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Abstract: In cattle, cryopreservation of semen and sex-sorting kill up to 50% of spermatozoa and decrease the success of assisted insemination (AI). Therefore, significant efforts are being carried out to improve the quality of semen prior to AI. In this work we used the Cell-SELEX technique to select single strand DNA aptamers able to recognize with high affinity and specificity damaged sperm cells generated by heat-treatment. We first isolated aptamers with a conserved two motifs of 6 nucleotides of length that bind to the membrane of heat-treated spermatozoa. Then, we used synthetic biotin-labeled aptamers containing the conserved motif to recognize membrane-damaged cells and separate them from viable cells by the use of avidin-coated superparamagnetic iron oxide nanoparticles (SPION). This procedure improved the quality of semen by significantly increasing the percentage of healthy sperm cells without affecting the rate of blastocyst cleavage. This technique was successfully applied to both unsorted and sex-sorted sperm suspension.

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Dear Editors
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We are submitting our manuscript "**IMPROVEMENT OF BOVINE SEMEN QUALITY BY REMOVAL OF MEMBRANE-DAMAGED SPERM CELLS WITH DNA APTAMERS AND MAGNETIC NANOPARTICLES**" to be considered for publication in Journal or Biotechnology.

This manuscript describes the use of a novel aptamer-based procedure coupled to the use of superparamagnetic iron oxide nanoparticles for the removal of membrane-damaged sperm cells from bovine sperm suspensions. This technique improved the quality of both unsexed and sex-sorted sperm demonstrating a potential application in *in vitro* fertilization procedures that are widely used in reproduction programs. We believe this manuscript will be of interest to the readers of the Journal or Biotechnology.

This manuscript has not been submitted elsewhere and the authors declare no conflict of interest.

As the corresponding author, I take full responsibility for the analyses, interpretation and conduct of the research. I have full access to the data and have the right to publish all the data included in this study.

Thanks in advance for your consideration.

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Highlights (for review)

- DNA aptamers recognize membrane-damaged bovine sperm cells
- Superparamagnetic iron oxide nanoparticles remove aptamer-bound damaged cells from sperm suspensions
- Improved semen quality was achieved in both unsorted and sex-sorted bovine sperm suspensions
- Removal of damaged sperm cells with DNA aptamers and nanoparticles does not affect embryo development

IMPROVEMENT OF BOVINE SEMEN QUALITY BY REMOVAL OF MEMBRANE-DAMAGED SPERM CELLS WITH DNA APTAMERS AND MAGNETIC NANOPARTICLES

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Running title*:* *Sorting sperm using SPION-aptamer*

Keywords: DNA Aptamer – Magnetic nanoparticles – Semen quality – Bovine – Cell-SELEX

Abstract

In cattle, cryopreservation of semen and sex-sorting kill up to 50% of spermatozoa and decrease the success of assisted insemination (AI). Therefore, significant efforts are being carried out to improve the quality of semen prior to AI. In this work we used the Cell-SELEX technique to select single strand DNA aptamers able to recognize with high affinity and specificity damaged sperm cells generated by heat-treatment. We first isolated aptamers with a conserved two motifs of 6 nucleotides of length that bind to the membrane of heat-treated spermatozoa. Then, we used synthetic biotin-labeled aptamers containing the conserved motif to recognize membrane-damaged cells and separate them from viable cells by the use of avidin-coated superparamagnetic iron oxide nanoparticles (SPION). This procedure improved the quality of semen by significantly increasing the percentage of healthy sperm cells without affecting the rate of blastocyst cleavage. This technique was successfully applied to both unsorted and sex-sorted sperm suspension.

Introduction

Semen cryopreservation and artificial insemination (AI) are routine procedures in reproduction programs, especially in cattle and the *in vitro* production of bovine embryos represents approximately 66% of embryos transferred in the world (Vian*a et al*., 2010). The *in vitro* fertilization (IVF) success rate of bovine embryos is affected by several factors inherent to the technique specially if sexed spermatozoa and/or freezing semen are used and have an important impact on the profitability of dairy and beef cattle production (Palm*a et al*., 2008). The cryopreservation process is an aggressive procedure specially for cell membranes (Mazur, 1984, Watson, 1995) that can kill up to 50% of the spermatozoa (Gravanc*e et al*., 1998). Therefore, preserving semen quality is a critical factor for improving IVF rates. In this regards several experimental methods have been tested such as separation of viable sperm by microfluidic sperm sorter (L*i et al*., 2015), reduction in centrifugation forces in discontinuous Percoll gradients (Guimarãe*s et al*., 2014), addition of caffeine to increase sperm motility (Baraka*t et al*., 2015), epinephrine and theophylline (Kan*g et al*., 2015). In humans removal of cells with apoptotic markers (e.g. annexin V) has been shown to improve acrosomal reaction (Grunewal*d et al*., 2001, L*e et al*., 2010, Sai*d et al*., 2006). The sperm sub-population recovered in this way from the annexin-MACS column was found to be superior, in terms of motility, viability, normal morphology and fertilization potential (Makke*r et al*., 2008, Sai*d et al*., 2008). Therefore, by selecting the sperm population with a higher fertilization potential, this technique allows to increase AI success rate (Sai*d et al*., 2006). However, due to the high cost its application is economically viable only in the sphere of human reproduction.

Aptamers are an emerging class small molecules of single-stranded DNA or RNA produced by chemical synthesis methods and *in vitro* selection that present affinity and specificity comparable to those of monoclonal antibodies but without the costs and the difficulty entailed to produce them. They have a central region with a random sequence by means of which they can bind to their target molecules. This central region, ranging between 40 and 50 nucleotides, is flanked by two 20-base regions with known sequences that allow amplification by PCR (Ellington and Szostak, 1990, Gol*d et al*., 2010).

Aptamers specific against a target molecule are selected by the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method . The selection process is normally initiated with a combinatorial aptamer

library containing around 10^{14}-10^{15} single-stranded oligonucleotide molecules that are presented to the chosen target molecule. In a variation of the SELEX procedure, called Cell-SELEX, the initial combinatorial library is incubated against whole cells carrying more than one target molecule of interest, has been used to recognize specific types of cells (Gu*o et al*., 2008, Sefa*h et al*., 2010).

In this study our aim was to improve bovine semen quality by removal of damaged spermatozoa by means of DNA aptamers specific for membrane-damaged sperm cells generated by the Cell-SELEX technique coupled to the use of superparamagnetic iron oxide nanoparticles (SPION). The impact of this procedure on sperm quality was tested in both unsexed and sex-sorted sperm samples followed by *in vitro* fertilization (IVF) experiments to determine embryo production.

