ビドロキシエチル・スターチを用いた犬赤血球の-80℃凍結保存法に関する研究

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ORIGINAL ARTICLE

A STUDY OF –80°C CRYOPRESERVATION OF CANINE ERYTHROCYTES BY USING HYDROXYETHYL STARCH (HES)

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ABSTRACT

In human medicine the freezing of erythrocytes at –196°C is already practiced. In this study the freezing of erythrocytes at –80°C was examined and the possible application of it in veterinary clinical medicine was pursued.

The washed and concentrated erythrocytes were mixed with the equal volume of cryoprotective solution, which contains 10%~40% (W/V) hydroxyethyl starch (HES) and 60 mmol/liter-NaCl. The mixture was put into plastic bags of vinyl chloride and quickly frozen to –196°C in liquid nitrogen. They were transferred to a –80°C deep freezer and preserved for 24 hours. After thawing, HES groups were washed one time, while GL group was deglycerolized. The effect of storage temperature on the erythrocytes was evaluated by measuring hemolysis and osmotic fragility. The recovery rates of HES groups that is 10%, 20%, 25%, 30%, and 40% HES were 65%, 86.2%, 87%, 88.9%, and 85.6%, while the recovery rate of GL groups was 84.4%. The frost damage protective ability of HES was not different from that of GL. The increase of osmotic fragility was noticed. It was considered, however, that the damage of the erythrocytes, which was caused by the formation of ice crystals associated with temperature change, was small.

In case HES is used as a cryoprotective solution, a process such as deglycerolization is necessary. It was suggested that a large quantity of dog erythrocytes could be cryopreserved at –80°C.

INTRODUCTION

In veterinary medicine, just like in human medicine, blood transfusion is indispensable as a treatment of diseases, when a large amount of blood is lost, such as bleeding at the time of an operation, traffic accidents, declining of blood production, reduction of other blood components, and progressive blood destruction. In spite of such circumstances, veterinary medicine is far behind human medicine regarding blood preservation. Therefore, it is important to investigate the cryopreserved dog red blood cells in veterinary medicine. The advantages of cryopreservation include the extended time and the condition of preservation. While the blood is preserved for about one month in liquefied preservation, it is possibly preserved for years in cryopreservation. The deterioration of the blood preserved in liquid state is rapid and considerable. On the other hand the deterioration of the blood preserved at –80°C is slight. Furthermore, it is demonstrated that the erythrocytes may be cryopreserved for 20 to 30 years. There are only a small number of reports on the cryopreservation of dog RBC by using a cryoprotectant, such as glycerol (GL).

Some major concerns of cryopreservation include the physical destruction of cell structure by growth of crystals, change of electrolyte solution and pH, precipitation of protein in the concentration solution by drying, and disappearance of structure water.
Acryoprotectant is used to avoid destruction of cell structure during freezing. Intracellular protectants can pass through erythrocyte cell membrane freely because of 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), Polyvinyl pyrrolidone (PVP), Polyglycol, and Dextran.

In GL, which is widely used as an intracellular protectant, hemolysis results from inadequate deglycerolization after thawing, because of the characteristic of the action and the toxic effect by GL itself. On the other hand, HES is degraded in the body by alpha-amylase and used as carbohydrate 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 on the cryopreservation of human erythrocytes, including a clinical test, including a clinical test. It is expected that HES can be used as a method of transfusing with no post-thaw washing. However, few reports about the possibility of the cryopreservation of canine erythrocytes. The possibility of the cryopreservation using HES is admitted by a research of autotransfusion. In order to be applied to veterinarian clinical medicine, however, safer cryopreservation and a larger volume of erythrocytes are needed. The purpose of this study is to examine the possibility of freezing a large quantity of dog erythrocytes at -80°C.

**MATERIALS AND METHODS**

**Donor dogs:**

Blood was collected from 10 healthy dogs, which were bred at Yamaguchi University, Faculty of Agriculture, Veterinary Medicine, Livestock Surgery Classroom. Prior to blood collection, physical and blood examinations of them were conducted, and they were in normal state. The weights of the dogs were from 7.4 to 17.2 kg, and their ages were from 2 to 5 years. Blood was sterilely collected from a cervical vein. As anticoagulant, CPD was added at the rate of 12.2%/V%.

**Washing of blood:**

The collected blood samples were poured into 50ml plastic test tubes. The plasma was separated by centrifugation at 3,000 rpm at 4°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°C for 5 minutes. After the centrifugation was performed three times, the value of hematocrit (Hct) was adjusted to approximately 80%.

