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GONADAL PHYSIOLOGY AND DISEASE

Association between neuropeptide oxytocin and male infertility

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Abstract
Purpose To investigate the relationship between oxytocin (OT) and male infertility, serum OT baseline concentration and oxytocin receptor (OTR) gene expression in fertile and infertile men were investigated.
Methods and patients Twenty obstructive azoospermia patients, twenty five idiopathic asthenozoospermia patients, twenty idiopathic oligozoospermia patients and twenty healthy subjects were taken into consideration. Serum OT baseline concentration was determined by radioimmunoassay. Moreover, serum concentration of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) were determined by chemoluminescence to evaluate the correlation with OT. OTR gene promotor and OTR mRNA expressions were determined by polymerase chain reaction and reverse transcriptase-polymerase chain reaction, respectively. OTR protein expression was also performed by Western Blot.
Results Serum OT baseline concentrations in infertile groups were significantly higher than in fertile group (F0.05/2(2,82)=8.29, $p<0.001$). Serum baseline concentration of OT was not correlated with that of LH, FSH and T. There was no significant difference in gene sequences of OTR gene promotor and OTR mRNA when comparing infertile patients with fertile. Human OTR was in the form of oligomers and monomers, and the oligomers were in the majority containing tetramers and hexamers. Monomer expression was significantly higher in idiopathic asthenozoospermia and idiopathic oligozoospermia than that in obstructive azoospermia and control group (F0.05/2(2,82)= 115.50, $p<0.001$). There was no significant difference in oligomer expression between different groups, but 20% of idiopathic asthenozoospermia cases showed a decrease.
Conclusions Significantly different OT baseline concentrations and OTR expressions between fertile and infertile men strongly suggest that OT/OTR system is likely to be linked with male infertility, providing new insights into the pathogenesis and treatment of male infertility.

Keywords Oxytocin · Male infertility · Polymerase chain reaction (PCR) · Reverse transcription PCR (RT-PCR) · Western blot

Capsule Oxytocin/oxytocin receptor system is linked with male infertility.

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Introduction

Neuropeptide oxytocin (OT) has been traditionally recognized as a 'female' hormone due to its roles in parturition and milk ejection. However, OT is recognized as having

endocrine and paracrine effects in male reproduction. A burst of OT is released into the systematic circulation at orgasm to stimulate contractions of the reproductive tract aiding sperm release [1]. Studies have shown that OT is synthesized within the mammalian testis [2], epididymis [3], prostate [4] and penis [5], and the presence of oxytocin receptor (OTR) through the reproductive tract supports a local action of this peptide.

OT plays the physiological roles with the interaction of its specific receptor, OTR. Human OTR is a G-protein-coupled-receptor (GPCR) consisted of 389 amino acid residues. Sarasija [6] reported that amino acid residues deletion of OTR COOH-terminal region would affect the affinity for OT and make the mutated receptor uncoupled from G_q mediated pathways. Miao [7] found that OTR expression would be reduced by 50% by deletion of RVSSVKL (residues 619–625) in the COOH-terminal region of the third intracellular domain without affecting affinity for OT. The ability to activate G protein-mediated actions of OTR would be also decreased by that. OTR gene is mainly consisted of four exons and three introns. Exon 2 encodes NH_2-terminal region of OTR while exon 1 encodes COOH-terminal region. Some mistakes occured in the process of exon 1 expression will be likely to cause COOH-terminal deletion or some specific amino acids mutation which will result in OTR dysfunction.

In view of OT roles in male reproduction and OTR function elucidated above, we speculate that there is an important role of OT in male infertility. In this study, we determined the serum OT baseline concentration and its correlation with LH, FSH and T in infertile and fertile men, and analyzed OTR gene expression characteristics aiming at providing further insights into the relationship between OT and male infertility.

