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ORIGINAL ARTICLE

First study of sperm mediated gene transfer in Egyptian river buffalo

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Abstract The present study was carried out to find the best treatments for enhancing the ration of insertion of a desired gene construct (pEGFP-N1) onto the sperm of buffalo as the first step for the production of transgenic buffalo using sperm mediated gene transfer (SMGT). The tested conditions were plasmid DNA concentration, sperm concentration, transfecting agent concentration: Dimethyle sulphoxide (DMSO) and time of transfection. The study proved that the best conditions for producing transgenic embryos were incubation sperm solution its concentration is 10^7/ml sperm with 3% DMSO: with 20 μg/ml from the linarized DNA, for 15 min at 4 °C are the best conditions to produce transgenic buffalo embryo using sperm mediated gene transfer.

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1. Introduction

Transgenesis is an important tool in experimental and applied biology, which could be used for altering the characteristics of animals by directly modifying the genetic material. In general, it is as a procedure by which a gene or part of a gene from one individual is incorporated into the genome of the other one [1]. It can be identified merely as a transfer of an exogenous gene into a host genome [2]. Transgenic animal production may facilitate the augmentation of production characteristics like growth, development, disease resistance, reproduction, lactation

Abbreviations: SMGT, sperm mediated gene transfer; DMSO, Dimethyle sulphoxide; EGFP, enhanced green fluorescent protein; OD, optical density; COCs, Cumulus oocyte complexes; PBS, Phosphate buffer saline; TCM199, tissue culture medium; ANOVA, analysis of variance; CCC, covalently closed circular; IVF, *in vitro* fertilization; MII, second meiotic division

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performance, feed efficiency and immune response [3,4]. Moreover, there are many methods for the production of transgenic animals, all of these techniques require expensive equipments, time consuming as well as the low efficiency [5]. The revolution came after the discovery of gene transfer through sperm mediated gene transfer (SMGT) [6]. Sperm mediated gene transfer is the most simple and cheap effective way to produce transgenic animals. It focuses on the natural ability of the sperm cells to carry, internalize and transport the foreign DNA into the oocyte during fertilization. Manipulation step is restricted into the head of the sperm, then nature will be allowed to fulfill its scheduled task of reproduction [7].

Sperm mediated gene transfer could provide the opportunity to carry out transgenesis on a mass scale using spermatozoa as vectors for exogenous DNA [8]. The sperm's ability to bind exogenous DNA molecules and internalize them into nuclei can be exploited by using them as vectors for delivering foreign genetic information to eggs during fertilization. Exploiting the possibility protocols for SMGT has been developed in a variety of animal species with extremely variable results [9].

One of the most exciting recent advances in cell biology is the possibility to use the green fluorescent protein and its various mutated forms as reporter proteins in studies carried out *in vitro* and *in vivo* [10]. The enhanced green fluorescent protein (EGFP) is widely used as a marker or evaluation of tissue-specific expression of transgenic animal production *in vitro* and *in vivo* after the born of transgenic animals [11].

Sperm mediated gene transfer could provide the opportunity to carry out transgenesis on a mass scale successfully succeeded in the production of transgenic farm animals including cattle [12]; pig [13]; goat [14]; rabbit [15] and sheep [16]. Based on a review done by Forabosco et al. [17], transgenic farm animals for food production include a large number of species engineered with the aim of improving economically important traits such as wool growth [18], growth rate [19], meat and milk quality [20,21], mastitis resistance [22], feed conversion [23], lactation and survival [24]. Several factors determine the success of SMGT including the donor of spermatozoa, incubation media, exogenous DNA size and type and the assisted reproductive technique used [25].

