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# *EFFECT OF TIME AND TEMPERATURE ON THE SURVIVAL RATE OF MOUSE SPERM (MUS MUSCULUS VAR. ALBINO) IN SHORT-TERM PRESERVATION WITHOUT CRYOPROTECTANT AGENTS*

## Abstract

In this study, we studied the use of physiological saline solution (NaCl 0,9%) or dulbecco's phosphate-buffered saline (D-PBS) for mature sperms short-term preservation. After being collected from epididymides, sperms were adjusted to desired concentration (2x106 sperms/ml) with NaCl 0.9% solution or D-PBS solution (the dishes containing sperms were covered by mineral oil) and stored at 4oC, 10oC and room temperature (RT/26oC). The survival rate of sperms were evaluated by morphological observation and staining methods (single staining and double staining) every 8 hours. The results showed that the mouse sperms can be stored in a short-term at 4oC or 10oC without cryoprotectant agents, the survival rate in D-PBS solution are higher than those in NaCl 0,9% solution. The lethal concentration and time (LCt50) to mouse sperm in short-term preservation is 10 hours in D-PBS or NaCl 0,9% solution without cryoprotectant agents.

**Key words**: epididymis, mouse, short-term preservation, spermatozoa.

From the late twentieth century, the development of biology is very strong with many great achievements, especially in the studies about transgenation, cell culture, cloning, *in vitro* fertilization. They have contributed importantly in improving human's life and preservation of genetic resources of many species. In particular, sperm preservation is the most effective method to preserve genetic resources of males which are valuable or in danger.

The mammalian sperms have been preserved by freeze-drying or vitrification methods with variety levels of success; the survival rate after thawing is quite high. In general, these techniques require elaborate protocols using expensive apparatuses. Moreover, cryopreservation has disadvantages when used for short-term storage, such as retaining the motility and fertility of the spermatozoa. In many cases, e.g. during the short interval between two experiments, or to be transferred from a farm to a lab, sperms need to be preserved only in a short time [10]. The using the fresh semen short-term preservation would be particularly convenient since it does not require bacteriological monitoring, which is often laborious and time-consuming; and it does not require complicated method (spermatozoa freezing-thawing) [3, 9]; spermatozoa can be preserved without cryoprotectants and do not require a supply of liquid nitrogen as in the case of long-term storage and shipment [1].

Most of short-term sperm preservation methods mainly used commercial media (M2, KSOM$^{AA}$, TYH medium) [9-11]. In mice, various methods of sperm storage without freezing have been tried, such as evaporative drying or storage in salt and sugars [7]. The mouse spermatozoa remains the fertilizing ability after being stored at 4-6$^{o}$C in TYH medium in 7 days (Sankai et al. [8]); mouse sperm could be preserved at 4$^{o}$C in KSOM$^{AA}$ medium with a high-salt concentration and osmolarity of 800 mOsm$^{-1}$ for 2 months (Van Thuan et al. [10]). The sperm preserved at

4°C in medium with trehalose supplemented for 1 week or at RT in salt or sugar can be used as donor in ICSI [3, 7]. In this study, we were going to evaluate the use of two common solutions NaCl 0,9% and dulbecco's phosphate-buffered saline (D-PBS) in short-term sperms preservation without cryoprotectant agents.

## Materials and methods

Animals

Adult male mice (8 to 12 weeks old) were obtained from Laboratory of Stem cell Research and Application, University of Science, Ho Chi Minh city and Pasteur institute Ho Chi Minh city. All experimental animals were maintained in an room temperature condition and light controlled room (14:10-hours light-dark cycle with lights on at 7 a.m.). All experiment were carried out in the Laboratory of Anatomy-Human and Animal physiology, the University of education in Ho Chi Minh city.

Sperm collection

Epididymides collected from males using the euthanasia method would be placed directly into 1.5 ml eppendorfs containing NaCl 0.9% or D-PBS (gentamicin supplemented). 2 epididymides were transferred into a petri dish (ϕ 35mm) containing 1ml NaCl 0,9% or D-PBS which were then punctured by a sterile needle to release the sperm into the solution. Amount of sperms were determined using hemocytometer. The final sperm concentration ranged about $2x10^6$ sperm/ml [5].

