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Bovine sperm cells viability during incubation with or without exogenous DNA

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Summary

The aim of this study was to assess the effect of exogenous DNA and incubation time on the viability of bovine sperm. Sperm were incubated at a concentration of 5×10^6/ml with or without plasmid pEYFP–NUC. Fluorescent probes, propidium iodide/Hoechst 33342, FITC–PSA and JC-1, were used to assess plasma membrane integrity (PMI), acrosome membrane integrity (AMI) and mitochondrial membrane potential (MMP) respectively at 0, 1, 2, 3 and 4 h of incubation. Exogenous DNA addition did not affect sperm viability; however, incubation time was related to sperm deterioration. Simultaneous assessment of PMI, AMI and MMP showed a reduction in the number of sperm with higher viability (integrity of plasma and acrosome membranes and high mitochondrial membrane potential) from 58.7% at 0 h to 7.5% after 4 h of incubation. Lower viability sperm (damaged plasma and acrosome membranes and low mitochondrial membrane potential) increased from 4.6% at 0 h to 25.9% after 4 h of incubation. When PMI, AMI and MMP were assessed separately we noticed a reduction in plasma and acrosome membrane integrity and mitochondrial membrane potential throughout the incubation period. Therefore, exogenous DNA addition does not affect sperm viability, but the viability is reduced by incubation time.

Keywords: Acrosomal membrane, Exogenous DNA, Fluorescent probes, Mitochondrial membrane potential, Plasma membrane

Introduction

In the past decades, the ability to modify gene expression through transgenesis, particularly in mammals, has become one of the most important advances in the field of applied and experimental biology (Celebi *et al.*, 2003). Sperm-mediated gene transfer (SMGT), a simple, low-cost technique, which, in theory, can be used in all species that reproduce through gametes, is drawing researchers' attentions. Nevertheless SMGT presents inconsistent results and is difficult to reproduce (Maione *et al.*, 1998).

A better understanding of how exogenous DNA molecules interact with sperm cells is crucial to optimize SMGT. Longer incubation time results in a higher binding rate of exogenous DNA and sperm cells followed by the internalization of the nucleus (Castro *et al.*, 1990; Francoline *et al.*, 1993). However, longer incubation time can affect sperm viability resulting in lower fertilization rates and consequently fewer transgenic embryos. Understanding how incubation time and exogenous DNA affect sperm viability might

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reduce the inconsistency of results and improve cell transfection rates.

In order to maximize the production of transgenic animals it is necessary to determine when, for how long, what type and what amount of exogenous DNA should be added to the sperm, so that exogenous DNA is efficiently bound and internalized by the majority of sperm cells. Therefore, understanding the relationship between exogenous DNA and sperm is crucial. Sperm viability influences the efficiency of exogenous DNA binding and internalization. However, SMGT protocols reveal that little is known about the effect of exogenous DNA on sperm viability.

The aim of this study was to assess the effect of exogenous DNA addition and incubation time on sperm motility, plasma membrane integrity, acrosome membrane integrity and mitochondrial membrane potential.

Material and methods

Preparation of sperm and incubation with exogenous DNA

Bovine semen was thawed in water at 37 °C for 30 s and subjected to Percoll gradient separation (45 and 90%) at 600 *g* for 30 min. To remove excess Percoll, the sediment was resuspended and washed in Sperm-TALP medium at 200 *g* for 5 min. Sperm was suspended in fertilization medium (Parrish *et al.*, 1988) without heparin at a concentration of 5×10^6/ml and incubated at 39 °C and in 5% CO_2 (v/v) in air and under high humidity for 0 (incubation time control), 1, 2, 3 and 4 h with or without (exogenous DNA control) 500 ng/ml of plasmid pEYFP–NUC (Clontech, BD Biosciences), linearized with *Stu*I restriction enzyme.

Assessment of motility, plasma and acrosome membrane integrity and mitochondrial membrane potential

Sperm motility was assessed by evaluating sperm placed on a slide, covered with a coverslip and examined under a light microscope. Fluorescent probes, propidium iodide (PI) and Hoechst 33342 (H342), were used to assess plasma membrane integrity. Fluorescently labelled *Pisum sativum* lectin (PNA–FITC) was used to assess acrosome integrity. Mitochondrial function was assessed with the JC-1 probe, which marks mitochondria that have high membrane potentials as fluorescencent red and mitochondria with low membrane potentials as fluorescencent green.