**Cryopreservation at 10%, 20%, 25%, 30%, and 40% (W/V) HES:**

Each HES solution consists of 10wt%, 20wt%, 25wt%, 30wt%, and 40wt% HES, and 60 mmol NaCl/liter. The washed RBC (Hct=80%) was mixed with the equal volume of HES solution. The mixtures were poured into freezing bags (CPL99 Plastic Charter Med Inc.) (each 5%, 10%, 12.5%, 15%, and 20% W/V of final HES concentration). They were kept for 15 minutes, and placed in an aluminum container. They were then submerged into the liquid nitrogen in which rapid freezing was carried out. They were kept in a -80°C deep-freezer for 24 hours.

**Thawing:**

In order to prevent blood from being destroyed by the recrystallization during warming, the mixtures were quickly transferred to the warm water of 43°C, well shaken, and thawed in less than 3 minutes. The thawed erythrocytes were mixed with the equal volume of physiological saline, and separated by centrifugation at 3,000 rpm at 4°C for 10 minutes. The remaining packed cells were used as HES erythrocyte solution.

**Cryopreservation at 30W/V% GL:**

The method introduced by Sumida et al. was applied to the composition and the freeze method of GL: 30% (W/V) glycerol solution (SF-30) (which consists of 30W/V% GL and 2.0 W/V% mannitol, 0.64 W/V% NaCl,
2.0W/V% sorbitol) was used. The washed RBC (Hct=80%) was mixed with the equal volume of GL solution. The mixture was poured into a freezing bag (CPL99 Plastic Charter Med Inc.) (15W/V% of final GL concentration). It was kept for 35 minutes, and placed in an aluminum container. It was then submerged into the liquid nitrogen where the rapid freezing was carried out. It was kept in a −80°C deep-freezer for 24 hours.

Thawing:
In order to prevent RBC from being destroyed by the recrystallization during warming, the bag was well shaken in the warm water of 43°C, and thawed in less than 3 minutes. As GL is an intracellular cryoprotectant, deglycerolization was performed after thawing.

Deglycerolization:
The 30 ml of GL was thawed, the blood was poured into a 50 ml plastic test tube, and the supernatant fluid was removed by centrifugation in 3,000 rpm at 4°C for 5 minutes. Then 1.5 times of 3.5% saline solution was added, and the supernatant fluid was removed by centrifugation in 3,000 rpm at 4°C for 5 minutes. After that, 1.5 times of 0.9% saline solution was added, and the supernatant fluid was removed by centrifugation in 3,000 rpm at 4°C for 5 minutes. The same operation was repeated one more time by using 0.9% saline solution. The packed cells were used as the deglycerolized GL erythrocytes solution.

Experiment groups:
The experiment groups were classified into 7 (seven) groups, that is, HES (10%, 20%, 25%, 30%, and 40%W/V), GL, and the control group. The sample number of each group was 5 dogs. The osmotic fragility test of the control group was conducted immediately after blood collection, and its recovery rate was measured after it was preserved at +4°C for 24 hours.

Measurement:

1) Recovery rate: The ratio of the quantity of hemoglobin of the packed cells and the amount of hemoglobin lost in the supernatant fluid was calculated as the hemoglobin recovery rate. It was used as an index, which shows the damage on erythrocytes caused by the freezing and thawing. The recovery rate was calculated from the following formula using the hemoglobin value:

\[
\text{Recovery rate} = 100 \times \frac{\text{1}}{(\text{1} + \text{2})}
\]

\(\text{1} : \text{Hemoglobin concentration of the packed cells after centrifugation} \times \text{Amount of the packed cells.}\)

\(\text{2} : \text{Hemoglobin concentration of supernatant fluid after centrifugation} \times \text{Amount of supernatant fluid.}\)

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

2) Osmotic fragility test: An osmotic fragility test was carried out on the basis of the basic research. Osmotic fragility was determined by adding 0.02 ml of blood solution to 5.0 ml of buffered NaCl solution in concentration ranging from 0.1 to 0.85%, and to 5.0 ml of distilled water. The contents were mixed, and allowed to stand at 37°C for 30 minutes. The tubes were 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 mμ. The percentage of hemolysis was calculated, assuming hemolysis in the biggest absorbance to bee equal to 100%. Also, the normal value of the dog was decided by using the new fresh blood of the five healthy dogs.

Statistical analysis: The results present the mean value ± standard deviation. Significant statistical difference was analyzed by t-test.
Difference by p<0.05 was considered significant.
RESULTS

(1) Recovery rate:

The recovery rates of the HES groups of 10%, 20%, 25%, 30%, and 40% were 65.01±2.09%, 86.15±3.60%, 86.99±2.93%, 88.93±1.90%, and 85.61±6.33%, respectively. The recovery rates of the control group and GL group were 100±0% and 84.41±5.16%. Between 10% HES group and the other HES groups, a significant difference of recovery (p<0.05) was observed.

