Final Report

Project Title

Distribution of the glucose transporters (GLUTs) for spermatozoa and specific extender for semen cryopreservation in Asian elephant

By

Somchai Sajapitak

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สำนักงานกองทุนสนับสนุนการวิจัย

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Distribution of the glucose transporters (GLUTs) for spermatozoa and specific extender for semen cryopreservation in Asian elephant

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LIST OF CONTENT

เอกสารแนบหมายเลข 2

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Executive Summary:

Report on Experiment to study of Distribution of the glucose transporters (GLUTs) for spermatozoa and specific extender for semen cryopreservation in Asian elephant

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Purpose of Experiment:

To understand the function of the sperm of an elephant, the energy from hexose of elephant's sperm need to be used as energy. It is necessary to study transporter proteins, particularly Glucose transporter proteins (GLUTs) at the surface of the sperm cell. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism, especially GLUT3. To our knowledge, there were a few reports about the GLUT proteins in the plasma membrane of elephant's spermatozoa. Therefore, the study of transporter proteins at the surface of the tissue as a kind of elephant sperm is essential for the development of the cooled elephant semen quality. In particular, the preparation was diluted semen containing the sperm of elephant energy for sperm to extend the life and performance quality of sperm after thawing of cooled semen. Therefore, this study aims to investigate the presence and localization of GLUT3 in freshly ejaculated Asian elephant spermatozoa with different quality of progressive motility to evaluate the effect of different Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen on the expression of GLUT3 on semen quality of spermatozoa motility. The present investigation was undertaken to study the effect of 3% glycerol in Tris-based extender on the variations in the temporal localization of the hexose specific transporters (GLUT3) after cold storage of elephant spermatozoa with respect to changes the GLUT3 in plasma membrane integrity and spermatozoa motility, both of which are indicators of sperm viability and metabolic intactness.

Experiment Materials and Methods:

Experimental I, The fresh semen samples were collected from 10 normal Asian elephants. The semen samples were classified according to the percentages of motile sperm by Group 1 (≤ 20%; n=4), Group 2 (> 20% - 60%; n=3) and Group 3 (> 60%; n=3).

Experimental II, The semen samples were collected from 6 Asian elephant bulls that have greater than 60% individual motility. The samples were suspended in TG extender or T extender and chilled in refrigerator at 4˚C for 48 h. The GLUT3 transporter was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. For the evaluation of sperm integrity and motility, statistical comparisons of the expression of immunolocalisation of GLUT3 samples were performed by STATA program. All results were expressed as mean with standard deviations (SD) and the level of significance was set at $p<0.05$.

Summary of Results:

Experimental I; The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3, strong GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail. Percentages of the expression of immunolocalisation of GLUT3 in the 3 type groups showed significant differences between the Group1 (21.46±10.25) and Group2 (84.37±8.70), Group1 and Group3 (99.40±0.69) and Group2 and Group3.

Experimental II; This study revealed that GLUT3 expression after cold storage were found in all parts of the head, middle piece, principal piece and end piece of the sperm tail in the TG extender group, but while the T extender group were expressed at middle piece, principal piece and end piece of the sperm tail. Percentages of the expression of immunolocalisation of GLUT3 of the head, middle piece, principal piece and end piece of the sperm tail in the 2 type of the extender groups showed significant differences between the TG extender group and T extender group ($p<0.05$). The percentages of the 3 type groups of motility in Asia elephant spermatozoa showed statistically significant difference at $p<0.05$.

Conclusion:

The present study indicated that the expression of GLUT3 was localized at the principal and end piece of the sperm tail and the motility of fresh elephant spermatozoa were affected by

GLUT3 expression and its expression may involve energy production via the glycolytic pathway. In addition, this result confirmed the reduction of the expression of GLUT3 and motility in elephant spermatozoa after cold storage of the T extender group when compared the TG extender group and reveals that the effect of glycerol on sperm function in the TG extender improved by notice spermatozoa motility and the expression of GLUT3 of spermatozoa better than the T extender group. This substance glycerol may be a result of the sperm cell membrane strength after cold storage.

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Distribution of the glucose transporters (GLUTs) for spermatozoa and specific extender for semen cryopreservation in Asian elephant

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Abstract

The objective of this study was to investigate the distribution of the glucose transporters (GLUTs) for spermatozoa and specific extender for semen cryopreservation in Asian elephant. Spermatozoa, as other eukaryotic cells, need hexoses to produce energy for moving along the female genital tract and maintaining membrane homeostasis. Glucose transporter 3 (GLUT3) proteins, as a whole, is mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism. The aims of this study were to determine the localization of GLUT3 in freshly ejaculated Asian elephant sperm with different quality of progressive motility, and to evaluate the effect of different extenders in cooled semen on the expression of GLUT3. For experiment I, the fresh semen samples were collected from 10 Asian elephants bulls, and were classified according to the percentages of motile sperm: Group 1 (≤ 20%; n=4), Group 2 (> 20% - 60%; n=3) and Group 3 (> 60%; n=3). In experiment II, six semen samples were collected from 3 Asian elephant bulls for 2 times, in which motile sperm were >60%. The samples were suspended in Tris extender with 3% glycerol (TG) and without 3% glycerol (T) and kept in a refrigerator at 4°C for 48 h. The GLUT3 was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. The results of experiment I showed that the GLUT3 were localized at the principal and end piece of the sperm tail. The percentages of sperm with GLUT3 expression were highest in Group 3, and lowest in Group 1. In experiment II, the sperm GLUT3 expressions after cold storage in T and TG extenders were different. The sperm of T group showed the localization of GLUT3 similar to those of fresh semen, while the sperm of TG group showed GLUT3 expressions at the head, middle piece, principal piece and end piece. Therefore, the present study demonstrated that GLUT3

expression was related with sperm motility and was affected by 3% glycerol in extender after cold storage.

Keywords: GLUT, Elephant, Sperm

Introduction

In recent years, the development of freezing techniques for semen cryopreservation has become a major resource for the preservation of genetic material in most wildlife and domestic species (Hickman et al., 1984; Morrell et al., 2006). Viability of spermatozoa of frozen-thaw semen depends on several factors, such as semen quality, type of extender/cryoprotectant (Nadir et al., 1993; Saacke et al., 1984) and storage condition (Parks and Graham, 1992; Graham et al., 2004). However, poor sperm motility is a common finding in domesticated Asian elephant bulls, which may restrict their suitability for semen preservation. In addition, the causes of poor sperm motility in Asian elephant remain un-clear, in other species glucose transporters (Gluts) has been proposed to play an important role in compromising sperm quality (Sancho et al., 2007). To understand the function of the sperm of an elephant, the energy from hexose of elephant's sperm need to be used as energy. It is necessary to study transporter proteins, particularly Glucose transporter proteins (GLUTs) at the surface of the sperm cell. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism, especially GLUT3 (Angulo et al., 1998; Glander et al., 1978; Rigau et.al., 2001; Simson 2008; Thorens and Mueckler, 2010; Vera et al., 1993).

There were several possible pathways for the use of energy substrates, for example when compared with glycolytic and aerobic pathways, to maintain both the cells needs and the active physiology of the spermatozoa cells. The flagellar function was related to sperm motility and the ATP consuming process. Flagellar movement was related to the local ability to produce ATP anaerobically by glycolytic pathway of the principal and end piece of the sperm tail (Mukai and Okuno, 2004), while the aerobic (e.g. mitochondrial) producing ATP was used for cell metabolism in the middle piece of the spermatozoa (Miki et al., 2004; Silva and Gadella, 2006; Peña et al., 2009). To our knowledge, there is no report about the GLUT proteins in the plasma membrane of elephant's spermatozoa. Therefore, the study of transporter proteins at the surface of the tissue as a kind of elephant sperm is essential for the development of the cooled and frozen elephant semen quality. In particular, the preparation was diluted semen containing the sperm of elephant energy for sperm to extend the life and performance quality of sperm after thawing of cooled semen.

The objective of this study aims to investigate the localization of GLUT3 in freshly ejaculated Asian elephant spermatozoa with different quality of progressive motility to evaluate the effect of different Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen on the expression of GLUT3 on semen quality of spermatozoa motility. The present investigation was undertaken to study the effect of 3% glycerol in Tris-based extender on the variations in the temporal localization of the hexose specific transporters (GLUT3) after cooling and thawing of elephant spermatozoa with respect to changes the GLUT3 in plasma membrane integrity and spermatozoa motility, both of which are indicators of sperm viability and metabolic intactness.

Materials and Methods

Animals and sample collections

Experimental I, Ten Asian elephants were used in this study. The experiment was carried out with the sperm-rich fraction of the ejaculation being manually collected once a week, using the long gloved-hand method, and analyzed to ensure the quality of the ejaculates. Two ejaculates were evaluated per elephant.

Experimental II, Six semen samples were collected from 3 Asian elephant bulls 2 times which individual motility over than 60%. Immediately after collection, Semen was brought into the laboratory within 3 min and the ejaculated spermatozoa were smeared to the slide glass and were fixed by 4% paraformaldehyde. Blood samples were collected from an ear vein approximately generally in the morning before semen collection. The blood samples were maintained at approximately 4°C by the tube containing ethylenediamine tetraacetic acid (EDTA), and then blood samples were stored at 4°C until analysis.

Blood evaluation

All samples were analyzed with an automated analyzer for animal (XT-2000iV/XT-1800iV, SYSMEX, Kobe, Japan). All blood parameters compost of red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (Hct), platelets (PLT), mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and plasma protein (PP) were assessed in whole of samples. Plasma

protein concentration analyzed with a refractometer (ATAGO portable hand held brix refractometer, Japan).

Sperm evaluation

Experimental I, ejaculates were immediately analyzed for volume, sperm concentration, progressive motility, sperm viability and pH (Kidd et al., 2001). Sperm concentration was determined by counting the sperm, with respect to the dilution and volume, in a counting chamber under a phase contrast microscope. Individual sperm motility was determined by phase contrast microscope examination by placing the slide on a drop of semen diluted sodium citrate (0.9%). A coverslip were then placed over them and observation performed under a phase contrast microscope with maximum magnification. The semen samples were classified according to the type of movement of the progressive individual sperm, as follows:

Group1: less than or equal to 20% progressive individual motility (n=4).