A volume (2 µl) of Hoechst 33342 (40 µg/ml in DMSO) was added to 150 µl of semen that had been diluted in TALP (5×10^6 sperm/ml) and incubated for 10 min at 37 °C. Then 3 µl propidium iodide (0.5 mg/ml in Dulbecco's phosphate-buffered saline solution – DPBS), 2 µl JC-1 (76.5 µM in DMSO) and 50 µl PNA–FITC (100 µg/ml added 10% of sodium azide solution at 10%) were added and incubated for an additional 10 min at 25 °C. Following incubation, 10 µl of the solution was spread on a slide and covered with a glass coverslip. At least 200 cells were examined under an epifluorescence microscope (Olympus) using 355, 490 and 520 nm excitation filters and 465, 252 and 610 nm emission filters, respectively. The association of the four probes allowed us to classify sperm into eight different groups (Celeghini *et al.*, 2007), described in Table 1.

Table 1 Classification of sperm according to fluorescence of propidium iodide, Hoechst, FITC–PSA and JC-1 probes.

Classification	Plasma membrane	Acrosome membrane	Mitochondria potential
Class 1	Integrity	Integrity	High
Class 2	Integrity	Integrity	Low
Class 3	Integrity	Damage	High
Class 4	Integrity	Damage	Low
Class 5	Damage	Integrity	High
Class 6	Damage	Integrity	Low
Class 7	Damage	Damage	High
Class 8	Damage	Damage	Low

Statistical analysis

For the purpose of this study we used semen samples from three bulls, in a total of 10 repetitions per treatment in a 5×2 factorial analysis. In all evaluations 200 cells were counted in nine quadrants of the slide. Experiment data were analysed using statistical software MINITAB Release 14. Prior to software analysis the homogeneity and distributions of variables was verified and data were subjected to analysis of variance and comparison using the Turkey test with a 5% significance level.

Results

After 4 h of incubation, exogenous DNA did not affect sperm motility (Table 2), plasma and acrosome membrane integrity and mitochondrial membrane potential. However, increased incubation time reduced sperm viability. In the group that was incubated with exogenous DNA and in the group incubated without exogenous DNA, sperm motility was high after 1 h of incubation ($p < 0.05$). Nevertheless, in both groups,

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Table 2 Average motility of sperm incubated with (+) or without (−) exogenous DNA.

	(% motility)				
Exogenous DNA	0 h	1 h	2 h	3 h	4 h
−	$79.5 \pm 8.0^{a,A}$	$58 \pm 16.2^{b,A}$	$35 \pm 14.3^{c,A}$	$15.5 \pm 10.4^{d,A}$	$4.5 \pm 5.0^{d,A}$
+	$79.5 \pm 8.0^{a,A}$	$57.5 \pm 16^{b,A}$	$34.5 \pm 13.2^{c,A}$	$15 \pm 8.5^{d,A}$	$4.5 \pm 5.0^{d,A}$

Different letters represent significant differences ($p < 0.05$). ^{A}The effect of exogenous DNA; comparison between lines in the same column. $^{a–d}$The effect of incubation time; comparison between columns in the same line.

Table 3 Percentage of sperm incubated with (+) or without (−) exogenous DNA at different time points and distributed in eight classes according to fluorescent probes: propidium iodide, Hoechst, FITC–PSA and JC-1.

