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The Effect of Cold Shock on Function and Morphology of Dog Epididymal Spermatozoa

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Abstract : Dog spermatozoa were recovered from the caudae epididymides of 23 domestic dogs which were 11 pure breed and 12 mix-breed dogs ranging in age from 0.6 to 3 years. The experimental designs were as follows: 1) the effect of chilling to 0, 10 or 37°C, 2) the kinetics of chilling injury at 0 or 4°C, and 3) the effect of sugars at 0°C. Viable spermatozoa were recovered by percoll gradient separation and adjusted to 5×10^7 spermatozoa/ml. In experiment 1, spermatozoa were diluted with 0.33 M glucose supplemented with 3% BSA (G-BSA) at 1:2 dilution. Spermatozoa were loaded into straws and exposed at 0, 10 or 37°C for 30 min. In experiment 2, spermatozoa were prepared as the experiment 1 and exposed for 0.5, 5, 15, or 30 min at 0 or 4°C. In experiment 3, spermatozoa were diluted with different sugars (0.33 M galactose, glucose, fructose, mannitol, lactose, sucrose, raffinose) and cooled to 0°C for 30 min. Sperm membrane integrity, motility and acrosome integrity were assayed after rewarming at 37°C for 5 min. Sperm motility and membrane integrity abruptly decreased with decreasing temperature but acrosome integrity gradually decreased ($P<0.05$). Sperm motility was more sensitive to cold shock than membrane integrity and acrosome integrity. Spermatozoa cooled to 0°C were more damaged than those at 4°C. Sperm motility was not different among exposed times at both 0 and 4°C. However, membrane integrity of spermatozoa exposed for 30 min at both 0 and 4°C was significantly lower ($P<0.05$). Spermatozoa diluted in 0.33 M fructose or galactose showed lower motility and higher morphological abnormality with coiled tail at 0°C. These sperm characteristics were strongly related. These results indicate that dog epididymal spermatozoa are relatively sensitive to rapid cooling and higher morphological abnormality at 0°C was shown in spermatozoa diluted in fructose and galactose.

Key words : *dog epididymal spermatozoa, cold shock, sugars, sperm membrane integrity, sperm motility.*

Introduction

One of the critical tools to preserve dog genetics is to cryopreserve dog spermatozoa. Cooling is an essential and important procedure in sperm cryopreservation. The determination of sperm sensitivity to rapid cooling (cold shock) is the first step to set appropriate cooling rates for cryopreservation of dog spermatozoa.

Many works about cryopreservation and artificial insemination of dog spermatozoa have been performed[7,9,13,19,28]. However, studies about cold shock and appropriate cooling method to improve frozen-thawed dog sperm function were rarely reported. The effects of slow cooling and fast cooling to 0°C on dog ejaculated spermatozoa were compared and sperm motility and viability of spermatozoa cooled quickly to 0°C were lower than those of spermatozoa cooled slowly[12]. Yubi *et al*[37] held dog ejaculated semen at different temperatures (45, 40, 37, 35, 32, 24 or 7°C) for 1 h, respectively and obtained lower sperm motility and viability at the higher temperature, 40 and 45°C.

A variety of compounds has been shown to be cryoprotective to spermatozoa from cold shock during freezing. These agents belongs to two groups-those that penetrate into spermatozoa (glycerol, ethylene glycol, dimethyl sulfoxide, methanol) and those that remain extracelluar (sugars, protein and polyvinyl pyrollidone)[6]. Glycerol has been used as a common cryoprotectant for dog sperm freezing[9,10,12,13,24].

The isotonic sugar media clearly offered a significant protection of bull sperm against fast-cooling damage at the very high cooling rate of 300°C/min[35]. Saito *et al*[26] recently preserved human ejaculated semen in 0.33 M glucose with 3% BSA at 4°C for 4 weeks and demonstrated human spermatozoa in the electrolyte-free solution survived for a long period of time. Kanno *et al*[16] also reported long-term preservation of human spermatozoa using 0.33 M glucose at 4°C for 2 weeks and more improved sperm viability and motility than those in electrolyte solution. These reports indicated that sugar can support the sperm metabolism at 4°C. However, there has not been reported whether dog spermatozoa could be preserved in only sugar without other electrolyte extender.

