Construction of ovine GH-pmKate2N expression vector and its uptake by ovine spermatozoa using different methods

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Abstract This study aims to produce transgenic ovine spermatozoa bearing Ossimi sheep growth hormone (Os_GH) cDNA using different methods. The complete coding sequence of Os_GH has been registered in GenBank accession no. KP221575. The sequence of Os_GH cDNA has been subcloned into pmKate2-N expression vectors to construct Os_GH-pmKate2-N vector. Five groups of sperm uptake were submitted. All groups were incubated at 37 °C for 1 h: Control (sperm cells were incubated without vector), Traditional incubation (sperm cells were incubated with vector), Heat shock (sperm cells were incubated with vector at 4 °C for 20 min and heated for 2 min at 42 °C), Heat shock + Dimethyl sulfoxide (DMSO) (sperm cells were incubated with vector and supplemented with 3% of DMSO and then submitted to heat shock regime) and DMSO (sperm cells were incubated with vector and supplemented with 3% DMSO). The sperm genomic DNA in groups was extracted. The Os_GH-pmKate2-N vector was introduced efficiently into the head of sperm cells in all treated groups. Adding DMSO either with or without heat shock increased the sperm uptake. The progressive motility was reduced (P < 0.05) by 29.9% in heat shock group compared to the control. Adding DMSO improved (P < 0.05) the total and progressive motilities by 8.2% and 19.8%, respectively in heat shock group compared to the heat shock group without DMSO. The results documented the ability of ovine spermatozoa to uptake the exogenous vector. Also, sperm incubation with 3% DMSO is the best method to introduce the exogenous vector into spermatozoa without notable adverse effects on sperm motilities.

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1. Introduction

The sperm mediated gene transfer (SMGT) is a technique that could be used to produce transgenic animals [26]. This technique utilizes the capacity of the spermatozoa to uptake the exogenous DNA and then introduces it into the oocyte during fertilization [11]. Growth hormone (GH) is a single-chain polypeptide hormone synthesized and secreted by the pituitary gland under hypothalamic control [16,28]. Growth hormone causes an increase in muscles and bone growth and milk production and decrease in fatness [19]. Concentration of blood GH had increased in cows genetically selected for high milk production [2] and in sheep selected for low fat [3]. Transgenic mice, rabbits, sheep and pigs have been used as models to investigate the growth performance of mammals [21,29]. The blood plasma growth hormone was 10–20-fold in transgenic GH-sheep compared to the control [15]. The objective of the present study was to investigate the capacity of ovine spermatozoa to uptake the exogenous Os_GH-pmKate2-N expression vector using different methods and its impact on sperm motility.

2. Materials and methods

2.1. Semen collection, evaluation and cryopreservation

A total of 18 semen samples were collected from three adult Rahmani rams twice a week during 9 weeks by an artificial vagina at Animal Production Dept., Faculty of Agriculture, Cairo University. The semen advanced motility (%), abnormalities (%), live sperm (%) and sperm concentration (×10⁹/ml) were evaluated according to Hafez and Hafez [7]. After evaluation, the semen samples were diluted with Tris-based extender to a final sperm concentration of 250 × 10⁶/ml according to Vivanco and Alarcon [27]. The semen samples were cryopreserved. The cryopreserved semen straws were thawed by immersion in water bath at 37 °C for 1 h with ladder marker range from 100 to 10,000 bp (New England, UK).

2.2. Isolation of ovine growth hormone cDNA

Total RNA was isolated from pituitary gland of Ossimi sheep (Os) using Booze reagent kit (Bioflux®) and converted into cDNA using Oligo dT18 and RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). The Os_GH cDNA was amplified using designed forward primer 5'-GCTCCACAGCTATGATTGCGT-3' and reverse primer 5'-TGGCACTAAGGCGGCAGCT-3'. The PCR reaction mixture consisted of 1 µl of 10× buffer containing MgCl₂ (25 mM), 1 µl of dNTPase mixture (200 µM), 1 µl of F-primer, 1 µl of R-primer, 0.2 µl of Taq polymerase (1 U/µl), 1 µl of Os_GH cDNA and water nuclease-free up to 10 µl. The PCR condition was denaturation for 1 min at 94 °C, annealing for 2 min at 63 °C and extension for 3 min at 72 °C for 29 cycles and final extension at 72 °C for 7 min. The PCR product was visualized using 1% agarose gel stained with ethidium bromide through 1× TAE buffer at 100 V for 1 h with ladder marker range from 100 to 10,000 bp (New England, UK).