Materials and Methods

Reagents and Chemicals

Solvents were supplied by Merck & Co., Inc (Merck, KG, AA), and the other reagents were provided by Sigma-Aldrich Corporation (Sigma-Aldrich, intl.). All other reagents were of analytical grade or the highest grade available.

Oligonucleotide Combinatory Library Construction

The starting synthetic combinatorial library of random-synthesized DNA oligonucleotides of molecules with a length of 87 bases, structured and a central region of a random-sequence of 50 nucleotides flanked with a known sequence region of 21 and 16 nucleotides at the 5' and 3' ends (5´CGGAATTCCGAGCGTGGGCGT-$(N)_{50}$-TACGCCCACGCTCGAG-3´), primers H1 (5´-CGGAATTCCGAGCGTGGGCGT-3´) and H2 (5'-CTCGAGCGTGGGCGTA-3´) were purchased from Integrated DNA Technologies International Inc. (IA, USA) and used with or without biotin at the 5' end, as specified. The library was purified by HPLC to eliminate incomplete strands (Moncaler*o et al*., 2011) and amplified by PCR with *Taq* polymerase for 25 cycles at 94 ºC, 30 sec.; 58 ºC 30 sec.; 72 ºC, 10 sec. in a thermocycler (Gene cycler BIO-RAD Laboratories Ltd., CA, USA). The reaction mix included: 1 X Buffer Go-Taq (50 mM KCl, 10 mM Tris-HCl pH 9, 1.5 mM $MgCl_2$, 0.1 % Triton X-100) (Promega, WI, USA). Before incubation, the amplification products were heated for 5 minutes at 95 ºC and then chilled on ice for 5 minutes to open the double DNA strands and obtain ssDNA aptamer structure.

Preparation of sperm suspension

Semen obtained from *Aberdeen Angus* cryopreserved in a liquid nitrogen were thawed in a water bath at 37 ºC for 30 seconds and used as a control (Januskauska*s et al*., 1999). In order to induce cell membrane damage, the frozen sperm was heat-treated by incubation for 15 minutes in a thermostatic water bath at 45 ºC (DeJarnett*e et al*., 2000). Membrane damage was confirmed by Hoechst and annexin V staining (Data not shown). Afterwards, the sample was diluted with 3 ml 0.26 M sucrose in PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) and then centrifuged for 5 min. at 1,500 rpm. The harvested spermatozoa were washed once with 2 ml 0.26 M sucrose in PBS and then fixed in 2 ml 2.5% glutaraldehyde in

PBS for 30 minutes at room temperature. Finally, the sperm was centrifuged and resuspended in 300 µl PBS. Sperm concentration was quantified in a Neubauer chamber. Final sperm concentration was adjusted to 3 x 10^7 spermatozoa/ml in PBS containing a final concentration of 5% BSA.

Selection of aptamers that recognize damaged sperm cells from heat-treated sperm

In order to eliminate aptamers that bind to healthy cells 300 µl of the amplified synthetic library (1 µM) was mixed with 300 µl of a suspension of heat-untreated sperm fixed with glutaraldehyde (Pintado Sanjuanbenito and Pérez Llano, 1992). Prior to incubation, aptamers were heated at 95 °C for 5 min and then quickly cooled in ice. After the incubation (3 hrs with gentle agitation), the sample was centrifuged and the unbound aptamers were collected from the supernatant. This aptamer library (amplified and substracted) were used to isolate aptamers specific for heat-treated (membrane-damaged) sperm cells by two cycles of selection and amplification (Figure 1A). In the first cycle the amplified and substracted library was incubated overnight under shaking at room temperature with heat-treated sperm (glutaraldehyde fixed). Afterwards, the sample was centrifugated (5 min at 900g) and washed three times (30 min each) with an equal volume of PBS containing 2 mg/ml Bovine Serum Albumin (BSA, pH 7.4) to remove unbound aptamers. Then, the sample was washed twice with PBS to remove the BSA and the final pellet was resuspended in 100 µl NaCl (1M) in order to detach the bound aptamers (elution step). The sample was centrifuged again, and the eluted aptamers recovered from the supernatant were precipitated with ammonium acetate (250 mM final concentration) and 2/3 volume isopropanol. The aptamers were recovered after centrifugation (14,000g for 20 min.) by washing the pellet twice with 70% ethanol and resuspended in 50 µl H_2O. The aptamer concentration was quantified by light absorption at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., MA, US). The eluted aptamers were amplified by PCR and subjected to a second cycle of selection and amplification.

Cloning and sequencing

The aptamer mixture eluted in the second selection cycle from heat-treated sperm (Figure 1A) was amplified by PCR and ligated in a pGEM-T Easy vector (Promega, WI, USA). The ligation mixture was used to transform *Escherichia coli* cells, which were plated on Luria Broth-agar (LB-agar) with 100 µg/ml ampicillin, 0.5

mM IPTG and 50 µg/ml X-GAL. The plasmids were purified by alkaline hydrolysis and the inserted DNA was amplified by PCR. The clones were tested in binding assays with suspensions of heat-treated spermatozoa and fixed with glutaraldehyde as described above. Clones able to recognize membrane-damaged (heat-treated) sperm cells were sequenced and analyzed by means of multiple sequence alignment program ClustalW2 (Multiple Sequence Alignment from the European Molecular Biology Laboratory-The European Bioinformatics Institute: http://www.ebi.ac.uk/Tools/msa/clustalw2/, Accessed July 2014) (Larki*n et al*., 2007). Prediction of the secondary structure of the aptamer molecules was performed with the *UNAFold* program (Unified Nucleic Acid Folding and hybridization package, Washington University, http://mfold.rna.albany.edu, Accessed July 2014), adjusting the folding parameters to incubation conditions: 137 mM NaCl, 1 mM $MgCl_2$ and 37 ºC. For each entered sequence, the program predicts possible secondary structures according to their Gibbs free energy values (dG). We only show the possible secondary structure presenting the lowest free energy value for each sequence .

Binding assays

The ability of the selected aptamers to recognize heat-treated sperm cells was measured in binding assays using i) biotin-labeled aptamers, ii) fuorescence-labeled aptamers or iii) fluorescence-labelled synthetic aptamers.