Evaluate the survival rate of sperm

The survival rate of sperm can be evaluated by morphological observation or via staining. After staining, survival and death sperm were counted.

- Morphological observation: the morphological good-quality sperms are the sperms can swim strong and straight, is not deformed, and its head is bright resource [4, 6].
- Staining observation: single staining (0.5% Eosin dye) or double staining (mixture of 1% Eosin and 10% Nigrosin). The survival sperms are not stained; the death sperm is stained red [4, 6].

The survival rate of spermatozoa was calculated every replicate by counting in 100 spermatozoa.

Preservation of spermatozoa

The survival rate of sperms during being preserved at 4°C, 10°C and room temperature (26°C) in NaCl 0.9% or D-PBS solution were calculated every 8 hours.

Experimental design

Each experiment had 4 replicates. In each experiment, number of input sperms at initial time (0 hour) are the same. At each examined temperature, we evaluated the survival rate of sperm at the different time points: 0, 8, 16, 24, 32, 40 hours. Based on final result, the effects of solution, duration of preservation, and temperatures on the survival rate of mouse sperm in short-term preservation without cryoprotectant agents were determined. Consequently, an equation was set up to predict the survival rate of sperm under the influence of these factors.

Statistical Analysis

All data obtained from this study were calculated by Minitab 16, R software. Data are given as the mean ± SE. For all statistical tests, differences were considered statistically different at $p < 0.05$. Logistic regression analysis method with Poission regression model was used to analyze the correlation between the survival rate of sperms and examined factors. This model is a function: $\log\left(\frac{\quad}{\quad}\right) = \alpha + \beta$; this means log of the survival rate of sperms is a function depend on x factor.

When the parameter $\alpha$ and $\beta$ was estimated by maximum likelihood-based method:

$$\begin{cases} \sum_{i=1}^{n} y_i = \sum_{i=1}^{n} (e^{(\hat{\alpha}+\hat{\beta}x_i)}) \\ \sum_{i=1}^{n} x_i y_i = \sum_{i=1}^{n} \mathrm{x}_i (e^{(\hat{\alpha}+\hat{\beta}x_i)}) \end{cases}$$ and

$$\begin{cases} \hat{p}(y \mid x) = e^{\hat{\alpha}+\hat{\beta}x} \\ RR(x_i \mid x_0) = \frac{\hat{p}_i}{\hat{p}_0} = \frac{e^{\hat{\alpha}+\hat{\beta}x_i}}{e^{\hat{\alpha}+\hat{\beta}x_0}} = e^{\hat{\beta}(x_i - x_0)} \end{cases}$$

$\hat{p}(y \mid x)$: Predicted the survival rate follow x

$RR(x_i \mid x_0)$: Risk ratio of the survival rate with $x_i$ versus $x_o$

**Results and discussions**

*Comparative results of evaluated methods about the survival rate*

The survival rate of sperms during preservation in all tests were evaluated by three methods (morphological observation, single staining and double staining). In 36 tests obtained, we randomly selected 2 tests in two solutions and two different temperature at 0 hour in order to examine the differences among three evaluated methods. The results are shown in table 1.

*Table 1*

**The survival rate of sperms were evaluated by three methods in 2 tests**

| Assessable Methods | Tests | | | |
|---|---|---|---|---|
| | I | | II | |
| | The survival rate (%) | 95% CI (%) | The survival rate (%) | 95% CI (%) |
| Morphology | 59.75± 2.45$^{ac}$ (239/400) | 54.94 - 64.56 | 67.50 ±2.34$^{a}$ (270/400) | 62.91 - 72.09 |
| Single staining | 65.25±2.38$^{ab}$ (261/400) | 60.58 - 69.92 | 68.75 ± 2.32$^{a}$ (275/400) | 64.21 - 73.29 |
| Double staining | 66.50 ± 2.36$^{b}$ (266/400) | 61.87 - 71.13 | 72.50 ± 2.23$^{a}$ (290/400) | 68.12 - 76.88 |

*a, b, c: significantly different (p < 0.05) follow column*
*I: NaCl, 10°C, 0 hour; II: D-PBS, 26°C, 0 hour*

In the first test, the survival rates of sperms are significantly different between morphology and double staining methods (p = 0.049). In the second test, all three evaluated methods are not statistically significantly different (p > 0.05). This results showed that we can use all methods to evaluate the survival rate of sperms.

