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# Effect of Exogenous DNA on Bovine Sperm Functionality Using the Sperm Mediated Gene Transfer (SMGT) Technique

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SUMMARY

Sperm mediated gene transfer (SMGT) could provide the opportunity to carry out transgenesis on a mass scale using spermatozoa as vectors for exogenous DNA. However, the efficiency of sperm-mediated DNA transfer is still questionable, and the mode of transmission to the egg has not yet been well understood. Our aim was to investigate the capacity of bovine spermatozoa to carry exogenous DNA and its relationship to sperm functionality. We studied these parameters using flow cytometry to measure viability (necrosis and apoptosis) and capacitation status, computer-assisted semen analysis (CASA) to measure motility parameters and in vitro fertilization (IVF) to assess fertilizing capacity. Furthermore, we studied the effect of capacitation status on interaction with exogenous DNA, and the role of heparin supplementation in this process. Bull spermatozoa showed a high capacity to bind DNA quickly and reached a maximum after 30 min, with approximately half of the DNA-bound spermatozoa being viable. Incubation with exogenous DNA induced a decrease in sperm viability and motility and increased the proportion of apoptotic cells, but did not affect the cleavage rate in IVF assay. Heparin increased high-lipid disorder and the number of sperm with DNA bound (viable and dead). In conclusion, this study shows that live spermatozoa can bind exogenous DNA with a slight negative effect in some parameters of sperm function that in our opinion, would not drastically compromise fertility.

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## INTRODUCTION

The sperm mediated gene transfer (SMGT) system is a technique that could provide the opportunity to carry out transgenesis on a mass scale (Spadafora, 1998). The first report showing that exogenous DNA could be introduced into sperm was provided by Brackett's group in rabbits (Brackett et al., 1971). Since then, this technique has been used by multiple laboratories, but controversial results have been obtained (reviewed by Smith and Spadafora, 2005). Although transgenic animals have been produced in various species, the efficiency of sperm-mediated DNA transfer is still questionable and the mode of transmission to the egg has not yet been well understood. In bulls the binding of exogenous DNA to spermatozoa has been shown by several authors (Castro et al., 1990; Atkinson et al., 1991; Camaioni et al., 1992; Schellander et al., 1995; Alderson et al., 2006; Anzar and Buhr, 2006) although the mechanism of binding and internalization of exogenous DNA is a question that has not been solved. Some studies have resulted in the creation of bovine transgenic embryos (Gagne et al., 1991; Sperandio et al., 1996; Rieth et al., 2000; Shemesh et al., 2000; Alderson et al., 2006; Hoelker et al., 2007; Pereyra-Bonnet et al., 2008) or calves (Perez et al., 1991; Schellander et al., 1995; Shemesh et al., 2000) using SMGT, although the efficiency of this technique is quite low and huge differences between studies were observed.

SMGT techniques use the capacity of sperm cells to spontaneously uptake or attach exogenous DNA, acting as a vector for transporting exogenous DNA to the oocyte (Lavitrano et al., 1989). A sperm endogenous nuclease activity that is triggered in a dose-dependent manner upon interaction of spermatozoa with foreign molecules has been identified as one barrier against intrusion of exogenous molecules (Maione et al., 1998). It would be reasonable to expect that mechanisms have evolved to protect the spermatozoa against the massive intrusion of foreign DNA molecules. These natural mechanisms against foreign DNA would result in a reduction in the ability of spermatozoa to efficiently act as carriers for exogenous DNA. Some authors have proposed sperm immobilization and nuclease activation (Maione et al., 1997; Smith, 2002; Alderson et al., 2006; Anzar and Buhr, 2006) as natural defenses activated in the sperm after the binding of exogenous DNA; also apoptosis has been proposed as a natural phenomenon to prevent the transmission of exogenous DNA to the next generation (Anzar and Buhr, 2006). However, clear studies about the effect of exogenous DNA on these parameters have not been reported in the bovine SMGT system. More information about bovine SMGT and the effect of exogenous DNA on sperm functionality would provide insight into the fertilizing capacity of sperm carrying exogenous DNA in conventional fertilization systems and would help to improve the efficiency of this technique.

For SMGT to be successful, the spermatozoa should incorporate or attach the exogenous DNA into the head as one critical step, but it is also necessary that the transfected spermatozoa keep their functionality to fertilize the oocyte. Some reports have proposed that a direct relationship exists between the binding of exogenous DNA to spermatozoa and the integrity of the plasma membrane (Perry et al., 1999; Anzar and Buhr, 2006) or capacitation status (Wang et al., 2003). It is known that capacitation involves changes in the distribution and composition of lipids, phospholipids (PL) and other molecules that trigger a destabilization process, and lead to an increase in the membrane fluidity and changes in its architecture, where cholesterol plays a key role (Cross, 1998; Flesch and Gadella, 2000; Cross, 2003) and this could impact the binding of exogenous DNA. However, it is also known that the destabilization process, named as ''capacitation status,'' occurs temporally near the acrosome reaction and total membrane degeneration, or cell death (Harrison, 1996; Gadella and Harrison, 2000).

Our purpose in this study was to investigate the capacity of bovine spermatozoa to carry exogenous DNA and the impact of this activity on sperm functionality. We studied these parameters using flow cytometry to measure viability (necrosis and apoptosis) and capacitation status, computer-assisted semen analysis (CASA) to measure motility parameters and in vitro fertilization (IVF) to assess fertilizing capacity. Furthermore, we studied the effect of capacitation status on the interaction with exogenous DNA, and the role of heparin supplementation in this process. The use of techniques such as flow cytometry and CASA allowed the evaluation of sperm functionality in a specific, objective, accurate, and reproducible manner compared to traditional microscopy-based methods (Graham, 2001; Verstegen et al., 2002).

## RESULTS

### Dynamics of Interaction Between Exogenous DNA and Bovine Spermatozoa

Bovine spermatozoa showed a particular binding profile for exogenous DNA, with a high capacity to bind quickly. After only 5 min of incubation, all bulls analyzed showed more than 20% of spermatozoa with exogenous DNA bound (Fig. 1A). Although significant differences between males were observed in the absolute capacity of DNA binding ($P < 0.01$), all of them followed similar dynamics, with a fast increase during the first 10 min and then a plateau with small oscillations at values close to 30% without significant differences after this time (Fig. 1B, Table 1).

During the incubation time with DNA, a reduction in viability was detected. Mean value for viable cells ranged from $78.69 \pm 1.51\%$ at 1 min to $73.59 \pm 1.91\%$ after 90 min of incubation, (Fig. 1B, $P < 0.01$). This could be due to the incubation time or represents a direct negative effect of the presence of DNA. To elucidate this question, Experiment 2 was designed and developed.

