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Effect of neostigmine on contractility of equine pre-ovulatory follicles: An *in vitro* study

M. Piccinno [a], A. Rizzo [a], G. Cariello [b], R.L. Sciorsci [a, *]

[a] Department of Emergency and Organ Transplantation, Section of Veterinary Medicine and Animal Production, University of Bari Aldo Moro, S.P. per Casamassima km. 3, 70010 Valenzano, BA, Italy
[b] ASL, Specialist Agreement, Siav, Putignano, Bari, Italy

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ABSTRACT

In this study, the Authors investigated the modulatory effect of three single doses (10^{-6}, 10^{-5}, and 10^{-4} M) of neostigmine on the spontaneous contractility of equine pre-ovulatory follicles in an isolated organ bath, to establish the relationship between this acetylcholinesterase inhibitor and ovulation, in the mare. The results indicate that neostigmine increases pre-ovulatory contractility in equine follicles at each dose, but in a different manner. Indeed, the rise in contractility induced by neostigmine at 10^{-6} M and 10^{-4} M was phasic, while at 10^{-5} M it was tonic. The data obtained indicate possible implications of these drugs in the pharmacological modulation of equine ovulation.

1. Introduction

In horse breeding, the main objective of the breeder and the veterinarian is to inseminate, at short notice, as many mares as possible, while achieving a high rate of pregnancy and reducing the possible cost of ownership [1]. This can be achieved through the use of substances such as *human chorionic gonadotropin, gonadotropin-releasing hormone agonist, kisspeptin* and *recombinant luteinizing hormone*, in order to induce ovulation, thus reducing the number of matings/artificial inseminations needed for conception [2,3].

Ovulation is determined by the *luteinizing hormone* (LH) peak, as well as by neurotransmitters, such as acetylcholine (ACh) [4]. In the ovary, this neurotransmitter acts as a signaling molecule for follicle growth; indeed, the inhibition of acetylcholinesterase induces a reduction in nutrition to the follicle [5] and, probably, a decline in ovarian contractility.

Previously, studies on the musculature of the mature Graafian follicle of the sow have pointed out the ability of a known anti-cholinesterase, physostigmine, to positively modulate their contractility [6]. Indeed, anticholinesterases act by binding to the enzyme acetylcholinesterase and inhibiting its activity [7]. This induces an accumulation of ACh around the cholinergic nerve terminals, thus increasing stimulation of the cholinergic receptor by activating the contractile mechanism [8]. Therefore, the aim of this study was to evaluate, *in vitro*, the contractility effect induced by another acetylcholinesterase inhibitor (neostigmine) on equine pre-ovulatory follicles. This substance was chosen, as it is already used, in horse and cow, to restore and coordinate gastrointestinal motility, in the event of gastrointestinal disorders [9].

2. Materials and methods

The study included 14 equine pre-ovulatory follicles identified at the slaughterhouse both *ante-mortem*, with a complete clinical examination, including rectal examination to evaluate follicle diameter (4–5 cm) and consistency (surface tension), and ultrasonography (SonoSite MicroMaxx Bothell WA, USA with 7.5 MHz linear probe; pre-ovulatory follicle measuring 4–5 cm with a large anechoic area) and by *post-mortem* examination [10]. From each mare, a blood sample from the jugular vein was also taken and transferred into pre-refrigerated vacutainer glass tubes. After transporting these samples to the laboratory, they were centrifuged at 1620 × g for 10 minutes at 4 °C. The sera were subsequently frozen at −20 °C for later analysis of progesterone (P_4) using a competitive immunoenzymatic colorimetric method (Progesterone EIA WELL; Radim SpA, Pomezia [Roma], Italy). The detection limit of

* Corresponding author.
E-mail address: raffaeleluigi.sciorsci@uniba.it (R.L. Sciorsci).

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the assay was 0.05 ng/mL. The intra-assay and interassay precisions had coefficients of variation of 2.9% and 4.8%, respectively. P_4 cutoff values for the follicular phase was set at 1 ng/mL [10].

After slaughter, ovaries containing a pre-ovulatory follicle were collected in about 20 ± 10 min. The ovary was excised and immediately placed in a flask containing pre-refrigerated Krebs solution (NaCl 113 mM, KCl 4.8 mM, $CaCl_2$ $2H_2O$ 2.2 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, glucose 5.5 mM, sodium-ascorbate 5.5 mM), which was prepared daily. The flask was then immediately transported (in 15 ± 5 min) to the laboratory in an insulated box.

