Transgene transmission in chickens by sperm-mediated gene transfer after seminal plasma removal and exogenous DNA treated with dimethylsulfoxide or *N*,*N*-dimethylacetamide

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Transgenic animals have been successfully produced by mass gene transfer techniques such as sperm-mediated gene transfer (SMGT). The aim of this work was to demonstrate transgene transmission by SMGT in chickens using dimethylsulfoxide (DMSO) or *N*,*N*-dimethylacetamide (DMAc) as transfectants after seminal plasma removal to prevent DNase activity. Sperm samples were prepared by repetitive washes, and after each wash sperm motility, seminal plasma proteins, exogenous DNA integrity and its uptake by spermatozoa were evaluated. Laying hens were inseminated using spermatozoa transfected with pEGFP-N1 vector in the presence of DMSO or DMAc. Transgene transmission in newborn chicks was evaluated by *in vivo* enhanced green fluorescent protein (EGFP) expression, RT-PCR and PCR analysis. DNA internalization was limited to sperm samples washed twice. The presence of DMSO or DMAc during transfection had no effect on fertilization or hatching rates. PCR analysis detected the presence of EGFP DNA in 38% of newborn chicks from the DMSO group and 19% from the DMAc group. EGFP mRNA was detected in 21% of newborn chicks from the DMSO group, as against 8.5% from the DMAc group. However, *in vivo* expression of EGFP was only observed in a single animal from the DMSO group. Our data revealed that the plasmid DNA–DMSO combination coupled with sperm washes can be an efficient method for transfection in chickens.

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1. Introduction

Over the past 20 years, several methods have been developed to produce transgenic animals (Wall 2002; Canovas *et al.* 2010). The most common methods include pronuclear microinjection, somatic cell nuclear transfer and transduction using retroviruses. The latter is the most widely used method to produce transgenic chickens. However, the retroviral method has several disadvantages such as a limit on the size of the inserted gene and the method has been considered to be dangerous (Ebara and Fujihara 1999; Han 2009). An alternative, low-cost strategy

Keywords. Chicken; DMAc; DMSO; seminal plasma; SMGT; transgenic

Abbreviations used: CMV, cytomegalovirus; DMAc, *N,N*-dimethylacetamide; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; PBS, phosphate buffered saline; REMI, restriction-mediated insertion; SMGT, sperm-mediated gene transfer; TMGT, testis-mediated gene transfer

is based on the introduction of foreign DNA into spermatozoa before the fertilization process (Lavitrano *et al.* 1989; Smith and Spadafora 2005; Collares *et al.* 2010).

Although transgenic animals have been produced using sperm-mediated gene transfer (SMGT), the efficiency of the process is low. This is mainly due to the poor uptake of exogenous DNA by the spermatozoa, thereby reducing the number of fertilized oocytes with transfected spermatozoa (Anzar and Buhr 2006). In addition, interspecies and intraspecies success variability remains a problem (Garcia-Vazquez *et al.* 2009). The poor reproducibility of SMGT was suggested to be due to the activation of defense mechanisms in the spermatozoa and seminal plasma, resulting in degradation of the exogenous DNA (Sato *et al.* 2003; Kang *et al.* 2008; Lanes *et al.* 2009). However, DNA uptake efficiency was improved using dimethylsulfoxide (DMSO) and nanocomposites (Kim *et al.* 2009; Campos *et al.* 2011). Cryoprotectants such as DMSO can improve DNA uptake by sperm cells as previously demonstrated in rabbits and mice (Li *et al.* 2006; Shen *et al.* 2006). Therefore, other cryoprotectants, such as *N*,*N*-dimethylacetamide (DMAc), recently used for the cryopreservation boar sperm cells (Bianchi *et al.* 2008), could improve the uptake of exogenous DNA by spermatozoa.