To understand the number/percentage of spermatozoa carrying exogenous DNA that could fertilize the oocyte, we calculated the parameter ''live spermatozoa with DNA bound'' with respect to the total spermatozoa. We found that $29.06 \pm 0.45\%$ of the cells were bound to DNA, and the percentage of live-DNA bound was $13.84 \pm 0.22\%$; the percentage for dead spermatozoa bound to DNA was $15.21 \pm 0.28\%$ (Table 1). Incubation time affected the total viability, but not the DNA binding parameters (Table 1). Although male effect is detected for all the parameters studied ($P < 0.01$, Table 1), all the bulls follow a similar pattern as is shown by interaction between bull and incubation time ($P \geq 0.72$, Table 1).

### Effect of Presence of Exogenous DNA on Sperm Viability, Motility, and In Vitro Fertilizing Capacity

In Experiment 1, we observed a reduction in viability during incubation with DNA over time, but we were not able to know if it was due to the time effect or DNA effect. In the present experiment, we confirmed that the incubation of bovine spermatozoa with exogenous DNA induced a decrease in the percentage of viable bovine spermatozoa versus the control group without DNA (mean values for DNA + group: $78.00 \pm 0.51\%$ vs. $79.86 \pm 0.45$ for control group, $P = 0.02$, Table 2, Fig. 2A). Moreover, mean values for sperm viability were also affected by the incubation time ($P < 0.01$, Table 2, Fig. 2A). Both factors have an independent effect on viability as is shown in the interaction ($P = 0.38$) and although differences between bulls were found ($P < 0.01$) the pattern of response to the treatment was similar for all of them (interactions bull–time and bull–treatment, $P > 0.05$).

Computer-assisted sperm analysis revealed that both the incubation of bovine spermatozoa with exogenous DNA and

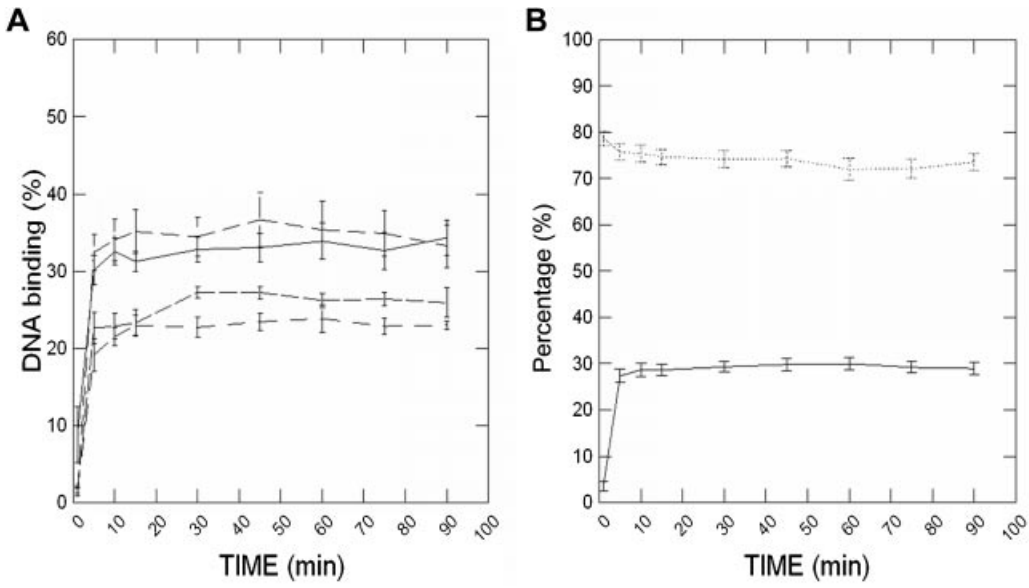


**Figure 1.** Kinetics of DNA binding between bull spermatozoa and exogenous DNA during a 90 min incubation, evaluated by flow cytometry. **A**: Percentage of DNA-bound spermatozoa for four bulls analyzed; each different line represents one male. **B**: Mean values for percentage of DNA-bound spermatozoa (continuous line) and percentage of viable spermatozoa (dotted line).

the duration of incubation affect the sperm motion parameters (Tables 2 and 3, Fig. 2). Time and presence of DNA are independent factors (interaction $>0.05$, Tables 2 and 3). Some motion parameters like VSL, VAP, LIN, and SRT were also reduced after incubation in the presence of DNA (Table 3, $P < 0.05$). Incubation induces a reduction in the values of viability and semen motion parameters. Specifically total motility, progressive motility, VAP, and VSL parameters suffered a decrease after 30 min of incubation (Fig. 2; control group).

In the IVF experiment, the results showed that bovine sperm incubated with exogenous DNA can fertilize oocytes and this treatment did not significantly affect the cleavage rate (control 81.43% vs. DNA 78.14%; $P = 0.38$). When the EGFP expression was evaluated in the putative embryos at 48 hr post-insemination, no fluorescence signal was detected.

## Effect of Sperm Capacitation Status on Exogenous DNA Binding Capacity

We established two groups of bovine spermatozoa, under the same conditions and origin with or without heparin, and they showed different values of merocyanine 540 fluorescence (mean values 8.67% vs. 28.25%; $P < 0.001$). The higher value represents a greater frequency of sperm with high lipid disorder (HLD). Under these conditions, we observed that the presence of heparin had the effect of increasing the total number of sperm with DNA bound (mean value $14.54 \pm 0.64\%$ vs. $21.11 \pm 0.71\%$, $P < 0.01$). This difference is more evident after 30 min of incubation. In the absence of heparin, the binding percentage increased until 30 min and then maintained a plateau near 20% (Fig. 3A). With heparin present in the media, however, the binding percentage increased during the whole incubation period and was significantly different after 60 min

**TABLE 1. Mean Values (Percentage) for Sperm Viability and DNA Binding to the Spermatozoa During 85 min of Incubation, in the Time Period Between 5 and 90 min, Measured by Flow Cytometry**

| | Live spermatozoa (%) | DNA-bound spermatozoa (%) | DNA-bound and live spermatozoa (%) | DNA-bound and dead spermatozoa (%) |
|---|---|---|---|---|
| Bull | | | | |
| A | $61.46 \pm 1.11^{a}$ | $32.56 \pm 0.62^{a}$ | $14.03 \pm 0.31^{a}$ | $18.53 \pm 0.41^{a}$ |
| C | $82.95 \pm 0.37^{b}$ | $22.60 \pm 0.43^{b}$ | $11.32 \pm 0.22^{b}$ | $11.29 \pm 0.26^{b}$ |
| G | $71.48 \pm 1.05^{c}$ | $34.50 \pm 0.94^{a}$ | $17.42 \pm 0.43^{c}$ | $17.08 \pm 0.64^{a}$ |
| S | $81.16 \pm 0.32^{b}$ | $25.54 \pm 0.47^{c}$ | $12.12 \pm 0.29^{d}$ | $13.43 \pm 0.27^{c}$ |
| Mean | $73.93 \pm 0.67$ | $29.06 \pm 0.45$ | $13.84 \pm 0.22$ | $15.21 \pm 0.28$ |
| ANOVA, source of variation | | | | |
| Bull | $<0.01$ | $<0.01$ | $<0.01$ | $<0.01$ |
| Time | 0.01 | 0.15 | 0.07 | 0.54 |
| Interaction | 0.72 | 0.99 | 0.99 | 0.94 |

Numbers within columns with different superscripts differ ($P < 0.05$). A, C, G, and S are the names for the different bulls.

