Original Article

Effect of cholecystokinin on learning and memory, neuronal proliferation and apoptosis in the rat hippocampus

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Abstract

Background: Cholecystokinin (CCK) has roles in learning and memory, but the cellular mechanism is poorly understood. This study investigated the effect of CCK on spatial learning and memory, neuronal proliferation and apoptosis in the hippocampus in rats.

Materials and Methods: Experimental groups were control and CCK. The rats received CKK octapeptide sulfated (CCK-8S, 1.6 μg/kg, i.p.) for 14 days. Spatial learning and memory were tested by Morris water maze and finally immunohistochemical study was performed; neurogenesis by Ki-67 method and apoptosis by Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay in hippocampal dentate gyrus (DG).

Results: Cholecystokinin increased Ki-67 positive cells and reduced TUNEL positive cells in the granular layer of hippocampal DG. CCK failed to have a significant effect on spatial learning and memory.

Conclusion: Results indicate neuroprotective and proliferative effects of CCK in the hippocampus; however, other factors are probably involved until the newly born neurons achieve necessary integrity for behavioral changes.

Key Words: Apoptosis, cholecystokinin, hippocampus, learning, memory, neurogenesis

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INTRODUCTION

Studies have shown that cholecystokinin (CCK), one of the digestive system hormones, is the most abundant peptide neurotransmitter in the brain and has roles in various physiological functions of the brain, including memory.[1] CCK and its receptors are characterized with high concentrations in the regions that are essential for memory, including the hippocampus.[2] CCK has different molecular forms; however, the most abundant form in the central nervous system is CCK sulfated octapeptide (CCK-8S). Physiologically, CCK-8S is probably the only form that acts as a neurotransmitter.[3] CCK has two kinds of receptors, CCK1, and CCK2.[4] Both receptors exist in the hippocampus with high concentrations, and CCK-8S has the same affinity for the two receptors.[2]

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Cholecystokinin-8S can rapidly cross the blood-brain barrier and appears in different brain regions, including the hippocampus.[5,6] Studies have shown that induction of long-term potentiation and learning and memory processes are severely damaged in rats that are genetically lacking CCK1 receptor.[7] In addition, the importance of the CCK2 receptors in learning and memory has been shown, and in equilibrium with the CCK1 receptor, the CCK2 receptor plays an important role in memory retention.[8] CCK levels and its receptors decrease dramatically in the brain with age.[9,10] In Alzheimer's disease that is associated with widespread neuronal apoptosis, there is a large reduction in brain CCK concentrations.[9] However, the mechanism by which CCK is involved in learning and memory is not fully clear.

Therefore, the aims of this study were to investigate whether CCK is involved in regulating mitotic process in hippocampal neurons in the adult brain; and through its neuroprotective effects, whether CCK can decrease neuronal apoptosis in dentate gyrus (DG). Using a behavioral test, we evaluated spatial learning and memory of rats after treatment with CCK-8S.

MATERIALS AND METHODS

Subjects and procedures

Male Wistar rats (280 ± 20 g) were housed four/cage and maintained on a 12 h light–dark cycle in an air-conditioned constant temperature (23°C ± 1°C) room, with food and water made available *ad libitum*. The Ethic Committee for Animal Experiments at Isfahan University approved the study and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health publications no. 80–23) revised 1996. The animals were divided into two groups ($n = 10$): Controls and CCKs.

In this study, the rats in the CCK group received CCK-8S (1.6 μg/kg, i.p., dissolved in saline; Sigma-Aldrich)[11] for 14 days and the rats in the control group received an equivalent volume of saline as placebo. To assess spatial learning and memory, Morris water maze test was carried out during 10th to 14th days, at least 3–4 h after injection of CCK-8S. Immediately after behavioral studies, rats were lightly anesthetized with ether and then rapidly decapitated, and the hippocampi were removed for histological study.