Avian species, particularly chickens, have been increasingly used in transgenic research due to their inherent advantages, such as short generation times, high semen production and potential applications as transgenic bioreactors for heterologous protein production (Lillico *et al.* 2007; Han 2009). Previous studies in chickens reported a low efficiency for SMGT (Gavora *et al.* 1991; Nakanishi and Iritani 1993). However, the production of transgenic chickens by SMGT using liposomes and restriction-mediated insertion (REMI) was recently demonstrated (Harel-Markowitz *et al.* 2009). Furthermore, transfection by testis-mediated gene transfer (TMGT) was recently established in chickens (Liu *et al.* 2010), showing the feasibility of SMGT in avian species.

The aim of this study was to evaluate the effect of seminal plasma removal and treatment with DMSO and DMAc on exogenous DNA uptake by rooster spermatozoa and transgene transmission through SMGT.

2. Materials and methods

2.1 *pEGFP vector preparation*

A commercial eukaryotic cell expression vector, pEGFP-N1 (Clontech, USA), containing the cytomegalovirus (CMV) immediate early promoter fused to the enhanced green fluorescent protein (EGFP) was used as the source of exogenous DNA. The plasmid was propagated in *Escherichia coli* DH5α cells and purified using the Perfectprep Plasmid Maxi kit (Eppendorf, Germany), according to the manufacturer's instructions.

2.2 *Animals groups and sperm collection*

A total of 24 fertile animals were used (8 roosters and 16 hens). The chickens were maintained in individual cages in the Central Animal House, Federal University of Pelotas, with a photoperiod of 15 h of light. All males were maintained in temperature-controlled facilities. The reproductive routine of the roosters was maintained with three semen collections per week, as described previously by Latorre *et al.* (1988). Briefly, semen samples from 8 roosters were collected into conical, polystyrene sample cups and transported to the laboratory within 15–20 min of collection.

2.3 *Evaluation of sperm motility, exogenous DNA integrity and uptake by spermatozoa*

To determine the impact of seminal plasma removal on DNA uptake by spermatozoa, the semen samples were pooled and 10^9 sperm cells were washed, by centrifugation at 600*g*, up to six times in Lake's diluent (Howarth 1983) containing 10 μg of plasmid DNA. Prior to centrifugation, samples were incubated for 10 min to allow DNA uptake by sperm cells. Following each wash, an aliquot of the supernatant was removed to test for DNase activity and the presence of seminal plasma proteins. DNase activity was measured by horizontal gel electrophoresis (1% agarose), and the DNA was visualized with ethidium bromide at 0.5 μg/ml. An aliquot of the supernatant collected after each sperm wash was analysed by electrophoresis in 15% SDS-PAGE gels, as described previously (Campos *et al.* 2010b), to verify the presence of seminal plasma proteins. Sperm motility and vigour was evaluated by phase contrast microscopy before and after each wash.

Exogenous DNA adsorbed to the sperm cell surface, but not internalized, was removed by treatment with 20 U of DNase I (Invitrogen, USA) for 30 min and three washes in phosphate buffered saline (PBS), 5 min at 600*g*. DNA extracted from the sperm cells was analysed by PCR to detect internalized exogenous DNA. Briefly, DNA was extracted using the PureLink Genomic DNA Mini kit (Invitrogen, USA). To detect pEGFP-N1 plasmid DNA, pEGFP-specific oligonucleotides (5'- CGGGACTTTCCAAAATGTCG and 5'-GAAGATGGTGCGCTCCTGGA) were used to amplify a 500 bp fragment (figure 1). PCR reactions were carried out with an initial denaturation step of 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by a final incubation of 7 min at 72°C. PCR products were analyzed in 1% agarose gels, stained