Materials and methods

Patients

This study enrolled 20 cases of obstructive azoospermia (azoospermia group, AZG), 25 idiopathic asthenozoospermia (asthenozoospermia group, ASG), 20 idiopathic oligozoospermia (oligozoospermia group, OZG) and 20 healthy subjects (control group, CG) between 20 and 40 years of age. They were recruited from the Center for Reproductive Medicine Outpatient Clinic of ChangZheng Hospital. The study protocol was approved by the Ethical Committee of Second Military Medical University and informed consent was obtained from every patient. The procedures of conventional semen analysis were performed twice with strict adherence to the World Health Organization guidelines [8]. The diagnoses were designated according to the following criterias: AZG: sperm count=0 ml^{-1}, total testicular volumn ≥30 ml, normal serum FSH level; ASG: sperm motility A <25% or A+B <50%, sperm count ≥20×10^6 ml^{-1}, no definite etiology; OZG: 0 ml^{-1} <sperm count <20×10^6 ml^{-1}, with or without attenuation of sperm motility, no definite etiology; CG: with childbearing history, sperm count ≥20×10^6 ml^{-1}, sperm motility A ≥25% or A+B ≥50%. There is no significant differences in their ages and semen PH between investigated groups enrolled in this study. Mean ages of AZG, ASG, OZG and CG were 29.7±3.3, 29.5±4.1, 30±3.7 and 30±2.5 years old, respectively; and mean PH is 7.4±0.1, 7.3±0.3, 7.4±0.3 and 7.3±0.1, respectively.

Samples

Because blood cells have been widely used as a model to examine hormone-receptor interactions in fundamental science research [9], we determined OTR expression in lymphocytes in infertile and fertile men to study the association between OT and male infertility. Semen and 6 ml peripheral venous blood were collected 2–7 days after sexual abstinence. Three thousand IU aprotinin was added into blood sample to inhibit protein degradation. Blood lymphocytes were isolated by using Ficoll-Paque™ PLUS (GE Healthcare, 71-1017-00-EG) according to the procedure. Serum was used for detection of OT, LH, FSH and T, and lymphocytes were used for analyses of OTR gene expression. OT level we measured will stand for OT serum concentration at that moment we drawn blood, which will represent OT serum baseline concentration.

Detection of hormones

Detection of the serum concentration of OT was performed by radioimmunoassay using Oxytocin (Human, Rat, Mouse, Bovine)—RIA Kit (PHOENIX PHARMACEUTICALS, INC. RK-051-01) [10]. Detection of the serum concentration of LH, FSH and T was performed by Chemoluminescence using Access 2 Immunoassay System (Beckman Coulter, Inc. 973111).

Determination of OTR gene promotor sequence by polymerase chain reaction

Based on the report by Kusui [11], we investigated nucleotides sequences on the CpG island, a region prone to be methylated, located in the OTR gene promotor (−140/1200). Genomic DNA was isolated using a Genomic DNA Extraction Kit (Generay, GRX006), and the quality of genomic DNA was assessed by gel electrophoresis. Nucleotides segment of 1,340 bp was amplified by PCR using DNA Thermal cycler (Eastwin life Science.Inc, EDC-

810). The primers used were: forward (5′ AGTTCCCTCCCTCTCGCAGTT3′) and reverse (5′AGTCCACCCTGAAACAAACCG3′). An aliquot of 50 μl reaction mixture was applied into the PCR system: PCR Master Mix 2X (Fermentas, #K0171) 25 ul, 10 μM forward primer 1 μl, 10 μM reverse primer 1 μl, template DNA 1 ug and 22 μl nuclease-free water. The amplification protocols were as follows: DNA polymerase activation at 94° C for 3 min, 30 cycles of amplification, denaturation at 94°C for 40 s, annealing at 60.8°C for 40 s, extension at 72°C for 90 s. Identification and purification of PCR products were assessed by gel electrophoresis and extraction. PCR product was sequenced and analyzed by software of DNASTAR-MegAlign.

Determination of OTR mRNA by reverse transcription-polymerase chain reaction

In view of the OTR gene expression features and OTR function researches above, we drew a segment of 490 bp mRNA(1425/1914) encoding COOH-terminal of OTR to view the sequences. Total RNA was isolated from lymphocytes by using TRIZOL Reagent (Invitrogen, 15596-026) according to manufacture's instructions. Organic phase was saved and stored at −80°C after chloroform added desiring for protein isolation. Dissolved total RNA in RNase-free water and reversed transcript PCR (RT-PCR) was performed by using ReverTra Ace-α kit (TOYOBO, FSK-100). Total RNA was reversed-transcribed and PCR on DNA thermal cycler was carried out in a 25 μl reaction containing: deionized water 14.25 μl, 10×KOD Buffer 2.5 μl, 10 mM dNTP 2.5 μl, 25 mMMg^{2+} 1.5 μl, DMSO 1.25 μl, 10 μM forward primer (5′CAAGATCCGCACGGTCAAGATGA3′) 0.75 μl, 10 μM reverse primer (5′GGAGGGGAGGGATA CAAACTGATAGGT3′) 0.75 μl, cDNA 1 μl, KOD-PLUS 0.5 μl. The thermal cycling conditions were initiated by DNA polymerase activation at 94°C for 3 min, then 30 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 40 s. Subsequently, PCR products were also sequenced and analyzed by DNASTAR-MegAlign.