**TABLE 2. Mean Values for Seminal Parameters After Incubation in the Presence (+) or Absence (−) of DNA During 5–60 min of Incubation**

| Bull | DNA | Live spermatozoa (%) | Progressive motility (%) | Motility (%) |
|---|---|---|---|---|
| C | − | 74.09 ± 0.47 | 66.56 ± 1.66 | 68.90 ± 1.49 |
| C | + | 70.70 ± 0.71 | 59.50 ± 1.61 | 61.90 ± 1.60 |
| G | − | 75.22 ± 0.57 | 67.22 ± 0.92 | 69.60 ± 0.83 |
| G | + | 73.03 ± 0.55 | 57.02 ± 1.01 | 59.87 ± 0.98 |
| P | − | 83.89 ± 0.57 | 74.50 ± 0.61 | 76.50 ± 0.63 |
| P | + | 81.95 ± 0.62 | 63.02 ± 1.43 | 66.25 ± 1.32 |
| Pr | − | 85.34 ± 0.43 | 67.97 ± 0.90 | 70.41 ± 0.97 |
| Pr | + | 84.45 ± 0.45 | 61.43 ± 1.34 | 64.70 ± 1.21 |
| Mean | − | 79.86 ± 0.45[a] | 69.07 ± 0.62[a] | 71.36 ± 0.57[a] |
| Mean | + | 78.00 ± 0.51[b] | 60.16 ± 0.71[b] | 63.08 ± 0.69[b] |

| | Live spermatozoa (%) | Progressive motility | Motility |
|---|---|---|---|
| ANOVA, source of variation | | | |
| DNA | 0.02 | 0.01 | 0.01 |
| Time | <0.01 | <0.01 | <0.01 |
| DNA × time | 0.38 | 0.12 | 0.06 |
| Bull | <0.01 | 0.05 | 0.03 |

Numbers within columns with different superscripts differ ($P < 0.05$). C, G, P, and Pr are the names for the different bulls.

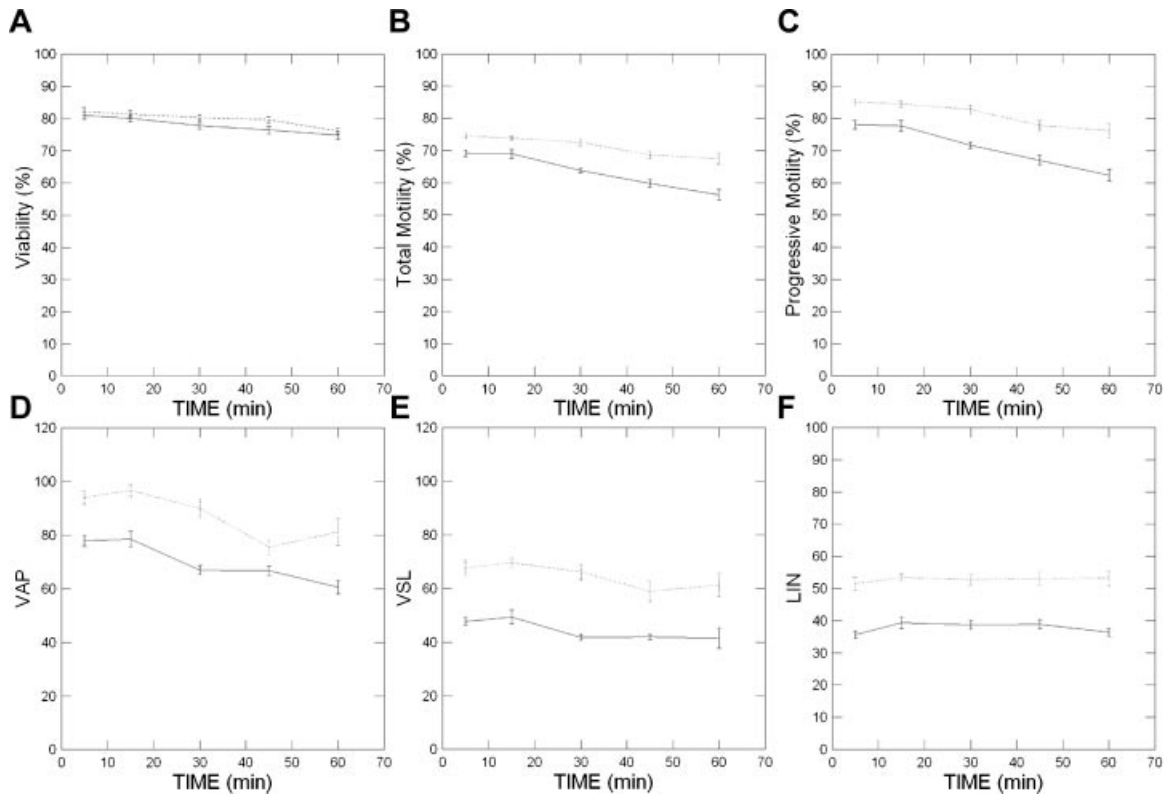


**Figure 2.** Effect of the presence of DNA in the incubation media on the sperm functionality. **A**: Viability (%); (**B**) total motility (%); (**C**) progressive motility (%); (**D**) VAP, average path velocity (μm/sec); (**E**) VSL, straight-line velocity (μm/sec); (**F**) LIN, linearity of the curvilinear trajectory (%); control group (dotted line) and DNA group (continuous line).

**TABLE 3. Mean Values for Sperm Motility Parameters After Incubation in the Absence (Control) or Presence of DNA During 55 min of Incubation, Measured in the Time Period Between 5 and 60 min**

| Group | VCL | VSL | VAP | LIN | STR | WOB | ALH | BCF |
|---|---|---|---|---|---|---|---|---|
| Control | 121.64 ± 1.98 | 64.80 ± 1.46[a] | 89.31 ± 1.73[a] | 52.86 ± 0.83[a] | 71.69 ± 0.67[a] | 72.64 ± 0.66 | 4.00 ± 0.08 | 6.58 ± 0.09 |
| DNA | 113.84 ± 1.79 | 44.06 ± 1.04[b] | 69.39 ± 1.12[b] | 37.96 ± 0.64[b] | 61.68 ± 0.51[b] | 66.33 ± 5.43 | 4.41 ± 0.09 | 6.28 ± 0.10 |

| | VCL | VSL | VAP | LIN | STR | WOB | ALH | BCF |
|---|---|---|---|---|---|---|---|---|
| ANOVA, source of variation | | | | | | | | |
| DNA | 0.48 | 0.03 | 0.04 | 0.02 | 0.02 | 0.15 | 0.21 | 0.21 |
| Time | 0.01 | 0.02 | 0.02 | 0.75 | 0.63 | 0.60 | 0.01 | 0.63 |
| DNA × time | 0.38 | 0.85 | 0.60 | 0.94 | 0.98 | 0.49 | 0.30 | 0.93 |
| Bull | 0.65 | 0.31 | 0.27 | 0.22 | 0.31 | 0.19 | 0.37 | 0.45 |

Numbers within columns with different superscripts differ ($P < 0.05$). VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF are CASA-derived kinematic parameters (see details in Material and methods).

