

## ***In Vivo* Activity of the Potent Oxytocin Antagonist on Uterine Activity in the Rat**

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**Abstract.** Oxytocin antagonist (OTA), TT-235, was developed by our group and shown to inhibit either spontaneous or oxytocin-induced uterine contractions in primates. The purpose of the present study was to confirm the duration of TT-235 to block oxytocin-induced uterine contractions in estrous rats. In Experiment 1, the time-response of the three OTAs on uterine contractility was examined. The rats were anesthetized and cannulas were placed in the jugular vein for infusing vehicle (sterile saline), Antag I, Antag II and TT-235. The uterine activity was monitored through a water-filled balloon-tipped cannula placed in the uterine horn. The uterine contractile activity was determined as the integrated area for 10 minutes. Each OTA was administered as a single bolus injection of 5 µg, followed by 100 mU of oxytocin 5 minutes later, also done as a single bolus. Oxytocin injection of the same dosage was repeated every hour for 5 hours. Experiment 2 determined the effect of the three OTAs on uterine oxytocin receptor number (Rn) and binding affinity (Kd). Rats treated with either OTA or vehicle were sacrificed at 0.5 and 4 hours for receptor assay. In Experiment 1, Antag I, Antag II and TT-235 inhibited the integrated uterine response to oxytocin at 5 minutes by 76%, 77% and 80%, respectively, compared to controls ( $p < 0.05$ ). Two hours after injecting Antag I, inhibition of uterine contractility was 55% lower than controls ( $p < 0.05$ ). At 3 hours, uterine contractility was no longer affected in rats treated with Antag I compared with controls. The suppressive uterine activity with Antag II continued up to 3 hours. However, uterine contractility remained lower (53%) in rats treated with TT-235 5 hours later. In Experiment 2, TT-235 induced a significant decrease

( $p < 0.05$ ) in oxytocin receptor number and binding affinity at both 0.5 and 4 hours compared with controls. Antag I and Antag II did not alter oxytocin receptor number or binding affinity significantly at each time point studied compared with controls. In conclusion, TT-235 may inhibit the uterine response to oxytocin by decreasing oxytocin receptor numbers and oxytocin binding affinity, which might explain the prolonged oxytocin antagonist activity of TT-235.

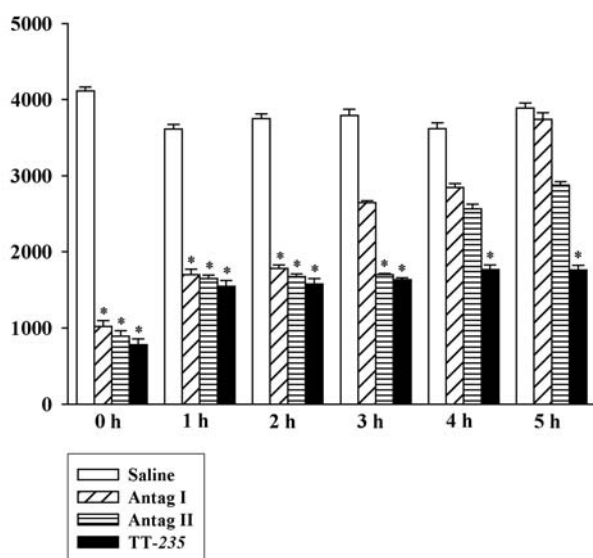
Since the molecular structure of oxytocin was introduced (1), many oxytocin-derived peptidyl analogs have been synthesized (2-5), with an ultimate goal of applying them in the clinical area. Although the role of oxytocin in the initiation and progress of labor has not always been clear, oxytocin and its receptors have been found to play an important role in the labor process (6,7).

Our laboratories have developed a number of potent and specific oxytocin antagonists (4,8) for studying their roles as potential inhibitors of preterm labor, which is one of the main factors contributing to newborn mortality. Among those compounds, Antag I disrupted labor (9) and suppressed uterine activity for 2 hours in response to exogenous oxytocin (10) in rats. In addition, Antag I inhibited spontaneous uterine contractions (11) and kept the uterus quiescent longer than Atosiban under the influence of oxytocin (12) in pregnant baboons. Antag II appears to be superior to Antag I from bioassay and receptor assay (13). The most potent oxytocin antagonist developed by our group, TT-235 (formerly known as Antag III) has significant clinical potentials based on data from bioassay (13), receptor assay affinity (12) and antagonist response interval (12). It has been shown that TT-235 decreased uterine contractions in subhuman primates for over 24 hours after they were exposed to physiological concentrations of oxytocin (12).