Demonstration of OTR expression by western blot

Frozen organic phase collected during RNA isolation was incubated in ice bath. Total protein was isolated according to TRIZOL Reagent instructions (www.invitrogen.com). The aqueous proteins were evaluated by the Bradford's method using Coomassie reagent (Generay, GRX001), and transferred into a fresh tube at −20°C until further use. Following protein measurements, solution containing 10 μg of protein was diluted in with/without reducing agent 2×SB (with reducing agent buffer=62.5 mM Tris pH 6.8, 10% glycerol, 20% SDS, 2.5% bromophenol blue and 100 mM Dithiothreitol; without reducing agent buffer=62.5 mM Tris pH 6.8, 10% glycerol, 20% SDS, 2.5% bromophenol blue) and loaded onto 7.5% SDS-PAGE. After separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes, which were blocked for 2 h at room temperature in 10% non-fat milk powder blocking buffer in TTBS (0.5‰ Tween-20, 20 mM Tris, 150 mM NaCl). Then the membrane was washed 5 min by 3 times in TTBS and incubated overnight with primary antibody and GAPDH [rabbit polyclonal antibody of OTR (Shanghai Genomics, Inc. SG4210-5012) and Monoclonal Mouse Anti-glyceraldehyde-3-phosphate Dehydrogenase diluted in proportion of 1:5000 and 1:10,000 in immunoreaction enhancer solution (TOYOBO CO.,LTD. NKB-101), respectively]. Peroxidase-conjugated secondary IgG was followed [Peroxidase-conjugated Affinity Purified Goat Anti-Rabbit IgG (KANGCHENG, KC-RB-035) and Peroxidase-conjugated Affinity Purified Goat Anti-Mouse IgG (KANGCHENG, KC-MM-035) diluted in proportion of 1:7500 and 1:10,000 in immunoreaction enhancer solution, respectively]. Finally, reacted proteins were revealed by chemiluminescence system using BeyoECL Plus (Beyotime, P0018). Mammary gland specimens from breast cancer patients received radical operation of mastocarcinoma were performed as positive control of Western Blot. Total protein extraction was performed by using ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas, #k0301) according to its protocol. The aqueous proteins from mammary gland specimens were managed as mentioned above.

Statistical analysis

Serum OT detections were repeated in duplicate. Experiments to demonstrate OTR gene expression were repeated in triplicate. Data are summarized as mean ± SE. ANOVA followed by the Student-Newman-Keuls test was used to determine differences between the mean level of OT in different groups. Correlative study was carried out to measure correlation between different hormones. Necleotide sequences were matched by DNA STAR-MegAlign. Western Blot data were analyzed by Image-Pro Plus, ratio of OTR-band-density-value/GAPDH-band-density-value was analyzed by ANOVA. $P<0.05$ is considered statistically significant.

Results

Serum OT baseline level and it's correlation with reproductive endocrine hormones

Serum OT baseline concentrations in infertile and fertile cases were as follows: AZG, 118.53±7.69 pg/ml; ASG, 108.81± 5.66 pg/ml; OZG, 103.71±4.54 pg/ml; CG, 79.30±3.83 pg/ml.

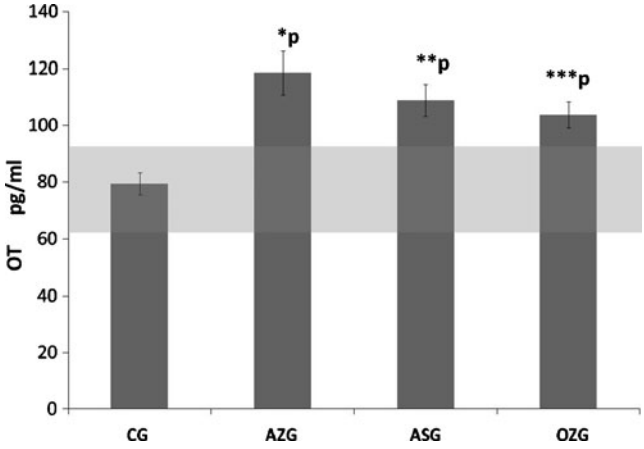

Fig. 1 Serum OT concentrations in infertile and normal cases. The gray region presents a physical baseline concentration range of OT in normal male. (*P<0.001 vs CG; **P<0.001 vs CG; ***P=0.004 vs CG)

Significantly higher serum concentrations of OT were observed in cases of AZG (p<0.001), ASG (p<0.001) and OZG (p<0.001) than in cases of CG ($F_{0.05/2\ (2,\ 82)}$=8.29, p<0.001) (Fig. 1). No correlation between OT and LH, FSH and T was noticed in hormone correlative study.