Effects of factors: solution, time and temperature on the survival rate of sperms after short-term preservation

Based on the results shown in table 1, we choose the single staining method to present the effect of three examined factors on the survival rate of sperms after short-term preservation as in table 2.

*Table 2*

**The survival rate of sperms in examined tests**

| Time (hour) | The survival rate (%) | | | | | |
|---|---|---|---|---|---|---|
| | NaCl | | | D-PBS | | |
| | 4°C | 10°C | 26°C | 4°C | 10°C | 26°C |
| 0 | 65.25±2.38 (261) | 65.25±2.38 (261) | 65.25±2.38 (261) | 68.75±2.32 (275) | 68.75±2.32 (275) | 68.75±2.32 (275) |
| 8 | 44.75±2.49 (179) | 54.75±2.49 (219) | 39.50±2.44 (158) | 54.50±2.49 (218) | 54.00±2.49 (216) | 40.05±2.45 (162) |
| 16 | 21.25±2.05 (85) | 18.00±1.92 (72) | 17.50±1.90 (70) | 29.25±2.27 (117) | 25.75±2.19 (103) | 18.50±1.94 (74) |
| 24 | 12.50±1.65 (50) | 12.00±1.62 (48) | 11.25±1.58 (45) | 19.50±1.98 (78) | 17.50±1.90 (70) | 12.50±1.65 (50) |
| 32 | 4.75 ±1.06 (19) | 7.50 ±1.32 (30) | 5.00 ±1.09 (20) | 9.25±1.45 (37) | 8.50±1.39 (40) | 5.50±1.14 (22) |
| 40 | 1.75 ± 0.66 (7) | 4.25 ± 1.01 (17) | 3.75 ± 0.95 (15) | 5.25±1.12 (21) | 3.50±0.92 (14) | 1.25±0.56 (5) |

*Effect of solution factor*

As shown in table 3, the survival rate of sperms after preservation was affected by the changes of solution factor, the difference is statistically significant (p = 0.0002). Specifically, when NaCl 0.9% was replaced by D-PBS, the survival rate of sperms increased 1.126 times (equivalent 12.6%, 95% confidence interval, change in the range from 5.7% to 20%). This can be explained that D-PBS solution has much more components than NaCl 0,9% solution. D-PBS solution consist of NaCl, KCl, $KH_2PO_4$, $Na_2HPO_4$, $MgCl_2.6H_2O$, $CaCl_2$, while NaCl 0,9% solution is only sodium chloride in water. According to Van Thuan et al. [10], mouse sperm could be preserved for 2 months in a high-salt solution, especially, increasing the concentration of NaCl in $KSOM^{AA}$ medium. When sperms are placed in a high concentration of NaCl, water is drawn out of the cell until equilibrium is reached [3, 10]. When epididymides were stored in salt at room temperature, about 30% of water was lost within 1 day, while epididymides were stored in glucose, about 70% of the water was lost within 1 day with no further changes [7]. So, oocyte activation capacity of spermatozoa after storage in the cauda epididymidis in salt or sugar at room temperature in 1 week, the percentage of surviving oocytes after injected was from 70% to 71%. Our results showed that the survival rate of sperms stored in D-PBS solution is higher than those in NaCl 0,9% solution.

