Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function

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Abstract

The objective was to confirm the presence of nerve growth factor (NGF) and its specific receptor, TrkA, in ejaculated bovine sperm, and to investigate the effects of NGF on specific aspects of bovine sperm function. Both TrkA transcripts and immunoreactivity typical of the translated protein were detected by RT-PCR and western blotting. However, only the NGF protein was detected in bovine sperm using western blotting, and there was no RT-PCR evidence for NGF transcripts in sperm. Using an immunofluorescent technique, NGF-immunoreactivity was localized to the sperm head and tail, whereas that of TrkA was detected in the acrosomal cap, nucleus, and tail regions. When sperm were treated with exogenous NGF, both leptin secretion and sperm viability were increased (P < 0.05); moreover, the percentages of late apoptotic and dead sperm were increased (P < 0.05). However, NGF had no effects on insulin secretion, mitochondrial activity, intracellular calcium levels, or the acrosome reaction of sperm (P > 0.05). In conclusion, the presence of TrkA transcript, as well as NGF and TrkA immunoreactivity were confirmed in bovine sperm. Furthermore, exogenous NGF had significant effects on the secretion of leptin, cell viability, and sperm apoptosis. This study provided strong evidence that NGF/TrkA may have roles in regulation of sperm physiology and perhaps male fertility and infertility.

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1. Introduction

Neurotrophins (NTs) are a family of proteins that regulate development and maintenance of sympathetic and sensory peripheral neurons, as well as central cholinergic neurons [1]; they include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and NT4 [2]. The functions of neurotrophins are mainly mediated via high-affinity tyrosine kinase (Trk) receptors, which are specific for various neurotrophins [3]. There is evidence that NTs may not only have effects on the nervous system, but also play important roles in regulating the development of cells in cardiovascular [4], immune [5], and endocrine [6] systems, as well as non-neuronal cells in reproductive systems [7]. The expression of NGF and
its receptor (TrkA) in human fetal and adult ovaries [8] implied a role for NGF in this organ, whereas the ability of NGF to stimulate E2 secretion by human preovulatory ovarian follicles, both directly and by increasing the formation of FSHR [9], provided indications of potential mechanisms of action. In addition, marked reductions in populations of primary and secondary follicles, accompanied by increased numbers of oocytes that failed to be incorporated into a follicular structure, were detected in the ovaries of homozygous NGF-null mutant animals, despite the presence of physiologic serum gonadotrophin concentrations [10]. Expression of NGF and TrkA was also detected in animal testes [11], and NGF increased Leydig cell steroid production [12]. Collectively, these observations provided growing evidence that NGF played important roles in animal reproduction.

Although the presence of RNA in mature sperm is still equivocal, sperm RNA could play important roles in early zygotic and embryonic development [13]. In the present study, we investigated the roles of NGF and its receptor in sperm physiology, and hence male fertility. To this end, we assessed the presence of NGF and TrkA protein and mRNA in ejaculated bovine sperm, and examined the effects of NGF on key aspects of sperm function.

2. Materials and methods

2.1. Sperm preparations

Motile bovine sperm from frozen semen of three Yanbian bulls were collected by the swim-up method, as described [14]. Unless otherwise stated, sperm were obtained using this method throughout this study. Briefly, 0.25 mL aliquots of thawed bovine semen were layered under 1 mL aliquots of Sp-TALP medium [14] in each of four, 5 ml plastic tubes. Sperm were then centrifuged (1000 × g for 6 min) and washed twice with Sp-TALP, then resuspended in this medium to a final concentration of 10 × 10^6/mL. Samples were examined microscopically to ensure that there was no contamination by leukocytes or other unwanted cellular material.

2.2. Extraction of RNA and reverse transcription polymerase chain reaction

Total RNA was extracted using an RNAprep Micro Kit (Tiangen Co., Beijing, China). To avoid DNA contamination, RNA samples were treated with DNase, then reverse transcription was carried out with a commercial kit (Promega Co., Madison, WI, USA) and the resulting cDNA was frozen until used. The PCR was carried out in mixtures with a final volume of 25 μL containing 150 ng cDNA, 2.5 μL of 10 × Buffer, each primer at a final concentration of 0.2 mol/L, and 0.20 units of Taq polymerase (TaKaRa Dalian Co., Dalian, China). The specific primers used for amplifying genes encoding NGF and TrkA are shown (Table 1). For negative controls, RNA samples were incubated without reverse transcriptase. The PCR products were electrophoretically separated in agarose gels, eluted, and sequenced using an ABI 377 DNA (Applied Biosystems, Foster, CA, USA) and using M-13F/R primers (TransGenBiotech Co., Beijing, China).