Group2: more than 20% to equal to 60% progressive individual motility (n=3).

Group3: greater than 60% progressive individual motility (n=3).

Experimental II, ejaculates were immediately analyzed for volume, sperm concentration, progressive motility, sperm viability and pH (Kidd et al., 2001). Sperm concentration was determined by counting the sperm, with respect to the dilution and volume, in a counting chamber under a phase contrast microscope. Individual sperm motility was determined by phase contrast microscope examination by placing the slide on a drop of semen diluted sodium citrate (0.9%). A coverslip were then placed over them and observation performed under a phase contrast microscope with maximum magnification. For the semen samples to be used for chilled semen were classified according to the type of individual sperm progressive motility by more than 60% progressive individual motility. The samples were suspended in Tris extender with 3% glycerol and without 3% glycerol and chilled in refrigerator at 4°C for 48 h.

Sperm viability was determined by microscope observation of a smear of semen subjected to special staining fluids, eosin-nigrosin. The method involves placing a drop of approximately 10 microlitres of pure semen on a prepared slide (cleaned and degreased at a temperature of 37°C on the hot plate). The assessment of sperm viability was performed under

a phase contrast microscope at x100 magnification (Olympus CX31, Olympus, Japan). Two hundred spermatozoa were counted per slide (BjÖrndahl et al., 2003).

Immunocytochemistry

Smears were prepared by spreading sperm suspensions of each sampling point on to superfrost polylysine coated slides, which immediately after being shortly air-dried, and fixed in buffered paraformaldehyde (0.5%) for 15 minutes at room temperature. The smears were then rinsed in PBS (pH 7.4) and incubated for 12 h at 4°C with rabbit anti-GLUT3 antibody (Gene Tex, Inc., Texas, USA) at a dilution of 1:50 (v/v) in TBS humid chambers. After extensive washing, sperm cells were incubated with a goat anti-rabbit GLUT3 (Gene Tex, Inc., Texas, USA) at a dilution 1:500 in TBS, Horseradish peroxidase (HRPO)-conjugated secondary antibody for 1 h under dark conditions at 37°C. Slides were then washed extensively with PBS and mounted with Vecta shield mounting medium with propidium iodide. Images were obtained using an Olympus digital camera installed on an Olympus microscope (Olympus BX51 and Digital camera DP50, Olympus, Japan).

Statistical analysis

For the evaluation of sperm integrity and motility, statistical comparisons of the expression of immunolocalisation of GLUT3 samples were performed by STATA program. The correlation between the expression of immunolocalisation of the GLUT3 in spermatozoa and percentage of sperm motility and live sperm of Asia elephants were analyzed by Pearson Correlation. All results were expressed as mean with standard deviations (SD) and the level of significance was set at $p<0.05$.

Results

Experimental I;

The means and standard deviation (mean±SD) of the haematological complete blood count (CBC) parameters and plasma protein concentration values of all elephant samples remained with in normal ranges as seen in the table 1.

The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3. Strong GLUT3 immunoreactivity was observed at the principal piece of

the sperm tail (Fig 1). Percentages of the expression of immunolocalisation of GLUT3 in the 3 type groups showed significant differences between the Group1 (21.46±10.25) and Group2 (84.37±8.70), Group1 and Group3 (99.40±0.69) and Group2 and Group3 (Fig 2). In addition, the results also showed the Mean±SD of semen characteristics of percentages of sperm concentrations, volumes, pH, percentages of GLUT3 spermatozoa and percentages of live sperm were showed in the table 2. The correlation between the expression of immunolocalisation of the GLUT3 in spermatozoa and percentage of sperm motility and live sperm of Asia elephants showed the significant correlation between the expression of immunolocalisation of GLUT3 spermatozoa and percentage of sperm motility and live sperm (R=0.960 and 0.938, p<0.05) (Table 3).

Experimental II;

The means and standard deviation (mean±SD) of the haematological CBC parameters and plasma protein concentration values of all elephant samples remained with in normal ranges as seen in the table 4.

The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3. Strong GLUT3 immunoreactivity was observed at the principal piece of the sperm tail (Fig 3, A) before cooled semen. While this study revealed that GLUT3 expression after 48 h cooled semen were found in all parts of the head, middle piece and principal piece of the sperm tail in the TG extender group (Fig 3, B), but while the T extender group were expressed at middle piece and principal piece of the sperm tail (Fig 3, C). In addition, the results also showed the Mean±SD of the percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, T and TG extender cooled semen after 48 h group (Table 5). Percentages of the expression of immunolocalisation of GLUT3 of the head and middle piece include principal piece of the sperm tail in the 2 type of the extender groups showed significant differences between the TG extender group and T extender group (p <0.05, Fig 4). The percentages of the sperm motility in the 3 type groups showed significant differences between the fresh semen group (67.5±9.87) and the cooled semen T extender group (33.0±18.02), the fresh semen group and the cooled semen TG extender group (47.0±23.28) and the cooled semen T extender group and the cooled semen TG extender group (p<0.05, Fig 5).

Discussion

This study showed that the Asia elephant spermatozoa expressed the family members of the facilitative hexose transporters (GLUTs). These results demonstrated that the GLUT3 proteins were localized on specific cellular compartments at the level of the principal and end piece of the sperm tail, with the exclusion of the middle piece. However, the localization was different from the other mammals such as in boar spermatozoa, the positive was evident in the acrosome and in a band across the middle of the sperm head (Medrano et al., 2006; Sancho et al., 2007; Bucci et al., 2011). A strong signal of stallion sperm cells was evident in the sperm tail, with a particular emphasized neck spot (Bucci et al., 2011). In bull spermatozoa, the positive signal was present only in the middle piece of the spermatozoa (Vera et al., 1993; Glander and Dettmer, 1978; Bucci et al., 2011). Therefore, GLUT3 might be a very effective glucose transporter on their localization in Asia elephant spermatozoa, as reported in domestic animals such as boar, bull, stallion and human spermatozoa (Angulo et al., 1998; Burant et al., 1992; Rigau et al., 2002; Haber et al., 1993; Glander and Dettmer, 1978; Bucci et al., 2011). However, the localization of GLUT3 expression after 48 h cooled semen were found in all parts of the head, middle piece and principal piece of the sperm tail in the TG extender group than T extender group. Percentages of fresh spermatozoa motility and the expression of immunolocalisation of GLUT3 of the head, middle piece include principal piece of the sperm tail in the 2 type of the extender groups showed significant differences between the cooled semen TG extender group and cooled semen T extender group after cooled semen 48 h.

Our results indicated that fresh elephant spermatozoa express the family members of the facilitative hexose transporter, GLUT3. These proteins were localized on specific cellular compartments at the level of the principal and end piece of the sperm tail, and their distribution was characteristic. Additionally, GLUT3 position had a relationship with hexokinase distribution in cytoplasm (Medrano et al., 2006): being that glycolytic enzyme bound line of the tail's fibrous sheath in Asia elephant spermatozoa as in mouse sperm cells (Krifalusi et al., 2006), the GLUT3 distribution was strictly related to enzymes involved in glycolytic chain, especially as related to their local in the sperm tail. This is logical, since the uptake of essential sugars, such as glucose and fructose, to maintain energy metabolism was mediated for both transporters. Thus, GLUT3 was a very effective glucose transporter, as has been already reported in bull (Angulo et al. 1998), boar (Medrano et al. 2006), dog (Rigau et al. 2002) and

human (Haber et al. 1993) spermatozoa. Therefore, this result indicated that the expression of GLUT3 was localized at the principal and end piece of the sperm tail and the motility of fresh elephant spermatozoa was affected by GLUT3 expression, and its expression may involve energy production via the glycolytic pathway.

One of the major findings in this study, in addition to the presence of GLUT3 on the plasma membrane and within the sperm of an elephant was the fact that the distribution of GLUT3 protein changes after the process of cooled semen, especially for the GLUT3 which reduced labeling after 48 h in the cooled semen T extender group. These changes occur simultaneously in the membrane will result in a reduction in the ability to use nutrients that causes a powerful movement that has been compromised and / or membrane integrity. It is very surprising after the cooled semen for 48 h with the expression of GLUT3 proteins were localized on specific cellular surface compartments of the sperm elephants, especially around the head, middle, principal and end piece by the expression of GLUT3 the entire header middle and end piece in the TG group compared with the T group after being chilled to 48 h may be due to the membrane of Glyceral, results in the preservation of the sperm with the effective function of the membrane (Wall and Foote, 1999) in elephant spermatozoa. This distribution of GLUT3 in the TG extender group of elephant spermatozoa differs from that seen in the T extender group, where GLUT3 was only moderately expressed on the principle piece of the tail region but strongly expressed along the tail and head region. Therefore, the expression of GLUT3 in the head, middle, principal and end piece of the sperm tail, which may be indicative of the presence of the inner workings of the sperm cells, which requires more energy by using the substance glucose or fructose (Bucci et al., 2011), resulting in the expression of GLUT3 on these area in the elephant spermatozoa of cooled semen TG extender group.

In conclusion, the study was strengthened by this result, demonstrating the importance of hexokinase I as a regulatory factor for glycolysis (Fernandez-Novell et al., 2004; Medrano et al., 2006) in Asia elephant sperm cells, together with the presence of GLUT3 that was localized on specific cellular compartments at the level of the principal and end piece of the sperm tail. In addition, the expression of GLUT3 spermatozoa numbers in each experimental groups were significantly different. The group with good progressive individual motility (group3) was the expression of GLUT3 spermatozoa numbers more than the others that were consistent with progressive motility of sperm. Therefore, this result indicated that the expression of GLUT3

was localized at the principal and end piece of the sperm tail and the motility of Asian elephant spermatozoa may be affected by GLUT3 expression, and its expression may involve energy production via the glycolytic pathway. In addition, this result confirmed the reduction of the expression of GLUT3 and motility in elephant spermatozoa after cooled semen 48 h of the T extender group when compared the TG extender group and reveals that the effect of glycerol on sperm function in the T extender improved by notice spermatozoa motility and the expression of GLUT3 of spermatozoa better than the T extender group. This substance glycerol may be a result of the sperm cell membrane strength after the cooled semen.