In this study, there were several reasons that we have used epididymal, rather than ejaculated spermatozoa. We are attempting to devise procedures that might be used for endangered species of animals that may die unexpectedly. Recovery, determination of cold shock, and cryopresevation of their epididymal spermatozoa would be one useful way to rescue the germplasm of dead animals.

The aim of this study was to determine 1) the effect of chilling on function of dog epididymal spermatozoa, 2) the kinetics of chilling injury at 0 or 4°C, and 3) the effect of various sugars on sperm function and morphology at 0°C.

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Materials and methods

Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and from Gibco (Life Technologies, Grand Island, NY, U.S.A.).

Preparation of Dog Testes

For this study, we used spermatozoa recovered from the epididymides of 11 pure breed and 12 mixed-breed dogs (n= 23) ranging in age from 0.6 to 3 years old; the dogs privately owned and were housed in various households. Pure breed used were Beagle, Boston Terrier, Dachshund, Doberman Pinscher, Labrador, Old English Sheepdog, Pekinese, Pomeranian, Poodle, Rottweiler. Testes with epididymides were obtained from the dogs at several local veterinary clinics during routine orchiectomy. Immediately upon removal, the testes with epididymides were placed into plastic bags with sterile saline supplemented with penicillin at 100 IU/ml and streptomycin at 0.1 mg/ml and were transported to the laboratory at 25°C.

Collection of Dog Epididymal Spermatozoa

As soon as the specimens were returned to the laboratory, the caudal epididymides of testes were dissected, rinsed with 0.9% saline solution and placed into a 35 mm petri dish containing 1.5~2 ml of modified Tyrode's salt solution (TALP: Tyrode's Albumin- Lactate-Pyruvate), as described by Parrish *et al*[23]. Epididymides were cut repeatedly with a surgical scissors and spermatozoa were allowed to swim out into the solution for 10 min at 38.5°C in a humidified atmosphere of 5% CO_2: 95% air. Each suspension of spermatozoa was filtered by cell strainer (Falcon®, NJ, U.S.A.) and transferred into a plastic tube with a sterile pipette.

Percoll gradient centrifugation for live spermatozoa

The isotonic percoll solution (90% v/v) was prepared by mixing 9 volumes of percoll with 1 volume of 10× TALP solution. This isotonic percoll solution formed the 100% fraction. The 90% (v/v) and 45% (v/v) percoll solution were obtained by diluting the isotonic 100% stock percoll solution with 1× TALP. Mean osmolarity of 90% and 45% percoll was 355 and 326 mOsm/kg, respectively.

The two-layer discontinuous gradient was formed by laying 1.5 ml of the 45% percoll on 1.5 ml of 90% percoll in a 15 ml conical centrifuge tube. After warming for 5 min at 37°C, 1~2 ml aliquots of semen was layered on the percoll gradients and centrifuged for 20 min at 300 g. After centrifugation, supernatant from the percoll was removed. The pellet was recovered and washed with 5 ml of TALP by centrifuging at 300 g for 5 min. The supernatant was aspirated off and pellet in a tube was kept in a water bath at room temperature.

Sperm concentration and motility

Sperm concentration of the pellet was determined using a Neubauer haemocytometer. The sperm concentration was adjusted to 5×10^7 spermatozoa/ml by dilution with TALP.

Progressive sperm percentage was assessed subjectively by microscopic examination at a magnification of 200× using on a scale of 0~4 described by Mortimer[21].

Sperm membrane integrity (viability) and acrosome integrity

The integrity of sperm plasma membranes was measured by the method of Garner and Johnson[11] using the fluorescent double stain Fertilight® (Molecular Probes Inc., Eugene, OR). This vital stain contains two nucleic acids dyes: (1) SYBR-14, which permeates cells with intact membranes that fluorescein green, and (2) propidium iodide (PI), which permeates cells with damaged plasma membranes, causing them to exhibit red flurorescence. For each of two straws of each sample, appropriately 200 spermatozoa were counted for each slide. For each sample, the number of spermatozoa with green or red fluorescence was counted, and the percentage of membrane-intact spermatozoa was calculated.