Class	Exogenous DNA	0 h (%)	1 h (%)	2 h (%)	3 h (%)	4 h (%)
Class 1	−	58.7 ± 5.6^{a}	35.7 ± 11.6^{b}	19.4 ± 10.7^{c}	$10.3 \pm 5.4^{c,d}$	7.5 ± 7.1^{d}
	+	58.7 ± 5.6^{a}	35.1 ± 11.5^{b}	18.3 ± 9.0^{c}	$11.2 \pm 8.6^{c,d}$	6 ± 3.2^{d}
Class 2	−	6.6 ± 4.4	9 ± 3.5	10.7 ± 6.1	10.3 ± 5.5	7.4 ± 5.6
	+	6.6 ± 4.4	8.1 ± 4.3	8.2 ± 6.5	9 ± 1.5	5.6 ± 4.0
Class 3	−	2.5 ± 3.4	1.9 ± 2.1	0.4 ± 0.9	1.1 ± 0.8	0.3 ± 0.4
	+	2.5 ± 3.4	1.7 ± 2.1	1 ± 1.1	0.6 ± 1.5	0.3 ± 0.5
Class 4	−	0.7 ± 0.7	1.2 ± 1.1	0.8 ± 1.5	0.8 ± 0.7	0.3 ± 0.4
	+	0.7 ± 0.7	1.1 ± 0.9	0.5 ± 0.8	0.3 ± 0.4	0.2 ± 0.3
Class 5	−	$14 \pm 6.1^{a,b}$	20.2 ± 5.7^{a}	18.1 ± 7.7^{a}	9.5 ± 4.6^{b}	7.6 ± 7.4^{b}
	+	14 ± 6.1^{a}	22.3 ± 4.0^{b}	13.1 ± 7.5^{a}	9.6 ± 3.9^{a}	7.8 ± 5.3^{a}
Class 6	−	6.3 ± 5.1^{a}	10.4 ± 3.9^{a}	28.1 ± 15.9^{b}	37.1 ± 12.7^{b}	45.7 ± 12.3^{b}
	+	6.3 ± 5.1^{a}	12.7 ± 4.8^{a}	27.9 ± 11.5^{b}	$40.1 \pm 12.6^{b,c}$	41.6 ± 1.8^{c}
Class 7	−	6.6 ± 6.2	10.5 ± 6.4	9.9 ± 7.1	8.3 ± 6.3	5.3 ± 4.4
	+	6.6 ± 6.2	10.2 ± 5.9	14.5 ± 8.7	6.1 ± 6.9	8.2 ± 5.8
Class 8	−	4.6 ± 3.2^{a}	$11.1 \pm 6.5^{a,b}$	12.6 ± 5.6^{b}	22.6 ± 6.8^{c}	25.9 ± 7.9^{c}
	+	4.6 ± 3.2^{a}	$8.8 \pm 3.7^{a,c}$	$16.4 \pm 5.9^{b,c}$	$23.1 \pm 8.2^{b,d}$	30.3 ± 8.9^{d}

$^{a–d}$Different letters represent significant differences ($p < 0.05$) between the effect of incubation time, comparison between columns in each class.

sperm motility was reduced when compared with the control group.

Class 1 sperm were more frequent after 1 h of incubation with and without exogenous DNA, but these results were still lower than results from the control group (0 h) ($p < 0.05$). Regarding classes 2, 3, 4 and 7, no variation was noticed throughout the incubation period. Class 5 sperm were more frequent at 0, 1 and 2 h of incubation without exogenous DNA and at 1 h incubation with exogenous DNA ($p < 0.05$). Class 6 sperm were more frequently observed at 2, 3 and 4 h incubation without exogenous DNA and at 3 and 4 h incubation with exogenous DNA ($p < 0.05$). Class 8 sperm were more frequently seen after 3 and 4 h of incubation ($p < 0.05$) with and without exogenous DNA (Table 3).

Sperm that presented plasma membrane integrity (PMI) (Σ classes 1, 2, 3 and 4), acrosome membrane integrity (AMI) (Σ classes 1, 2, 5 and 6) and high mitochondrial membrane potential (HMMP) (Σ classes 1, 3, 5 and 7) were evaluated separately (Fig. 1). Sperm that presented plasma membrane integrity were more frequently observed at 0 and 1 h incubation with and without exogenous DNA ($p < 0.05$). A greater number of sperm with acrosome membrane integrity was observed at 0 and 1 h of incubation with and without exogenous DNA and at 2 h incubation without exogenous DNA ($p < 0.05$). The number of sperm with high mitochondrial membrane potential was also higher at 0 and 1 h incubation ($p < 0.05$).

Discussion

This study assessed the effect of incubation time and exogenous DNA addition on sperm motility, plasma membrane integrity, acrosome membrane integrity and mitochondrial membrane potential. According to our results, exogenous DNA has no influence on sperm viability (exogenous DNA uptake by sperm cells was

