

EFFECT OF *CENTELLA ASIATICA* LEAVES EXTRACT ON HIPPOCAMPAL CA3 NEURONS: A MORPHOLOGICAL STUDY IN MICE

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ABSTRACT

Introduction: *Centella asiatica* is a perennial herbaceous creeper, growing in moist soil. It has anti-thrombotic, antibacterial and antiinflammatory properties. In Ayurvedic medicine, *Centella asiatica* is used extensively as brain tonic to enhance the neural function, learning and memory. In the present study, the neuroprotective role of *Centella asiatica* (CeA) leaves extract on hippocampal CA3 neurons against stress induced neurodegeneration in mice was investigated.

Materials and Methods: Three months old albino mice were divided into four groups and treated as follows: (i) Normal control (NC) - remained without any treatment, (ii) Saline control (SC) - treated with saline, (iii) Stressed group (S) - stressed in a wire mesh restrainer for 6 hours/day for 6 weeks, (iv) Stress + CeA treated group (S+CeA) - stressed and treated orally with CeA leaves extract throughout the stress period (n=6 in all groups). At the end of the experimental period, mice in all groups were anesthetised, perfused transcardially with 50ml of saline followed by 100ml of 10% formalin. Brain was removed and processed for paraffin sections, sections were stained with cresyl violet stain. Number of neurons in the hippocampal CA3 region was counted in ten randomly selected fields (1000 μ^2 area each).

Results: Neurons in the hippocampal CA3 region, S group was found to be shrunken, irregular in shape with pyknotic nuclei compared to NC and SC groups. However such neurodegenerative changes were not seen in S+CeA group. Number of neurons in S group was significantly less compared to control groups and S+CeA group (P<0.001).

Conclusion: Oral intubation of *Centella asiatica* leaves extract can protect the hippocampal CA3 neurons from degeneration in stressed mice.

KEY WORDS: Restraint stress, Hippocampal CA3 neurons, Neurodegeneration.

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INTRODUCTION

Hippocampus belongs to the limbic system and plays important roles in the integration of information from short-term memory to long term

memory and spatial memory. Hippocampus is one of the important areas of the brain concerned with learning, memory and emotional behaviour of the individual [1]. It is also involved in the

control of adrenocortico trophic hormone (ACTH) secretion through hypothalamo pituitary adrenal (HPA) axis [2]. Damage to the neurons of the hippocampus is seen in some of the neurological disorders like Alzheimer's disease and epilepsy [3,4]. People with extensive, bilateral hippocampal damage may experience amnesia-the inability to form and retain new memories.

On the other hand, certain experiments with plant extracts like *withania somnifera*, *semicarpus anacardium* and *asparagus racemosus* protects the neurons from stress induced neurodegeneration [5,6].

Centella asiatica leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats [7,8]. But the cytoprotective property of *Centella asiatica* on the morphology of the hippocampal CA3 neurons has not been reported. The effect of restraint stress on the hippocampal CA3 neurons has been reported leading to neurodegeneration [9]. So in the present study mice were stressed in a wire mesh restrainer 6hr/day for 6 weeks and received the *Centella asiatica* leaves extract throughout the stress period. Our aim was to determine the cytoprotective effect of *Centella asiatica* leaves extract on the morphology of the hippocampal CA3 neurons.

MATERIALS AND METHODS

Male and female albino mice of 3 months of age weighing 30-36 grams were included in the present study. The mice were bred and maintained in the central animal house. The mice were maintained in 12hrs light and 12 hrs dark cycles in a well-ventilated room. Four to six mice were housed in each polypropylene cage, paddy husk was used as the bedding material, which was changed on alternate days. Mice were given ad libitum access to food and water except during stress period of the experimental study.

Extraction procedure: Fresh *Centella asiatica* leaves were collected, cleaned and sunshade dried. It was then powdered. Dry powder was weighed and mixed with distilled water at 1:10 ratio and boiled over a low flame for 30 minutes, cooled and decanted. The above procedure

was repeated twice. Clear supernatant obtained each time was decanted and then centrifuged (300 rpm for 5 minutes). And supernatant was evaporated on a low flame to get a thick paste like extract, which was later dried in a desiccator.