In the past decade, Egyptian buffalo genetic improvement took its way using traditional animal breeding methods like the rest of the world. These methods depend on selecting individuals with the desired phenotype as parents for the next generation. Classical breeding usually taking much time, moreover the animals have some disadvantage like low milk yield as compared to cattle. In addition, these methods are based on the use of statistical methodology under a wide range of assumptions. The buffalo reproduction problems such as delayed puberty, higher age at first calving, long post-partum anoestrus period, long inter-calving period, silent heat coupled with poor expression of estrus, seasonality in breeding and low conception rate and poor semen freezing ability [26]. Many recent molecular studies proved that all Egyptian buffalo are one breed and the difference in productivity of meat and milk is due to climate difference especially between south and north of Egypt [27–30]. Transferring genes from cattle or other species to buffalo genome for production of transgenic buffalo may help in overcoming these obstacles. Several groups have developed new procedures or used chemical treatments to improve gene transfer within spermatozoa before fertilization [31,32].

Only one previous study aimed to produce transgenic buffalo through microinjection of linear DNA onto the pronuclei of the fertilized Chinese swamp buffalo oocytes [33]. Therefore, the objective of the present study was to find the best treatments for enhancing the ratio of insertion of a desired gene onto the sperm of buffalo as the first step for the production of transgenic buffalo embryos using sperm mediated gene transfer (SMGT).

2. Materials and methods

2.1. Chemical and medium solutions preparations

All chemicals and media used in the present work were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of gene constructs

2.2.1. Plasmid Preparation and DNA extraction

A commercial bacterial plasmid pEGFP-N1 (BD Biosciences, Bedford, MA, #6085-1) contained a gene construct form of green fluorescent protein, was purchased and kindly provided by Prof. Wei Shen (Laboratory of Germ Cell Biology, College of Animal Science and Technology, Qingdao Agricultural University, China).

2.2.1.1. Bacterial propagation. The plasmid was amplified in DH5α competent *E. coli* bacteria. The propagation was done in 50 ml LB medium containing kanamycin & neomycin antibiotics for 18 h at 37 °C.

2.2.1.2. Plasmid isolation. Plasmid DNA was isolated from selected transformed *E. coli* using Quick and easy Kit for Bacterial Plasmids by Khalil [34]. Pellets of overnight culture were collected by centrifugation. More than 40 Eppendorf tubes (1.5 ml) containing selected transformed plasmid pellets were subjected to the plasmid isolation procedure. At the end of the plasmid isolation steps, plasmid pellets were dissolved in 30 μl TE buffer.

2.2.1.3. Plasmid linearization (cutting by AseI restriction enzyme). The plasmid was linearized after digestion with AseI restriction enzyme (Fastdigest®, Fermentas). For the confirmation of the successful linearization, sample was run on agarose gel electrophoresis with the plasmid isolated. The purity of genomic DNA was determined by measuring the ratio of optical density (OD) at 260 and 280 nm with a UV spectrophotometer (Nano Drop ND-1000-USA). The concentration of genomic DNA was estimated using the following formula. The amount of DNA (ng/μl) = ($OD260 \times 50 \times$ dilution factor). The isolated genomic DNA samples were diluted to get 100 ng of DNA/μl for all samples, divided into aliquots and stored at −20 °C until use in sperm transfection experiments.

2.3. Sperm vitality assessment by using the one-step eosin-nigrosin staining technique

Semen samples were thawed at 37 °C and transferred onto five Eppendorf, which contain DMSO at different concentrations (0% control, 0.3%, 1%, 3% and 5%) ($n = 3$ replicate observa-

tions for any concentration). Every fifteen minutes approximately equal volumes of semen (50 μl) were mixed in a ceramic well with one droplet of the eosin-nigrosin stain (50 μl) and the suspension was incubated for 30 s at room temperature (20 °C). Stained semen smeared, air dried and examined directly. At least 200 sperms were assessed at 1000*g* magnification under oil immersion with a high-resolution 100 bright field objective as recorded by Ahmed et al. [35]. Live spermatozoa were seen to be white in color because they were eosin-impermeable, while, dead spermatozoa were pink because they became eosin-permeable [36].