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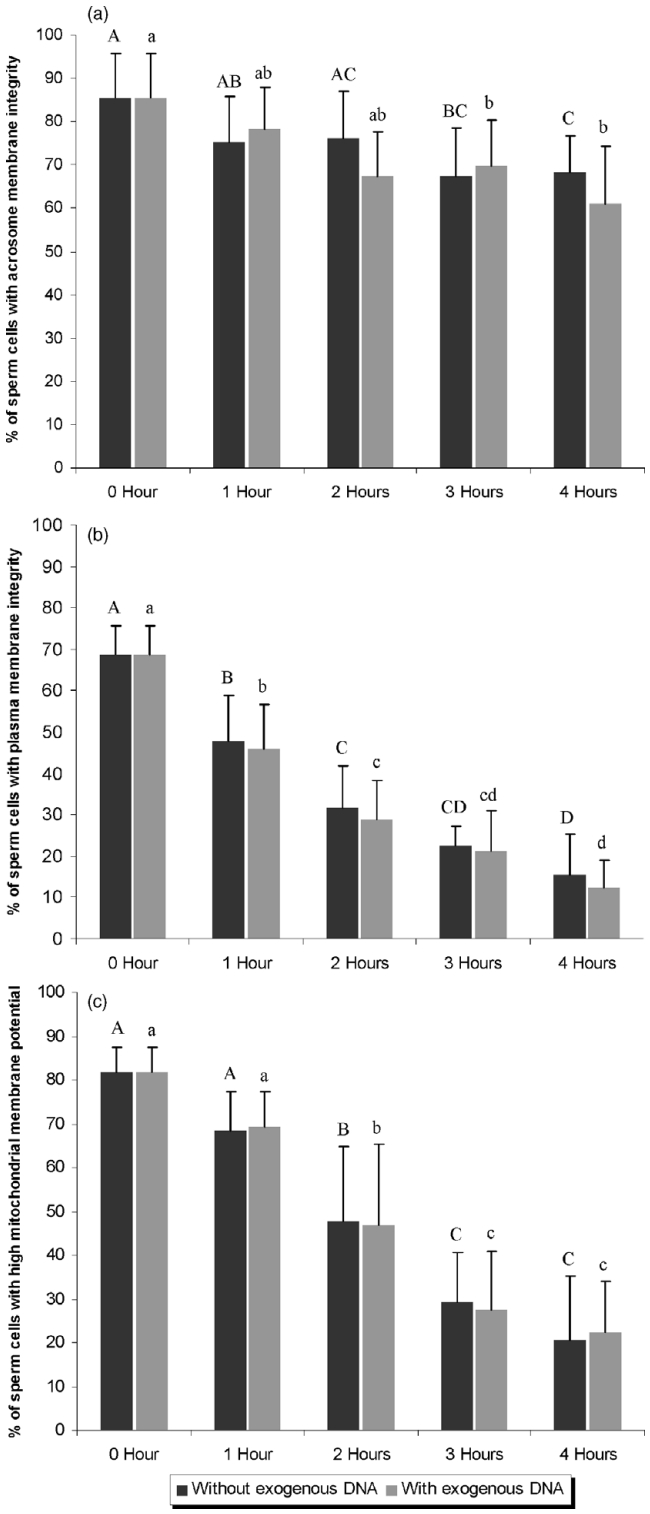

Figure 1 Percentage of bovine sperm with: (*a*) acrosome membrane integrity; (*b*) plasma membrane integrity; and (*c*) high mitochondrial membrane potential after different incubation times without or with exogenous DNA. Different letters represent significant difference ($p < 0.05$). Uppercase letters represent the effect of incubation time on sperm cell viability after incubation without exogenous DNA; lowercase letters represent the effect of incubation time on sperm cell viability after incubation with exogenous DNA.

confirmed by PCR in all incubation times, data not shown), which, on the other hand, is affected by the incubation time.

In SMGT, exogenous DNA interacts with sperm cells after 15 min of incubation and reaches a maximum level of binding at 40–45 min, followed by a plateau (Castro *et al.*, 1990; Camaioni *et al.*, 1992). Results of the present study agree with data presented in other studies (Castro *et al.*, 1990; Camaioni *et al.*, 1992) and might explain the existence of a plateau phase in SMGT. According to these studies, following 1 h of incubation sperm presented reduced motility, plasma and acrosome membrane disruption and a reduction in mitochondrial membrane potential.

