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Investigation of relationship between expression of the glucose transporter 3 (GLUT3) and sperm quality in Asian elephants (*Elephas maximus*)

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Abstract: The aims of this study were to determine the relation of glucose transporter 3 (GLUT3) expression on the spermatozoa membrane with the quality of semen in Asian elephants. Fresh semen samples were collected from 10 Asian elephants and classified according to the percentage of motile sperm into Group 1 (≤ 20%; n = 4 males), Group 2 (>20% to 60%; n = 3 males), and Group 3 (>60%; n = 3 males). The immunocytochemistry results showed the presence of GLUT3 at the principal and end piece of the sperm tail. GLUT3 expressions in each group were significantly different ($P < 0.05$). The group with good sperm motility (Group 3) significantly ($P < 0.05$, $R = 0.960$) expressed higher GLUT3 (99.40 ± 0.69%) than Group 1 (21.46 ± 10.25%) and Group 2 (84.37 ± 8.70%). Moreover, the percentage of live sperm of this group was also significantly highest ($P < 0.001$, $R = 0.938$) at 82.00 ± 4.00% compared to Group 1 (20.75 ± 3.30%) and Group 2 (53.00 ± 8.02%). Therefore, the motility of Asian elephant spermatozoa and percentage of live sperm may be influenced by GLUT3 expression, and GLUT3 expression may be involved in energy production via the glycolytic pathway.

Key words: Glucose transporter 3 (GLUT3), elephant, sperm quality

1. Introduction

Semen cryopreservation has become a major resource for the preservation of genetic material in most domestic species (1). However, viability of frozen-thawed spermatozoa depends on several factors such as semen quality, type of extender/cryoprotectant (2), and storage condition (3). In domesticated Asian elephants, poor sperm motility commonly presents (4), which may restrict the suitability of their sperm for semen preservation. In addition, the causes of poor sperm motility in Asian elephants remain unclear; in other species, glucose transporters (GLUTs) have been proposed to play an important role in compromising sperm quality (5,6). 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 (7–10). GLUT3 is a low-Km transporter that can work well with low-substrate concentrations of glucose (11), and seminal plasma contains large amount of fructose and small amount of glucose (9). Thus, glucose is transported into a sperm cell via GLUT3 and used for ATP production and cell metabolism including motility of spermatozoa. To our knowledge, there is no report concerning GLUT proteins in the plasma membrane of elephant sperm. Therefore, the aims of this study were to determine the localization of GLUT3 on which part of the sperm structure and the correlation between its expression and sperm motility in Asian elephants.

2. Materials and methods

2.1. Animals and sample collection

Experiment procedures were approved by the Animal Usage and Ethics Committee, Kasetsart University, Thailand (ID no. ACKU 01858). Ten Asian elephants were used in this study. The experiment was carried out with the sperm-rich fraction of the ejaculate, being manually collected once a month, by transrectal massage of the ampullary region (12). Two ejaculates per elephant,

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uncontaminated by urine, were studied. A blood sample from each elephant was collected from an ear vein after semen collection. Each blood sample was stored separately at 4 °C in a tube containing ethylenediamine tetraacetic acid until analysis.

2.2. Blood evaluation

All samples were analyzed with an automated analyzer for animal blood cells (XT-2000iV/XT-1800iV, SYSMEX, Kobe, Japan). The analyzed blood parameters were: red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), hematocrit (Hct), platelets (PLTs), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and plasma protein (PP). The plasma protein concentration was analyzed using a refractometer (ATAGO, Tokyo, Japan).

2.3. Sperm evaluation

Ejaculates were immediately analyzed for volume, sperm concentration, progressive motility, sperm viability, and pH according to the method of Kidd et al. (13). The sperm concentration was determined manually using a hemocytometer. Individual sperm motility was determined by two independent technicians under a phase-contrast microscopy (Olympus CX31, Tokyo, Japan) at 400× magnification using a sperm motility examination plate (Dongjin, Seoul, South Korea) and maintaining it on a warm plate (37 °C; Fisher Scientific, Pittsburgh, PA, USA). Sperm motility was scored in percentage. The semen samples were classified according to the progressive movement of individual sperm, as follows:

Group 1: poor motility; less than or equal to 20% progressive individual motility (4 males, 8 ejaculates).

Group 2: moderate motility; more than 20% to 60% progressive individual motility (3 males, 6 ejaculates).

Group 3: high motility; greater than 60% progressive individual motility (3 males, 6 ejaculates).