2.4. In vitro production of buffalo embryos

2.4.1. Oocytes collection and in vitro maturation

Buffalo ovaries were collected at a local abattoir and transported to the laboratory within 2 h in a thermos containing physiologic saline (0.9% NaCl) at 30–35 °C. Cumulus oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated with Phosphate buffer saline (PBS) (136.89 mM NaCl, 2.68 mM KCl, 8.03 mM Na_2HPO_4, 0.66 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.47 mM KH_2PO_4, 5.55 mM glucose, and 1 mM sodium pyruvate, 4 mg bovine serum albumin/ml PBS and 50 μg/ml gentamycin), using an 18-gauge needle attached to a 10 ml disposable syringe. Aspiration contents were collected in 15 ml Falcon tubes and kept in water bath at 37 °C for 15 min. Under a stereomicroscope, intact COCs were selected and washed at least 3 times in PBS then one time in maturation tissue culture medium (TCM199 + 10% fetal calf serum + 10 μg/ml FSH + 50 IU equine chorionic gonadotropin + 50 μg/ml gentamycin). Maturation was performed for 22–24 h at 38.5 °C, under 5% CO_2 in humidified air.

2.4.2. Buffalo semen preparation and incubation with exogenous DNA

Buffalo frozen spermatozoa from fertile bulls were obtained from Al Harm Animal Reproduction Research Institute farm, Al Harm, Egypt. Frozen semen samples were thawed at 37 °C for 40 s and the content was diluted with sperm-TALP medium (100 mM NaCl, 3.1 mM KCl, 25.0 mM $NaHCO_3$, 0.3 mM NaH_2PO4, 2.16 mM lactate (sodium salt), 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1.0 mM pyruvate. Motile spermatozoa were separated by laying out the thawed semen to a gradient separation by centrifugation on a 45/90 discontinuous Percoll density gradient for 30 min at 2000 rpm according to Parrish et al. [36]. Sperm pellet was re-suspended in sperm-TALP medium (100 mM NaCl, 3.1 mM KCl, 25.0 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 2.16 mM lactate (sodium salt), 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1.0 mM pyruvate) supplemented with 6 mg/ml BSA + 50 μg/ml gentamycin and centrifuged for 10 min at 1500 rpm. The Final pellet was re-suspended in fertilization-TALP (Fert-TALP) medium (114.0 mM NaCl, 3.2 mM KCl, 25.0 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 10.0 mM Lactatic acid (sodium salt), 2.0 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgCl_2$. $6H_2O$ and 0.2 mM sodium pyruvate supplemented with 6 mg/ml BSA (Fraction-V), 50 μg/ml gentamycin, 20 mM penicillamine, 1 mM epinephrine and 10 mM hypotaurine) [37] to give a final concentration of 1×10^7 spermatozoa/ml, then was incubated with 20 μg/ml plasmid DNA in 3% DMSO at 4 °C for 15 min. The control group was incubated under the same conditions but without DNA.

2.4.3. In vitro fertilization

After 22–24 h of IVM, matured oocytes were washed three times in IVF medium. (114.0 mM NaCl, 3.2 mM KCl, 25.0 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 10.0 mM Lactatic acid (sodium salt), 2.0 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgCl_2$. $6H_2O$ and 0.2 mM sodium pyruvate and supplemented with 6 mg/ml BSA (Fraction-V), 50 μg/ml gentamycin, 20 mM penicillamine, 1 mM epinephrine and 10 mM hypotaurine), then groups of 25–30 matured oocytes were transferred to each well of a Nunc 4-well culture dish containing 300 μl bicarbonate buffered TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 10 μg/ml FSH, 50 IU/ml eCG and 50 μg/ml gentamycin. Just before fertilization, SMGT treated spermatozoa with or without exogenous DNA were washed with IVF medium and then oocytes and spermatozoa (1×10^7 spermatozoa/ml) were co-incubated for 18–20 h at 38.5 °C under atmosphere of 5% CO_2 in humidified air. In order to evaluate the efficiency of the SMGT technique, the resulting presumptive zygotes were removed from the cumulus mass by gentle pippeting several times according to [38] to show presence of a transgenic construct.