Morris water maze test

The circular tank (180 cm in diameter) was filled with water (22°C ± 2°C) made opaque and was surrounded by a variety of extra-maze cues. The tank was divided into four quadrants, and four start positions were located at the interactions of the quadrants. Data were recorded using custom software (Radiab 1). Twenty-four hours before water maze testing, all rats were habituated to the water and apparatus. In the spatial acquisition phase, the rats learned to find a submerged platform using extra-maze cues. A transparent Lucite platform (10 cm) was submerged 2 cm underneath the water in north-east quadrant of the tank, where it remained for all spatial trials. Each rat participated in 16 trials, which were organized into daily block of four trials (1 trial/start position within a block) for 4 consecutive days. For each trial, the rat was given a maximum time of 60 s to locate the platform, after which the rat remained there for 30 s; if the rat did not locate the platform within 60 s, it was guided to it by the experimenter. The next trial started immediately after removal of rat from the platform. Escape latencies (s) and path lengths (cm) were recorded.[12]

In the retention phase, a 60-s probe trial was conducted to examine how well the rats had learned the exact location of the platform. During this trial, the platform was removed from the tank. The swim time was measured inside a circular area (70 cm diameter) around the center of the platform.

Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling test

After scarification, the right hippocampus of the brains were fixed in 10% neutral buffered formalin, dehydrated and cleared through ethanol and xylene, respectively, and embedded in paraffin.

Coronal slices were made from the beginning to end of the right hippocampus and for every 500 μ a slice was selected and mounted on poly-l-lysine-coated (Sigma) glass slides. For the detection of apoptotic cells, Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay was used. The *in situ* cell death detection kit POD (Roche) was applied on paraffin sections according to the manufacturer's instructions. Briefly, shortly after the dewaxing, the slices were treated with proteinase k (Roche) and incubated at 37°C for 30 min. Then the slices were washed with PBS solution (25°C), and were left in Tris HCl solution (0.1 mM, pH 7; Roche) for 30 min at room temperature and then were washed with PBS solution (Roche). TUNEL reaction mixture was then added, and the slices were incubated in a humidified chamber for 60 min at 37°C. The labeled product was visualized using diaminobenzidine (DAB) (Roche) for 15–30 min at room temperature, which yielded brown granules

mainly localized to apoptotic cells. Then, they were rinsed with running water and counterstained using hematoxylin (Sigma). Comparisons of levels of neuronal cell death were determined from the number of morphologically intact cells and the number of deep brown-stained, TUNNEL positive, damaged cells in the hippocampal DG region. The samples were photographed by a camera attached to a microscope and computer software, Motic Image, and then the cells were counted in the images.

Ki-67 immunohistochemistry

The slices of hippocampus were prepared as above and were put in boiling epitope retrieval solution (citrate buffer) (Sigma) for 10–15 min (based on kit instructions). Then, samples were washed 2-times with washing buffer solution (Sigma). Hydrogen peroxide solution was put on the slices for 15 min. Samples were washed again with washing buffer solution. Then, slices were incubated in anti-rat Ki-67 primary antibody (concentration: 1/100) (Abcam; ab 16667) at 37°C for 30 min, and again washed with washing buffer solution. Then, they were incubated in secondary antibody + washing buffer solution for 20 min at room temperature and were washed in washing solution and incubated in Novolink Polymer (Leica) + washing buffer solution for 10 min at room temperature and washed again. Chromogen solution substrate (DAB + substrate) was put on the samples for 15 min and washed in the washing solution. Then, they were washed with running water and stained with hematoxylin (detection kit: Leica).

Statistical analysis

All data collected were analyzed using the SPSS for Windows (version 16). The escape latencies and path lengths in the water maze were analyzed by two way ANOVA for between-subjects differences between controls and CCKs ("CCK" effect) and repeated measures (within subjects) effects across block interval 1–4 ("BLOCK" effect). The probe trial data for the swim time spent inside a circular area around the center of the platform were analyzed by the independent sample *t*-test. For the histological analysis (neuronal proliferation and apoptosis), the nonparametric independent *t*-test (Mann–Whitney test) was applied to test for any differences between the controls and CCKs. A $P < 0.05$ is designated to be significant. All data are presented as mean ± standard error of the mean.

