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Nitric oxide in the bovine oviduct: Influence on contractile activity and nitric oxide synthase isoforms localization

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Abstract

The oviducts of 64 Holstein cows in luteal (early I, early II and late) and follicular phases were evaluated to determine the protein expression and mRNA transcription of different nitric oxide synthase isoforms (eNOS, iNOS, nNOS) as well as the effect of nitric oxide (NO) on spontaneous contractility *in vitro*. The expression patterns of nitric oxide synthase (NOS) isoforms in isthmus and ampulla (n = 6 for each phase) were determined by immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis. In the contractility studies, longitudinal and circular isolated strips of isthmus and ampulla (n = 10 for each phase) of oviducts located ipsilateral to the luteal structure or preovulatory follicle were treated as follows: a) L-arginine, an endogenous NO donor (10^{-8} to 10^{-3} M), b) N^{ω}-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor (10^{-5} M) and L-arginine (10^{-3} M), c) methylene blue (MB), an inhibitor of soluble guanylate (10^{-5} M) and L-arginine (10^{-3} M) and d) sodium nitroprusside (SNP), an exogenous NO donor (10^{-8} to 10^{-4} M). Immunohistochemical evaluation revealed that endothelial NOS (eNOS) expression detected in epithelial layer of isthmus and ampulla was strong in early I luteal phase, moderate in follicular phase and weak in other phases. Neuronal NOS (nNOS) immunoreactivity was strong in isthmus and moderate in ampulla, and staining of nerve fibers was observed mostly in early I luteal and follicular phases. All eNOS, nNOS and inducible NOS (iNOS) isoforms were detected by RT-PCR. eNOS and iNOS proteins were evident, whereas nNOS was undetectable by Western blot analysis in the tissue examined. L-arginine applied alone or after L-NAME did not alter or increase the contractile tension of the strips in most tissues examined. However, L-arginine applied after MB increased contractile tension in the strips of ampulla and longitudinal isthmus from early I luteal phase and circular isthmus from follicular phase but decreased it in isthmus from early II luteal phase. SNP differentially modulated oviductal contraction depending on the type of muscular strips and period examined. These results showed the estrous phase-dependent changes related to endogenous NO system which might be of physiological importance to the oviduct for secretory and ciliary functions involved in gametes and embryo(s) transportation.

Keywords: Cow; Oviduct; Nitric oxide; Nitric oxide synthase

1. Introduction

The oviduct plays a key role in the reproductive process by regulating the transport of gametes and embryo(s), providing an optimum microenvironment for the fertilization process and the primary stages of

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embryonic development [1,2]. Smooth muscle contractility, ciliary beat and secretory activity are the essential functions of the oviduct. Ovary-derived hormones [3–5], prostaglandins (PGs) [4], cytokines [6,7], endothelial growth factors [8] and nitric oxide (NO) [7,9] are effective in regulating these functions.

Nitric oxide is a short half-life, highly reactive free radical gas [10] and can pass through membranes by diffusion and cause relaxation in smooth muscle cells after being released from the endothelium [11]. Nitric oxide is synthesized by nitric oxide synthase (NOS) enzyme group, which utilizes the semiessential amino acid L-arginine as a substrate [12]. Similarly, an exogenous NO effect can be derived from nitrate containing compounds, such as sodium nitroprusside (SNP) [13] which induces vasodilatation at the vascular smooth muscle cells by activating guanylate cyclase (GC), catalyzes cGMP accumulation and the pathways resulting in smooth muscle relaxation [14]. Nitric oxide synthase is an enzyme group and has inducible (iNOS) and constitutive (cNOS), which includes neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms [15,16]. The cNOS activity requires Ca^{2+}/calmodulin complex and can be stimulated by mechanical factors, hormones, such as estrogens and agonists, such as endothelin [11,15,17]. The iNOS does not require Ca^{2+} [18] and can be expressed in response to cytokines and lipopolysaccharides [10,11,19]. The NO has a variety of physiological actions in the reproductive system, such as ovarian function [20], implantation, maintenance of pregnancy and delivery [21]. It has been reported that bovine oviduct epithelial cells synthesize endothelin *in vitro* [22] and the contractile effects of endothelin-1 are enhanced by the presence of *N*-monomethyl-l-arginine monoacetate [23]. Therefore, it has been suggested that endogenously produced NO may be effective in oviductal functions [23]. The NOS has been identified by using different methods in the rat [24], human [25,26], bovine [25,27], and porcine [28] oviducts. Before ovum leaves the oviduct, NOS activity and expression decrease and therefore, contractile molecules, such as endothelin-1 [23] and $PGF_{2\alpha}$ [29] evoke motility in the oviduct. It has been reported that the injection of NOS inhibitors into the bursa ovarica in rats causes a decline in the number of ovum in oviduct, even though spermine NONOate (NO donor) does not affect ovum transport in pregnant rats [24].

Endogenous NO production occurs in relation to NOS activity and can affect the oviduct motility caused by oviduct smooth muscle relaxation. It has been suggested that NO can play a key role in the regulation of oviduct functions. However, the effects of SNP and L-arginine-NOS-NO-sGC pathway on spontaneous bovine oviduct contractility in different phases of estrous cycle are not clearly established *in vivo* and/or *in vitro*. Therefore, the objective of this study was to determine the protein expression and mRNA transcription of different NOS isoforms (eNOS, iNOS, nNOS) as well as the influence of NO on spontaneous contractility of the bovine oviduct (isthmus and ampulla) throughout the estrous cycle.

2. Materials and methods

2.1. Animals

Oviducts of multiparous, 3 to 4 years old, non-pregnant Holstein cows (n = 64) slaughtered in a private abattoir were used. Before slaughter, uterus and ovaries were examined by transrectal palpation to determine the phase of estrous cycle. Moreover, blood samples were taken, centrifuged, and sera were frozen at -20 °C for later analysis of NO and progesterone (P4) concentration to verify the estrous cycle. Immediately after slaughter, uterus and ovaries were visually examined by macroscopic observation. Cows that had healthy genital tracts and active ovaries were used in the study. The stages of the estrous cycle were defined by post-mortem examination of the ovaries (follicle and corpus luteum), and the oviducts were separated into four groups: early I luteal (Days 2–3), early II luteal (Days 5–6), late luteal (Days 15–17), and follicular (Days 19–21) stages according to the criteria documented by Miyamoto, et al [30]. If the concentration of P4 did not correspond to transrectal palpation findings, the data were excluded from analysis.

2.2. Progesterone and nitric oxide determination

Progesterone concentration was determined by electrochemiluminiscence immunoassay (ECLIA) with a commercial test kit (Elecsys progesterone II, cobas e, Roche Diagnostics, GmbH, Germany) according to the manufacturer's instructions, in an immunologic test analyzer (cobas e 601, Roche Diagnostics, GmbH, Germany). The standard curve ranged from 0.03 to 60.00 ng/ml and the sensitivity was 0.15 ng/ml. The intra- and interassay coefficients of variation were on an average 3% and 6%, respectively.

The NO concentrations were determined by the spectrophotometric method, as described previously [31]. The standard curve ranged from 0.5 to 200 μmol/l and the sensitivity was 0.119 μmol/l. The intra- and

interassay coefficients of variation were 5.6% and 6.3%, respectively.

2.3. Oviduct preparation for NOS evaluation by immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis

Immunohistochemistry, RT-PCR and Western blot analysis were performed on the oviducts of 24 cows (n = 6 for each period of the estrous cycle) located ipsilateral to the luteal structure or preovulatory follicle. Oviduct histologic sections were stained with hematoxylin-eosin before performing immunohistochemistry, RT-PCR and Western blot assays [32]. Tissues with no histopathological lesions were included in the study.

2.3.1. Immunohistochemistry

Cow oviducts were dissected into isthmus (n = 24) and ampulla (n = 24) and samples were fixed by immersion in 4% paraformaldehyde for 2 h. Then, they were rinsed with phosphate buffered saline (PBS) (pH 7.4) overnight and finally transferred to and stored in 18% buffered (pH 7.4) sucrose solution containing 0.01% sodium azide (NaN_3; Sigma, Chemical Co., USA, Cat # S2002) until further processing.

