International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

The Effect of Maltose in Tris-Aminomethane Egg Yolk Base Extender on Semen Quality of Taiwan Native Rooster Following Frozen Storage

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***Abstract*—** *The success of freezing chicken semen is desired for preserving Taiwan native chickens. Semen samples from three Taiwan roosters were collected using massage technique twice a week. Two Cryoprotectant Maltose concentrations, 0.6 and 1.2 g/ml, were added in Tris Aminomethane+20 % egg yolk as the basic semen extender. The purpose of this study was to observe the effect of Tris Aminomethane extender supplemented with maltose on post thawing quality (mass spermatozoa motility, spermatozoa motility, spermatozoa abnormality, and viability) following frozen storage on Taiwan Native Rooster spermatozoa. The experiment was conducted in Animal Embriology Engineering Laboratory of The National Pingtung University of Science and Technology, Taiwan. During the period of one month experiment, feed and water were provided ad libitum for the roosters. All semen samples were diluted (semen:diluent=1:10) with warmed diluents at 30ºC in a water bath. The diluted semen samples were gradually cooled to 4ºC for 4h. After cooling, spermatozoa motility was examined. The samples were packed in 0.25 ml straws and plugged with the straw powder. The straws were kept at 4ºC until frozen, which was carried out by placing the samples in liquid nitrogen vapor (-125 to -130ºC) for 20 minutes, and stored in liquid nitrogen (-196ºC) until examination after 3 hours. spermatozoa motility, abnormality and viability showed significant differences (P < 0.05) when preserved with cryoprotectant when compared to the control treatment. It can be concluded that maltose is a potential protector for improving spermatozoa quality following frozen storage.*

***Keywords*—** *Cryoprotectant, Maltose, Taiwan Native Rooster, Tris Aminomethane*

I. INTRODUCTION

One effort to improve the quality of native chicken is to conduct crossbreeding process the local chicken and chicken that have superior genetic qualities such as having large in size and carries more meats than other chicken breeds using artificial insemination (AI) technology.

The Success of AI using both liquid and frozen semen require good quality semen with high life ability, the use of frozen semen can be stored for long periods of time. The problem that often arises on the process of freezing semen is cold shock to spermatozoa cell that is frozen and changes in intra-cellular [1]. Semen dilution is aimed to obtain a higher amount of semen before the AI and maintain the quality of the semen before being inseminated into the female reproductive organs. Semen dilution depends on the semen volume obtained, the concentration of semen, the percentage of life and the semen dose to be inseminated.

The essential requirements for any diluents are: (1) non-toxic substances to spermatozoa, (2) containing energy sources, (3) isotonic, (4) containing buffers, (5) protecting from influence rapid cooling, (6) inhibits bacterial growth, (7) and increases the volume so that it can be used several times of AI process [2].

One widely used diluent and proven to maintain the quality of liquid semen is the Tris-aminomethane egg yolk. The Benefits of egg yolk was found in lipoproteins and lecithin contain in Tris-aminomethane which works to maintain and protect the integrity of the lipoprotein sheath of spermatozoa cells [3]. Egg yolks also contain better glucose used by rooster spermatozoa for their metabolism than the fructose contain in semen, various proteins, water-soluble or soluble vitamins and have viscosities that may benefit for spermatozoa [4].

Storage of semen at low temperatures that is commonly done is at temperatures of around 3 to 5ºC and at temperatures of -196ºC in a frozen storage (in liquid nitrogen containers). However, storing semen at low temperatures can affect in damage to the spermatozoa thereby reducing the quality of the semen. Spermatozoa damaged generally begins in the cell plasma membrane caused by the influence of cold shock. Damage to the cell plasma membrane will adversely affect overall cell survival. Efforts to reduce damaged to spermatozoa stored for a long time at low temperatures can be done by adding various compounds in semen diluents. [1]

One effort to overcome this problem was by using cryoprotectant substances into a semen dilution medium so that only a few spermatozoa were damaged during the freezing process [5]. One of the compounds that can be used for this purpose is sugar. Maltose is one type of sugar that has improved the quality of semen in various types of animal. Maltose is one of the disaccharide sugars that can function as extracellular cryoprotectant for spermatozoa by reducing negative effects such as cold shock and the formation of ice crystals that appear during the process of cooling and freezing of semen [6].