Sperm viability was detected by eosin-nigrosin staining method. A sample of 200 spermatozoa per slide was counted according to the method of Björndahl et al. (14).

2.4. Immunocytochemistry

Fresh semen smears were done on Superfrost polylysine coated slides, then air-dried and fixed in buffered paraformaldehyde (0.5%) for 15 min at room temperature. The smears were then rinsed in phosphate buffered saline (PBS; pH 7.4) and incubated for 12 h at 4 °C with rabbit anti-GLUT3 antibody (Gene Tex, Inc., Irvine, CA, USA) at a dilution of 1:50 (v/v) in Tris-buffered saline (TBS). The same was done in a negative control group, but without a primary antibody. After extensive washing, sperm cells were incubated with a secondary antibody (goat antirabbit IgG horseradish peroxidase (HRPO)-conjugated (KPL, Gaithersburg, MD, USA)) for 1 h at 37 °C. Slides were then counter-stained with hematoxylin; dehydrated with 80%, 95%, and 100% alcohol; and mounted with mounting medium (Amersham Phamacia Biotech, Little Chalfont, UK). Images were obtained from an Olympus digital camera (Olympus E330) installed on a phase-contrast Olympus microscope (Olympus CX31) at 1000× magnification.

2.5. Statistical analysis

For the evaluation of sperm integrity and motility, statistical comparisons of the expression of GLUT3 samples were performed using the STATA program (Version 8.2, Stata Corp., College Station, TX, USA). The correlation between sperm motility and integrity with the percentage of sperm that expressed GLUT3 of each group was analyzed using the Pearson correlation statistic. The data of each individual group were also analyzed by analysis of variance (ANOVA) and Tukey's multiple range test. A significance level of $P < 0.05$ was used.

3. Results

3.1. Blood parameters evaluation

The means and standard deviations (mean ± SD) of the hematological complete blood count parameters and plasma protein concentration values of all elephant samples were within normal ranges, as seen in Table 1. Their values were not significantly different among groups, except for the plasma protein, such that the high motility group had a greater value than the moderate and poor motility groups ($P = 0.01$).

3.2. Semen characteristics

The semen characteristics of each group are represented in Table 2. There were significant differences among groups in terms of the percentage of sperm motility and live sperm ($P < 0.001$). The high motility group had higher values than the moderate and poor motility groups.

3.3. Sperm GLUT3 expression

The positive results from immunocytochemistry indicated that the elephant sperm expressed GLUT3. Strong GLUT3 immunoreactivity was observed at the principal and end piece of the sperm tail (Figure 1). The percentages of GLUT3-expressing sperm (mean ± SD) in Group 1 (poor motility), Group 2 (moderate motility), and Group 3 (high motility) were 21.46 ± 10.25%, 84.37 ± 8.70%, and 99.40 ± 0.69%, respectively, which were significantly different among individual groups (Figure 2). Moreover, the correlation between the percentage of GLUT3-expressing sperm and the percentages of sperm motility and live sperm was quite high ($R = 0.960$ and 0.938, respectively).

4. Discussion

This study demonstrated the expression of GLUT3 in elephant sperm and its high correlation with sperm motility and viability.

Table 1. Mean values (±SD) of the hematological parameters and plasma protein concentration of Asian male elephants in the study.

Parameters	Sperm motility group*			P-value	Ref. range**
	Poor	Moderate	High		
No. of males	4	3	3		
HCT (%)	35.33 ± 3.99	33.73 ± 2.97	32.55 ± 1.05	0.53	29–49
WBC ($\times10^3$/μL)	12.49 ± 1.73	14.38 ± 2.79	14.63 ± 1.14	0.34	11.1–16.1
RBC ($\times10^6$/μL)	3.22 ± 0.49	3.03 ± 0.39	2.8 ± 0.25	0.43	2.13–3.85
Hb (g/dL)	13.63 ± 2.03	12.73 ± 1.29	12.10 ± 0.70	0.46	9.7–16.4
MCV (fL)	110.15 ± 4.74	111.70 ± 4.91	117.00 ± 6.50	0.29	81–158
MCH (pg/cell)	42.35 ± 1.30	42.17 ± 1.50	43.40 ± 1.30	0.51	40.0–45.5
MCHC (g/dL)	38.18 ± 1.33	37.73 ± 0.50	37.15 ± 0.95	0.47	27.7–40.0
PLT ($\times10^3$/μL)	164.50 ± 24.13	229.00 ± 56.82	221.50 ± 4.50	0.08	80–400
PP (mg/dL)	7.70 ± 0.48[a]	7.67 ± 0.31[a]	8.8 ± 0.20[b]	0.01	6–11

a, b: Values with different superscripts show statistically significant differences at $P < 0.05$.
*Sperm motility groups: Poor, ≤20%, Moderate, >20% to 60%, and High, >60%.
**From Lewis et al. (29) and Silva and Kuruwita (30).