Table 1 Mean values (± SD) and range of the haematological parameters and plasma protein concentration of ten Asia elephants; Gr. 1 = the percentages of motile sperm (≤ 20%), Gr. 2 = the percentages of motile sperm (> 20% - 60%) and Gr. 3 = the percentages of motile sperm (> 60%)

parameters	Ref. range*	Gr.1 (mean±SD)	Gr.2 (mean±SD)	Gr.3 (mean±SD)
HCT (%)	29-49	35.67±4.81	33.87±2.44	32.55±1.48
Wbc ($x10^3$/µl)	11.1-16.1	12.65±2.08	13.79±2.57	14.62±1.60
Rbc ($x10^6$/µl)	2.13-3.85	3.26±0.60	3.05±0.32	2.8±0.35
HGB (g/dl)	9.7-16.4	13.80±2.45	12.83±1.06	12.1±0.99
MCV (fl)	81-158	110.23±5.80	111.25±4.11	117.0±9.19
MCH (pg/cell)	40.0-45.5	42.47±1.57	42.12±1.23	43.4±1.84
MCHC (g/dl)	27.7-40.0	38.17±1.63	37.85±0.46	37.15±1.34
PLT ($x10^3$/µl)	80-400	168.67±27.74	209.75±60.29	221.5±6.36
PP (mg/dl)	6-11	7.6±5.3	7.75±0.3	8.8±0.28

Reference range* from (Silva and Kuruwita, 1993; Lewis et al., 1974)

Table 2 Mean values (±SD) of the semen characteristics and the expression of immunolocalisation of GLUT3 in the 3 type groups of Asia elephants; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%)

Semen parameters	Mean±SD		
	Group 1	Group 2	Group 3
Sperm motility (%)	15±4.08	48.33±7.64	73.33±5.77
Sperm concentration ($x10^6$ sperms/ml)	1120.00±254.66	1153.33±244.56	1271.67±95.04
Volume (ml)	21.25±13.00	16.67±6.11	13.33±4.16
Semen pH	7.25± 0.5	7.17±0.76	7.33±0.58
Live sperm (%)	20.75±3.30	57.00±4.58	82.00±4.00
GLUT3 sperm (%)	21.46±10.25	84.37±8.70	99.40±0.69

Table 3 Correlation between the expression of immunolocalisation of the glucose tranporter3 (GLUT3) in spermatozoa and percentage of sperm motility and live sperm of Asia elephants

Semen parameters	*R*-squared	Correlation	*P* value
Semen motility (%) (n=10)	0.960	0.9798	0.001
Live sperm (%) (n=10)	0.938	0.9685	0.001

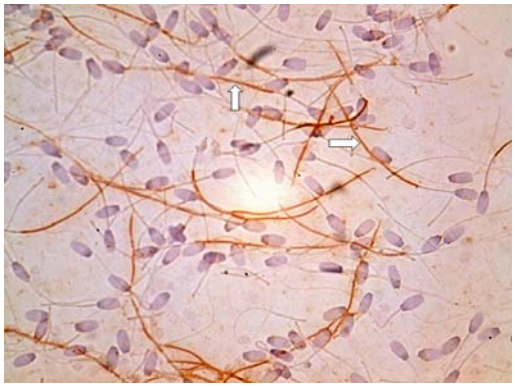
Table 4 Mean values (± SD) and range of the haematological parameters and plasma protein concentration of six Asia elephants

parameters	Ref. range*	Mean values (±SD)
HCT (%)	29-49	32.47±1.32
Wbc ($x10^3$/µl)	11.1-16.1	14.4±1.99
Rbc ($x10^6$/µl)	2.13-3.85	2.83±0.26
HGB (g/dl)	9.7-16.4	12.13±0.73
MCV (fl)	81-158	115.25±6.39
MCH (pg/cell)	40.0-45.5	43.00±1.56
MCHC (g/dl)	27.7-40.0	37.32±0.83
PLT ($x10^3$/µl)	80-400	222.25±55.99
PP (mg/dl)	6-11	8.1±0.66

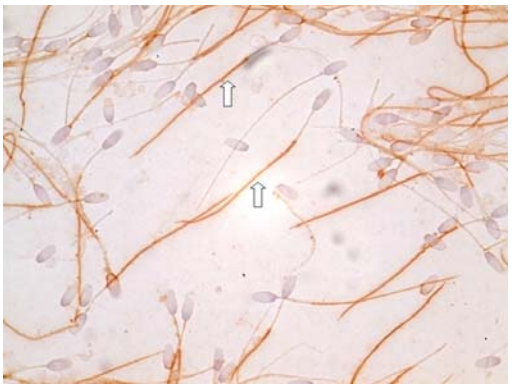
Reference range* from (Silva and Kuruwita, 1993; Lewis et al., 1974)

Table 5 Mean values (±SD) of the percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 h group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa

Position of immunolocalisation of the GLUT3 expression in spermatozoa (%)	Mean±SD		
	Fresh semen gr.	T extender gr.	TG extender gr.
Head+MP+PP+EP	0	0	99.25±7.71
MP+PP+EP	0	9.10±13.52	0
PP+EP	99.35±3.50	83.40±16.54	0
No	0.65±0.82	7.50±5.43	0.75±0.52



A



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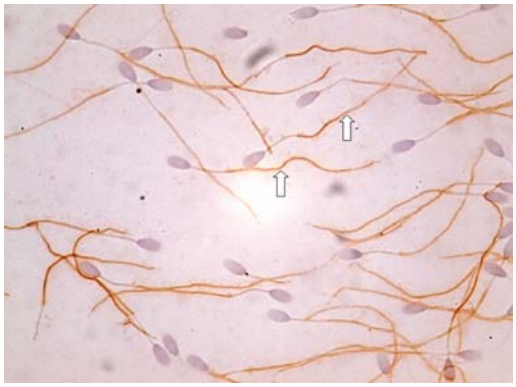


C

Fig 1 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) group1 = the percentages of motile sperm (≤ 20%), (B) group2 = the percentages of motile sperm (> 20% - 60%) and (C) group3 = the percentages of motile sperm (> 60%)

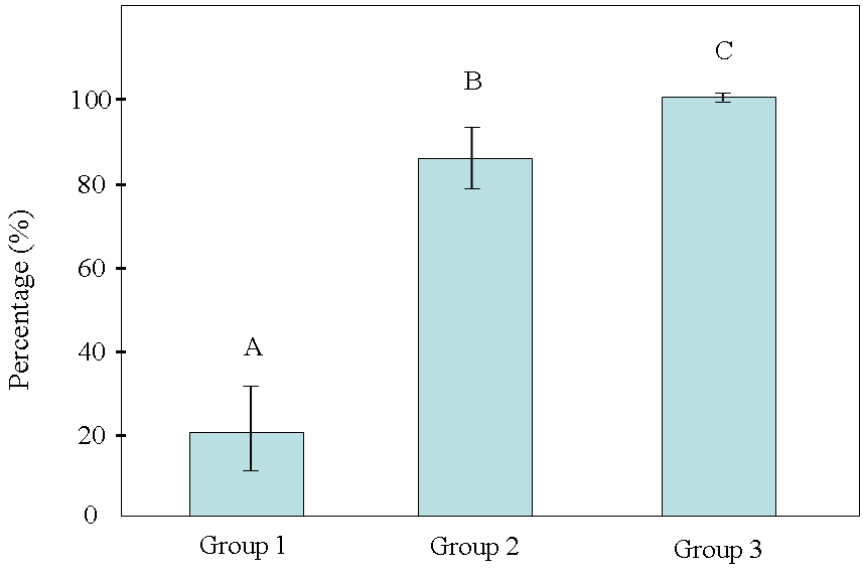
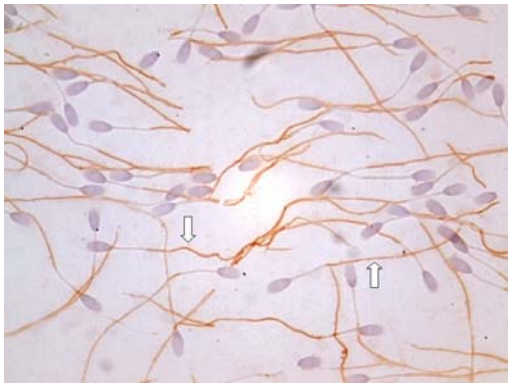
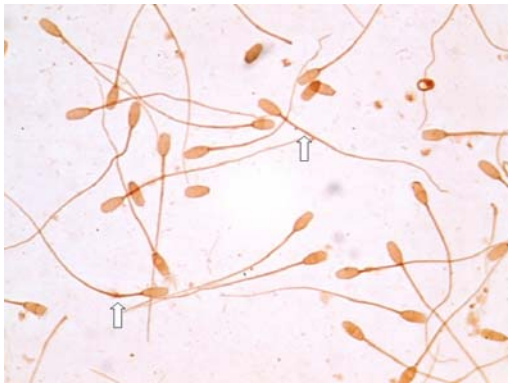


Fig 2 The graft showed percentages of the 3 type groups of immunolocalisation of the GLUT3 in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%)



A



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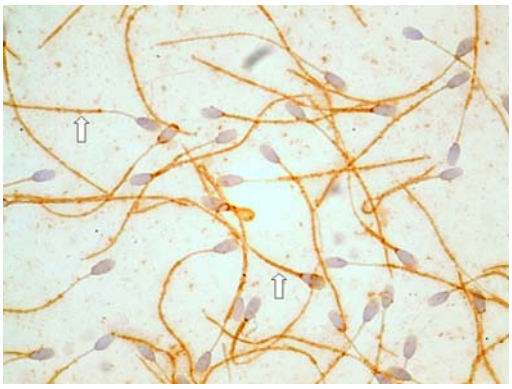

C

Fig 3 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) the GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail in the fresh semen, (B) the expression of immunolocalisation of GLUT3 of head and the middle piece include principal piece and end piece of the sperm tail in the Tris extender with 3% glycerol (TG) group and (C) the expression of immunolocalisation of GLUT3 of the middle piece, principal piece and end piece or the principal piece and end piece of the sperm tail in the Tris extender without 3% glycerol (T) group

