the direct escape of hemoglobin after thawing. It may also be damaged by the fall of the membrane function caused by the reduction of ATP in erythrocytes after freezing-thawing. The consequence is the possible increase of osmotic fragility and hemolysis. The concentration of NaCl producing 50% hemolysis is a criterion for judgment. In this study no significant differences were observed among the storage periods (p > 0.05). The deficit of the membrane function by the decrease of ATP in erythrocytes after freezing-thawing was conceivable as one factor. It was also conceived that the membrane of erythrocytes was simultaneously damaged by the formation of the ice crystals associated with freezing. However, osmotic fragility is influenced by both exogenic factors (pH, temperature, and oxygenation) and intrinsic factors (age of the animal, kind, lipemia, aging of erythrocytes). It was presumed that the injury of erythrocytes by the above-mentioned factors was limited to the minimum when they were preserved at $-80^\circ C$.

The erythrocyte changes the form of its membrane by the freezing damage and the exogenic factors. The surface of erythrocytes will protrude if the membrane injury advances. In this study the erythrocytes were classified into three types. The comparison of ratio was used as a guideline to judge the injury of erythrocytes. When the control group was compared with the cryopreserved groups, a significant difference was observed in the ratio of discocytes and echinocytes. Among the storage periods no significant differences were noticed in the ratio of erythrocytes. Therefore, the results of our study indicate that the injury caused by the prolongation of cryopreservation may be minor.
From the results of the recovery rate, the osmotic fragility test, and the scanning electron microscopy, we discovered that canine erythrocytes can be cryopreserved at $-80^\circ C$ as long as three months, which is longer than the period of liquefied preservation. This may contribute to the possible creation of canine ozoenblood banks.

ACKNOWLEDGMENT

The authors sincerely appreciate the guidance and support extended by Ms. Kana SHIRAISHI, Mr. Akihisa ITO, Mr. Natsuki MORIMOTO, Ms. Kumiko NOMA, Mr. Masayuki KAWAI, Mr. Yusuke SASAKI, Ms. Mio MATOBA, and Mr. Tatsuhumi YAMASHITA of Veterinary Surgery, Faculty of Agriculture, Yamaguchi University.

REFERENCES


FIGURES

Table 1  Recovery rate

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<tr>
<td>Control group</td>
<td>98.9±0.9%</td>
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<tr>
<td>24 hours group</td>
<td>97.8±1.6%</td>
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<tr>
<td>1 month group</td>
<td>95.6±3.6%</td>
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<tr>
<td>2 month group</td>
<td>96.3±3.3%</td>
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<td>3 month group</td>
<td>95.9±3.3%</td>
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24 hours Group

Fig. 1 Osmotic Fragility Test (24 hours Group)
Fig. 2 Osmotic Fragility Test (1 month group, 2 month group)
Fig. 3 Osmotic Fragility Test (3 month group, All group)
Fig. 4 Scanning electron microscopy
Discocyte (magnification ×3000)
Echinocyte-1, Echinocyte-2 (magnification ×4000)
Fig. 5 Scanning electron microscopy
Control, 24 hours and 1 month group (magnification × 1000)
Fig. 6 Scanning electron microscopy  
2 months group and 3 months group (magnification × 1000)

Table 2 Scanning electron microscopy Ratios of Discocyte, echinocyte-1 and echinocyte-2

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<td>Control group</td>
<td>98.5±1.7%</td>
<td>1.7±2.0%</td>
<td>0%</td>
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<tr>
<td>24 hours group</td>
<td>82.3±8.1%</td>
<td>12.9±5.0%</td>
<td>4.9±3.8%</td>
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<tr>
<td>1 month group</td>
<td>82.3±8.1%</td>
<td>13.3±5.8%</td>
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<td>2 month group</td>
<td>79.3±7.2%</td>
<td>15.7±4.7%</td>
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<td>3 month group</td>
<td>80.5±7.0%</td>
<td>14.8±5.4%</td>
<td>4.6±2.5%</td>
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原著

ビドロキシエチル・スターチを用いた犬赤血球の－80℃凍結保存法の保存時間による影響

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[受付：2007年12月25日]

畜医臨床の進歩により、輸血の必要性は高まる一方であるが、十分なドナー血液を入手するのは困難な現状である。赤血球の保存法として液状保存と凍結保存があげられる。液状保存では簡易に保存が可能であるが1ヵ月ほどしか保存できない。凍結保存ではより長期間の保存ができるが、凍結保存における必要となる、現在の医学においては凍結保存は凍害保護物質としてGlycerolを用い、－196℃で行われている。しかし、Glycerolは解凍後に洗浄除去せねばならず、液体窒素での保存はコストや使用管理面から問題がある。このため畜医学領域への応用は難しいと考えられた。そこで、非洗浄で輸血が可能なビドロキシエチル・スターチ(Hydroxyethyl starch, HES)を用い、－196℃で急速冷却後に－80℃超低温フリーザーに移す2ステップ冷凍保存法による実験を行ったところ、ヘモグロビン（Hb）回収率、浸透圧脆弱試験はともにGlycerol群よりもHES群の方が良好であった。さらに、－80℃凍結保存血の自動輸血における再利用の発現は認められなかった。そこで、本研究では－80℃冷凍保存赤血球の輸血用としてin vitroでの変化を観察することにより、－80℃冷凍保存での保存可能な期間について検討をおこなった。

実験群は保存期間、24時間、1ヵ月、2ヵ月、3ヵ月の保存群と対照群（凍結保存を行っていない血液）の5群とした。6頭の健康実験犬から採取後作製した濃厚赤血球と25%HESを等量混合し、液体窒素内で急速凍結し、その後－80℃超低温フリーザー内で24時間、1ヵ月、2ヵ月、3ヵ月冷凍保存後、急速解凍し、Hb回収率、浸透圧脆弱試験、走査電子顕微鏡による観察を実施した。

24時間、1ヵ月、2ヵ月、3ヵ月保存群のHb回収率はそれぞれ97.8±1.6％、95.6±3.6％、96.3±3.3％、95.9±3.3％となった。Hb回収率、浸透圧脆弱試験の50％溶血濃度、走査電子顕微鏡による観察による赤血球の形態的な変化は凍結保存時間による有意差は認められなかった。

以上の結果により、－80℃でHESを用いた2ステップ冷凍保存法が犬赤血球を長期に保存する方法になりうることが示唆された。さらに長期間の保存の評価を行うことにより、犬の自己血液バンク設立の一助になりうると考えられた。

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