RESULTS

Morris water maze test

All rats showed a reduction in escape latencies [BLOCK effect, $F(3,54) = 16.537$, $P < 0.001$; Figure 1a] and a reduction in the distance swam to locate the platform [BLOCK effect, $F(3,54) = 17.047$, $P < 0.001$; Figure 1b] across blocks of trials, indicating spatial acquisition. The pattern of reduction in distance to locate the platform across the blocks was significantly more in CCK group [CCK * BLOCK effect interaction, $F(3,54) = 2.852$, $P < 0.05$; Figure 1b]. Although CCK group showed more reductions of escape latencies across the blocks than control group, but this different wasn't significant [CCK * BLOCK effect interaction, $F(3,54) = 1.715$, $P = 0.17$; Figure 1a]. However, the CCK group found the platform more quickly than control group [30.21 ± 3.62 s and 33.29 ± 3.62 s, respectively; $F(1,18) = 0.36$, $P = 0.55$; Figure 1a] and took shorter distance to the platform [836.61 ± 101.11 cm and 866.42 ± 101.12 cm, respectively; $F(1,18) = 0.043$, $P = 0.84$; Figure 1b], these differences weren't significant.

The swim speed was faster in the CCKs than controls, but it wasn't significant [$F(1,18) = 3.15$, $P = 0.09$; Figure 1c]. Furthermore, swim speed had no changes across the blocks and no changes in the pattern of swim speed between the groups.

For the results of probe trial as measured by the mean time spent inside a circular area (70 cm diameter) around the center of platform, between group comparison indicated that, although the CCK group spent more time (17.35 ± 2 s) around the area, where the platform was previously located, than the control group (13.85 ± 2.42 s), but this different wasn't significant [$P = 0.14$; Figure 2].

Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling-test and Ki-67 immunohistochemistry

In histological studies, under the light microscope, in CCK group, granular neurons in the DG subfield of the hippocampus were almost in normal appearance. The number of apoptotic cells in the DG had decreased in CCK group [$P < 0.01$; Figures 3 and 4].

Cholecystokinin significantly increased proliferation in the granular layer of DG, and there was a significant number of Ki-67 positive cells in this subfield of the hippocampus than control group [$P < 0.001$; Figures 5 and 6].

DISCUSSION

The results showed that CCK increases Ki-67 positive cells and also, it reduces the number of TUNEL positive cells in the granular layer of hippocampal DG. Nevertheless, CCK failed to have a significant effect on spatial learning and memory in rats.

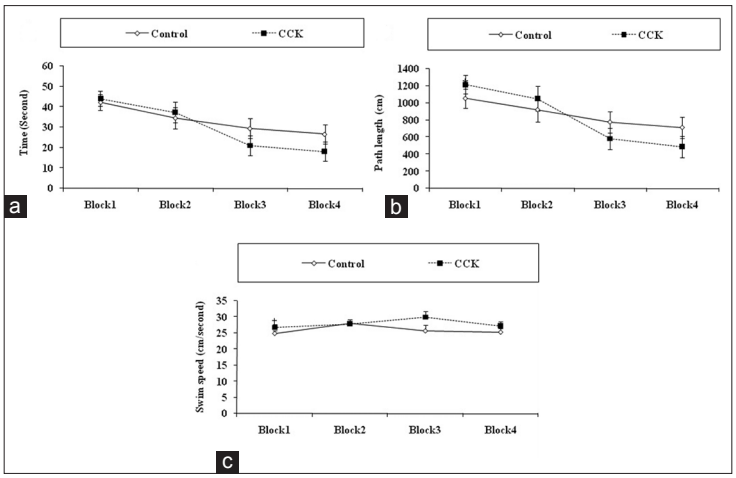

Figure 1: Effects of cholecystokinin sulfated octapeptide (CCK-8S) on performance during the spatial acquisition of Morris Water Maze test in rats. The escape latency (a), the path length (b) and the swim speed at different days to reach the platform (c). Each point represents the day mean ± standard error of the mean of 4 swims. For latency and path length, lower numbers indicate better performance $n = 10$)