(21.47 ± 1.25% vs. 32.47 ± 2.43%, $P < 0.01$, Fig. 3A). However, the heparin treatment also decreased the percentage of viable cells during the total period studied (Fig. 3B, $P < 0.01$).

To determine specifically whether the increase in the total number of sperm with DNA bound was due to an increase in the number of live or dead (unviable) cells with DNA bound, we analyzed binding and viability simultaneously. The results showed that heparin increased the proportion of both groups, viable (5.91 ± 0.28% vs. 9.47 ± 0.31%; $P < 0.01$) and dead cells (8.63 ± 0.41% vs. 11.64 ± 0.45%; $P < 0.01$) with DNA bound. That indicates that at 60 min, the percentage of viable spermatozoa carrying DNA is increased by heparin to 8.16 ± 0.69% in control group to 12.94 ± 1.18% ($P < 0.01$, Fig. 3C).

Heparin treatment was useful to induce capacitation, as the results showed an increase in the percentage of HLD taken as an indication of capacitation having occurred, as expected. The most interesting results are related to the increase in the percentage of cells with HLD (capacitated) that was observed in the heparin group ($P < 0.01$, Fig. 3D) and the similar increase observed for viable sperm bound to DNA during the incubation time (Fig. 3C,D).

The results confirm that in bull spermatozoa, heparin increases the lipid disorder of the membrane and can increase the proportion of viable bovine spermatozoa with DNA bound.

### Effect of Presence of Exogenous DNA on Sperm Apoptosis

With the combined use of Yo-Pro®-1 and propidium iodide (PI), we studied apoptosis in bovine spermatozoa and the effect of exogenous DNA incubation at 5 and 15°C (Fig. 4). We confirmed that the presence of DNA in the media and the duration of incubation reduced the viability of the sperm at 15 and 5°C as two independent factors (Table 4 and Fig. 5). As was expected, the number of dead cells was higher in the group at 5 versus 15°C.

With respect to the values for early apoptosis, the apoptotic cells represented a low percentage in all the experimental groups (<10%); nevertheless, the flow cytometry technique is a very accurate methodology that allowed us to count a high number of cells in short time, permitting detection of significant differences between groups. In our study, the presence of DNA increased the proportion of apoptotic cells at 5 and 15°C, but during the incubation period the percentage of apoptotic cells decreases as the same time as the percentage of dead cells increases.

## DISCUSSION

SMGT is a technique for producing transgenic animals with huge potential in different fields, including modeling human disease and agricultural applications (revised by Lavitrano et al., 2006; Robl et al., 2007; Niu and Liang, 2008). In the case of bovine SMGT, binding of exogenous DNA to spermatozoa was first showed in 1990 (Castro et al., 1990) with subsequent reports elucidating additional aspects of the process (Atkinson et al., 1991; Gagne et al., 1991; Camaioni et al., 1992; Schellander et al., 1995; Sperandio et al., 1996; Alderson et al., 2006; Hoelker et al., 2007; Feitosa et al., 2009).

Several hypotheses have emerged that are leading us closer to an understanding of the impact of exogenous DNA on sperm functionality (Maione et al., 1997; Smith, 2002; Alderson et al., 2006; Anzar and Buhr, 2006). However, detailed understanding of this process at the molecular level remains to be completed. To the best of our knowledge, the present study of bovine SMGT is the first one that analyzes the dynamics of binding and viability simultaneously, using flow cytometry. It allows us to know the number of live spermatozoa carrying exogenous DNA that could fertilize in conventional IVF or AI systems. The current study showed that live spermatozoa can also bind exogenous DNA to a maximum of around 15% (Table 1) of the sperm population, in contrast with previous reports in pigs (Garcia-Vazquez et al., 2009a,b) showed that most of the exogenous DNA bound to dead or altered cells, a difference that could reflect different DNA binding patterns between species.

Previous studies considered that sperm DNA binding could require an incubation time longer than 30 min (Castro et al., 1990; Camaioni et al., 1992; Sperandio et al., 1996); however, our results showed that bovine spermatozoa were able to bind exogenous DNA much more