Drug Dosage: Dry CeA leaves extract was done and stored in air tight bottle. For each mice 500 mg/kg body weight of CeA leaves extract was administered orally throughout the experimental period (6 weeks). Plant extract was dissolved in saline to get the appropriate dilution. Drug was administered orally just before the stress exposure on each day.

Oral intubation: The required dose of drug was taken in a syringe attached with a capillary tube and tube was introduced gently into the oral cavity of the mice and the drug was delivered slowly.

Restrainer and stress procedure: A wire mesh restrainer, fabricated locally consisting of 12 compartments was used for restraint stress. Each compartment has 2" (length) X 1.5" (breadth) X 1.4" (height) dimension. Mice were stressed individually by placing within the restrainer for 6hrs/day for 6 weeks. Stress induction and its severity were assessed by measuring the suprarenal gland weight at the time of sacrifice.

Experimental design: i. Normal control group (NC) - they remain undisturbed in their home cage. ii. Saline control (SC) – mice in this group received equivolume of normal saline during the experimental period (6 weeks). iii. Stress group (S) – mice in this group were stressed in a wire mesh restrainer 6hr/day for 6 weeks. iv. Stress + *Centella asiatica* (S+CeA) - mice in this group were stressed in the same way as in the group-iii, and treated with 500mg/kg/day of aqueous leaves extract of CeA throughout the stress period (i.e 6 weeks). Drug was administered orally just before the stress exposure on each day.

A day after the last dose or equivalent day in control group, mice in all the groups were deeply anesthetized with ether and fixed on to a dissection board. The heart was exposed and 50ml of 0.9% NaCl solution was perfused through the left ventricle followed by 10%

formalin. The animal was decapitated and brain was removed and post fixed in 10% formalin for 48hrs. The brain tissue was processed for paraffin sectioning. The fixed tissue was dehydrated in the ascending grades of ethyl alcohol and then cleared with xylene. Tissues were embedded in paraffin after infiltration with the molten paraffin. Serial coronal sections of the brain through hippocampus was cut using microtome and then stained with 0.1% cresyl violet stain. Briefly, sections were deparaffinised in xylene, and hydrated in the descending grades of ethyl alcohol, and sections were brought to distilled water. Sections were stained with 0.1% cresyl violet stain at 60°C for 15 minutes. Sections were dehydrated with the ascending grades of ethyl alcohol, cleared with xylene, and mounted with DPX. Stained sections were observed under light microscope.

Quantification of neurons: Number of neurons in unit volume (cells/mm³) was quantified in hippocampus CA3 region. Number of cells in ten randomly selected 1000 μ² area were counted in each section. A total of 12-15 sections were selected from each brain (every 15th section). Finally number of cells/mm³ was calculated using stereoinvestigator principle.

Measurement of cell diameter, cross sectional area, circumference: Above parameters were measured using the Scion image analysis software. From each animal 10 sections were selected (inter-section interval was 50 microns). From each section two randomly selected fields from hippocampal CA3 region were photographed using a digital camera. These digital images were saved as bitmap image (BMP) and opened in Scion image software. After calibration various parameters were measured. Data was entered in the Excel worksheet for further statistical analysis.

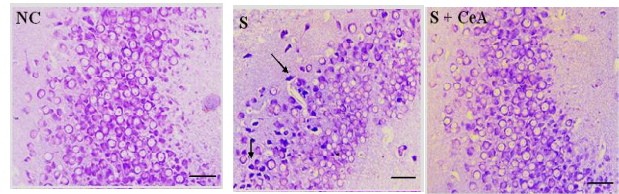
RESULTS

Quantitative neuromorphology of hippocampal CA3 neurons:

