

A Cohort Study of the Level of Plasma Oxytocin associated with Autism Spectrum Disorder in Japanese Males, Females and Pregnant Females

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Abstract

Oxytocin (OXT), which is a nonapeptide hormone with amidation of the C-terminal carboxyl and an internal disulfide bridge between two cysteines, is secreted mainly from the posterior pituitary gland. Recently, OXT was shown to play important key roles in social interactions and social behavior. Specifically, a series of studies investigated the relationship between autism spectrum disorder (ASD) and OXT. Few large-scale research reports have been published on plasma OXT levels in Japanese individuals. Investigation of a possible association between ASD and plasma OXT levels will be valuable for OXT research. Here, we measured plasma OXT levels in about 200 pregnant Japanese females during early gestation, the second trimester of pregnancy, the last trimester of pregnancy, and in the funiculus umbilicalis shortly after delivery utilizing an OXT enzyme immunoassay kit. As a results, the average plasma OXT levels of 11 Japanese males 22-65 years old and 11 non-pregnant females 23-59 years old were 31.7 ± 10.2 and 25.3 ± 6.1 pg/mL, respectively. Moreover, OXT levels in plasma in early gestation, the second trimester of pregnancy, the last trimester of pregnancy, and the funiculus umbilicalis were 27.88 ± 10.88 (n=43), 33.06 ± 16.06 (n=111), 42.97 ± 35.96 (n=91) and 34.66 ± 22.42 pg/mL (n=130), respectively. Little information regarding plasma OXT levels among the Japanese population is available. We suggest that these results may be valuable on a broad scale in prospective cohorts when examining an association between the mother and the formative influence on her child with ASD, after conducting a follow-up determination of OXT levels in Japanese individuals.

Keywords: Oxytocin; Autism spectrum disorder; Enzyme immunoassay; Peptide hormone

Introduction

Oxytocin (OXT), which is composed of nine amino acids with amidation of the C-terminal carboxyl and an internal disulfide bridge between two cysteines, is secreted mainly from the posterior pituitary gland after synthesis in the paraventricular and supraoptic nuclei of the hypothalamus [1-3].

Recently, the role of OXT in social behavior including social recognition, pair bonding, and maternal care, which are involved in autism spectrum disorder (ASD), was examined [4,5]. Autistic children tend to have low OXT levels in plasma, [6] and thus, infusion of OXT may diminish repetitive behaviors in autistic patients [7].

ASD is a neurodevelopmental disorder with symptoms including impaired social interaction and communication and repetitive and restricted behavior [8]. The worldwide frequency of ASD is >0.6% and the disorder is widely prevalent in children [9-11]. Diagnosis of ASD in clinical psychiatry has been established according to the definition of the disorder in DSM-IV since 1993 [12,13]. However, providing a definitive diagnosis of ASD is difficult, because the characteristics of ASD are very similar to those of schizophrenia and social anxiety disorder. If a child develops ASD in early childhood, does not receive treatment, and remains ignorant of his or her condition until adulthood, he or she has a high risk of secondary disorders such as depression. Thus, development of a simple general method of examination utilizing a biomarker based on clinical laboratory test results for ASD is necessary for early detection and rapid cure of the disorder.

Few large-scale research reports have been published on plasma OXT levels in Japanese individuals. Investigation of a possible association between ASD and plasma OXT levels will be valuable for OXT research. Here, we measured plasma OXT levels in about 200 pregnant Japanese females during early gestation, the second

trimester of pregnancy, the last trimester of pregnancy, and in the funiculus umbilicalis shortly after delivery utilizing an OXT enzyme immunoassay kit.

Materials and Methods

Materials

Acetonitrile and trifluoroacetic acid (TFA) were purchased from WAKO Pure Chemical industries Ltd. (Osaka, Japan). Sep-Pack C18 was purchased from Waters Corp. (Milford, Massachusetts, USA). The OXT enzyme immunoassay kit was purchased from ENZO Life Sciences (Farmingdale, NY, USA) and contained the following reagents: assay buffer, washing buffer, standard sample for OXT, anti-OXT antibody, alkaline phosphatase conjugated to OXT, substrate for alkaline phosphatase, p-nitrophenylphosphate (pNpp), and a 96-well plate. All other reagents were analytical grade.

