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Effect of clinical doses of buserelin on in vivo bovine uterine activity at estrus

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Abstract: For successful fertilization, a transfer system named "rapid sperm transport" generated by uterine contractions plays a crucial role. The purpose of this in vivo study was to investigate the effect of buserelin, a GnRH agonist widely used for ovulation induction after mating or artificial insemination, on bovine uterine contractility which supports rapid sperm transport at estrus. In vivo uterine contractile activity was measured by intrauterine pressure recording technique. In the study, the spontaneous uterine contractile activities of 28 cows at estrus were observed for 30 min. The cows were randomly divided into 4 treatment groups which have 7 cows each of and were injected appropriate agents as follows; two different clinical doses of buserelin (10.5 µg, 21 µg), oxytocin (50 IU) and control (placebo). Following treatment, drug-induced uterine activity was measured for 60 min. There were no differences in terms of frequency in all groups observed. Oxytocin which is known as an effective uterotonic increased the amplitude and area under curve of contraction at estrus. Two different doses of buserelin were observed to have no effect on uterine contractile activity in cows throughout 60 min. It was concluded that GnRH has no function on bovine uterine contractility which plays a role in sperm transportation at estrus.

Key words: Buserelin, GnRH, uterine contractility, estrus, cow

1. Introduction

Uterine contractility plays crucial roles for rapid sperm transport, a passive transfer system which supports the spermatozoon movements in the female genital tract [1]. In follicular phase, the contractile activity of bovine uterus has been reported to be higher compared to the luteal phase because of varying steroid hormones profile and their receptor expression [2,3]. Peak levels of uterine contractile activity are observed, particularly in the second half of estrus, then it weakens again with the ovulation. It is also reported that the propagating waves of contractions move towards the uterine tubes in the second half of the estrus in a regular manner [4]. This contraction pattern plays an important role in the rapid transport of spermatozoa during the second half of the estrus when the cows are subjected to mating or artificial insemination [4, 5].

Hormonal modulation of uterine contractility is proven by steroid hormones, oxytocin and B_2 adrenergic agonists and their receptor expression [6]. There are several in vitro and in vivo studies investigating the dose-dependent, species-specific and in different sexual phase effect of various reproductive hormones including estradiol [3], progestogens [7,8], chorionic gonadotrophin [9,10], prostaglandins [11,12] and oxytocin [13,14] on uterine contractile activity. However, there is limited information about the effects of GnRH and synthetic GnRH agonists on uterine contractility. In vitro studies [15,16] showed that GnRH can modulate the uterine contractile activity. It was shown that while dose-dependent and cycle phase related stimulation was occurred in bovine uterine strips [15,16], increasing doses of GnRH caused direct inhibition in rat uterine stripsin dose and time dependent manner [17]. Giammarino et al. [15] also reported that GnRH was more effective in uterine strips in follicular phase than in luteal phase because of more biologically active GnRH receptors. Additionally, this study highlighted that when GnRH receptors were invaded by GnRH antagonists, the increased doses of GnRH did not have an impact on uterine contractility in strips both follicular and luteal phase.

Endogenous GnRH, a decapeptide produced from hypothalamus, is secreted to hypophyseal portal circulation in a pulsatile manner, thus it is known that GnRH could not join the peripheral circulation [18]. However, in recent years, GnRH may have different functions in some reproductive tissues and organs besides the pituitary, and the presence of GnRH receptor has been observed in several reproductive tissues in various species [19–22]. Moreover, the presence of GnRH receptors in bovine uterus and oviduct have been proved for the first time

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and revealed that there has been no difference in GnRH receptors' expression pattern compared to the receptors in the pituitary [20].

However, there is no clear information about the function of synthetic GnRH agonists with GnRH receptors in these nonpituitary regions. Buserelin administration was estimated to affect the increase in uterine contractility by increasing the intracellular level of Ca^{++} through a series of intracellular pathways similar to oxytocin, which supports the direct effect on the paired receptor using agonistic (buserelin) heptahelical G protein (pharmaco-mechanical coupling) [23]. Although in vitro studies [15,16] have shown an inductive effect on uterine contractility, especially in the follicular phase, the in vivo effect of supraphysiological doses of GnRH is unknown. We hypothesize that administration of clinical doses of synthetic GnRH agonist at estrus stimulate the bovine uterine activity which supports rapid sperm transport. For this reason, we aimed to investigate the clinical efficacy of buserelin on in vivo bovine uterine contractile activity at estrus.