Acrosome integrity was determined using a methods modified from that described by Cross *et al*[1]. For each replicate sample, two slides were examined, and approximately 200 spermatozoa were counted for each slide. Percentage of spermatozoa with intact acrosome were calculated. Spermatozoa were considered to have an intact acrosome if bright green fluorescence was distributed over the entire acrosome. Spermatozoa with either slight fluorescence on the equatorial segment or no fluorescence were considered as having damaged acrosomes.

Sperm Morphology

Five microliter of spermatozoa and 10 ul of Eosin Green B stain were gently mixed on a clean glass slide. A drop of mixture was smeared on a clean glass slide and allowed to dry on a warm plate. Sperm morphology was evaluated by microscopic examination at magnification of 1000×. Each spermatozoon was evaluated for abnormalities arising in the head, neck, middle piece, and tail[15]. A minimum of 250 spermatozoa was counted.

Experimental designs

Experiment 1: The effect of rapid cooling (cold shock) on sperm function

The effect of cooling temperatures on sperm motility, membrane integrity and acrosome integrity

Dog spermatozoa were diluted with 0.33 M glucose supplemented with 3% (w/v) bovine serum albumin (G-BSA) at 1:2 dilution, loaded into 0.25 ml straw and sealed using heat sealer. Mean osmolarity of G-BSA was 352 mOsm/kg. Two sets of 2 straws were exposed at 0 or 10°C for 30 min using a freezer (Biocool III, FIS System, Stone Ridge, NY). In addition, two straws (control) were kept at 37°C for 30 min. After being removed from 0 or 10°C, The straws were rewarmed to 37°C in a water bath for 5 min. Sperm motility, membrane integrity and acrosome integrity were assessed.

Cooling rates to 0 or 10°C was measured with a thermo-

couple (Diqui-sense®, Coleparmer, USA). The overall cooling rate from 37 to 0°C was ~85°C/min, and the overall cooling rate from 37 to 10°C was ~65°C/min.

Experiment 2: The kinetics of chilling injury on sperm function

The effect of exposed times on sperm motility and membrane integrity

Dog spermatozoa were diluted with G-BSA at dilution of 1:2, loaded into 0.25 ml straw and sealed. One set of 2 straws was exposed for different times (0.5, 5, 15 or 30 min) at 0 and 4°C, respectively. The straws were rewarmed to 37°C in a water bath for 5min. Sperm motility and membrane integrity were assessed.

Experiment 3: Effect of sugars on sperm function and morphology

The effect of sugars on sperm motility and membrane integrity

0.33 M galactose, glucose, fructose, lactose, mannitol, sucrose, and raffinose were prepared, respectively and stored at -80°C. All solutions were warmed to 37°C before use. Ten microliter of sperm pellet was suspended in 90 ul of each sugar solution in a tube (1.5 ml). Sperm suspension was loaded into 0.25 ml straw, sealed and exposed to 0°C for 30 min. The straw were rewarmed at 37°C for 5 min. Sperm motility and membrane integrity were assessed.

The effect of sugars on sperm morphology

The straws of spermatozoa were prepared, exposed to 0°C for 30 min and rewarmed at 37°C for 5 min. Spermatozoa were stained using Eosin Green B and evaluated for morphological abnormalities.

Statistical analysis

The percentages of motility, and membrane integrity and acrosome integrity were compared by one-way analysis of variance (ANOVA). One-way ANOVA was performed using GraphPad Instat version 3.00, GraphPad Software, San Diego, CA, USA. Differences were considered significant when $P < 0.05$.

Results

Dog epididymal spermatozoa were exposed for 30 min at different temperatures (0, 10, 37°C). Sperm motility, membrane integrity, and acrosome integrity were assessed. Sperm motility, membrane integrity and acrosome integrity decreased (Fig 1). Sperm motility was the most sensitive to rapid cooling among sperm functions.

