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—Original Article—

Effect of neurotensin on cultured mouse preimplantation embryos

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Abstract. Previously, we revealed that neurotensin (NTS) derived from the oviduct and uterus can function during fertilization. However, little is known about NTS actions on the pre-implantation embryo after fertilization. Here, we found that pro-*Nts* mRNA is expressed in the oviduct and uterus during when preimplantation embryos develop and an increase in mRNA level in the uterus is induced by human chorionic gonadotropin (hCG) treatment. Expression of mRNA for two NTS receptors, *Ntr1* and *Ntr3,* was found throughout these stages, whereas *Ntr2* mRNA was not detected, suggesting that NTS signaling occurred through NTR1 and NTR3. Supplementation of 1, 10, 100 or 1000 nM NTS to embryo culture medium after fertilization showed that 100 nM NTS significantly improved the blastocyst formation. In comparison, the total number of cells and inner cell mass ratio of blastocysts was not significant different between the 0 nM and 100 nM NTS treatment groups. These results indicate that NTS has a positive effect upon preimplantation embryo development *in vitro*.
Key words: Blastocyst, Neurotensin (NTS), NTR1, NTR3

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Neurotensin (NTS) is a peptide hormone that was first isolated from the bovine hypothalamus and shown to have hypotensive actions [1]. Neurotensin consists of 13 amino acids, processed from the NTS precursor, pro-NTS [2, 3]. Neurotensin performs various functions, and its interaction with the dopamine system has been well studied [4, 5]. Neurotensin is also localized in gut endocrine cells [6], and recent work reported that it is secreted from secretory cells of the small intestine, with findings from gene-deficient mice showing it is involved in fat absorption [7]. NTS signaling may also play a role in tumor growth [8]. Despite reported expression in the oviduct [9], the function of NTS in the female reproductive system remains incomplete. Previously, we reported that NTS mRNA expression and secretion increased in cumulus cells after ovulation [10]. Consistent with this finding, transcriptome analysis in the bovine oviduct revealed that pro-*Nts* mRNA expression was markedly increased in the follicular phase of the estrous cycle, suggesting a specialized role for NTS in ovulation or fertilization [11]. In addition, NTS receptor 1 (NTR1) was expressed in sperm, and the addition of NTS increased sperm intracellular calcium levels, promoted the acrosome reaction and tyrosine phosphorylation. A past study also showed that NTS immunoreactivity was detected in the epithelial cells of the oviduct and uterus [10]. However, the involvement of NTS on preimplantation embryonic development remains unclear. Therefore, the current experiments investigated the following: 1) pro-*Nts* mRNA expression patterns in the oviduct and uterus during the preimplantation period; 2) temporal expression profiles of NTS receptor mRNAs in the embryo; 3) effects of NTS supplementation upon preimplantation embryo developmental competence *in vitro*.

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Materials and Methods

Animals

C57BL/6N mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All experiments using animals were approved and conducted in accordance with the Guide for the Care and Use of Laboratory animals published by Tohoku University.

Quantitative real-time PCR (qRT-PCR)

Sexually mature female mice (6–8 weeks) were intraperitoneally administered 7.5 IU pregnant mare serum gonadotropin (PMSG), and 48 h later 7.5 IU human chorionic gonadotropin (hCG). Females were then mated with males, checked for a vaginal plug and sacrificed at 18, 48, 72, and 96 h after hCG administration. For other groups, mice were administered PMSG and 48 h later were divided three groups, treated with saline, hCG, or further mated after hCG, and sampled after 18 h from individuals confirmed the presence of plug. Oviducts and uteri were separated at the uterus oviduct junction and collected and stored in RNA later stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA) until total RNA extraction.