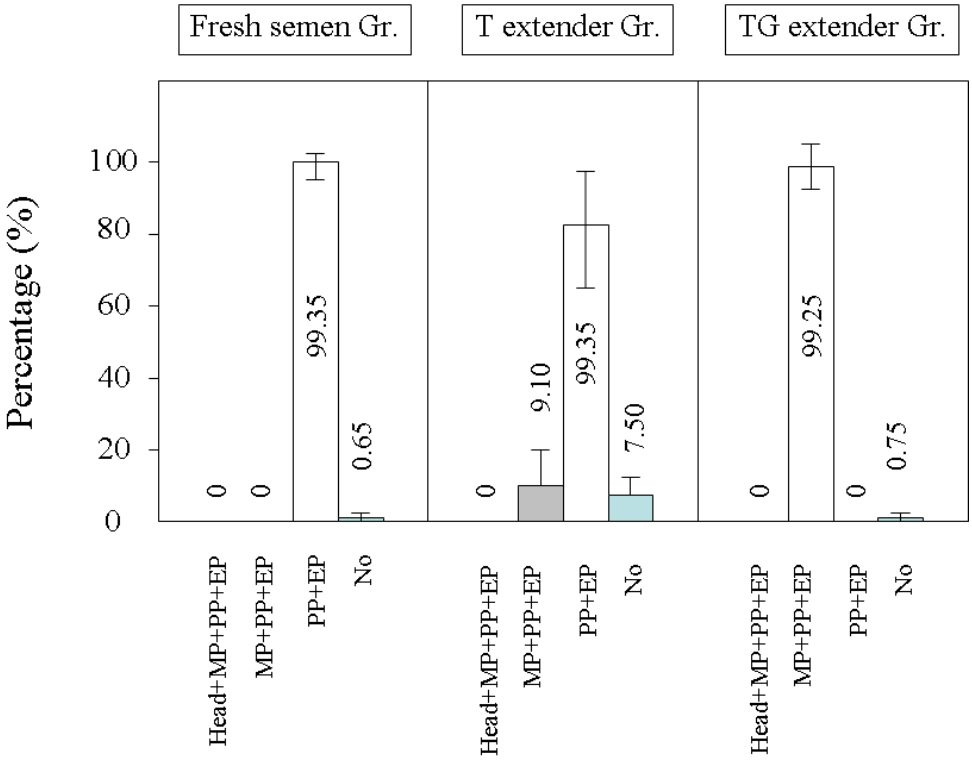

Fig 4 The graft showed percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 h group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa

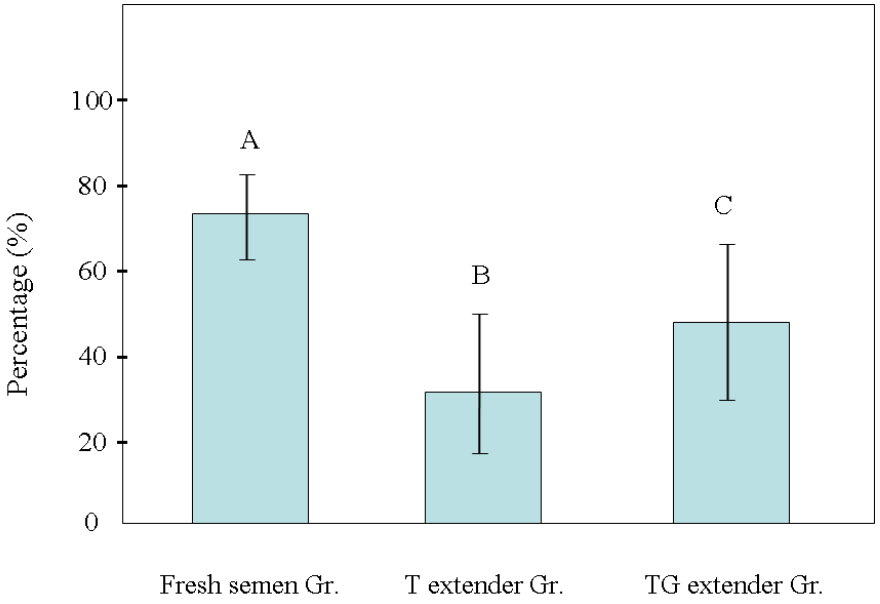

Fig 5 The graft showed percentages of the 3 type groups of motility in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; TG = Tris extender with 3% glycerol, T = Tris extender without 3% glycerol

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เอกสารแนบหมายเลข 3

Output (Acknowledge the Thailand Research Fund)

- International Journal Publication

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. Distribution of the glucose transporter3 (GLUT3) in Asian elephant spermatozoa

Preparation for submit to Journal of Reproduction and Development.

(Appendix A)

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. The effect of different extenders of Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen on the expression of GLUT3 of Asian elephant spermatozoa

Preparation for submit to Journal of Reproduction and Development or Kasetsart Journal.

(Appendix B)

- Application

- Others e.g. national journal publication, proceeding, international conference, book chapter, patent

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. Distribution of the glucose transporter-3 (GLUT3) for spermatozoa and the effect of different extenders of TG and T in cooled semen on the expression of GLUT3 in Asian elephant. Proceeding of The 13th การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. Conference, 16-18 October, 2013. Phetchaburi, Thailand.

(Appendix C)

Appendix A

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. Distribution of the glucose transporter3 (GLUT3) in Asian elephant spermatozoa

Preparation for submit to Journal of Reproduction and Development.

Appendix B

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. The effect of different extenders of Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen on the expression of GLUT3 of Asian elephant spermatozoa

Preparation for submit to Journal of Reproduction and Development or Kasetsart Journal.

Appendix C

Sajapitak, S., Kornkaewrat, K., Suthunmapinanta, P., Boodde, O., Mahasawangkul, S., Pinyopummin, A. Distribution of the glucose transporter-3 (GLUT3) for spermatozoa and the effect of different extenders of TG and T in cooled semen on the expression of GLUT3 in Asian elephant. Proceeding of The 13th การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. Conference, 16-18 October, 2013. Phetchaburi, Thailand.

Title: Distribution of the glucose transporter3 (GLUT3) in Asian elephant spermatozoa

Running head: GLUT3 in Asian elephant spermatozoa

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Abstract

Spermatozoa, as other eukaryotic cells, need hexoses to produce energy for moving along the female genital tract and maintaining membrane homeostasis. Glucose transporter 3 (GLUT3) proteins, as a whole, is mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism. The aims of this study were to determine the presence of GLUT3 in freshly ejaculated Asian elephant spermatozoa with different quality of progressive motility and to describe its localization. The fresh semen samples were collected from 10 Asian elephants. The semen samples were classified according to the percentages of motile sperm by Group 1 (≤ 20%; n=4), Group 2 (> 20% - 60%; n=3) and Group 3 (> 60%; n=3). The GLUT3 transporter was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. The results showed the presence of GLUT3, and were localized on specific cellular compartments at the principal and end piece of the sperm tail. GLUT3 expressions in each group were significantly different. The group with good sperm motility (Group 3) had more expression of GLUT3 than the others. Therefore, the motility of Asian elephant spermatozoa and percentage of live sperm may be affected by GLUT3 expression, and its expression may involve energy production via the glycolytic pathway.

Keywords: GLUT, Elephant, Sperm

Introduction

In recent years, the development of freezing techniques for semen cryopreservation has become a major resource for the preservation of genetic material in most domestic species [9, 15]. Viability of spermatozoa of frozen-thaw semen depends on several factors, such as

semen quality, type of extender/cryoprotectant [17, 22] and storage condition [7, 18]. However, poor sperm motility is a common finding in domesticated Asian elephant bulls, which may restrict their suitability for semen preservation. In addition, the causes of poor sperm motility in Asian elephant remain un-clear, in other species glucose transporters (Gluts) has been proposed to play an important role in compromising sperm quality [23]. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism, especially GLUT3 [1, 6, 20, 26, 28]. To our knowledge, there is no report about the GLUT proteins in the plasma membrane of elephant's spermatozoa. Therefore, the aims of this study were to determine the presence of GLUT3, and any variations in the temporal localization of its expression in Asian elephant spermatozoa with different motile quality and percentage of live sperm. This study may provide some information on the cause of Asian elephant poor semen quality.

Materials and Methods

Animals and sample collections

Ten Asian elephants were used in this study. The experiment was carried out with the sperm-rich fraction of the ejaculation being manually collected once a week, using the long gloved-hand method, and analyzed to ensure the quality of the ejaculates. Two ejaculates were evaluated per elephant. Immediately after collection, Semen was brought into the laboratory within 3 min and the ejaculated spermatozoa were smeared to the slide glass and were fixed by 4% paraformaldehyde. Blood samples were collected from an ear vein approximately generally in the morning before semen collection. The blood samples were maintained at approximately 4°C by the tube containing ethylenediamine tetraacetic acid (EDTA), and then blood samples were stored at 4°C until analysis.

Blood evaluation

All samples were analyzed with an automated analyzer for animal (XT-2000iV/XT-1800iV, SYSMEX, Kobe, Japan). All blood parameters compost of red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (Hct), platelets (PLT), mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and plasma protein (PP) were assessed in whole of samples. Plasma protein concentration analyzed with a refractometer (ATAGO portable hand held brix refractometer, Japan).

Sperm evaluation

Ejaculates were immediately analyzed for volume, sperm concentration, progressive motility, sperm viability and pH [10]. Sperm concentration was determined by counting the sperm, with respect to the dilution and volume, in a counting chamber under a phase contrast microscope. Individual sperm motility was determined by phase contrast microscope examination by placing the slide on a drop of semen diluted sodium citrate (0.9%). A coverslip were then placed over them and observation performed under a phase contrast microscope with maximum magnification. The semen samples were classified according to the type of movement of the progressive individual sperm, as follows:

Group1: less than or equal to 20% progressive individual motility (n=4).

Group2: more than 20% to equal to 60% progressive individual motility (n=3).

Group3: greater than 60% progressive individual motility (n=3).