2. Materials and methods

2.1. Animals

This study was carried out in Agriculture and Livestock Research Farm. The permission of the Animal Experiments Local Ethics Committee was received before the study (2014/8 - 85). Cows used in this study were fed with same total mixed ration and had ad libitum access to water in a free-range barn. Cows had body condition scores ranging 3.25 to 3.75. Milking was performed twice a day. Average milk yield was approximately 2l.0 L. In the study, 28 Brown Swiss cows, in pp Day 70–120, aging between 3–8 and that were not pregnant and completed the voluntary waiting period (until pp Day 60) without problems, were used as subjects.

Experimental protocol was applied at the second half of the spontaneous estrus which were determined as 6–8 h after the first observation of external estrus signs. However, before this spontaneous estrus, to eliminate possible luteolysis delay or luteal phase elongation, an estrous cycle, called control cycle, was monitored without any intervention. Reproductive records of cows were examined, their transrectal ultrasonographic examinations were carried out and the stage of their sexual cycle was determined. Follow-up of the estrus signs in cows was performed with careful observation of the external signs of the estrus and lasted 30 min 3 times a day. Cows exhibiting estrus symptoms (standing to be mounted) were confirmed as described by Roelofs et al. [24] by performing rectal and transrectal ultrasonographic examinations. Accordingly, the cow is considered to be in estrus in the presence of a corpus luteum with a diameter of less than 10 mm or not detectable in rectal and ultrasonographic examination, in the presence of a dominant follicle with a diameter of 12–25 mm, if the uterus is turgid, its tonocyte is increased and it is contractile to the touch, if vaginal discharge (cervical mucus) is clear, slimy and clean.

2.2. Equipment used to evaluate in vivo uterine contractility

In vivo uterine contractility was evaluated with an intrauterine pressure (IUP) measuring system with an open-ended fluid-filled catheter. This system consists of a data acquisition unit (MP36 Data Acquisition Unit, Commat Ltd.), a noninvasive external pressure sensor (SS13L pressure transducer, Commat Ltd.), a software program (BSL 4.01 Software, Commat Ltd.), a notebook PC, a thin open-ended polyethylene catheter, transfer hoses, and a 3-way valve. The system was prepared following the procedure described by Bajcsy et al. [25] and calibrated by using the software before each measurement. The electrical signals (mV) were transformed into pressure units (mmHg) by the data acquisition unit. The data acquisition sampling frequency was set to 4 Hz.

2.3. Experimental protocol

The experimental protocol applied in the study is schematized in Figure 1. Blood samples were collected from cows defined at estrus to subsequently confirm of cycle phase with measurement of serum progesterone ($P_{4)}$ levels and centrifugated at 3500 g for 10 min. to extract their serum. The sera were kept at –20 °C until measuring their P_4 level.

The tail of the cow at estrus was tied to one side to prevent erroneous measurements due to tail-wagging motion. After cleaning the perineal region using an antiseptic solution, the previously calibrated catheter was transcervically placed into the uterus. The external pressure sensor was fixed to the root of the tail with an adhesive material to prevent reactions of the cow such as moving foot, urination, and defecation. The catheter was washed by using a saline solution of 10 mL for preventing possible catheter blockages. After the adaptation period (30 min on average), spontaneous IUP changes were recorded continuously for 30 min. The reactions of the animal such as coughing, itching, urination, defecation, hunching, and other significant actions were recorded. These artefacts were carefully examined and eliminated during the evaluation and analysis.

Following the spontaneous IUP recording, cows (n = 28) divided into 4 equal treatment groups consist of two clinical doses of buserelin treatment (Bus1 and Bus2), an oxytocin treatment (Oxt) (in order to monitor the uterotonic effect to check the operability of the IUP system) and a control group (Con). In Bus1 group (n = 7), 10.5 µg buserelin (0.0042 mg/mL buserelin acetate, 2.5 mL Receptal, MSD Animal Health), in Bus2 group 21 µg