Study of OTR gene expressions characteristics in male fertility and infertility

No significantly aberrant gene transcription was observed in analyses of OTR gene promotor and OTR mRNA nucleotides sequencing (Fig. 2). Consecutive investigation into OTR expressions characteristics were performed by Western Blot. The results indicated that human OTR in the lymphocyte was in the form of oligomers and monomers (60 KD–70 KD), and the oligomers expressed in the majority contained tetramer (about 250 KD) and hexamer (300 KD–350 KD) (Fig. 3a). Monomers mean band density values were as follows: ASG, 0.41±0.03; OZG, 0.13±0.01; AZG, 0.05±0.004; CG, 0.05±0.003. There were more monomers in cases of ASG and OZG than those in cases of AZG and CG (F0.05/2(2,82)=115.50, p<0.001) (Fig. 3a and b). Oligomer expression showed no significant differences between each group, whereas 20% cases of ASG decreased obviously (Fig. 3c).

Discussion

Results of this study have demonstrated the association between serum OT basal concentration and OTR expression in infertile men. The evidence is demonstrated in two ways. First, study on serum OT baseline concentration manifested that it was higher in infertile cases (AZG, ASG, OZG) than in fertile cases (CG). Second, to determine the potential of OTR expressions in associate with male infertility, western blot was performed to analyze OTR expressions in infertile and fertile patients. More frequent in OTR monomers of ASG and OZG was observed than in those of AZG and CG. Moreover, oligomers exhibit obviously decreased in frequency in 20% cases of ASG.

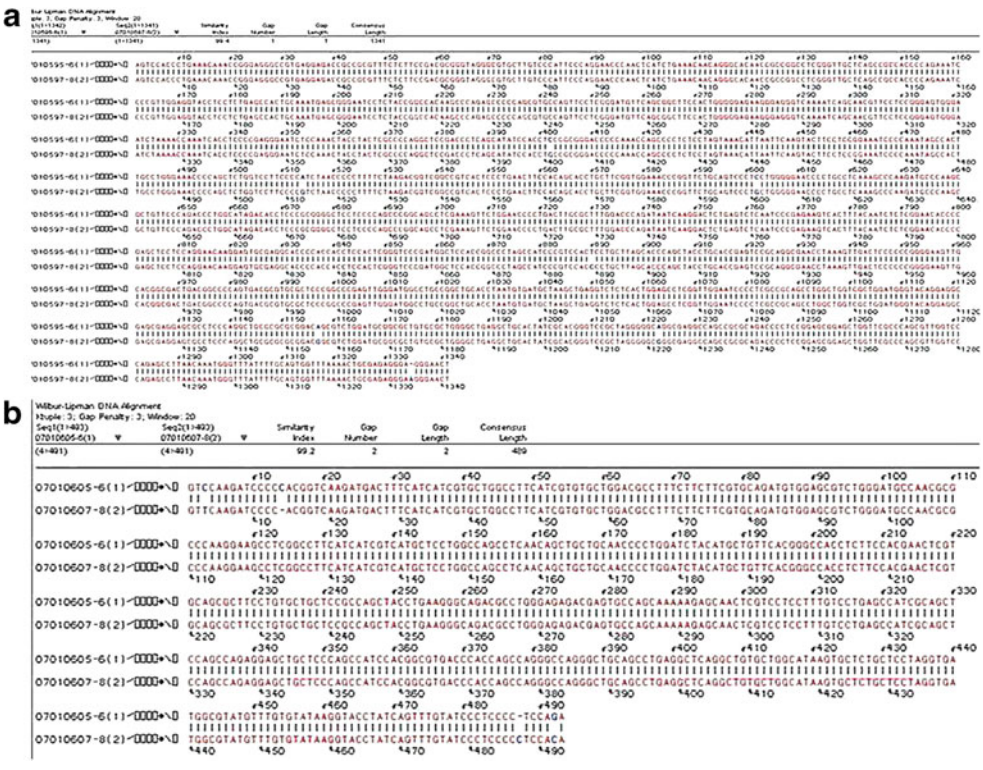

Fig. 2 Analyses of OTR gene transcription. **a** gene sequences match diagram of OTR gene promotor between cases of fertility and infertility (infertile vs control group) No significant gene mutation is observed. **b** gene sequences match diagram of OTR mRNA between cases of fertility and infertility (infertile vs control group) No significant gene mutation is observed

