Spinal Antinociceptive Effect of Neostigmine in Donkeys

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Abstract: Recent research on the pharmacology of nociception has shown the involvement of cholinergic transmission and its safe use at the spinal level to be among the numerous candidates for spinal pain modulation. Intrathecal (IT) injection of neostigmine represents a cholinergic mechanism of spinal analgesia. It inhibits the breakdown of the endogenous neurotransmitter, acetylcholine that has been shown to cause analgesia. This study was carried out in well-defined animal model (donkeys) over a range of doses to provide information on the analgesic effect as well as the adverse reactions of neostigmine 0.25% by the spinal route. Neostigmine methyl sulfate 0.25% was used in different doses to induce lumbosacral intrathecal (IT) analgesia in 32 non-medicated clinically healthy donkeys of both sexes. The animals were classified randomly into 2 groups, a main or neostigmine group (Group I, of 20 donkeys) and a control group (Group II, of 12 donkeys). Each group was further subdivided into 4 equal subgroups. IT injections of 1 ml (2.5 µg) and 2 ml (5 µg) neostigmine in subgroups Ia & Ib respectively did not alter sensory perception in these donkeys. IT injections of 3 ml (7.5 µg) and 4 ml (10 µg) resulted in analgesia that was started after 12.60 + 5.85 min and 10.70 + 3.04 min from the end of injections and lasted for 33.4 + 2.40 min and 52 + 8.63 min. in subgroups Ic & Id respectively. The control group was injected by normal saline intrathecally. Analgesia involved the triangular flank regions, hind quarters, buttocks, tail, perineum, vulva or scrotum, lateral aspect of the thigh and area over the last 3rd – 7th ribs in different animals. Recumbancy occurred in 3 donkeys in subgroup Id (4 ml). Prolapse of penis was observed in 2 and 3 donkeys in subgroup Ic and Id respectively. There were symptoms of frequent diarrhea, lacrimation, increased salivation, nasal discharge as well as tremors in different animals. There was significant increase in body temperature in all animals. Pulse rate showed significant increase in all subgroups except for subgroup Id, as it exerted significant decrease in pulse rate. IT neostigmine produced significant increase in respiratory rate in all subgroups Ia, Ib, Ic and Id. The changes of these parameters (body temperature, pulse rate, respiratory rate) were of no clinical importance. IT neostigmine 0.25% in doses of 7.5 µg and 10 µg gives good analgesia that could be satisfactory for surgical operations caudal to the last rib in donkeys.
Keywords: Neostigmine, Intrathecal, Analgesia, Donkey.

INTRODUCTION

The effective management of acute and chronic severe pain remains one of the major challenges in both human and veterinary medicine. Spinal analgesia is a useful anesthetic technique in donkeys as well as it is easily performed and costs less than most methods of general anesthesia. Recent research on the pharmacology of

nociception has shown the involvement of cholinergic transmission and its safe use at the spinal level to be among the numerous candidates for spinal pain modulation[1, 2, 3]. Intrathecal (IT) injection of neostigmine represents a cholinergic mechanism of spinal analgesia. It inhibits the breakdown of the endogenous neurotransmitter, acetylcholine that has been shown to cause analgesia[4, 5].

The aim of the present work is to assess the analgesic effect of IT neostigmine in donkeys and the effects of this agent on vital parameters of body including body temperature, pulse rate and respiratory rate.

MATERIALS AND METHODS

Animals: A 32 non-medicated clinically healthy donkeys of both sexes (17 males, 15 females), weighing from 80 - 120 kg and aged between 4 - 9 years were used in this study. The animals were divided randomly into two groups according to the drugs used. A main or neostigmine group (Group I, of 20 donkeys) and a control group or normal saline group (Group II, of 12 donkeys). Each group was further subdivided into 4 equal subgroups: a, b, c and d. The animals were injected with 1 ml (25 µg), 2 ml (50 µg), 3 ml (75 µg) and   4 ml (100 µg) neostigmine 0.25 % sterile solution (Epistigmine, 5 ml sterile vial, 2.5 mg / ml, EPICO) intrathecally in subgroups Ia, Ib, Ic and Id respectively in the main group. The animals in the control group were injected with 1 ml, 2 ml, 3 ml and 4 ml normal saline (0.9 % sodium chloride) sterile solution intrathecally in subgroups IIa, IIb, IIc and IId respectively.