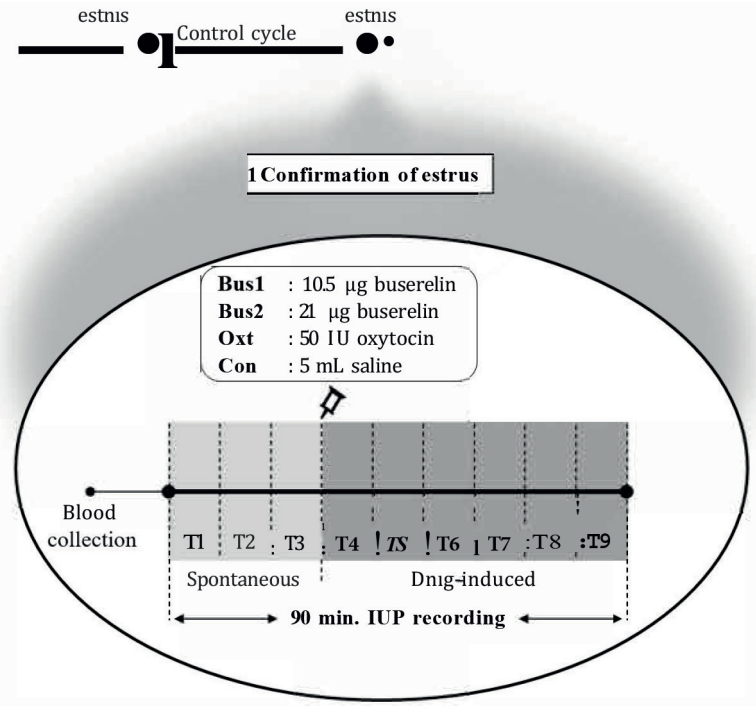

Figure 1. Schematic diagram of experimental protocol.

buserelin (n = 7) (5 mL, using the same preparation), in Oxt group (n = 7) 50 IU oxytocin (10 IU/mL oxytocin, Provet Oksitosin, Provet), and in Con group (n = 7), 5 mL saline solution were intravenously injected to cows. After administration of these agents to cows, IUP changes were recorded for 60 min.

Serum P_4 levels were measured using a commercial ELISA kit (Progesterone ELISA Kit, Cayman Chemical Company, Catalog No: 582601) following the procedure recommended by the manufacturing company.

2.4. Data management

For ensuring accuracy and more reliable statistical analysis of the mathematical data obtained from the IUP recording graphs, the experimental protocol period was divided into 9 time periods (TP), each of which lasted 10 min and named TP1-TP9 as described by Hirsbrunner et al. [11,12]. The periods of the first 30-min spontaneous contractions were named TP1, TP2, and TP3, respectively and the contraction periods following the administration of the agents (60 min) were named TP4-TP9, respectively. An increase in the IUP graph was considered a pressure wave in case the peak exceeded the threshold pressure by at least 7 mmHg for 20 s as described in the literatures [4,12].

The software program (BSL 4.0.1 version, Commat Ltd.) was used to analyse the frequency (FREQ, number of contractions/10 min), amplitude (AMP, mmHg, the pressure difference between IUP peak and resting period), and area under curve (AUC, mmHg × s., the total area under the curve in 10 min.) of the contractions.

2.5. Statistical analysis

In this study, the sample size was determined by statistical power analysis with using G*Power 3.1.9.4 version software program. The required minimum sample size was determined as 28, taking the effect size (f) 0.23, type I error (α) 0.05 and power (1-β error) 0.80 (using Cohen guidelines).

The data obtained by processing the IUP records were analyzed using SPSS 22.0 software package (Statistical Package for the Social Sciences for Windows, SPSS 22.0 Edition for Windows, Chicago, IL, USA). In this study, two-way ANOVA with replication test were used, which allows to test both factors treatment (TR) and time period (TP) and their interaction (TR × TP) on independent variables (FREQ, AMP and AUC). Tukey's multiple-comparison posttest were also used to find real-time differences within treatment groups. A difference was considered to be statistically significant when $p < 0.05$. The example of raw graphical tracing was reproduced by using the BSL 4.0.1 and the graphs were built in by GraphPad Prism 8.0.2 version.

3. Results

Serum progesterone levels of all cows subjected to this study were measured to be <1 ng/mL and the mean serum

progesterone level was determined to be 0.62 ± 0.2 ng/mL. Therefore, all cows used in the study were confirmed to be at estrus. Some artefacts were observed due to overactivity of two cows. However, IUP changes of all cows were recorded successfully. As IUP changes were rapid and instantaneous in artefacts, uterine contractions could easily be distinguished from these erroneous measurements.

3.1. IUP changes of spontaneous uterine contractility at estrus

The data of the first three periods (TP1-TP3) of all cows (n = 28) were used to calculate IUP parameters of spontaneous uterine contractile activity at estrus. The mean ± SEM of FREQ, AMP and AUC of TP1 to TP3 periods were found to be 5.11 ± 0.10 contraction cycle, 30.23 ± 0.94 mmHg and 773.43 ± 30.45 mmHg × s., respectively. The contraction cycles were observed to be nearly uniform and regular.