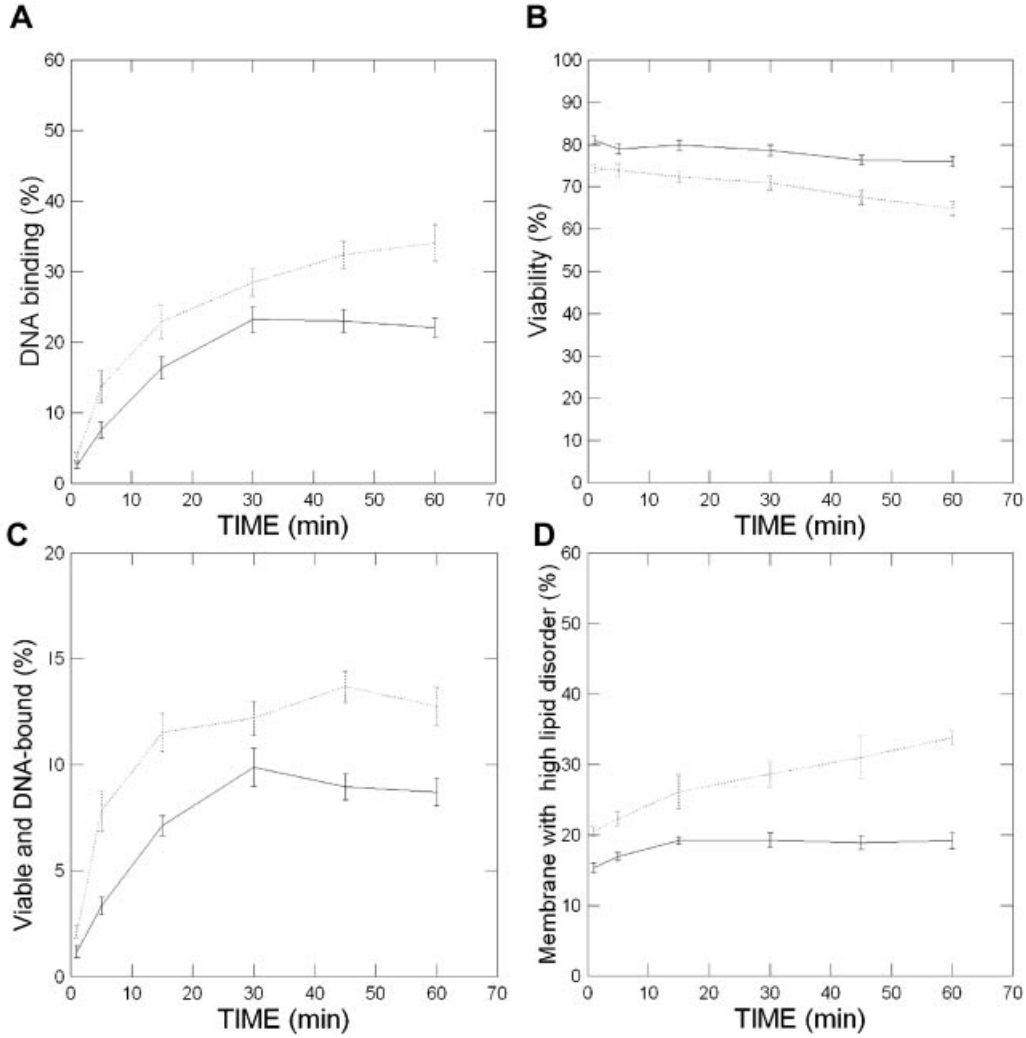


**Figure 3.** Kinetics of DNA binding between bull spermatozoa and exogenous DNA during a 60 min incubation, evaluated by flow cytometry in the presence (heparin group, dotted line) or absence (control group, continuous line) of heparin in the Sperm-TALP media. **A**: Percentage of DNA-bound spermatozoa; (**B**) percentage of viable spermatozoa; (**C**) percentage of viable spermatozoa with DNA bound; (**D**) percentage of spermatozoa with high lipid membrane disorder.

rapidly, and that in 30 min, the binding process was complete. These results suggest that long incubations with exogenous DNA before fertilization is not necessary for maximal fertilization results, particularly when considering that prolonged incubation negatively affects sperm viability. However, it is well known that the efficiency of the binding depends on the kind of construct used and the amount of DNA used (Sperandio et al., 1996; Lavitrano et al., 2003). In future studies, the effects detected with this construct CVM-EGFP must be compared with others under the same conditions to know if it is specific for this construct.

We also observed that the pattern of binding was highly male-dependent. Significant differences between males were observed in the absolute capacity of sperm DNA binding, although the dynamics of binding followed a similar pattern. In contrast, previous authors (Anzar and Buhr, 2006) did not find that donor-dependent spontaneous uptake of DNA, but they used fluorescent microscopy to evaluate a very limited numbers of cells. Our results are closer to agreement with results seen in earlier reports using pigs (Lavitrano et al., 2006; Kang et al., 2008) that showed that sperm cells from boars differed greatly in their capacity to take up exogenous DNA, although it was also observed that the dynamics were similar between males (Lavitrano et al., 2006).

Although nearly 100 papers have been published concerning SMGT over the last 20 years, the effect of exogenous DNA on sperm functionality remains unclear. Motility is a special characteristic of spermatozoa that is absolutely necessary for its biological function. A putative negative effect of DNA binding on motility could explain the failure of SMGT reported by numerous authors. Previous studies

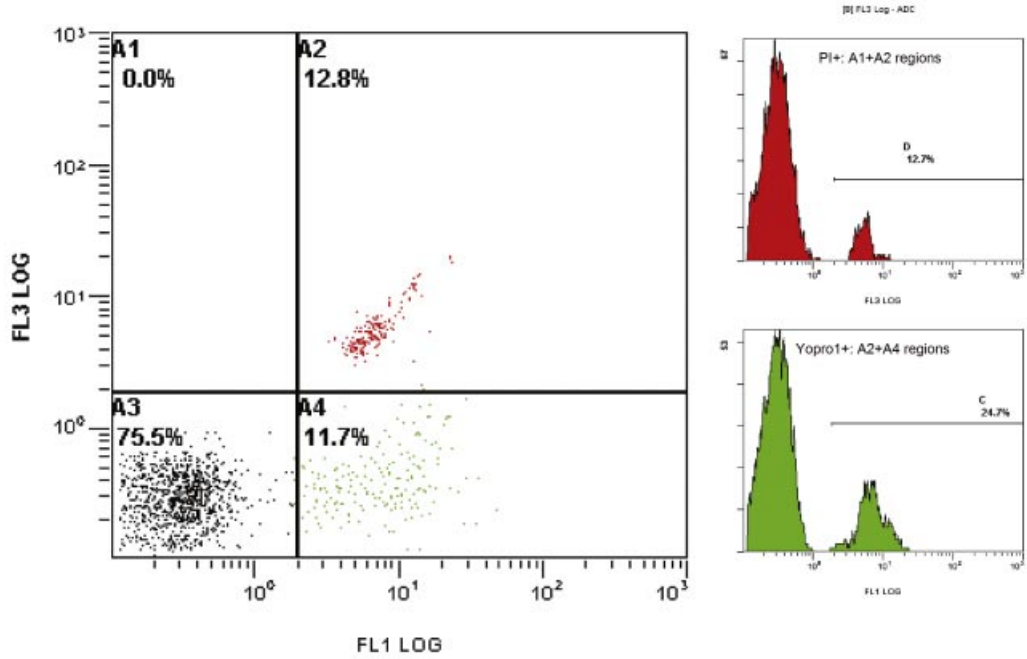


**Figure 4.** Flow cytometric plotting (left) showing Yo-Pro1 (FL1, green) expression plotted against propidium iodide (FL3, red). Three different populations were identified using channels FL1 (Yo-Pro1: green) and FL3 (propidium iodide, PI: red). A2 region: double staining (PI and Yo-Pro1 positives cells), dead cells; A3 region: no stained cells, viable cells. A4 region: Yo-Pro1 positive cells but no PI staining, apoptotic cells. Right-hand histograms show a one-color plot of PI:FL3 (upper) or Yo-pro1:FL1 (below) with low and high staining populations. PI high staining population correspond to region A1 plus A2 and Yo-Pro1 high staining population is equivalent to region A2 plus A4 of the plotting.