The aptamers eluted after the second cycle of selection were amplified with biotin-labeled nucleotides and incubated (15 min at 37 ºC) with heat-treated sperm fixed with glutaraldehyde. The samples were centrifuged (5 min at 1,500 rpm), the supernatant was discarded, the pellet was washed twice with PBS and resuspended in an equal volume of streptavidin-FITC or streptavidin-Cy3 (1/100) in PBS containing 2% BSA. After incubation (15 min at 37 ºC), the samples were washed twice with PBS (5 min at 900g), the pellet was resuspended in PBS containing Hoechst 33258 solution (0.5 µg/ml) (de Leeu*w et al*., 1991). After incubation (5 minutes) the sperm cells were mounted on a glass slide and observed under a fluorescence microscope. This procedure was applied for experiments shown in Figure 1B.

Binding assays were later performed with sperm suspensions thawed under optimum conditions without glutaraldehyde fixation, so as to preserve the percentage of viable sperm. The selected aptamers were labeled with a fluorescent nucleotide by PCR amplification. This allows minimizing procedure times, since it avoids the binding step with avidin-FITC or avidin-Cy3. PCR conditions were as follows: Go-Taq buffer 1X (50

mM KCl, 10 mM Tris-HCl pH 9, 1.5 mM MgCl2, 0.1 % Triton X-100) (Promega, WI, USA), 100 µM dNTPs 50% T, 1 µM dUTP-rhodamine, 1 µM primer H1, 1 µM primer H2, and 2.5 U/100 µl Taq polymerase (Go-taq, Promega, WI, USA). 25 cycles were performed at 94 ºC, 30 sec.; 58 ºC, 30 sec.; 72 ºC, 10 sec. This procedure was applied for experiments shown in Figure 2.

Binding assays with synthetic aptamers (Macrogen Inc., Korea) were performed with the forward or reverse complementary sequences of the selected region of aptamers.

Sequences:

Af: 5'-GAC TAT GTT AGG-3'

Ar: 5'-CCT AAC ATA GTC-3'

Bf: 5'-GGA TTG TAT CAG-3'

Br: 5'-CTG ATA CAA TCC-3'

Control: 5'-CGT AGA GAC AAA TAT CGC AAT AGT C-3'

PSII: 5'-AAA GAC-3'

Synthetic aptamers were resuspended in H_2O (100 µM). For the binding assays, DNA was previously denaturalized for 5 min. at 95 ºC and cooled for 5 min. in ice. The resuspended strands were incubated for 10 minutes with the sperm suspensions at a final concentration of 5 µM in PBS with 2% of albumin and processed for fluorescence microscopy as described above.

Synthesis of avidin-coated superparamagnetic iron oxide nanoparticles (SPION):

Superparamagnetic Fe_3O_4 nanoparticles (SPION) were synthesized by applying the method of chemical coprecipitation using Fe^{2+} and Fe^{3+} salts in the presence of a base. The gamma-Fe_2O_3 nanoparticles (NP) obtained were coated with silica by the sol-gel process used in the Stöber technique (Masalov *et al*., 2011, Stöber *et al*., 1968). Briefly, 267.7 µl (containing 16.6 mg iron) were added to 25 ml isopropyl alcohol and stirred by sonication for 5 min. Then, 0.5 ml 98% tetraethyl orthosilicate (TEOS) and 750 µl 25% NH_3 were added dropwise during 5 minutes under constant ultrasonic stirring and 5 ml of 97% 3-aminopropyl-triethoxysilane (APTES) were added to generate nanoparticles coated with a free amino (NP-NH_2) . NP-NH_2 were pelleted by a permanent magnet from a 3 ml aliquot (concentration 15.5 mg/ml). The pellet was washed twice with 5 ml 99.8%

anhydrous DMF (N, N-dimethylformamide), resuspended in 3 ml DMF and mixed with 25 ml 1M succinic anhydride in DMF and 10 ml pyridine, by adding dropwise under constant stirring. After overnight incubation at room temperature the nanoparticles were washed twice DMF, once with 20 ml 5% HCl (v/v) for 5 minutes under stirring, and then with distilled until a constant pH of 5.5. The precipitated NP-NH_2 was resuspended in 2 ml purified water. To obtain NP-COOH the precipitated NP-NH_2 (100 µl of 15.5 mg/ml) was mixed with 100 µl 0.1 M 3-dimethylaminopropyl-carbodiimide (EDC) in phosphate buffer (10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl pH 7.4) and 10 µl 25mM N-hydroxysuccinimide (NHS) in phosphate buffer 0.1M pH 7, stirred for 30 minutes and then washed 3 times with 500 µl 0.1M borate buffer (0.1M H_3BO_3, pH = 8.5).

The liquid phase was discarded as before by action of the magnet , the precipitate (1.5 mg) resuspended with 63 µg avidin (1 mg/ml) in 0.1M borate buffer, pH 8.5, stirred for 2 hrs and incubated at 4 °C for 24 hours. The liquid phase was discarded by action of the magnet, and the precipitate was re-suspended in 100 µl TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7.6), obtaining a 63 µg/1.5 mg NP ratio. The final binding capacity was 12.5 nmol oligonucleotide-biotin/mg NP. As control group, Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs; Cat. # TB246, Promega, WI, USA), with 1.0±0.5 µm diameter, 1.0 mg/ml concentration and 0.75-1.25 nmol oligonucleotide-biotin/ mg microparticles binding capacity were used.

Characterization of nanoparticles

Fourier transform infrared (FT-IR) spectra for the nanoparticles were recorded using a Nicolet 6700 FT-IR spectrometer. The particle size, structure and morphology of the were investigated by scanning electron microscopy (SEM) (Carl Zeiss Supra 40 Scanning Electron Microscope, GE, UE) operated at 2.2, 2.3 and 2.8 Kv.

Removal of damaged sperm cells using aptamers and SPION

Prior to the assay the synthetic aptamers were denaturalized at 95º C for 5 min and cooled in ice for 5 min and the magnetic nanoparticles were washed three times with PBS. Cryopreserved bovine semen samples were thawed, diluted with 3 ml PBS containing 2 mg/ml BSA and then centrifuged for 5 min. at 900g. The pellet was re-suspended in 100 µl of 2 mg/ml BSA and Hoechst 33258 0.5 µg/ml in PBS. The sperm concentrations usually

obtained with this protocol are $5x10^7$ cells/ml approximately. From these suspensions, 20 µl aliquots (10^6 spermatozoa) were taken and incubated for 15 min. at 37º C with 10 µM 5'-biotin-labeled synthetic aptamers. After incubation, 100 µl commercial magnetic Streptavidin-coated microbeads or 25 µl avidin-coated SPION were added and incubated for 15 min at 37º C. The particles were captured and the unbound spermatozoa were recovered from the supernatant. The samples were mounted on a slide, and the percentage of viable sperm was assessed. The viability studies were conducted by visual examination of the samples with fluorescence and phase contrast simultaneously with 1,000 X magnifications under a fluorescence microscope (Olympus IX-71 microscope, Olympus Corporation, Japan). Each assay was performed in triplicate, and at least 300 spermatozoa were analyzed. The same procedure was applied to low-quality semen samples (obtained by incubation at 45 ºC for 15 minutes) which have a viability percentage lower than 45%.