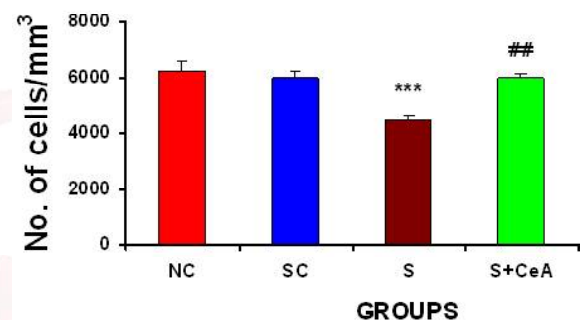
i. Numerical cell density: Numerical cell density of neurons was found to be decreased in the hippocampal CA3 neuron in the stressed mice. (6250.00 ± 335 cells/mm³ in NC vs 4500.00 ± 150.00 cells/mm³ in stressed, P < 0.001). It was found to be increased in treated groups

compared to the stressed group 6000.00 ± 150 cells/mm³ in S+CeA group Vs 4500.00 ± 150.00 cells/mm³ in stressed P < 0.01).

Fig. 1: photographs showing hippocampal CA3 neurons – cresyl violet stained. Note the S group neurons with shrunken, irregular in shape with pyknotic nuclei. (Dark cells, showing peripheral or central chromatolysis, apoptotic bodies, vacuolated cytoplasm).



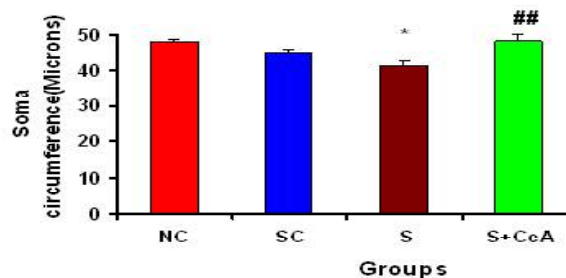
Graph 1: Showing the CA3-Numerical cell density.



Hippocampal CA3 Neuronal cell density in different groups. Note there is a decrease in numerical cell density in stressed group, which was increased in Centella asiatica leaves extract treated groups. NV Vs S - **P < 0.001; S Vs CeA-## P < 0.01 (One way ANOVA, Bonferroni's Test).

ii. **Soma Circumference:** Soma circumference was found to be decreased in the hippocampal CA3 neuron in the stressed mice. (48.05 ± 0.65 μm in NC vs 41.26 ± 1.26 μm in stressed, P < 0.05). It was found to be increased in treated groups, compared to the stressed group (48.33 ± 1.67 μm in S+CeA group Vs 41.26 ± 1.26 μm in stressed, P < 0.01).

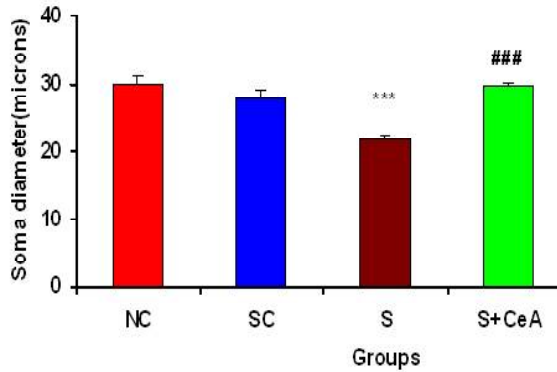
Graph 2: CA3- Soma Circumference.



Hippocampal CA3 Neuronal cell soma circumference in different groups. Note there is a decrease in circumference in stressed group, which was increased in Centella asiatica leaves extract treated groups. NV Vs S - *P < 0.05; S Vs CeA-## P < 0.01 (One way ANOVA, Bonferroni's Test).

iii. Soma diameter: Soma diameter was found to be decreased in the hippocampal CA3 neurons in the stressed mice. ($29.87 \pm 1.17 \mu\text{m}$ in NC vs $21.81 \pm 0.34 \mu\text{m}$ in stressed, $P < 0.001$). It was found to be increased in treated groups, compared to the stressed group ($29.64 \pm 0.49 \mu\text{m}$ in S+CeA group Vs $21.81 \pm 0.34 \mu\text{m}$ in stressed, $P < 0.001$).