2.3. Western blotting

Total protein was extracted from isolated sperm using a commercial kit, following the manufacturer’s instructions (Applygen Co., Beijin, China), approximately 50 μg protein was separated by SDS-PAGE on a 12% polyacrylamide gel and the separated proteins were transferred onto PVDF membranes (Millipore Co., Billerica, MA, USA) using a suitable transfer system at 80 V for 2 h. Membranes were blocked by incubation with blocking solution (5% non-fat dried milk in TBST) for 2 h at room temperature. They were then incubated with rabbit anti-human antisemur (anti-NGF or TrkA, as appropriate) overnight at 4 °C, washed with TBST (3 × 15 min) and incubated with HRP-conjugated goat anti-rabbit secondary antibody (diluted 1:5000 in TBST) for 2 h at room temperature. Membranes were then washed several times and proteins were detected using SuperSignal substrate (Pierce Co., Rockford, Il, USA) and exposure to X-ray films.

2.4. Immunofluorescence

Motile sperm were spread onto microscope slides, air dried at room temperature, fixed with absolute methanol for 10 min at -20 °C, and treated with 0.3% Triton X-100 (Sigma Co., St. Louis, MO, USA) for 5 min at room temperature. The slides were then blocked with normal goat serum (10%) in PBS at room temperature
for 30 min and incubated with rabbit anti-human antibodies (anti-NGF or TrkA, as appropriate) at 4 °C for 24 h. Following three washes with PBS, slides were exposed to goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC, 1:100; Boster Co., Wuhan, China) for 60 min at room temperature, and then washed with PBS. For negative controls, slides were incubated with BSA diluted with PBS instead of primary antibody. All slides were examined under a fluorescence microscope (Olympus Co., Tokyo, Japan; 400× magnification).

2.5. Effects of NGF on sperm insulin and leptin secretion, viability, mitochondrial activity, apoptosis and calcium concentration

Four triplicate sets of collected motile sperm samples were incubated with NGF at various concentrations (0, 5, 20, 40, or 80 μg/L) in Sp-TALP medium for 2 h (38.5 °C, 5% CO₂) to assess effects of NGF on sperm insulin and leptin secretion, viability, mitochondrial activity and calcium concentration, and a further set for 3 h to assess its effects on sperm apoptosis.

After 2 h, culture medium was recovered by centrifugation (1500 × g, 10 min) from one set of samples incubated with NGF at all the above concentrations, and supernatants were frozen until concentrations of insulin and leptin in the supernatants were determined using bovine insulin and leptin ELISA kits (Cusabio Co., Carlsbad, CA, USA), respectively, according to the manufacturer’s protocols.

After 2 h, the viability of the sperm in another set of samples (incubated with 0, 20, 80, or 120 μg/L NGF) was determined using a Live/Dead® sperm viability kit (Invitrogen Co., Foster, CA, USA), following the manufacturer’s instructions, to stain live sperm green with SYBR-14 and dead sperm red by propidium iodide, then assessing their proportions by flow cytometry. A total of 10,000 gated events were analyzed per sample.

A further set of samples incubated with 0, 20, 40, 80, or 120 μg/L NGF was diluted in Sp-TALP medium containing 2 μM JC-1 (Beyotime Co., Hangzhou, China) and incubated at 37 °C for a further 20 min. The samples were then washed once with TALP medium, and percentages of cells with high and low mitochondrial membrane potentials were evaluated using flow cytometry. A total of 10,000 gated events were analyzed per sample.

Another set of samples incubated in Sp-TALP media with 0, 20, 80, or 120 μg/L NGF for 3 h (38.5 °C, 5% CO₂) was collected by centrifugation, then Annexin V/PI (Beyotime) was used to quantify viable and apoptotic cell populations by flow cytometry, following the manufacturer’s instructions. Again, in total, 10,000 gated events were analyzed per sample.

Flow cytometry was also used to determine calcium concentrations of sperm following incubation with 0, 20, 80, or 120 μg/L NGF (38.5 °C, 5% CO₂) for 2 h, using Fluo-3 AM (Beyotime), again following the manufacturer’s recommendations and analyzing 10,000 gated events per sample. Calcium levels in sperm were expressed as an average of the intensity of the recorded fluorescence.