Intrathecal injection was carried out under complete aseptic precautions. With the thumb and middle fingers, sacral tubersities were palpated and with the index finger the depression leading to the lumbosacral foramen was felt. The tip of the spinal needle was directed about 5 degrees cranially from perpendicular plane to the spinal cord. The needle was slowly advanced with the bevel point directed cranially until the subarachnoid space was identified by free flow of clear cerebrospinal fluid from the needle hub after removal of the stylet or aspirating it with a sterile syring. The determined dose of neostigmine or normal saline was slowly injected (1 ml / 1 min) at body temperature. The stylet of the needle was replaced in its position again. While withdrawal of the needle, the skin was pressed by a piece of cotton soaked in tincture of iodine 3 % to control bleeding and infection. Animals thereafter were put under observation and the efficiency of analgesia was determined by observing reflex movement in response to painful stimuli elicited by pin pricks in the skin and the deeper tissues[6.] Onset and duration of the analgesic effect, the desensitized area, limb status and adverse effects were recorded in every case. Rectal temperature, Pulse rate and respiratory rate were recorded before injection and at 5, 15, 25, 40 and 60 minutes post-injection.

Statistical analysis: The experimental design was the complete randomized design (CRD). The data were statistically analyzed using GLM procedures of SAS[7.] Dunnett's test was used for comparisons of all treatments (body temperature, pulse rate and

respiratory rate) against a control. Comparisons significant at 0.05 levels were indicated by (***).

RESULTS

Analgesic effect of IT injection of neostigmine 0.25% in donkeys:

IT injection of neostigmine 0.25% in doses of 1 ml (25 µg) and 2 ml (50 µg) did not alter sensory perception in donkeys in subgroup Ia and Ib respectively. IT injection of neostigmine 0.25% in a doses of 3 ml (7.5 µg) and 4 ml (10 µg) and 4 ml (10 mg) in subgroup Ic & Id respectively, resulted in analgesia that was started after 12.60 ± 5.85 min and 10.70 ± 3.04 min from the end of injection and lasted for 33.4 ± 2.40 min and 52 ± 8.63 min. respectively, table (1), fig.(1).

The desensitized area:

Uniform bilateral analgesia started in the triangular flank regions in animals of both groups, Ic and Id. Analgesia extended to involved the hind quarters, buttocks, tail, perineum, vulva or scrotum 10 – 15 min and 20 – 25 min later in subgroup Ic and Id respectively. The analgesia covered area over the last 3 - 5 ribs (in 4 donkeys) and extended to cover the area over the last 7 ribs (in one donkey) in subgroup Ic. The desensitized area extended to cover the area over the last $6^{th} – 7^{th}$ ribs and lateral aspect of the thigh in subgroup Id.

Limb status:

Donkeys remained in standing position in both subgroups Ia and Ib. Three donkeys showed weakness and incoordination in pelvic limbs but without recumbancy in subgroub Ic. Three donkeys in subgroup Id showed tremors and incoordination in the pelvic limbs that was followed by recumbency 35 – 40 min post injection and lasted for 15 - 20 min.

Adverse effects:

Signs of irritation and irritability noticed in all animals following IT injection of neostigmine.

All animals had frequent passage of the fecal matter (5 – 7 times) that had changed from its normal consistency to become profuse watery diarrhea at the $3^{rd} – 4^{th}$ passage throughout the observation time (60 min.). profuse salivation, lacrimation, watery nasal discharges as well as dilatation in the anal sphincter, contractions in the vulva as well as penis prolapse was noticed in some donkeys in subgroups Ic and Id

Effect of IT neostigmine 0.25% and on body temperature in donkeys:

The base line value of body temperature was $37.08 \pm 0.25\,°C$, $37.32 \pm 0.18\,°C$, $37.34 \pm 0.21\,°C$ and $37.30 \pm 0.21\,°C$ in subgroups Ia, Ib, Ic and Id respectively. There was a non-significant increase in body temperature in all main subgroups in the first 15 min. post-injection except for subgroup Ib as it recorded significant increase ($37.56 \pm 0.15\,°C$). All animals in subgroups recorded significant increase in body temperature 25 min. post-injection and till the end time of observation (60 min.). Table (2), fig. (2).

Effect of IT injection of neostigmine 0.25% on pulse rate in donkeys:

The base line value of pulse rate was 33.60 ± 4.72, 37.40 ± 4.67, 35.40 ± 4.34 and 40.80 ± 3.19 beat/min. in subgroups Ia, Ib, Ic and Id respectively. There was a significant increase in pulse rate in the main subgroups Ia, Ib and Ic throughout the observation

time. Animals in subgroup Id recorded significant decrease in pulse rate, its lowest value (28.40 $\pm$ 8.17 beat/min) was at the end of the experiment (60 min). Table (3), fig. (3).

