EFFECTS OF THREE DIFFERENT CRYOPROTECTANTS ON OXIDATIVE STRESS OF RABBIT SEMEN DURING CRYOPRESERVATION

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ABSTRACT

Glycerol, DMSO and acetamide are three cryoprotectants commonly used in the process of cryopreservation of rabbit semen. In this study, these three cryoprotectants with concentration of 4% were tested for their effect on oxidative stress of rabbit sperm. After thawing, the oxidative stress was evaluated by five indicators: level of carbonyl groups and malondialdehyde, activity of superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GSHPX). The results showed that glycerol provided the lowest protection to GR ($P<0.05$) and SOD ($P<0.01$) activity. While acetamide provided lowest protection to GR ($P<0.01$), the performance of DMSO was between those of glycerol and acetamide. Glycerol, DMSO and acetamide can provide moderate protection for oxidative stress change of rabbit sperm. Nevertheless, acetamide, appears to be the best cryoprotectant for freezing rabbit semen, because it contributes in maintaining the integrity of membrane and leads to the lowest oxidation damage.

Key words: Rabbit, sperm cryopreservation, oxidative stress, carbonyl groups, superoxide dismutase (SOD)

INTRODUCTION

Although research on the cryopreservation of rabbit semen was conducted at an early time, the unsatisfactory cryoprotective effects, such as the quality loss of the semen, reduced fertility rate and number of newborn rabbits per litter, make the application of this technique impossible in the production of domestic rabbits. This unsatisfactory effects of cryopreservation could be attributed to the characteristics of the rabbit semen, such as the high ratio of cholesterol and lecithin (0.88-1.56) and low ratio of polyunsaturated fatty acid and fatty acid (0.8) in sperm plasma membrane (Darin-Bennet *et al.*, 1977; Castellini *et al.*, 2006), which should be taken into consideration when choosing the types and concentration of the cryoprotectants.

Researchers have studied many cryoprotectants and found that glycerol, DMSO and acetamide are the most valuable ones. Numerous studies have shown that glycerol is not an ideal cryoprotectant for the cryopreservation of rabbit semen. Since Polge discovered the cryoprotective property of glycerol in 1949 (Polge *et al.*, 1949), some researchers have applied it to the cryopreservation of rabbit semen. However, it was found that glycerol could not provide rabbit spermatozoa with adequate cryoprotective protection, for the sperm motility rate remains very low after thawing and even when glycerol is used at a concentration of 5%, obvious toxicity or side effects could be witnessed. Research by Smith and Polge (1950) demonstrated that when using glycerol as a cryoprotectant, fertilization ability of rabbit sperm dropped significantly and only 2% of sperm could fertilize the ovum after thawing (Smith and Polge, 1950). Emmens and Blackshaw (1950) tested the cryoprotective effect of different types of sugar and alcohol which were found to exhibit more toxicity to spermatozoa of rabbit than to that of cattle or rat.

Curry *et al.* (1995) argued that glycerol was not an ideal cryoprotectant for cryopreservation of rabbit semen due to the poor permeability of sperm plasma membrane and high activation energy needed. Therefore, smaller molecular weight and high-osmolarity are required for an ideal cryoprotectant. Compared with glycerol, acetamide and DMSO are of better choice (Curry *et al.*, 1995). Kashiwazaki (2006) reported that acetamide and lactamide were best, DMSO fallen in the middle and glycerol was the worst as cryoprotectants when all of them were at the same concentration (1 M). Okuda (2007) obtained similar results that the freezing process could cause oxidative damage to sperm at a lower concentration of antifreezes (2% for acetamide and glycerol). Oxidative damage to sperm during freezing was reported previously (McCarthy *et al*., 2010). There were also reports about the increase of activity of swine sperm after adding antioxidant (Zhang *et al*., 2011). In the present research, we tried to explain the strengths and weaknesses of different cryoprotectants in terms of oxidative damage.