2.5. Detection of sperm mediation transgenic success using fluorescent microscope

Number of embryos keep in embryo culture solution were put inside the circular sticker on glass slide, covered with cover slip and kept to dry at room temperature prior to the examination. The slides were examined on 20× magnification using Nikon Fluorescent microscope (Axio star Z2 Imager Carl Ziess) at blue filter (450–490 nm length). Photos were taken with the aid of (Zen 2011Blue Edit ion) Software attached with the microscope.

2.6. Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's Multiple Range Test with $p \leq 0.05$ being considered statistically significant [39]. Statistical analysis was conducted using SPSS software (v16; Lead Technologies, Inc., IL USA) program. Descriptive values of data were represented as means ± standard deviation.

3. Results

3.1. Plasmid DNA purity and concentration

Plasmid DNA purity and quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant, there is some additional OD, which decreases the ratio between OD 260 and OD 280 nm. Clean DNA has an OD260 between 1.8 and 2.0. Different isolated samples were measured at OD280 & OD260 and the measured value used to calculate the DNA quality. Results in Table 1 showed that all samples have high purity (1.93, 1.82 and 1.93) and high concentrations (7.25,

Table 1 Plasmid DNA purity by calculating the ratio of the A_{260}/A_{280} and DNA Concentration at A_{260}.

DNA Purity				DNA Concentration	
Gel Band	Sample	Dilution	A260/A280	A260	μg/μl
2 band	1	1:250	1.93	145	7.25
	3	1:1000	1.80	232	11.6
1 Band	1	1:1000	1.93	297	14.85

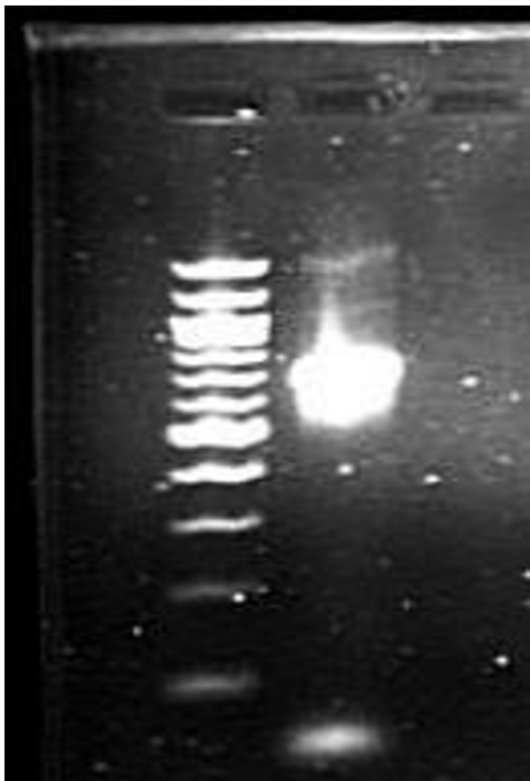

Figure 1 Agarose gel of plasmid after elution and electrophoresis running.