Dog epididymal spermatozoa were exposed for different times (0.5, 10, 15, 30 min) at both 0 and 4°C, respectively. Sperm motility and membrane integrity were assessed to determine the kinetics of chilling injury at both 0 and 4°C. Sperm motility and membrane integrity of spermatozoa decreased regardless of exposed times at both 0 and 4°C (Figs 2a and 2b, respectively). Membrane integrity of spermatozoa exposed for 30 min was significantly lower than those of

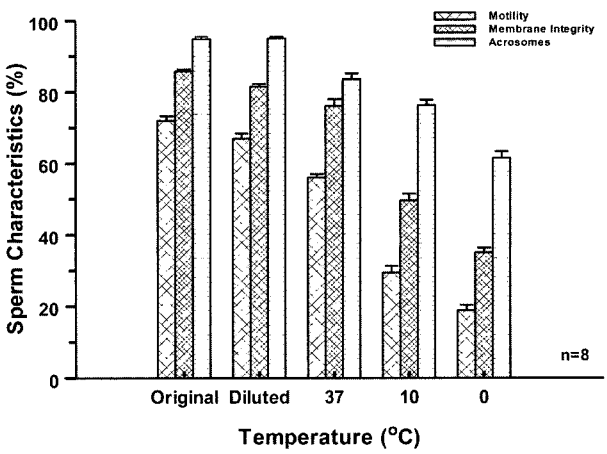

Fig 1. Effect of chilling on dog epididymal spermatozoa. Original and diluted mean spermatozoa after percoll gradient and after dilution with G-BSA, respectively. The data represent mean±SE ($P < 0.05$).

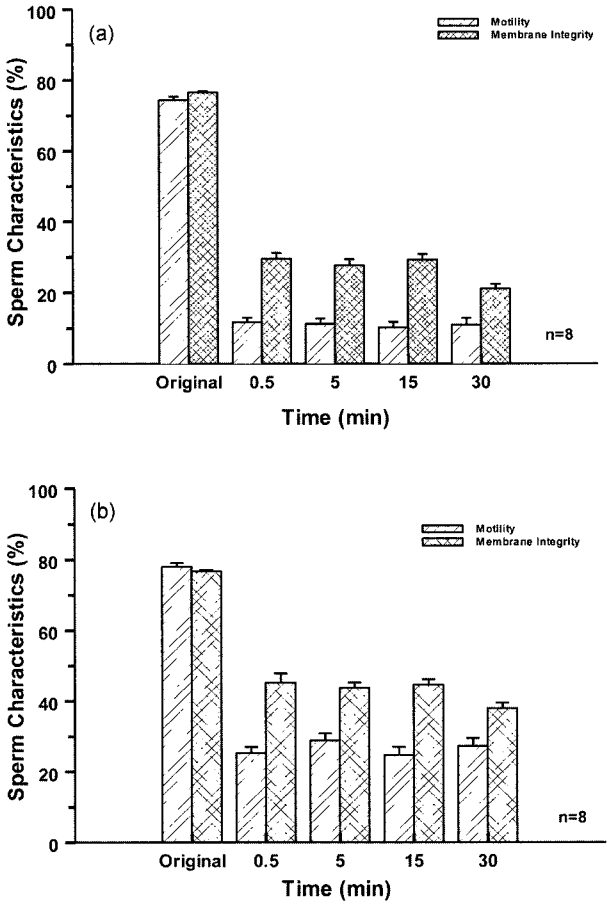

Fig 2. (a) Kinetics of chilling injury at 0°C. Original means spermatozoa after percoll gradient. The data represent mean± SE ($P < 0.05$). (b) Kinetics of chilling injury at 4°C. Original means spermatozoa after percoll gradient. The data represent mean± SE ($P < 0.05$).