2.3. Cloning and expression vectors construction

The visualized band of Os_GH cDNA was extracted from gel using GeneElute™ Gel Extraction Kit (Fermentas, USA) and cloned into pTZ57R/T cloning vector (Fig. 1) according to the kit instructions (2886 bp, Fermentas, USA). The constructed Os_GH-pTZ57R/T cloning vector and pmKate2-N expression vector digested with two restriction enzymes SacI and SacII (Sangon Biotechnology Co., Ltd., Shanghai, China). The digested Os_GH cDNA sequence was ligated into the digested pmKate2-N expression vector in reaction volume 20 µl containing 1 µl of digested pmKate2-N expression vector, 2.5 µl of digested Os_GH cDNA, 2 µl of 10× ligase buffer, 1 µl T4 DNA ligase, and 13.5 µl sterile water and was incubated at 22 °C for 1 h for ligation. The constructed Os_GH-pmKate2-N vector was transformed into DH10B cells according to the kit instructions (Fermentas, USA). The constructed Os_GH-pTZ57R/T vector was extracted from DH10B cells using GeneJet plasmid Miniprep kit. The efficiency of transformation was determined using PCR and electrophoresis.

The pmKate2-N is a mammalian expression vector (Fig. 2) that encodes far-red fluorescent protein (4700 bp, Evrogen, Cat. No. FP182, Russia). The Os_GH-pTZ57R/T cloning vector and pmKate2-N expression vector digested with two restriction enzymes SacI and SacII (Japan Bioscience GmbH, Germany). The digested Os_GH cDNA sequence was ligated into the digested pmKate2-N expression vector in reaction volume 20 µl containing 1 µl of digested pmKate2-N expression vector, 2.5 µl of digested Os_GH cDNA, 2 µl of 10× ligase buffer, 1 µl T4 DNA ligase, and 13.5 µl sterile water and was incubated at 22 °C for 1 h for ligation. The constructed Os_GH-pmKate2-N vector was transformed into DH10B cells according to the kit instructions (Fermentas, USA) and extracted using GeneJet plasmid Miniprep kit. The transformation was confirmed by PCR and electrophoresis. The construction method of Os_GH-pmKate2-N expression vector was summarized in Fig. 3.

2.4. Semen preparation

The cryopreserved semen straws were thawed by immersion in water bath at 37 °C for 1 min. The pooled semen samples (7 straws) were kept in water bath at 37 °C for 5 min and then assayed using Computer Assisted Sperm Analysis (CASA) instrument (SpermVision™ software MiniTube, version 3.0, USA) connected to Olympus BX 51 microscope (Olympus, Japan). The sperm motion parameters recorded were total motility (%), progressive motility (%), distance curved line (DCL, μm), distance average path (DAP, μm), distance straight line (DSL, μm), velocity average line (VAP, μm/s), velocity curved line (VCL, μm/s), velocity straight line (VSL, μm/s), straightness (STR = VSL/VAP, %), linearity (LIN = VSL/VCL, %), wobble (WOB = VAP/VCL), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). The pooled semen that has high percentage of advanced motility, live sperm, straightness and linearity was washed three times using 500 µl of 0.9 NaCl by centrifugation at 4000 rpm for 5 min to remove the seminal plasma that contains inhibitory factors. The seminal plasma inhibitory factors may compete with exogenous DNA for the same binding region on the sperm surface according to [12]. The washed
semen was re-suspended with Tris extender to a final concentration of $40 \times 10^6$ spermatozoa and then assayed using CASA.

2.5. Sperm uptake experiments

A total of 160 µl ovine sperm cell suspension ($40 \times 10^6$ sperm) was incubated with 200 ng of Os_GH-pmKate2-N expression vector according to Kuznetsov et al. [10]. A total of five groups were submitted in sperm uptake experiments using pmKate2-N expression vector; control group 1 (sperm cells incubated at 37 °C for 1 h without vector), Traditional incubation group 2 (sperm cells incubated at 37 °C for 1 h with vector), Heat shock group 3 (sperm cells incubated with vector at 4 °C for 20 min and heated for 42 °C for 2 min then incubated at 37 °C for 1 h), Heat shock + Dimethyl sulfoxide (DMSO) group 4 (sperm cells incubated with vector and supplemented with 1% of DMSO, then vortexed and incubated at room temperature for 10 min and supplemented with 2% DMSO and then submitted to heat shock regime as group 1) and DMSO group 5 (sperm cells incubated with vector and submitted to DMSO regime as group 4 without heat shock then incubated at 37 °C for 1 h). All incubated groups were washed three times with 500 µl of 0.9% saline by centrifugation at 4000 rpm for 5 min to remove the unbind Os_GH-pmKate2-N vector. The sperm motion parameters were performed for washed semen groups using CASA.

