Original Article

The Effects of Salmon Calcitonin on the Concentrations of Periaqueductal Gray Monoamines in the Formalin Test

Rahimi et al. Monoamines After ICV Injection of sCT

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Background: The receptors of salmon calcitonin, located in such areas of brain as periaqueductal gray matter (PAG), are responsible for pain modulation.
Aims: In the current study, the effects of intracerebroventricular (ICV) injection of sCT on the behavioral response to pain and on the levels of monoamines in PAG are explored in a biphasic animal model of pain.
Study Design: Animal experimentation study.
Methods: 45 male rats in four groups were considered (n=6). sCT was infused into the lateral ventricle of the brain (1.5 nmol, with a volume of 5 µl). After twenty minutes, formalin 2.5% was injected (SC) into the right leg claw and pain behavior was recorded on a numerical basis. At the time of the formalin test, the PAG area was microdialized. High-performance liquid chromatography (HPLC) method was used to gauge the levels of monoamines and their metabolites.
Results: ICV injections of sCT led to the reduction of pain in the formalin test ($p<0.05$). Dialysate concentrations of serotonin, dopamine, norepinephrine, HIAA, DOPAC, and HMPG increased in the PGA area in different phases of the formalin pain test ($p<0.05$).
Conclusions: sCT reduced pain by increasing the concentrations of monoamines and the metabolites derived from them in the PAG area.
Keywords: sCT, formalin test, microdialysis, HPLC, monoamines.

The PAG area has a key role in descending pain control pathways in the brain stem (1). Downstream signals from the nuclei in the brain stem to the spinal cord constrain the dispatch of pain signals through the spinal cord (2). Monoamines play an important role in antinociceptive systems and have a close proximity to one another in the anesthetic centers of the brain (3). Different regions of the brain receive monoaminergic neurons from the PAG area (4).
The c-cells of the thyroid gland synthesize calcitonin (CT) (5, 6). It has also been reported that CT may be secreted by some cells in the brain (7). CTRa and CTRb are two types of CT receptors whose ability to connect to calcitonin is not very different from each other (8, 9). Calcitonin has different species. Salmon calcitonin receptors in rats have been distributed with a high density in such brain areas as the periaqueductal gray (10-13). The intracerebroventricular injection of calcitonin leads to hypocalcemia which demonstrates that the nervous system controls the blood calcium (14). Salmon calcitonin (sCT) which is a non-opioid peptide has a particular antinociceptive effect. The injections of calcitonin into the middle part of the PAG in the brain of rats reduce acute pain in the thermal withdrawal test (15). The ICV injection of salmon calcitonin causes analgesic effects in tail-flick and hot-plate tests (16). When salmon calcitonin is injected into the brain of mice, the pain caused by the formalin test is reduced (17). Calcitonin provides an interesting analgesic effect in a series of painful conditions. However,

the mechanism of its performance is not well-known. In the current research, the objective is to explore the impact of the ICV injection of sCT on the level changes of monoamines and the behavioral responses of pain in the PAG area in the formalin pain test.

MATHERIALS AND METHODS

Animals

In the current research, 45 male rats of Sprague-Dawley race (250-300 g) were studied. The rats had free access to water and food and were preserved at the temperature of 23 ± 2℃ and in a 12-h light/dark cycle. The protocol of the present study was authorized by the Ethics Committee of the School of Veterinary Medicine of Shiraz University, Shiraz, Iran.

In this study, four groups (n=6) were considered. In the first group, serum physiology (SP) was ICV (with a volume of 5 □l) and subcutaneously (SC) injected in the right leg claw (with a volume of 50 □l). In the second group, serum physiology was ICV injected (with a volume of 5 □l) and then formalin (2.5%) was SC injected in the right leg claw (with a volume of 50 □l). In the third group, 1.5 nmol of sCT was ICV injected (with a volume of 5 □l) and serum physiology was SC injected in the right leg claw (with a volume of 50 □l). In the fourth group, 1.5 nmol of sCT (Sigma-Aldrich, USA) (dissolved in serum physiology) was ICV injected (with a volume of 5 □l) and formalin (2.5%) was SC injected in the right leg claw (with a volume of 50 □l).

