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Efficacy of evaluation of mitochondrial membrane potential in equine spermatozoa using JC-1

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Evaluation of semen including sperm motility associated with fertility is important in a number of species, including the horse (Jasko *et al.* 1991, Hafez 2000). The evaluation of sperm motility by visual, subjective assessment is burdened with a large component of variability (Varner *et al.* 1991) while visual or computer-assisted sperm analysis (CASA) assessment of spermatozoal motility has been poorly correlated with the estimated fertility of a stallion. Energy for motility is provided by the mitochondria of spermatozoa, which are localized on the midpiece. Mitochondrial membrane potential plays a vital role in determining sperm cell competence because of its relationship with the energetic status of the cell and motility and has been related to fertility (Martinez-Pastor *et al.* 2004). Hence, any changes in the mitochondrial function may be reflected in sperm motility and fertility (Ericson *et al.* 1993). Due to inherent variability of subjective analyses, such as motility, detection of alterations in the mitochondrial membrane potential of spermatozoa may provide more reliable information. Therefore, development of such assays for individual species is of importance.

Previously, fluorochrome probes like Rhodamine 123 and MitoTracker have been used to evaluate mitochondrial function of spermatozoa, but no distinction could be made between spermatozoa exhibiting different respiratory rates (Gillan *et al.* 2005). On the contrary, the potentiometric dyes, such as JC-1 probe, permit a distinction between spermatozoa with poor and high functional mitochondrial potential by the determination of the inner mitochondrial membrane potential since this molecule can exist in two different forms (aggregates or monomers) each with a different emission spectra (Allesandract *et al*. 2010). If the JC-1 molecule remains in the monomeric form, it will fluoresce in green (that is, ø m low) after passage through mitochondrial membrane. If it converts into aggregate form, it will fluoresce in orange (that is, ø m high) (Reers *et al.* 1991; Mocé and Graham, 2008). The aggregate green monomeric form has absorption/emission of 510/527 nm, and the aggregate red form has absorption/emission 585/590 nm (Smiley *et al.* 1991). Indeed, the use of fluorescence ratio detection provides a means to compare measurements of membrane potential while also assessing the percentage of mitochondrial depolarization occurring in a pathological condition (e.g. cellular stress, apoptosis, etc.). The red to green fluorescence intensity ratio is only dependent on the membrane potential and no other factors such as shape, mitochondrial size, and density, which may influence single component fluorescence signals. Thus JC-1 dye can be used both as qualitative (considering the shift from green to red fluorescence emission) and quantitative (considering only the pure fluorescence intensity) measure of mitochondrial membrane potential (Cossarizza and Salvioli, 1998). Keeping the advantages in view, JC-1 staining was applied to the horse and donkey stallion spermatozoa, analysed, and compared the differences in the measurement of mitochondrial membrane potential for both the species.

Apparently, four healthy Marwari stallions, 4–6 year-old, maintained at a Government Research organization, Bikaner, Rajasthan were used for the present study. All the experiments were carried out in accordance with the guidelines set out by the Institute Ethics Committee. All the experiments were conducted during breeding season.

The semen samples were collected once weekly using a colorodo model of Artificial Vagina (AV) on estrus mare as dummy. Total nine collections from each stallion were collected. Each stallion was given one false mount before actual collection. The semen samples were collected directly into a clean dry graduated bottle attached to the latex cone of the AV. The tubes containing semen were marked and placed in a water bath at 37 °C immediately after collection.

A Computer Assisted Semen Analyzer (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA) equipped with a thermostage (MiniTherm®, Hamilton Thorne Inc. Beverly, MA, USA, Fig. 8) was used to analyze the progressive sperm motility. 4 µl diluted aliquots of semen sample (50 µl of semen sample dissolved in 1 ml of 2.9% sodium citrate diluting fluid to make a 1:20 dilution) was loaded in disposable chambers having a 20 µm chamber depth (Leja® Standard Count 4 Chamber Slide, 20 µm,

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Leja® Products BV, Netherlands). The HTB CEROS II system was set to measure 30 frames per field at a frame rate of 60 Hz. A minimum of seven fields and 500 spermatozoa were measured for each sample. Sperm tracks with a straightness value less than 60% were considered non-progressive (Yogesh *et al.* 2018).

Mitochondrial membrane potential was assessed by using JC-1 (5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethyl-benzimidazolylcarbocyanine iodide). Three micro liters of a prepared solution (200 µM JC-1 in dimethyl sulfoxide) was added to a 100–mL sample of sperm (100×10^6/ml) and incubated for 30 min at 37°C and counterstaining of nuclei was performed with 10 mL of PI (Propodeum Iodide) stock solution (0.27 mg/ml in phosphate buffer). Then, cells were washed once in PBS and resuspended in PBS, smeared and analysed with epifluorescence microscope (Nikon 80i, Japan). A minimum of 300 spermatozoa was observed in a 400× magnification. The cells with green fluorescence (monomer) mitochondria were considered as lowest membrane potential and yellowish to orange fluorescence (aggregates) were considered as highest membrane potential. The proportion of cells exhibiting yellowish to orange fluorescence was considered as cells having mitochondrial membrane potential.

Data were subjected to ANOVA using the Post hoc procedure from Statistical software package, version 20 (SPSS 20, SPSS Inc., Chicago, IL, US). For fresh, unextended semen, the Paired t-test model was used in order to see the variability between stallions as well as between the two groups.