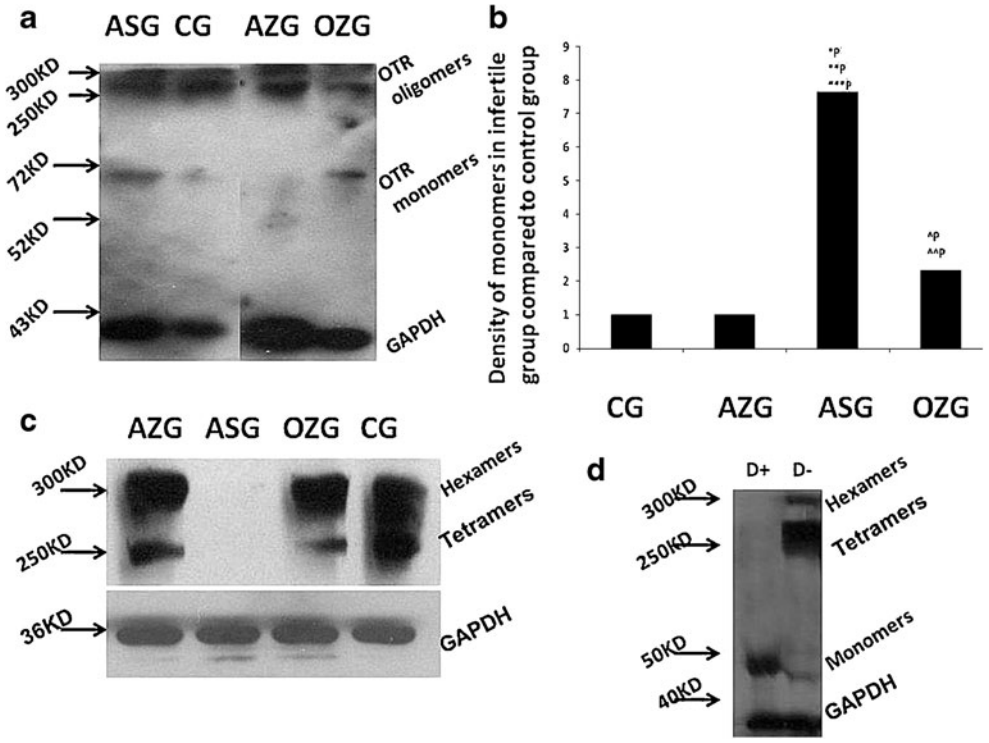

Fig. 3 OTR expression in human lymphocytes in cases of each group and mammary gland. **a** Western blot detection of OTR in cases of each group. Oligomers expressions are significantly higher than thoses of monomers. Significant increase in monomers in cases of ASG and OZG compared to AZG and CG. **b** OTR monomers expressions in cases of each group. Density value of CG is identified as 1, and the others were compared with it. **P*<0.001 vs CG; ***P*<0.001 vs AZG; ****P*<0.001 vs OZG; ^*P*=0.002 vs CG; ^^*P*=0.003 vs AZG. **c** Western blot detection of OTR oligomers in cases of each group. Significant decrease in oligomers in cases of ASG. **d** Positive control of human mammary gland performed by Western Blot. D+: loading buffer with reducing agent; D−: loading buffer without reducing agent