(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 result in the termination of structure maintenance. In order to find out the damage of erythrocytes during cryopreservation, an osmotic fragility test was carried out. The results of the tests of the control group, every HES group, and GL group are shown in Fig. 1 to Fig. 3. Compared with the mean value measured by using the new fresh blood of the five healthy dogs clinically, in all the HES groups and GL group, the curve of the graph moved to the left. This shows that the osmotic fragility increased. In addition, the concentration of NaCl producing 50% hemolysis is one of evaluation methods which determine the degree of the damage of erythrocytes membrane. The concentration of NaCl producing 50% hemolysis was measured by using a regression line which was made from the NaCl density of the front and back 2 points of the concentration of NaCl producing 50% hemolysis. The results were as follows: the control group; 0.37%, 10% HES group; 0.46%, 20% HES group; 0.43%, 25% HES group; 0.42%, 30% HES group; 0.43%, 40% HES group; 0.49%, and GL group; 0.50%. In comparison with the osmotic fragility value in the control group, the values in every HES group and GL group increased rather significantly. In comparison with GL group, 10%~30% HES showed fine results significantly.

DISCUSSION

The preservation of blood in the liquid state is useful, but it is generally limited up to 21 days. Many attempts have been made to extend the preservation period of blood in the liquid state. So far, a method of preserving blood in the liquid state for a prolonged period has not been established, though. At present cryopreservation is the sole method which enables preservation of the red blood cells for a prolonged period.

Blood transfusions are performed in the field of veterinary medicine. However, unlike those in human medicine, neither the blood donation system nor the blood bank is established. Accordingly, the needed amount of blood is not always available, and the less chance is to prepare the fresh blood of the matching type 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 situation is far from being satisfactory to deal with the cases which need an autotransfusion.10

Even when the blood transfusion to animals is necessary due to their chronic anemia or other long-term medical treatment, an autotransfusion cannot be applied clinically, because the period of the conventional refrigeration preservation of blood is no more than 21 days. 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.11,12,14,15,20 When red blood cells are kept under −80°C (especially at −196°C), the structure is retained in the normal range and the deterioration is minor.10 They are suited to the prolonged preservation.

The protection mechanism of glycerol used in human medicine is as follows. Water, which participates in the formation of ice crystals by carrying out a hydrogen bond, is decreased, and the formation of ice crystals is restricted. The intra-extra-cellular liquid decreases, the hydration of the surface of cells is stabilized, and the cells are protected from the damage caused by saline water and mechanical destruction according to colligative effect.20 In short, glycerol osmotically replaces the intracellular water, and allows the cells to freeze without dehydration. The cells prepared in such a manner are hyperosmolar with respect to the intravascular environment. They must be deglycerolized before transfusion.
Hydroxyethyl starch (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. One opinion is that the holes on the surface of the cell membrane are closed by HES, so that the cells are protected from the ice crystals. Another opinion is that the dehydration of the cells prevents the growth of the ice crystals, which results in the prevention of frost damage.

It is reported that quick freezing is effective for the cryopreservation of human erythrocytes, as well as the quality of the cryoprotectant of the blood platelets. It is necessary to freeze them quickly and control the molecular movement, because the extracellular (non-penetrating) cryoprotective effect of HES is weak.

HES, which is the polysaccharide of molecular weight 10^1 to 10^3, is biodegraded by alpha-amylase in the body, and is used as a carbohydrate. There is still less antigen, so clinical application is carried out with PVP or Dextran as a plasma expander at the time of bleeding. The frozen 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 hydroxyethyl starch (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 in sterile solution and removed first. If deglycerolization is inadequate, hemolysis will occur and GL toxicity will be suspected. RBC frozen with HES can normalize oxygen transport and blood volume loss in case of extended hemorrhage. For this reason the cryopreserved erythrocytes with HES can be transfused after thawing without washing. And HES may be suitably used for cryopreservation of dog erythrocytes. Attempts have been made to use HES for a cryoprotectant as an alternative to glycerol. It is reported that a small quantity of canine erythrocytes was used with HES as a cryoprotectant and that the possibility of large units of RBC cryopreservation in −196°C was examined. However, the demerit of the cryopreservation in liquid nitrogen is to damage the freezing bag at the time of thawing. In this study, therefore, the cryopreservation in the −80°C deep-freezer was conducted in order to establish a safer cryopreservation. 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 experiment 10% HES group showed a significantly lower value (P<0.05) than the other HES groups. It is conceivable that the density of HES was too low to defend erythrocytes from frost damage. The existence rate within a vital body is another important guideline that shows the usefulness of erythrocytes. The existence rate within the living body is measured with ^51Cr method. With this method the blood transfusion effect index is decided by the product of the collection rate and the erythrocytes existence rate of 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 in cryopreservation of erythrocytes rises by ice crystals which are formed inside and outside the cells of the frozen erythrocytes. Aholon erythrocytes membrane bigger than the diameter of 64 Å of a hemoglobin molecule may bring about some sort of damage on erythrocyte membrane, such as the direct escape of hemoglobin after thawing. Moreover, damage may happen by the decrease of the membrane function brought by the reduction of ATP of erythrocytes after freezing-thawing.