Stereotaxic surgery and microdialysis probe implantation

With the intraperitoneal injection of pentobarbital sodium (50 mg/kg), the rats were anesthetized. The guide cannula (with the coordinates: Anteroposterior: -0.8, Laterality: +1.5, Dorsoventral: -3.5) was implanted in the lateral ventricle of the brain. Brain microdialysis probes, with an appropriate length for the PAG area, were constructed based on the Paxinos Atlas. The microdialysis probes were implanted into the PAG area (with the coordinates: Anteroposterior: -7.6, Laterality: 0.6, Dorsoventral: -5.8) (18).

Pain assessment and the microdialysis of the PAG area

To assess pain sensation, the formalin test is employed. In this test, if the rat does not exhibit any unusual behavior, it is given score 0; if the rat's leg claw is on the floor of the chamber and it does not put its weight on the leg claw, it is given score 1; if the rat strikes the floor of the chamber with a leg claw or pulls up its legs in the abdominal area, it is given score 2; and lastly, if the rat bites or licks the injection location, score 3 is recorded for it (19). The SC injection of formalin in the leg claw causes a two-phase response to pain in the rat. The first five minutes after formalin injection comprise the first phase. The second phase begins from the end of the fifteenth minute to the end of the sixtieth minute. In addition, the interphase starts from the end of the fifth minute to the end of the fifteenth minute (20).

After twenty-four hours, the rats were put in a micro-dialysis chamber and were permitted to adjust to the environment for fifteen minutes. A Hamilton syringe (10 µl) was employed for the ICV injections of sCT. The pain test was performed twenty minutes after the injection of sCT. The PAG area was perfused with artificial cerebrospinal fluid (aCSF) with the flow rate of 2.0 µl/min using a syringe pump (WPI, SP 210). The dialysis samples were gathered at fifteen-minute intervals [(S1): the base sample; (S2): base sample with sCT or serum physiology effects; (S3-S6): the four samples corresponding to the various times of the formalin test with sCT or serum physiology effects; and (S7, S8): the two samples collected after finishing the pain test]. The combination of the aCSF was (in mM): $CaCl_2$ (1), KCl (3), NaH_2PO_4 (1.25), NaCl (114), $NaHCO_3$ (26), $MgSO_4$ (2), NaOH (1), glucose (10), and pH=7.40.

Chemical assays

High performance liquid chromatography-electrochemical detection (HPLC-ECD) method was employed to determine the concentrations of dopamine, serotonin, norepinephrine, and their metabolites in the dialysis of the PAG area (n=6). After the preparation and addition of the internal standard (14.3µl), the samples were infused into an HPLC column (Eurospher reverse-phase column, 100-5 C18, 250×4.6 mm), a pump (Knauer), and an electrochemical detector (Amperometric detector EC 3000). The oxidizing potential of the working electrode was set at +750 mV versus the Ag/Cl reference electrode. The mobile phase comprised a combination of Ethylenediaminetetraacetic acid (30 mg), sodium phosphate (8.4 g), 1-octane-sulfonic acid (360 mg), and 16% methanol (in 1000 ml of water with HPLC grade (pH=4.5)). The mobile phase had the flow rate of 1.0 ml/min.

Histological substantiation

The rats were put down using a high dose of diethyl ether (MERK, Germany) after completing each experiment. After 72 hours, the rats' brains were placed in formalin 10%. The positions of the guide cannula in the ventricle and the probes of microdialysis in the PAG area of all the brains were verified according to the Paxinos Atlas (18).

Statistical analyses

The SPSS software (version 16) was employed for analyzing the data. According to the distribution and homogeneity of variances, a normalization test was performed on the data (SPSS Kolmogorov-Smirnov Test). Since

the data were normal, one-way ANOVA was used to evaluate the groups. Duncan's tests were performed as post-hoc analyses. P<0.05 was taken as the significance level.

RESULTS

Pain assessment

The results of the pain behavior responses at various phases of the formalin pain test are shown in Table 1. Compared to the second group (2.4±0.05), injecting sCT into the ventricle of the brain in the fourth group (1.46±0.08) significantly decreased the nociceptive behavioral score in the first phase of the formalin pain test (Fig. 1). Compared to the second group (1.92±0.03), sCT in the interphase of the formalin pain test of the fourth group (0.35±0.06) significantly decreased the nociceptive behavioral score (Fig. 1). Furthermore, compared to the second group (2.05±0.01), the injection of sCT in the chronic phases of the formalin pain test of the fourth group (1.87±0.03) significantly reduced the nociception following formalin injection (Fig. 1).