Table 2. Mean values (±SD) of the semen characteristics of Asian male elephants in the study.

Semen parameters	Sperm motility group*			P-value
	Poor	Moderate	High	
No. of ejaculates	8	6	6	
Sperm motility (%)	15.00 ± 4.08[a]	48.33 ± 7.64[b]	73.33 ± 5.77[c]	<0.001
Concentration ($\times10^9$ sperms/mL)	1.12 ± 0.25	1.15 ± 0.24	1.27 ± 0.09	0.66
Volume (mL)	21.25 ± 13.00	16.67 ± 6.11	13.33 ± 4.16	0.56
pH	7.25 ± 0.5	7.17 ± 0.76	7.33 ± 0.58	0.95
Viability (%)	20.75 ± 3.30[a]	53.00 ± 8.02[b]	82.00 ± 4.00[c]	<0.001

a, b, c: Values with different superscripts show statistically significant difference at $P < 0.05$.
*Sperm motility groups: Poor, ≤20%, Moderate, >20% to 60%, and High, >60%.

All elephants were apparently healthy, as indicated by hematological parameters. Thus, this indicated that males with poor semen quality could be ones that look healthy. However, the significantly high value of plasma protein in the high motility group might be related to semen motility, as previously reported for seminal plasma protein in elephants (15) and humans (16) since seminal plasma contains proteins and peptides that are unique and partly originated from the blood plasma by exudation through the lumen of the male genital tract (17,18). Therefore, the plasma protein might be also used as an additional indicator for the screening and selection of males prior to making an attempt to collect and evaluate the semen.

This study showed that the Asian elephant sperm expressed GLUT3, which is one of the members of the facilitative hexose transporter family. These GLUT3 proteins were localized at the principal and end piece of the sperm tail, with the exclusion of the middle piece. However, the localization was different from the sperm of other mammals; for example, in boar, positive GLUT3 was evident in the acrosome and in a band across the middle of the sperm head (5,6,19,20). In bull sperm,