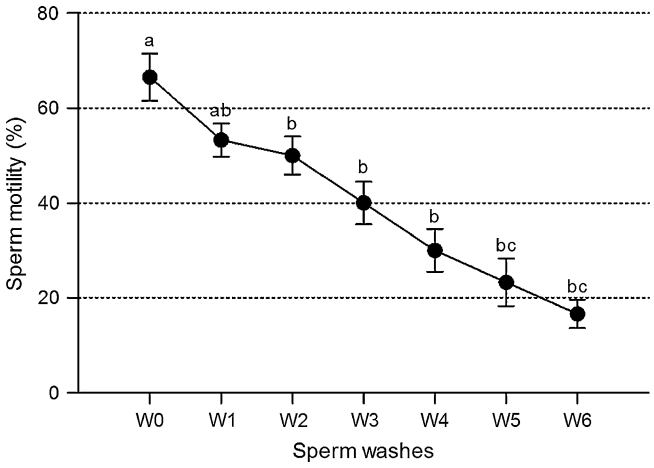

Figure 1. Sperm motility after successive washes. Data are expressed as means ± SEM ($N = 3$). Different letters indicate significant differences among means.

with ethidium bromide at 0.5 μg ml^{-1}. This experiment was repeated three times.

2.4 *Transgene transmission of exogenous DNA treated with DMSO or DMAc*

2.4.1 *Experimental design:* To evaluate transgene transmission, four groups comprising four fertile laying hens were established: Treatment I – Control (washed semen without DNA); Treatment II – Control (washed semen with DNA); Treatment III – DMSO (washed semen incubated with DNA plus 3% DMSO) and Treatment IV – DMAc (washed semen incubated with DNA plus 3% DMAc). After DNA transfection, the treated spermatozoa were used to inseminate the chickens in each group. Seven consecutive artificial inseminations at 3-day intervals were carried out. Following insemination, eggs were collected to evaluate the rate of fertilization and hatching. Eggs were incubated in an automated incubator at 37°C and at approximately 55% humidity (Barbosa *et al.* 2008). All incubated eggs were submitted to evaluation (candling) on the 10th day of incubation for identification of clear eggs, which were considered unfertilized. After 21 days of incubation, the hatching rate was determined. Fertility (FR) and hatching rate (HR) were calculated as follows:

$$FR(\%) = [(\text{number of fertile eggs}) \times 100]/(\text{number of incubated eggs})$$
$$HR(\%) = [(\text{number of chicks born}) \times 100]/(\text{number of fertile eggs})$$

All eggs were evaluated for EGFP expression (fluorescence), EGFP mRNA transcripts (RT-PCR) and presence of EGFP plasmid DNA (PCR) followed by sequencing analyses.

2.4.2 *Sperm DNA transfection:* The EGFP plasmid DNA (10 μg) was treated with either 3% DMSO (Sigma-Aldrich, USA) or 3% DMAc (Sigma-Aldrich, USA) for 30 min at room temperature. Sperm was collected; spermatozoa motility and vigour were evaluated in the samples from each of the roosters and the samples were pooled. Approximately 10^9 spermatozoa were diluted in 600 μl of Lake's extender and washed twice to remove seminal plasma. The spermatozoa were incubated with the transfectant solutions (in a total volume of 1 ml) as described in section 2.4, including DNA alone and sperm without exogenous DNA for 10 min at 5°C (Shen *et al.* 2006). After DNA transfection, sperm motility and vigour was evaluated to verify the sperm quality.

2.4.3 *Artificial Insemination:* Insemination was carried out by deep artificial insemination, using a catheter for the introduction of the treated sperm 6 cm inside the uterus of the hen (Latorre *et al.* 1988). The insemination dose was 250 μl (25×10^6 cells) of treated sperm.