Based on this background, it was necessary to observe the semen quality of Taiwan native roosters using Tris aminomethane egg yolk with the addition of maltose following frozen storage.

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ISSN (Online): 2455-9024

II. Materials and Method

A. Place and Time of Research

This experiment was performed at the Testing Station located in chicken farm of Animal Science Department, semen quality evaluation was conducted at reproductive laboratory Department of Animal Science and Animal Embriology Engineering laboratory of National Pingtung University of Science and Technology Taiwan and this research was conducted from March to April 2019.

B. Animals and Semen Collection

A total of three mature Roosters, 13.9±2.13 months of age and 3-3.5 kg weight were used in this study. The technique of semen storage was conducted by massage method.twice a week for 4 weeks. The cloaca and its surroundings were cleaned of dirt with tissue paper that has been moistened with alkanol 70 %. Then the Eppendorf tube container was used as an artificial cloaca tied to the back of the roosters. Furthermore, the semen collector provided stimulation from the rooster's abdomen to the end of the tail in a few seconds, ejaculation occurred and semen was contained in the Eppendorf tube.

The semen samples were evaluated the quantity and quality of fresh semen. Fresh semen was dropped on glass objects for microscopic quality observation. Only ejaculates between 0.3 until 0.5 mililitres volume with a concentration of greater than $0.05x10^9$ spermatozoa/ml, having ≥45% spermatozoa motility, ≥ 2+ mass motility and ≥80% of the spermatozoa with normal morphology were selected for cryopreservation.

C. Extender Preparation

All chemicals were reagent grade and were purchased from Sigma–Aldrich, St. Louis, MO. The base extender consisted of 1.36 g Tris aminomethane, 0.76 g citric acid, 0.2 g lactose, 0.5 g fructose, 20 ml egg yolk, 0.5 g raffinose, 0.1 g streptomycin, 0.1 g penicillin and 80 ml distilled water, modified from [2]. Semen was diluted (semen:extender =1:10) each treatments (warmed in water bath 37˚C in temperature), there were three treatments, control, treatment 1 and treatment 2. Three extender were prepared for control group (without adding maltose), treatment 1 group (adding the concentration of 0.6 g/ml maltose) and treatment 2 (adding the concentration of 1.2 g/ml maltose).

D. Semen freezing

Semen was equilibrated at 4-5ºC for 2 hours, evaluation the quality of semen after equilibration include in motility scale, viability and abnormality. Pre-freezing of semen was carried out by filled packaged in French straws (0.25 ml). The end side of the filled straws was sealed with polyvinyl chloride powder, and continue to equilibrate at -135°C for 4 h before being exposed to liquid nitrogen vapor (7-10 cm from the liquid nitrogen surface level) for 20 min. Then the freezing of the semen was carried out by putting straw into liquid nitrogen which has a temperature of -196ºC for a minimum of 3-4 hours. After that, thawing was carried out on water bath with a temperature of 37-38ºC for 30 seconds and evaluation the quality of semen after thawing.

E. Variables Measured

The variables in this study were semen quality evaluations which included macroscopic and microscopic evaluations of semen. Macroscopic evaluation of fresh semen from Taiwan native roosters included semen volume, smell, semen color, semen consistency and pH. The volume of fresh semen was measured by looking at the size of the storage tube with the smallest line of 1 ml.

The color of semen was seen with certain colors including white, milky white, yellowish white and beige. The normal semen smell had a typical semen smell. The pH of semen was observed using a pH meter. The consistency of fresh semen can be evaluated with guidelines that include thick, medium and dilute. Normal semen had a specific smell.

The microscopic evaluation was a quality evaluation of semen using a microscope consisting of mass motility evaluation, spermatozoa motility, concentration with spectrophotometer test method, viability (percentage of life), and abnormalities of spermatozoa.

a.) Spermatozoa motility scale

The percentage of individual motility was the percentage of spermatozoa that move forward compared to all observed spermatozoa. Observation of mass spermatozoa motility was observed by determining one drop of semen on the object glass and observed using a microscope 100x magnification while for observation of individual motility using 400x magnification. Mass motility was characterized by very good values (+++), (++) good, (+) poor and (0) bad, individual motility was marked by percentages.