Total RNA was extracted from the collected tissues using Sepasol RNA I super G (Nacalai Tesque, Kyoto Japan). Embryos at 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stages were collected 12, 28, 48, 55, 72 and 96 h after incubation with sperm, respectively. Total RNA from embryos was extracted using RNeasy micro (Qiagen, Hilden, Germany). cDNA was synthesized using reverse transcriptase (Revertra ace-α; TOYOBO, Osaka, Japan). Quantitative analyses of targeted transcripts were performed using TaKaRa TB Green® *Premix Ex Taq*™ II on a TaKaRa Thermal Cycler Dice Realtime System II (TaKaRa, Tokyo, Japan). The PCR program was as follows: 30

sec at 94°C, 45 cycles of 5 sec at 94°C, 10 sec at 60°C and 30 sec at 72°C. Specific primers sequences are listed in Table 1. Internal control genes were *β-actin* in the oviduct and uterus, and *H2afz* in the embryo [12]. Quantitative evaluation of the expression of target genes relative to the expression level of internal standard genes was calculated by the ΔΔct method. The sizes of obtained amplicons were checked by agarose gel electrophoresis.

Embryo culture

The ovulated cumulus oocyte complexes (COCs) were collected 16 h after hCG administration from animals sacrificed by cervical dislocation. Collected COCs from the oviduct ampulla were transferred to a 200-µl drop of human tubal fluid (HTF) medium containing 0.1% bovine serum albumin (BSA; Nacalai Tesque). Then COCs were incubated with sperm for 4 h, and two-pronuclei confirmed embryos after 6 h insemination were collected and randomly divided into 50-µl drops of KSOM covered by mineral oil. Stock NTS solution was dissolved in saline and added to KSOM at final concentrations of 1, 10, 100 and 1,000 nM to assess the effects of NTS on developmental competence. Embryos were checked daily to determine the 2-cell, 4-cell and blastocyst formation and hatching rates. Each ratio was calculated by dividing the number of embryos by the number of 2PN embryos first served. The hatching rate of blastocysts was calculated after 96 h culture. The embryos were cultured at 37°C under a humidified atmosphere and 5% CO_2.

Counting blastocyst cell numbers

To evaluate the effect of NTS on blastocyst cell numbers, blastocysts were fixed after 96 h culture and the control (0 nM) or 100 nM NTS samples were immunostained. Briefly, blastocysts were treated with acetic Tyrode solution (pH 2.5) to dissolve zona pellucida and fixed with 4% paraformaldehyde-phosphate buffered saline (PFA-PBS) with 0.2% Triton-X for 30 min at RT. After washing with 1% BSA-PBS, the specimens were blocked with 10% fetal bovine serum for 1 h and incubated with anti-OCT4 mouse monoclonal antibody (1:100 dilution; #sc-5279, SantaCruz, Dallas, TX, USA) at 4°C overnight. Then specimens were treated with donkey anti-mouse Alexa-555 antibody (1:200) and Hoechst 33452 (0.5 µg/ml) counterstaining for 1 h at RT. The washed samples were mounted on glass slides with a small amount of 1% BSA-PBS, covered with a glass coverslip and pressed to spread the cells. Cell numbers were determined using an Olympus BX63 fluorescence microscope (Tokyo, Japan). For counting, positive cells were defined by Hoechst staining, and cells in the inner cell mass (ICM) were those positive for OCT4, calculated as a percentage of the total cells.

Statistical analysis

For multiple comparisons with the control group, significant differences were compared by the Dunnett's or Tukey-Kramer test, comparisons between two groups used the unpaired Student *t*-test (GraphPad Prism 8). Values are represented as mean ± SEM. A P value < 0.05 was considered a significant difference.