2.4.4 *pEGFP plasmid DNA detection, sequencing and EGFP expression:* Three days after hatching, blood was collected from the newborn chicks for DNA and RNA extraction. Genomic DNA was purified using the PureLink Genomic DNA Purification Kit (Invitrogen, Carlsbad, USA). PCR for pEGFP-N1 was performed as described above and an internal control reaction was performed using primers to amplify a region of the *OVR* gene (5′-TGGTGATGAAGACTGTTCAGACGG and 5′-CACAC ACTGACCACTGTTACACAC) (Li *et al.* 2008). This primer set amplifies a 970 bp fragment present in the chicken genome. PCR reactions were carried out with an initial denaturation step of 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by a final incubation of 7 min at 72°C. All products were analysed by horizontal gel electrophoresis as described above and the PCR products were sequenced using the MegaBACE 1000 DNA sequencer (GE Healthcare, USA). Samples that contained OVR and EGFP-specific PCR products were classified as PCR positive for transfection.

After hatching, the *in vivo* expression of EGFP was determined by fluorescence using goggles (GFsP-5, BLS, Hungary) containing a filter set to detect EGFP fluorescence and a light source to excite fluorescence (excitation maximum=488 nm; emission maximum = 507 nm). EGFP expression was also evaluated at the transcript level by RT-PCR. RNA was extracted from blood samples collected from the newborn chicks. Blood was frozen and stored in liquid nitrogen until analysis. Total RNA extraction and cDNA synthesis was carried out as previously described (Campos *et al.* 2010a). Briefly, RNA samples were isolated

using TRIzol Reagent (Invitrogen, Carlsbad, USA) and samples were DNase-treated with a DNA-free kit (Ambion, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 200 ng of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. PCR reactions were conducted using primers for EGFP (5′-TATATCATGGCCGACAAGCA and 5′-GAACTCCAG CAGGACCATGT, 219 bp product, GenBank # U55762) and β-actin (5′-TAAGGATCTGTATGCCAACACAGT and 5′-GACAATGGAGGGTCCGGATTCATC, 241 bp product, GenBank # NM_205518). PCR conditions for both genes were: 35 cycles of 94°C for 15 s, 50°C for EGFP and 60°C for β-actin for 30 s and 72°C for 30 s, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. PCR products were analysed on a 1% agarose gel and visualized by staining with ethidium bromide.

2.5 *Statistical analyses*

ANOVA was used to evaluate the effect of the treatments in sperm motility (SM), and vigour. Fisher's exact tests and Pearson's chi-square were used to evaluate fertility rate (FR), hatching rate (HR), EGFP expression and PCR analyses. Differences were considered to be statistically significant at 95% confidence level ($P<0.05$).

3. Results

3.1 *Effect of repeated washes on sperm motility, seminal plasma protein profile, exogenous DNA fragmentation and uptake by sperm cells*

Our results demonstrated that sperm motility was reduced after successive washes. After three successive washes, sperm motility was significantly reduced in comparison to fresh sperm ($P<0.05$) (figure 1).

The effects of the washes on the seminal plasma protein profile were evaluated using a seminal plasma protein band with 72 kDa as a marker. This protein was selected on the basis of its high concentration in the seminal plasma samples (figure 2A). This protein was present at decreasing amounts in samples W0, W1, W2 and W3. From the fourth wash onwards, this protein was only weakly visualized, demonstrating the low concentration of seminal plasma proteins and consequently reduced seminal plasma DNase activity.

It was observed that plasmid DNA was extensively degraded in the presence of fresh sperm and after the first and second washes, since pEGFP-N1 DNA could not be detected by agarose gel analysis. Following the third and fourth washes, the presence of EGFP DNA was detected and a shift from supercoiled to the relaxed conformation