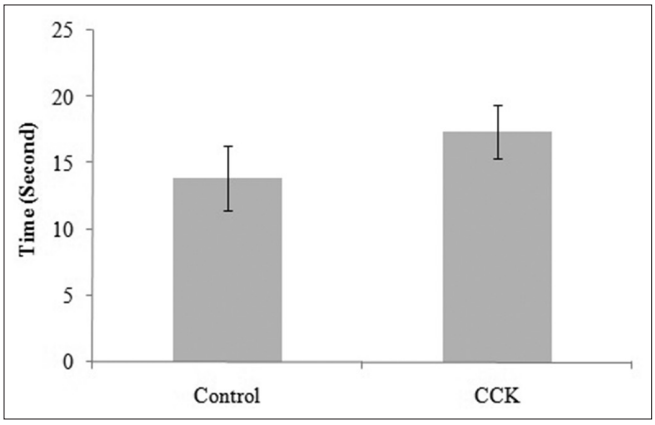

Figure 2: Effects of cholecystokinin sulfated octapeptide (CCK-8S) on performance during the probe trial as measured by the mean time spent inside a circular (70 cm diameter) around the center of platform, 1-day after spatial acquisition phase ($n = 10$)

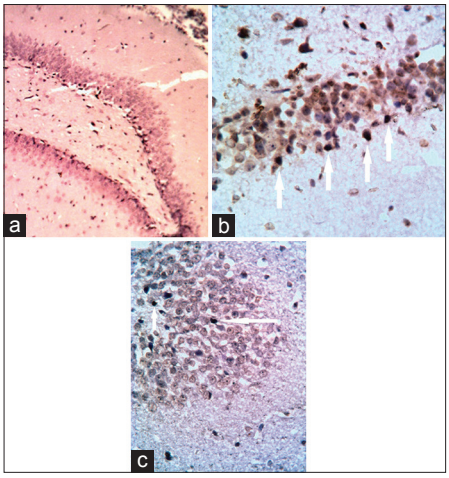

Figure 4: Photomicrograph of the optical microscope from granular layer of hippocampal dentate gyrus of rats (a) (M = ×4). Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling-positive cells in a granular layer of the studied groups; control (b) and cholecystokinin (c) are visible on the arrow tip (M = ×40)

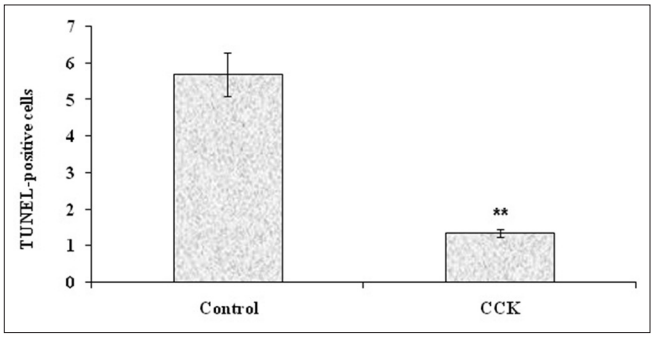

Figure 3: Numbers of Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling-positive cells in granular layer of hippocampal dentate gyrus of rats after receiving CCK-8S for 2 weeks ($n = 7$, $^{**}P < 0.001$)

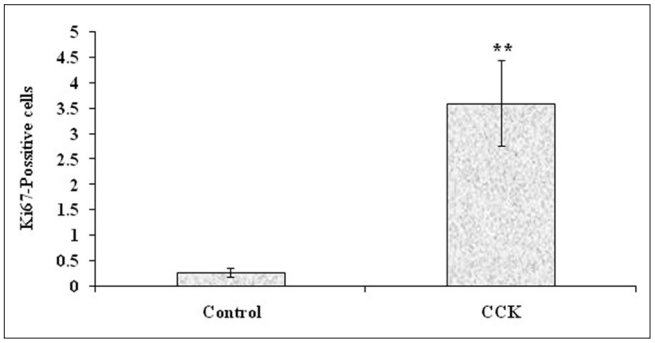

Figure 5: Numbers of Ki-67-positive cells in a granular layer of hippocampal dentate gyrus of rats after receiving cholecystokinin-8S for 2 weeks ($n = 7$, $^{***}P < 0.001$)

Studies have shown that programmed cell death or apoptosis exists in the central nervous system of adult mammalian and this programmed cell death often presents in the area where neurogenesis is seen even in adulthood; like DG of hippocampal formation.[13] Since a very high number of new neurons are made during adult neurogenesis and only some of them