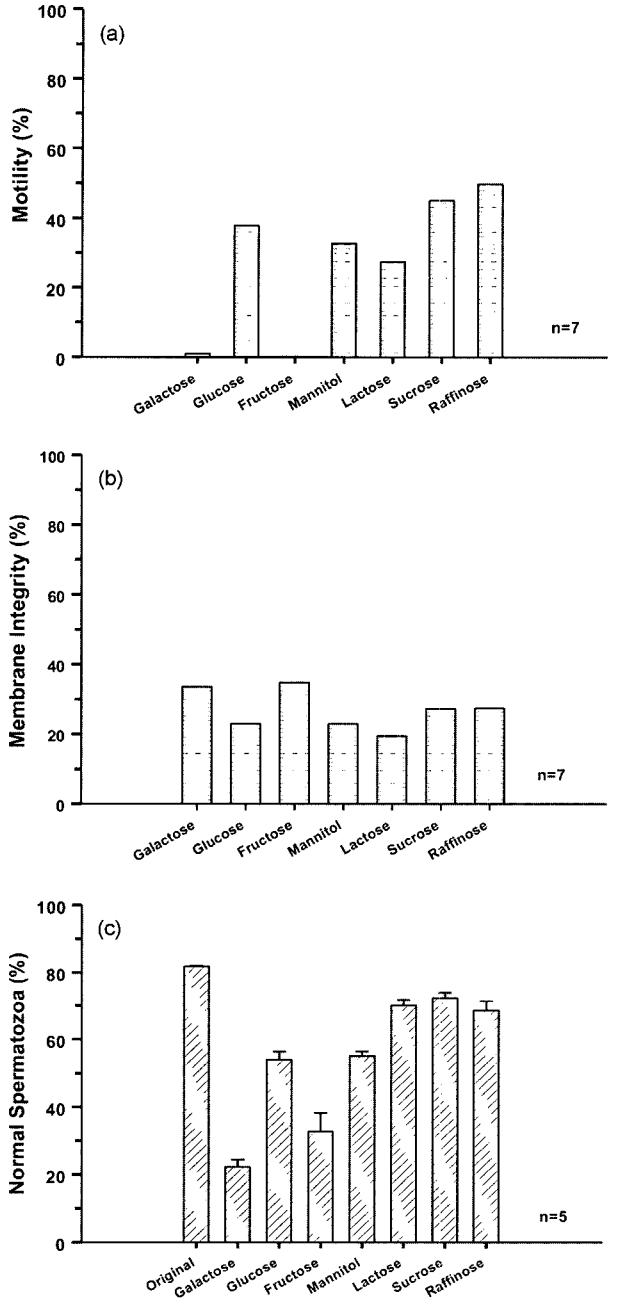

Fig 3. (a) Effect of sugars on sperm motility. The normalized data is shown ($P<0.05$). (b) The effect of sugars on sperm membrane integrity. The normalized data is shown ($P<0.05$). (c) Effect of sugars on morphology of spermatozoa at 0°C. Original means spermatozoa after percoll gradient. The data represent mean±SE ($P<0.05$).

spermatozoa exposed for other different times at 0°C ($P<0.05$). Sperm functions at 4°C showed the similar pattern as resulted at 0°C.

Dog epididymal spermatozoa were diluted with each sugar solution and exposed for 30 min at 0°C. Sperm motility and membrane integrity were determined and normalized (Figs 3a and 3b, respectively). Spermatozoa diluted with 0.33 M fructose or galactose were rarely motile but their membrane integrities were higher than those of spermatozoa diluted with other sugars ($P<0.05$).

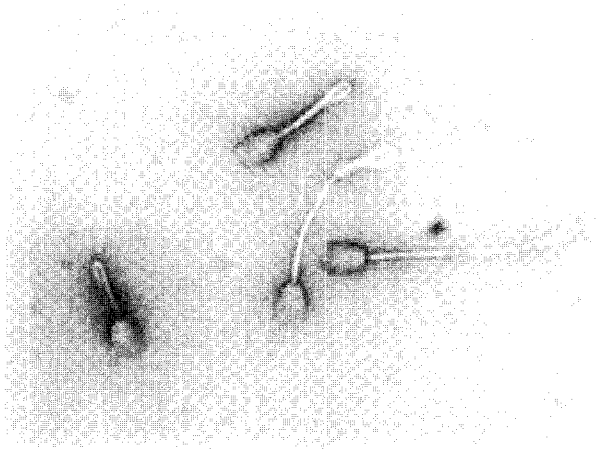

Fig 4. Sperm abnormality (coiled tail) examined at magnification of 1000×. The spermatozoa were stained with Eosin Green B stain.

