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Neuroprotective effect of spiradoline and naloxone in focal cerebral ischemia: Promising behavioral and biochemical changes in Wistar rats

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ABSTRACT

Brain ischemia modulates endogenous opioids production and the expression of opioid receptors. The present investigations aim to determine the neuroprotective effect of Spiradoline and Naloxone, administered intraperitoneally, against carotid artery occlusion induced cerebral ischemia in adult male Wistar rats. The ischemic assessment was carried out by evaluation of behavioral (cognitive and sensorimotor), and biochemical (antioxidants, acetylcholinesterase, brain swelling and hemispheric water content) parameters. The groups included sham-operated control, vehicle control, spiradoline-treated and naloxone-treated rats. Carotid artery occlusion induced behavioral impairment as per 8-arm radial maze, elevated plus maze, sensorimotor deficits, free radical production and biochemical changes. The results revealed that spiradoline significantly ($p<0.05$) antagonized the cognitive and sensorimotor deficits, indicated by reduced working memory errors, increased transfer latency time, and neurologic scores on 24 h and day 7 after ischemia, while naloxone produced transient improvement. The drugs significantly ($p<0.05$) restored the levels of antioxidants, and acetylcholinesterase on day 7. The brain edema development was significantly antagonized by spiradoline while naloxone showed lesser improvement. The results implied that the opioid agonist spiradoline has better efficacy than opioid antagonist naloxone for brain stroke therapy at tested doses, and spiradoline may be used in future combination therapies as a neuroprotective drug.

Keywords: Spiradoline, naloxone, occlusion, behavior, antioxidants.

INTRODUCTION

Cerebral ischemia is a condition used to describe the loss of oxygen and nutrients for brain cells due to inadequate blood flow. The prevalence of brain stroke in India varies in different regions and, ranges from 50 to 300 per 100,000 population and about 20% of heart patients are susceptible to it (Ginsberg, 2007). Except alteplase, recombinant tissue-type plasminogen activator, currently no effective therapy exists for acute ischemic stroke. Among more than 1000 drugs which have been studied and found effective in animal stroke models, none has proved efficacious on the basis of a positive phase III trial (Gupta and Briyal, 2004). Previous findings have suggested that inactivation of the μ opioid receptor or activation of the δ or κ opioid receptor has a beneficial effect against brain injury (Chen *et al.,* 2008). Endogenous opioids have been demonstrated to play a role in the pathogenesis of ischemic brain damage and in the CNS (Araki *et al.*. 1993).

Pretreatment with kappa-opioid receptor (KOR) agonists significantly reduced global and focal ischemic damage in rodents. The precise mechanism of the neuroprotective action of KOR agonists is presently unknown. The κ-opioid receptor agonists have been shown to decrease brain edema and reduce brain injury (Zeylanov *et al.,* 2006). KOR agonists have especially received attention in terms of clinical drug treatments for stroke, because dynorphin-A-(1-13), an endogenous KOR agonist, prolonged survival time in a focal ischemia model in cats, and because dynorphin-A immunoreactivity is reduced in the hippocampus of ischemic gerbils (Genovese *et al.,* 1994). The selective KOR agonist, BRL 52537 provides significant neuroprotection when given as a pretreatment and at the onset of reperfusion in the rat middle cerebral artery occlusion model (Zhang *et al.,* 2003). Furthermore, reports have indicated that selective KOR agonists such as U-50,488H and GR89696 can produce neuroprotective effects in animal models of cerebral ischemia (Ukai *et al.,* 1993). In addition to the protective effect in animal models of spinal cord trauma, hemorrhagic and endotoxic shocks, opiate antagonists has been found to ameliorate the neurologic deficit due to ischemia. Several reports indicated that naloxone can exert a protective influence against cerebral ischemia consequences in gerbils, cats (Chen *et al.,* 2001). Opioid receptor antagonists have been shown to be effective in reducing injury due to both CNS trauma and ischemia, but their mechanism of action remains unclear. It has been observed that naloxone administration reverses the neurologic and metabolic deficits in animals following ischemic/reperfusion injury (Kuo *et al.,* 2000). Naloxone improved cerebral blood flow (CBF) and reduced seizure activity. Various studies have also showed that in experimental models of brain ischemia opioid antagonists were quite effective in reducing the deficits.[9] Relevant studies have also identified the opioid receptor-independent, anti-inflammatory actions of naloxone (Liu and Hong, 2003). In consideration of the alterations in opioidergic receptors, the present studies are aimed to investigate whether Spiradoline (U-62,066), a selective KOR agonist, and Naloxone, a non-selective antagonist, can provide a neuroprotective effect against focal cerebral ischemia in bilateral carotid artery occlusion model followed by reperfusion assessing neurobehavioral, and biochemical changes in male Wistar rats. The study also aims to find a possible mechanism of action of these drugs against cerebral ischemic injury.