This study was designed to compare the duration of these three compounds *in vivo* to block oxytocin-induced uterine

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\*Significantly different from the control ( $p < 0.05$ ).  $n = 6$  at each time point of each group

Figure 1. Illustrated is the *in vivo* uterine response to 100 mU of oxytocin given every hour for 5 hours in estrous rats following infusion of 5 mg of Antag I, Antag II, TT-235 and saline.

contractions in estrous rats. The effects of the oxytocin antagonists on uterine receptor number and binding affinity were also determined.

## Materials and Methods

**Animals.** Virgin female Holtzman rats (200 to 270 grams) were used in this study. The rats were housed in rooms with controlled light cycles (14 hours light and 10 hours dark) with lights on at 6 a.m. The estrus was confirmed with vaginal smear. They were given water and food as desired. The protocols for the animal studies were approved by the Animal Care Committee at Chosun University Medical School, Korea, which has established guidelines for the care and use of animals.

**Oxytocin antagonists.** Three oxytocin antagonists were synthesized with the solid phase method (4,8) and used in this study. They are (a) [PMP<sup>1</sup>, D-Trp<sup>2</sup>, Phe<sup>3</sup>, Ile<sup>4</sup>, Arg<sup>8</sup>]-oxytocin (Antag I, PMP = beta-mercapto-beta, beta-cyclopentamethylene propionic acid), (b) [PMP<sup>1</sup>, D-Trp<sup>2</sup>, Arg<sup>8</sup>]-oxytocin (Antag II) and (c) [PMP(s)<sup>1</sup>, D-Trp<sup>2</sup>, Pen<sup>6</sup>, Arg<sup>8</sup>]-oxytocin (TT-235).

**Animal cannulation.** Rats in natural estrus were anesthetized with chloral hydrate (500 mg/kg) intraperitoneally for over 5 hours. Core body temperature, monitored in the rectum, was maintained at 37-38°C throughout the study using a body temperature control system composed of a DC current heat pad and an infrared lamp. A catheter (PE-50) was placed into a jugular vein for infusing oxytocin antagonists (5 µg) or vehicle (sterile saline) as a bolus injection, essentially as described by Park *et al.* (10).

To monitor uterine contractions, a PE-50 balloon-tipped and water-filled cannula was placed into one uterine horn at the ovarian end. Integrated intrauterine pressure changes were measured over 10 minutes using the Grass polygraph device (Grass Instruments, Quincy, MA, USA) and recorded with a Gould P23id pressure transducer (Gould Electronics, Oxnard, CA, USA). Animals were sacrificed by overdosing with chloral hydrate at the end of the study.

**Experiment 1: Effect of Antag I, Antag II and TT-235 on oxytocin-induced uterine contractions.** The experimental treatments were saline and 5 mg (14) of each oxytocin antagonist. Five minutes after infusing each oxytocin antagonist, 100 mU (14) of oxytocin was given and repeated every hour for 5 hours. The 5-minute time point is referred to as the 0 hour in Figure 1. Six animals were used at each time point of each treatment.

**Experiment 2: Effect of Antag I, Antag II and TT-235 on oxytocin receptor.** Rats were injected with vehicle (sterile saline), Antag I, Antag II or TT-235 of 5 µg *i.v.* for each, then sacrificed at 0.5 and 4 hours. The uteri were collected and prepared for analysis of oxytocin receptor number and affinity. These time points were chosen based on the results of the *in vivo* oxytocic assay shown in Figure 1. Six animals were used for each experimental group. The new methodology of the receptor assay has been described recently (15). A typical standard curve for the radioreceptor assay for TT-235 was shown with a sensitivity of 10 pg/well and the curve had a range from 10 to 1,000 pg (15). Furthermore, Antag I and Antag II were highly correlated with TT-235 for  $K_a$  values from receptor assays and  $pA_2$  values from uterine oxytocic bioassays (13).

**Data analysis.** Data from Experiment 1 were analyzed by repeated measures of analysis of variance and, when significant, differences were determined by Duncan's Multiple Range test. Data from Experiment 2 were analyzed by two-way analysis of variance with factors of treatment, time and their interaction. Significance was set at  $p < 0.05$ .

## Results

The effects of oxytocin antagonists against oxytocin challenge, expressed as an integrated response over a 10-minute period, are shown in Figure 1. Antag I, Antag II and TT-235 reduced oxytocin-induced uterine contractions by 76%, 77% and 80%, respectively, compared to controls ( $p < 0.05$ ) at 5 minutes. Antag I reduced oxytocin-induced uterine contractions up to 50% ( $p < 0.05$ ) for 2 hours, supporting our previous data (10). Antag II suppressed uterine activity for 3 hours ( $p < 0.05$ ). In contrast, TT-235 exhibited an anti-oxytocic effect throughout the 5-hour duration of the experiment. The inhibitory effect of TT-235 was 58% (1 hour post TT-235), 60% (2 hours post TT-235), 58% (3 hours post TT-235), 54% (4 hours post TT-235) and 53% (5 hours post TT-235) lower than the control, and all points showed significant differences ( $p < 0.05$ ). Based on this result, we designed Experiment 2 to look at the oxytocin receptor number and binding affinity at 0.5 and 4 hours. TT-235 induced a significant decrease ( $p < 0.05$ ) in oxytocin receptor number (approximately 50%) and binding

affinity (approximately 50%) at both the 0.5- and 4-hour time points compared to the control (Table I). Antag I and Antag II did not differ significantly ( $p>0.05$ ) from the control in either oxytocin receptor number or binding affinity at each time point studied (Table I).