**TABLE 4. Effect of the Presence of DNA in the Incubation Media on Sperm Apoptosis and Death**

| DNA | Temperature | Viable cells (%) | Apoptotic cells (%) | Death cells (%) |
|---|---|---|---|---|
| DNA | 15 | $49.96 \pm 0.83^{a}$ | $4.43 \pm 0.24^{a}$ | $45.61 \pm 0.70^{a}$ |
| Control | 15 | $55.08 \pm 0.84^{b}$ | $2.27 \pm 0.24^{b}$ | $42.65 \pm 0.72^{b}$ |

| | Viable cells (%) | Apoptotic cells (%) | Death cells (%) |
|---|---|---|---|
| ANOVA, source of variation | | | |
| DNA | <0.01 | <0.01 | <0.01 |
| Time | 0.19 | <0.01 | <0.01 |
| DNA × time | 0.83 | 0.43 | 0.98 |

| DNA | Temperature | Viable cells (%) | Apoptotic cells (%) | Death cells (%) |
|---|---|---|---|---|
| DNA | 5 | $19.86 \pm 0.49^{x}$ | $5.54 \pm 0.42^{x}$ | $74.59 \pm 0.71$ |
| Control | 5 | $22.44 \pm 0.59^{y}$ | $3.51 \pm 0.35^{y}$ | $74.05 \pm 0.80$ |

| | Viable cells (%) | Apoptotic cells (%) | Death cells (%) |
|---|---|---|---|
| ANOVA, source of variation | | | |
| DNA | <0.01 | <0.01 | 0.21 |
| Time | <0.01 | <0.01 | <0.01 |
| DNA × time | 0.39 | 0.65 | 0.29 |

Sperm incubated at 15 (upper) and 5°C (lower). Mean values for viable, apoptotic and dead cells for 55 min of incubation, measured in the time period between 5 and 60 min. Numbers within columns with different superscripts differ ($P < 0.05$).

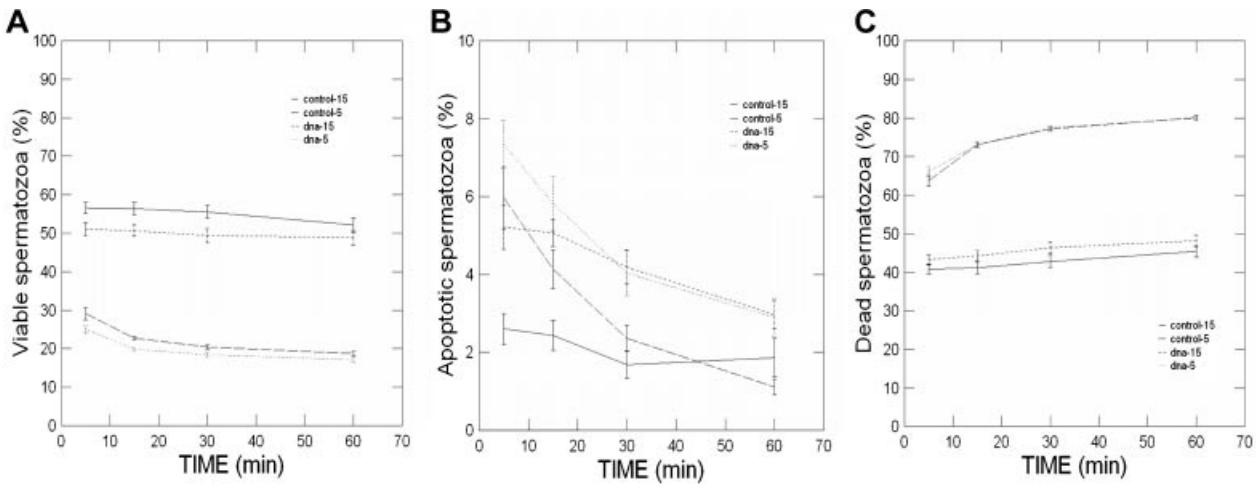


**Figure 5.** Effect of the presence of DNA in the incubation media on sperm apoptosis and cell death. Sperm were incubated for 60 min at 5 (control -5 and dna-5) and 15°C (control-15 and dna-15). The labels dna-5 (· · · · ·) and dna-15 (- - -) correspond to the groups incubated with DNA at 5 or 15°C, respectively. Control-5 (———) and control-15 (—) represent the groups incubated without DNA at 5 or 15°C, respectively. **A**: Viable spermatozoa (%). **B**: Apoptotic spermatozoa (%). **C**: Dead spermatozoa (%).

reported opposing results regarding a DNA effect: one group of works described a negative effect of the exogenous DNA on the motility of bovine spermatozoa (Schellander et al., 1995; Anzar and Buhr, 2006; Feitosa et al., 2009), while others did not report any effect (Rieth et al., 2000; Alderson et al., 2006). This could be partially explained by the variations between observers during subjective assessments. In our study, the reproducibility and objectivity of measurements by CASA allowed us to affirm that incubation with exogenous DNA decreases the total and progressive motility in bull spermatozoa (Table 2). Moreover, VAP, LIN, STR, and VSL motion parameters (Table 3) were reduced as well. Total and progressive motility are two parameters commonly related to the fertilizing ability of sperm, and it has been proposed that straight-line velocity (VSL) may play a role in sperm transport through the female reproductive tract and penetration of the oocyte vestments (Gillan et al., 2008). Nevertheless, although the registered decline in these parameters may have some impact on sperm fertility, in our opinion it would not be enough to totally compromise fertilizing potential.

Using flow cytometry, our results showed that incubation with exogenous DNA induced a slightly negative effect on sperm viability. In contrast, recently Feitosa et al. (2009) did not report any DNA effect regarding viability using microscopy examination. The negative effect regarding motility and viability, which was higher after a long incubation time, could partially explain the frequent failure of IVF and AI with exogenous DNA reported by some authors. Sperm motility and viability are very sensitive properties as they could be affected by chemical agents, temperature, light, and other factors (reviewed by Vishwanath and Shannon, 1997).

Success using SMGT was only reported in some papers using techniques that require the spermatozoa to maintain full functionality to reach the oocyte during AI (Perez et al., 1991; Schellander et al., 1995; Sperandio et al., 1996) or IVF (Shemesh et al., 2000). Two hypotheses could explain this fact. Firstly, that sperm incubated with exogenous DNA have decreased fertilizing capacity and they can not fertilize. However, in our work, sperm incubated with exogenous DNA did not show a significant decrease in the fertilizing potential using cleavage rate as marker. Moreover, others authors (Rieth et al., 2000; Hoelker et al., 2007) showed no differences in embryo production between sperm incubated with or without exogenous DNA. Another possible explanation, which seems more feasible, could be that sperm carrying exogenous DNA are at a disadvantage when fertilizing the oocyte compared to sperm without DNA. The apoptotic cascade that is triggered after contact with exogenous DNA could be one such disadvantage. In this case, the majority of oocytes would be fertilized by sperm without exogenous DNA, resulting in a high percentage of nontransgenic embryos.