MATERIALS AND METHODS

Chemicals

Spiradoline and naloxone hydrochloride were purchased from Sigma, USA. All other chemicals, reagents and solvents used were of analytical grade.

Animals

Healthy, adult, male Wistar rats weighing 200-250 g were used in the study. The animals were acclimatized to the laboratory conditions prior to start of the experiment to adapt to the conditions of light-dark cycle (12:12 h), relative humidity and temperature. The animals were housed in spacious cages; food and water *ad libitum* were made available throughout the experimental period. The experiments were done according to CPCSEA guidelines with the prior approval of Institutional Animal Ethics Committee (JSSCP/IAEC/PH.COLOGY/03/2010-11).

Induction of focal cerebral ischemia

Briefly, under ketamine-xylazine combination anesthesia, a midline incision in neck was given to rats. For the study, rat brains were made ischemic by occluding common carotid artery with clamps for 15 min along with ligation by means of a monofilament thread which was tied around the vessel. Body temperature was maintained with proper heating conditions (Iwasaki *et al.,* 1989). Skin incision was sutured with stitches. Sham-operated controls underwent the same surgical manipulation without the occlusion of arteries.

Allocation and treatment

Thirty six Wistar rats were divided into four groups with nine animals in each group:

Group 1: served as sham-operated control and received no injection,

Group 2: animals subjected to carotid artery occlusion and received normal saline i.p. as treatment,

Group 3: animals received spiradoline (10 mg/kg, i.p.) 30 min before and 2 h after occlusion,

Group 4: animals received naloxone (5 mg/kg, i.p.) at 30 min, 60 min and 120 min after occlusion.

Cognitive (spatial memory) evaluation

Elevated plus maze test

Elevated plus maze is an established task to evaluate spatial memory functions in rats (Ukai *et al.,* 1993). The plus maze was made of plywood and consisted of two open arms (25×8 cm) and two enclosed arms (25×8×20 cm). The arms extended from a central platform (8×8 cm). The maze was 50 cm above the floor. A rat was placed at the end of one open arm facing away from the central platform, and the time it took for the rat to move from the open arm to either of the enclosed arms (transfer latency) was recorded. If the rat did not enter the enclosed arm within 90 s, it was pushed gently on the back into the enclosed arm and a transfer latency of 90 s was scored. After a 10 s gap, the rat was placed in the central square and the number of entries into both open and enclosed arms were recorded for a period of 5 min. Trials were continued for 1 week before carrying out the experimental studies.

8-arm radial maze test

The 8-arm radial maze is a frequently used behavioral model of spatial learning and memory (Iwasaki *et al.,* 2006). Eight arms (55×10 cm) radiate outward from alternate side of a central polygonal platform (71cm across, 16 sides). At the end of each arm, a space provides access to a food cup containing food pellets, serving as a refuge for the rat (the goal box). Of the 8 arms, only one contained the true refuge. Rails (2.5cm high) bordered each arm to prevent the animal from falling. Several extra-maze cues