The concentrations of monoamines in the PAG area

The third dialysis samples (S3) which included the aCSF gathered from the PAG area in 0-15 min time intervals after formalin injection correlated to the first phase and the interphase of the formalin pain test. The fourth, fifth, and sixth dialysis samples (S4-S6) obtained within the time intervals of 15-30, 30-45, and 45-60 min, respectively, after formalin injection correlated to the second phase of the formalin pain test.

Serotonin

Serotonin concentrations in groups 1 and 3 did not have significant differences at any of the tested times. The serotonin concentration (pg/ml) in dialysis sample 3 in the second group (155.42±31.12) was significantly lower than that of the fourth group (369.11±51.92) (p<0.05). Serotonin concentrations in dialysis samples 4 and 5 in the second group (101.33±11.36 and 90.16±17.61) were significantly lower than those of the fourth group (198.27±17.06 and 136.23±19.32) (p<0.05). Serotonin concentration in dialysis sample 6 in the second group was lower than that of the fourth group. However, the difference was not significant (Fig. 2).

5-hydroxyindileacetic acid (HIAA)

HIAA concentrations did not have significant differences in groups 1 and 3 at any of the time intervals. HIAA concentration (pg/ml) in dialysis sample 3 in the second group (1416.142±79.74) was significantly less than that of the fourth group (6166.580±16.28) (p<0.05). HIAA concentration in dialysis sample 4 in the second group (1115.312±66.18) was significantly less than that of the fourth group (2061.548±40.01) (p<0.05). HIAA concentrations in dialysis samples 5 and 6 in the fourth group did not show any significant differences compared to those of the second group (Fig. 3).

Dopamine

Dopamine concentrations in groups 1 and 3 did not have significant differences at any of the tested times. Dopamine concentration (pg/ml) in dialysis sample 3 in the second group (182.25±32.58) was significantly lower than that of the fourth group (390.119±12.29) (p<0.05). Dopamine concentration in dialysis sample 4 in the second group (88.27±18.42) was lower than that of the fourth group (183.30±19.68) (p<0.05). Dopamine concentrations in dialysis samples 5 and 6 in the second group were lower than those of the fourth group. Nevertheless, the difference was not significant (Fig. 4).

3,4-dyhidroxyphenylacetic (DOPAC)

DOPAC concentrations in groups 1 and 3 did not have significant differences at any of the time intervals. DOPAC concentration (pg/ml) in dialysis sample 3 in the second group (630.97±97.02) was significantly less than that of the fourth group (1214.169±29.01) (p<0.05). Dopamine concentration in dialysis sample 4 in the second group (298.76±38.34) was less than that of the fourth group (709.166±40.25) (p<0.05). DOPAC concentrations in dialysis samples 5 and 6 in the fourth group did not show any significant differences compared to those of the second group (Fig. 5).

Norepinephrine

Norepinephrine concentrations in groups 1 and 3 did not have significant differences at any of the time intervals. Norepinephrine concentration (pg/ml) in dialysis sample 3 in the second group (110.34±19.13) was significantly lower than that of the fourth group (213.39±17.85) (p<0.05). Norepinephrine concentration in dialysis sample 4 in the second group (53.14±19.79) was lower than that of the fourth group (140.39±25.61) (p<0.05) (Fig. 6).

4-hydroxy-3-methoxyphenylglycol (HMPG)

HMPG concentrations in groups 1 and 3 did not have significant differences at any of the time intervals. HMPG concentration (pg/ml) in dialysis sample 3 in the second group (248.51±41.78) was significantly less than that of the fourth group (514.95±42.89) (p<0.05). HMPG concentration in dialysis sample 4 in the second group (110.13±10.05) was less than that of the fourth group (213.49±16.09) (p<0.05). HMPG concentrations in dialysis samples 5 and 6 in the second group were less than those of the fourth group. However, the difference was not significant (Fig. 7).