Effect of IT injection of neostigmine 0.25% on respiratory rate in donkeys:

The control value of respiratory rate was 10.60 $\pm$ 1.52, 10.80 $\pm$ 1.64, 9.80 $\pm$ 0.84 and 11.00 $\pm$ 1.58 breath/min. in all main subgroups. There was an increase in respiratory rate in all animals through the entire time of the experiment (60 min.). The increase in respiratory rate was significant at 15 & 25 min. post-injection (12.00 $\pm$ 1.73 breath/min. & 12.00 $\pm$ 1.71 breath/min. respectively) in subgroup Ia where both subgroups Ib & Ic recorded significant increase only at the first 5 min. post-injection. There was significant increase in respiratory rate throughout the observation time in subgroup Id. table (4), fig.(4).

Table. Analgesic effect of IT injection of neostigmine in donkeys

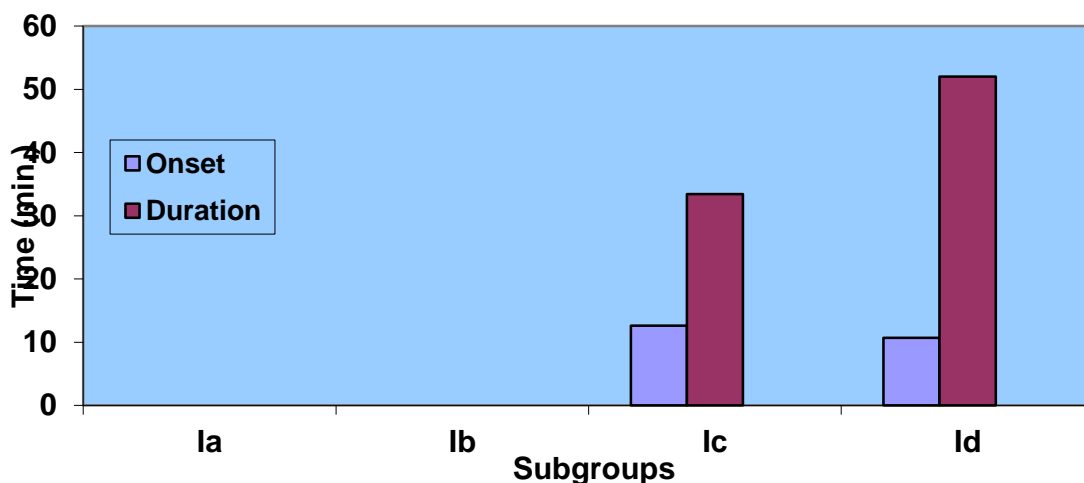| Main subgroups	Onset (min.)			Duration (min.)		
	Min.	**Max.**	**Mean $\pm$ SD**	**Min.**	**Max.**	Mean $\pm$ SD
Ia	---	---	---	---	---	**---**
Ib	---	---	---	---	---	**---**
Ic	4	20	12.60 $\pm$ 15.24	30	36	**33.4 $\pm$ 2.40**
Id	**6**	**12**	**10.70 $\pm$ 3.04**	**38**	**60**	**52 $\pm$ 8.63**

Fig. 1. Analgesic effect of IT injection of neostigmine in donkeys Conclusions

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Table 2. Effect of IT neostigmine 0.25% and on body temperature in donkeys