(e.g. posters on the walls) were available in the room. The animals were trained so that they would become habituated to the apparatus. A 10 min period of habituation was repeated two times a day, at intervals of 2 h. In each training session, the animal was placed directly in the center of the maze and allowed to move freely. The trial continued until the animal had either entered all 8 arms or 10 min had elapsed. When the rat found and entered the goal box, it was allowed to remain in the goal box for 1 min. Animals that proceeded through the maze using non-spatial strategies, i.e., repeatedly choosing the arm adjacent to or the arm that was three arms away from the one currently visited, were excluded because they were considered to not have acquired spatial memory. During the acquisition and retention phases, behavioral performance was measured by the numbers of working memory errors and reference memory errors. A reference memory error was registered when the rat visited an arm containing a false goal box (open-ended box) for the first time within a trial. However, if the rat returned to an arm which had been previously visited during that trial, a working memory error was recorded. An arm was considered visited when the rat entered half way down the arm.

Sensorimotor evaluation

Neurological scores were measured 24 h and 7 days after cerebral ischemia. Scoring was done with a modified Garcia's neurological scoring system (Garcia *et al.,* 1995). The scores assigned to each rat at the end of the examination is the sum of all the test scores. The minimum neurological score is three and the maximum is 18. It consisted of six tests:

Spontaneous activity: The animal was observed for 5 min in its normal environment (cage).

Symmetry in the movement of four limbs: The rat was held in the air by the tail to observe symmetry in the movement of the four limbs.

Forepaw outstretching: The rat was brought up to the edge of the table and made to walk on forelimbs while being held by the tail. Symmetry in the outstretching of both forelimbs is observed while the rat reaches the table and the hindlimbs are kept in the air.

Climbing: The rat was placed on the wall of a wire cage. Normally the rat uses all four limbs to climb up the wall. When the rat was removed from the wire cage by pulling it off by the tail, the strength of attachment was noted.

Body proprioception: The rat was touched with a blunt stick on each side of the body, and the reaction to the stimulus was observed.

Response to vibrissae touch: A blunt stick was brushed against the vibrissae on each side; the stick was moved toward the whiskers from the rear of the animal.

Preparation of brain homogenate

Animals were sacrificed by decapitation, immediately after behavioral assessment for the biochemical analysis. The brains were dissected out, cleaned and weighed. The brain homogenate was prepared in phosphate buffered saline (pH 7.4). The homogenate was centrifuged (Remi, R-8C, India) and aliquots of supernatant was separated and used for biochemical estimation.

Superoxide dismutase

Assay mixture containing 0.1ml of supernatant, 1.2ml of sodium pyrophosphate buffer (pH 8.3), 0.1ml of phenazine methosulphate, 0.3ml of nitroblue tetrazolium and 0.2ml of NADH was incubated at 30ºC for 90s, the reaction was stopped by addition of 0.1ml of glacial acetic acid. The reaction mixture was stirred with 4.0ml of *n*-butanol, colour (blue) intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as enzyme concentration required to inhibit the absorbance at 560 nm of chromogen production by 50% in 1 min under assay conditions and expressed as specific activity in units/mg of protein (Kakkar *et al.*, 1984).

Catalase

Catalase measurement was carried out by the ability of catalase enzyme to oxidize hydrogen peroxide (H_2O_2). 2.25ml of potassium phosphate buffer (65mM, pH 7.8) and 100µl of the brain homogenate were incubated at 25ºC for 30 min. A 650µl H_2O_2 (7.5mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240nm for 2–3 min and the results were expressed as catalase µmol/min mg of protein (Mukherjee *et al.,* 2007).