To analyze whether or not the destabilization of the plasma membrane (increase of HLD) that occurs during capacitation could affect DNA binding, we used heparin supplementation to induce an increase in HLD in sperm membranes and to thereby trigger capacitation. Our results showed that heparin increased the percentage of sperm with HLD and this population, which is starting the capacitation process, can bind exogenous DNA at higher rates. Moreover, for the first time, we showed that heparin supplementation increases the percentage of viable spermatozoa with DNA bound, a finding that is potentially of great value to improving the success of IVF or AI systems using SMGT. In contrast, other reports showed in bull (Camaioni et al., 1992), rabbit (Kuznetsov et al., 2000) and *Xenopus laevis* (Jonak, 2000) spermatozoa, that heparin decreased the DNA binding capacity. They proposed that glycosaminoglycans (such as heparin) present in the seminal plasma might interfere with the binding of foreign DNA to spermatozoa. Camaioni and coworkers (Camaioni et al., 1992) incubated

the bull spermatozoa with heparin 30 min before the addition of DNA. However, we combined spermatozoa, heparin, and DNA simultaneously, which could explain the different outcomes given that the DNA binding process occurs very quickly according to our results.

During sperm-mediated gene transfer in mice, Maione et al. (1997) reported the activation of endogenous nucleases in mature sperm cells, which cleaved exogenous and genomic DNA and could activate apoptosis, a physiological cell death mechanism that can be initiated by many stimuli from outside or inside the cell (Marti et al., 2008). In bull spermatozoa, different authors have reported the expression of apoptotic markers in response to stress such as the cryopreservation process (Anzar et al., 2002; Martin et al., 2004; Chaveiro et al., 2007). Our results showed that the incubation of bull spermatozoa with exogenous DNA specifically increased the rate of apoptotic spermatozoa (Yo-Pro-1 positive). The results obtained in the control group were slightly lower that previously described (Martin et al., 2004). The observed increase in apoptosis could be a consequence of the activation of the Fas apoptotic pathway. Although different pathways participate in the apoptotic process, Fas/Fas Ligand is one of the best known and its presence has been reported in bull spermatozoa (Meggiolaro et al., 2006; Porcelli et al., 2006). Moreover, this pathway has been implicated in the increased sensitivity to apoptosis in fibroblasts after plasmid transfection or simple exposure to naked DNA, which induce its upregulation (de Carvalho Bittencourt et al., 2002). If involvement of Fas/Fas L apoptotic activity is confirmed in the near future, it would be interesting to investigate the effect of different factors regarding this pathway and its possible relationship with the success of SMGT in IVF or AI systems.

In conclusion, this study showed a slight negative effect of exogenous DNA on viability, motility and apoptosis in bull spermatozoa but it did not dramatically compromise fertilizing potential. The kinetic study reported that DNA binding happens quickly in bovine sperm and that live spermatozoa can carry exogenous DNA, making it possible to use SMGT in IVF or AI systems. Moreover, this population—live spermatozoa with DNA bound—can increase with heparin supplementation, possibly due to HLD of the sperm membrane favoring DNA binding in viable sperm. Culture conditions that allow reaching a high proportion of viable and capacitated cells to be reached during a reasonable time period could be useful to improve the output of SMGT IVF or artificial insemination systems.

## MATERIALS AND METHODS

### Media and Chemicals

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma–Aldrich Quimica S.A. (Madrid, Spain).

### Transgene Construction and Labeling

The enhanced green fluorescent protein (EGFP) plasmid construct (pEGFPN1, 5.4 kb; Clontech Laboratories, Inc., Palo Alto, CA) used for our experiments containing the human cytomegalovirus (CMV) immediate early promoter and the enhanced GFP gene. This construct was linearized with AflII (gene from Anabaena flos-aquae) prior to use. The transgene was purified using an Elu-Quit DNA Purification Kit (Schleicher & Schuell, Dassel, Germany) following the manufacturer's instructions. DNA was resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 8).

Linear plasmid was labeled with fluorescein-12-dUTP (Roche, Mannheim, Germany). DNA labeling was carried out by means of random primer. Briefly, mold DNA (linearized plasmid) was denatured by incubation at 95°C for 5 min; nucleotides (1 mM of dATP, dCTP, and dCTP; 0.65 mM of dTTP; and 0.35 mM of fluorescein-12-dUTP marked nucleotide) were added later as well as 5× buffer and 1 U/μl Klenow (Promega, Madrid, Spain). Subsequently, the mixture was incubated for an hour at 37°C, and the reaction was stopped by adding 2 μl of 0.2 MEDTA (pH 8). Marked DNA was precipitated out with cold ethanol and finally resuspended in TE microinjection buffer. Verification of plasmid marking was determined in an agarose and dying gel with ethidium bromide. The incorporation of the labeled nucleotide to synthesized DNA diminishes its electrophoretic mobility in relation to nonmarked DNA (Gutierrez-Adan and Pintado, 2000).

### Preparation and Incubation of Spermatozoa With Exogenous DNA

Frozen bull spermatozoa from fertile bulls of autochthonous Spanish breed "Asturiana de los Valles" generously provided by the Breeders Association (ASEAVA, Gijón, Asturias, Spain) were used. Frozen semen samples (0.5 ml straws) were thawed in a water bath at 37°C for 30 sec and the content was diluted in Tyrode's sperm medium (sperm-TALP; Parrish et al., 1988). This was then subjected to a gradient separation by centrifugation on a 45/90 discontinuous Percoll® gradient (Pharmacia, Uppsala, Sweden) for 10 min at 900*g*. The pellet was resuspended in 10 ml Sperm-TALP medium (Parrish et al., 1986) and washed again for 8 min at 300*g*. The final pellet was resuspended in Sperm-TALP medium and sperm concentration adjusted to $10^8$ cells/ml. Then, spermatozoa were incubated with linearized EGFP transgene ($10^8$ cells/ml and 5 μg DNA/ml) in Sperm-TALP medium. The control group was incubated under the same conditions but without DNA.

### Analysis of the Motion Parameters

Motion parameters were determined using a CASA system (Sperm Class Analyzer, Microptic, Barcelona, Spain) following the methodology previously described for bull spermatozoa (Gadea et al., 2008).

The CASA-derived kinematic parameters recorded for each spermatozoon were total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross-frequency (BCF, Hz).

A 7 μl drop of the sample was placed on a warmed (37°C) slide and covered with a 24 × 24 $mm^2$ cover slip. The setting parameters were: 25 frames in which spermatozoa had to be present in at least 15 in order to be counted, images were obtained at 200× magnification using a contrast phase microscope. Spermatozoa with a VAP < 20 ml/sec were considered immotile. A minimum of five fields per sample was evaluated, counting a minimum of 200 spermatozoa per sub-sample.

### Analysis of Seminal Parameters by Flow Cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter, Inc., Miami, FL). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from

10,000 events per sample were collected in list mode, and three measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter, Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

### In Vitro Oocytes Maturation and In Vitro Fertilization

In vitro oocyte maturation and IVF were made as we have previously described (Canovas et al., 2009), with minor modifications. Groups of 30–40 mature bovine oocytes were transferred to wells containing IVF media and 25 μl of penicillamine–hypotaurine–epinephrine mix was added to each well ~30 min before insemination.