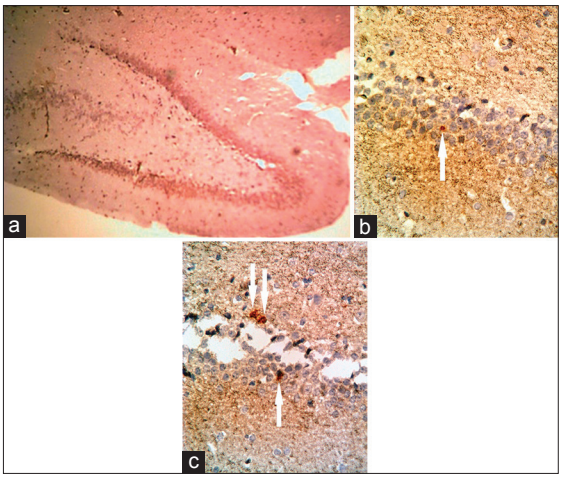

Figure 6: Photomicrograph of the optical microscope from granular layer of hippocampal dentate gyrus of rats (a) (*M* = ×4). Ki-67-positive cells in a granular layer of the studied groups; control (b) and cholecystokinin (c) are visible on the arrow tip (*M* = ×40)

survive, and others encounter apoptosis,[14] therefore it has been suggested that apoptosis is actually dependent on neurogenesis.[15] In the hippocampus, neural progenitors in the subgranular zone of the DG proliferate, and the new neurons migrate to the DG granular layer and participate in the building of hippocampal neuronal circuits. Studies have shown that both types of CCK receptors are expressed by immortalized rat brain neuroblasts. CCK through both receptors may contribute in neuronal proliferation and adult neurogenesis.[16,17] CCK through type-A receptors probably regulates neuronal proliferation in adult mice hippocampus because in mice lacking this receptor proliferation of neuronal was significantly reduced.[17] Furthermore, CCK has important effects on neuronal developmental processes. CCK-8S has raised filopodia and spines density in hippocampal neurons in intact animals and also had favorable effects on animal models of Alzheimer's disease.[18]

In our study, CCK reduced TUNEL-positive cells [Figures 3 and 4] and increased Ki-67-positive cells [Figures 5 and 6], thus it can be concluded that this neuropeptide induced proliferation and prevented death of newborn cells. It has been observed that CCK has both neuroprotective and proliferative effects, and CCK and its receptors are widely existing in the hippocampus.[19] CCK can prevent the degeneration of cholinergic neurons in animal models of Alzheimer's disease.[20] Because, in the adult neurogenesis, half of newborn cells die within the next few days, through its neuroprotective effects, probably CCK can increase the neuroblasts viability.[17]

It has been demonstrated that CCK through NGF (Nerve growth factor)-mediated effects performs neuroprotection and regeneration in the central and peripheral nervous systems.[21] Its neuroprotective effects against neurotoxicity induced by glutamate in the culture medium are shown.[19] CCK probably provides this protection by inhibiting the formation of nitric oxide and activation of astrocytes.[22]