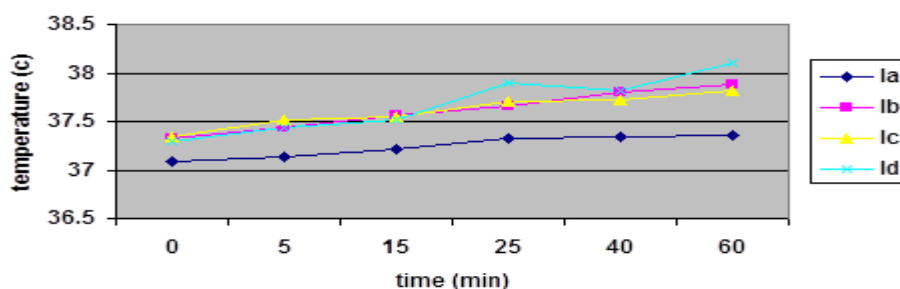| Time (Min.)	Subgrouos											
	Ia			**Ib**			**Ic**			Id		
	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD
0 (control)	36.90	37.50	37.08 ± 0.25	37.10	37.50	37.32 ± 0.18	37.10	37.60	37.34 ± 0.21	37.00	37.50	**37.30 ± 0.21**
5 (post Inj.)	36.90	37.50	37.14 ± 0.23 n.s	37.20	37.50	37.44 ± 0.13 n. s	37.30	37.70	37.52 ± 0.15 n.s	37.20	37.70	**37.44 ± 0.21 n.s**
15 (post Inj.)	37.00	37.50	37.22 ± 0.19 n.s	37.40	37.80	37.56 ± 0.15 ***	37.40	37.70	37.54 ± 0.11 n.s	37.30	37.90	**37.52 ± 0.27 n.s**
25 (post Inj.)	37.20	37.60	37.32 ± 0.16 ***	37.50	37.80	37.66 ± 0.15 ***	37.60	37.90	37.70 ± 0.12 ***	37.50	38.80	**37.90 ± 0.53 ***
40 (post Inj.)	37.10	37.80	37.34 ± 0.27 ***	37.70	38.00	37.80 ± 0.12 ***	37.50	37.90	37.72 ± 0.15 ***	37.70	38.00	**37.82 ± 0.11 ***
60	**37.00**	**38.00**	**37.36±0.38 ***	**37.60**	**38.00**	**37.88±0.16 ***	**37.30**	**38.00**	**37.81 ± 0.29 ***	**37.80**	**38.80**	**38.10 ± 0.40 ***

Fig. 2 Effect of IT neostigmine 0.25% and on body temperature in subgroups Ia, Ib, Ic and Id

Table 3. Effect of IT neostigmine 0.25% and on pulse rate in subgroups donkeys

Time (min).	Subgrouos											
	Ia			Ib			Ic			Id		
	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD
0	29	40	33.60+4.72	30	42	37.40 + 4.67	30	40	35.40 + 4.34	37	45	**40.80 + 3.19**
5	35	45	39.40+5.18 ***	33	44	39.60 + 4.39 n.s	24	55	40.80 + 11.26 n.s	23	36	**32.00 + 5.34 *****
15	35	50	42.40+6.73 ***	40	50	44.80 + 4.55 ***	38	50	45.40 + 4.72 n.s	29	36	**31.40 + 2.79 *****
25	38	56	43.80+7.29 ***	39	53	47.40 + 5.50 ***	40	56	48.40 + 6.02 ***	28	37	**30.80 + 3.70 *****
40	38	50	42.00+4.69 ***	39	48	43.00 + 3.67 n.s	47	58	53.40 + 5.41 ***	22	38	**28.60 + 6.19 *****
60	**35**	**49**	**41.00+5.15 *****	**39**	**46**	**42.60 +3.05 n.s**	**48**	**58**	**51.60+3.85 *****	**20**	**39**	**28.40 + 8.17 *****

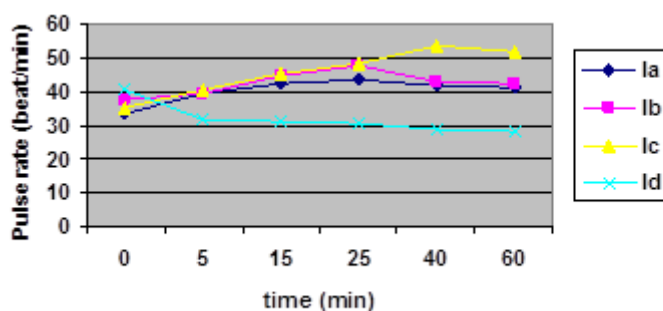**Figure 3.** Effect of IT neostigmine 0.25% and on pulse rate in subgroups donkey

Table 4. Effect of IT neostigmine 0.25% on respiratory rate in donkeys

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Time	Subgrouos											
(min).	Ia			Ib			Ic			Id		
	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD	Min.	Max.	Mean+SD
0	9	13	10.60 ± 1.52	9	13	10.80 ± 1.64	9	11	9.80 ± 0.84	9	13	**11.00 ± 1.58**
5	10	14	11.80 ± 1.48 n.s	12	17	14.80 ± 1.79 ***	9	21	14.00 ± 4.58 ***	16	23	**19.00 ± 2.74 *****
15	11	15	12 ± 1.73 ***	10	15	12.00 ± 2.00 n.s	10	14	11.80 ± 1.48 n.s	15	18	**16.20 ± 1.30 *****
25	11	13	12 ± 0.71 ***	10	13	12.00 ± 1.41 n.s	11	14	12.60 ± 1.14 n.s	13	17	**15.20 ± 1.48 *****
40	10	13	11.40 ± 1.34 n.s	10	13	11.60 ± 1.52 n.s	12	14	13.00 ± 0.71 n.s	14	16	**14.80 ± 1.10 *****
60	**10**	**12**	**10.40 ± 0.89 n.s**	**10**	**16**	**12.20±2.39 n.s**	**11**	**15**	**12.40±1.52 n.s**	**13**	**16**	14.20 ± 1.30 ***