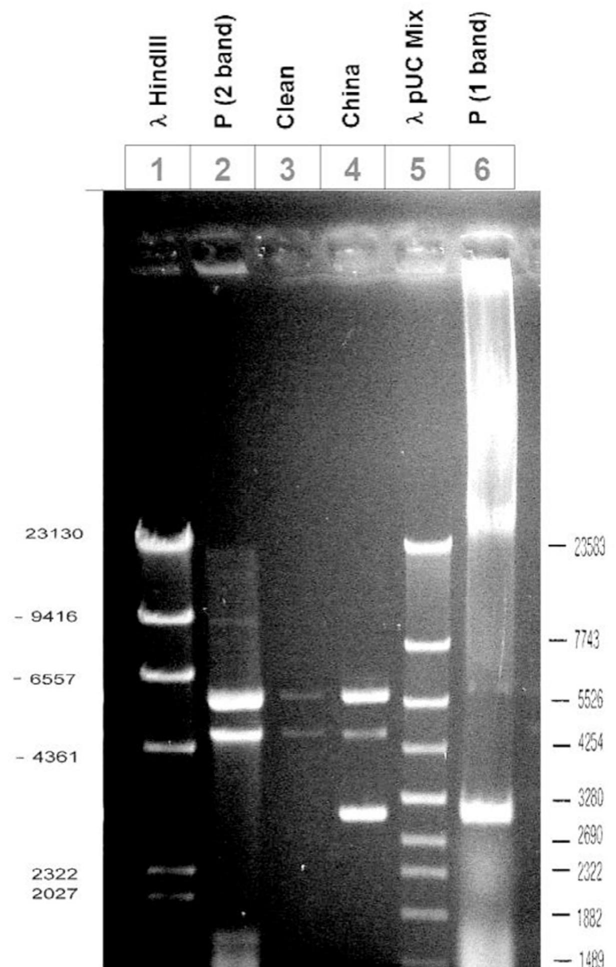

Figure 2 Agarose photos of plasmid all forms and sources after running. (1) HindIII as size markers, (2) P2 plasmid contains two main bands (two forms of plasmid), (3) plasmid after gene cleaning contains two bands, (4) China plasmid contains three bands, one of them represent CCC form of plasmid, (5) DNA λpUC Mix size markers and (6) P1 plasmid in one main band represent CCC form.

11.6 and 14.85) μg/μl respectively. Fig. 1. is showing agarose gel of plasmid after elution and electrophoresis. In contrast, Fig. 2. Is showing agarose photos of plasmid in all forms and sources after electrophoresis running.

3.2. Effect of DMSO on sperm viability

The data of sperm viability test was presented in Table 2. Results showed that sperm viability was significantly decreased ($p \leq 0.05$) by time. There was no significant difference in the percentage of sperm viability of control and the different concentrations of DMSO from zero until 45 min while DMSO concentrations of 3% and 5% at 60 and 75 min incubation significantly decreased sperm viability ($p \leq 0.05$) as compared to control or the 0.3 and 1% DMSO groups. In addition, data showed that DMSO at all concentrations significantly decreased the sperm viability at 90 min of incubation. Accordingly, DMSO at 3% concentration and 15 min incubation were selected as acceptable dose and time that can be used in fluorescence gene transfection. Sperm transfection was done by using 3% DMSO and (1 μg/ml & 20 μg/ml) plasmid DNA for 15 min at 4 °C.

Table 2 Effect of DMSO on sperm viability percentage for different time intervals.

DMSO conc.%	Sperm viability						
	0.0 (min)	15 (min)	30 (min)	45 (min)	60 (min)	75 (min)	90 (min)
Control	84.83 ± 3.06 a	79.83 ± 0.24 b	77.83 ± 1.17 bc	74.33 2.36 cd	70.83 ± 1.17 de	67.33 0.01 ef	63.66 ± 2.82 f
0.3%	84.83 ± 3.06 a	78.50 ± 2.12 b	75.16 ± 3.53 bc	71.83 ± 1.17 cd	68.66 ± 1.41 de	66.99 ± 0.47 de	65.99 ± 0.94 e
1%	84.83 ± 3.06 a	73.49 ± 1.64 b	71.5 ± 2.12 bc	67.33 ± 1.88 bcd	66.49 ± 4.00 cde	64.33 ± 3.77 de	59.99 ± 0.47 e
3%	84.83 ± 3.06 a	75.99 ± 3.76 b	71.16 ± 0.70 bc	70.491.18 bc	69.491.64 bc	66.50 ± 0.71 c	65.16 ± 5.42 c
5%	84.83 ± 3.06 a	72.16 ± 3.53 b	68.83 ± 1.17 bc	67.00 ± 4.24 bc	64.66 ± 1.88 c	63.165 ± 2.59 c	62.001.41 c

Different superscripts within the same column and row designate significant differences ($p \leq 0.05$).