Methods

Preparation of plasma samples for assay: The assay for OXT plasma level was that pregnant woman during early gestation (11th-

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Received February 16, 2016; **Accepted** February 29, 2016; **Published** March 07, 2016

Citation: Sano Y, Watanabe N, Suzuki E, Shimodaira K, Kato N, et al. (2016) A Cohort Study of the Level of Plasma Oxytocin associated with Autism Spectrum Disorder in Japanese Males, Females and Pregnant Females. Clin Med Biochemistry 2: 113. doi:10.4172/2471-2663.1000113

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13th week of gestation), the second trimester of pregnancy (26th-28th week of gestation), or the last trimester of pregnancy (36th-37th week of gestation) or healthy Japanese males and non-pregnant females was of reproductive age (20-50), lived in Tokyo area, and had no known diseases. They were recruited at Showa University Hospital during 2009-2011 and voluntarily participated in this study after giving informed consent. The ethics committees of the Showa University Hospital approved the research. To take plasma samples for assay, the blood samples were collected in heparinized tubes from above pregnant woman and separated by centrifugation and then the collected plasma were stored at -80°C until the assay.

Measurement of OXT in plasma on extraction procedure with the OXT enzyme immunoassay kit: The extraction procedure was performed according to the manufacturer's instructions in the OXT enzyme immunoassay kit. For extraction, 350 µL of plasma was added to 350 µL of 0.1% TFA and centrifuged at 17,000 × g for 15 minutes. After centrifugation, 700 µL supernatant was applied to the Sep-Pack C18 after equilibrating with 0.1% TFA. To remove unbound sample, the Sep-Pack C18 was washed three times with 350 µL 0.1% TFA. After washing, 3 mL of 90% acetonitrile containing 0.1% TFA was applied. The eluate was collected, evaporated to dryness, and dissolved in 350 µL assay buffer from the OXT enzyme immunoassay kit.

Measurement of OXT with the OXT enzyme immunoassay kit: A 100 µL plasma sample extracted with Sep-Pack C18 or prepared the standard way for OXT (15.6-1,000 pg/mL), anti-OXT antibody (50 µL), and alkaline phosphatase-labeled OXT (50 µL) were added to the well of an anti-rabbit goat IgG-coated 96-well plate. After 18 hours, wells were washed three times with 400 µL washing buffer. Then, 200 µL substrate solution (pNpp for alkaline phosphatase) was added to each well and incubated at room temperature for 1 hour for development. After addition of 50 µL stop solution (trisodium phosphate solution), the optical density of each well was measured at 405 nm with a 405WALLAC ARVO™ SXzd 1420 MULTILABEL COUNTER (WALLAC).

Results

The standard curve for OXT is shown in Figure 1. The detection limit of OXT was 15.6 pg/mL, and the result was reproducible. The standard curve for measurement of OXT ranged from 15.6-1000 pg/mL, which was comparable to the manufacturer's instructions in the OXT enzyme immunoassay kit. The intra- and interassay variance were 0.15-3.6 the coefficients of variation (CV) % and 29.6 ± 9.6 (Mean ± SD; 32.5 CV%), respectively, for this assay.