2.6. Effect of NGF on the acrosome reaction

Frozen semen was thawed and pooled. Motile sperm were collected and then incubated with 60 μg/mL heparin (39 °C, 30 min) in Sp-TALP medium to allow capacitation. Sperm were then treated with 0, 20, 80, or 120 μg/L NGF for 2 h at 38.5 °C. In positive controls, the acrosome reaction was induced by egg yolk lysophosphatidylcholine (Sigma). Subsequently, sperm were evaluated for their acrosomal status with Coomassie blue stain, as described [15]. Stained sperm were examined under bright field microscopy at 600× magnification. A minimum of 200 sperm were observed on each slide. Sperm with acrosomes intact stained darkly near the apical portion of the sperm head (i.e. the location of the acrosome). However, acrosome-reacted sperm stained

![RT-PCR analysis of NGF and TrkA mRNA expression in ejaculated bovine sperm. Although NGF mRNA was not detected in sperm (lane 2), it was detected in testis (lane 1). An expected fragment of TrkA was obtained from sperm (lane 2) and testis (lane 1). Negative controls are shown in lane 3 (without reverse transcriptase) and lane 4 (without cDNA); M = DNA marker. Both NGF and TrkA proteins were detected in bovine testis (T) and sperm (S). In negative controls, antibodies were replaced by normal goat serum (N).](image-url)
very faintly or not at all in the acrosome region. All experiments were performed in duplicate on four separate occasions.

2.7. Statistical analyses

The experiments for RT-PCR, Western blot, and Immunofluorescence were repeated at least three independent occasions, whereas ELISA analysis, Flow cytometry analysis, and acrosome reaction analysis were performed at least four replicate experiments. The data were presented as the means ± SEM. The differences in mean values were calculated using one-way ANOVA (as implemented in SPSS 13.0. software), and Dunnet’s test was applied for multiple comparison. A value of $P < 0.05$ was taken to indicate a statistically significant difference between means.

3. Results

3.1. RT-PCR and western blotting

The RT-PCR did not yield a product of predicted size for NGF (150 bp) from sperm with NG-F and NG-R primers. Interestingly, however, a specific fragment of TrkA was obtained with TR-F and TR-R primers (Fig. 1A).

The presence of the NGF and TrkA proteins in ejaculated bovine sperm was investigated using the western blotting technique and applying rabbit antihuman NGF or TrkA polyclonal antibodies, respectively, to membranes after protein transfer. Although specific bands were detected with our antibodies on membranes displaying total sperm protein, no bands were detected in negative controls (antibodies replaced by normal goat serum; Fig. 1B).

3.2. Immunolocalization of NGF and TrkA in ejaculated bovine sperm with immunofluorescence

To corroborate the presence of NGF and TrkA proteins in bovine sperm, and determine the cellular localization of these proteins, sperm smears were stained immunofluorescently with rabbit antihuman anti-NGF or anti-TrkA polyclonal antibodies. The NGF protein was identified in the head and tail regions, whereas the TrkA protein was mainly localized to the acrosomal cap, nucleus, and tail of the sperm (Fig. 2).

Fig. 2. Immunolocalization of NGF and TrkA in ejaculated bovine sperm by confocal microscopy, following immunofluorescent staining. (A) The NGF-immunoreactivity was localized to the head and tail of sperm. (B) Immunoreactivity of the TrkA protein was mainly localized to the acrosome, nucleus, and tail of bovine sperm. (C, D) Sperm incubated with BSA (in lieu of antibodies) served as negative controls.
3.3. Effects of NGF on sperm insulin and leptin secretion, viability, mitochondrial activity, apoptosis, and calcium concentration

The secretion of leptin by sperm was increased significantly when exogenous NGF (40 or 80 μg/L) was added to the incubation medium. However, production of insulin by sperm was not significantly affected by NGF (Fig. 3).

Based on flow cytometry, sperm viability was increased (P < 0.05, Fig. 4) when exogenous NGF was added to the incubation medium, at a range of concentrations (20 to 80 μg/L). Mitochondrial activity was also higher when similar concentrations of exogenous NGF (20–120 μg/L) were added, but not significantly (P > 0.05). In addition, the mean percentages of live and early apoptotic sperm were not altered (P > 0.05) following exposure to NGF at a range of concentrations. However, the percentages of late apoptotic and dead sperm increased (P < 0.05) when concentrations of NGF were increased from 20 to 120 μg/L (Fig. 5).

3.4. Effects of NGF on sperm calcium concentration and the acrosome reaction

Intracellular calcium levels in sperm were not significantly affected by treatment with NGF at concentrations of 20–120 μg/L (Fig. 6A), and no differences were detected in the acrosome reaction when sperm were treated with NGF at various concentrations (P > 0.05). However, in positive controls, an acrosome reaction was induced at a high rate with egg yolk lysophosphatidylcholine (Fig. 6B).