Spermatozoa exposed in 0.33 M galactose or fructose at 0°C yielded higher abnormalities in morphology (Fig 3c). The rate of normal spermatozoa was higher in 0.33 M lactose, sucrose, and raffinose. Coiled abnormality was observed in most of spermatozoa exposed in 0.33 M galactose or fructose at 0°C (Fig 4).

Discussion

The effect of cold shock on function of dog spermatozoa has been rarely reported, while the effect of cold shock on survival of ram and hamster spermatozoa has been published[8,22,29].

The critical effect of cold shock on spermatozoa is reduction of motility[14,34]. As expected, motile spermatozoa decreased as cooling temperature was lower. This result indicated that injury after rapid cooling depends on rate of cooling rates[20,33]. In this study, the overall cooling rate from 37 to 0°C was ~85°C/min, and the overall cooling rate from 37 to 10°C was ~65°C/min. Dog spermatozoa cooled at the same cooling rate showed similar motility regardless of exposed times. This result proves that cold shock is dependent on rate of cooling as well as the final temperature to which semen is cooled[20,25].

Sperm membrane integrity (viability) abruptly decreased as temperature decreased. Loss of sperm viability after cold shock or freezing involves disruption of the sperm membranes and, as membrane components, phospholipids and cholesterol may be important to sperm in maintaining the structural integrity of the various highly organized membrane systems[34]. The mechanism for cold shock is linked to phase changes in the lipids in the spermatozoal membranes[5,18]. Cold shock may be related to the lipid composition of the membrane bilayer affecting the fluidity of the plasma membrane[32]. Darin-bennett[4]

divided several groups related with sensitivity on cold shock based on the ratios of phospholipid bound polyunsaturated : saturated fatty acids. The susceptibility of sperm to cold shock is linked with a high ratio of unsaturated : saturated fatty acids in the phospholipids and a low cholesterol content. The polyunsatured : saturated fatty acids ratios of dog ejaculated sperm phospholipids was low (~1.0). Dog ejaculated spermatozoa are relatively resistant on cold shock. However, dog epididymal spermatozoa in this study were relatively sensitive to rapid cooling. Only 33% of spermatozoa were viable, if dog epididymal spermatozoa were cooled to 10°C.

Membrane integrity of spermatozoa exposed at 0 or 4°C for 30 min was lower than that of spermatozoa cooled for other different times (0.5, 5, 15 min). We suggest that extension over certain times can cause severe damage on sperm membrane. Hay *et al*[12] diluted ejaculated semen with egg yolk extender, exposed them for 30 min at 0°C and examined motility. Sperm motility was not different with that of fresh semen at collection. On the other hand, the viability and motility of spermatozoa exposed for 3 h at 0°C were lower.

Sugars add osmotic pressure to the medium and act as cryoprotectants[31]. Sugars form hydrogen bonds with the polar head groups of membrane lipids and this property helps to stabilize the membrane during transition through the critical temperature zones[2,3,35]. In this study, dog spermatozoa were still motile and viable in only sugar without electrolyte extender if there was difference in motility and viability among sugars.

Woelders *et al*[35] demonstrated trehalose and sucrose has a significant interaction with cooling rates. The sugars of higher molecular weight, such lactose and raffinose, have low permeability and are generally considered to be cryoprotectants[6]. In this study, monosaccharides (galactose, glucose, fructose), disaccharides (lactose, sucrose), trisaccharides (raffinose) were compared to determine the effect of cold shock on function of dog spermatozoa. Spermatozoa diluted with 0.33 M sucrose or raffinose and cooled to 0°C showed higher motility than those diluted with monosaccharides.

Reversely, membrane integrity of spermatozoa exposed in fructose and galactose was higher than those of spermatozoa exposed in other sugars. Sugars were prepared without electrolyte solution and bovine serum albumin. In sperm membrane integrity, sugar, itself does not seem to protect membrane integrity and has synergy with other macromolecular components. Yikdiz *et al*[36] determined the influence of different sugars supplemented to the extender on the motility, viability and intact acrosome rates of dog spermatozoa during dilution, equilibration and freezing.