Immunohistochemistry was performed on 12-μm thick cryostat sections from the isthmus and ampulla mounted on chrome alum-gelatin coated glass slides. After air-drying at room temperature (rt) for 30 min, the sections were submerged for 20 min in PBS and subsequently incubated with a blocking solution containing 0.1% Triton X-100 (Sigma, Cat # 93,443), 0.1% bovine serum albumin (Sigma, Cat # A2153), 0.05% thimerosal (Sigma, Cat # T5125), as an antibacterial agent, 0.01% NaN_3 and 10% normal goat serum (Sigma, Cat # G9023) in PBS for 1 h (rt). The blocking solution was then removed and sections were incubated overnight with primary antisera directed against three isoforms of the NOS, the rabbit polyclonal anti-human nNOS diluted at 1:5,000 (Chemicon International, USA, Cat # AB5380), the mouse monoclonal anti-human eNOS diluted at 1:100 (BD Transduction Laboratories, USA, Cat # N30020) or rabbit polyclonal anti-mouse iNOS diluted at 1:800 (Sigma, Cat # N7782), respectively. Following triple washing in PBS (rt) the slides were incubated for 60 min (rt) with Alexa 488-conjugated goat anti-rabbit IgG for nNOS and iNOS (Invitrogen, UK, Cat # A11010) or goat anti-mouse IgG for eNOS (Invitrogen, Cat # A11003), both of them diluted at 1:500 in a blocking solution. The staining procedure was finished by rinsing sections thrice with PBS (rt). Finally, the slides were coverslipped with phosphate-buffered glycerol (pH 7.4). The samples were then analyzed under a fluorescent microscope (Axiophot, Zeiss, Germany). The distribution of structures immunoreactive to the studied enzymes was described. The semiquantitative assessment of relative immunoreactivity of positive cells was done by two independent observers giving a score as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; and 4, very strong reaction according to the procedure described elsewhere [33]. Arbitrary evaluation of the number of nerve fibers in studied tissues was performed using a score: (+) sporadically observed single fibers, + single fibers, ++ few fibers, +++ moderate number of fibers, ++++ numerous fibers, − not observed [34].

Slides were photographed with a confocal microscope (Bio-Rad MR2A, UK). Omission of the primary antisera or their replacement by non-immune sera abolished the immunostaining proving specificity of the immunoreaction.

2.3.2. RT-PCR and Western blot analysis

Bilateral tissue samples of isthmus and ampulla from each oviduct were dissected out and stored in RNAlater RNA protection solution (Ambion, USA, Cat # AM7024) for further analysis. The tissues were processed to isolate total RNA and proteins as described previously [35].

RT-PCR was performed as a two-tube reaction separately for isthmus and ampulla. The cDNA synthesis was performed as described previously [35]. Resulting cDNA preparations were used for subsequent PCR with primers specific for bovine nNOS, iNOS, eNOS and 18S rRNA as an internal control (Sigma), to assess the uniform efficiency of reverse transcription and PCR in each sample. The composition of PCR mix was the same as described previously [35]. Primers sequences, annealing temperatures and expected sizes of RT-PCR products are listed in Table 1. RT-PCR products were analyzed in 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer with M1 molecular size marker (DNA Gdańsk, Poland). DNA fragments were visualized with ethidium bromide (Sigma, Cat # 46,067) and 302-nm transluminator (Pharmacia LKB, Sweden). Gels were photographed with 100 ASA, 35-mm black and white film.

Immunoblotting was performed according to the procedure described by Laemmli [36]. The procedure was described elsewhere [35]. Primary antibodies were the same as used for immunohistochemistry: rabbit polyclonal anti-human nNOS (Chemicon International),

Table 1
Data of primers used for reverse transcriptase polymerase chain reaction.

mRNA	PCR primers	Size	Temp.	Gen Bank Acc. No.
nNOS	5′-gtcatttctgtccgtctcttcaaac-3′ 5′-gctgggtcacacggatggtcttg-3′	329 BP	66°C	XM_002694585.1
iNOS	5′-agcacgggaatgagtctcc-3′ 5′-cgtcagctggtaggttcctg-3′	394 BP	63°C	NM_001076799.1
eNOS	5′-ccttccgctaccagccaga-3′ 5′-cagagatcttcaccgcgttggcca-3′	104 BP	67°C	NM_181037.1
18 S rRNA	5′-gtaacccgttgaaccccatt-3′ 5′-ccatccaatcggtagtagcg-3′	151 BP	58°C	NR_036642.1

mouse monoclonal anti-human eNOS (BD Transduction Laboratories) and rabbit polyclonal anti-mouse iNOS (Sigma) diluted at 1:500, 1:100 and 1:1,000, respectively.

2.4. Isolated oviduct smooth muscle strips preparation for contractility experiments

Contractility experiments were performed on 40 oviducts (n = 10 for each period of the estrous cycle), ipsilateral to the luteal structure or preovulatory follicle.

Oviducts were collected about 15 min after exsanguinations, separated from uterine body and transported on ice to the laboratory within 30 min. Then, oviducts (infundibulum, ampulla and isthmus) were put into a dissecting Petri dish containing Krebs' solution (KS: NaCl 118 mmol/l KCl 4.7 mmol/l, $CaCl_2$ 2.5 mmol/l, $MgSO_4$ 1 mmol/l, KH_2PO_4 1 mmol/l, glucose 11 mmol/l, $NaHCO_3$ 25 mmol/l), which were continuously ventilated with a gas mixture (95% O_2 and 5% CO_2). The surrounding tissues of oviduct were carefully removed. Five mm-long ring strips of isthmus and ampulla were dissected from the ampulla-isthmus junction, which was identified by advancing a blunt probe (1.5 mm) from the fimbrial end until resistance was met [37] and incised longitudinally. Thereafter, longitudinal and circular smooth muscle strips were carefully isolated and one edge of each oviduct tissue preparation was fixed to platinum ring electrodes. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY, Commat, Ankara, Turkey). Isolated oviduct strips were placed in a four chambers organ baths (IOBS 99 Isolated Tissue Bath Stand Set, Commat) filled with 20 ml KS (pH 7.4), which were continuously oxygenated (95% O_2 and 5% CO_2) at 37 °C. The isometric smooth muscle activity of the oviduct samples were monitored and recorded by computer via the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab PRO Software, Biopac Systems, Commat).

2.4.1. Recording of isometric oviduct contractility

Oviduct samples in organ baths were kept in KS for at least 1 h before the recordings to enable the tissues to adapt to the environment and the solution was refreshed at 15 min intervals. The appropriate resting tension for the strips was determined in initial experiments. The strips were placed under progressive increments of tension. Optimal tension relationships were achieved with resting tensions of 1 g for the oviduct strips. Therefore, a resting tension of 1 g was applied to the tissues. After the completion of the 30 min baseline period, contractions of isthmus and ampulla of longitudinal and circular strips for each animal were visualized and recorded to determine normal spontaneous contractions. Thereafter, the strips were treated with (a) L-arginine (Sigma, Cat # A8094) at increasing concentrations (10^{-8} to 10^{-3} M) to determine endogenous NO activity; (b) N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M, Sigma, Cat # N5501), an NOS inhibitor, and followed by L-arginine (10^{-3} M) 10 min later to determine the effect of endogenous NO on the enzymatic pathway; (c) methylene blue (MB; 10^{-5} M, Sigma, Cat # M9140) and 30 min later L-arginine (10^{-3} M) to evaluate the effect on the soluble GC pathway and (d) SNP (Sigma, Cat # S0501) at increasing concentrations (10^{-8} to 10^{-4} M) for the evaluation of the exogenous NO pathway (increasing quantities of both L-arginine and SNP were added every 10 min). Concentrations of all reagents were chosen based on the previous studies [33,38] and confirmed by the preliminary experiments (data not shown). All treatments were performed on the same samples. The mean tension of spontaneous contractions for each strip calculated for a 10-min period before administration of examined substances was set as 100% (control period). Thereafter, changes in oviductal contractions caused by the examined substances were recorded and compared to the control period.