2.6. Recognition of pmKate2-N vector in the spermatozoa

The genomic DNA of the spermatozoa was extracted according to Jerzy et al. [9]. The specific primer for pmKate2-N red fusion protein sequence has been designed to confirm the presence of pmKate2-N vector in the extracted genomic DNA of sperm cells; the forward primer was 5'-CCACTCGCTCGAC TAATTCC-3' and the reverse primer was 5'-GATCCCTC
The PCR mixture contained 1 μl of 10× buffer containing MgCl₂ (25 mM), 1 μl of dNTPase (dATP, dTTP, dCTP and dGTP) (200 μM), 1 μl of forward primer, 1 μl of reverse primer, 0.2 μl of Taq polymerase (1 U/μl), 1 μl (50 ng) of total semen DNA and water nuclease-free up to 10 μl. The PCR conditions were denaturation for 1 min at 94°C, annealing for 30 s at 59°C, and extension for 3 min at 72°C, and then final extension at 72°C for 5 min after 35 cycles. The PCR product of mKate2 red fusion sequence (690 bp) was electrophoresed and visualized as mentioned above.

3. Statistical analysis

Data of sperm motion parameters after sperm uptake experiments were analyzed using the general linear model [20] according to the following model:

\[ Y_{ij} = \mu + T_i + E_{ijk} \]

where:

- \( Y_{ij} \) = the observation \( ij \).
- \( \mu \) = Overall mean.
- \( T_i \) = Treatment (\( i = 1 \), Control; \( i = 2 \), Traditional incubation; \( i = 3 \), Heat shock; \( i = 4 \), Heat shock + DMSO, \( i = 5 \), DMSO only).
- \( E_{ijk} \) = Experimental error, \( ij \) observation assumed to be randomly distributed (0 – 6²).

The differences among means were tested [3].

4. Results and discussion

4.1. Growth hormone cDNA isolation and subcloning

The electrophoretic band of Os_GH cDNA was 690 bp (Fig. 4). The uncut forms of Os_GH-TZ57R/T (3575 bp) and Os_GH-pmKate2-N (5390 bp) vectors are shown in Figs. 5 and 6, respectively. The PCR electrophoresis bands of Os_GH-TZ57R/T cloning and Os_GH-pmKate2-N expression vectors were 690 bp (Figs. 7 and 8, respectively) that might indicate that the sequence of Os_GH cDNA was subcloned into pTZ57R/T and pmKate2-N vectors successfully.

4.2. Sperm uptake

The physical characteristics of Rahmani semen samples before cryopreservation were 84.2% for advanced motility, 369 x 10⁹ for sperm concentration, 4.8% for sperm abnormalities and 81.8% for live sperm. All tested semen characteristics in the present study were in normal range as reported previously [24]. After cryopreservation, the advanced sperm motility was reduced from 84.2% to 70.2%. The PCR electrophoresis of spermatozoa after incubation with Os_GH-pmKate2-N expression vector (Fig. 9) illustrated that Os_GH-pmKate2-N expression vector was introduced efficiently into the head of spermatozoa in all treated groups. The Os_GH-pmKate2-N vector was spontaneously uptaken by spermatozoa incubated at 37°C for 1 h.
**Fig. 4** Electrophoretic pattern of Os_GH cDNA band. M refers to DNA marker given in Kbp; Os_GH cDNA, Ossimi sheep growth hormone cDNA; 1, 2 and 3 are the samples of Os_GH cDNA.

**Fig. 5** Electrophoretic pattern of uncut forms of Os_GH-TZ57R/T cloning vector. M refers to DNA marker given in Kbp; 1, 2 and 3 are the samples of uncut Os_GH-TZ57R/T cloning vector.
Fig. 6  Electrophoretic pattern of uncut forms of Os_GH-pmKate2-N expression vector. M refers to DNA marker given in Kbp; 1 and 2 are the samples of uncut forms of Os_GH-pmKate2-N expression vector.

Fig. 7  Electrophoretic pattern of Os_GH-TZ57RT cloning vector (PCR). M refers to DNA marker given in Kbp; 1, 2 and 3 are the samples of Os_GH-TZ57R/T cloning vector.
Fig. 8  Electrophoretic pattern of Os_GH-pmKate2-N expression vector band (PCR, 740 bp). M refers to DNA marker given in Kbp; 1, 2 and 3 are the samples of Os_GH-pmKate2-N vector.