Behavioral results of this study did not show the desired effect of CCK [Figures 1 and 2]. On the assumption that the animals in this study were healthy rats, therefore, more time probably is needed until the newly born neurons achieve necessary integrity for behavioral changes. Although further studies maybe necessary to understand the mechanism through which CCK-8S acts, but the fact that i.p. injection with CCK stimulates neuronal proliferation and inhibits apoptosis in the hippocampus, prospects the possibility that CCK may be useful for a therapeutic approach to neurodegenerative diseases.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Li XL, Aou S, Hori T, Oomura Y. Spatial memory deficit and emotional abnormality in OLETF rats. Physiol Behav 2002;75:15-23.
2. Schneider R, Osterburg J, Buchner A, Pietrowsky R. Effect of intranasally administered cholecystokinin on encoding of controlled and automatic memory processes. Psychopharmacology (Berl) 2009;202:559-67.
3. Acosta GB. A possible interaction between CCKergic and GABAergic systems in the rat brain. Comp Biochem Physiol C Toxicol Pharmacol 2001;128:11-7.
4. Lo CM, Samuelson LC, Chambers JB, King A, Heiman J, Jandacek RJ, *et al.* Characterization of mice lacking the gene for cholecystokinin. Am J Physiol Regul Integr Comp Physiol 2008;294:R803-10.
5. Acosta GB. Administration of cholecystokinin sulphated octapeptide (CCK-8S) induces changes on rat amino acid tissue levels and on a behavioral test for anxiety. Gen Pharmacol 1998;31:637-41.
6. Dolatabad LK, Reisi P. Acute effect of cholecystokinin on short-term synaptic plasticity in the rat hippocampus. Res Pharm Sci 2014;9:331-6.
7. Nomoto S, Miyake M, Ohta M, Funakoshi A, Miyasaka K. Impaired learning and memory in OLETF rats without cholecystokinin (CCK)-A receptor. Physiol Behav 1999;66:869-72.
8. Lemaire M, Böhme GA, Piot O, Roques BP, Blanchard JC. CCK-A and CCK-B selective receptor agonists and antagonists modulate olfactory recognition in male rats. Psychopharmacology (Berl) 1994;115:435-40.
9. Greenstein RJ, Ybanez MM, Zhang RL, Bauman WA. Is aging preprogrammed? Observations from the brain/gut axis. Mech Ageing Dev 1991;61:113-21.
10. Harro J, Oreland L. Age-related differences of cholecystokinin receptor

binding in the rat brain. Prog Neuropsychopharmacol Biol Psychiatry 1992;16:369-75.
11. Voits M, Hasenöhrl RU, Huston JP, Fink H. Repeated treatment with cholecystokinin octapeptide improves maze performance in aged Fischer 344 rats. Peptides 2001;22:1325-30.
12. Hamidi G, Arabpour Z, Shabrang M, Rashidi B, Alaei H, Sharifi MR, *et al.* Erythropoietin improves spatial learning and memory in streptozotocin model of dementia. Pathophysiology 2013;20:153-8.
13. Arabpoor Z, Hamidi G, Rashidi B, Shabrang M, Alaei H, Sharifi MR, *et al.* Erythropoietin improves neuronal proliferation in dentate gyrus of hippocampal formation in an animal model of Alzheimer's disease. Adv Biomed Res 2012;1:50.
14. Gould E, Vail N, Wagers M, Gross CG. Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A 2001;98:10910-7.
15. Schlessinger AR, Cowan WM, Gottlieb DI. An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat. J Comp Neurol 1975;159:149-75.
16. Langmesser S, Cerezo-Guisado MI, Lorenzo MJ, Garcia-Marin LJ, Bragado MJ. CCK1 and 2 receptors are expressed in immortalized rat brain neuroblasts: Intracellular signals after cholecystokinin stimulation. J Cell Biochem 2007;100:851-64.
17. Sui Y, Vermeulen R, Hökfelt T, Horne MK, Stanic D. Female mice lacking cholecystokinin 1 receptors have compromised neurogenesis, and fewer dopaminergic cells in the olfactory bulb. Front Cell Neurosci 2013;7:13.
18. Zhang LL, Wei XF, Zhang YH, Xu SJ, Chen XW, Wang C, *et al.* CCK-8S increased the filopodia and spines density in cultured hippocampal neurons of APP/PS1 and wild-type mice. Neurosci Lett 2013;542:47-52.
19. Akaike A, Tamura Y, Sato Y, Ozaki K, Matsuoka R, Miura S, *et al.* Cholecystokinin-induced protection of cultured cortical neurons against glutamate neurotoxicity. Brain Res 1991;557:303-7.
20. Sugaya K, Takahashi M, Kubota K. Cholecystokinin protects cholinergic neurons against basal forebrain lesion. Jpn J Pharmacol 1992;59:125-8.
21. Manni L, Lundeberg T. Effects of cholecystokinin-8 in peripheral neuropathies: A nerve growth factor mediated action? Arch Ital Biol 2003;141:117-26.
22. Müller W, Heinemann U, Berlin K. Cholecystokinin activates CCKB-receptor-mediated Ca-signaling in hippocampal astrocytes. J Neurophysiol 1997;78:1997-2001.