Table 2
Serum progesterone (P4) and nitric oxide (NO) concentrations (mean ± SD; n = 16 in each group) in the bovine serum.

Phases of the estrous cycle				
Parameters	Early I luteal	Early II luteal	Late luteal	Follicular
P4 (ng/ml)	1.29 ± 0.14^{a}	3.03 ± 0.12^{b}	8.24 ± 1.52^{c}	0.44 ± 0.06^{a}
NO (μmol/l)	17.22 ± 0.82^{a}	12.01 ± 0.68^{b}	14.03 ± 0.73^{b}	18.12 ± 1.12^{a}

Letters (^{abc}) in the same line indicate significant differences among different groups ($P < 0.001$).

2.5. Statistics

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test (SPSS 13.0). All values are presented as mean ± SD. Group differences were considered significant at $P < 0.05$.

3. Results

3.1. Serum progesterone and nitric oxide concentrations

Mean serum P4 and NO values are given in Table 2. Circulating concentrations of P4 were greater in late luteal (8.24 ± 1.52 ng/ml) than in early I luteal (1.29 ± 0.14 ng/ml), early II luteal (3.03 ± 0.12 ng/ml) and follicular (0.44 ± 0.06 ng/ml) phases ($P < 0.001$). There was no significant difference between early I luteal and follicular phases while P4 concentrations of early II luteal were higher than those of early I luteal and follicular phases ($P < 0.001$). Concentrations of P4 confirmed previous estrous cycle classification.

The NO concentrations in early I luteal (17.22 ± 0.82 μmol/l) and follicular (18.12 ± 1.12 μmol/l) phases were higher than in early II luteal (12.01 ± 0.68 μmol/l) and late luteal phases (14.03 ± 0.73 μmol/l) ($P < 0.001$).

3.2. Immunohistochemistry (qualitative)

The endothelium of blood vessels in serosa, muscular layer and lamina propria of both isthmus and ampulla showed various levels of eNOS immunoreactivity (ir) (Fig. 1A, C, E). Moreover, eNOS-ir and nNOS-ir (Fig. 1F) were present in epithelial cells, whereas nNOS-ir was found in nerve fibers of muscular layer (Fig. 1B), serosa (Fig. 1D), and lamina propria. In isthmus and ampulla of all groups, eNOS-ir was principally localized at the luminal surface of epithelium (Fig. 2A–D), whereas nNOS-ir extended from luminal surface of epithelium to lamina propria (Fig. 1 F and Fig. 2A–D). Indeed, strong eNOS- and nNOS-specific staining was observed in isthmus. Neuronal NOS-ir in nerve fibers within the serosa, muscular layer and lamina propria was mainly detected in follicular phase in the isthmus and ampulla (Table 3, Fig. 1). Omission of the primary antibody or their replacement by non-immune sera abolished the immunostaining proving specificity of the immunoreactions for eNOS (Fig. 1G, I) and nNOS (Fig. 1H, J).

Inducible NOS-ir staining was not detected in any of the tested oviduct samples.

3.3. Immunohistochemistry scoring for epithelium (semiquantitative)

3.3.1. Endothelial and neuronal NOS immunoreactivity

Immunohistochemical scoring for eNOS-ir and nNOS-ir between groups in isthmus and ampulla are listed in Table 4. The strongest reaction for eNOS-ir at luminal surface of epithelium in both isthmus and ampulla was observed in the early I luteal phase (Fig. 2). This reaction showed a significant difference between early I luteal and other groups ($P < 0.001$). However, eNOS-ir in epithelium of isthmus and ampulla in follicular phase was stronger than in early II and late luteal phases ($P < 0.001$), whereas immunoreactivity was weaker than in early I luteal phase ($P < 0.001$) (Fig. 2).

The reaction for nNOS-ir in epithelium of isthmus was strong in early I luteal and follicular phases (Fig. 3), whereas it was weak in early II luteal and moderate in late luteal phase ($P < 0.001$). However, nNOS-ir was stronger in the early I luteal and follicular phases (Fig. 3) than in early II and late luteal phases ($P < 0.001$).

3.4. RT-PCR and Western blot analysis

The screening of eNOS, nNOS and iNOS mRNA in bovine oviduct by RT-PCR showed different amounts of amplification products, as shown in Fig 4. All enzymes were expressed in both the ampulla and isthmus during the estrous cycle. The expression of eNOS and iNOS mRNA was similar but both were stronger than that of nNOS mRNA.

To better characterize the presence of NOS during the estrous cycle, the expression of all three NOS isoforms (eNOS, nNOS, iNOS) were also evaluated by employing Western blot in oviducts. Western blot showed the protein bands which corresponded to ex-

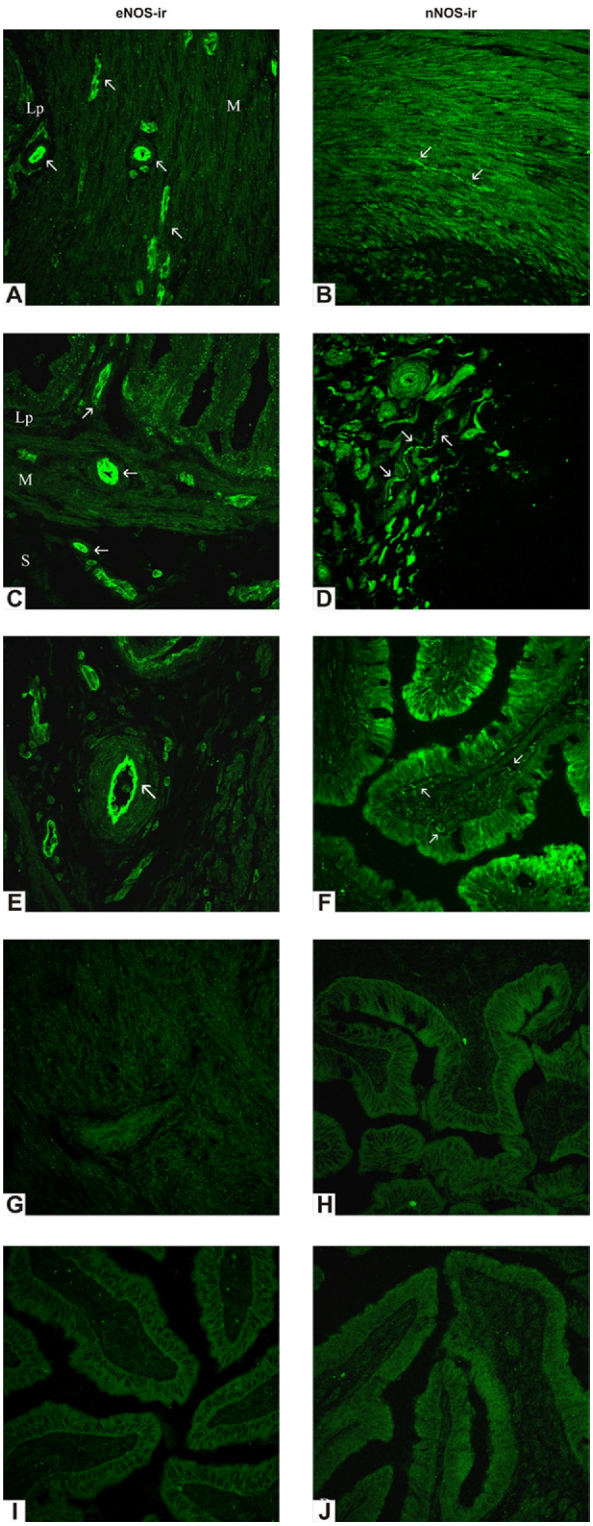

Fig. 1. Representative images of endothelial nitric oxide synthase immunoreactivity (eNOS-ir), neuronal nitric oxide synthase immunoreactivity (nNOS-ir) and negative control for both NOS isoforms. eNOS-ir was principally detected in endothelium of blood vessels (arrows) in lamina propria (A), muscular layer (A) and serosa (arrow) (E) of isthmus and in lamina propria, muscular layer and serosa of ampulla (C). nNOS-ir was expressed in nerve fibers (arrows) in muscular layer (B), in serosa (D) and lamina propria (F) of isthmus. Control staining by omission of the primary antibody or their replacement by non-immune sera abolished the immunostaining proving specificity of the immunoreactions for eNOS (G–I) and nNOS (H–J). M, Muscular layer; S, Serosa; Lp, Lamina propria (magnification: 40 X).

pected molecular weight of eNOS (140 kDa) and iNOS (130 kDa) (Fig. 5), whereas nNOS was not detectable.