DISCUSSION

The nociceptive behavioral scores in the first, inter, and second phases of the formalin pain test were significantly reduced by The ICV injection of sCT. sCT enhanced the concentrations of serotonin, dopamine, norepinephrine, HIAA, DOPAC, and HMPG in the first and inter phases of the formalin pain test in the PAG area. sCT also enhanced the concentrations of serotonin, dopamine, norepinephrine, and their metabolites at the start of the second phase of the formalin pain test. In our previous study, the other calcitonin family peptide (calcitonin gene-related peptide (CGRP)) showed similar effects. Subsequently, the ICV injection of CGRP reduced pain after the injection of formalin. The dialysis of PAG also showed that the ICV injection of CGRP increased the concentrations of serotonin, norepinephrine, dopamine, HIAA, HMPG, and DOPAC in the periaqueductal gray in the formalin pain test (21).

Salmon calcitonin has analgesic effects both on somatic pains such as muscle and bone pains and on visceral pains such as migraine headaches. Salmon calcitonin has recently been shown to have the ability to attenuate migraine-like pains by c-fos expression and regulating the release of CGRP at different levels (22). eCT, a synthetic derivative of eel calcitonin, exhibits analgesic effects on radicular pain via the modulation of mRNA-expression of voltage-gated sodium channels. Thus, it can be stated that patients who have radicular pain or need long-term treatments prefer calcitonin for their therapy (23). sCT can inhibit the progression of facet joint syndrome in ovariectomized rats. This potential is ascribed to the inhibitory impacts of sCT on apoptosis, cartilage metabolism imbalance, and bone remodeling (24). PCR analysis shows that the chronic constriction injury causes the upregulation of tetrodotoxin (TTX)-sensitive Nav.1.3 mRNA. It also leads to the downregulation of mRNA of Nav1.8 and Nav1.9 on the dorsal root ganglion (DRG). This will, in turn, enhance the excitability of peripheral nerves. Elcatonin can play an important role in reversing these changes (25).

The results of the current study demonstrated that the ICV injection of sCT significantly decreased nociception during the first phase and the interphase of the formalin pain test. Candeletti and Ferri also reported similar effects in mice (17). Different mechanisms have been attributed to the analgesic effects of calcitonin. However, no study has shown a change in the concentration of monoamines in the analgesic systems of the brain. Our results indicate that the concentrations of serotonin, dopamine, norepinephrine, and their metabolites have enhanced in the PAG area in the first phase and the interphase of the formalin pain test. Therefore, it seems that the monoaminergic pathways in the brain mediate the analgesic effects observed in the first phase and the interphase of the formalin pain test.

The central serotonergic system might have a role in the analgesia caused by calcitonin (26). The ICV injection of calcitonin leads to an increase of serotonin contents in several regions of the brain (27). Serotonin has an analgesic effect on chronic pain in humans and animals (28). Calcitonin in ovariectomized rats can cause the release of glutamate from C-afferent fibers and can also normalize the expression of sodium channel in damaged peripheral nerves (29). Immunohistochemical studies have also shown that the PAG area containing dopaminergic neurons is involved in the analgesic system. The injection of the dopamine antagonist in the vPAG reduces the analgesic effects of heroin and morphine (30). Also, in the formalin test, norepinephrine and HMPG concentrations are enhanced in the locus coeruleus (LC) (31). LC has projections to different areas of the brain such as the PAG area (32).

In the second phase of the formalin pain test, pain-related behaviors were significantly decreased with the ICV injection of sCT. The concentrations of serotonin and HIAA increased at 15-45 min and 15-30 min time intervals after formalin injection, respectively. The serotonergic system in the PAG area plays a key role in pain modulation. The administration of serotonin agonist in the PAG area reduces pain (33). Dopamine and its metabolite (DOPAC) concentrations increased at a 15-30 min time interval in the second phase of the formalin pain test. It is noteworthy that a network of dopaminergic neurons is distributed throughout the mesencephalon (34). sCT also enhanced the concentrations of norepinephrine and its metabolite at a 15-30 min time interval after the injection of formalin. The administration of norepinephrine in the dorsal part of the PAG area showed that norepinephrine plays a role in pain perception (34).