Figure 4. Effect of IT neostigmine 0.25% and on respiratory rate in donkeys
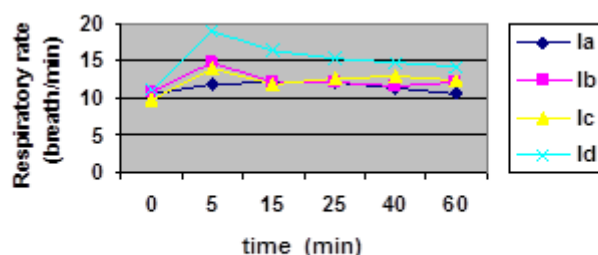
DISCUSSION

A number of anti-cholinesterase agents are used for the relief of various abnormalities in cholinergic transmission, such as in the maintenance of muscle strength in patients with myasthenia gravis, in the treatment of glaucoma, and in the control of certain types of cardiac arrhythmias[8]. Spinally mediated analgesia can be produced by several mechanisms. Local anesthetics cause non - specific axonal

blockade. More specific blockade of nociception may be accomplished by injection of direct agonists, such as opioids or α₂-adrenergic agonists, which stimulate receptors involved in nociceptive processing in the spinal cord.

Unlike these direct acting agonists, neostigmine, a cholinergic mechanism of spinal analgesia, it inhibits the breakdown of an endogenous neurotransmitter, acetylcholine which has been shown to cause analgesia in animals and human[9, 10, 11, 12]. The cholinesterase inhibitory activity of neostigmine has been reported to be due to its ability to act as a competitive inhibitor that binds to acetyl cholinesterase enzyme. By serving as alternative substrate with a similar binding orientation as Ach, it gives rise to the carbamoylated enzyme. Sequestration of the enzyme in its carbamoylated form, thus precludes the enzyme – catalyzed hydrolysis of Ach for extended periods of time[13].

This study showed that ant nociceptive effects of neostigmine were produced in dose - related manner with an effective dose of 3 ml (7.5 mg) & 4 ml (10 mg) of IT neostigmine 0.25% in donkeys. This agreed with who reported that IT neostigmine resulted in adose – dependent analgesia[11, 14, 15, 2, 12, 16, 17]. On the other hand this was in disagreement with [18] who reported that these drugs (anticholinestrases) are relatively ineffective when noxious stimuli is high as well as who stated that IT neostigmine alone is unlikely to produce complete analgesia after surgery[19].

The analgesic effect of neostigmine is obtained by activating cholinergic mechanisms[20]. However the analgesic mechanisms and site of actions of cholinergic agents in the spinal cord are not fully cleared[21]. The activation of muscarinic sites in the lumbar spinal cord may result in either inhibition of the activity of nociceptive dorsal horn neurons or reduced transmitter release from small diameter nociceptive primary afferents in the spinal cord dorsal horn, or both[10]. Neostigmine in sufficient dosage will produce an acetylcholine - induced block. The preserved Ach will accumulate at the muscle end -plate and produce a depolarization block. The end - plate potentials are prolonged and during high frequency nerve stimuli, they summate and block the neuromuscular function by a persistent depolarization of the postsynaptic membrane [22]. The analgesic effect may be explained by a muscarinic presynaptic inhibition of glutamatergic afferents[23, 24]. Lamina II neurons receive both glutamatergic excitatory and GABAergic inhibitory inputs. Ach can inhibit glutmate release through presynaptic muscarinic receptors located on the glutamatergic terminals. Also, Ach can activate muscarinic receptors on the GABAergic terminals to evok synaptic GABA release[21].

In the present study we used the intrathecal route of injection, because the site of action is spinal. Concerning to the onset latency for the effect of neostiogmine (12.60 ± 5.85 and 10.70 ± 3.04 min. for 3 ml and 4 ml subgroups respectively), it could be explained by the lower lipophilicity of neostigmine resulting in longer time to its penetration to spinal cord tissues. This agreed with[25] as well as[5] reported that penetration of drugs from CSF to spinal sites of action affected by lipid solubility.