3.5. Contractility experiments

L-arginine at the concentrations of 10^{-8}, 10^{-7} and 10^{-6} M did not alter the contractile tension in all examined strips when compared to the control period (Fig. 6). Moreover, the most effective dose of L-arginine in increasing oviduct contractility was 10^{-3} M. The contractile tension was increased by the highest dose of L-arginine in longitudinal isthmus from early II luteal phase ($P < 0.001$) (Fig. 6A). L-arginine did not affect the contractility in circular isthmus from early II luteal phase (Fig. 6B), whereas the contractile tension was increased in other phases ($P < 0.001$). In longitudinal ampulla, the contractility was increased in the strips from early I and II luteal ($P < 0.001$) and follicular ($P < 0.01$) phases (Fig. 6C), whereas the contractile tension in circular ampulla was increased ($P < 0.001$) only in early I and II luteal phases (Fig. 6D).

L-NAME administered alone had no effect on contractile tension of examined strips (data not shown). In longitudinal isthmus L-arginine administered after L-NAME significantly increased ($P < 0.001$) contractile tension in early I luteal phase as compared to control period and L-arginine administered alone (Fig. 7A). Moreover, an insignificant increase was also observed in early II luteal phase as compared to control period but this increase was significantly lower ($P < 0.001$) as compared to L-arginine administered alone (Fig. 7A). In circular isthmus, L-arginine administered after L-NAME significantly increased ($P < 0.05$) contractile tension in early I luteal phase as compared to control period and this increase was similar to the effect exerted by L-arginine administered alone (Fig. 7B). Moreover, a significant increase ($P < 0.001$) was also observed in late luteal and follicular phases as compared to control period but in late luteal phase this increase was significantly higher ($P < 0.05$) and in follicular phase significantly lower ($P < 0.001$) as compared to L-arginine administered alone (Fig. 7B). In

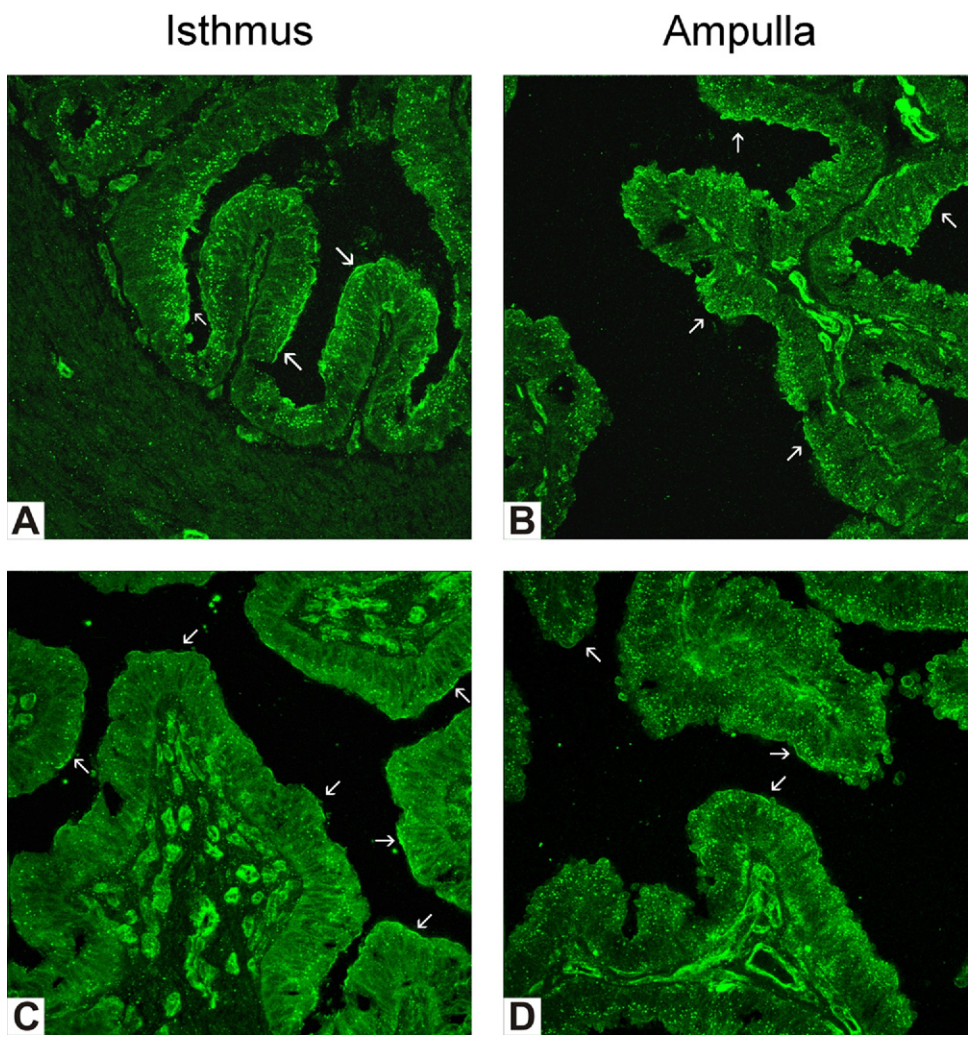

Fig. 2. Representative images of endothelial nitric oxide synthase immunoreactivity localized in luminal surface of epithelium of isthmus (A, C) and ampulla (B, D). Strong reaction was detected in early I luteal phase of isthmus (A) and ampulla (B), while moderate reaction was observed in follicular phase of isthmus (C) and ampulla (D) (magnification: 40 X).

Table 3
Distribution and co-localization of positive neuronal nitric oxide synthase immunoreactivity in nerve fibers of isthmus and ampulla of the bovine oviduct.

Phase of the Estrous cycle	Nerve-fibers surrounding Blood vessels						Nerve-fibers					
	Isthmus			Ampulla			Isthmus			Ampulla		
	S	M	Lp	S	M	Lp	S	M	Lp	S	M	Lp
Early I luteal	(+)	−	−	+	−	−	+	(+)	−	+	+	−
Early II luteal	+	−	+	−	−	−	(+)	(+)	−	+	−	−
Late luteal	++	−	−	(+)	−	−	+	++	+	−	−	−
Follicular	++++	−	−	+++	−	−	+++	++	++++	++	++	+++

S, Serosa; M, Muscular layer; Lp, Lamina propria; (+), sporadically observed single fibres; +, single fibres; ++, few fibres; +++, moderate number of fibres; ++++, numerous fibres; −, not observed.

Table 4
Evaluation of endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase immunoreactivity (mean ± SD) in epithelium of isthmus (n = 24) and ampulla (n = 24) of the bovine oviduct according to the score: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, very strong reaction.

Phase of the Estrous cycle	eNOS		nNOS	
	Isthmus	Ampulla	Isthmus	Ampulla
Early I luteal	3.83 ± 0.16[a]	3.50 ± 0.22[a]	3.66 ± 0.21[a]	2.83 ± 0.16[a]
Early II luteal	1.50 ± 0.22[b]	1.33 ± 0.21[b]	1.83 ± 0.16[b]	1.00 ± 0.25[b]
Late luteal	1.66 ± 0.16[b]	1.50 ± 0.22[b]	2.66 ± 0.21[c]	1.83 ± 0.16[c]
Follicular	2.66 ± 0.16[c]	2.33 ± 0.33[c]	3.33 ± 0.21[a]	2.66 ± 0.42[a]

[abc] superscript letters in the same column indicate significant differences among the groups $P < 0.001$).