Spermatozoa were obtained by centrifugation of two 0.5 ml straws of frozen–thawed semen on a 45/90 discontinuous Percoll gradient. Sperm were incubated with or without exogenous DNA for 30 min in the same conditions reported previously. Sperm concentration was adjusted, and cells added at a final concentration of $1.5 \times 10^6$ spermatozoa/ml to the wells containing the oocytes. At 18–20 hr post-insemination, putative zygotes were washed to remove loosely attached sperm and cultured in fresh medium until 48 hr post-insemination. Then, putative zygotes were examined under stereomicroscope to measure the cleavage rate. Using epifluorescence microscopy, the EGFP expression was also examined in the embryos.

### DNA Binding Capacities and Cellular Viability Assessment

Spermatozoa were incubated with linear plasmid labeled with fluorescein (FITC-DNA), as previously described above and simultaneously stained with PI (final concentration 2.5 μg/ml) for evaluating DNA binding capacities and cellular viability (Garcia-Vazquez et al., 2009a). Fluorescence was measured using an FL-1 sensor, a 525-nm band-pass filter to detect FITC-DNA, an FL-3 sensor, and a 575-nm band-pass filter to detect PI. Cells were classified into four categories: (1) living spermatozoa without DNA bound (no sign of fluorescence), (2) living spermatozoa with DNA bound (only green fluorescence), (3) dead spermatozoa with DNA bound (red and green fluorescence), and (4) dead spermatozoa without DNA bound (red fluorescence).

This methodology has been validated with fluorescent microscope observation and the use of multispectral imaging flow cytometry (ImageStream; Amnis Corporation, Seattle, WA), a combination of quantitative image analysis and flow cytometry (unpublished data).

### Assessment of Plasma Membrane Lipid Packing Disorder

To detect an increase in plasma membrane lipid packing disorder, sperm samples were stained with Merocyanine 540 (M540) and Yo-Pro 1 (Yo-Pro®-1 iodide) (Harrison et al., 1996; Gadea et al., 2008). Stock solutions of M540 (1 mm) and Yo-Pro 1 (25 μm; Molecular Probes, Eugene, OR) in DMSO, were prepared. For each 1 ml diluted semen sample, 2.7 μl M540 stock solution (final concentration of 2.7 μm) and 1 μl of Yo-Pro (25 nm final concentration) were added. M540 fluorescence was collected with an FL2 sensor using a 575 nm band-pass filter and Yo-Pro 1 with an FL1 sensor using a 525 nm band-pass filter. Cells were classified into three categories: (1) low merocyanine fluorescence (viable, low disorder, no sign of fluorescence), high merocyanine fluorescence (viable, high disorder, only red fluorescence) or Yo-Pro-1 positive (dead, green fluorescence).

### Assessment of Apoptosis Incidence

In apoptotic cells during the degradation phase, the cytoplasmic membrane becomes slightly permeable to Yo-Pro-1 green fluorochrome, but it stays impermeable to PI. Thus, combined Yo-Pro-1 and PI dyes have been used as an indicator for apoptosis in bull spermatozoa (Martin et al., 2004, 2007). The sperm cells were incubated with PI (2.5 μg/ml final concentration) and Yo-Pro-1 (50 nM final concentration). The samples were gently mixed and incubated for 30 min in darkness at room temperature before flow cytometry analysis. Cells were classified into three categories: (1) necrotic cells, labeled with PI (red fluorescence); (2) viable cells without apoptosis marker, with low Yo-Pro-1 permeability membranes (no sign of fluorescence); and (3) apoptotic cells (viable cells with modified membranes that are permeable only for Yo-Pro-1, only green fluorescence).

### Statistical Analysis

Data are expressed as the mean ± SEM and analyzed by ANOVA, considering the sperm addition of exogenous DNA, temperature of incubation, time of incubation and bulls, as the main variables. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test. Differences were considered statistically significant at $P < 0.05$.

### Experimental Design

**Dynamics of interaction between exogenous DNA and bovine spermatozoa** Our goal was to evaluate the DNA binding capacity of bovine spermatozoa and the sperm viability simultaneously. With this approach, we sought to answer the question of when (dynamics) and what kind of cells (viable or not viable) bind the exogenous DNA.

Frozen-thawed semen samples from four fertility-tested bulls were processed by a Percoll gradient, diluted on Sperm-TALP medium and incubated with exogenous DNA at 15°C. DNA binding (labeled with FITC) and sperm viability (stained by PI) were simultaneously measured by flow cytometry after 1, 5, 10, 15, 30, 45, 60, 75, and 90 min of incubation. Cells were classified into four categories according to viability and DNA binding capacity. Three replicates per bull were analyzed.

**Effect of presence of exogenous DNA on sperm viability, motility, and in vitro fertilizing capacity** Semen samples from four fertility-tested bulls were processed by a Percoll gradient, diluted on Sperm-TALP medium and incubated with or without exogenous DNA at 15°C. Then, viability (by PI staining and flow cytometry measurements) and motility parameters (by CASA) were evaluated after 5, 15, 30, 45, and 60 min of incubation.

For the IVF experiment, the semen samples were processed by a Percoll gradient and incubated with or without exogenous DNA before being used in our standard IVF system (Canovas et al., 2009). In 3 replicates, 237 oocytes in the control group and 215 oocytes in the DNA group were inseminated. Cleavege rate and EGFP fluorescence expression were measured in the putative embryos at 48 hr post-insemination. In this experiment, pools of frozen samples from three different bulls were used together to avoid the individual effect.

**Effect of sperm capacitation status on exogenous DNA binding capacity** We determined if the capacitation status, measured in terms of sperm membrane lipid disorder, modulated the dynamics of DNA binding in frozen-thawed bull spermatozoa. For this analysis, frozen-thawed samples were selected by a Percoll gradient and diluted in Sperm-Talp medium (Parrish et al., 1986) containing 1.75 UI/ml heparin (Heparin +) that induces the sperm capacitation, or diluted in Sperm-Talp without heparin (Heparin −) where the capacitation process would occur in a slower manner

(Bergqvist et al., 2007). Then, semen samples were incubated at 15°C in the presence of DNA plasmid labeled with FITC (binding and viability measurements) or unlabelled DNA (membrane lipid disorder measurements) and evaluated by flow cytometry at 1, 5, 15, 30, 45, and 60 min of incubation. It is well known that the heparin effects can be different between bulls (Parrish et al., 1986, 1988; Blottner et al., 1990). For this reason, we used pools of frozen samples from three different bulls to avoid the individual effect; six replicates were analyzed.

**Effect of presence of exogenous DNA on sperm apoptosis** Semen samples were processed by a Percoll gradient, diluted on Sperm-Talp medium and incubated with or without exogenous DNA at 5 and 15°C. Then, membrane permeability to Yo-Pro-1 was measured as an apoptosis index after 5, 15, 30, and 60 min of incubation. In this experiment, pools of frozen samples from three different bulls were used together to avoid the individual effect; four replicates were analyzed.

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