Sperm viability was determined by microscope observation of a smear of semen subjected to special staining fluids, eosin-nigrosin. The method involves placing a drop of approximately 10 microlitres of pure semen on a prepared slide (cleaned and degreased at a

temperature of 37°C on the hot plate). The assessment of sperm viability was performed under a phase contrast microscope at x100 magnification (Olympus CX31, Olympus, Japan). Two hundred spermatozoa were counted per slide [2].

Immunocytochemistry

Smears were prepared by spreading sperm suspensions of each sampling point on to superfrost polylysine coated slides, which immediately after being shortly air-dried, and fixed in buffered paraformaldehyde (0.5%) for 15 minutes at room temperature. The smears were then rinsed in PBS (pH 7.4) and incubated for 12 hours at 4˚C with rabbit anti-GLUT3 antibody (Gene Tex, Inc., Texas, USA) at a dilution of 1:50 (v/v) in TBS humid chambers. After extensive washing, sperm cells were incubated with a goat anti-rabbit GLUT3 (Gene Tex, Inc., Texas, USA) at a dilution 1:500 in TBS, Horseradish peroxidase (HRPO)-conjugated secondary antibody for 1 hour under dark conditions at 37˚C. Slides were then washed extensively with PBS and mounted with Vecta shield mounting medium with propidium iodide. Images were obtained using an Olympus digital camera installed on an Olympus microscope (Olympus BX51 and Digital camera DP50, Olympus, Japan).

Statistical analysis

For the evaluation of sperm integrity and motility, statistical comparisons of the expression of immunolocalisation of GLUT3 samples were performed by STATA program. The correlation between the expression of immunolocalisation of the GLUT3 in spermatozoa and percentage of sperm motility and live sperm of Asia elephants were analyzed by Pearson Correlation. All results were expressed as mean with standard deviations (SD) and the level of significance was set at $p<0.05$.

Results

The means and standard deviation (mean±SD) of the haematological complete blood count (CBC) parameters and plasma protein concentration values of all elephant samples remained with in normal ranges as seen in the table 1.

The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3. Strong GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail (Fig 1). Percentages of the expression of immunolocalisation of GLUT3 in the 3 type groups showed significant differences between the Group1 (21.46±10.25) and Group2 (84.37±8.70), Group1 and Group3 (99.40±0.69) and Group2 and Group3 (Fig 2). In addition, the results also showed the Mean±SD of semen characteristics of percentages of sperm concentrations, volumes, pH, percentages of GLUT3 spermatozoa and percentages of live sperm were showed in the table 2. The correlation between the expression of immunolocalisation of the GLUT3 in spermatozoa and percentage of sperm motility and live sperm of Asia elephants showed the significant correlation between the expression of immunolocalisation of GLUT3 spermatozoa and percentage of sperm motility and live sperm (R=0.960 and 0.938, p<0.05) (Table 3).

Discussion

All elephants used in this study were apparently healthy as indicated by the hematological parameters. This study showed that the Asia elephant spermatozoa expressed the family members of the facilitative hexose transporters (GLUTs). These results demonstrated that the GLUT3 proteins were localized on specific cellular compartments at the level of the principal and end piece of the sperm tail, with the exclusion of the middle piece. However, the localization was different from the other mammals such as in boar spermatozoa, the positive was evident in the acrosome and in a band across the middle of the

sperm head [3, 13, 23]. A strong signal of stallion sperm cells was evident in the sperm tail, with a particular emphasized neck spot [3]. In bull spermatozoa, the positive signal was present only in the middle piece of the spermatozoa [3, 6, 28]. Therefore, GLUT3 might be a very effective glucose transporter on their localization in Asia elephant spermatozoa, as reported in domestic animals such as boar, bull, stallion and human spermatozoa [1, 3, 4, 6, 8, 21].

There were several possible pathways for the use of energy substrates, for example when compared with glycolytic and aerobic pathways, to maintain both the cells needs and the active physiology of the spermatozoa cells. The flagellar function was related to sperm motility and the ATP consuming process. Flagellar movement was related to the local ability to produce ATP anaerobically by glycolytic pathway of the principal and end piece of the sperm tail [16], while the aerobic (e.g. mitochondrial) producing ATP was used for cell metabolism in the middle piece of the spermatozoa (14, 19, 25]. Additionally, GLUT3 position had a relationship with hexokinase distribution in cytoplasm [13]: being that glycolytic enzyme bound line of the tail's fibrous sheath in Asia elephant spermatozoa as in mouse sperm cells [11], the GLUT3 distribution was strictly related to enzymes involved in glycolytic chain, especially as related to their local in the sperm tail. This fragmentation of GLUT3 was characteristic. This was logical since the absorption of sugars, such as glucose and fructose, which are important for maintaining energy metabolism mediated for this transporter [27]. This study was strengthened by this result, demonstrating the importance of hexokinase I as a regulatory factor for glycolysis [5, 13] in Asia elephant sperm cells, together with the presence of GLUT3 that was localized on specific cellular compartments at the level of the principal and end piece of the sperm tail. In addition, the expression of GLUT3 spermatozoa numbers in each experimental groups were significantly different. The group with good progressive individual motility (group3) was the expression of GLUT3

spermatozoa numbers more than the others that were consistent with progressive motility of sperm and percentage of live sperm.

In conclusion, the correlation between the expression of immunolocalisation of the GLUT3 in spermatozoa and percentage of sperm motility and live sperm of Asia elephant semen could help indicate the effect of the expression of GLUT3 on Asia elephant semen quality. In addition, this result showed that the expression of GLUT3 was localized at the principal and end piece of the sperm tail. The motility of Asian elephant spermatozoa may be affected by GLUT3 expression, and its expression may involve energy production via the glycolytic pathway.

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Table 1 Mean values (± SD) and range of the haematological parameters and plasma protein concentration of ten Asia elephants; Gr. 1 = the percentages of motile sperm (≤ 20%), Gr. 2 = the percentages of motile sperm (> 20% - 60%) and Gr. 3 = the percentages of motile sperm (> 60%).

Table 2 Mean values (±SD) of the semen characteristics and the expression of immunolocalisation of GLUT3 in the 3 type groups of Asia elephants; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%).

Table 3 Correlation between the expression of immunolocalisation of the glucose tranporter3 (GLUT3) in spermatozoa and percentage of sperm motility and live sperm of Asia elephants.

Fig 1 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) group1 = the percentages of motile sperm (≤ 20%), (B) group2 = the percentages of motile sperm (> 20% - 60%) and (C) group3 = the percentages of motile sperm (> 60%).

Fig 2 The graft showed percentages of the 3 type groups of immunolocalisation of the GLUT3 in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%).

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parameters	Ref. range*	Gr.1 (mean±SD)	Gr.2 (mean±SD)	Gr.3 (mean±SD)
HCT (%)	29-49	35.67±4.81	33.87±2.44	32.55±1.48
Wbc ($x10^3$/µl)	11.1-16.1	12.65±2.08	13.79±2.57	14.62±1.60
Rbc ($x10^6$/µl)	2.13-3.85	3.26±0.60	3.05±0.32	2.8±0.35
HGB (g/dl)	9.7-16.4	13.80±2.45	12.83±1.06	12.1±0.99
MCV (fl)	81-158	110.23±5.80	111.25±4.11	117.0±9.19
MCH (pg/cell)	40.0-45.5	42.47±1.57	42.12±1.23	43.4±1.84
MCHC (g/dl)	27.7-40.0	38.17±1.63	37.85±0.46	37.15±1.34
PLT ($x10^3$/µl)	80-400	168.67±27.74	209.75±60.29	221.5±6.36
PP (mg/dl)	6-11	7.6±5.3	7.75±0.3	8.8±0.28

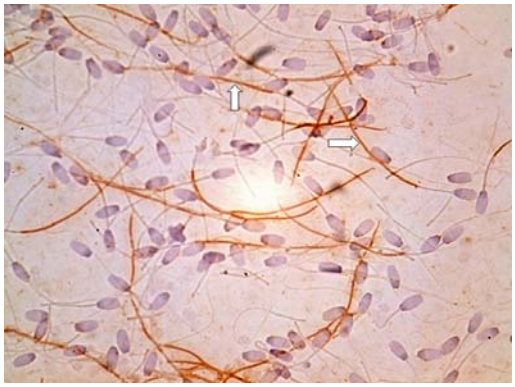
Reference range* from (Silva and Kuruwita, 1993; Lewis et al., 1974)

Table 2 Mean values (±SD) of the semen characteristics and the expression of immunolocalisation of GLUT3 in the 3 type groups of Asia elephants; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%)

Semen parameters	Mean±SD		
	Group 1	Group 2	Group 3
Sperm motility (%)	15±4.08	48.33±7.64	73.33±5.77
Sperm concentration ($x10^6$ sperms/ml)	1120.00±254.66	1153.33±244.56	1271.67±95.04
Volume (ml)	21.25±13.00	16.67±6.11	13.33±4.16
Semen pH	7.25± 0.5	7.17±0.76	7.33±0.58
Live sperm (%)	20.75±3.30	57.00±4.58	82.00±4.00
GLUT3 sperm (%)	21.46±10.25	84.37±8.70	99.40±0.69

Table 3 Correlation between the expression of immunolocalisation of the glucose tranporter3 (GLUT3) in spermatozoa and percentage of sperm motility and live sperm of Asia elephants

Semen parameters	*R*-squared	Correlation	*P* value
Semen motility (%) (n=10)	0.960	0.9798	0.001
Live sperm (%) (n=10)	0.938	0.9685	0.001



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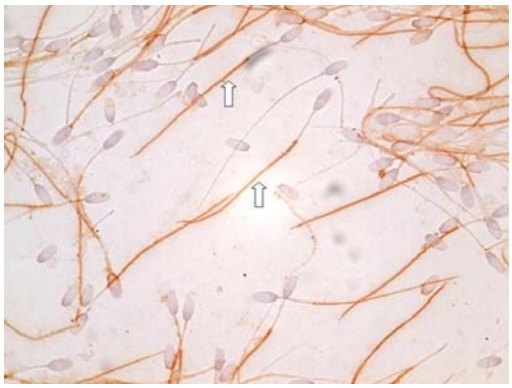