MATERIALS AND METHODS

Reagents

Pooled semen samples were diluted in Tris-citric-glucose (TCG) (Roca *et al.*, 2000) formulated with 4 % glycerol, DMSO or acetamide. The formulation of TCG was as shown in Table 1.

Table 1: TCG formulation

Constituents	Tris	Citric acid	Glucose	Kanamycin(mg/L)	pH	Osmotic pressure(mOsmol/L)
Concentration (mM)	313.79	103.07	33.3	80	6.9	336

Animals and collection of semen

Fifteen male Hyla rabbits 1-year-old which have a normal libido and no genital disease were included in this experiment. Semen was collected with a pre-heated artificial vagina. Colloid materials in the semen were removed immediately after the collection.

Pretreatment of the semen samples

The colloid-removed semen was observed under the phase contrast microscope. Semen samples with motility greater than 70% were chosen. Eight samples of semen were selected. Each sample was diluted 1:4 in preheated freezing diluents and balanced at 37°C for 1 h. The samples were placed at 4°C for 1 h and then frozen in liquid nitrogen.

Freezing and thawing of semen

Sperm cryopreservation tube was filled with the cooled semen. Then, the tube was put 5 cm above the surface of the liquid nitrogen vapors for 10 min, before immersing it into the liquid nitrogen. When thawing, the sperm cryopreservation tube was gently shaked for 10 s in the preheated water at 37°C and then decanted into a tube and incubated at 37°C.

Indicators

After incubation for 30 min, biochemical method was used to quantify the level of carbonyl groups (CG) and malondialdehyde (MDA), as well as the activity of SOD, GR, GSHPX in the samples. The quantification method used was ELISA and all testkits are purchased from Nanjing Jiancheng BiotechCompany.

Data Processing

Data was first subjected to analysis of variance and then paired with t-test if there was significant difference.

RESULTS AND DISCUSSION

Table 2: Effect of three cryoprotectants on oxidative damage of rabbit semen

Cryoprotectant	CG (mg/ml)	MDA (nmol/ml)	SOD (U/ml)	GR (U/L)	GSHPX
Glycerol	2.13±0.61[a]	3.23±0.82[a]	100.60±19.17[b]	33.96±18.32[a]	176.04±53.74[a]
DMSO	1.38±0.87[a]	2.35±0.78[ab]	112.97±9.06[a]	14.87±7.91[ab]	214.04±21.68 [a]
Acetamide	1.44±0.60[a]	1.74±0.53[b]	114.10±11.22[a]	13.87±9.25[b]	213.75±37.98 [a]

Note：The superscripts “a”, “b” and “c” denote the significance at the 5%level.

Protein carbonyl groups (CG)

Protein is important component of rabbit semen. Many enzymes play important roles in the survival and metabolism of spermatozoa. Once oxidized, the affected protein would not function well. All the amino acids that make up protein are vulnerable to the attack of reactive oxygen species (ROS); therefore protein is a major target of radicals. In the present study, there was no significant difference in the level of carbonyl groups between the three groups, suggesting that these three cryoprotectants could not provide adequate protection against protein oxidization.

Malondialdehyde (MDA)

Malondialdehyde is one of the end products of lipid peroxidation. The level of MDA can reflect the degrees of the lipid peroxidation in the organism. Lipid vesicles are found in large amounts in the rabbit semen. Their function is still not clear, yet they play an important role in maintaining the quality of the rabbit semen. In the present study, there was no significant difference in the level of MDA between glycerol or DMSO treated group. The level of MDA in these two groups was significantly higher than that of acetamide treated group, the latter of which was very low, indicating that acetamide could cause a lesser lipid oxidation.

Superoxide dismutase (SOD)

SOD, an important antioxidase in living organisms, is involved widely in many antioxidative reactions. It could catalyze the superoxide radical into hydrogen peroxide. In the present study, there is no significant difference in the level of SOD between DMSO group and acetamide group. The level of SOD in both groups is significantly higher than in the glycerol group. The level of SOD in acetamide group was very significantly higher than in glycerol group.