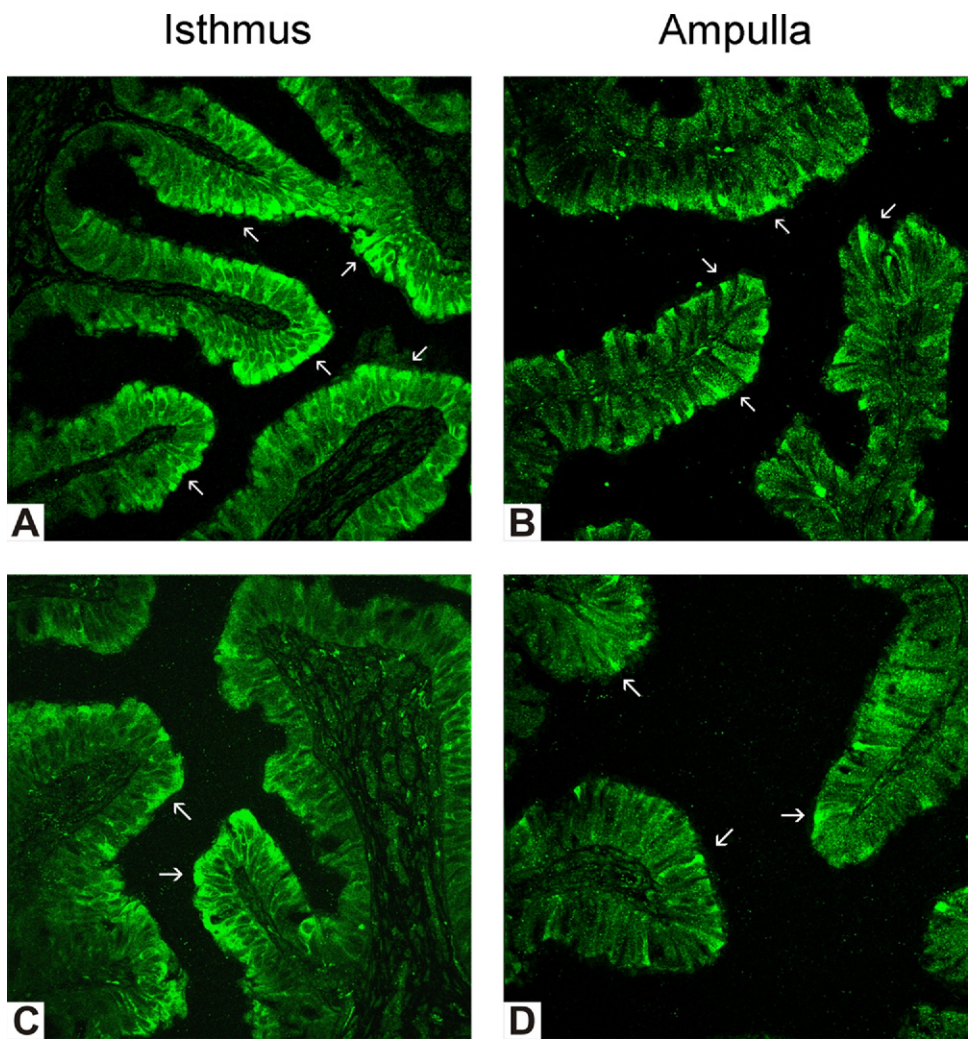

Fig. 3. Representative images of neuronal nitric oxide synthase immunoreactivity localized from luminal surface of epithelium to lamina propria of isthmus (A, C) and ampulla (B, D). Strong reaction was detected in early I luteal phase (A) and follicular phase (C) of isthmus while moderate reaction was observed in early I luteal (B) and follicular phase (D) in ampulla (magnification: 40 X).

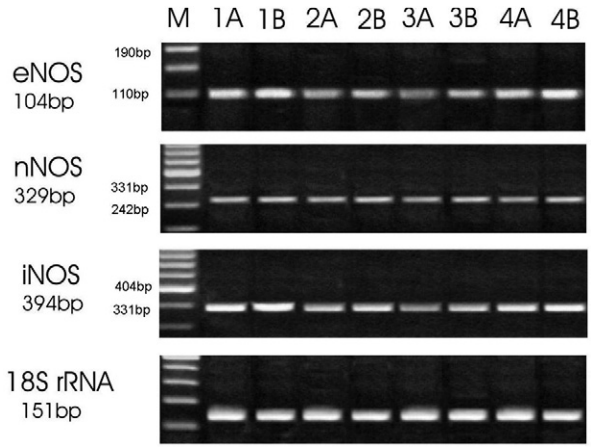

Fig. 4. RT-PCR products of the housekeeping gene 18S and the target genes of endothelial, neuronal and inducible nitric oxide synthase immunoreactivity in the isthmus and ampulla throughout the estrous cycle. M, Marker; 1, Early luteal I phase; 2, Early II luteal phase; 3, Late luteal phase; 4, Follicular phase; A, Ampulla; B, Isthmus.

longitudinal ampulla administered after L-NAME significantly increased contractile tension in early I ($P < 0.05$), early II ($P < 0.01$) and late luteal ($P < 0.001$) phases as compared to control period but in early II luteal phase this increase was significantly lower ($P < 0.01$) and in late luteal phase significantly higher ($P < 0.001$) as compared to L-arginine administered alone (Fig. 7C). In circular ampulla L-arginine administered after L-NAME significantly increased contractile tension in all phases examined ($P < 0.001$, $P < 0.05$, $P < 0.01$ and $P < 0.05$ in early I, early II, late luteal and follicular phases, respectively) as compared to control period and this increase was significantly higher ($P < 0.001$) only in late luteal phase as compared to L-arginine administered alone (Fig. 7D).

The MB administered alone had no effect on contraction of examined strips (data not shown). In longitudinal isthmus, L-arginine administered after MB significantly increased ($P < 0.01$) contractile tension in early I luteal phase as compared to control period, but this increase was insignificant as compared to L-arginine administered alone (Fig. 7A). Moreover, a significant decrease was observed in early II luteal phase as compared to control period ($P < 0.01$) and L-arginine administered alone ($P < 0.001$) (Fig. 7A). In circular isthmus, L-arginine administered after MB significantly decreased ($P < 0.001$) contractile tension in early II luteal phase as compared to control period and L-arginine administered alone (Fig. 7B). In late luteal phase contractile tension was similar as compared to control period but significantly lower ($P < 0.001$) as compared to L-arginine administered alone (Fig. 7B). In follicular phase contractile tension was significantly higher ($P < 0.001$) as compared to control period but similar as compared to L-arginine administered alone (Fig. 7B). In longitudinal ampulla, L-arginine administered after MB significantly increased ($P < 0.05$) contractile tension in early I luteal phase as compared to control (Fig. 7C). However, contractile tension was significantly lower in early II ($P < 0.001$) and follicular ($P < 0.05$) phases as compared to L-arginine administered alone (Fig. 7C). In circular ampulla, L-arginine administered after MB significantly increased ($P < 0.001$) contractile tension in early I luteal phase as compared to control period and L-arginine administered alone (Fig. 7D). However, contractile tension was significantly lower ($P < 0.01$) in early II luteal phase as compared to L-arginine administered alone (Fig. 7D).

Application of SNP resulted in relaxation ($P < 0.001$) of isthmus and ampulla from early I luteal and follicular phases (Fig. 8). Moreover, SNP decreased ($P < 0.001$) contractile tension in the strips of circular isthmus from early II luteal phase (Fig. 8B) and increased contractility ($P < 0.05$) in longitudinal ampulla from the late luteal phase (Fig. 8C).