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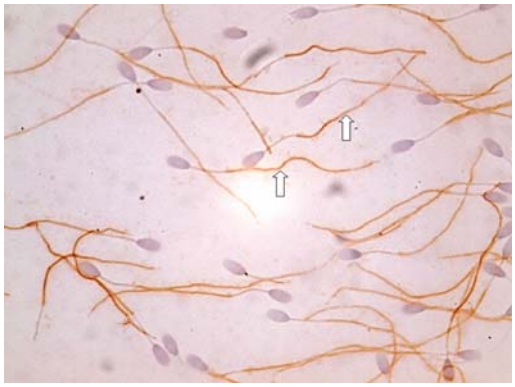

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Fig 1 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) group1 = the percentages of motile sperm (≤ 20%), (B) Group 2 = the percentages of motile sperm (> 20% - 60%) and (C) Group 3 = the percentages of motile sperm (> 60%)

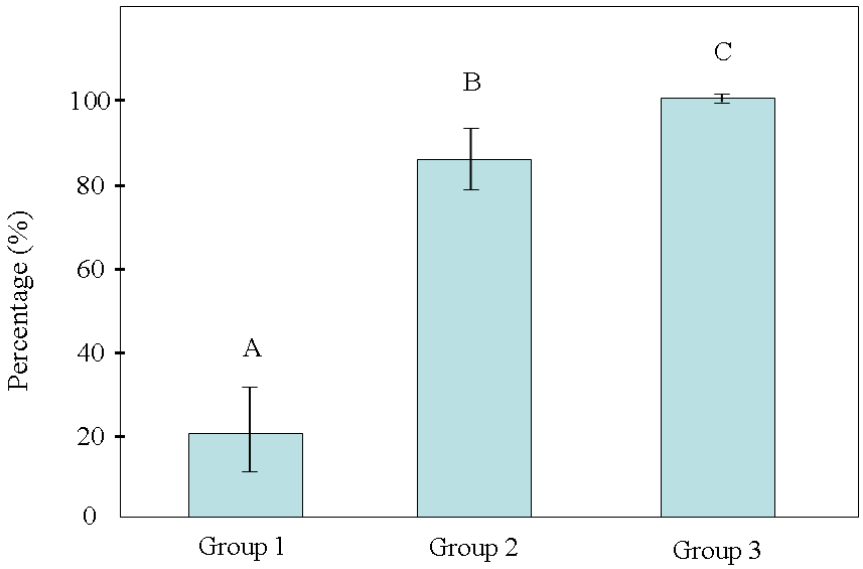

Fig 2 The graft showed percentages of the 3 type groups of immunolocalisation of the GLUT3 in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; Group 1 = the percentages of motile sperm (≤ 20%), Group 2 = the percentages of motile sperm (> 20% - 60%) and Group 3 = the percentages of motile sperm (> 60%)

Title: The effect of different extenders of Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen on the expression of GLUT3 of Asian elephant spermatozoa

Running head: GLUT3 in Asian elephant spermatozoa

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Abstract

The aims of this study were to evaluated the effect of cooled semen on semen quality of spermatozoa motility, expression of glucose transporter 3 (GLUT3) and to describe its localization in spermatozoa from Asian elephant. The semen samples were collected twice weekly from 6 Asian elephants bulls. The samples were suspended in Tris extender with 3% glycerol (TG) and without 3% glycerol (T) and chilled in refrigerator at 4°C for 48 hours. The GLUT3 transporter was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. The results showed the presence of GLUT3, and were localized on specific cellular compartments at the principal and end piece of the sperm tail before the refrigerator. GLUT3 expressions in T and TG extender groups were significantly different. The TG extender group showed the presence of GLUT3 that were localized on specific cellular compartments at the head, end piece and the middle piece of the sperm tail after chilled in refrigerator at 4°C for 48 hours when compared with the T extender group. Therefore, the expression and localization of GLUT3 in Asian elephant spermatozoa may be affected by T with 3% glyceral in the extender, and its localization may involve energy production via the oxidative phosphorylation and glycolytic pathway.

Keywords: GLUT, Elephant, Sperm

Introduction

To understand the function of the sperm of an elephant, the energy from hexose of elephant’s sperm need to be used as energy. It is necessary to study transporter proteins, particularly Glucose transporter proteins (GLUTs) at the surface of the sperm cell. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across

mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism, especially GLUT3 [1, 4, 6, 15, 20, 21, 22]. To our knowledge, there were a few reports about the GLUT proteins in the plasma membrane of elephant's spermatozoa. Therefore, the study of transporter proteins at the surface of the tissue as a kind of elephant sperm is essential for the development of the cooled elephant semen quality. In particular, the preparation was diluted semen containing the sperm of elephant energy for sperm to extend the life and performance quality of sperm after thawing of cooled semen. Therefore, this study aims to investigate the transporter proteins on the surface of the tissue that is responsible for sperm elephant energy into the sperm cells. The present investigation was undertaken to study the effect of 3% glycerol in Tris-based extender on the variations in the temporal localization of the hexose specific transporters (GLUT3) after cooling and thawing of elephant spermatozoa with respect to changes the GLUT3 in plasma membrane integrity and spermatozoa motility, both of which are indicators of sperm viability and metabolic intactness.

Materials and Methods

Animals and sample collections

Six semen samples were collected from 3 Asian elephant bulls 2 times which individual motility over than 60%. The experiment was carried out with the sperm-rich fraction of the ejaculation being manually collected once a week, using the long gloved-hand method, and analyzed to ensure the quality of the ejaculates. Two ejaculates were evaluated per elephant. Immediately after collection, Semen was brought into the laboratory within 3 min and the ejaculated spermatozoa were smeared to the slide glass and were fixed by 4% paraformaldehyde. Blood samples were collected from an ear vein approximately generally in the morning before semen collection. The blood samples were maintained at approximately

4°C by the tube containing ethylenediamine tetraacetic acid (EDTA), and then blood samples were stored at 4°C until analysis.

Blood evaluation

All samples were analyzed with an automated analyzer for animal (XT-2000iV/XT-1800iV, SYSMEX, Kobe, Japan). All blood parameters compost of red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (Hct), platelets (PLT), mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and plasma protein (PP) were assessed in whole of samples. Plasma protein concentration analyzed with a refractometer (ATAGO portable hand held brix refractometer, Japan).

Sperm evaluation

Ejaculates were immediately analyzed for volume, sperm concentration, progressive motility, sperm viability and pH [7]. Sperm concentration was determined by counting the sperm, with respect to the dilution and volume, in a counting chamber under a phase contrast microscope. Individual sperm motility was determined by phase contrast microscope examination by placing the slide on a drop of semen diluted sodium citrate (0.9%). A coverslip were then placed over them and observation performed under a phase contrast microscope with maximum magnification. For the semen samples to be used for chilled semen were classified according to the type of individual sperm progressive motility by more than 60% progressive individual motility. The samples were suspended in a Tris extender with and without of 3% glycerol and chilled in refrigerator at 4°C for 48 hours.

Sperm viability was determined by microscope observation of a smear of semen subjected to special staining fluids, eosin-nigrosin. The method involves placing a drop of

approximately 10 microlitres of pure semen on a prepared slide (cleaned and degreased at a temperature of 37°C on the hot plate). The assessment of sperm viability was performed under a phase contrast microscope at x100 magnification (Olympus CX31, Olympus, Japan). Two hundred spermatozoa were counted per slide [2].

Immunocytochemistry

Smears were prepared by spreading sperm suspensions of each sampling point on to superfrost polylysine coated slides, which immediately after being shortly air-dried, and fixed in buffered paraformaldehyde (0.5%) for 15 minutes at room temperature. The smears were then rinsed in PBS (pH 7.4) and incubated for 12 hours at 4°C with rabbit anti-GLUT3 antibody (Gene Tex, Inc., Texas, USA) at a dilution of 1:50 (v/v) in TBS humid chambers. After extensive washing, sperm cells were incubated with a goat anti-rabbit GLUT3 (Gene Tex, Inc., Texas, USA) at a dilution 1:500 in TBS, Horseradish peroxidase (HRPO)-conjugated secondary antibody for 1 hour under dark conditions at 37°C. Slides were then washed extensively with PBS and mounted with Vecta shield mounting medium with propidium iodide. Images were obtained using an Olympus digital camera installed on an Olympus microscope (Olympus BX51 and Digital camera DP50, Olympus, Japan).

Statistical analysis

For the evaluation of sperm integrity and motility, statistical comparisons of the expression of immunolocalisation of GLUT3 samples were performed by STATA program. All results were expressed as mean with standard deviations (SD) and the level of significance was set at $p<0.05$.

Results

The means and standard deviation (mean±SD) of the haematological complete blood count (CBC) parameters and plasma protein concentration values of all elephant samples remained with in normal ranges as seen in the table 1.

The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3. Strong GLUT3 immunoreactivity was observed at the principal piece of the sperm tail (Fig 1, A) before cooled semen. While this study revealed that GLUT3 expression after 48 hours cooled semen were found in all parts of the head, middle piece and principal piece of the sperm tail in the TG extender group (Fig 1, B), but while the T extender group were expressed at middle piece and principal piece of the sperm tail (Fig 1, C). In addition, the results also showed the Mean±SD of the percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, T and TG extender cooled semen after 48 hours group (Table 2). Percentages of the expression of immunolocalisation of GLUT3 of the head and middle piece include principal piece of the sperm tail in the 2 type of the extender groups showed significant differences between the TG extender group and T extender group (p <0.05, Fig 2). The percentages of the sperm motility in the 3 type groups showed significant differences between the fresh semen group (67.5±9.87) and the cooled semen T extender group (33.0±18.02), the fresh semen group and the cooled semen TG extender group (47.0±23.28) and the cooled semen T extender group and the cooled semen TG extender group (p<0.05, Fig 3).