Glutathione reductase (GR) and glutathione peroxidase (GSHPX)

GR and GSHPX are two important enzymes in the antioxidant system of semen. In the present study, there was no significant difference in GR activity between DMSO group and glycerol group. The similar result was also found between DMSO group and acetamide group. However, GR activity in glycerol group was significantly higher than that in the acetamide group. These results indicate that glycerol could provide more protection for the maintenance of the activity of GR than acetamide. There was no significant difference in GSHPX activity between the three groups.

ROS produced during freezing will affect the sperm motility as well as the activity of some intra- and extracellular enzymes (Baumber *et al.*, 2001). The level of the oxidative stress can be determined by measuring either the activity of ROS or the content of antioxidant, or indirectly measuring the level of products of oxidative stress. The high level of ROS can cause protein oxidation which could result in a rise in the level of carbonyl groups. The antioxidant system comprised of superoxide dismutase, glutathione reductase, glutathione peroxidase and other antioxidases plays an important role in the antioxidation of host cells. Previous studies have shown that ROS produced during freezing could cause damage to sperms (Bucak *et al.*, 2009). Though much work had been done on the comparison of cryoprotective effect between glycerol, DMSO and acetamide in terms of motility, vitality and integrity of membranes of thawed sperms, there is no report concerning the comparison of the

oxidative stress level between them.

ROS plays important roles in the structure and function of related proteins: On the one hand, ROS can oxidize proteins to generate carbonyl groups. On the other hand, ROS could affect the function of proteins by oxidizing polysaccharide or lipids. In this study, such three cryoprotectants probably protected against the oxidation of proteins so that no significant difference of oxidative damage level was observed.

There exist abundant vesicles in rabbit sperms, which makes it obviously different from other domestic animals. These vesicles are similar to human prostasome and contain large amounts of lipids which are essential for the semen quality. In this study, the value of malondialdehyde measured in the acetamide treated group, was significantly lower than that in the glycerol-treated group, indicating that acetamide is better for the protection of lipids existing in the rabbit semen.

Compared to other animals, studies of oxidation system in rabbit semen are few. Here we determined three enzymes, SOD, GR and GSHPX produced during the procedure of semen oxidation. SOD can catalyze the reduction of superoxide anion to hydrogen peroxide (Li *et al.*, 2010). Selenium-containing GSHPX can remove peroxy group from molecules such as H_2O_2, while GR can reduce the oxidation state of glutathione. In this study, it was found that the activity of SOD in glycerol treated group, was inhibited obviously. No difference was measured for the activity of GSHPX in all groups treated with cryoprotectant, while the activity of GR was much higher in glycerol treated group than that in DMSO treated group. According to their effect on different antioxidases, though it could not fully meet the requirement of freezing, acetamide seems to be the best of the studied cryoprotectants.
We also found that the protection effect of DMSO against oxidation damage was between glycerol and acetamide but closer to the later.

Taken together, we found that acetamide may be preferable to glycerol as a cryoprotectant for rabbit semen from the perspective of oxidative stress. Glycerol would result in oxidation of more lipids and depression of the activity of SOD. However, acetamide is still not an ideal cryoprotectant, since its damage to GR is greater than that of glycerol. Therefore, oxidation stress level should be considered in further studies as an indicator for cryoprotective effect. Furthermore, adding some antioxidative materials, such as glutathione and unsaturated fatty acid, into the freezing diluent is also one of the prospective research areas. It is reported that vitamin E added in the feed of male rabbit could highly improve the quality of semen, suggesting that its effect on the cryopreservation of rabbit semen should be further studied.

CONCLUSION

Compared to glycerol and DMSO, acetamide is the best cryoprotectant for freezing rabbit semen, because it contributes in maintaining the integrity of membrane and leads to the lowest oxidation damage. Glycerol provided the lowest protection to glutathione reductase and superoxide dismutase activities. But, none of the three cryoprotectants could provide enough protection to protein oxidation and glutathione peroxidase.

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