The reasons for significantly different serum OT baseline concentrations can be understood from the roles of OT in male reproductive system. OT plays many important physiological roles in male sexual behaviors and male reproduction. Although the serum OT baseline level is very low normally in the male, it transiently rises rapidly to stimulate reproductive tract contraction aiding sperm release when man is at orgasm [1]. It is also confirmed by the evidence that exogenous administration of OT in infertile men will increase the ejaculated sperm counts in semen [3]. Male volunteers whose OT roles are attenuated by antagonist naloxone have a complaint of low pleasure at orgasm compared to the former and control cases [12]. OT also has a local action in male reproductive system supported by it's synthesis within the mammalian testis [2], epididymis [3], prostate [4], penis [5] and OTR expression through the reproductive tract. Interestingly, OT also affects on conversion of testosterone to DHT by modulating 5-α reductase [13]. In this study, significantly low concentrations of OT were observed in cases of CG as compared to those in infertile cases. The finding shows an opposite result to the OT roles elucidated above. However, to the best of our knowledge, no other literatures have demonstrated OT concentrations in male infertility. This finding may suggest that, in male infertility, OT functions by its interaction with specific receptor OTR, and OTR doesn't work effectively, which cause OT baseline level to rise by a possible feedback mechanism.

When we refer to OTR, we know that human OTR is a GPCR in the form of monomers and oligomers, which has critical functions in intercellular communication. So far as we know, no conclusive evidence has proven that functional OTR is in the form of monomers or oligomers. Signals transcellular are always intergrated, and the ability of GPCR to form dimers or oligomers is thought to generate such signal intergration [14, 15]. In this experiment, we have found that monomers expression was significantly higher in cases of ASG and OZG than that in cases of AZG and CG. Based on the significant difference of receptor expressions and OT basal concentrations, we speculate that OT may play roles mainly with the interaction of OTR oligomers instead of monomers in male reproduction. If the monomers are in the majority and oligomers are in the minority of OTR as presented in this study, OT mediated physiological functions will decrease. On condition that this speculation is established, the association between OT/OTR and male infertility can be understood well. Therefore, further research into OTR function are needed. In addition,

significantly decreased in expression of oligomers was observed in cases of ASG (20%), which further supports the speculation.

As to OT role besides reproduction, it also refers to its neurobiology in psychology, social behavior, recognition and so on [16]. It seems to exist a common neurobiological mechanism which involves dopamine, morphine and other endogenous signalling molecules, e.g. OT and other opioid receptor agonists act via NO releases, and this share seems to be of critical importance for regulation and management of psychology and social behavior [17]. OT is also associated with increased trust and keep a good mood [18]. Perceptions of trustworthiness are associated with detection of positive facial affect, which suggests that OT may enhance the recognition of positive facial affect. In this study, OT level in AZG was significantly higher than in CG, whereas no OTR expression difference was observed. We suspect that azoospermic patients' state of self-regulation and management of stress may take part in causing OT level risen, however, this need to be further studied.

Explanation to different expressions in oligomers and monomers can also be understood from view of congenital and acquired factors. Genomic polymorphism can cause abnormal expression of OTR gene, which is heritable [19, 20]. In this research, nucleotide sequences analyses by gene sequencing did not show any mutation. However, only the OTR gene promoter region prone to be methylated and 490 bp of mRNA encoding COOH-terminal of OTR were studied. Other genomic polymorphisms analyses are still needed for further investigation. Also receptor function could be affected by acquired factors, and specific amino acids will get mutated, which are non-heritable.

As to LH and FSH roles in male reproduction and interactions with OT, it is well known that, they are secreted mainly by pituitary. LH promotes leydig's cells proliferation and stimulates the synthesis and secretion of testerone (T) by leydig's cells to provide for spermatogenesis; FSH plays roles in process of sperm maturation, mediates prespermatid developed into spermoblast and sperm [21–24]. Additionally, there are some controversial issues regarding the interaction between OT and FSH, LH. Some early studies have shown that OT can inhibit the release of LH and FSH [25], whereas other reports have not [26, 27]. In this research, OT plasma concentration was not correlated with that of LH and FSH. Large sample and profound studies are needed to be performed to present the evidence for this issue. Besides, the monomer weight of 60 KD–70 KD expressed in lymphocytes was higher than reported deglycosylation monomer weight of 50 KD–60 KD [28]. The molecular weight of monomers expressed in the mammary glands were similar to that [28, 29]. The samples we used are human lymphocytes which may be not sufficient to elucidate the role of OTR in male infertility, although blood cells have been widely used as a model to examine hormone-receptor interactions in fundamental science research. Because OT always has local actions in male reproduction, specimens from reproductive system are needed for further research.

In summary, results of this study have demonstrated association between OT/OTR and male infertility. Increase in serum OT baseline concentration, decrease in expression of OTR oligomers and increase in monomers provide new insights into the pathogenesis and treatment of male fertility disorders. The further research on this issue might help in understanding the etiology of male infertility.

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