3.2. IUP changes after drug administration

The two-way ANOVA (treatment × time period) on each of uterine contractility parameters (FREQ, AMP and AUC) are presented in Table 1. In visual examination of the raw graphical tracings, there was only a striking IUP increase after the injection of 50 IU oxytocin (Figures 2 and 3). In other treatment groups (Con, Bus1 and Bus2), no significant activity was seen throughout the 90 min recording period. Similar findings were encountered in the mathematical analysis of graphical data. Graphical data of recording period obtained from the software program is presented in Figure 3. The differences in frequency between mean spontaneous (TP1-TP3) and drug-induced (TP4-TP9) contraction periods were not found to be statistically significant ($p > 0.05$) for all groups. While the differences in amplitude of contraction were not found to be statistically significant for Con, and Bus2 groups, they were found to be statistically significant for Bus1 and Oxt group, in which the mean amplitude of contraction increased by 51.12% following the intravenous administration of 50 IU oxytocin. This effect of oxytocin lasted for 13 min on average in the periods TP4 and TP5. In the Bus1 group, it was determined that the statistical difference between the time periods in terms of amplitude and AUC was caused by the TP1 period, and there was no effect in the TP4-TP9 period as a result of 10.5 µg buserelin injection. Similarly, the differences in AUC values were not found to be statistically significant for Con, and Bus2 groups. However, an increase of 51.30% in the AUC of the trace was observed in the Oxt group.

4. Discussion

It is well-known that in the presence of a preovulatory follicle, synthetic GnRH agonists induce ovulation by providing LH surge [26]. In addition to this well-known conventional effect, recent studies [19–22] have reported that GnRH had also local regulatory and different physiological functions in the reproductive system. As is known, buserelin is a widely used synthetic GnRH agonist for ovulation induction after artificial insemination at estrus. And in this process, uterine contractility contributes to rapid sperm transfer. Previous studies [2–4, 11–14, 27] investigating the in vivo bovine uterine activity have focused on changes in contractile activity throughout the sexual cycle and in postpartum period and effects of some hormones on uterine contractile activity. In this study, the main goal is to understand the effect of GnRH (synthetic agonist) on uterine activity which plays a role in rapid sperm transport after artificial insemination during estrus, rather than merely determining the effect of uterine activity. Therefore, we investigated the effects of two clinical doses of buserelin on bovine uterine contractility at estrus from a different perspective.

The data regarding spontaneous IUP changes of cows at estrus were collected to compare contraction patterns after buserelin administration. The analysis revealed that the data were similar to those of previous in vivo studies [3, 4, 14, 27], in which spontaneous uterine contractions were analyzed using different techniques.

In the study, the effect of buserelin treatment on uterine contractility have been observed for 60 min. In the determination of this period, the time of the spermatozoa to reach the fertilization site and the pharmacokinetic properties of buserelin were taken into consideration. Buserelin was reported to be 25 times more potent compared to gonadorelin [28], to have higher receptor affinity [29], and to stimulate LH surge approximately 120 min after intramuscular injection [30]. Its elimination of half-life is approximately 80 min. Considering the said pharmacokinetic effects of buserelin, we think that a period of 60 min would be sufficient to observe the effects of supraphysiological doses of GnRH on uterine contractile activity.

We hypothesized that buserelin has a dose dependent effect on in vivo bovine uterine contractility when administered at estrus. In order to test this hypothesis, the effects of treatment (two clinical buserelin dose, oxytocin and placebo) and time period (TP1 to TP9) and their interaction on FREQ, AMP and AUC, a two-way ANOVA (Treatment: 4 × Time period: 9) analysis was conducted. There were no significant effect of treatment and time period on FREQ (Table 1). Regarding to AMP, a significance was determined in Time period. After the recording of spontaneous uterine contractions, administration of 50 IU oxytocin, to test the eligibility of the IUP measuring system, increased up AMP in TP4 and in TP5 (Figure 2). However, the difference in TP1 in the BUS2 group (21 µg buserelin) is thought to be insignificant and possibly due to adaptation process or a system-related condition. There is no significant difference in AMP after buserelin administration.

Table 1. Two-way ANOVA (Treatment (TR) × Time period (TP)) on FREQ, AMP and AUC of uterine contractility.