In the present study, the motility parameters through CASA and the mitochondrial membrane potential evaluation through JC-1 staining were studied and presented in Table 1 and Table 2. The percent of total motile spermatozoa in the fresh semen of donkey stallions was found to be higher to that of the horse stallions and they were found to be non-significant. The mean values of progressive motility varied individually within the donkey and horse stallion groups and was found be varied significantly ($P < 0.05$) which explains the reason for overall significant differences between the two groups (horse and donkey groups).

The mitochondrial membrane potential varied significantly between the species and also between the individuals of species. The mitochondria of sperm provide the energy source for motility and any changes in mitochondrial function may be reflective of changes in sperm motility. With the use of JC-1 two distinct sperm populations were observed. Green sperm head with an orange- yellow midpiece indicated viable spermatozoa with high MMP (Fig. 1). The percentage of cells fluorescing orange indicates the amount of sperm in the sample that contains functioning mitochondria.

Correlation between the motility parameters and MMP was also assessed for combining the both the groups and was presented in Table 3. The expected percentage of spermatozoa forming JC-1 aggregates (higher MMP) was correlated with the actual percentage of orange labeled sperm cells (r^2=0.48). A positive and significant (r^2=0.64, r^2=0.92) correlation was observed between the total and progressive motility and high MMP. A higher percentage of functioning mitochondria in the sample provided information that the sperm are able to produce the energy needed for motility. Indeed the use of fluorescence ratio detection provides similar workers with a means to compare measurements of membrane potential while also assessing the percentage of mitochondrial depolarization occurring in a pathological conditions (e.g. cellular stress, apoptosis, etc.).

In routine practice, fertility in stallions used in breeding programs is lower and more variable than in other domestic animal species primarily because current selection procedures are based on pedigree, phenotype, and/or athletic performances, with little consideration of fertility or fertility potential (Neild *et al.* 2005). The modest consideration of stallion fertility assessment could be notably improved by the introduction of objective methodologies such as fluorescence and CASA system (Vamer *et al.* 1991).

SUMMARY

The present study was conducted to standardize the technique of JC-1 staining for equine spermatozoa and to assess the MMP in the equine spermatozoa. A positive

Table 1. Seminal motility parameters and MMP levels in horse and donkey stallions (mean±SE) (n=9)

Parameter	Horse stallions (3) (n=9)			Donkey stallions (3) (n=9)		
	1	2	3	1	2	3
Total motility (%)	80.5±8.05^a	82.5±8.05^a	78±8.05^a	85.5±8.05^a	88.12±12.08^a	89.12±12.08^a
Progressive motility (%)	68.10±8.12^a	66.5±8.12^a	62.5±8.12^a	78.5±8.12^a	79.37±12.19^a	78.37±12.19^a
VAP (mm/s)	135.5±8.59^a	128±8.59^a	144.5±8.59^a	124.5±8.59^a	134.87±12.89^a	138.87±12.89^a
VSL (mm/s)	80.4±9.6^a	72.6±9.6^a	70.4±9.6^a	92.95+9.6^a	91.25±14.4^a	91.45±14.4^a
VCL	209±13.67^a	247±13.67^a	217.5±13.67^a	279±13.67^{ab}	259.87±20.5^a	263.87±20.51^{ac}
Mitochondrial Membrane potential high (%)	64.01±1.05^a	69.17±0.08^b	68.20±1.24^b	74.14±1.24^a	72.12±0.17^a	78.14±1.54^b
Mitochondrial Membrane potential low (%)	26.41±0.24^a	28.34±0.27^a	27.47±1.87^a	22.17±0.47^{ab}	20.47±0.41^a	21.22±0.08^b

Means bearing different superscripts in a row differ significantly (P<0.05).

Table 2. Seminal motility parameters and MMP levels in horse and donkey stallions (mean±SE) (n=9)

Parameter	Horse stallions (3)	Donkey stallions (3)
Total motility (%)	80.33±0.17	87.58±1.36*
Progressive motility (%)	65.7±1.24	78.74±1.02**
VAP (mm/s)	136±2.27	132.75±2.57
VSL (mm/s)	74.46±1.57	91.88±0.24**
VCL	224.5±4.68	267.58±2.97*
Mitochondrial Membrane potential high (%)	67.127±0.34	74.8±1.24**
Mitochondrial Membrane potential low (%)	27.40±0.35	21.28±0.02*

*P<0.05 and **P<0.001.

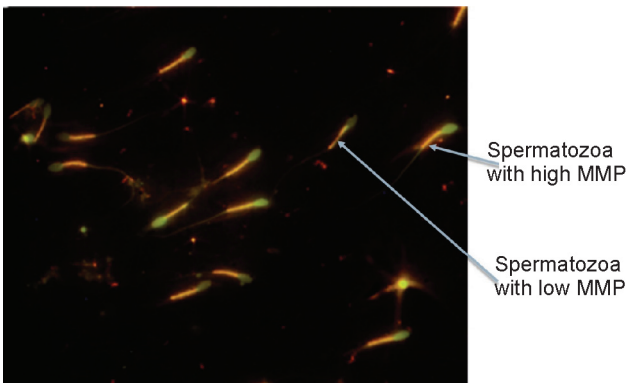

Fig. 1. Stallion spermatozoa depicting high and low MMP.

Table 3. Pearson correlation results of MMP with the seminal parameters in equine spermatozoa

	Total Motility	Progressive motility	VAP	VSL	VCL
High MMP	0.64*	0.92*	–0.21	0.33	0.18

*P<0.05.

correlation was observed between the progressive motility and MMP of the equine spermatozoa. And there were significant differences were observed between the MMP levels of donkey and horse stallion groups in their fresh semen and individual variation was noticed within the group, which was found to be non-significant. The technique of JC-1 was found to be most reliable and suitable technique for assessing the mitochondrial membrane potential of equine spermatozoa.

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