b.) Spermatozoa viability

The percentage of viability was the percentage of life spermatozoa. Life spermatozoa was marked with a head that did not absorb color (transparent) while the dead spermatozoa was marked with a red head. Viability was performed using a modification of the eosin-nigrosin stainning procedure, the number of life spermatozoa was calculated by dripping semen on a clean object glass, then eosin negrosine pigment was dripped using ose wire and made a smear preparation with a slope of 45° A mixture of 10 µl of diluted spermatozoa and 10 µl eosin-nigrosin stain was smeared on a slide and allowed to air dry in a room storage. Two hundred spermatozoa from different microscopic fields were examined under a bright-field microscope using a 400× objective, and the number of non-stained (viable) spermatozoa was counted [2]. A life spermatozoa was not be colored by eosin negrosine and dead spermatozoa due to damage was be purplish red.

c.) Spermatozoa abnormality

Observation of abnormalities using a smear technique that used eosin-negrosine solution. One drop of semen was taken by ose inoculation loop and mixed with eosin negrosin solution (10µl NE solution mixed with 5µl fresh semen). Calculations and reviewing were the same as how to calculate viability, only compared between normal and abnormal spermatozoa. Abnormal spermatozoa could be seen from the morphological form of the sperm itself, abnormal spermatozoa forms including the head was too big or small, the tail was broken, forked, circular and so on, Two hundred spermatozoa from

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different microscopic fields were examined under a bright-field microscope using a 400× objective. Taking pictures of abnormalities of sperm using a micro object photo 1000x magnification.

F. Statistical analysis

Data obtained first tested normality by using Shapiro Wilk test. The data was statistically analized by the analyses of variance using one-way ANOVA with completely Randomized Block Design (CBD) in SPSS statistic 24. The differences between the means of groups were separated by Duncan Multiple Range Test ($P<0.05$).

III. Results and Discussion

A. Fresh Semen Evaluation

The quality of fresh semen obtained was very determine whether the semen was feasible to do the dilution process or freezing. The amount of semen volume resulting effect on the number of doses semen that can be disseminated, so that if the volume produced was a little therefore the number of doses semen produced was also a little. Before dilution, semen was evaluated to determine the quality of semen in a fresh condition.

TABLE I. Fresh semen caracteristics

Fresh Semen Caracteristics	Mean
age (month)	13.9±2.13
Macroscopic evaluation	
Volume (ml)	0.41 ± 0.07
Color	Milky White
pH	6.70 ± 0.14
Viscocity	Medium
Microscopic evaluation	
Mass motility	2+
Motility scale (%)	59±5.68
Viability (%)	83.64 ± 7.85
Abnormality (%)	6.77 ± 3.32
Membrane integrity (%)	84.37 ± 1.03
Concentration (10^6 spermatozoa/ml)	507.25 ± 66.89

The results showed that semen volume of Taiwan native roosters averaged 0.41±0.07 ml. The difference in the volume of semen per ejaculate was influenced by differences in breed, age, body size, feed nutrition, semen collection frequency, changes in reproductive health, semen collection techniques and methods, long time period of day time and ambient temperature, in this study the roosters was 13.9±2.13 weeks old. The difference in the volume of semen per ejaculate was influenced by differences in breed, age, body size, feed nutrition, semen collection frequency, changes in reproductive health, semen collection techniques and methods, long time period of daytime and ambient temperature [7].

The results indicated fresh spermatozoa of native roosters had average of volume, colour, consistency, concentration, motility; viability and abnormality. Results of this research was still normal, which was volume ranges from 0.1 to 0.9 ml [8]; viscous white color and concentrations 312.6±842.2 x 10^6 spermatozoa/ml [9]; viability average around 96.64% and abnormality around 8% [10].

Based on the results of the study it was obtained that the consistency of semen was medium and milky white. Based on the research that has been done, the concentration of spermatozoa was 507.25 ± 66.89 million/ml with medium consistency and milky white color. Concentration is the degree of consistency that is closely related to the concentration of spermatozoa. Quality semen both beige and translucent with light that shows high concentration. The spermatozoa concentration of Burmese native chicken was 347x10^6 spermatozoa/ml, with a thick and creamy consistency [11]. Quality semen both beige and translucent with light that showed high concentration.