Discussion

All elephants used in this study were apparently healthy as indicated by the hematological parameters. This study showed that the Asia elephant spermatozoa expressed the family members of the facilitative hexose transporters (GLUTs). These results demonstrated that the GLUT3 proteins were localized on specific cellular compartments at

the level of the principal and end piece of the sperm tail, with the exclusion of the middle piece before cooled semen. However, the localization of GLUT3 expression after 48 hours cooled semen were found in all parts of the head, middle piece, principal piece and end piece of the sperm tail in the TG extender group than T extender group. Percentages of fresh spermatozoa motility and the expression of immunolocalisation of GLUT3 of the head, middle piece include principal piece and end piece of the sperm tail in the 2 type of the extender groups showed significant differences between the cooled semen TG extender group and cooled semen T extender group after cooled semen 48 hours.

Our results indicated that fresh elephant spermatozoa express the family members of the facilitative hexose transporter, GLUT3. These proteins were localized on specific cellular compartments at the level of the principal and end piece of the sperm tail, and their distribution was characteristic. This is logical, since the uptake of essential sugars, such as glucose and fructose, to maintain energy metabolism was mediated for both transporters. Thus, GLUT3 was a very effective glucose transporter, as has been already reported in bull [1], boar [10], dog [16] and human [6] spermatozoa. Therefore, this result indicated that the expression of GLUT3 was localized at the principal and end piece of the sperm tail and the motility of fresh elephant spermatozoa was affected by GLUT3 expression (un-public observation), and its expression may involve energy production via the glycolytic pathway.

One of the major findings in this study, in addition to the presence of GLUT3 on the plasma membrane and within the sperm of an elephant was the fact that the distribution of GLUT3 protein changes after the process of cooled semen, especially for the GLUT3 which reduced labeling after 48 hours in the cooled semen T extender group. These changes occur simultaneously in the membrane will result in a reduction in the ability to use nutrients that causes a powerful movement that has been compromised and / or membrane integrity. It is very surprising after the cooled semen for 48 hours with the expression of GLUT3 proteins

were localized on specific cellular surface compartments of the sperm elephants, especially around the head, middle, principal and end piece by the expression of GLUT3 the entire header middle principal and end piece in the TG group compared with the T group after being chilled to 48 hours may be due to the membrane of Glyceral, results in the preservation of the sperm with the effective function of the membrane [5, 13, 23] in elephant spermatozoa. This distribution of GLUT3 in the TG extender group of elephant spermatozoa differs from that seen in the T extender group, where GLUT3 was only moderately expressed on the principle piece and end piece of the tail region but strongly expressed along the tail and head region. Therefore, the expression of GLUT3 in the head, middle and tail pieces of the spermatozoa, which may be indicative of the presence of the inner workings of the sperm cells [1, 3, 8, 11, 12, 14, 17, 19], which requires more energy by using the substance glucose or fructose [3], resulting in the expression of GLUT3 on these area in the elephant spermatozoa of cooled semen TG extender group.

In conclusion, the present study indicated that the expression of GLUT3 was localized at the principal and end piece of the sperm tail and the motility of fresh elephant spermatozoa were affected by GLUT3 expression and its expression may involve energy production via the glycolytic pathway. In addition, this result confirmed the reduction of the expression of GLUT3 and motility in elephant spermatozoa after cooled semen 48 hours of the T extender group when compared the TG extender group and reveals that the effect of glycerol on sperm function in the T extender improved by notice spermatozoa motility and the expression of GLUT3 of spermatozoa better than the T extender group. This substance glycerol may be a result of the sperm cell membrane strength after the cooled semen.

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Table 1 Mean values (± SD) and range of the haematological complete blood count (CBC) parameters and plasma protein concentration of six Asia elephants.

Table 2 Mean values (±SD) of the percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 hours group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa.

Fig 1 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) the GLUT3 immunoreactivity was observed at the principal piece of the sperm tail, (B) the expression of immunolocalisation of GLUT3 of head and the middle piece include principal piece and end piece of the sperm tail in the TG extender group and (C) the expression of immunolocalisation of GLUT3 of the middle piece, principal piece and end piece or the principal piece and end piece of the sperm tail in the T extender group.

Fig 2 The graft showed percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 hours group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa.

Fig 3 The graft showed percentages of the 3 type groups of motility in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; T = Tris extender without 3% glycerol, TG = Tris extender with 3% glycerol.

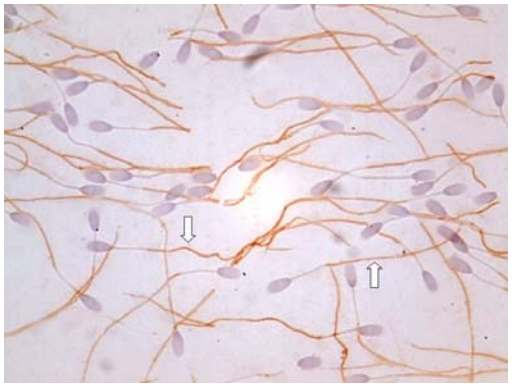
Table 1 Mean values (± SD) and range of the haematological parameters and plasma protein concentration of six Asia elephants

parameters	Ref. range*	Mean values (±SD)
HCT (%)	29-49	32.47±1.32
Wbc ($x10^3$/µl)	11.1-16.1	14.4±1.99
Rbc ($x10^6$/µl)	2.13-3.85	2.83±0.26
HGB (g/dl)	9.7-16.4	12.13±0.73
MCV (fl)	81-158	115.25±6.39
MCH (pg/cell)	40.0-45.5	43.00±1.56
MCHC (g/dl)	27.7-40.0	37.32±0.83
PLT ($x10^3$/µl)	80-400	222.25±55.99
PP (mg/dl)	6-11	8.1±0.66

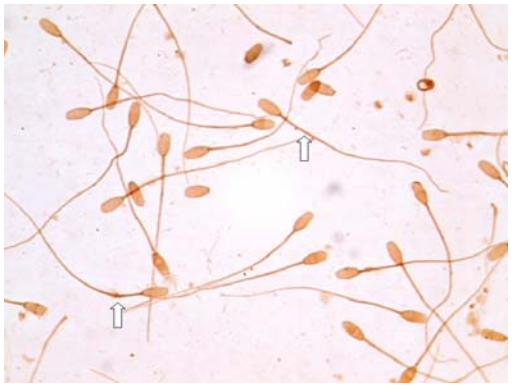
Reference range* from [9, 18]

Table 2 Mean values (±SD) of the percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 hours group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa

Position of immunolocalisation of the GLUT3 expression in spermatozoa (%)	Mean±SD		
	Fresh semen gr.	T extender gr.	TG extender gr.
Head+MP+PP+EP	0	0	99.25±7.71
MP+PP+EP	0	9.10±13.52	0
PP+EP	99.35±3.50	83.40±16.54	0
No	0.65±0.82	7.50±5.43	0.75±0.52



A



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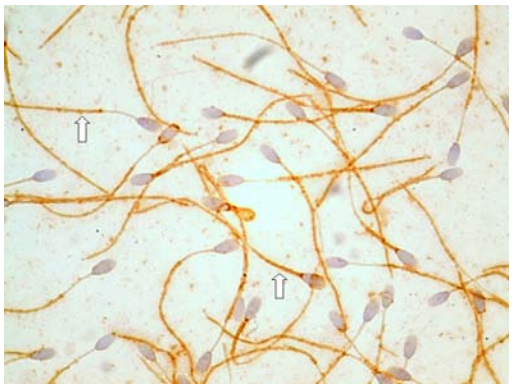

C

Fig 1 Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) the GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail in the fresh semen, (B) the expression of immunolocalisation of GLUT3 of head and the middle piece include principal piece and end piece of the sperm tail in the Tris extender with 3% glycerol (TG) group and (C) the expression of immunolocalisation of GLUT3 of the middle piece, principal piece and end piece or the principal piece and end piece of the sperm tail in the Tris extender without 3% glycerol (T) group

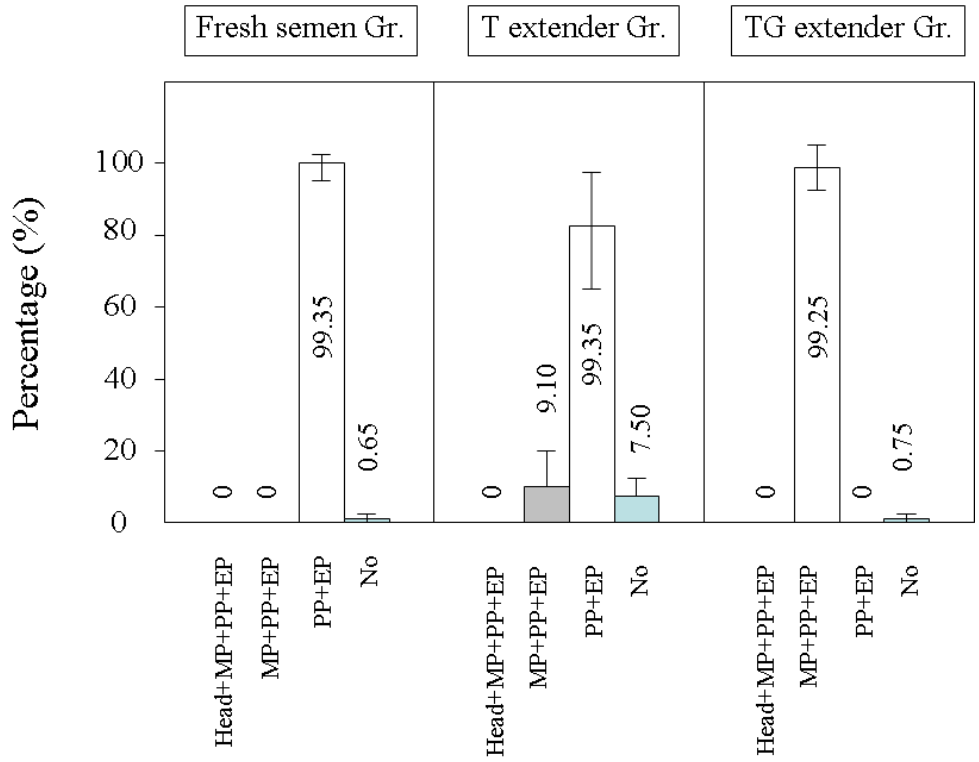

Fig 2 The graft showed percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, Tris extender with 3% glycerol (TG) and without 3% glycerol (T) in cooled semen after 48 hours group; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa, No = Negative expression of GLUT3 in spermatozoa