Lipid peroxidation

The reaction of malondialdehyde (MDA), a secondary product of lipid peroxidation, with thiobarbituric acid (TBA) is a sensitive assay method for lipid peroxidation in animal tissues (Ohkawa *et al.,* 1978). To samples less than 0.2ml of 10% (w/v) brain homogenate were added, 0.2ml of 8.1% sodium dodecyl sulphate, 1.5ml of 20% acetic acid solution adjusted to pH 3.5, and 1.5ml of 0.8% aqueous solution of TBA. The mixture was made up to 4.0ml with distilled water and heated in an oil bath at 95ºC for 60min. After cooling with tap water, 1.0ml of distilled water and 5.0ml of the mixture of n-butanol and pyridine (15:1 v/v) were added and shaken. Subsequent to centrifugation at 4000 rpm for 10min, the organic layer was taken and its absorbance at 532nm was measured. 1,1,3,3-tetramethoxy propane was used as an external standard, and the level of lipid peroxides were expressed as mol of MDA.

Acetylcholinesterase

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed (Ellman *et al.*, 1961). Briefly, 0.1ml of 0.01M DTNB was added to 2.6ml of 0.1M phosphate buffer (pH 8.0). 0.04ml of brain homogenate was added to the above mixture followed by incubation for 5min. Then 0.04ml of substrate (0.075M acetylthiocholine iodide) was added to the reaction mixture. The readings were taken at 420nm continuously for 5min at 1min

intervals. The results were expressed in μmol^{-1} min^{-1} mg $protein^{-1}$ using a molar extinction coefficient 1.36×10^4 M^{-1} cm^{-1}.

Brain swelling and hemispheric water content

On day 7 after removal of the brains in total, the hemispheres were separated along the inter-hemispheric plane. Both hemispheres were weighed to judge their wet weight. Then they were dried for 24 h at 110ºC for determination of the dry weight (Zausinger *et al.,* 2002). Based on the gravimetrical differences between both hemispheres, swelling of the ischemic hemispheres and water content in both ischemic and non-ischemic hemispheres was calculated as follows:

- Hemispheric brain swelling (%) = [(WW_{isc} – $WW_{non-isc}$)/$WW_{non-isc}$] X 100
- Hemispheric water content (%) = [(WW - DW)/WW] X 100
- Where WW is wet weight of ischemic or non-ischemic hemisphere (g); DW is dry weight (g).

Statistical analysis

Data are expressed as mean ± S.E.M. Kruskal-Wallis test was used to evaluate neurobehavioral parameters with subsequent group comparisons by Dunn's multiple comparison test. The results for biochemical tests were subjected to One-way ANOVA with Dunnett post hoc test using GraphPad Prism, version 5.01, USA. A *P* value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effects of spiradoline and naloxone by cognitive (spatial memory) and sensorimotor evaluation in rats

In the present study, carotid artery occlusion in Wistar rats showed significant impairment in learning, gait, reflexes and working memory. The cognitive deficit progressively and significantly worsened on repeated evaluations after 24 h and 7 days accompanied by sensorimotor modifications, cerebral infarcts and specific changes in the hippocampus in vehicle control rats. Working memory is a part of the memory system that by temporarily maintaining, manipulating, and integrating various sources of information permits the performance of complex cognitive activities. Working memory is seriously impaired in cerebral ischemia (Liang *et al.,* 1997). Previous studies have shown that this effect is related to hippocampal damage. Similarly, escape behavior in the elevated plus maze is thought to be based on long-term memory. Training sessions and explored cognitive domains, such as reference memory and learning, which rely on the integrity of the hippocampus have been focused in behavioral studies. A significant and progressive decline of performances in vehicle control rats was observed on the elevated plus maze test, 8-arm radial maze test and sensorimotor evaluation at 24 h and 7 days. Spiradoline treated rats showed significant improvement in cognition as indicated by results of transfer latency time at 24 h ($p<0.05$) and on day 7 ($p<0.01$) respectively, while naloxone treated group showed detectable yet insignificant improvement. The treated groups did not show significant improvement in number of arm entries as compared to vehicle control at the tested doses (Table 1).

Table. 1: Evaluation of spatial memory in rats treated with Spiradoline and Naloxone in elevated plus maze.