Results

Temporal expression patterns of pro-Nts mRNA in the oviduct and uterus

The pro-*Nts* transcript was targeted because NTS is enzymatically processed from pro-NTS. The expression levels of pro-*Nts* mRNA in the oviduct and uterus during the preimplantation period were compared by qPCR, as shown in Figs. 1A and B. Compared with diestrus stage expression, there were no significant differences in pro-*Nts* mRNA levels in the oviduct until 96 h after hCG treatment. In contrast, a significant increase in uterine pro-*Nts* mRNA expression was observed after 18 h of hCG administration. To clarify whether the increase in uterine expression was due to the hCG treatment or mating, pro-*Nts* mRNA levels were compared between three groups in which administered saline, hCG, further crossed, after PMSG treatments. (Fig. 1C). The expression of pro-*Nts* mRNA was significantly increased by hCG, but no difference was observed between hCG treatment and further mated group.

Temporal expression of NTS receptor transcripts in preimplantation embryos

The mRNA expression patterns of NTS receptors in developing embryos were examined by qPCR. The relative mRNA expression

Table 1. Primer sequences used in qPCR

Gene	Sequences (5' to 3')	Amplicon (bp)	Accession ID
pro-Nts	F: GCCCTGGAGGCAGATCTATTG	163	NM_024435
	R: TGCCAACAAGGTCGTCATCAT		
Ntr1	F: AGAGCACAGCACGTTCAACA	82	NM_018766
	R: CACAGCACGTAAGACGAGGA		
Ntr2	F: GGTGAGACACAAGGATGCCA	149	NM_008747
	R: CAGTCCATCCATCATCGGGG		
Ntr3	F: GTGGGCATAGTCATCGCTCA	106	NM_019972
	R: CTAGCATCTTCGCCCAGGAG		
β-actin	F: AGCCTTCCTTCTTGGGTATGGA	99	NM_007393
	R: TGGCATAGAGGTCTTTACGGATG		
H2afz	F: GTGGACTGTATCTCTGTGAA	89	NM_016750
	R: GGTTGGTTGGAAGGCTAA		