Salmon calcitonin also has clinical applications. Since the 1970s, sCT has been applied as a nasal spray or injection for treating osteoporosis and other metabolic bone diseases (35). sCT has also been employed for treating postmenopausal osteoporosis. Although the nasal application is less effective than the injectable formulation, it has been more frequently employed. Salmon calcitonin increases bone mineral density (36, 37). Nasal calcitonin spray has been more effective than gabapentin in treating patients with lumbar spinal stenosis (38). Moreover, nasal sCT has been recommended for ameliorating acute osteoporotic distal radius fractures (39). A study focused on a novel oral sCT (SMC021) and found that this sCT failed to meet its primary objective, i.e. reducing vertebral fractures in postmenopausal women suffering from osteoporosis. The study suggested that more researches be done on the delivery of peptides (40). Despite its analgesic effects, oral sCT does not have renewable benefits for patients suffering from knee osteoarthritis (41). Studies suggest that calcitonin can mitigate the back pain of patients suffering from neuropathic pain or osteoporosis by altering the expression of channels or receptors (29, 42).

In the current study, the intracerebroventricular injection of salmon calcitonin reduced pain in the formalin pain test. The observed effect may be due to an increase in serotonin, dopamine, norepinephrine, and their metabolites in the PAG area or the related nuclei.

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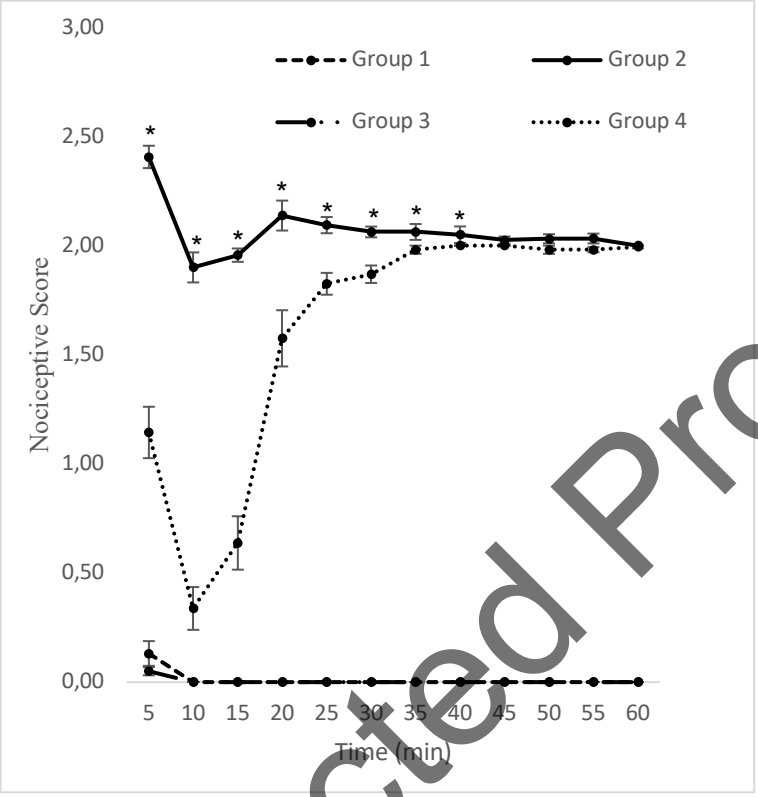
Table 1. Pain-related behaviors (mean±SD) in formalin test

	Min5	Min10	Min15	Min20	Min25	Min30	Min35	Min40	Min45	Min50	Min55	Min60
G 1	0.13±0.16 [a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]
G 2	2.41±0.14 [d]	1.90±0.07 [c]	1.96±0.86[c]	2.14±0.19[c]	2.09±0.10[c]	2.06±0.69[c]	2.06±0.10[c]	2.05±0.10[c]	2.03±0.46[c]	2.03±0.02[c]	2.03±0.05[c]	2±0[c]
G 3	0.05±0.16 [a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]	0±0[a]
G4	1.14±0.22 [b]	0.34±0.10 [b]	0.64±0.20[b]	1.58±0.48[b]	1.83±0.32[b]	1.87±0.68[b]	1.98±0.04[b]	2±0.03[b]	2±0.10[b]	1.98±0.02[b]	1.98±0.03[b]	1.99±0.13[b]