To investigate plasma OXT levels in healthy Japanese males and non-pregnant females, their plasma was measured utilizing OXT immunoenzyme kit. As a result, the average plasma OXT levels of 11 Japanese males 22-65 years old and 11 non-pregnant females 23-59 years old were 31.7 ± 10.2 and 25.3 ± 6.1 pg/mL, respectively (Figure 2 and Table 1). Plasma OXT levels in males were higher than in females by about 125%. Moreover, Plasma OXT levels in about 200 pregnant Japanese females were investigated as below. Plasma OXT levels during early gestation, the second trimester of pregnancy, the last trimester of pregnancy, and in the funiculus umbilicalis were 27.88 ± 10.88 (n=43), 33.06 ± 16.06 (n=111), 42.97 ± 35.96 (n=91), and 34.66 ± 22.42 pg/mL (n=130), respectively (Figure 3 and Table 2). Levels in early gestation were about 1.5 fold lower than in the last trimester of pregnancy. Significant differences (P<0.05) were seen between the average OXT levels in early gestation and the second trimester of pregnancy, early gestation and the last trimester of pregnancy, and early gestation and the funiculus umbilicalis.

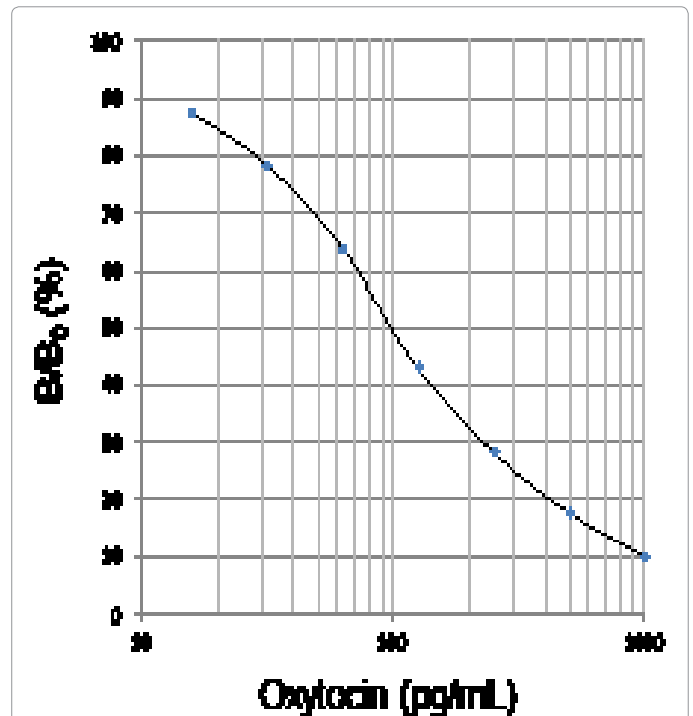


Figure 1: The standard curve for OXT using reagents from the OXT immunoassay kit (ENZO).

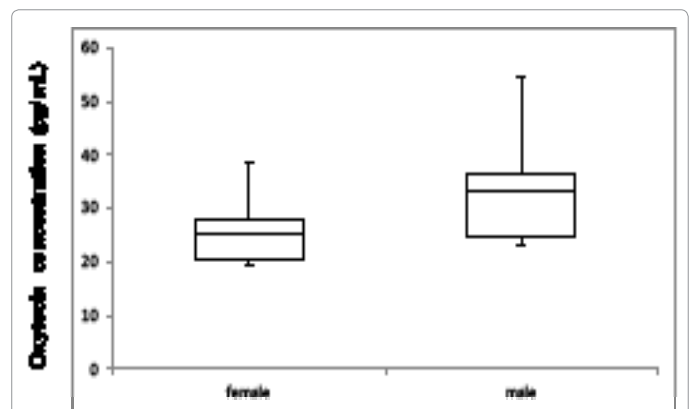


Figure 2: OXT plasma levels in Japanese males and non-pregnant females.