## Discussion

The incidence of preterm birth remains high in spite of efforts invested in prediction and early detection of preterm labor. The available tocolytic agents may stop labor for 24 to 48 hours, but their ability to prolong pregnancy significantly is ineffective once true preterm labor has begun. This explains why oxytocin antagonists, which seem to be organ-specific, are receiving much attention and are a source of hope as a new tocolytic.

The results of this study showed that 5 mg bolus infusion of the oxytocin antagonist, TT-235, inhibits the uterine contractile response to 100 mU of exogenous oxytocin for up to 5 hours in estrous rats. The selection of a single dose of oxytocin and a single dose of TT-235 were from the previous study (14). TT-235 also decreased oxytocin receptor number and receptor binding affinity for 4 hours when compared with controls. These data suggest that the *in vivo* tocolytic activity of the oxytocin antagonist, TT-235, is long acting and probably acts *via* competitive inhibition of oxytocin receptors like Atosiban (16). At present, it is unknown whether oxytocin receptors are modulated or internalized by oxytocin antagonists *in vivo*. Our previous *in vitro* bioassay studies do not support this possibility because the uterus returned to normal after washing out the oxytocin antagonists from the organ bath with new buffer (13). Other than down-regulation of the oxytocin receptors and post-receptor effects from the present results, there are several other possible explanations for the prolonged action of TT-235, including high affinity to the oxytocin receptors (13), long half-life, reduced metabolic clearance rate and distribution volume, and resistance to enzymatic degradation (17). Desensitization of oxytocin receptors has also been reported in the human myometrium (18).

The development of a potent oxytocin antagonist would be useful for studying the contribution of endogenous oxytocin to nocturnal and labor-induced uterine contractions during pregnancy. The physiological role of oxytocin in the initiation and progress of parturition is not fully understood despite the fact that oxytocin, along with other factors, promotes uterine contractions (19). In pregnant women (20) and the rhesus monkey (21), an oxytocin antagonist prolonged pregnancy. Another oxytocin antagonist was resistant to oxytocinase in blood from pregnant women (17). In addition, the number of myometrial oxytocin receptors before parturition dramatically increases, correlating with increased contractile

Table I. *The effect of oxytocin antagonists on receptor number and binding affinity<sup>a</sup>.*

Group	Rat #	Parameter	
		Rn (fmole/mg) <sup>b</sup>	Kd (nM) <sup>c</sup>
0.5 h			
Saline	6	3,934±2.5	0.91±0.4
Antag I	6	3,345±1.5	0.77±0.2
Antag II	6	3,089±1.7	0.72±0.4
TT-235	6	2,050±2.8 <sup>d</sup>	0.47±0.1 <sup>d</sup>
4 h			
Saline	6	3,422±1.5	1.03±0.5
Antag I	6	2,979±2.5	1.00±0.4
Antag II	6	2,842±1.9	0.98±0.3
TT-235	6	1,689±2.5 <sup>d</sup>	0.71±0.1 <sup>d</sup>

<sup>a</sup>All values are mean±SEM

<sup>b</sup>Rn: oxytocin receptor number

<sup>c</sup>Kd: dissociation constant as binding affinity

<sup>d</sup>significantly different ( $p<0.05$ ) compared to saline control

responsiveness to oxytocin at the end of pregnancy (22, 23). Furthermore, parturition was associated with a high level of uterine oxytocin receptor gene expression (24). These studies clearly indicate that oxytocin plays an important role in the mechanism of uterine contractility during gestation.

Antag I, Antag II and TT-235 are about 2.5, 5 and 50 times more potent than Atosiban, respectively, as determined by an *in vitro* rat oxytocin bioassay and an oxytocin receptor assay (13). Atosiban was the first oxytocin antagonist approved for clinical use in Europe with the trade name of Tractocile (8). At least in a murine model, TT-235 was found to be the most potent agent to have been developed in our laboratory. Based on the present results, it is suggested that our oxytocin antagonists are long-acting inhibitors of oxytocin action *in vivo* in rats. They have the potential to be potent tocolytic agents for prevention of preterm labor in human.

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