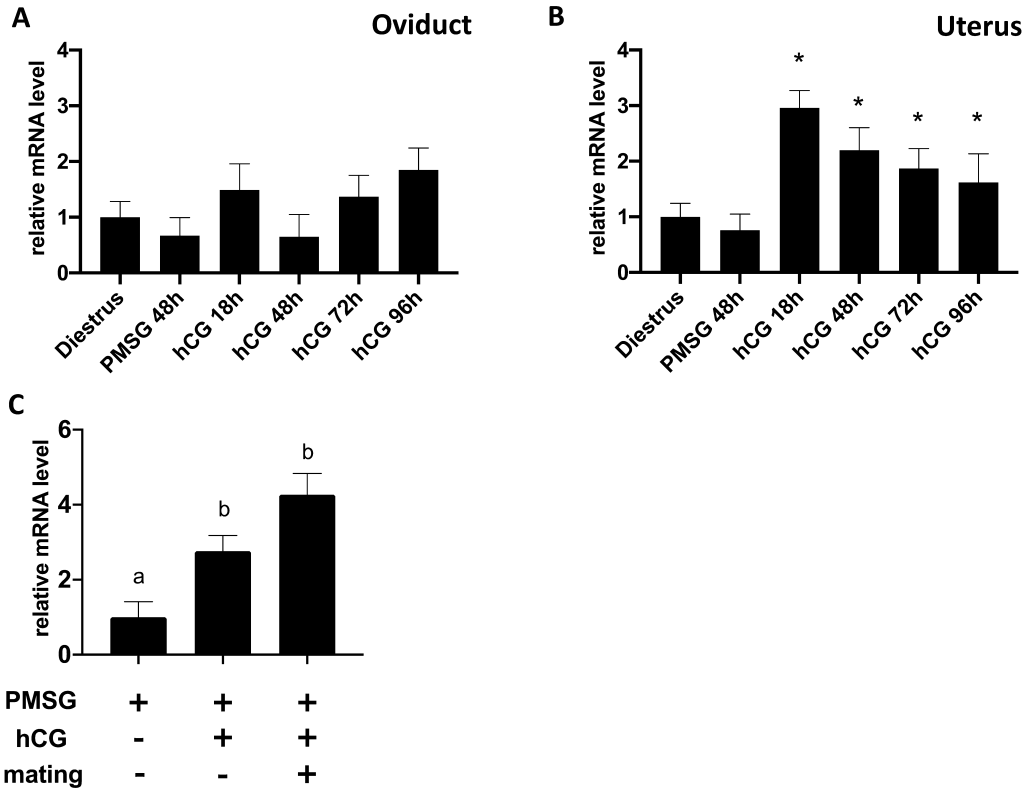

Fig. 1. Temporal expression profiles of pro-neurotensin (pro-*Nts)* transcripts in the preimplantation oviduct and uterus. Quantitative PCR analysis of pro-*Nts* transcripts in the oviduct (A) and uterus (B) during preimplantation periods (n = 3). Pregnant mice were those with a confirmed vaginal plug. Asterisks represent significant differences compared with the diestrus group, as analyzed by the Dunnett's test. Effects of human chorionic gonadotropin (hCG) and mating on uterine pro-*Nts* mRNA expression (C) (n = 7). Values within a column with different superscripts or asterisks represent significant differences, as analyzed by the Tukey-Kramer test. Values are mean ± SEM. $P < 0.05$ was considered significant by either statistical analysis.

levels of *Ntr1* and *Ntr3* were both markedly reduced at the 2-cell compared with the 1-cell stage, although expression remained in the 4-cell, 8-cell, morula and blastocyst stages, as shown in Fig. 2. *Ntr2* transcript expression was not detected during any of the stages analyzed (data not shown).

Effects of NTS on preimplantation embryo development

To investigate the effects of NTS on preimplantation embryos, fertilized embryos were cultured in the presence of different concentrations of NTS for 96 h. There were no significant differences in the 2-cell cleavage and 4-cell rate between control and NTS treatment (1, 10, 100 and 1,000 nM) groups, but a significant increase in blastocyst formation was observed with the 100 nM NTS group (60.6 ± 1.7% *vs*. 75.6 ± 3.4%, P = 0.03), as shown in Table 2. At this treatment group, the hatching rate was 28.1 ± 3.8% versus that of control group (13.0 ± 4.1% ; P = 0.08). To further examine the effect of NTS on blastocyst cell number and the ICM ratio, blastocysts were supplemented with 0 nM or 100 nM NTS and then fixed, immunostained and counted. There were no significant differences in total cell number (P = 0.13) and the ICM ratio (P = 0.09) between both groups (Fig. 3).

Discussion

The present study demonstrates that NTS has a positive effect on early embryonic development. Pro-NTS is processed by prohormone convertase (PC), which has multiple subtypes expressed in several tissues [13]. Of the known PC1, PC2 and PC5-A subtypes, PC5A mRNA was previously found to be expressed in the uterus. This suggests that processed and functional NTS is likely to be secreted into the lumen. The current results indicate that NTS may continue to be secreted into oviduct or uterine fluid during the preimplantation stage. Unlike the report of Cerny *et al.* in bovine [11], no significant change in pro-*Nts* expression was observed in mouse up to 96 h after hCG administration in oviduct. However we showed the protein expression in the oviduct epithelial cells, 14 h after hCG treatment [10]. There is supposed to be a species difference that the mice oviduct doesn't show remarkable change of mRNA level compared to bovine. Meanwhile, a significant increase in uterine pro-*Nts* mRNA expression was observed after 18 and 48 h hCG administration in mated mice. Because the expression level of pro-*Nts* mRNA did not significantly change in the presence or absence of mating after hCG treatment, we propose that hCG was the main factor inducing elevated pro-*Nts* mRNA levels. Our previous work showed that hCG