Spermatozoa diluted with 0.33 M galactose or fructose at 0°C showed lower motility and higher abnormality of coiled tail. The tail abnormality was strongly related to decrease of sperm motility. For the future research, the effect of sugar as an additive to electrolyte solution on function of dog spermatozoa should be determined. We suggest that other additive should be supplemented to cryoprotectant to improve sperm motility if monosaccharide is used.

The egg yolk improved sperm motility and viability, if the data is not shown. Dog spermatozoa diluted with G-BSA supplemented with 20% egg yolk showed higher motility than those diluted with only G-BSA at both 0 and 4°C. Fisher and Fairfull[8] demonstrated the addition of egg yolk into initial extender in ram semen had a beneficial effect on percentage of motile spermatozoa particularly after rapid cooling of semen to 10 and 5°C. The importance of the role of egg yolk was revealed during cooling or freezing in other species[17,27,30].

Conclusion

The effect of cold shock on function of dog spermatozoa was determined in chilling temperature, kinetics of chilling injury, and sugars as a cryoprotectant. Dog epididymal spermatozoa were relatively sensitive to cold shock. Sperm motility was more sensitive to cold shock than membrane integrity and acrosome integrity.

Cold sensitivity was depended on which sugar was used as a cryoprotectant. Spermatozoa diluted with 0.33 M galactose or fructose at 0°C showed lower motility and higher abnormality of coiled tail. Sperm abnormalities and motility were strongly correlated.

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개에서 cold shock이 정소상체유래의 정자의 기능과 형태에 미치는 영향

유 일 정[1]
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요 약 : 냉각이 개 정자의 기능(생존력, 활력, 첨체고유성)과 형태(기형율)에 미치는 영향을 알아보기 위해 23마리 개의 정소상체에서 정자를 회수하여 다음과 같은 실험에 사용하였다. 정자를 3%BSA가 첨가된 0.3 M glucose (G-BSA)에 희석한 후 실험 1에서 서로 다른 온도, 0, 10, 37°C에 30분동안 냉각하여 37°C에서 가온한 후 정자의 활력, 생존력, 첨체고유성을 검사하였다. 실험 2에서 0°C와 4°C에서 정자를 서로 다른 시간(5, 10, 15, 30분)동안 노출시켜 37°C에서 가온한 후 정자의 활력 및 생존력을 검사하였다. 또한 sugars가 개정자의 기능과 형태에 미치는 영향을 알아보기 위해 여러 종류의 sugars (0.3 M)를 준비하고 0°C에서 정자를 sugar용액에 노출시켜 37°C에서 가온한 후 정자의 활력, 생존력 및 기형정도를 관찰하였다. 본 실험결과 개 정소상체유래의 정자는 냉각온도가 감소할수록 정자의 기능이 급격하게 감소하여 냉각에 민감하게 나타났다($P<0.05$). 특히 정자의 활력이 다른 정자의 기능, 생존력과 첨체고유성에 비해 냉각에 민감하게 나타났다. 정자를 0°C와 4°C에서 서로 다른 시간(5, 10, 15, 30분)동안 냉각하였을 때 30분 동안 냉각된 정자가 그 외의 시간 동안 냉각된 정자에 비해 생존력이 저하되었다($P<0.05$). Sugars가 정자의 기능과 형태에 미치는 결과는 fructose와 galactose에 노출된 정자의 활력이 다른 sugars에 노출되었던 정자의 활력에 비해 현저히 낮았으며, 이와 관련되어 정자의 기형율이 높게 나타났다($P<0.05$). 이상의 결과 개 정소상체유래의 정자는 냉각에 비교적 민감하였으며 정자의 냉각에 대한 민감성을 완화시키는 첨가체로 단당류뿐만 아니라 다당류에 대한 연구가 이루어져야 할 것으로 생각된다.

주요어 : 개 정소상체의 정자, Cold shock, Sugars, 정자의 생존력, 정자의 활력