4. Discussion

The data obtained in this study support previous *in vitro* and *in vivo* observations [5,7,23,25,27] and provide new evidence that NO may play an important role in the functioning of bovine oviduct. In the present study, we have demonstrated that serum NO levels were the highest in early I luteal and follicular phase. Accordingly, it has been well documented that during periovulatory period bovine ovary [39] and blood ves-

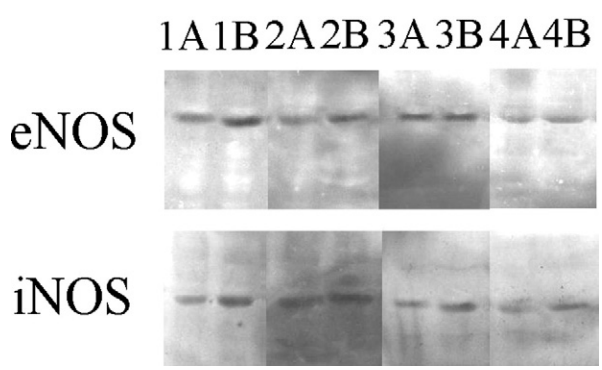

Fig. 5. Endothelial and inducible nitric oxide synthase bands in isthmus and ampulla of the bovine oviduct in different phases of the estrous cycle. 1, Early I luteal phase; 2, Early II luteal phase; 3, Late luteal phase; 4, Follicular phase; A, Ampulla; B, Isthmus.

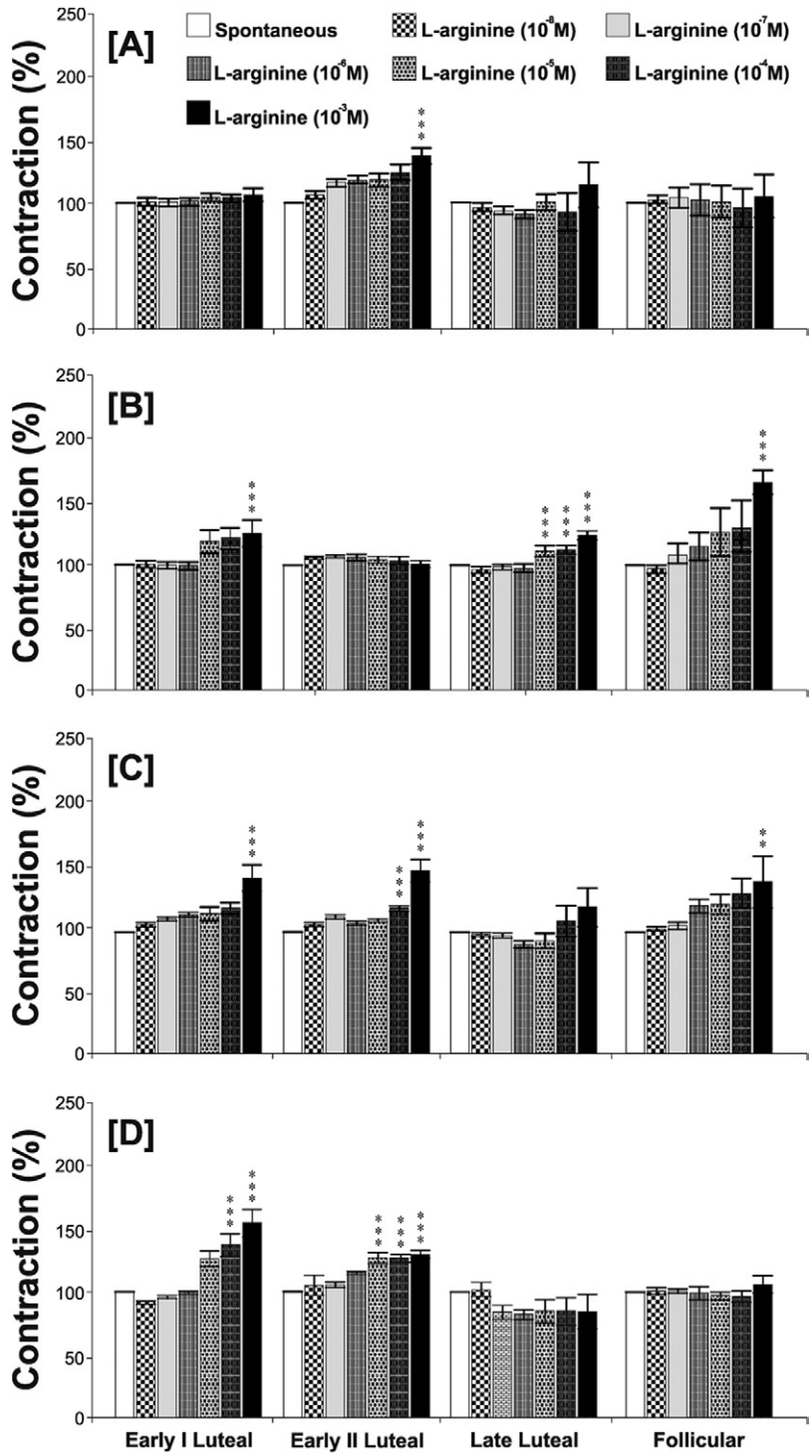

Fig. 6. Influence of increasing concentrations (10^{-8} to 10^{-3} M) of L-arginine on the contractile tension of smooth muscle of longitudinal isthmus (A), circular isthmus (B), longitudinal ampulla (C) and circular ampulla (D) in the bovine oviduct collected from early I, early II and late luteal and follicular phases of the estrous cycle. Data are expressed as percentage of mean tension calculated during 10-min period of spontaneous contractions of each strip before the administration of examined substances and accepted as 100% (control period). ** $P < 0.01$, *** $P < 0.001$ as compared to the control period.