Fig. 9  Electrophoretic pattern of Os_GH-pmkate2-N vector after sperm uptake using mkate primer (PCR, 690 bp). Line1, 1 M refers to DNA marker given in Kbp, Line2, Negative control (spermatozoa incubated at 37 °C/1 h without vector), Line3 (1), Traditional incubation group (spermatozoa incubated at 37 °C/1 h with vector), Line4 (2), Heat shock group (spermatozoa incubated at 4 °C/20 min with vector, then at 42 °C/2 min and finally at 37 °C/1 h), line5 (3), Heat shock + DMSO group (spermatozoa supplemented with 3% DMSO and incubated with vector at 4 °C/20 min, then at 42 °C/2 min and finally at 37 °C/1 h) and line6 (4), DMSO group (spermatozoa supplemented with 3% DMSO and incubated with vector at 37 °C/1 h).
The enhancement effect of DMSO on sperm uptake is supported by the results reported by Jacob and Herschler [8], where the DMSO has physiological and technical characteristics of replacing the water in living cells, which interferes with the production of intracellular free oxygen radicals, increasing the permeability of cell membranes, so all molecules are transported across cell membranes of sperm. In the present study, the results of sperm uptake efficiency are supported by Kuznetsov et al. [10], where transgenic rabbits were produced using sperm mediated gene transfer.

4.3. Computer assisted sperm analysis (CASA)

The cryopreserved semen straws have been pooled and washed to remove the seminal plasma inhibitory factors that might prevent the dangerous molecules such as foreign DNA from gaining access to spermatozoa [12]. The seminal plasma inhibitory factors such as Polyamines are basic molecules presented in the most mammalian species and strongly binding to acidic or negatively charged molecules, such as DNA [23].

A second seminal plasma inhibitory factor is glycosaminoglycans that might interfere with the binding of foreign DNA to spermatozoa by competing for the same binding sites [13]. The incubation effect of Os_GH-pmKate2-N vector and treatments on sperm motility traits is shown in Table 1. The total sperm motility was reduced significantly by 5.5% in spermatozoa that incubated with vector at 37 °C for 1 h and by 15.9% in Heat shock group compared to the control (60.5% vs. 53.8%, respectively). The Heat shock group had a significant adverse effect on the progressive motility reduced by 29.9% compared to the control (36.3% vs. 51.8%, respectively). The Heat shock + DMSO group and did not differ (P<0.05) with other groups. In DMSO group, the progressive motility was increased (P<0.05) by 19.8% when adding 3% DMSO compared to Heat shock without DMSO. In DMSO group, the progressive motility was reduced by 29.9% compared to the control (36.3% vs. 51.8%, respectively).

In Heat shock group, the progressive motility was enhanced by 19.8% when adding 3% DMSO compared to Heat shock group without adding DMSO (43.5% vs. 36.3%, respectively). Furthermore, the progressive sperm motility was higher (P<0.05) in DMSO group than other treated groups. This enhancement in total and progressive motilities may be due to the effect of the physiological characteristics of DMSO. The percentage of sperm STR was significantly (P<0.05) higher in Heat shock group than Heat shock + DMSO but did not differ significantly with other groups. In DMSO group, the WOB value was higher (P<0.05) than Heat shock + DMSO group and did not differ (P<0.05) compared to the other treated groups.

The reduction in Wob value in Heat shock group with or without DMSO may be attributed to the adverse effect of heat shock on the movement of head sperm. In traditional incubation and heat shock groups, the value of BCF was increased (P<0.05) compared to other experimental groups. The values of VSL, VCL, ALH, STR and LIN were increased (P<0.05) in all treated groups compared to the control. The semen parameters such as VSL, VCL, ALH, STR and LIN were positively correlated with bull fertility [4,17]. The motility and velocity parameters of spermatozoa reflect their mitochondrial function and energy status indirectly. The higher values of VCL and ALH indicate that there is a major bending of the mid piece and large amplitude of lateral head displacement. This signifies the hyper-activation of the spermatozoa that in

Table 1 Effect of Os_GH-pmKate2-N vector uptake on sperm motility (mean ± SEM).