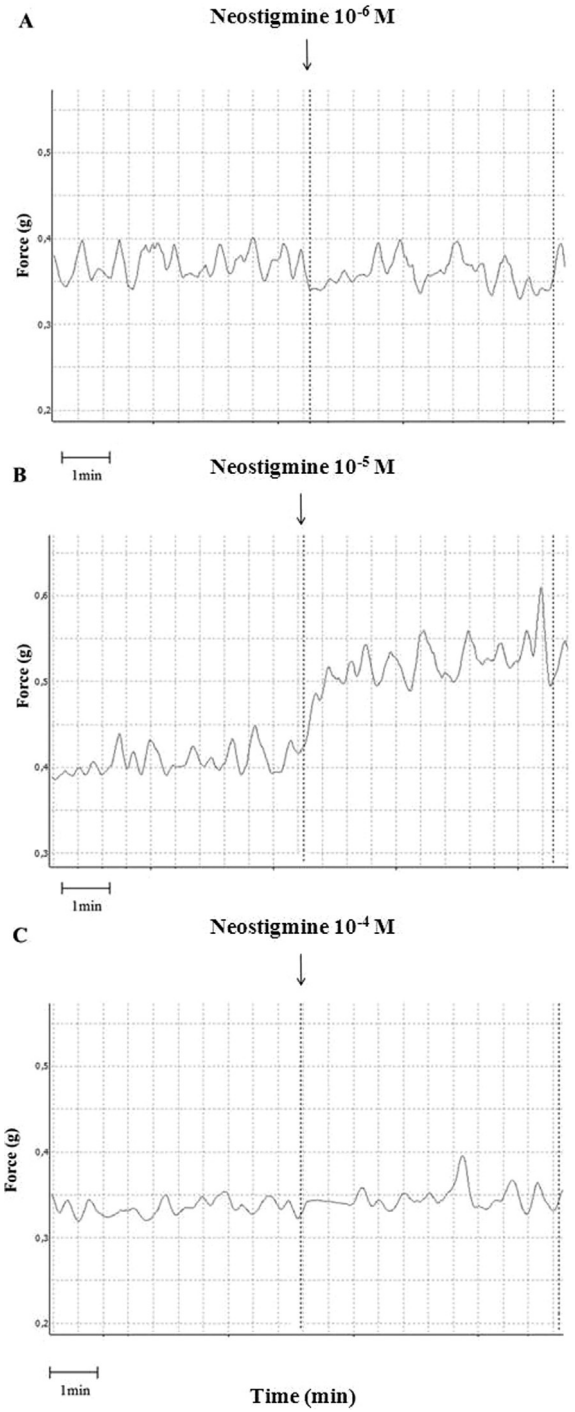

Fig. 1. Representative tracing of the effects induced by the three single doses of neostigmine (10^{-6} M, 10^{-5} M and 10^{-4} M, respectively A, B and C) on contractility of equine pre-ovulatory follicles. The force (y-axis) is expressed in grams.

Table 1
Effect of three single neostigmine concentrations on the contraction amplitude of equine pre-ovulatory follicles. Data are expressed as the mean ± SEM. The table also shows, in bold, the percentage basal value. In column: A,B: $p < 0.01$.

Neostigmine (amplitude-g)	Pre-ovulatory follicles	
	Mean ± SEM	Mean ± SEM
Basal	0.65 ± 0.69	+**3.74**^{B}
10^{-6} M	0.67 ± 0.23	
Basal	0.73 ± 0.23	+**16.39**^{A}
10^{-5} M	0.84 ± 0.23	
Basal	0.67 ± 0.23	+**3.33**^{B}
10^{-4} M	0.68 ± 0.22	

Full-thickness strips (5-mm in length and 3-mm in width) were cut from the wall of the pre-ovulatory follicle, as reported by [11]. The strips were immediately placed in a jacketed organ bath (mod. 4050 Ugo Basile, Milan, Italy) containing 10 ml of Krebs solution and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The pH was kept at 7.4, and the temperature was maintained at 37 °C. A silk thread was used to attach the follicular strips to a fixed hook and an isometric force displacement transducer (FORT25; AD Instruments, Castle Hill, NSW, Australia). Contractile activities were recorded using the PowerLab 4/35 acquisition software (AD Instruments). During the first 60 min, the strips were allowed to stabilize in the organ baths without applying tension. Subsequently, the strips were allowed to equilibrate under a constant tension of 1 g for about 30 min. After the equilibration period, the functionality of the strip throughout the experiment was evaluated by two/three doses of carbachol (10^{-5} M), as described by [12].

The experimental protocol included those strips that were exposed to single concentrations of neostigmine (10^{-6}, 10^{-5}, and 10^{-4} M) dissolved in distilled water.

Because there are no studies in this regard, the choice of concentrations used *in vitro* was made starting from the lowest concentration (10^{-6} M), and then ascending to reach *in vitro* concentrations that are closest to the *in vivo* concentration used in equine species (0.5, 1, or 2 μg/kg) [13]. The neostigmine was left in the bath for 10 minutes and then removed by washing. Finally, to evaluate the functionality of the strip throughout the experiment, the registration period was followed by a dose of carbachol (10^{-5} M). The response of the strip had to be repeatable (within 20%) compared to that for the previous administration [12].

Amplitude, frequency and area under the curve (AUC) of contractions for each strip were determined by analyzing the sequence corresponding to the last 10 min of contraction, from the recording section. The time interval over which such determinations were made was identified after observing the effect of neostigmine on basal contractility. For each administration, the percentage index of increase or decrease from baseline (basal *vs* neostigmine 10^{-6} M, basal *vs* neostigmine 10^{-5} M and basal *vs* neostigmine 10^{-4} M) was evaluated using the following formula: $(T_{\text{Second value}} - T_{\text{first value}}/T_{\text{first value}}) \times 100$ [12,14].

2.1. Statistical analysis

For motility studies, all amplitude, frequency and AUC values were expressed as mean ± SEM and were subjected to statistical analysis by SPSS® Statistics 19 (IBM®, NY).

The effects of single concentrations of neostigmine (10^{-6}, 10^{-5}, and 10^{-4} M) on basal contractility were analyzed with Student's *t* test. Intergroup variations as well as the percentage increases or decreases in amplitude and frequency of contractions and AUC