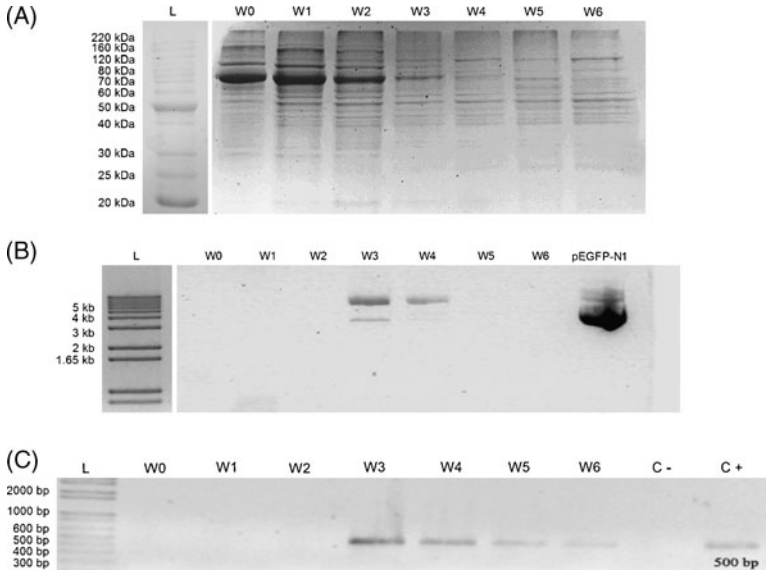

Figure 2. Effect of sperm washes on seminal plasma protein concentration, exogenous DNA integrity and uptake. L: BenchMark™ Protein Ladder (Invitrogen®, USA). W0-W6 represents the number of sperm washes. (**A**) SDS-PAGE analysis in the polyacrylamide gel of the rooster seminal plasma protein in different washes. (**B**) Agarose gel of the vector in the rooster seminal plasma after each wash. (**C**) PCR products of sperm cells after each wash; (−) negative control (+) positive control. (**B** and **C**) L: 1 Kb Plus DNA Ladder (Invitrogen®, USA). The same volume sample (5 μl) for each treatment was running on gels to permit comparison among treatments since the same exogenous DNA concentration (10 μg) was previously used for incubation with sperm.

Table 1. Evaluation of motility and vigour of treated sperm before artificial insemination and fertilization hate (FR), haching rate (HR), EGFP expression and PCR positivity rates for born chicks in the experiment 2

	Motility (%) ± SE (n = 6)	Vigour (0–5) ± SE (n = 6)	FR (%)	HR (%)	*In vivo* EGFP Fluorescence (%)	PCR (%)	RT-PCR (%)
TI	63.7 ± 10.0[a]	3.2 ± 0.7	85.7 (72/84)	81.9 (59/72)	0 (0/59)	0 (0/59)[c]	0 (0/59)[c]
TII	50.70 ± 13.5[a]	3.1 ± 0.3	96.7 (90/93)	90 (81/90)	0 (0/81)	5 (4/81)[b]	0 (0/81)[c]
TIII	38.5 ± 9.3[b]	2.7 ± 0.5	85.5 (71/83)	92.9 (66/71)	1.23 (1/66)	38 (31/66)[a]	21 (14/66)[a]
TIV	54.6 ± 13.4[ab]	3.5 ± 0.5	58.6 (34/58)	67.6 (23/34)	0 (0/23)	19 (5/23)[b]	8.5 (2/23)[b]

Treatments: Treatment I – Control (washed sperm without DNA); Treatment II – Control (washed sperm with DNA); Treatment III – DMSO (washed sperm incubated with 3% of DMSO) and Treatment IV – DMA (washed sperm incubated with 3% of DMA). Parameters: sperm motility; sperm vigour; fertilization rate (FR); hatching rate (HR); embryo GFP expression (GFP) and PCR positive analyses (PCR). a,b differ significantly ($P < 0.05$) in the same column.

was observed from W3 to W4 (figure 2B). In washes W5 and W6, the presence of the vector in agarose gels was again not observed. PCR analysis using DNA extracted from treated spermatozoa showed the presence of exogenous DNA from the third to the sixth wash (figure 2C), demonstrating that after three washes exogenous DNA in incorporated by spermatozoa.