The average pH of semen obtained in this experiment was 6.70 ± 0.14 which the pH of semen used can be said to be normal. The mass motility of fresh semen was estimated by Taiwan native roosters at the time of the study obtained at 2+. So from the result of this study spermatozoa quality was still feasible to use as frozen semen.

The results of the microscopic examination showed that the average percentage of motility scale in Taiwan native roosters was 59±5.68 %. For motility scale this was still in the normal range of 50-80%. The motility of spermatozoa is also influenced by increasing age of poultry, breed, individual, amount of ejaculation and changes in ambient temperature. The individual motility of the results of this study was lower due to the age factor of roosters. Research on the motility of individual spermatozoa using a microscope and seeing progressive movement or active movement forward was the best result but circular motion or backward movement was a sign of cold shock or a medium that is less isotonic of semen.

Viability is one of the determinants of semen quality and sign of survival of spermatozoa. The percentage of viability of fresh semen of Taiwan native rooster from the results of the study was 83.64 ± 7.85%. The viability of semen in this result was in a very good category, the percentage of viability was above 70% or the percentage of viability should be above the percentage of motility. Living and dead spermatozoa can be distinguished from their reactions to certain colors, spermatozoa cells that are not motile and are considered to suck color and spermatozoa cells that are motile and which live colorless, the coloring agent commonly used is eosin negrosin. Semen viability is in a good category because viability is above 70% and is still considered good if it has a range of values between 50-69% [12].

Percentage of fresh semen abnormality of Taiwan native rooster spermatozoa at the time of observation was 6.77±3.32%, the percentage of spermatozoa abnormalities should not exceed 15% and if spermatozoa abnormalities of more than 25% would reduce fertility.

Observation of pH in this study obtained an average of 6.70 ± 0.14. The degree of acidity or pH needs to be known to ensure that the holding liquid semen has normal character, while the pH range of rooster chicken semen was 6.4-7.8 [2].

B. Motility Scale

One of the factors influencing spermatozoa fertility is individual spermatozoa motility, motility is one of the important indicators for determining semen quality. The

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ISSN (Online): 2455-9024

results of the observation showed that the evaluation of semen quality after dilution was not significantly different (P>0.05) in each treatment (Table II).

TABLE II. Motility scale percentage of Taiwan native rooster spermatozoa after dilution, after equilibration and after thawing with the addition of different maltose doses

Treatment	After dilution	After equilibration	After thawing
T0 (control)	48.83±0.73 [a]	42.17±0.22 [a]	33.92±0.87 [a]
T1 (Maltose 0.6 g)	49.17±1.10 [a]	42.25±0.95 [ab]	34.92±0.36 [ab]
T2 (Maltose 1.2 g)	49.58±1.10 [a]	43.50±0.52 [b]	35.92±0.36 [b]

[a, b] Mean ± SE followed by the same letters within the same column do not differ significantly (Duncan test; P < 0.05). T0= Tris aminomethane+egg yolk 20%, T1=Control+maltose 0.6 g, T2= Control+maltose 1.2 g/ml

While the motility evaluation after equilibration treatment T1 (42.25±0.95%) which was the addition of maltose 0.6 g showed a significant difference (P <0.05) higher than control groups (42.17±0.22%) in maintaining the motility scale of spermatozoa but treatment T1 was not significantly different (P> 0.05) on T2 treatment which was the addition of maltose 1.2 g by 43.50±0.52%. In the T2 treatment, the addition of maltose 1.2 g gave a significant difference (P<0.05) during the spermatozoa motility evaluation at the stage after thawing, the T2 treatment gave the best results when compared to T1 and control, which were T2, T1 and control results of 35.92±0.36%, 34.92±0.36% and 33.92±0.87% respectively.

The high percentage of motility in the treatment maltose compared to controls caused by maltose as a disaccharide sugar can function as an energy source. The motility of spermatozoa is very depends on the supply of energy in the form of metabolic adenosine triphosphate (ATP). Maltose as disaccharide can be used as raw material to avoid silve energy through glycolysis in addition to monosacarides (glucose, fructose and mannose). Before entering into the glycolysis process, disaccharides are hydrolyzed first by the disaccharidase enzyme [13]. The enzyme that breaks down maltose is maltase (α-glucosidase) and produce two molecules of glucose [14]. Spermatozoa will be easier to use the glucose in its metabolism compared to that of other energy sources contained in plasma semen, which is fructose [15].