In the present study, when compared with sperm incubated without exogenous DNA, the addition of exogenous DNA to the incubation medium did not influence plasma and acrosome membrane integrity or mitochondrial membrane potential. These results conflict with results presented by Anzar & Buhr (2006), who reported that DNA uptake reduces plasma membrane integrity. However, our results confirm data presented by Lavitrano *et al.* (1989) who reported that incubation with exogenous DNA did not cause morphologic changes in sperm. Despite the finding that exogenous DNA uptake did not influence sperm viability, other studies reported that sperm viability influences fertilization and binding of exogenous DNA molecules within sperm cells (Lavitrano *et al.*, 1989; Castro *et al.*, 1990; Horan *et al.*, 1991; Anzar & Buhr, 2006).

Increased incubation time reduced average motility of sperm incubated with and without exogenous DNA. Motility reduction during incubation might play an important role in the transfection of sperm cells. Furthermore, sperm with good motility showed higher exogenous DNA binding rates than sperm with no motility (Horan *et al.*, 1991). However, it is not known whether this increased binding rate is the consequence of more efficient DNA interaction and internalization mechanisms or of greater exposure of motile sperm to exogenous DNA molecules, which would favour the interaction of these molecules with the sperm cells.

In vivo and *in vitro* studies reported that reduced motility also affects fertilization (Kjaestad *et al.*, 1993; Holt *et al.*, 1997; Tanghe *et al.*, 2002). Thus, an incubation time with reduced effect on sperm motility not only improves transfection efficiency but also prevents the reduction of fertilization rates.

The interaction, internalization and integration of exogenous DNA within sperm cells is not merely the consequence of a passive and uncontrolled process, it is regulated by factor-specific mechanisms that metabolically activate the cells (Smith & Spadafora, 2005). Thus, SMGT might involve energy consumption. Mitochondrial membrane potential of sperm was not

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affected by exogenous DNA addition. Initially these results suggested that the mechanism that regulates SMGT is an isolated event that does not consume mitochondrial energy, or that energy consumption is extremely low and is not detected by the fluorescent probe JC-1.

However, flagellum motility does not depend exclusively on ATP derived from mitochondrial oxidative phosphorylation. Several studies have shown that glucolysis throughout the main piece is the most important ATP source for the flagellum (Bradley *et al.*, 1996; Westhoff & Kamp, 1997; Bunch *et al.*, 1998; Mori *et al.*, 1998; Travis *et al.*, 1998). Therefore, considering that SMGT is an energy-demanding process, energy might be derived from glucolysis instead of from the mitochondria.

The plasma membrane also plays an important role in sperm and exogenous DNA interaction. Atikinson *et al.* (1991) did not observe any difference in the percentage of intact sperm with exogenous DNA molecules (18.54%) and disrupted sperm (19.2%). However, they observed a difference in the exogenous DNA-binding site, which suggests that plasma membrane integrity might influence SMGT. Even though the plasma membrane affects exogenous DNA uptake, our results showed that exogenous DNA not affect plasma membrane integrity. On the other hand, Lavitrano *et al.* (1989) observed that only live cells bind to exogenous DNA molecules. This information was later confirmed by Castro *et al.* (1990) and Anzar & Buhr (2006). Furthermore, a sperm with functional disruption of the plasma membrane is considered deteriorated (dead) and is incapable of *in vivo* or *in vitro* fertilization (Silva & Gadella, 2006).

In this study exogenous DNA did not affect acrosome membrane integrity. However, incubation time played a role, causing acrosome membrane disruption during increased incubation periods. Acrosome membrane integrity does not influence exogenous DNA binding to sperm cells without a difference in exogenous DNA uptake by sperm with and without acrosome reaction (Horan *et al.*, 1991).

Nevertheless, despite being no correlation between acrosome membrane integrity and exogenous DNA uptake rates, acrosome membrane integrity is crucial for fertilization. The acrosome must remain intact before and during the passage of the sperm through the isthmus of the uterine tube and until it reaches the zona pellucida. Early acrosome reaction (Silva & Gadella, 2006) or absence thereof (Bielfeld *et al.*, 1994) hinders zona pellucida penetration and consequently reduces fertilization capacity.

Our study shows that whereas exogenous DNA does not influence sperm viability, longer incubation times reduce sperm viability. According to the literature, reduced sperm viability affects binding of exogenous DNA to sperm cells (Lavitrano *et al.*, 1989; Castro *et al.*, 1990; Atkinson *et al.*, 1991; Horan *et al.*, 1991; Anzar & Buhr, 2006). Thus, increasing the incubation time and reducing sperm viability might reduce SMGT efficiency and fertilization.

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