3.2 *Transgene transmission*

Motility and vigour measurements after incubation with exogenous DNA and respective treatments are presented in table 1. There was no significant difference in motility and vigour of the spermatozoa in treatment groups I (control), II (pEGFP) and IV (pEGFP+DMAc). There was also no significant difference in motility between semen incubated with pEGFP+DMSO and pEGFP+DMAc. However, the DMSO treated sperm had a lower motility in comparison to the control. Moreover, no significant differences were observed with respect to fertilization and hatching rates among the treatment groups.

PCR analysis revealed the presence of the EGFP gene in the genomes of newborn chicks from all treatment groups apart from the control (table 1; figure 3). However, the DMSO group demonstrated a significant improvement of transgene transmission with 38% of the samples positive for EGFP DNA, compared to DMAc (19%) and EGFP DNA alone (5%). The 970 bp fragment of *OVR* gene (internal control) was detected in all genomic DNA samples. Thus, a negative PCR for EGFP was considered negative for the presence of EGFP and was not due to inhibition of the PCR reaction. All EGFP PCR products were sequenced and the sequences compared with the *Aequorea victoria* EGFP DNA using the BLAST program (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) and sequence identity ranged from 97% to 100%.

Analysis of transcription levels by RT-PCR showed EGFP expression in 21% of newborn chicks from the DMSO treatment group, 8.5% in the DMAc group, and no EGFP mRNA was detected in DNA only group (table 1; figure 4). However, only one animal from the DMSO treatment group was positive for *in vivo* EGFP expression (figure 5).

4. Discussion

The data presented in this study demonstrate that treatment with DMSO or DMAc, combined with seminal plasma removal, was capable of producing transfected spermatozoa and that the sperm samples could transmit the transgene to offspring by direct artificial insemination. The protocol is notable for its simplicity, no special training or equipment was required and it is applicable to animals, such as chickens, where micromanipulation is not feasible (Koo *et al.* 2006; Harel-Markowitz *et al.* 2009).

During SMGT the exogenous DNA must overcome a series of obstacles, including degradation by endogenous DNases. Previous reports on the DNase activity in the seminal plasma of roosters (Sato *et al.* 2003) and fish (Lanes *et al.* 2009) demonstrated their deleterious effects on SMGT

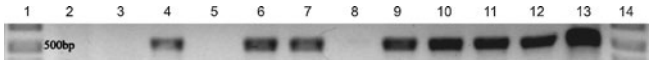

Figure 3. Genomic DNA PCR analysis from chicks. Line 1 and 14: 1 kb plus DNA ladder – Invitrogen®, Carlsbad, USA; lines 2, 5 and 8: C- negative control; line 3: TI – Control (animal from wash semen without DNA); line 4: TII – animal from wash semen with DNA; lines 6 and 7: TIII – animal from pEGFP-DMSO complex; line 9–12: TIV – animal from pEGFP-DMA complex; line 13: C+, positive control.

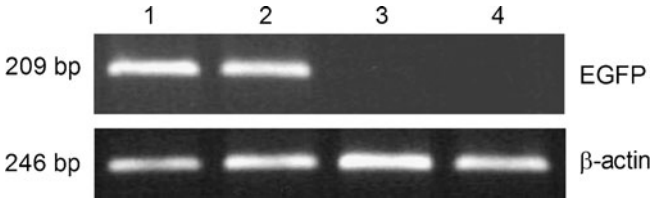

Figure 4. Gene expression analyses by RT-PCR of F0 transgenic chicks. Upper panel shows EGFP amplification and lower panel shows β-actin amplification. Lane 1 – DNA alone; lane 2 – DMSO; lane 3 – DMA; and lane 4 – control (PBS).

success. Our results suggest that two washes are needed to remove or reduce seminal plasma DNase activity and allow uptake of exogenous DNA by the spermatozoa. However, following four or more washes, sperm motility was reduced significantly and compromised fertilization. After the fifth and sixth washes, plasmid DNA was not detected in the seminal plasma, possibly due to improved transfection of the spermatozoa. However, further experiments are needed to elucidate the dynamics of this observation.