C. Viability

Viability of spermatozoa depends on the integrity of the spermatozoa membrane. Spermatozoa membrane damage will cause disruption of the intracellular metabolic processes of spermatozoa so that spermatozoa will weaken and can even cause death.

The results showed that the viability result was not significant differences (P> 0.05) in the treatment T0 and T1 at the spermatozoa evaluation stage after dilution because the adjustment time of the spermatozoa to the new diluent was added less than 2 hours and this was due to a decrease in cooling temperatures that have not been optimum (from normal temperature to refrigerator temperature of 4-5˚C) so that the protective effect has not been seen (table III).

TABLE III. Spermatozoa viability percentage of Taiwan native rooster spermatozoa after dilution, after equilibration and after thawing with the addition of different maltose doses

Treatment	After dilution	After equilibration	After thawing
T0 (control)	68.37±1.52 [a]	57.57±2.58 [a]	45.47±1.20 [a]
T1 (Maltose 0.6 g)	69.45±1.90 [a]	62.86±2.22 [b]	47.23±1.62 [b]
T2 (Maltose 1.2 g)	73.19±3.30 [b]	64.71±0.89 [b]	56.47±0.95 [b]

[a, b] Mean ± SE followed by the same letters within the same column do not differ significantly (Duncan test; P < 0.05). T0= Tris aminomethane+egg yolk 20%, T1=Control+maltose 0.6 g, T2= Control+maltose 1.2 g

The results of the statistical analysis showed that viability had a significant different (P <0.05) on the spermatozoa evaluation stage after dilution in the T2 treatment (maltose 1.2 g), meaning Tris aminomethane egg yolk + 1.2 g maltose gave a difference in viability that increased or was higher than the diluent Tris aminomethane egg yolk + 0.6 g maltose and diluted Tris aminomethane egg yolk +0% Maltose.

Based on data from statistical analysis showed that the spermatozoa evaluation after dilution showed Tris aminomethane egg yolk + 1.2 g maltose obtained a viability percentage of 73.19±3.30%, whereas in the treatment of Tris aminomethane yolk + 0% maltose and Tris aminomethane yolk + 0.6 g maltose each had a percentage of viability of T0 (68.37±1.52%) and T1 (69.45±1.90%) respectively indicating that T2 treatment (Tris aminomethane egg yolk + 1.2 g maltose) has an effective influence in maintaining the viability of Taiwan native roosters spermatozoa.

While the percentage of viability of spermatozoa on the extender without maltose (T0) has decreased dramatically during after dilution, after equilibration and after thawing, the decrease can be caused by damage to the plasma membrane and acrosome membrane due to the influence of cold shock during storage at low temperatures.

The results of the statistical analysis showed that viability had a significant different (P <0.05) on the spermatozoa evaluation stage after equilibration in the T1 (maltose 0.6 g) and T2 treatment (maltose 1.2 g), meaning Tris aminomethane egg yolk+maltose gave a difference in viability that increased or was higher than the diluent Tris aminomethane egg yolk without maltose supplementation.

Based on the table the percentage of semen viability showed that the addition of 0.6 g and 1.2 g maltose was able to maintain viability (P <0.05). The results of the statistical analysis showed that viability had a significant different (P <0.05) on the spermatozoa evaluation stage after thawing in the T1 (maltose 0.6 g) and T2 treatment (maltose 1.2 g), meaning Tris aminomethane egg yolk+maltose gave a difference in viability that increased or was higher than the diluent Tris Aminomethane egg yolk without maltose supplementation.

The result showed that T2 treatment, T1 treatment and control after thawing were 56.47±0.95%, 47.23±1.62%, 45.47±1.20% respectively. The result in table spermatozoa viability evaluation showed that T2 treatment, T1 treatment and control after equilibration were 64.71±0.89 %, 62.86±2.22 %, 57.57±2.58 % respectively. The observation process of

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room temperature viability it is suspected that lipid peroxidation has occurred in spermatozoa before the addition of antioxidant and sugar which results in damage to the spermatozoa membrane resulting in leakage and free intracellular enzymes needed for metabolic processes.

Good quality semen, the addition of antioxidants will maintain the viability of spermatozoa, but this was not the case for low quality semen because the peroxidation process that has taken place cannot be stopped by giving antioxidants [16]. Disaccharide acted to protect the spermatozoa in the process freezing of semen especially during the critical period freezing and thawing again [17].