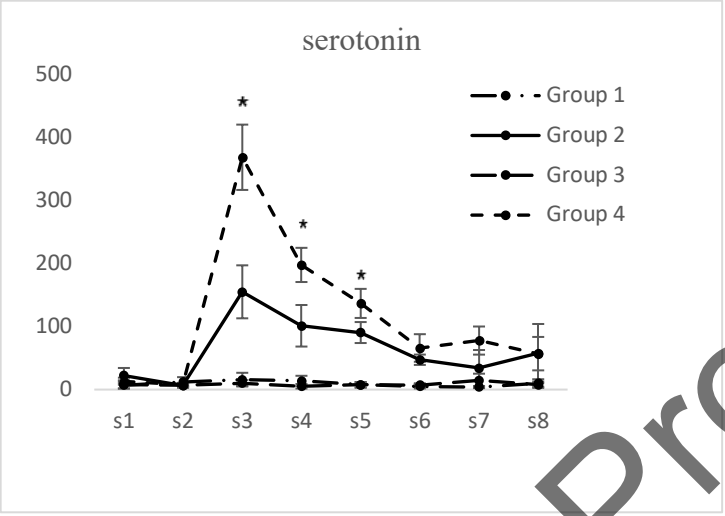
Dissimilar letters (a, b, c, d) indicate significant difference between groups. ($p<0.05$)
(Group 1: NS was ICV injected and normal saline was injected subcutaneously (SC) in the hind paw. Group 2: NS was ICV injected and formalin 2.5% was injected SC. Group 3: sCT with a dose of 1.5 nmol was ICV injected and normal saline was injected SC. Group 4: sCT with a dose of 1.5 nmol was ICV injected and formalin 2.5% was injected SC).
(G) group, (min) minute

Fig. 1. The nociceptive score in different groups.



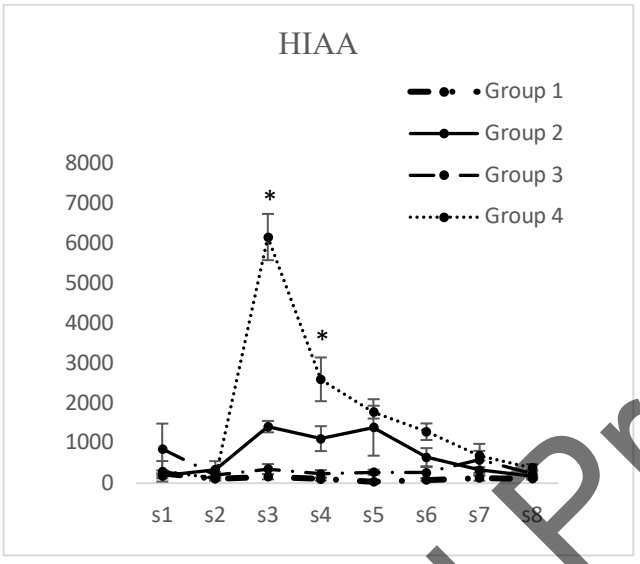
(Group 1: normal saline (NS) was intracerebroventricular (ICV) injected and normal saline was injected subcutaneously (SC) in the hind paw. Group 2: NS was ICV injected and formalin 2.5% was injected SC. Group 3: sCT with a dose of 1.5 nmol was ICV injected and normal saline was injected SC. Group 4: sCT with a dose of 1.5 nmol was ICV injected and formalin 2.5% was injected SC) * Significant differences between group 2 and group 4 ($P<0.05$) (mean±SD).

Fig 2. Concentrations of serotonin in different groups.



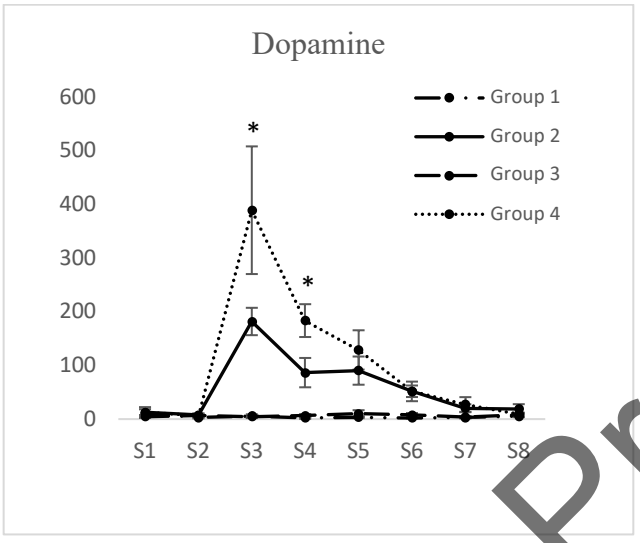
[S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).