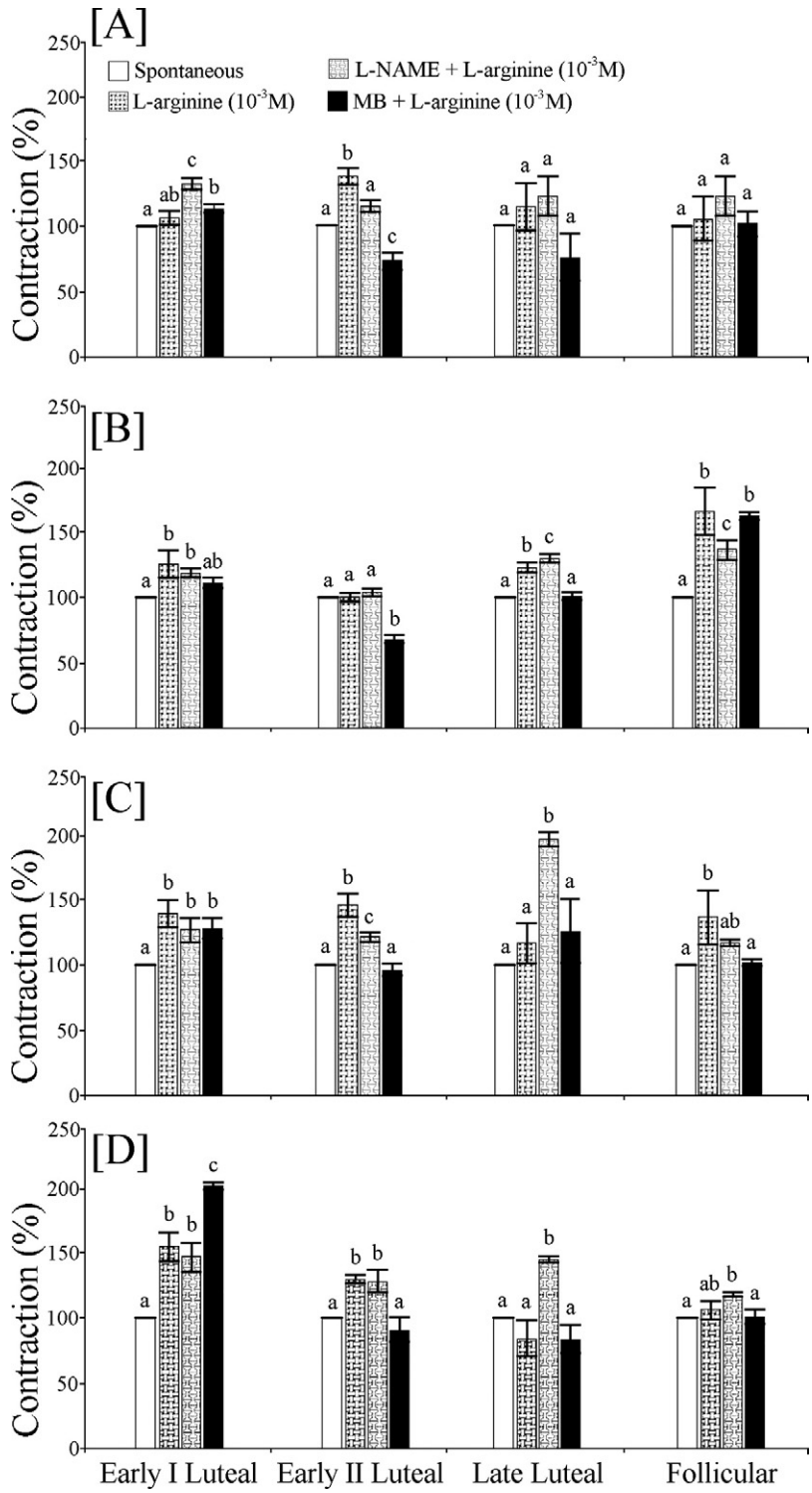

Fig. 7. Influence of L-arginine (10^{-3} M) administered alone and after N^{ω}-nitro-L-arginine (L-NAME, 10^{-5} M) or MB (10^{-3} M) on the contractile tension of smooth muscle of longitudinal isthmus (A), circular isthmus (B), longitudinal ampulla (C) and circular ampulla (D) in the bovine oviduct collected from early I, early II and late luteal and follicular phases of the estrous cycle. Data are expressed as percent of mean tension calculated during 10-min period of spontaneous contractions of each strip before administration of examined substances and accepted as 100% (control period). a,b,c, different letters indicate significant differences between groups.

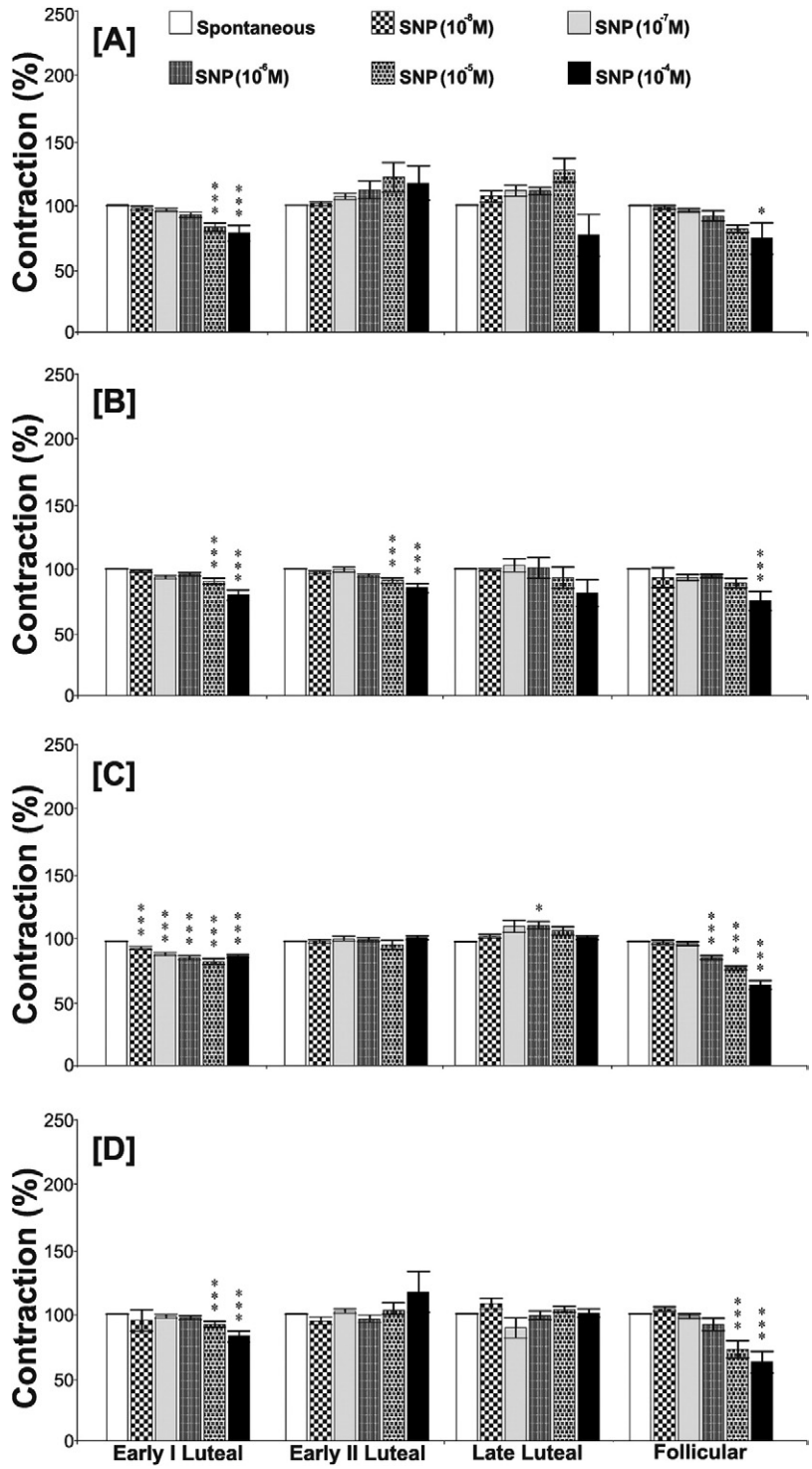

Fig. 8. Influence of increasing concentrations (10^{-7} to 10^{-4} M) of SNP on the contractile tension of smooth muscle of longitudinal isthmus (A), circular isthmus (B), longitudinal ampulla (C) and circular ampulla (D) of the bovine oviduct collected from early I, early II and late luteal and follicular phases of the estrous cycle. Data are expressed as percentage of mean tension calculated during 10-min period of spontaneous contractions of each strip before SNP administration and accepted as 100% (control period). * $P < 0.05$, *** $P < 0.001$ as compared to control period.

sels supplying reproductive tract [40] produce more NO than in other phases of the estrous cycle. In the present study, we demonstrated by using different methods, that the NOS isoforms were expressed in this specific organ of the female reproductive tract throughout the estrous cycle. Importantly, we detected by immunohistochemistry that the reaction for eNOS-ir in the luminal surface of epithelium in both isthmus and ampulla was strong in early I luteal phase and moderate in the follicular phase. Our results were consistent with the previous data reported by Lapointe, et al [9], that the eNOS expression in bovine oviduct was maximal at the periovulatory period (Days 0–3, and 18–20 of the estrous cycle). However, in another study eNOS activity was predominantly detected in secretory cells of bovine oviduct on Day 18 of the estrous cycle [27]. Observations in other species have indicated that eNOS activity in the epithelium of porcine oviduct was low in estrus [28], higher in follicular phase in the rat oviduct [41] or showed no difference in the rat oviduct throughout the estrous cycle [42]. Discrepancies in the above-mentioned observations indicate that differences in eNOS activity may exist between species. However, observed discrepancies could also be a consequence of differences in methodologies.

In our study, we found that nNOS immunoreactivity displayed a strong reaction in isthmus epithelium and moderate reaction in ampulla from early I luteal and follicular phases of the estrous cycle. However, nNOS was not detected by Western blot analysis. Lapointe, et al [9] reported that nNOS mRNA was highly expressed in the isthmus region of bovine oviduct throughout the estrous cycle, while protein level of nNOS was higher in the isthmus during luteal phase and in ampulla during the periovulatory period. In contrast, Ekerhovd, et al [26] reported that nNOS was not detected in human isthmus by immunohistochemistry and Western blot. The reason of this could be sensitivity of primary antibody and species differences between human and bovine.