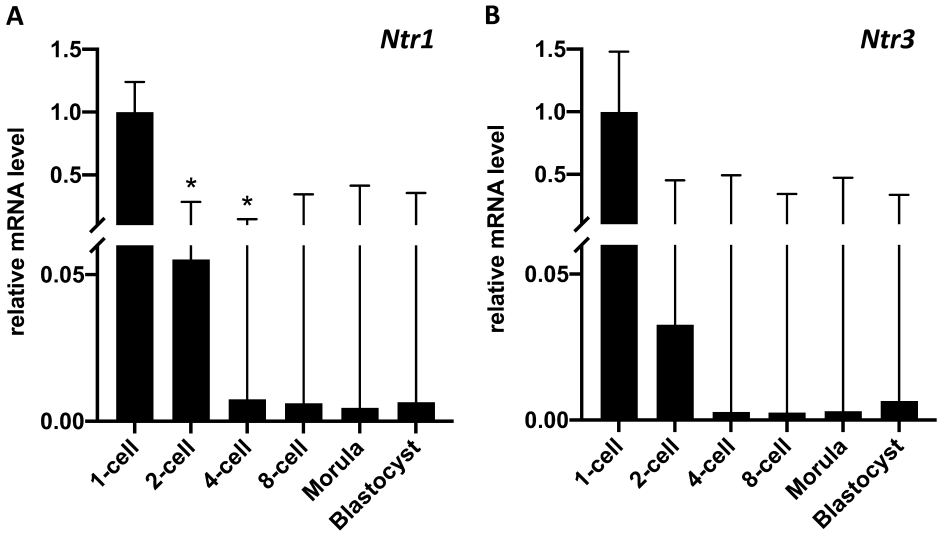

Fig. 2. Analysis of neurotensin receptor (*Ntr*) transcripts in embryos. Relative mRNA expression levels of *Ntr1* (A) or *Ntr3* (B) were normalized to the 1-cell stage. Twenty-five embryos per stage were used for analysis. Values are mean ± SEM. Asterisks represent significant differences compared with the 1-cell group, using the Dunnett's test. * $P < 0.05$. n = 3.

Table 2. Developmental competence of embryos cultured with neurotensin (NTS)

NTS (nM)	N	2-cell (%)	4-cell (%)	Blastocyst (%)	Hatching blastocyst (%)
0	137	120 (87.8 ± 4.4)	116 (85.0 ± 4.5)	83 (60.6 ± 1.7)	18 (13.0 ± 4.1)
1	143	127 (89.1 ± 1.9)	118 (82.2 ± 2.0)	99 (68.6 ± 3.9)	26 (18.7 ± 3.2)
10	145	135 (90.8 ± 3.4)	128 (87.7 ± 4.4)	107 (73.4 ± 4.2)	31 (20.7 ± 4.2)
100	150	129 (85.2 ± 3.2)	120 (79.6 ± 3.8)	114 (75.6 ± 3.4) *	42 (28.1 ± 3.8)
1000	147	126 (85.7 ± 3.1)	119 (81.3 ± 2.9)	93 (64.7 ± 5.0)	23 (18.4 ± 6.4)

N = Total number of 2PN embryos. Data were showed as the mean ± SEM of five replicates. Asterisk represents significant difference to NTS 0 nM group ($P < 0.05$) .

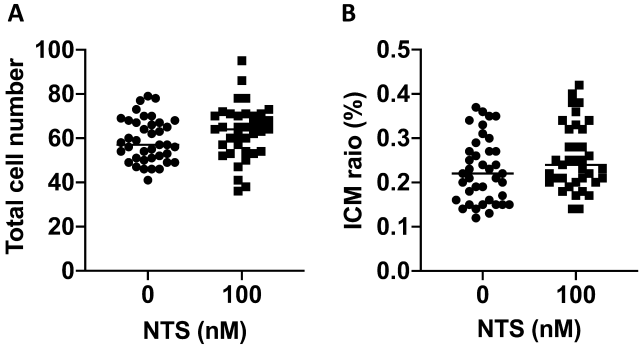

Fig. 3. Effects of neurotensin (NTS) on the number of total cells and inner cell mass (ICM) ratio of blastocysts. The blastocyst total cell numbers (A) and percentage of ICM cells per total cells (B) were compared between the 0 nM NTS (n = 39) and 100 nM NTS (n = 37) groups after 96 h culture. The bars represent mean values.