Ovarian puncture and in vitro maturation of oocytes

Bovine ovaries obtained from slaughtered animals were washed with warm, sterile physiological solution. Follicles of intermediate size, i.e. between 2 and 8 mm, were punctured with a 10-ml syringe filled with approximately 1 ml prewarmed (37°C) phosphate buffer (DPBS, 14287-072; GIBCO BRL, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS) (Internegocios, BA, Argentina) and 1 % antibiotic-antimycotic (15240, GIBCO BRL, Grand Island, NY, USA). The follicles were punctured by directing the needle from the inside of the ovarian parenchyma. Syringe contents were discharged in a 50-ml centrifuge tube with 1 ml searching medium in a water bath at 25º C. Once all ovaries had been punctured, the supernatant was discarded and the decanted cumulus-oocyte complexes (COCs) were placed on a 10-mm Petri dish for examination and selection under a stereo microscope. COCs containing immature oocytes (those completely surrounded by at least three layers of compact cumulus cells, with homogeneous cytoplasm and intact membrane) were rinsed and placed on 100 µl droplets of maturation medium covered with mineral oil (Sigma-Aldrich, Cat. # M-8410). Maturation medium was TCM-199 (Cat. # 31100-035; GIBCO BRL, Grand Island, NY, USA), containing 2 mM glutamine (Sigma-Aldrich, Cat. # G-8540), 10% FBS, 2 µg/ml FSH (NIH-FSH-P1, Folltropin, Bioniche, Belleville, Ontario, Canada), 0.3 mM 34 sodium pyruvate (Sigma-Aldrich, Cat. # P-2256), 100 µM cysteamine (Sigma-Aldrich, Cat. # M-9768) and antibiotic-antimycotic (100 U/ml penicillin, 100 µg/ml streptomycin and

250 ng/ml amphotericin B; Cat. # 15240-096, Gibco). In vitro maturation conditions were 6.5 % CO_2 in an incubator (Thermo-Fisher) for 22 hs at 37°C with humidified atmosphere (Vichera *et al*., 2011).

In vitro fertilization and evaluation of embryo development

After 22 hs of maturation, the COCs were washed in Tyrode's albumin lactate pyruvate medium buffered with 15 mM HEPES (TALP-H) without removal of the cumulus cells (Bavister and Yanagimachi, 1977). Subsequently, the oocytes were quickly deposited on droplets of 40 µl Fert-TALP medium supplemented with 0.5 mg/ml hypotaurine (Sigma-Aldrich, Cat. # H1384) and 20 I.U./ml heparin (Sigma-Aldrich, Cat. # H3149) in a gas stove. Frozen bovine semen was thawed in a water bath at 37°C for 30 s. The semen sample was placed on top of two equal volume layers of 90% and 45% in a Percoll gradient and centrifuged at 700 g for 15 min.. Afterwards, the precipitate was resuspended in Fert-TALP medium ($CaCl_2$ 2mM, KCl 3.2 mM, $MgCl_2$, 0.5 mM, NaH_2PO_4, 0.4 mM, Lactic Acid (Sodium salt; 60% w/w syrup) 11 mM, NaCl 114 mM, $NaHCO_3$ 25 mN, Na pyruvate 0.2 mM, penicillamine 20 mM, hypotaurine 10 mM, epinephrine 1 mM, BSA (Fraction V) 6 mg/ml, Gentamycin 50 µg/ml). The final sperm concentration was adjusted to 1-2 x 10^7 spermatozoa/ml. Finally, 10 µl of the concentrated sperm suspension were added to the 40-µl droplets containing the oocytes, and incubated at 39°C with an atmosphere of 5% CO_2 for 18 h. The presumptive zygotes were washed 3 times in TALP-H and cultured as described (Vichera *et al*., 2011). Fertilized oocytes were cultured in droplets of 100 µl of synthetic oviductal fluid medium (NaCI 107.70 mM, KCI 7.16 mM, KH_2PO4 1.19 mM, $NaHCO_3$ 25.07 mM, $CaCl_2$ 1.17 mM, $MgCl_2$ 0.49 mM, Na lactate (60% syrup) 3.3 mM, Na pyruvate 0.4 mM, BSA (fatty acid-free) 8.0 mg/ml, penicillin 100 mg/ml, streptomycin 100 mg/ml, amino acids BME amino acid solution 10 ml/ml, MEM non-amino acid solution (20 ml/ml) and FBS 2.5%) in a humidified atmosphere of 5% O_2 at 39ºC. 50% of the culture medium was renewed on Day 2 of culture and on Day 5 the medium was supplemented with 10% FBS. Cleavage was evaluated on Day 2, and the blastocyst rate on Day 7 post-IVF.

Ethic Statement

This study was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martín (N° 06/2014), which also approved protocol development under the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Research animals were kept in sanitary condition in accordance with the Argentinean regulations (National Service of Health and Agro-Food Quality, SENASA (http://www.senasa.gov.ar)).

Statistical Analyses

For the calculations of average and standard deviated each assay was performed in triplicate and in each replica more than 100 sperm per sample were analyzed. Correlation among different parameters of cryopreservation was performed by Pearson correlation test. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

Results

Aptamers recovered from heat-treated sperm are able to discriminate between healthy and membrane-damaged sperm cells.