Transgene transmission was significantly increased using sperm washes followed by DNA transfection with DMSO. These results are consistent with those obtained with rabbits (Kuznetsov *et al.* 2000): sperm cell permeability to DNA increased from 28% to 81%, but the factors involved in this high success rate were not elucidated. More recently, Shen *et al.* (2006) and Li *et al.* (2006) demonstrated the efficient production of transgenic rabbits and mice by TMGT using DMSO-treated DNA. Although the DMSO reduced sperm motility, fertilization and hatching rates were not affected (Li *et al.* 2006). In addition, the percentage of animals expressing EGFP increased when the spermatozoa were incubated with DMSO-treated DNA.

As DMSO is a cryoprotectant, we hypothesized that DMAc, used in semen cryopreservation (Bianchi *et al.* 2008), could improve transfection rates in avian SMGT. The addition of this amide to sperm cells did not affected motility, vigour or the fertilization and hatching rates. In addition using DMAc on SMGT procedure, 19% of newborn chicks were PCR positive for EGFP and 8.5% of animals expressed EGFP gene on blood cells. Although treatment with DMAc was not as efficient as DMSO, DMAc has a potential application in SMGT. However, further studies focused on optimizing concentration, sperm cells exposure time, exogenous DNA concentration, incubation temperature and sperm damage are necessary to improve its efficiency towards producing transgenic chickens. *In vivo* EGFP expression was only observed in one newborn chick and this originated from the DMSO treatment group. However, EGFP expression at the transcript level was detected in 21 animals. This result of mRNA expression on blood cells corroborates previous reports, showing that CMV promoter can drive EGFP expression in leucocytes of transgenic chickens produced by SMGT (Harel-Markowitz *et al.* 2009).

One of the main questions in the production of transgenic animals is the final location of the transgene – is it integrated into the host genome or is episomal? In SMGT, integration into the host genome occurs in a random manner (Wu *et al.* 2008). In this study we did not evaluate the transgene location by Southern blot analysis. However, Harel-Markowitz *et al.* (2009) showed high rates of transgene integration using SMGT associated to REMI and liposomes to produce transgenic chickens. This study confirmed that transgene integration could be achieved in transgenic chickens produced by SMGT in the future.

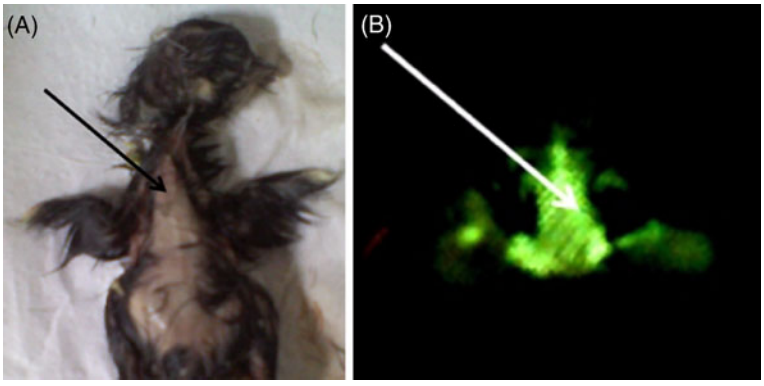

Figure 5. Transgenic chick expressing EGFP. (**A**) Bright field; (**B**) dark field.

The application of transgenic technology to domestic poultry offers an alternative to conventional practices for the improvement of this highly productive agricultural species and its use as a bioreactor model (Lillico *et al.* 2007). Our results show that DMSO treatment is viable alternative to the use of retroviruses, lipids and electroporation, thereby overcoming the safety concerns associated with retroviruses, the cost of commercial liposomes, and the difficulties inherent in using electroporation in mammals *in vivo* (Shen *et al.* 2006).

In conclusion, our study demonstrated the transgene transmission of exogenous DNA treated with DMSO or DMAc after seminal plasma removal in chickens through SMGT technology.

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