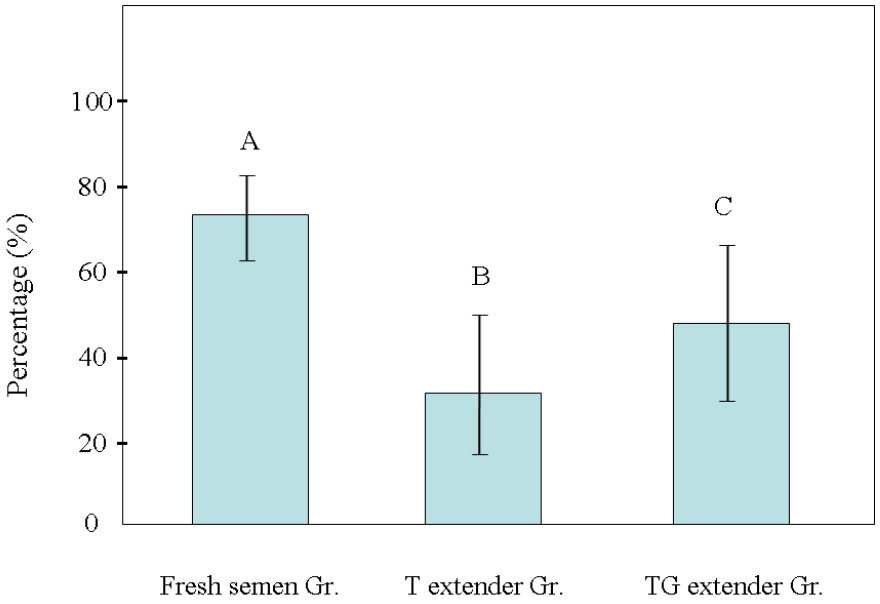

Fig 3 The graft showed percentages of the 3 type groups of motility in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$; T = Tris extender without 3% glycerol, TG = Tris extender with 3% glycerol

Distribution of the glucose transporter-3 (GLUT3) for spermatozoa and the effect of different extenders of TG and T in cooled semen on the expression of GLUT3 in Asian elephant

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Abstract

The aims of this study were to determine the presence and localization of glucose transporter-3 (GLUT3) in freshly ejaculated Asian elephant sperm with different quality of progressive motility, and to evaluate the effect of different extenders in cooled semen on the expression of GLUT3. For experiment I, the fresh semen samples were collected from 10 Asian elephants bulls, and were classified according to the percentages of motile sperm: Group 1 (≤ 20%; n=4), Group 2 (> 20% - 60%; n=3) and Group 3 (> 60%; n=3). In experiment II, six semen samples were collected from 3 Asian elephant bulls for 2 times, in which motile sperm were >60%. The samples were suspended in Tris extender with (TG) and without (T) of 3% glycerol and kept in a refrigerator at 4˚C for 48 h. The GLUT3 was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. The results of experiment I showed that the GLUT3 were localized at the principal and end piece of the sperm tail. The percentages of sperm with GLUT3 expression were highest in Group 3, and lowest in Group 1. In experiment II, the sperm GLUT3 expressions after cold storage in T and TG extenders were different. The sperm of T group showed the localization of GLUT3 similar to those of fresh semen, while the sperm of TG group showed GLUT3 expressions at the head, middle piece, principal piece and end piece. Therefore, the present study demonstrated that GLUT3 expression was related with sperm motility and was affected by glycerol in extender after cold storage.

Keywords: GLUT, Elephant, Sperm

Introduction

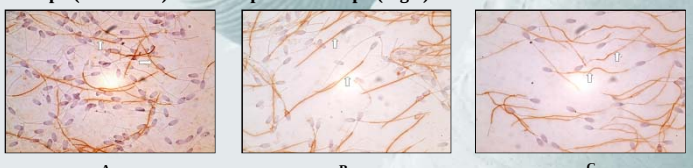
To understand the function of the sperm of an elephant, the energy from hexose of elephant's sperm need to be used as energy. It is necessary to study transporter proteins, particularly Glucose transporter proteins (GLUTs) at the surface of the sperm cell. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across mammalian sperm membranes and play a major role in the regulation of sperm glucose and fructose metabolism, especially GLUT3 (1-5). To our knowledge, there were a few reports about the GLUT proteins in the plasma membrane of elephant's spermatozoa. Therefore, the study of transporter proteins at the surface of the tissue as a kind of elephant sperm is essential for the development of the cooled elephant semen quality. In particular, the preparation was diluted semen containing the sperm of elephant energy for sperm to extend the life and performance quality of sperm after thawing of cooled semen. Therefore, this study aims to investigate the presence and localization of GLUT3 in freshly ejaculated Asian elephant spermatozoa with different quality of progressive motility to evaluate the effect of different Tris extender with (TG) or without (T) of 3% glycerol in cooled semen on the expression of GLUT3 on semen quality of spermatozoa motility. The present investigation was undertaken to study the effect of 3% glycerol in Tris-based extender on the variations in the temporal localization of the hexose specific transporters (GLUT3) after cold storage of elephant spermatozoa with respect to changes the GLUT3 in plasma membrane integrity and spermatozoa motility, both of which are indicators of sperm viability and metabolic intactness.

Methods

Experimental I, The fresh semen samples were collected from 10 normal Asian elephants. The semen samples were classified according to the percentages of motile sperm by Group 1 (≤ 20%; n=4), Group 2 (> 20% - 60%; n=3) and Group 3 (> 60%; n=3). For the experimental II, The semen samples were collected from 6 Asian elephant bulls that have greater than 60% individual motility. The samples were suspended in TG extender or T extender and chilled in refrigerator at 4˚C for 48 h. The GLUT3 transporter was determined by immunocytochemical localization using the rabbit anti-GLUT3 polyclonal antibody. For the evaluation of sperm integrity and motility, statistical comparisons of the expression of immunolocalisation of GLUT3 samples were performed by STATA program. All results were expressed as mean with standard deviations (SD) and the level of significance was set at $p<0.05$.

Results

Experimental I; The expression of immunolocalisation of GLUT3 clearly showed that the spermatozoa expressed the GLUT3. Strong GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail (Fig 1). Percentages of the expression of immunolocalisation of GLUT3 in the 3 type groups showed significant differences between the Group1 (19.44±10.57) and Group2 (84.39±8.84), Group1 and Group3 (99.40±0.07) and Group2 and Group3 (Fig 2).



A **B** **C**

Fig 1. Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) group1, (B) group2 and (C) group3

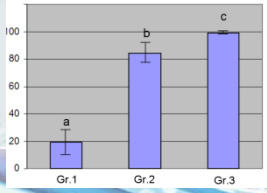
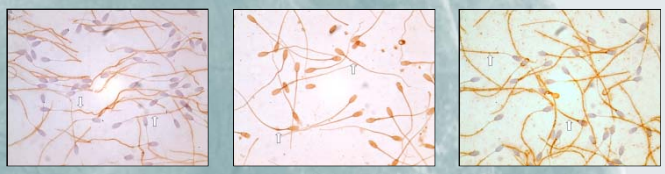


Fig 2. The percentages of the 3 type groups of immunolocalisation of the GLUT3 in Asia elephant spermatozoa; a, b, c values with the superscript show statistically significant difference at $p<0.05$

Experimental II, this study revealed that GLUT3 expression after cold storage were found in all parts of the head, middle piece, principal piece and end piece of the sperm tail in the TG extender group (Fig 3, B), but while the T extender group were expressed at middle piece, principal piece and end piece of the sperm tail (Fig 3, C). Percentages of the expression of immunolocalisation of GLUT3 of the head, middle piece, principal piece and end piece of the sperm tail in the 2 type of the extender groups showed significant differences between the TG extender group and T extender group ($p<0.05$, Fig 4). The percentages of the 3 type groups of motility in Asia elephant spermatozoa showed statistically significant difference at $p<0.05$ (Fig 5).



A **B** **C**

Fig 3. Representative photographs of immunolocalisation of the glucose tranporter3 (GLUT3) in Asia elephant spermatozoa (arrow); (A) the GLUT3 immunoreactivity was observed at the principal piece and end piece of the sperm tail, (B) the expression of immunolocalisation of GLUT3 of head, middle piece principal piece and end piece of the sperm tail in the TG extender group and (C) the expression of immunolocalisation of GLUT3 of the middle piece, principal piece and end piece or the principal piece and end piece of the sperm tail in the T extender group

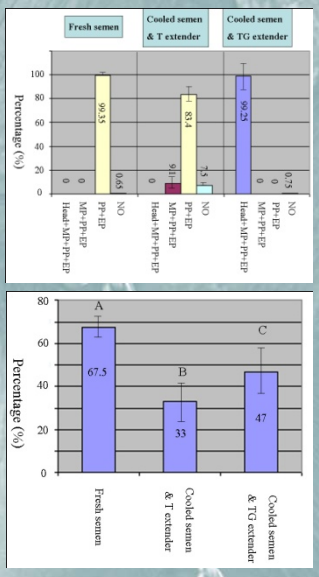


Fig 4. The percentages of immunolocalisation of the GLUT3 expression in Asia elephant spermatozoa in the fresh semen, TG and T extender after cold storage; Head = head of spermatozoa, MP = middle piece of spermatozoa, PP = principal piece of spermatozoa, EP = end piece of spermatozoa No = Negative expression of GLUT3 in spermatozoa

Fig 5. The percentages of the 3 type groups of motility in Asia elephant spermatozoa; A, B, C values with the superscript show statistically significant difference at $p<0.05$

Conclusions and Discussion

The present study indicated that the expression of GLUT3 was localized at the principal and end piece of the sperm tail and the motility of fresh elephant spermatozoa were affected by GLUT3 expression and its expression may involve energy production via the glycolytic pathway. In addition, this result confirmed the reduction of the expression of GLUT3 and motility in elephant spermatozoa after cold storage of the T extender group when compared the TG extender group and reveals that the effect of glycerol on sperm function in the TG extender improved by notice spermatozoa motility and the expression of GLUT3 of spermatozoa better than the T extender group. This substance glycerol may be a result of the sperm cell membrane strength after cold storage.

Acknowledgements

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