| Parameters                      | Control (37 °C/1 h)       | Os_GH-pmKate2-N expression vector groups |
|---------------------------------|---------------------------|------------------------------------------|
|                                 | Traditional incubationA   | Heat shockC + DMSO                       | DMSO2D |
| Total motility (%)              | 64.0 ± 0.8b               | 60.5 ± 0.8c                              | 58.2 ± 0.7d |
| Progressive motility (%)        | 51.8 ± 0.8b               | 45.0 ± 0.9d                              | 43.5 ± 0.6d |
| DAP (µm)                        | 23.8 ± 0.3d               | 23.9 ± 0.4ed                             | 25.0 ± 0.3ab |
| DCL (µm)                        | 38.0 ± 0.4ed              | 38.0 ± 0.5e                              | 40.4 ± 0.4ed |
| DSL (µm)                        | 20.6 ± 0.3d               | 20.8 ± 0.4ed                             | 22.0 ± 0.3a |
| VAP (µm/s)                      | 51.8 ± 0.6ed              | 51.8 ± 0.8ed                             | 54.2 ± 0.7e |
| VCL (µm/s)                      | 82.6 ± 0.9ed              | 82.4 ± 1.2ed                             | 87.2 ± 0.8e |
| VSL (µ/s)                       | 44.7 ± 0.6e               | 45.1 ± 0.8e                              | 47.5 ± 0.6e |
| STR (VSL/VAP, %)                | 0.86 ± 0.0ab              | 0.86 ± 0.0ab                             | 0.87 ± 0.0ab |
| LIN (VSL/VCL, %)                | 0.54 ± 0.0bc              | 0.54 ± 0.0ab                             | 0.53 ± 0.0ad |
| WOB (VAP/VCL)                   | 0.62 ± 0.0b               | 0.62 ± 0.0b                              | 0.61 ± 0.0bc |
| ALH (µm)                        | 2.9 ± 0.0e                | 2.8 ± 0.0b                               | 2.9 ± 0.0e |
| BCF (H2)                        | 28.8 ± 0.2b               | 29.2 ± 0.3ab                             | 29.7 ± 0.3a |

Means having different superscript letters (a, b, c, d, e) within the same row differ (P<0.05). DAP, Distance Average Path (microns); DCL, Distance Curved Line (microns); DSL, Distance Straight Line (microns); VAP, Velocity Average Line (microns/sec); VCL, Velocity Curved Line (microns/sec); VSL, Velocity Straight Line (microns/sec); STR, Straightness (VSL/VAP); LIN, Linearity (VSL/VCL); WOB, side to side movement of the sperm head (Wobble, VAP/VCL); ALH, Amplitude of Lateral Head Displacement (microns); BCF, Beat Cross Frequency (H2).

A Traditional incubation group, spermatozoa incubated at 37 °C/1 h with vector.
B Heat shock group, spermatozoa incubated with vector at 4 °C/20 min then at 42 °C/2 min and finally at 37 °C/1 h.
C Heat shock + DMSO group, spermatozoa supplemented with 3% DMSO and incubated with vector at 4 °C/20 min then 42 °C/2 min and finally at 37 °C/1 h.
D DMSO group, spermatozoa supplemented with 3% DMSO and incubated with vector at 37 °C/1 h.
turn implies high energy state of the spermatozoa, which is essential for sperm penetration through cervical mucus, zona pellucida, fuse with the oocytes, and successful fertilization [1].

According to the current study, the sperm hyperactivity was observed in Heat shock, Heat shock + DMSO and DMSO groups. Sebastian et al. [22] reported that some sperm motions such as VSL (44.06% vs. 64.80%), VAP (69.39% vs. 89.31%), LIN (37.96% vs. 52.86%), and SRT (61.68 vs. 71.69) were reduced \( (P < 0.05) \) after semen incubation with exogenous DNA compared to the control group. On the other hand, they concluded that such reduction in VSL, VAP, LIN, and SRT did not affect the ability of the sperm to fertilize the oocyte in vitro.

5. Conclusions

The Os_GH-pmKate2-N expression vector has successfully uptaken by ovine spermatozoa in all groups: Traditional incubation group, Heat shock group, Heat shock + DMSO group and DMSO group without significant adverse effects on sperm motion.

Authors’ contributions

Waleid Shakweer, design of experimental trials, writing the paper, construction of cloning and expression vector, semen collection, evaluation, cryopreservation and sperm uptake experiment and Computer assisted sperm analysis (CASA). Yassein Hafez, design of experimental trials, statistical analysis of results and paper revision. Ashraf EL-Sayed, paper revision. Ibrahim Awadalla and Mamdough Mohamed, providing the animals for semen collection and animals care.

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