*Table 3*

**Effect of solution on the survival rate of sperms**

| Predictor | The estimated coefficient | p-value | Risk Ratio | 95% CI |
|---|---|---|---|---|
| **Constant - α** | -1.377 | $< 2e^{-16}$ | 1.126 | 1.057 - 1.200 |
| **Solution - β** | 0.119 | 0.0002 | | |

*Effect of temperature factor*

The survival rate of sperms after preservation was affected by the changes of temperature factor, the difference is statistically significant ($p = 2.27e^{-5}$) (table 4). Specifically, the temperature was increased 1°C, the survival rate of sperms was decreased 0.993 times (equivalent 0.7%, 95% confidence interval, change in the range from 0.4% to 1.1%). Sankai et al. [8] reported that sperm motility decreased with increasing storage temperature (5-20°C). The decrease was significant difference between 15 and 20°C, suggesting that a change in the metabolism activity of spermatozoa occurs between 15 and 20°C and that some factors are involved in the maintenance of sperm motility at the different temperature. Spermatozoa stored at 5°C had bent tails, possibly due to damage to the plasma membranes and to the spermatozoa's hardening in the phospholipid by exposure to the low temperature [8]. Moreover, Sankai et al. indicated that when spermatozoa stored at 10°C are used for IVF and embryo transfer, the delivery rate might be higher than when spermatozoa are stored at 5°C.

*Table 4*

**Effect of temperature on the survival rate of sperms**

| Predictor | The estimated coefficient | p-value | Risk Ratio | 95% CI |
|---|---|---|---|---|
| **Constant - α** | -1.218 | $< 2e^{-16}$ | 0.993 | 0.989 - 0.996 |
| **Temperature - β** | -0.007 | $2.27e^{-5}$ | | |

Many studies also confirmed that 4°C is an optimal temperature for the preservation of freezedried spermatozoa [2, 3]. Our results showed that the survival rate of sperms stored at 4°C or 10°C is higher than those at 26°C (Table 2). Based on the results obtained, we plotted a chart to predict the survival rate of sperms in response to change in temperature (Fig 1). Using this chart, we can predict the survival rate of sperms during short-term preservation in NaCl 0.9% or D-PBS solution corresponding to the change of temperature in the range from 4°C to 26°C.