led to increased pro-*Nts* mRNA expression in cumulus cells, and was regulated by mitogen-activated protein kinase (MAPK) [10]. A similar hCG-induced pathway for the regulation of the pro-*Nts* system likely to be present in the uterus. Whether ovulated COCs affected pro-NTS expression in the oviduct is also needs to be considered if paracrine factors from oocyte or cumulus cells regulates its expression. It is required to verify that whether pro-NTS mRNA expression levels in the oviducts differ with or without ovulation or whether they correlate with the number of ovulatory COCs. We profiled the expression patterns of NTS receptor transcripts from the 1-cell to blastocyst stages. *Ntr1* and *Ntr3* were the major receptors expressed through the preimplantation period. To our knowledge, this is the first report of NTS receptor expression patterns in preimplantation embryos. The relative levels of *Ntr1* and *Ntr3* transcripts were markedly decreased in 2-cell or later stages compared with that of the 1-cell stage, suggesting that both genes are likely to be maternally regulated. However, transcripts were also detected throughout all stages, indicating that NTS is available to act on embryos at each developmental stage.

NTR1 and NTR2 are G protein-coupled receptors, whereas NTR3 is a single-pass transmembrane receptor [14]. The function of NTR3,

also called as sortilin, was reported to involve protein transport from the trans-golgi network into lysosomes [15]. In cultured CHO and HT29 cells, NTR3 has been reported to form heterodimers with NTR1 to modulate MAPK phosphorylation and phosphoinositol production, respectively, which modulates the responsiveness of NTS [16]. In the present study, it remains unclear whether NTR1 and NTR3 form a complex, or which receptor plays a dominant role in NTS-induced signal transduction in the embryo. Previous work reported that NTR3 alone functions as a receptor without forming heterodimers with other receptor types [17, 18]. Future experiments should investigate the receptor responsible for NTS signaling using antagonists to NTR1 and NTR3, noting antagonists to NTR3 are not currently available, or specific knock-out mice.

The present study showed that 100 nM NTS increased the blastocyst formation rate *in vitro*. Considering the qPCR findings, NTS may be available to embryos throughout all stages, but the uterine phase appears more likely to involve the effect of NTS on embryo development and implantation potential. Embryo transfer model could reveal this possibility. The significance of higher mRNA expression of *Ntr1* and *Ntr3* at the 1-cell stage and its contribution to developmental competence remains unclear. A significant increase in the blastocyst rate was obtained using 100 nM NTS, but no differences were observed at other concentrations, indicating that a limited concentration range for an effect. In future studies, the physiological level of NTS should be evaluated *in vivo* during the preimplantation period.

Several studies have demonstrated the stimulating effects of NTS upon cell proliferation, using tumor cell lines as reviewed in Carraway *et al*. [19]. In human pancreatic cancer cells, NTS activated the MAPK pathway involved with proliferation [20]. However, in our current results, 100 nM NTS treatment produced no significant increase in the number of blastocyst cells, so we propose that NTS promoted differentiation, rather than proliferation.

To summarize, we have shown that NTS mRNA was maternally expressed in the preimplantation stage, whereas the *Ntr1* and *Ntr3* receptors were expressed in the embryo. *In vitro*, NTS increased blastocyst formation through receptors expressed in the embryo, but had no significant effect upon the total cell number and ICM cell ratio of blastocysts. These findings indicate that NTS can contribute to efficient *in vitro* culture systems for blastocysts, and advance our understanding of the novel mechanisms of NTS-mediated interactions between maternal and early embryonic tissue.

Conflict of interest: We declare no competing financial interests.

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