Treatment		Transfer latency time (sec)	No. of entries in arms	
			Open	Enclosed
Sham-operated control	24 h	32.00± 1.88	2.89± 0.26	3.67± 0.24
	7 days	32.33± 1.46	2.90± 0.20	3.44± 0.29
Vehicle control	24 h	79.86± 1.92$^{###}$	1.14± 0.14$^{##}$	2.57± 0.20
	7 days	73.00± 2.20$^{###}$	2.14± 0.14	2.43± 0.20
Spiradoline (10 mg/kg)	24 h	46.75± 1.36*	1.63± 0.18	2.75± 0.16
	7 days	40.75± 0.84**	2.50± 0.19	2.87± 0.23
Naloxone (5 mg/kg)	24 h	58.25± 0.84	1.50± 0.18	2.25± 0.16
	7 days	48.63± 0.73	2.37± 0.18	2.63± 0.18

Data expressed as mean ± S.E.M.; n=9; Statistical significance: $^{###}p<0.001$, $^{##}p<0.01$ vs sham-operated control; **p<0.01, *p<0.05 vs vehicle control.

Spiradoline administered group also revealed significant improvement in spatial memory as per the results of correct responses before first error and working memory errors ($p<0.05$ after 24 h; $p<0.01$ after 7 days) when compared to vehicle control group. However, naloxone treated group showed significant improvement in cognitive behavior only after 7 days in working memory errors ($p<0.05$). The data demonstrated that spiradoline possess better activity than naloxone in improving learning and memory at tested dose levels (Table 2).

Table. 2: Evaluation of spatial memory in rats treated with Spiradoline and Naloxone in 8-arm radial maze.

Treatment		Correct responses before first error	Working memory errors
Sham-operated control	24 h	4.00± 0.28	4.11± 0.87
	7 days	4.78± 0.28	4.56± 0.44
Vehicle control	24 h	1.71± 0.18$^{##}$	9.57± 0.57$^{###}$
	7 days	2.57± 0.20$^{###}$	8.86± 0.63$^{##}$
Spiradoline (10 mg/kg)	24 h	3.50± 0.33*	5.25± 0.37*
	7 days	4.37± 0.32**	4.38± 0.42**
Naloxone (5 mg/kg)	24 h	3.25± 0.25	5.75± 0.37
	7 days	3.75± 0.25	5.0± 0.27*

Data expressed as mean ± S.E.M.; n=9; Statistical significance: $^{###}p<0.001$, $^{##}p<0.01$, $^{#}p<0.05$ vs sham-operated control; **p<0.01, *p<0.05 vs vehicle control.

The results also depicted that spiradoline administered rats had significant ($p<0.05$) improvement from neurological deficit after 24 h and 7 days, while, naloxone administration did not influence significantly as compared to vehicle control (Figure 1). Reversal of these deficits by spiradoline suggests that it improves memory retention and/or memory recall process in aged rats. Although, it seems that naloxone may possess therapeutic potential against behavioral alterations caused by brain ischemic injury.

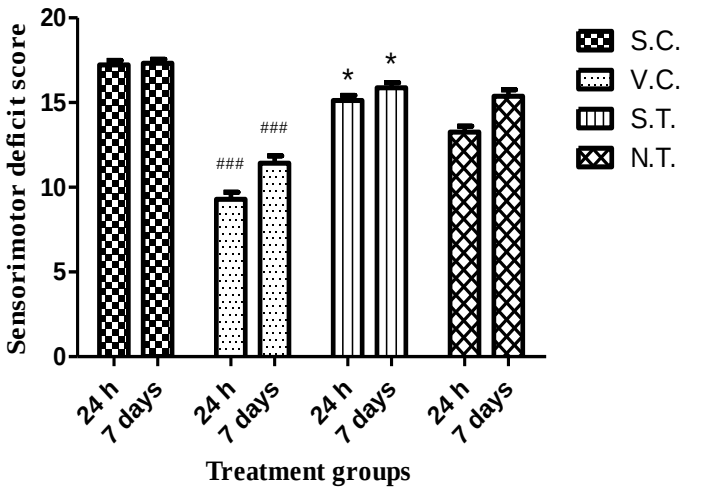

Fig. 1: Evaluation of sensorimotor deficits in rats treated with Spiradoline and Naloxone.
Data expressed as mean ± S.E.M.; n=9; Statistical significance: ###p<0.001vs sham-operated control; *p<0.05 vs vehicle control.
S.C.–Sham-operated control, V.C.–Vehicle control, S.T.–Spiradoline treated group, N.T.–Naloxone treated group.