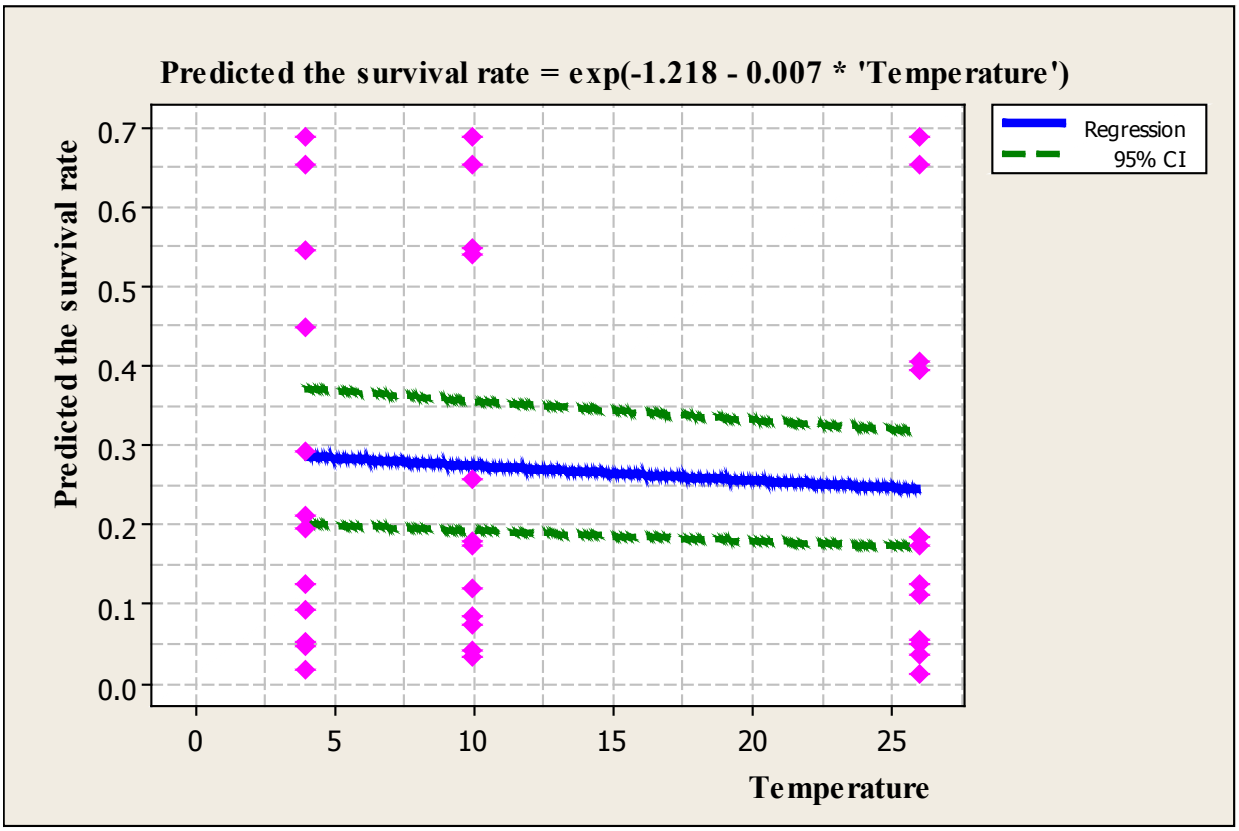


*Fig 1. A chart predict the survival rate of sperms in response to change in temperature*

*Effect of time factor*

The results in table 5 showed that the survival rate of sperms after preservation was affected by the changes of time factor, the difference is statistically significant ($p < 2e^{-16}$). Specifically, every one hour extended, the survival rate of sperms was decreased 0.932 times (equivalent 6.8%, 95% confidence interval, change in the range from 6.6% to 7.1%). This can be explained that two preservation solutions do not include any nutrition ingredients (only different kinds of salt), so the longer the storage time is, the more the survival rate of sperms is. Sato et al. [9] reported that the percentage of spermatozoa motility decreased when the preservation extended in the longer time (TYH media at 22°C and room temperature).

*Table 5*

**Effect of time on the survival rate of sperms**

| Predictor | The estimated coefficient | p-value | Risk Ratio | 95% CI |
|---|---|---|---|---|
| **Constant - α** | -0.327 | $< 2e^{-16}$ | 0.932 | 0.929 - 0.934 |
| **Time - β** | -0.071 | $< 2e^{-16}$ | | |

This result is consistent with the theory and previously reported studies. Based on the results obtained, we plotted a chart to predict the survival rate of sperms in response to the change of time (Fig 2). Using the chart, we can calculate the survival rate of sperms during short-term storage in NaCl 0.9% or D-PBS solution corresponding to the time prolongated. For example, the figure 2 shows that the survival rate of sperms was predicted at 0 hour is 72% and at 9.76 hours is 36%. This means the lethal concentration and time ($LCt_{50}$) to mouse sperm in short-term storage is 10 hours in D-PBS or NaCl 0,9% solution without cryoprotectant agents.