The consequence is the possible increase of the osmotic fragility and hemolysis. A judgment standard is the concentration of NaCl producing 50% hemolysis. In this experiment the osmotic fragility increased in HES groups and GL group more than in the control group. The decline 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, the osmotic fragility is influenced by both exogenic factors (pH, temperature, oxygenation) and intrinsic factors (age of the animal, kind, lipemia, aging of erythrocytes). According to Jain, the range and the mean values for the osmotic fragility of erythrocytes for the dog were 0.36 to 0.48, and 0.43. Judging from the concentration of NaCl producing 50% hemolysis of each HES group, it was conceivable that the degree of the damage to the membrane of the red blood cells was not so great. The osmotic fragility of 30% GL group increased more than that of HES groups. In human medicine usually cryopreservation at −80°C is carried out with the slow freezing method by using 60% GL. In our experiment it was carried out with the rapid freezing method using 30% GL. It is indicated,
however, that sufficient protection ability is not obtained by freezing at $-80^\circ$C if 30%GL is used. It is said that there is some correlation among the osmotic pressure of erythrocytes, the physical fragility abnormalities, and the life shortening of erythrocytes\(^{10}\). Therefore, in case that the red blood cells frozen with HES are applied to clinical medicine, the possibility of the life shortening of erythrocytes will remain, because the increase of the osmotic fragility of erythrocytes is admitted. In this experiment the possibility of freezing at $-80^\circ$C using 20%HES~30%HES was examined. As a complicated process such as deglycerolization washing after thawing, is unnecessary, the transfusion becomes less difficult. In addition, at the time of thawing, cryopreservation at $-80^\circ$C is safer than that at $-196^\circ$C. If the cryopreservation method of freezing at $-80^\circ$C is completely established, various applications of it will be expected in veterinary medicine.

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Fig. 1 Osmotic Fragility Test (10% HES group, 20% HES group)
Fig. 2 Osmotic Fragility Test (25% HES group, 30% HES group)
Fig. 3 Osmotic Fragility Test (40%HES group, All HES group)
原 著

ビドロキシエチル・スターチを用いた犬赤血球の－80℃凍結保存法に関する研究

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今日、獣医学領域において十分なドナー血液を入手するのは困難な現状である。その解決策として考えられる方法の1つに赤血球の凍結保存が上げられる。赤血球の凍結保存を行う場合に必要である凍害保護物質として細胞内性、細胞外性のものがある。前者で汎用されるGlycerolは現在の人医学では－196℃で使用されているが、凍結時の衝撃的なGlycerolが必要である。後者のビドロキシエチル・スターチ（Hydroxyethyl starch, HES）は抗原性が少なく、生体内で分解されることからその応用が期待されている。HESの凍害保護作用のためには急速に冷却、解凍する必要があり、in vitroで－196℃液体窒素のHES保存血を用いた自己輸血ではGlycerolと比較して輸血反応が少なかった。しかし、液体窒素での保存はコストや管理面から問題が残った。そこで本研究では、獣医臨床の応用のための次段階としてプラスチックバッグを使用した－80℃凍結保存法、すなわち、－196℃で急速冷却後に－80℃超低温ディープフリーザーに移す2ステップ法の可能性についてin vitroで検討した。

平均分子量20万のHESを用い最終濃度が5％～20% W/Vの保存液を作製し、洗浄濃厚赤血球と混和後、塩化ビニル製のプラスチックバッグに封入、－196℃の液体窒素内で急速凍結後、－80℃超低温ディープフリーザーに移し24時間保存した。凍結解凍後、HES群は一回洗浄し、GL群は脱グリセロール操作を行い測定に用いた。

その結果、解凍後の回収率は10%HES群65.0%，20%HES群86.2%，25%HES群87.0%，30%HES群88.9%，40%HES群85.6%，GL群84.4%であった。したがってHESの凍害保護能はGLと同様であり、浸透圧脆弱性の亢進は認められたが、温度変化に伴う氷晶の形成による赤血球の障害はわずかであると思われた。

以上より、HESを凍結保存物質として用いた場合、GLのような脱グリセロール操作は必要とせず、犬赤血球の－80℃多量凍結保存の可能性が示唆された。

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