Effect of spiradoline and naloxone on oxidative stress parameters in rats

Catalase and superoxide dismutase (SOD) are enzymes present endogenously in the brain for antioxidant defense against free radical action. Ischemic injury leads to the formation of several cellular toxic mediators, which contribute to oxidative damage (Xia and Zweier, 1995). Since, lipids are the most susceptible macromolecules to oxidative stress causing membrane damage, the results depicted that toxic lipid peroxides measured in terms of malondialdehyde (MDA), significantly increased during ischemia reperfusion.

The results revealed decreased levels of SOD and catalase, while increased level of lipid peroxidation significantly (p<0.001) in vehicle treated occluded rats as compared to sham-operated control. The data on day 7 showed both spiradoline and naloxone administered groups had significant (p<0.001) increase in SOD and catalase levels, significant (p<0.001) decrease in lipid peroxide level (Table 3).

Table. 3: Biochemical changes in brain of rats treated with Spiradoline and Naloxone on day 7.

Treatment	SOD (µmol/min/mg protein)	Catalase (µmol/min/mg protein)	Lipid peroxide-tion (mol MDA/mg protein)	Acetylcholine-sterase (µmol/min/mg protein)
Sham-operated control	31.17 ± 0.60	25.17 ± 0.60	2.00 ± 0.36	8.83 ± 0.48
Vehicle control	14.17 ± 0.60###	11.67 ± 0.76###	5.00 ± 0.37###	5.50 ± 0.43###
Spiradoline (10 mg/kg)	28.17 ± 0.60***	20.67 ± 0.67***	2.83 ± 0.31***	7.83 ± 0.31**
Naloxone (5 mg/kg)	22.50 ± 0.56***	16.83 ± 0.60***	2.67 ± 0.33***	7.17 ± 0.31*

Data expressed as mean ± S.E.M.; n=6; Statistical significance: ###p<0.001 vs sham-operated control; ***p<0.001vs vehicle control **p<0.01, *p<0.05 vs vehicle control.

Effect of spiradoline and naloxone on acetylcholinesterase level in focal ischemic rats

The key role that the cholinergic system plays in normal brain functions and in memory disturbances of several pathological processes has been well documented. A factor contributing to the decrease in acetylcholinesterase (AChE) specific activity might be the inhibition of proteosynthesis associated to ischemic deficits, but also a modulator effect of ischemia on AChE expression (Cena *et al.,* 2003).

The results demonstrated a significant (p<0.001) decrease in acetylcholinesterase level in vehicle control group as compared to sham control. Spiradoline administration significantly restored the level of acetylcholinesterase (p<0.01) at tested doses. Naloxone treatment also revealed a significant (p<0.05) increase in the acetylcholinesterase level on day 7. The data indicated that spiradoline is more efficacious than naloxone (Table 3).

Effect of spiradoline and naloxone on brain swelling and hemispheric water content in rats

Cerebral ischemia commonly causes development of edema and swelling due to accumulation of water. Brain edema after focal/global cerebral ischemia in humans develops over long periods (Zausinger *et al.,* 2002).

The spiradoline treated group on day 7 showed significant (p<0.001) decrease in brain swelling (Figure 2) and water content (Figure 3) as compared to vehicle control. Naloxone treatment did not produce any significant changes in brain swelling although water content was significantly (p<0.05) decreased. The effects of naloxone may not be sufficient to restore cerebral blood flow after ischemia.