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This is the first study on the use of IT neostigmine in donkeys, so we used various graded doses of neostigmine in the present study. This agreed with[26, 13] who reported that initial clinical trials of any new agent are generally performed using an open label dose escalating design.

The dose of neostigmine used in subgroups Ia (1 ml) & Ib (2 ml) was likely too small to have any analgesic effect. Increase the dose in subgroups Ic (3 ml) & Id (4 ml) resulted in satisfactory and uniform bilateral analgesia and an increase in duration of action from (33.4 $\pm$ 2.40 min) in subgroup Ic to (52 $\pm$ 8.63 min) in subgroup Id. These results were similar to that recorded by[18, 16, 14, 20, 27, 28].

The desensitized area in both subgroups Ic (3 ml) & Id (4 ml) was nearly similar. It was involved the flank regions, hind quarters, buttocks, tail, perineum, vulva or scrotum, area over the last 3rd – 5th ribs (in subgroup Ic), but extended to cover the last 6th – 7th ribs as well as the lateral aspect of the thigh in all animals of subgroup Id (4 ml). This may be attributed to increasing the dose of neostigmine that resulted in an extension in the desensitized area. This was in agreement with[29].

There was increase in body temperature following IT injection of neostigmine in donkeys. It may be attributed to vasoconstriction of the cutaneous blood vessels secondary to excitatory action on the sympathetic outflow. This agreed with[26]. This also was similar to results that were recorded by[15] who noticed increase in body temperature in dogs after IT injection of neostigmine (4 mg / 4 ml), but these changes were not of clinical significant.

There was an increase in pulse rate with the small doses of neostigmine (1, 2 and 3 ml), and this could be attributed to local spinal action due to excitatory actions on preganglionic sympathetic neurons, while the decrease in pulse rate that was recorded with the maximum dose (4 ml) used in the present study, could be due to central distribution of the drug and its action at cholinergic sites in the brain, or due to its systemic absorption which are related to CSF neostigmine concentration and amplification of the action of vagally released Ach. This was with an agreement with [13]. On the other hand the findings in this investigation disagreed with[30, 31, 2] who reported that relatively large doses of spinally administered cholinergic agonists or cholinesterase inhibitors increase blood pressure and heart rate. Also this was disagreed with[15] reported a bradycardiac effect after IT injection of neostigmine in dogs.

The present study revealed that the IT neostigmine has stimulatory respiratory effect. It may be attributed to cephald distribution in CSF. This was in agreement with findings of[26, 15]. In support to this observation is the respiratory stimulant effects of cholinergic agonists or cholinesterase inhibitors near pontine centers of respiratory control[32]. Also this agreed with[33] who reported that either no effects of spinal neostigmine or mild stimulation from cephalad distribution in CSF and this is in marked contrast to other clinically used spinal analgesics, α_2-adrenergic agonists and opioids which can cause mild or severe respiratory depression respectively.

The spinal delivery of neostigmine resulted in a dose-dependant adverse reactions (motor weakness, diarrhea, increased salivation, lacrimation and nasal discharge). It is

believed that those actions reflect on enhancement of cholinergic activity secondary to the inhibition of cholinesterases. Similar findings were recorded by previous researches [8, 18, 2, 12, 15, 16, 34, 19, 13, 28].

Motor effects that were observed in animals receiving spinal neostigmine are thought to be due to direct actions on motor neurons outflow in the ventral horn. This was in agreement with Yaksh et al. [9, 26, 2].

The present study revealed stimulatory effects of neostigmine on GIT. It could be attributed to the potent muscarinic effects of neostigmine on GIT. This agreed with[35]. On the other hand, the neostigmine induced increase in gut motility might be beneficial and lead to a reduction in postoperative ileus[34].

Sexual responses noticed in the present study involve both sympathetic & parasympathetic influences. Valvular contractions may reflect spinal sympathetic stimulation. Penis prolapse (erection) may reflect spinal parasympathetic stimulation. Similar findings were recorded by[2]

Atropine was not used to modify these side effects since it might have interfered with the analgesic effect of neostigmine which is known to be mediated by spinal muscarinic receptors. This was in agreement with[11, 18, 34].

We concluded that IT neostigmine 0.25% in doses of 3 ml (7.5µg) & 4 ml (10 µg) gives good analgesia in donkeys that could be satisfactory for surgical operations caudal to the last rib.

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