3.3. Oocytes maturation

The total number of collected ovaries was 200, 58 of them were smooth while the rest (142 ovaries) were cyclic animals. In total, 313 excellent and good quality cumulus-oocyte complexes (COCs) were aspirated. Maturation of oocytes successfully took place and 282 matured oocytes were obtained.

3.4. Transgenic embryos produced by IVF of EGFP transfected spermatozoa

Buffalo spermatozoa were incubated with two different concentrations of gene construct (1 µg/ml and 20 µg/ml) or without gene when subjected to *in vitro* fertilization (IVF) resulted in successful fertilization of oocytes and cleaved embryos. In the case of oocytes fertilization using sperm transfected with 1 µg/ml linearized plasmid DNA, totally 85 MII oocytes of 144 were fertilized with 59.03 ± 0.23% fertilization rate and the percentage of cleavage embryos was 23.6 ± 0.41%. Nevertheless, the detection with fluorescent microscopy confirmed that there were no transgenic embryos as presented at Table 3.

As regards to mature oocytes fertilization using sperm incubated with gene at concentration of 20 µg/ml linearized plasmid DNA. A total of 97 MII oocytes of 138 were fertilized with fertilization rate 70.3 ± 0.15% and 9 out of 97 embryos were cleavage and reached the two-cell stage with cleavage rate 9.3 ± 0.15%. Detection with fluorescent microscopy indicated that 72 out of 97 embryos were transgenic with ratio 74.2 ± 0.17% (Figs. 3A, B and 4A, B).

4. Discussion

Transgenic animal production is needed for many purposes including: genetic improvement for some economically important characters such as increasing the quality of special product such as producing cows gives less lactose or cholesterol and high omega-3 fatty acids milk [40]. Sperm mediated gene transfer depends mainly on sperm transfection with the desired gene construct. It has been reported that incubation with exogenous DNA led to the activation of sperm nucleases and a significant decrease in sperm motility. Sperm immobilization and nuclease activation might indicate the presence of natural defenses activated in the sperm after the binding of exogenous DNA. This was reasonable to poor reproducibility of SMGT [41,42]. Selection of good quality sperm, removal of seminal plasma, time and temperature of incubation and quantity of exogenous DNA are important to maximize DNA uptake in transgenic animal production and gives better chances to fertilize the oocytes [43,25]. Therefore, the present study was performed in order to find the best conditions for enhancing the ration of gene insertion to buffalo sperm as the first step for the production of transgenic buffalo using sperm mediated gene transfer (SMGT). The construct with enhanced form of the green fluorescent protein was used. The green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) is finding wide use as a genetic marker or a reporter gene that can be directly visualized in the living cells of many heterologous organisms [44]. This method has a great advantage that transgenic fertilized oocytes or embryos can be implanted into recipient mother directly ensuring the success of transgenesis [45].

Dimethylsuphoxide (DMSO) was used for sperm transfection, since it was reported that DMSO as a transfecting agent is better than or able to replace the use of retroviruses, lipids and electroporation in SMGT techniques [46]. Collares et al. [47] reported that the percentage of animals expressing enhanced green fluorescent protein (EGFP) increased when the spermatozoa were incubated with DMSO-treated DNA. As DMSO is a cryo-protectant, it helps to transfer foreign DNA to spermatozoa as it has a permeable effect on cell membranes [48,49]. The results of the present study were consistent with the research on rabbits and mice that was carried out by [15,46], whereas, 3% DMSO spermatozoa treatment has been successful for *in vitro* fertilization in rabbit and mouse. In addition, it significantly increased the percentage of transgenic embryo production.