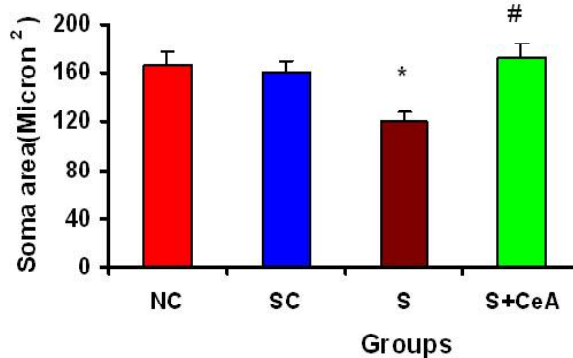
Graph 3: CA3- Soma Diameter.



Note there is a decrease in soma diameter in stressed group, which was increased in *Centella asiatica* leaves extract treated groups. NV Vs S - $*P < 0.001$; S Vs CeA-## $P < 0.001$ (One way ANOVA, Bonferroni's Test).

iv. Soma area: Soma area was found to be decreased in the hippocampal CA3 neuron in the stressed mice. ($165.93 \pm 11.7 \mu\text{m}^2$ in NC vs $120.50 \pm 7.96 \mu\text{m}^2$ in stressed, $P < 0.05$). It was found to be increased in treated groups, compared to the stressed group. ($173.00 \pm 12.07 \mu\text{m}^2$ in S+CeA group Vs $120.50 \pm 7.96 \mu\text{m}^2$ in stressed, $P < 0.05$).

Graph 4: CA3- Soma area.



Hippocampal CA3 Neuronal soma area in different groups. Note there is a decrease in soma area in stressed group, which was increased in *Centella asiatica* leaves extract treated groups. NV Vs S - $*P < 0.05$; S Vs CeA-## $P < 0.05$ (One way ANOVA, Bonferroni's Test).

DISCUSSION

In the present study, we have observed neurodegeneration in the hippocampal CA3 neurons in the stress group which may affect the various functions of hippocampus. Neurons degenerated are not replaced resulting in cognitive loss, dementia, Alzheimer's disease. The possible mechanisms of such neuronal atrophy may be due to:

Excitotoxicity: The excitotoxicity due to increased release of excitatory neurotransmitter, glutamate may be responsible for the dendritic atrophy of hippocampus CA3 neurons. Elevations in the circulating corticosterone levels can increase basal glutamate levels in the hippocampus [10]. Restraint stress has been shown to increase glutamate release in the hippocampus [11]. The major excitatory inputs to the hippocampus originates in the entorhinal area, an ipsilateral (perforant path) pathway activates the granule cells of the dentate gyrus, which in turn innervates the CA3 pyramidal neurons [12] and glutamate is the neurotransmitter involved in this pathway [13]. Entorhinal cortex lesioning protects stress induced atrophy of hippocampal CA3 neurons [14] which was claimed to be excitotoxic in nature.

Glutamate is not the only neurotransmitter involved in dendritic atrophy. Other participating neurotransmitters include GABA and serotonin, also causes neuronal damage [15,16].

Glucocorticoid toxicity: Glucocorticoid treatment induces dendritic atrophy, and this can be blocked by treatment with an adrenal steroid synthesis blocker, cyanoketone [17] indicating a role for endogenous glucocorticoids in stress-induced dendritic atrophy.

Both stress and glucocorticoid increase glutamate concentrations in the hippocampal synapse [18,19]. Glucocorticoids selectively increase glutamate accumulation in response to excitotoxic insults both in hippocampal cultures and in the hippocampus in vivo [20].

Apoptosis of neurons: Apoptosis is an organized, energy dependent process, which leads to cell death. Apoptotic cells break up into membrane bound apoptotic bodies and they are phagocytosed by resident tissue cells [21]. Repetitive perforant path stimulation in the rat

induces apoptosis and necrosis in different hippocampal neuron populations[22].

Brain-derived neurotrophic factor (BDNF): Brain-derived neurotrophic factor, a major neurotrophic factor in the brain, is critical for the survival and guidance of neurons during development, but is also required for the survival and function of neurons in the adult brain[23]. Stress and glucocorticoids are reported to decrease the expression of BDNF in the hippocampus and dentate gyrus[24]. Decreased levels of BDNF in response to stress could lead to loss of normal plasticity and eventually damage and loss of the neurons. Corticosterone induces damage to cultured hippocampal neurons via reducing their BDNF synthesis and this is attenuated by exogenously added BDNF[25].