In this study, we first obtained aptamers with high specificity for membrane-damaged sperm cells by the CELL-Selex procedure: a synthetic library made up by more than 10^{15} molecules of different oligonucleotide sequences was amplified by PCR in order to reach the necessary optimized concentration for selection (Moncaler*o et al*., 2011). The amplified library was heated to open its strands and incubated overnight with 1.2 x 10^7 healthy sperm cells fixed with glutaraldehyde. After incubation, the sperm cells and were centrifuged, the pellet was discarded and the supernatant containing oligonucleotides was used in a second incubation with heat-treated sperm fixated with glutaraldehyde. In this way, those aptamers that bind to the surface proteins of healthy cells are subtracted from the library. This substracted library was amplified and subjected to two rounds of selection and amplification as despicted in Fig 1A. For the first cycle the amplified subtracted combinatorial library was incubated with heat-treated sperm. After incubation, bound aptamers were eluted and amplified by PCR. The amplified product was incubated again with heat-treated sperm, eluted and amplified (second cycle). The PCR product of the second round of selection and amplification yielded a more concentrated mixture of aptamers with increasing specificity toward heat-treated sperm. The amount of aptamers recovered from untreated and treated sperm was 3 and 167 ng/µl (total volume of 50 µl) respectively). These results are consistent with those expected for a successful selection. In order to confirm the specificity of these aptamers we evaluated their ability to discriminate between live (untreated sperm) and dead sperm (heat-treated) by fluorescence microscopic examination using biotin-labeled aptamers (revealed with avidin-FITC). As shown in Figure 1B, while the fluorescence signals of control aptamers were weak in both untreated and heat-treated sperm cells, the mixture (polyclonal) of selected aptamers showed intense signal only in heat-treated sperm.

Cloned aptamers contain the consensus sequences TATGTT and bind to heat-treated sperm

In order to identify the specific sequence of the oligonucleotides, the aptamer (polyclonal) mixture eluted from heat-treated sperm in the second selection cycle (Fig. 1A) was amplified by PCR and cloned in a pGEM-T Easy vector (Promega, WI, USA). We obtained seventeen clones shown in Table I. Their sequences were analyzed using the ClustalW software program. In the analysis, only the variable sequences, which are unique to each clone, were considered. Sequence analysis sorted them into three groups: group A contains the ACTATGTTA consensus motif and group B has the TTGTAT motif that is the reverse of the first motif (Figure 2A). Group C does not share any apparent motif (not shown). Of the 17 analyzed sequences, four belong to the Group A where clones 3 and 4 are repeated, eight sequences belong to Group B, and 5 belong to Group C. The predicted secondary structure of these molecules revealed that in most of the analyzed sequences the consensus motifs of group A and Group B aptamers are single strands (Figure 2B). The ability to bind heat-treated sperm was evaluated for 8 clones using biotin-labeled oligonucleotides and we found that each individual cloned aptamer produced a strong signal similar to the positive control (Figure 2C). As positive and negative control we used the aptamer mixture eluated from the second selection step and the amplified synthetic library, respectively.

Short synthetic aptamers containing the consensus sequences from group A and B bind to heat-treated sperm

To further confirm that the consensus motifs from group A and B were the sequence important for recognizing the marker of heat-treated sperm we synthesized short (12 bases) aptamers containing the consensus motifs with three extra bases added in each side. The reverse complement of each sequence was labeled with biotin (Aptamers Ar and Br) and used for in binding experiment. As negative control, we used a synthetic biotin-labeled aptamer containing part of the sequence of clone 2 excluding the consensus motif. All synthetic aptamers containing the consensus motif presented a high correlation with Hoechst when incubated with heat-treated spermatozoa (Figure 3 and Table II).

Removal of damaged sperm cells with aptamers improves semen quality

To test whether sperm quality can be improved by removal of damaged sperm cells with biotin-labeled synthetic aptamers we incubated sperm suspension of low and high quality with Ar or Br aptamers. After incubation, avidin-coated superparamagnetic Fe_3O_4 were added and aptamer-bound cells were removed by centrifugation. This procedure eliminates unbound aptamers as well as aptamer-bound cells and also yields a suspension containing healthy sperm cells: when this procedure was applied to high quality sperm there was no significant difference between untreated and Ar or Br treated sperm suspension. In contrast, when the procedure was applied to low quality sperm, a significant improvement in the percentage of viable cells was observed. Table III summarizes the percentage of viable cells measured in low and high quality sperm subjected to the removal process with Ar or Br aptamers.

Removal of aptamer-bound damaged sperm cells with superparamagnetic nanoparticles does not perturb early embryo development.

In order to the safety of the removal process of damaged sperm cells by means of nanoparticles and aptamers, we compared *in vitro* the development rates of embryos generated by IVF using sexed or unsexed spermatozoa, selected by means of a Percoll gradient (Somfa*i et al*., 2002) or the nanoparticle-associated aptamers. Development was observed up to the blastocyst stage. All experimental groups showed high embryo cleavage rates on Day 2 and no statistical differences were observed in the cleavage rates or in the blastocyst rates between the groups of embryos produced with either sex-sorted or unsexed semen. As expected, the expanded blastocyst rates on Day 7 were lower in the groups where sexed semen was used, but with no significant differences between both preparations techniques (Table IV). Our results suggest that sperm selection using the system of nanoparticles and aptamers improves sperm quality without affecting at later stages embryo development in *in vitro* fertilization procedures.

Discussion

We have used DNA aptamers because they are more stable than RNA aptamers, and they can be directly amplified by PCR (Moncaler*o et al*., 2011). Using the Cell-SELEX technique we selected and amplified a mixture of DNA aptamers able to specifically bind to heat-treated damaged sperm cells (Figure 1). From this mixture 17 different aptamers were cloned, sequenced and analized for their predicted secondary structure which revealed two consensus motifs: **ACTATGTTA** and **TTGTAT** (Figure 2A). Eight individual clones containing these motifs were able to label heat-treated sperm cells with similar intensity compared to the "polyclonal" mixture (Figure 2B). Furthermore, 12-mer synthetic aptamers containing 6 bases of each sequence motif were able to label heat-treated sperm cells (Figure 3). The results obtained showed that the consensus motifs were the main responsible region for the binding of the different clones to the damaged sperm cells. For the separation of damaged cells from sperm suspension we produced avidin-coated superparamagentic nanoparticles of an average size of 136 ± 13 nm (Figure 4A) which have superior number of binding sites compared to commercial particles of sizes ranging between 1 to 3 μm (MagneSphere® Magnetic Separation Products Data sheet, Promega, WI, USA). On the other hand, coating the particles with silica and avidin bound on the amino end lends stability to the conjugate (Chauha*n et al*., 2011), making it possible to lyophilize and reconstitute them a long time later at least 1 year (data not shown). We showed for the first time that separation of damaged sperm cells by binding to specific aptamer coupled to precipitation with superparamagnetic nanoparticles improves the quality of semen (Table III), reaching the required fertility levels (Amann and Hammerstedt, 1993, Vincen*t et al*., 2012). Furthermore, neither the silica-coated nanoparticles nor the aptamers have caused alterations to sperm viability. The procedure has no deleterious effect on embryo development (Table IV) and it has shown to be as effective as the techniques described for the annexin V columns (L*ee et al*., 2010, Makke*r et al*., 2008, Sai*d et al*., 2006, Sai*d et al*., 2008) .