Disaccharide made the structure of the cell plasma membrane spermatozoa become flexible and regulate such a process for removing glycerol so that the spermatozoa cell membrane is not experiencing pressure that is too heavy on both critical stages [18].

D. Abnormality

Spermatozoa abnormalities increase every hour, are influenced by spermatogenesis from poultry and semen treatment after ejaculation such as handling fresh semen, mixing semen with diluents and at the techniques of staining spermatozoa, the length of storage time also affects the number of abnormal spermatozoa.

TABLE IV. Spermatozoa abnormality percentage of Taiwan native rooster spermatozoa after dilution, after equilibration and after thawing with the addition of different maltose doses

Treatment	After dilution	After equilibration	After thawing
T0 (control)	9.90±0.66 [c]	11.04±0.61 [b]	14.32±0.17 [b]
T1 (Maltose 0.6 g)	8.17±0.31 [b]	9.90±0.55 [ab]	14.11±0.51 [ab]
T2 (Maltose 1.2 g)	7.08±0.22 [a]	9.13±0.84 [a]	13.54±0.34 [a]

[a, b,c] Mean ± SE followed by the same letters within the same column do not differ significantly (Duncan test; P< 0.05). T0= Tris aminomethane+egg yolk 20%, T1=Control+maltose 0.6 g, T2= Control+maltose 1.2 g

The average percentage of Taiwan native roosters spermatozoa abnormalities at storage temperatures of 5˚C to -196˚C was significant different on evaluation after equilibration to the stage after thawing (P<0.05). This showed that the spermatozoa abnormality was in line with the storage time and temperature changes. It can be seen that during storage the temperature of 5˚C to -196˚C Taiwan native rosters spermatozoa abnormalities fluctuate at various concentrations of maltose.

While The results of the study of spermatozoa abnormalities at the evaluation stage after dilution showed that the treatment of the addition of maltose 1.2 g gave a lower percentage of abnormalities when compared with the addition of 0.6 g maltose and control treatment at T2 (7.08±0.22%), T1 (8.17±0.31%) and T0 (9.90±0.66%), the abnormality after cooling at -196°C until evaluation after thawing still showed a result less than 20% in all treatments, so it is still feasible to use artificial insemination. The abnormality evaluation result showed that T2 treatment, T1 treatment and control after thawing were 13.54±0.34%, 14.11±0.51%, 14.32±0.17% respectively.

Abnormalities of spermatozoa in rooster approximately 20% of fertility will decrease, abnormalities can be divided into primary, secondary and tertiary. Primary abnormalities associated with the head and acrosome. Secondary abnormalities occur when there is cytoplasmic droplet in the mid piece of the tail. Tertiary abnormalities are abnormalities in the spermatozoa tail [2].

Abnormalities have increased every hour, influenced by spermatogenesis of rooster and treatment of semen after ejaculation such as handling fresh semen, mixing semen with diluents and at the time of making the review, the length of storage time also affects the number of abnormal spermatozoa [19]. Abnormal morphology rates of 8-10% did not have a significant effect on fertility, but if the abnormalities were more than 25% of one ejaculate, fertility decline could not be anticipated [20]. The use of mono and disaccharide combinations such as the use of fructose and maltose together can have a significant effect in protecting semen plasma membrane, abnormality and maintaining spermatozoa quality compared with only use mono or disaccharide separately [21].

IV. Conclusions

This study found that from the quality of frozen semen produced, the study concluded that additions 1.2 g maltose/ml extender was supplemented in Tris Aminomethane-20 % egg yolk extender can produces the highest semen quality of Taiwan native rooster, it was significantly different (P <0.05) compared control and T1 (maltose 0.6 g/ml extender). This situation showed that maltose has been shown to function as external cryoprotectant role in protecting spermatozoa in the semen freezing process. To get the optimal quality of frozen Taiwan Native Rooster semen is recommended add maltose at a dose of 1.2 g/ml of frozen semen extender Tris aminomethane-egg yolk 20%.

Acknowledgment

This research has been supported by reproductive laboratory of National Pingtung University of Science and technology Taiwan. Our special thanks to reproductive laboratory officer and various people for providing the animals, tools, research materials and all of general help in semen cryopreservation.

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