Table 3 Summary of embryo production experiment with 1 and 20 µg/ml liniarized plasmid DNA.

Group	Fertilized oocytes %	Cleavage embryos %	Transgenic embryos %	
Control	59.03 ± 0.23 b	23.75 ± 0.21 a	0	b
1 µg/ml pEGFP-N1	59.40 ± 0.44 b	23.6 ± 0.41 a	0	b
20 µg/ml pEGFP-N1	70.30 ± 0.15 a	9.3 ± 0.15 b	74.2 ± 0.17 a	

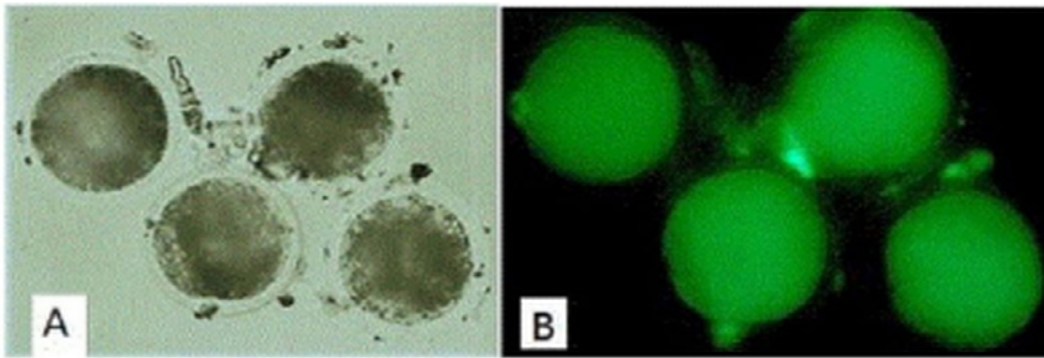

Figure 3 photograph showing (A) transfected fertilized oocytes under inverted microscope and (B) the same fertilized oocytes under fluorescent microscope (×200).

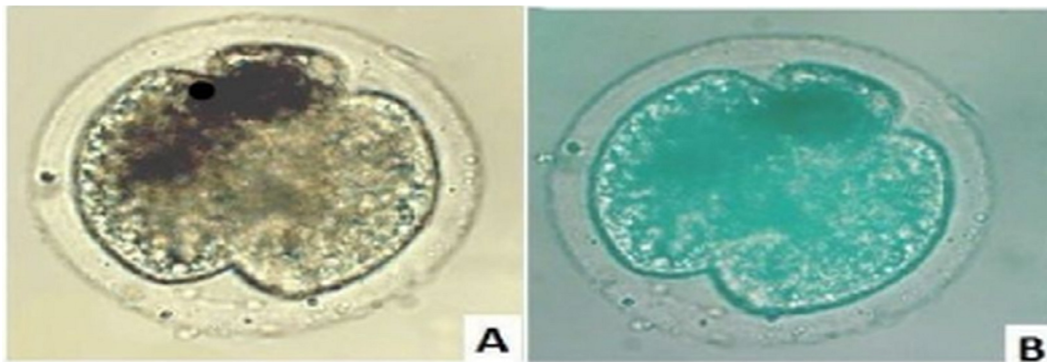

Figure 4 Photograph showing (A) two-cell transgenic embryo under light microscope; (B) the same embryo under fluorescent light microscope (×200).