Source of variation	SS	df	MS	F	p
FREQ					
Time period (TP)	5.798	8	0.7248	1.112	p = 0.850
Treatment (TR)	9.580	3	0.3193	4.901	p = 0.355
Interaction (TR × TP)	10.92	24	0.4549	0.6982	p = 0.002
Residual (error) Total	140.7 167.0	216 251	0.6516		
AMP					
Time period (TP)	2466	8	821.9	9.593	p < 0.0001
Treatment (TR)	385.8	3	48.22	0.5628	p = 0.807
Interaction (TR × TP)	1434	24	59.74	0.6973	p = 0.851
Residual (error) Total	18505 22790	216 251	85.67		
AUC					
Time period (TP)	298865	8	37358	3.804	p = 0.0003
Treatment (TR)	2531521	3	843840	85.92	p < 0.0001
Interaction (TR × TP)	933498	24	38896	3.960	p < 0.0001
Residual (error) Total	2121478 18592215	216 251	9822		

FREQ: frequency of contractions, **AMP:** amplitude of contractions, **AUC:** area under curve of contractions, **TP:** time period, **TR:** treatment (BUS1, BUS2, OXT, and CON)
SS: sum of squares, **df:** degrees of freedom, **MS:** mean square, **F:** Fisher value.

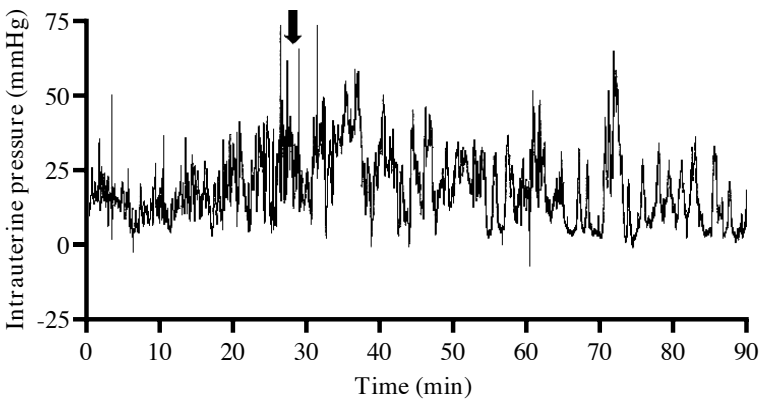

Figure 2. Raw graphical tracings of spontaneous and oxytocin-induced intrauterine pressure (IUP) changes (system control). Arrow represents the injection of 50 IU oxytocin. After oxytocin injection, there is a noticeable increase in IUP.

In the literature, there are a few studies on the effects of GnRH and synthetic agonists on uterine contractility, however their results are contradictory because of the difference about species-specific effects. GnRH was reported to inhibit contractility in isolated rat myometrial strips [17], however, Giammarino et al. [15] and Manera et al. [16] reported that increased doses of GnRH stimulated contractile activity. The researchers also stated that the inductive effect was more in uterine strips during the follicular phase and this effect was probably provided