Our method was tested in both non-sorted and sex-sorted sperm suspension with similar outcomes. As expected, in sex-sorted samples the fertilization rates were 10–20% lower compared to IVP using non-sorted sperm and traditional artificial insemination (AI; Seidel et al., 1999). Sex-sorting is another technique that affects the fertilizing capacity of spermatozoa (Arrud*a et al*., 2012, Carvalh*o et al*., 2010, Palm*a et al*., 2008, Wilso*n et al*., 2006) and is widely used for producing optimal proportions of males and females of farm animals (Rat*h et al*., 2013).

In conclusion, by separating membrane-damaged spermatozoa from viable sperm cells by using biotin-labeled DNA aptamers with avidin-coated nanoparticles we have improved the sperm selection process without damaging the capacity to form embryos. Due to the relative low cost of aptamers, this procedure has the potential to be used in large scale for the sustainable production of animals.

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Legends to the Figures

Figure 1. *Aptamers selected from heat-treated sperm are able to discriminate between live and dead sperm*, A) Aptamers selection schedule. B) Evaluation of the specificity of selected aptamers (mixture obtained after the second selection step) by binding assays. Untreated or heat-treated sperm were incubated with control (top) or selected (Bottom) biotin-labeled aptamers and revealed with avidin-FITC. Nuclei were stained with Hoechst. Magnification: 1,000X.

Figure 2. *Cloned aptamers bind to heat-treated sperm*. A) Alignment of sequences. For the alignment, the sequences in the variable region were analyzed, which are unique for each clone. Based on their consensus motif, sequences were classified into two groups. Group A (Top panel) contains motif TATGTTA, Group B (bottom panel) contains motif TTGTAT. B) Predicted aptamer secondary structure. In most structures, consensus motifs (green) are found as single strands. Only the secondary structure presenting the lowest free energy value for each analyzed DNA strand is shown. C) Binding of heat-treated sperm with the polyclonal mixture of aptamers (positive control) and 8 selected cloned aptamers from Group A (clones 3 and 7) and group B (Clones 1, 2, 8, 12, 13, 15).

Figure 3. Short (12 bases) synthetic aptamers containing the consensus motif from group A or B bind to heat-treated sperm. Binding of heat-treated sperm with biotin-labeled synthetic aptamers containing consensus motif from group A (Synthetic Ar), group B (Synthetic Br) or a negative control aptamer (Clone 2r). Staining was revealed with avidin-FITC.

Figure 4. Binding of nanoparticles coated with silica and conjugated to avidin to aptamer A) Scanning electron microscopy (SEM) image of synthesized nanoparticles (Top left panel) with average diameter of 136 ± 13 nm (top right panel). The figure (bottom panel) shows the infrared spectrum obtained for these derivatized particles with carboxyl groups to couple the avidin. The spectrum confirms the presence of silica on the coating, of the

amide and carboxyl functional groups. B) Phase contrast microscopy images of untreated or heat-treated sperm cells incubated for 15 minutes with biotin-labeled aptamer and avidin-coated nanoparticles. The same samples are shown under UV light to visualize Hoechst-positive cells. Nanoparticles are observed only in heat-treated cells (arrows).

Table I. *Sequences of cloned aptamers*.

Clone #	Sequence (N=50)
1 (*)	GAGCCCACCTG**ATACAA**CATGACGTCGGCCTGCGGAGTTAAAGTTGACGG
2 (*)	CGCCCACTGTGAGG**ATACAA**CTTTAGTCGACTATTGCGATATTTGTCTCG
3 (**)	GCATAGCGAGGTNCAGACTAGAC**TATGTT**AGGTGTTTATGCCCATTTTGN
4 (**)	GGCATAGCGAGGTNGCAGACTAGAC**TATGTT**AGGTGTTTATGCCCTTTTG
5 (*)	GCGTTAGCACGCCCACCTCTATAAAATCGGT**AACATA**GGACCGATTTCCG
6	TACGTCAAAGTGAGGTGACATGTCTGATATTGACTTCGATGGCTGCTTGG
7 (**)	AGANACGNNNCTGTTCTAGAAAC**TATGTT**ACCCGCTTGTTTTTAGGCATG
8 (*)	ACACA**ATACAA**TACGNCTGGCTTAACGTTCATACAAGGTTGGCGTGTGAG
9 (**)	TAGGGNNNCC**TTGTAT**CATCGGGGATCAGGTCTTCATTGGNGTGAANAGA
10 (*)	GAAAGGCCCAGGATTATTGGTC**ATACAA**TAAAGTTTCATCATGCAGCCTG
11	TCTAGACCACGCGACCCAACACGGTTTCAGACCGGTAGCCACAACAAGCG
12 (**)	ATGGAGGCGGCATAAGCGAGGG**TTGTAT**TAGACAGATTCCTCACGTGTGC
13 (**)	GAAAGCACGCGACTGATGGAA**TTGTAT**GTTAATGTGATTCCGGGACTATA
14	ACAAATGATGCTATACTTACACTCTGATCCTATTTGGTCTTGCCTGGGCT
15 (**)	ACGGAGTACGCCTAAGCGAGGG**TTGTAT**TGAACAGATTCCTCNCGTGTGC
16	ACTGCGCCGCCCACCCTGCTTGGGGAAGGGAATCCTCAGTCGAAATTCTG
17	GGACGCCTACAAGTGTCTATTAAACTTAATTATGAATACGCACTTAACGG

* and ** indicates clones containing the conserved motif **TATGTT** and **TTGTAT**, respectively.

Table II. *Correlation between Aptamers and Hoechst 33258 staining*.

	(%) Aptamer/ Hoechst 33258 stained cells	Correlation (Pearson)
Polyclonal mix	91.57 ± 2.99	0.91
Clones	89.82 ± 1.01	0.97
Synthetic aptamers		
Af	91.40 ± 3.44	0.87
Bf	90.97 ± 0.43	0.98
Ar	93.19 ± 1.45	0.98
Br	91.43 ± 3.82	0.99

Table III. *Removal of damaged cell by aptamers/nanoparticles improves sperm quality*. The results express the % (mean ± standard deviation, n=3) of viable sperm cells. Samples 1-6 indicate different bull sperm samples.