4. Discussion

Based on expression analyses of NGF and TrkA proteins, and corresponding mRNA species, NGF and TrkA were expressed in ejaculated bovine sperm; this was corroborated by immunolocalization of these proteins in sperm. In addition, NGF had significant effects on leptin secretion, sperm viability, and apoptosis. Based on these novel findings, in addition to previous reports of NGF and TrkA expression in animal reproductive systems [16,17], we inferred that NGF may
play an important role in animal reproduction. It was noteworthy that we previously demonstrated that NGF and TrkA were expressed in ejaculated human sperm, and that both NGF protein in seminal plasma and TrkA mRNA in spermatozoa are low in samples from oligoasthenozoospermic men [18].

Using RT-PCR, a fragment of the TrkA gene was amplified, but no amplified products of the NGF gene were obtained. However, both NGF and TrkA proteins were detected by western blotting and immunofluorescent techniques. Although ejaculated mammalian sperm are considered highly differentiated terminal cells, in which no mRNA transcription or translation occurs, mRNA has been identified in ejaculated mammalian sperm in both this and other studies[19]. One hypothesis as to why a terminally differentiated cell should retain mRNA is that these transcripts may be merely residues that reflect the shutting down of transcription during spermiogenesis [20]. However, multiple researchers have shown that some specific RNAs in sperm are associated with embryo development[21]. Furthermore, there is recent evidence that reverse transcriptase is active in mature sperm, as well as that sperm can translate nuclear-encoded proteins by mitochondrial-type ribosomes [22,23], indicating that sperm RNAs are functional.

Furthermore, NGF mRNA has been localized to sperm and early spermatids of adult mice using in situ hybridization, and NGF-like immunoreactivity has been de-
tected in germ cells of rat and mouse testis, suggesting that NGF may be involved in sperm maturation [16]. Based on the current results, we inferred that NGF may be a regulator of sperm function, perhaps through a paracrine mechanism.

Several lines of evidence suggest that insulin and leptin have roles in the regulation of male reproduction. For instance: insulin can promote spermatogonial differentiation into primary spermatocytes [24]; mice lacking the leptin gene have small testes, azoospermia, and multinucleated spermatids [25]; and ejaculated sperm can modulate their metabolism independently with insulin and leptin through autocrine mechanisms [26,27]. Furthermore, NGF increased glucose-induced insulin secretion by modulating electrical activity in adult beta cells [28], indicating that NGF could regulate metabolism by affecting expression of insulin, leptin, or both. Therefore, whether NGF could influence the secretion of these proteins from bovine sperm was studied. Although the secretion of insulin from sperm was not affected significantly by treatment with exogenous NGF, secretion of leptin was increased, indicating that NGF could affect sperm metabolism by regulating leptin secretion.

Specific factors (including freezing and the presence of reactive oxygen species) affect sperm viability and mitochondrial activity while promoting sperm apoptosis [29–31]. Exogenous NGF rescued Sertoli cell viability, indicating that NGF may regulate spermatogenesis through paracrine mechanisms [32]. In the present study, exogenous NGF increased bovine sperm viability, but had no effect on mitochondrial activity in sperm. The functional impact of programmed cell death in human sperm is poorly understood [33], so the mechanisms of action of hormones in sperm apoptosis remain elusive. The phosphoinositide-3 kinase (PI3K) signaling pathway represents an important intracellular mediator of cell survival and antiapoptotic signals [34]. It is, therefore, interesting that both insulin and leptin can increase PI3K activity in uncapacitated sperm [26,27]. However, in the present study, although exogenous NGF increased secretion of leptin by bovine sperm, it also increased the percentage of late apoptotic sperm. Perhaps NGF influenced the survival of bovine sperm via an alternative signaling pathway. In that regard, NGF has enhanced the survival and differentiation of neuronal cells [35]. Recently, there were indications that NGF can also induce the apoptosis of some cells (neuronal, retinal) via the p75 receptor [36,37]. Further studies are required to elucidate mechanisms involved in NGF-induced sperm apoptosis.

Capacitation of sperm is essential for successful fertilization. It involves several molecular events, including changes in intracellular calcium levels. Several lines of physiological and pharmacological evidence indicate that the opening voltage-dependent Ca$^{2+}$ channels played a critical role in inducing the acrosome reaction in mammalian sperm [38], and it has been previously reported that NGF can increase intracellular calcium levels in 3T3-Trk cells by acting on TrkA [39]. However, in the present study, there were no significant effects of exogenous NGF on either intracellular calcium levels in sperm or the acrosome reaction, indicating that NGF did not directly influence sperm capacitation.

In conclusion, we demonstrated the expression of NGF and TrkA in bovine ejaculated sperm and showed that NGF affected leptin secretions, viability and apoptosis of sperm. We concluded that NGF was a potentially significant regulator of sperm physiology and could play a role in male infertility, although further experiments are required to elucidate its role more clearly, and the mechanisms involved.

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