Effects of *Centella asiatica* leaves extract on neurons: *Centella asiatica* (CeA) induce changes in the morphology of hippocampal neurons. They have protected the neurons from death and reduced the dendritic atrophy in stress condition.

The probable mechanisms involved in protection against stress induced neuronal injury are:

Neurogenesis:

CeA extract may induce neurogenesis in the hippocampus involved in stress. Enhancement of numbers of new hippocampal neurons have been observed in the dentate gyrus of adult mice and rats living in enriched environment compared to those living in standard laboratory environments[26]. The continued addition of immature neurons could allow restructuring of the hippocampus, thus providing structural plasticity. This neurogenesis has been shown to be influenced by several factors, hormones and environment and may be even certain constituents in CeA extract. Neurogenesis has been reported in the hippocampus in relation to learning or training a task[27,28]. Conversely, aversive experience like stress seems to decrease the production of new cells[29].

Neuroprotectors and antioxidants: Aqueous extract of CeA may act as an anti-oxidant and an enhancing effect on cognitive functions [30,31]. The derivatives of Asiatic acid, a triterpene extracted from *Centella asiatica* are

efficacious in protecting neurons from oxidative damage caused by exposure to excess glutamate[32]. Accordingly, in the present study the cytoprotective and antioxidant property of CeA may be responsible for the neuroprotection against cell death and the deleterious effects of stress and hence increased dendritic arborization.

CONCLUSION

From the results of our study we conclude that, oral intubation of *Centella asiatica* plant extract in stressed albino mice leads to the following:

- i. CeA leaves extract significantly protected the neurons from neurodegeneration in stressed mice.
- ii. The extract can be used in some of the neurodegenerative diseases to reduce the neuronal death since it has the neuroprotective property.

Conflicts of Interests: None

REFERENCES

- [1]. Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 1993;361:31-9.
- [2]. Knigge KM, Hays M. Evidence of inhibitive role of hippocampus in neural regulation of ACTH release. *Proc Soc Exp Biol Med* 1963;114:67-9.
- [3]. Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL. Alzheimer's disease: cell specific pathology isolates the hippocampal formation. *Science* 1984;225:1168-70
- [4]. Sloviter RS. Epileptic brain damage in rats induced by sustained electrical stimulation of the perforant path. 1. Acute electrophysiological and light microscopic studies. *Brain Res Bull* 1983;10:765-97.
- [5]. Shukla S D, Jain S, Sharma K and Bhatnagar M. Stress induced neuron degeneration and protective effects of *Semecarpus anacardium* Linn. and *Withania somnifera* Dunn. in hippocampus of albino rats: an ultrastructural study. *Indian J Exp Biol.*2000;38(10):1007-1013.
- [6]. Saxena G, Mamta Singh, Meena P, Barber S, Sharma D, Shukla S, Bhatnagar M. Neuroprotective effects of asparagus racemosus linn root extract: An experimental and clinical evidence. *Annals of Neurosciences.* 2007;14(3).
- [7]. Mohandas Rao KG, Muddanna Rao S, Gurumadhva Rao S. *Centella asiatica* (L.) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats. *Evid Based Complement Alternat Med.* 2006 Sep;3(3):349-57.