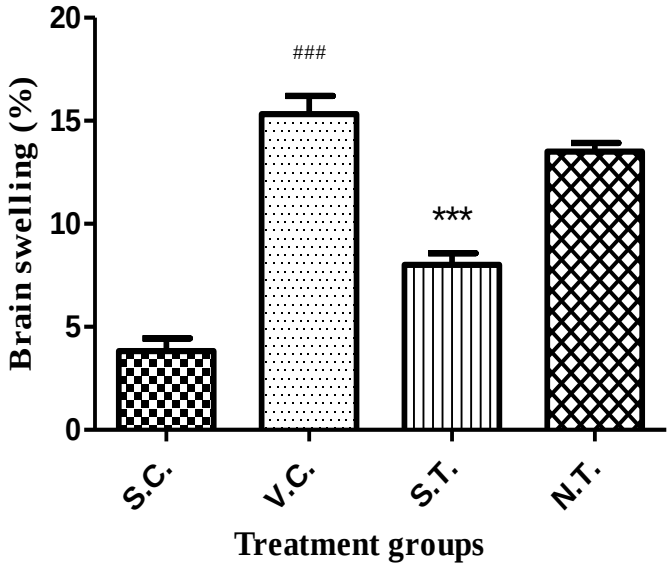

Fig. 2: Determination of brain swelling in rats treated with Spiradoline and Naloxone.
Data expressed as mean ± S.E.M.; n=6; Statistical significance: ###p<0.001 vs sham-operated control; ***p<0.001 vs vehicle control.
S.C.–Sham-operated control, V.C.–Vehicle control, S.T.–Spiradoline treated group, N.T.–Naloxone treated group.

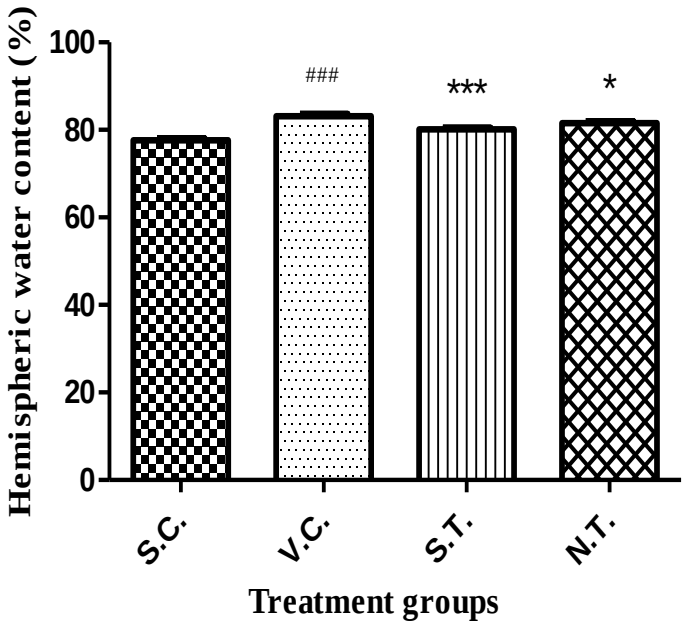

Fig. 3: Determination of hemispheric water content in rats treated with Spiradoline and Naloxone.
Data expressed as mean ± S.E.M.; n=6; Statistical significance: ###p<0.001vs sham-operated control; ***p<0.001, *p<0.05 vs vehicle control.
S.C.–Sham-operated control, V.C.–Vehicle control, S.T.–Spiradoline treated group, N.T.–Naloxone treated group.

CONCLUSION

Taken together, the results imply that the selective kappa opioid receptor agonist, spiradoline has better efficacy than the opioid antagonist naloxone at tested dose levels as indicated by neurobehavioral and biochemical alterations in rat brain and hence may be included in future combination therapies for brain stroke therapy. The mechanism by which these drugs act comes to knowledge, although detailed underlying mechanisms require further investigation. Additional studies should be planned to evaluate the time course and dose-effect functions for spiradoline to determine the conditions resulting in maximum therapeutic efficacy. Furthermore, in-depth studies of naloxone on other ischemic animal models should be carried out and it might be fruitful to explore in the present model the activity of long-acting opiate receptor blockers.

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