	No removal	Removal with Ar	Removal with Br
Untreated sperm	67.8 ± 5.0	75.4 ± 4.5	74.1 ± 0.9
Heat-treated sperm	39.2 ± 5.1	61.9 ± 1.4	54.9 ± 10.1
Sample 1	55.3 ± 2.9	75.4 ± 1.5	
Sample 2	58.5 ± 1.3	66.6 ± 0.6	
Sample 3	56.2 ±3.7	63.4 ± 2.5	
Sample 4	62.3 ± 3.1	71.5 ± 1.2	
Sample 5	53.8 ± 2.2	85.1 ± 0.8	
Sample 6	44.7 ± 1.8	74.1 ± 0.6	

Table IV. *Embryo formation using unsorted or sex-sorted sperm*.

Selection Method	Sperm Sex-sorted	n	Cleaved Embryos (%)	Blastocyst (%)	Blastocyst Expanded (%)
Percoll	-	131	113 (86.3)	33 (25.2)	22 (16.8)
	+	31	23 (74.1)	6 (19.4)	2 (6.5)
Aptamers-SPION	-	121	97 (80.2)	26 (21.5)	24 (19.8)
	+	26	21 (80.8)	5 (19.2)	2 (7.8)

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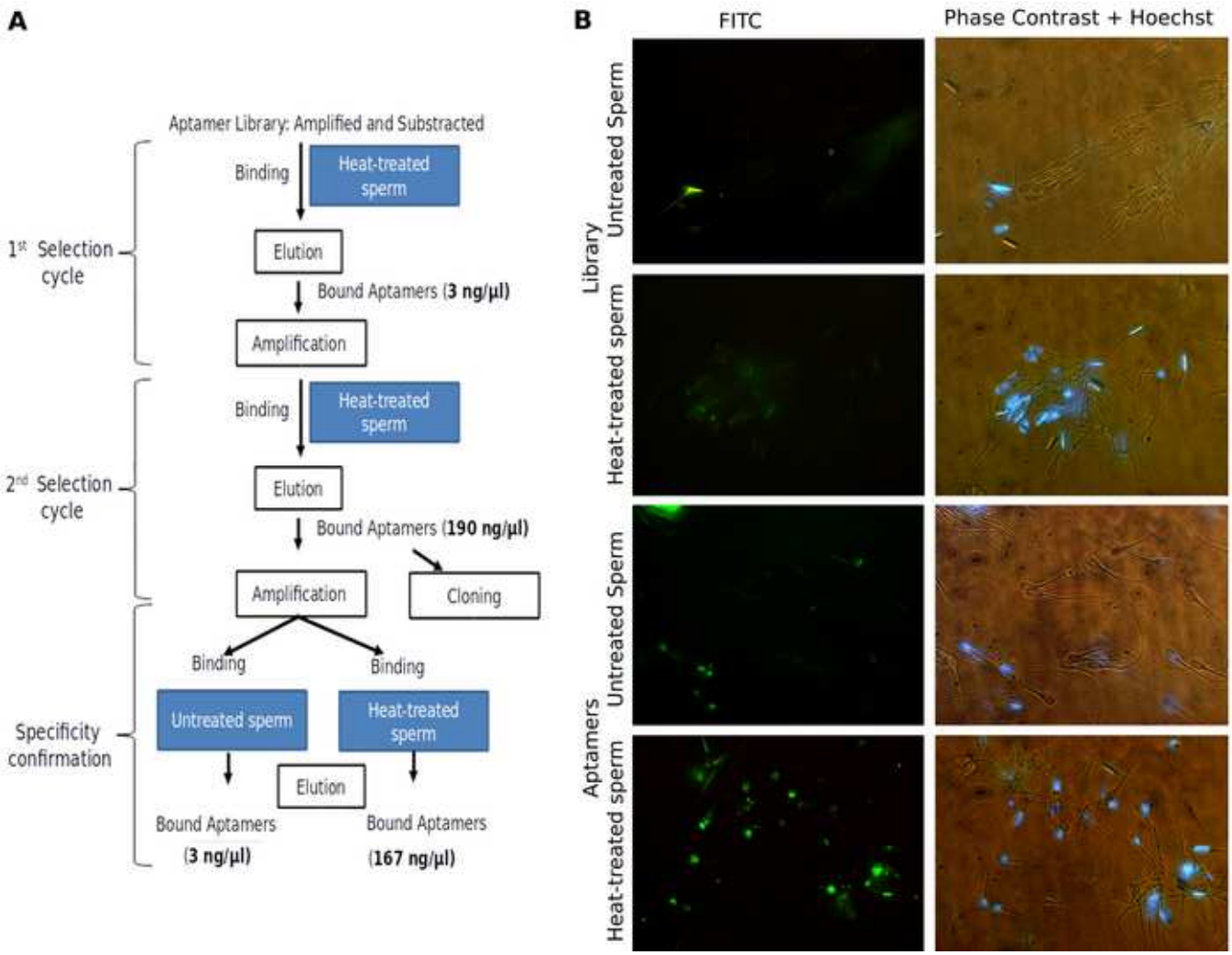