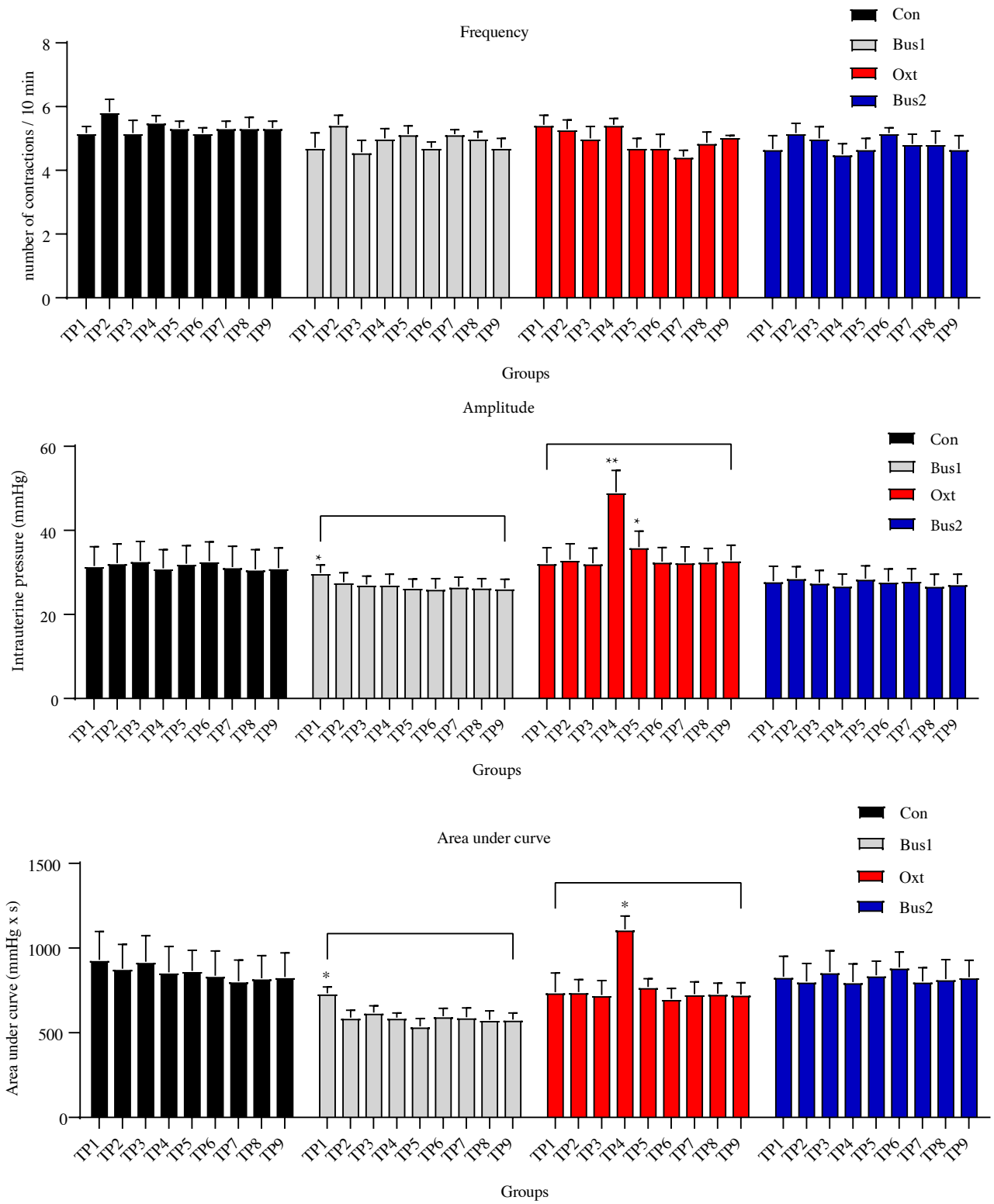

Figure 3. Mean contractility parameters, 95% confidence interval, of spontaneous and drug-induced IUP recordings through TP1 to TP9 in treatment groups. There is no significance in terms of frequency of contractions among time periods in groups (a). Statistical significance was detected in amplitude (b) and area under curve (c) in Bus1 and Oxt groups. Note the difference at TP4 period (just after the oxytocin injection) in amplitude and AUC of Oxt group. Statistical analyses were calculated by Tukey posthoc test following significant two-way ANOVA.* < 0.05, ** < 0.01.

by biologically active GnRH receptors. Additionally, the same studies highlighted that when GnRH receptors were invaded by GnRH antagonists, the increased doses of GnRH did not have an impact on uterine contractility in strips both follicular and luteal phase.

This in vivo study revealed that two different clinical doses of buserelin (10.5 µg, 21 µg) did not affect the uterine activity. Although increased doses of GnRH were proved to stimulate the contractile activity in isolated bovine myometrial strips in vitro [15,16], the buserelin as a GnRH agonist could be stated to have no impact on stimulating the uterine activity by contributing to rapid sperm transport for 60 min in oestrous cows in vivo.

The studies investigating the drug and dose-response effects on uterine activity suggest that the contraction parameters exhibit a harmonic attitude after drug administration considering the time of action and elimination of half-life (30). In our findings, TP1-TP3 periods during which spontaneous uterine activity was monitored, and TP4-TP9 periods during which the groups were monitored after administration of appropriate agents were observed to be uniform in terms of all contractility parameters in Con and Bus2 groups. In Oxt group, which was designed to test the eligibility of the IUP measuring system, a harmonious increase was observed in the amplitude and AUC of the contraction cycle after the injection of 50 IU oxytocin. The inconsistency observed in the Bus1 group in the TP1 period resulted in statistical differences between periods (Figures 2b and 2c). The reason for this difference might be due to incomplete adaptation process after placing the catheter for IUP measuring, however, no effect has been observed after the administration of 10.5 µg.

According to results of this study, it was concluded that buserelin administered in clinical doses (10.5, 21 µg) to cows in estrus did not show any direct effect on in vivo uterine contractile activity contributing to the rapid sperm transport, however, more detailed studies should be carried out to provide sufficient findings on this process.

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