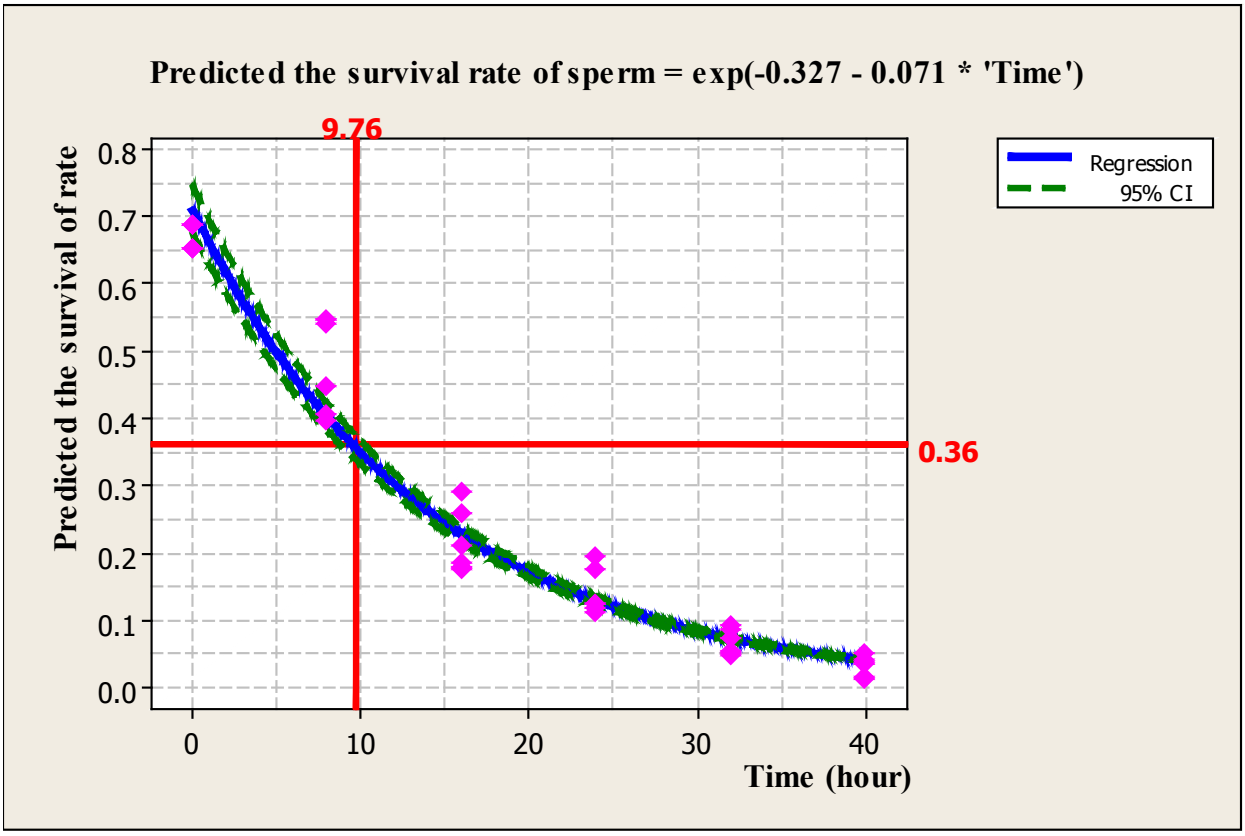


*Fig 2. A chart predict the survival rate of sperms in response to change in time*

*Effect of three evaluated factors*

The survival rate of sperms after preservation was affected by the changes of three factors (solution, temperature, time), the difference is very statistically significant ($p < 0.001$) (table 6).

When all three factors examined at the same time, the results are highly similar to those obtained when examined every factor separately. Consequently, an equation was set to predict the survival rate of sperms follow effect of examined factors:

$$A = \exp^{(\alpha + \beta 1 * solution + \beta 2 * Temperature + \beta 3 * Time)}$$

A: Predicted the survival rate

Apply the equation to the data collected from our analysis, in the case of: $\alpha = -0.291$; $\beta_1 = 0.119$; $\beta_2 = -0.007$; $\beta_3 = -0.071$; Solution = 0 for NaCl; Solution = 1 for D-PBS, we have a prediction equation for our experiments

$$\Rightarrow A = \exp^{(-0.291 + 0.119 * solution - 0.007 * Temperature - 0.071 * Time)}$$

*Table 6*

**Effect of three evaluated factors on the survival rate of sperms**

| Predictor | The estimated coefficient | p-value | Risk Ratio | 95% CI |
|---|---|---|---|---|
| **Constant - α** | -0.291 | < 2e-16 | - | - |
| **Solution - $\beta_1$** | 0.119 | 0.0002 | 1.126 | 1.057-1.200 |
| **Temperature - $\beta_2$** | -0.007 | 2.27e-05 | 0.993 | 0.989-0.996 |
| **Time - $\beta_3$** | -0.071 | < 2e-16 | 0.932 | 0.929-0.934 |

**Conclusion**

The short-term preservation of mouse sperms can be implemented at 4°C or 10°C in NaCl 0,9% or D-PBS solution without cryoprotectant agents. The survival rate of sperm in D-PBS solution is higher than those in NaCl 0,9% solution.

The lethal concentration and time ($LCt_{50}$) to mouse sperm in short-term preservation is 10 hours in D-PBS or NaCl 0,9% solution without cryoprotectant agents.

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