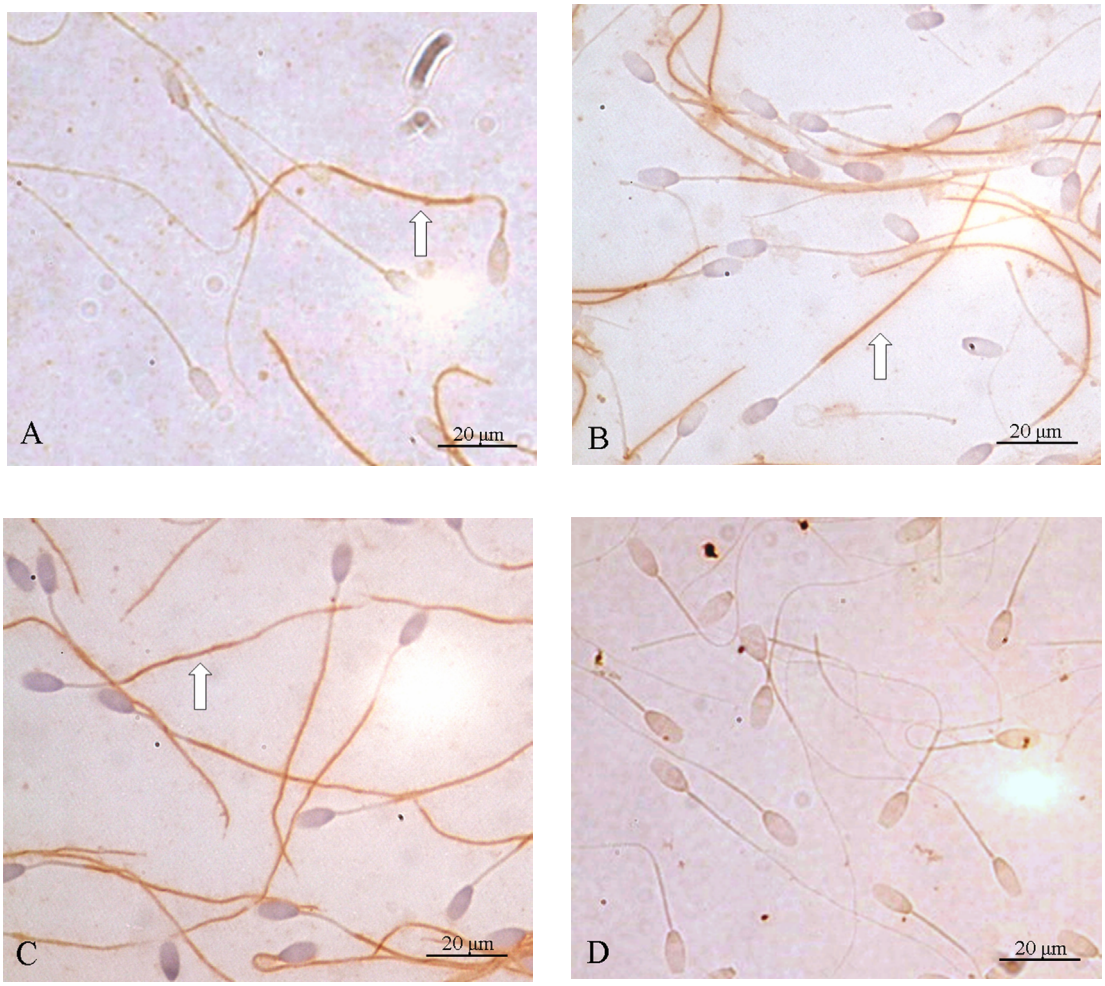

Figure 1. Immunocytochemistry results of glucose transporter 3 (GLUT3) on Asian elephant sperm with different percentages of motility group: Poor, ≤20% (A); Moderate, >20% to 60% (B); High, >60% motility (C); and negative control (D). The arrows show positive immunoreactivity of GLUT3.

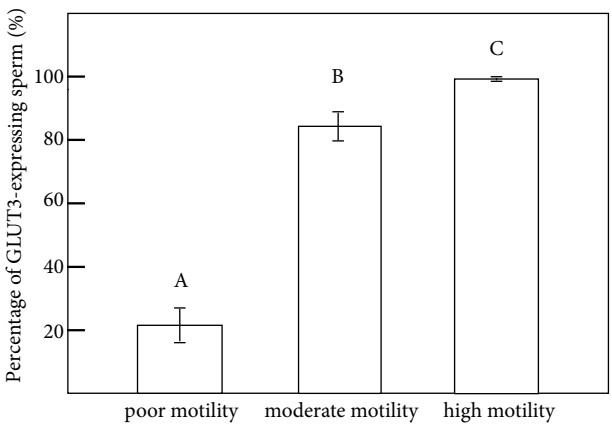

Figure 2. The percentages of GLUT3-expressing sperm in Asian elephant sperm with different percentages of motility group: Poor, ≤20%; Moderate, >20% to 60%; and High >60% motility. A, B, and C show significant differences at $P < 0.05$; error bars indicate the standard error.

the positive GLUT3 signal was present only in the middle piece (8,19). Therefore, the location of GLUT3 is expressed differently among species. However, its function as a glucose transporter as reported in other mammalian sperm (7,8,11,19,21,22) could also be speculated for Asian elephant sperm.

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 the glycolytic pathway of the principal and end piece of the sperm tail (23), while the aerobic-producing ATP was used for cell metabolism in the middle piece of the sperm (24–26). Additionally, the GLUT3 position had a relationship with the hexokinase distribution in the cytoplasm (20) of the glycolytic enzyme-bound lining of the tail's fibrous sheath in Asian elephant sperm, as also evidenced in mouse sperm cells (27). Thus, the GLUT3 distribution was strictly related to enzymes involved in

the glycolytic chain as indicated by their location at the sperm tail. The localization of GLUT3 was a characteristic of elephant that was logical since the absorption of glucose is important for maintaining energy metabolism (10). The current study demonstrated the importance of hexokinase I as a regulatory factor for glycolysis in Asian elephant sperm cells as in other species (20,28), with the presence of GLUT3 localized at the principal and end piece of the sperm tail. In addition, the percentages of GLUT3-expressing sperm were significantly different among groups and had a high correlation with sperm motility and viability. This might confirm the role of GLUT3 expression in sperm cell motility and metabolism in elephant.

In summary, this study showed that expression of GLUT3 was localized at the principal and end piece of the tail of Asian elephant sperm. GLUT3 expression had a high correlation with sperm motility and viability.

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