The expression of nNOS, eNOS and iNOS mRNA was highly expressed in periovulatory period in isthmus and ampulla in bovine oviduct [9]. In our study, we did not detect iNOS-ir by immunohistochemistry while iNOS mRNA was detectable by RT-PCR. It has been reported that iNOS is present in epithelium, muscular layer and lamina propria in bovine oviduct [9], whereas in the human oviduct it is present in muscular layer, epithelium, vascular endothelium and connective tissue [43] and only in epithelial layer in rat oviduct [42]. In the porcine oviduct iNOS positive reaction was restricted to the endothelium of lymph and blood vessels but was not detected by Western blot analysis [28]. Besides, the specificity of primary antibody of iNOS in our study, the differences with the colocalization of iNOS can be associated with species differences and poor antigenicity of primary antibody. All the above-mentioned findings clearly indicate the presence of NOS in the bovine oviduct. Moreover, the activity of this enzyme is increased mainly during periovulatory period, the time which is very important for transport of gametes and early embryo development.

In agreement with our RT-PCR and/or Western blot analysis for either eNOS and nNOS, the immunohistochemistry revealed that the expression in epithelium was higher in isthmus than ampulla, mostly in early I luteal and follicular phases of the estrous cycle. According to our current knowledge, the tubal epithelium consists of monostratified epithelium with cylinder, basal, secretory and ciliary cells. Secretory cells are the major cell type of the isthmic segment, while ciliated cells dominate in the ampulla [44]. In mammals, the respiratory tract, some parts of reproductive tract and ventricles of central nervous system have ciliary epithelial cells. It has been reported that the L-arginine-NO-cGMP pathway increased the ciliary beat frequency of ciliary cells in respiratory system [45,46]. Furthermore, Zhan, et al [47] demonstrated that L-arginine increased NO via NOS in cultured ciliary epithelial cells and eNOS staining was evident in ciliary epithelium, whereas non-ciliary epithelium was not stained, thus revealing the role of NO in ciliary motility [47]. In the present study, we showed intensive eNOS immunoreactivity in luminal surface of epithelium in both isthmus and ampulla during early I luteal and follicular phases of the estrous cycle. Therefore, we suggest that NO derived from eNOS has a role for arrangement of ciliary activity before and after ovum leaves the oviduct. By contrast, nNOS immunoreactivity was observed from luminal surface of epithelium to lamina propria. We have shown more intensive nNOS immunoreactivity in isthmus than ampulla in early I luteal and follicular phase in our study. This may explain that secretory cells in isthmus have more affinity for NO activity derived from nNOS than in ampulla and therefore, NO plays a role in the secretory activity. Furthermore, it is consistent with the previous observation of Siemieniuch, et al [7] that NO, when stimulated by TNF-α, seems to play a role in the secretory function in the oviduct during early I luteal phase and it is suggested that intratubal formation for ovum and spermatozoid transport, can be associated with NO.

Besides the ciliary and secretory activity of oviduct, contraction and relaxation of smooth muscle cells in the oviduct are the major factors involved in promoting and regulating the transport of gametes and embryo(s) [48]. Therefore, the present study focused on the effect of endogenous and exogenous NO on contractile activity of bovine oviduct throughout the estrous cycle. It has been observed that L-arginine (an endogenous NO donor) inhibits *in vitro* smooth muscle contractility [13]. Moreover, L-arginine has been reported to decrease the contractile tension in human oviduct [43]. Enzymatically synthesized NO following L-arginine administration [49] activates smooth muscle sGC and increases cGMP level and following the activation of protein kinase, smooth muscle relaxation occurs [50]. Therefore, if an endogenous NO system is involved in oviduct contractility, smooth muscle relaxation would be expected after *in vitro* L-arginine treatment. Nevertheless, in this work, L-arginine did not alter or increased contractile tension which was an unexpected result. It has been reported that L-arginine increased the contractile tension in human oviduct. However, an initial and transient increase in muscle tone was followed by reduced contractility [43]. The reduced contractility might depend on the resting period of the tissue followed by the contraction period. However, in the present study the increase was not transient and was not preceded by a decrease in contractility. Therefore, observed differences can occur due to either using oviducts of different species or dose ranges of L-arginine. Moreover, it has been reported that NO increases the contractile activity when motility is low and increased prostaglandin synthesis mediates this effect in rat myometrium [51]. It suggests that this mechanism may also exist in the oviduct and therefore increases contractile tension. Moreover, the present immunohistochemistry studies revealed that there was no eNOS-ir in smooth muscle cells of isthmus and ampulla, whereas nNOS immunoreactivity was evident in nerve fibers in serosa and lamina propria. These results indicate that NO may not have a direct relaxant effect on oviduct smooth muscle and that L-arginine increases the contractile tension via a NO independent pathway, which partially mediates TNF-α action and modulates prostaglandin secretion [5,7]. Siemieniuch, et al [7] demonstrated that TNF-α and NO increased $PGF_{2\alpha}$ secretion in the ampulla and PGE_2 secretion in the isthmus. Moreover, it was shown that $PGF_{2\alpha}$ stimulated contractility in both ampulla and isthmus, while PGE_2 was a potent inhibitor of oviduct motility. These results show possible linkage between many factors that may be important in regulation of oviduct contractions.

It has been reported that NO production is prevented by inhibition of NOS enzyme activity with competitive analogues of L-arginine [13,52]. Therefore, in our study the most effective dose was applied together with L-NAME to evaluate NOS activity. It was observed that L-arginine administered after L-NAME did not alter or increase contractile tension as compared to the spontaneous contraction. As L-NAME is an NOS inhibitor, it suggests that the contractility of oviduct smooth muscle caused by L-arginine is not mediated by nitric oxide. By contrast, these results may be explained by a prevailing endogenous saturation of the L-arginine for NO synthesis [53] or low NOS activity in the examined tissues.

The synthesis of NO was abolished not only following inhibition of NOS activity but also because of inhibition of sGC by MB [13,33]. In the present study, L-arginine administered after MB, decreased the contractile tension in both longitudinal and circular isthmus from early II luteal phase as compared to spontaneous contraction. This observation suggests that contraction occurs via an sGC-dependent manner. By contrast, the contractile tension was not altered or increased during the early I luteal and follicular phase. It has been reported that before ovum leaves the oviduct, contractile agents, such as endothelin-1 [23] and $PGF_{2\alpha}$ [29] may affect motility in the oviduct.

The SNP is an exogenous NO donor, which blocks spontaneous uterine contractility [13] and causes vasodilatation in uterine and ovarian arteries [38]. The present study shows that SNP inhibits spontaneous oviduct contractions at early I luteal and follicular phases but increases contractile tension in longitudinal strips of ampulla from late luteal phase. Yallampalli, et al [13] reported that there was a lag period in rat uterus before the onset of relaxation by SNP, indicating a possible requirement of metabolic activation. Moreover, it was assumed that the uterus required relatively higher doses of SNP compared to vascular tissue, indicating that this conversion system might be less active in the uterus [13]. It may suggest that such conversion system may be also less active in the oviduct during the late luteal phase. Word and Cornwell [18] stated that SNP doses lower than 10^{-4} M were not effective at inhibiting oxytocin-stimulated uterus contractility, and higher doses cause irreversible inhibition due to toxicity. Moreover, NO has been shown to activate cyclooxygenase and to stimulate uterine contractility in rat [54]

that confirmed the important role of PGs in the process of contraction.

In conclusion, our results showed different expression patterns of eNOS, nNOS and iNOS in the bovine oviduct. It appears that NO does not affect spontaneous oviduct contractility directly via NOS-NO pathway. However, endogenous NO system can be of physiological importance to the oviduct in secretory and ciliary functions involved in gametes and embryo(s) transportation.

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