Fig 3. Concentrations of 5-hydroxyindileacetic acid (HIAA) in different groups.



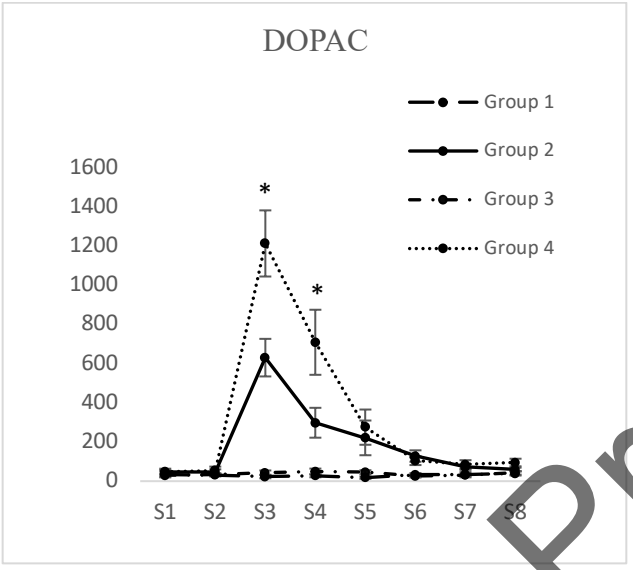
[S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).

Fig 4. Concentrations of dopamine in different groups.



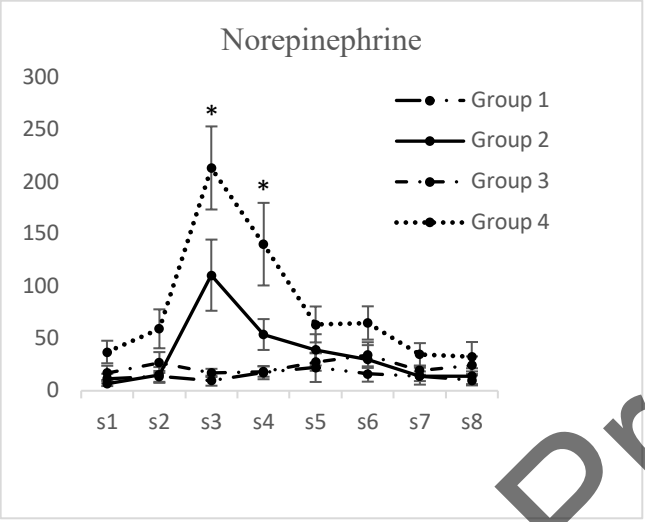
[S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).

Fig 5. Concentrations of 3,4-dyhidroxyphenylacetic (DOPAC) in different groups.



[S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).

Fig 6. Concentrations of norepinephrine in different groups.



[S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).

Fig 7. Concentrations of 4-hydroxy-3-methoxyphenylglycol (HMPG) in different groups.

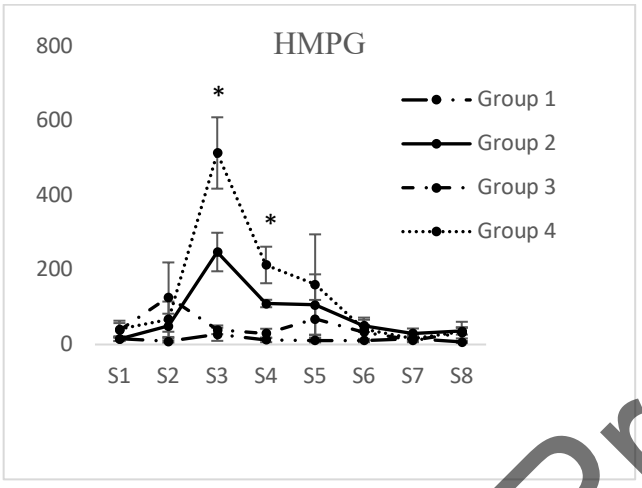


Fig 7. Concentrations of HMPG in different groups. [S1 to S8 represents the collected samples of dialysis from the PAG at different times. Base sample without medication effect (S1), Base sample with medication effect (S2), four samples related to different times of the formalin test (S3-S6) and two samples after completion of formalin test (S7, S8)]. * Significant differences between group 2 and group 4. ($P<0.05$) (mean±SD).