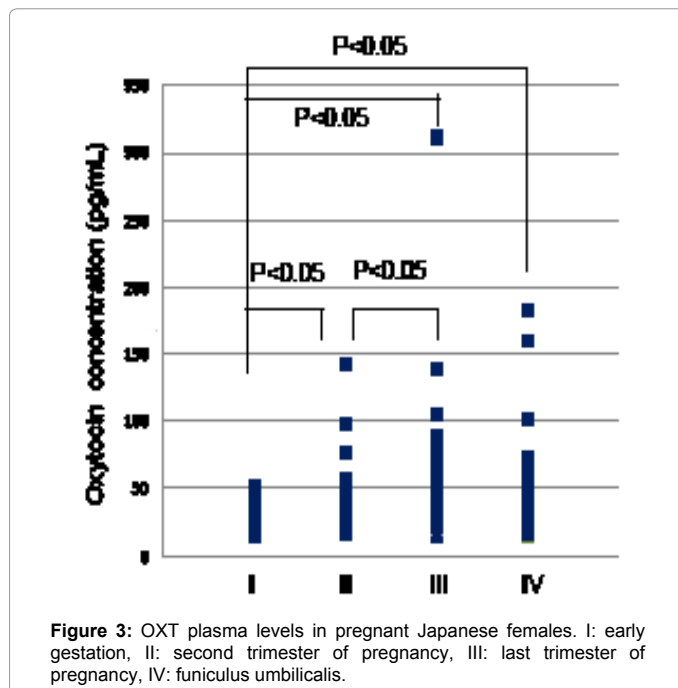
Sex	Number	Age	Mean ± SD (pg/mL)
Male	11	22-65	31.7 ± 10.2
Female	11	23-59	25.3 ± 6.1

Table 1: Comparison of OXT levels according to sex and age.

Stage	n	Mean ± SD (pg/mL)	Age
I	43	27.88 ± 10.88	39.0
II	111	33.06 ± 16.06	48.6
III	91	42.97 ± 35.96	83.7
IV	130	34.66 ± 22.42	64.7

I: early gestation; II: second trimester of pregnancy; III: last trimester of pregnancy; IV: funiculus umbilicalis

Table 2: Comparison of OXT levels during each stage of gestation.



Discussion

Most published reports for OXT have used the OXT immunoassay kit from ENZO Life Science [14] but different plasma OXT levels have been reported in many published studies. Christensen et al. and Ebstein et al. [14,15] reported serious problems in the different published OXT levels in studies utilizing the OXT immunoassay kit from ENZO Life Science [16]. OXT levels were very high without use of the extraction protocol as recommended in the manufacturer's instructions for the OXT immunoassay kit (ENZO Life Science) [14,15]. In addition, the recovery test of the exogenous spike was not correctly confirmed as shown by representative sample handling in the protocol for the ENZO kit [14]. Because the extraction procedure is recommended, samples that were not extracted may show an incorrect plasma OXT level. Therefore, we suggest that the development of a high sensitivity and specificity assay that does not require an extraction protocol for OXT will be very important in OXT research as a biomarker of psychiatric disorders for ASD etc. [16]. Furthermore, many studies of OXT as a biomarker have reported to social recognition and memory [17], romantic love, trust, and fear in humans [18], and mind-reading in humans [19]. Recently, Taurines et al. have reported that OXT plasma concentration was correlation with autism symptomatology in children with ASD or attention deficit hyperactivity disorder (ADHD) [20]. Early detection and rapid cure of ASD or ADHD are important, and OXT might be potential use in those disorders diagnosis as its biomarker in child development. However, there is little clear of the effect of OXT in child development or the influence of transported OXT from mother to fetus via placenta in fetal period. Additionally, measurement of plasma OXT level in baby in fetal life is very difficult including blood withdrawal from fetus. Therefore, we suggest that investigation of plasma OXT level in pregnant females is significant for studies of the effect of OXT on child development.

Finally, we reported significant differences in plasma OXT levels among samples obtained during early gestation, the second trimester of pregnancy, the last trimester of pregnancy including the funiculus umbilicalis, and in healthy males and non-pregnant females as controlled comparison in about 200 Japanese individuals utilizing

the OXT enzyme immunoassay kit with the extraction protocol as recommended by ENZO.

We suggest that this report of these plasma OXT levels provides useful data for OXT research investigating an association between plasma OXT levels in the mother and the formative influence for the child. Follow-up studies in Japan are needed.

Acknowledgements

We sincerely appreciate the subjects who participated in this study and to the support and technical staff and clinical staff of Showa University Hospital.

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