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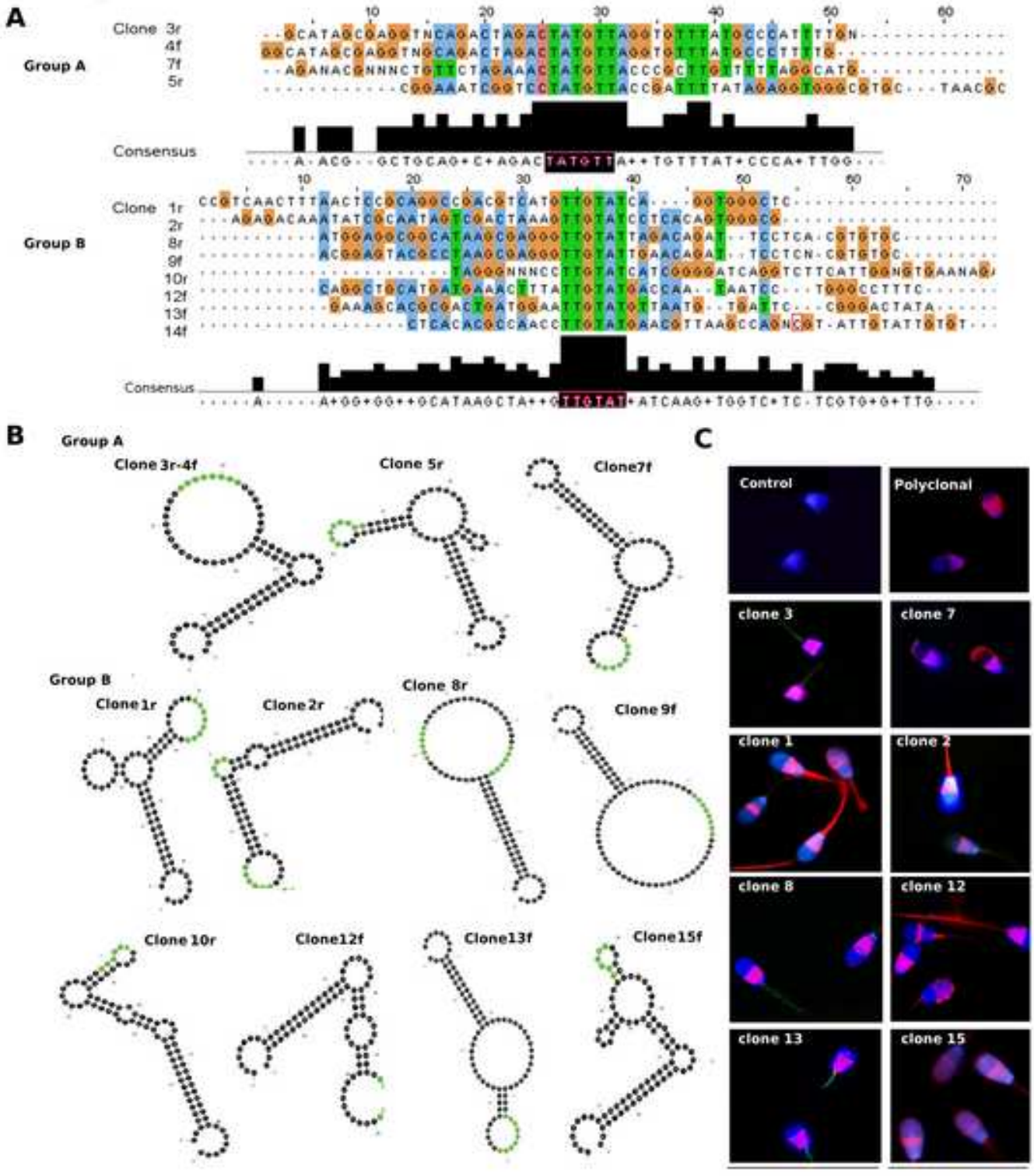

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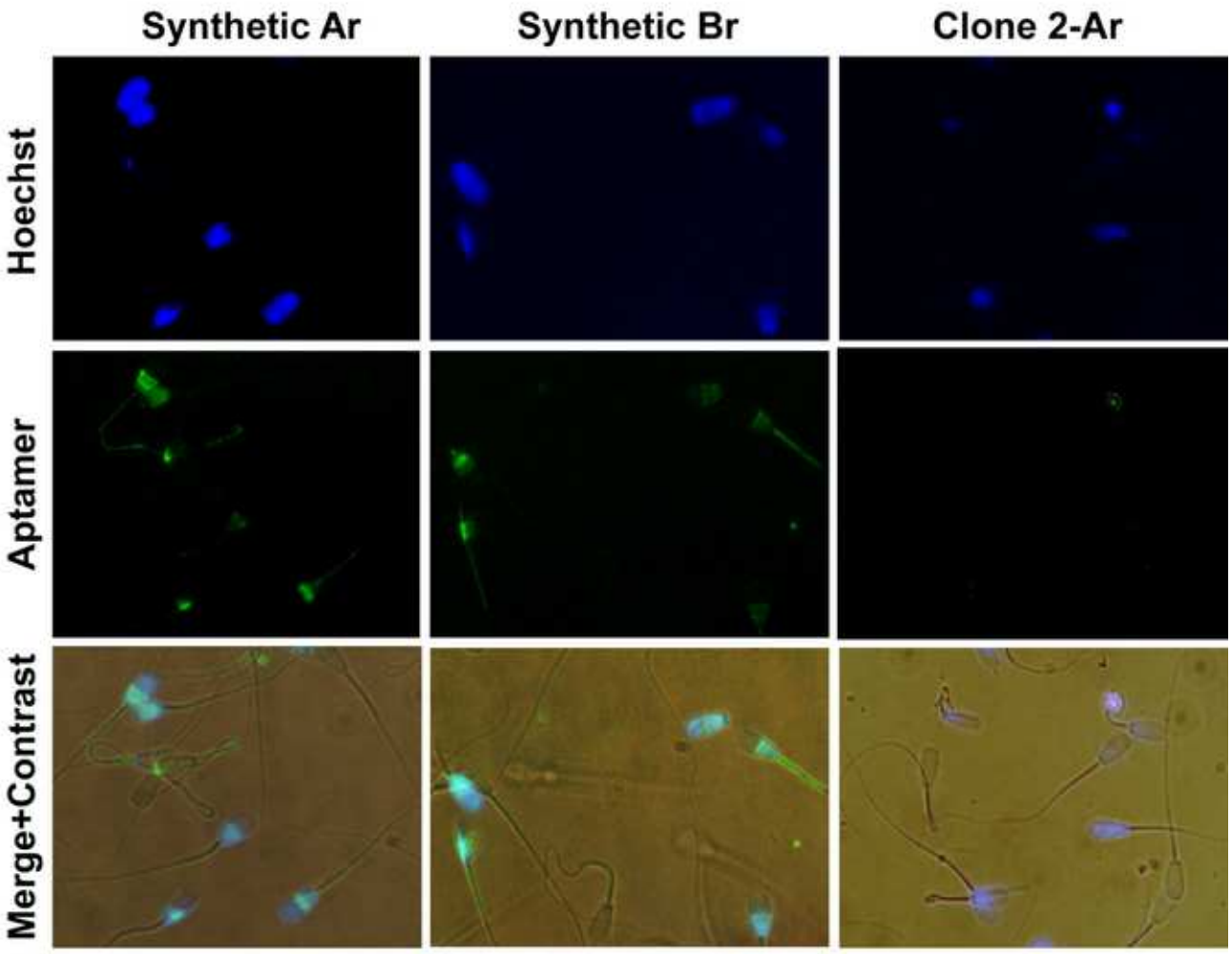

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