In our experiments, we found that gene construct (pEGFP-N1) at concentration 20 μg/ml of medium concentration is the optimum for producing transgenic buffalo embryos. Shen et al. [15] reported similar result earlier on their study for producing transgenic mice and rabbits using DMSO as a transfecting agent. Moreover, Kumar et al. [50] reported similar result of gene construct (pbIFN tau-EGFP) at concentration of 1 μg/ml and 20 μg/ml, when incubated with buffalo spermatozoa, and got successful transgenic cleavage embryos. This means that the optimum concentration of linearized plasmid in buffalo is around 20 μg/ml. However, our result did not agree with the study of [6,46,51] for porcine, rabbit and rat transgenic production. As the EGFP DNA, concentrations examined ranged from 0.5 μg/ml to 1 μg/ml, and provided evidence that resulted in no embryonic development beyond the four-cell stage in an *in vitro* study. The reason for this variation of DNA concentration may be due to the length of the plasmid itself, the transfecting agent or the concentration of sperm. Lavitrano et al. [25] reported that the efficiency of the binding depends on the kind of construct used and the amount of DNA used.

In the present study, results showed that the optimal time of incubating linearized plasmid DNA with buffalo sperm ranged from 10 to 15 min. Similar result was reported earlier by [46,15] on their study for producing transgenic mice and rabbits using DMSO as a transfection agent. Canovas et al. [8] reported that bovine spermatozoa showed a particular exogenous DNA binding profile after 5 min of incubation and bulls analyzed showed more than 20% of spermatozoa with exogenous DNA bound. However, Zhao et al. [52] considered that sperm DNA binding could require an incubation time longer than 30 min. Prolonged incubation before fertilization may increase the concentration of exogenous DNA associated with spermatozoa, but it may negatively affect sperm viability. Consequently, fertilization rates and the efficiency of exogenous DNA uptake was compromised, thereby reducing the rate of formation of viable transgenic embryos [53,54].

In addition, it was found that the best temperature for incubation of sperm along with DNA and the help of DMSO was 4 °C. Similar result was reported earlier by [46,15] on their study for producing transgenic mice and rabbits using DMSO as a transfecting agent. There are many conflicting reports about the optimum temperature needed for successful sperm transfection with linearized plasmid DNA. Hoelker et al. [55] on their study incubated bovine spermatozoa with liposome or FuGene 6 with CMV-IFNt-IRES-EGFP (10 μg/ml) DNA for 75 min at 39 °C. Kim et al. [56] incubated pig spermatozoa with pCXEGFP/NEO DNA (40–4 × 10^5 pg/ml) and liposome for time that ranged from 10 to 180 min at different temperatures (4, 17, 25, 38.5, 40 °C) and reported that 17 °C is the best incubation temperature. GarcíaVázquez et al. [49] incubated pig spermatozoa (10^8 cells/ml) with pEGFP-N1 (5 μg DNA/ml) and DMSO at 16 °C for 15 min. Zhao et al. [52] incubated goat spermatozoa with 1000 ng/ml DIG-labeled DNA for 60 min at 20 °C. Kumar et al. [50] incubated buffalo spermatozoa with 1 and 20 μg/ml of gene (pbIFN tau-EGFP) at 37 °C

for one and two hrs. These differences in temperature may be due to some factors like the species of animals, concentration of plasmid DNA and type of the construct (animal source and length), incubation time and the transfecting agent. In bulls the successful binding of exogenous DNA to spermatozoa has been reported by several authors [56,40], although the mechanism of binding and internalization of exogenous DNA is in question that has not been addressed. Some reports are available with respect to development of bovine transgenic embryos [57,8] or calve [12] using SMGT technique. Finally, exogenous DNA under optimal conditions could sufficiently be introduced into spermatozoa by DMSO, and then transfect spermatozoa could be used for *in vitro* fertilization to generate transgenic buffalo.

5. Conclusion

We conclude from the previously described results that the optimum incubation conditions of sperm solutions (concentration of 10^7/ml with 3% DMSO with 20 μg/ml exogenous DNA for 10–15 min at 4 °C) are the best conditions to produce transgenic buffalo embryo using sperm mediated gene transfer technique. Farther study is needed to follow up the integration of gene into buffalo genome through evaluation of newborn transgenic calves.

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