- [8]. Mohandas Rao K G, Muddanna Rao, Gurumadhva Rao. Enhancement of hippocampal CA3 neuronal dendritic arborization by *Centella asiatica* (Linn) fresh leaf extract treatment in adult Rats. *Journal of the Chinese Medical Association*. 2008;71(1):6-13.
- [9]. Mizoguchi K, Kunishita T, Chui DH, Tabira T. Stress induces neuronal death in the hippocampus of castrated rats. *Neurosci Lett* 1992;138:157-60.
- [10]. Stein-Behrens B A, Lin WJ, Sapolsky RM. Physiological elevations of glucocorticoids potentiate glutamate accumulation in the hippocampus. *J Neurochem* 1994a;63(2):596-602
- [11]. Gilad GM, Gilad VH, Wyatt JR, Tizobers Y. Region selective stress induced increase of glutamate uptake and release in rat forebrain. *Brain Res*. 1990; 525: 335-338.
- [12]. Steward O. Topographic organization of the projections from the entorhinal area to the hippocampal formation of the rat. *J Comp Neurol*.1976;167(3):285-314.
- [13]. Crawford IL, Connor JD. Localization and release of glutamic acid in relation to the hippocampal mossy fibre pathway. *Nature*.1973;244(5416):442-3
- [14]. Sunanda, Meti BL, Raju TR. Entorhinal cortex lesioning protects hippocampal CA3 neurons from stress-induced damage. *Brain Res*; 1997:770(1-2):302-6.
- [15]. McEwen BS. Stress and hippocampal plasticity. *Annu Rev Neurosci*. 1999;22:105-22.
- [16]. McEwen BS, Sapolsky RM. Stress and cognitive function. *Curr Opin Neurobiol*. 1995;5(2):205-16.
- [17]. Magarinos AM, McEwen BS. Stress induced atrophy of apical dendrites of hippocampal CA3 neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. *Neurosci*. 1995a;69: 89-98.
- [18]. Moghaddam B. Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. *J Neurochem*. 1993;60(5):1650-7.
- [19]. Moghaddam B, Bolinao ML, Stein-Behrens B, Sapolsky R. Glucocorticoids mediate the stress-induced extracellular accumulation of glutamate. *Brain Res* 1994;655(1-2):251-4.
- [20]. Chou YC, Lin WJ, Sapolsky RM. Glucocorticoids increase extracellular [3H]D-aspartate overflow in hippocampal cultures during cyanide-induced ischemia. *Brain Res*. 1994;654(1):8-14.
- [21]. Hiramane C. [Definition and morphological features of apoptosis]. *Rinsho Byori*. 1997;45(5):459-69.
- [22]. Sloviter RS, Dean E, Sollas AL, Goodman JH. Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. *J Comp Neurol*. 1996 Mar 11;366(3):516-33.
- [23]. McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. *Annu Rev Neurosci*. 1999;22:295-318.
- [24]. Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci*. 1995;15(3 Pt 1):1768-77.
- [25]. Nitta A, Ohmiya M, Sometani A, Itoh M, Nomoto H, Furukawa Y, Furukawa S. Brain-derived neurotrophic factor prevents neuronal cell death induced by corticosterone. *J Neurosci Res* 1999;57(2):227-35.
- [26]. Kempermann G, Kuhn HG, Gage FH. Experience-induced neurogenesis in the senescent dentate gyrus. *J Neurosci* 1998;18(9):3206-12.
- [27]. Gould E, Tanapat P. Stress and hippocampal neurogenesis. *Biol Psychiatry*. 1999 Dec 1;46(11):1472-9.
- [28]. Lemaire V, Aourousseau C, Le Moal M, Abrous DN. Behavioural trait of reactivity to novelty is related to hippocampal neurogenesis. *Eur J Neurosci*.1999;11(11):4006-14.
- [29]. Gould E, Tanapat P, Rydel T, Hastings N. Regulation of hippocampal neurogenesis in adulthood. *Biol Psychiatry*. 2000 Oct 15;48(8):715-20.
- [30]. Ramanathan M, Sivakumar S, Anandvijayakumar PR, Saravanababu C, Pandian PR. Neuroprotective evaluation of standardized extract of *Centella asiatica* in monosodium glutamate treated rats. *Indian J Exp Biol*. 2007;45(5):425-31.
- [31]. Gnanapragasam A, Ebenezer KK, Sathish V, Govindaraju P, Devaki T. Protective effect of *Centella asiatica* on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. *Life Sci*. 2004;76(5):585-97.
- [32]. Lee MK, Kim SR, Sung SH, Lim D, Kim H, Choi H, Park HK, Je S, Ki YC. Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. *Res Commun Mol Pathol Pharmacol*.2000;108(1-2):75-86.

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