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Effect of *Centella Asiatica* Leaves and *Clitoria Ternatea* Root Extracts on Dendritic Spine Density of Hippocampal CA3 Pyramidal Neurons in Restraint Stressed Albino Mice

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ABSTRACT

Introduction: *centella asiatica* (CeA) and *Clitoria ternatea* (CTR) are two widely used herbs since ages in traditional ayurvedic medicine. The various properties of both plants have been reported by many authors. The neuroprotective property has also been studied extensively in Alzheimer's disease, Parkinson's disease, cognition and memory enhancement, neurotoxicity, depression, and epilepsy. However, stress-induced neuroprotective efficacy of CeA and CTR plant extracts were not reported in the literature. In the present study, the effect of restraint stress and the neuroprotective property of CeA leaves and CTR root extracts on the dendritic spine density of hippocampal CA3 neurons was investigated.

Materials and Methods: three-month-old albino mice were divided into five groups. Group (i) was normal control - remained undisturbed in the cage throughout the experiment, Group (ii) was saline control, remained undisturbed but received an equal volume of saline, Group (iii) was the stress group, mice were stressed in a wire mesh restrainer for 6 hours/day for 6 weeks. Group (iv) was stress + CeA treated group. Group (v) was stress + CTR treated group. Group (iv) and Group (v) mice were stressed like group (iii) but they received oral CeA and CTR extracts throughout the stress period. After 6 weeks, the brain was removed and processed for Golgi staining.

Results: showed a significant decrease in the dendritic spine density in the stressed group, which was increased in the CeA and CTR extract-treated groups.

Conclusion: CeA and CTR extracts protected the neurons from stress-induced neurodegeneration.

Keywords: *Centella asiatica*; *Clitoria ternatea*; Restraint stress; Dendritic spine density.

Introduction

Centella asiatica (CeA) is a small perennial herbaceous creeper, growing in moist soil belonging to the umbelliferae family. CeA commonly called Mandukaparni in Sanskrit, has small fan-shaped leaves with white or pink flowers and it bears small fruits which are compressed sideways. The whole plant is reported to have the capacity to improve memory. In Ayurveda, CeA is one of the main ingredients used in products for revitalizing nerves and neurons of the brain. Mandukaparni and Mentat, are products by the Himalaya drug company, used to improve mental abilities such as improvement of memory, attention, and concentration in children with learning disability. *Clitoria ternatea* (CTR) is a vigorous, perennial herbaceous legume belonging to papilionaceae. Roots are tough having a strong smell. Root extract mixed with honey is used as a tonic given to children to boost their mental abilities. Baidyanath Shankapushpi contains an extract of CTR and it is prescribed especially for children to improve memory.

Gohil KJ *et al.* reported the anxiolytic, antidepressant, antiepileptic, cognitive, and antioxidant properties of CeA on CNS[1]. In contrast, another study by Orhan IE reported the neuroprotective properties of CeA in in-vitro, in-vivo, and clinical studies[2]. Lokanathan Y *et al.* reported the recent updates in the neuroprotective and neuro-regenerative properties of CeA[3]. CTR also exhibits almost the same properties as CeA as reported by various studies[4,5]. However, stress-induced neuroprotective efficacy of CeA and CTR were not reported. We prepared aqueous CeA leaves and CTR root extracts separately to see the neuroprotective properties in restraint-stressed mice and not use the readymade products that are available in the market. In the present study the effect of restraint stress, CeA leaves, and CTR root extracts on the dendritic spine density of hippocampal CA3 neurons was investigated.

The hippocampus has been involved in many important functions such as learning and memory processing, emotional behavior, and also in diseased conditions like Alzheimer's and epilepsy[6,7]. Repetitive stimulation, exposure of neurons to excess glucocorticoids released by the suprarenal gland due to stress, transient ischemia causes atrophy of the hippocampal CA3 neurons[8]. Studies have shown that

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increased levels of glucocorticoids, secreted during stress destroy hippocampal CA3 neurons since they are rich in glucocorticoid receptors[9,10]. Other factors that can also destroy hippocampal neurons, dendritic arborization, and spine density include alcohol, drugs, and malnutrition[11,12,13].

The dendritic spines are small projections on the dendrites for excitatory synapses between the hippocampus, the cortex, and other brain areas. The axons of the granule cells are called mossy fibers which project to the CA3 neurons of the hippocampus and synapse with the dendritic spines located on the basal portion of the apical dendrites[14] whereas fibers from the entorhinal cortex and commissural fibers form synapses on the distal portion of the apical dendrites[15]. The effect of restraint stress for 21 days showed a significant increase in the dendritic spine density of hippocampal CA3 neurons[16]. Vyas *et al*., reported dendritic remodeling in the neuronal morphology of hippocampal and amygdaloid neurons in rats due to chronic stress[17]. However, the effect of CeA and CTR plant extracts on restraint stress on dendritic spine density for six weeks has not been reported.

Materials and Methods

This study was conducted in Kasturba Medical College, Manipal, Central Animal House after obtaining ethical clearance from IEC, KMC, Manipal. IEC No-IAEC/KMC/02/2002-2003, Dated 21st Oct 2002. Three-month-old albino mice weighing 30-36 grams of both sexes were used in the present study. The mice were bred and maintained in the central animal house. Mice were given ad libitum access to food and water except during the stress period of the study.

Extraction procedure:

CeA and CTR plants were first correctly identified by pharmacy colleagues and aqueous extract was also prepared in Manipal College of Pharmaceutical Sciences, Manipal. Fresh CeA leaves were collected, cleaned and sunshade dried separately. It was then powdered. Dry powder was weighed and mixed with distilled water at a 1:10 ratio 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. The same procedure was repeated for the CTR root extract.

Extract Dosage:

Group (iv) mice received 500 mg/kg body weight of CeA leaf extract orally throughout the experimental period (6 weeks) The dose was selected from previous publications from our laboratory and preliminary study using different doses[18]. The plant extract was dissolved in saline to get the appropriate dilution. The extract was administered orally just before the stress exposure on each day. The same procedure was repeated to CTR group (v) [100 mg/kg body weight][19].

Oral intubation:

The required dose of extract was taken in a syringe attached to a capillary tube and the tube was introduced gently into the oral cavity of the mice and the extract was delivered slowly. The extract was administered very carefully in such a way as to prevent the leakage or backflow of the administered extract from the mouth.

Restrainer and stress procedure:

A wire mesh restrainer, fabricated locally consisting of 12 compartments was used for restraint stress (Fig.M1). Each compartment has a 2" (length) X 1.5" (breadth) X 1.4" (height) dimension. Mice were stressed individually by placing them within the restrainer for 6 hours/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 group (SC) – mice in this group received an equivolume of normal saline during the experimental period (6 weeks). The saline control group was used just to practice how to administer the dose into the mouth so that it wouldn't leak back from the mouth.

iii. Stress group (S) – mice in this group were stressed in a wire mesh restrainer 6 hours/day for 6 weeks.

iv. Stress + *Centella asiatica* group (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 (6 weeks). The extract was administered orally just before the stress exposure on each day.

v. Stress + *Clitoria ternatea* group (S+CTR) - stressed in the same way as in group-iii, and treated with 100mg/kg/day of aqueous CTR root extract throughout the stress period (6 weeks). The extract was administered orally just before the stress exposure on each day.

A day after the last dose or equivalent day in the control group, mice in all the groups were sacrificed with ether anesthesia. The brain was removed and processed for rapid Golgi staining (n=8 in each group).

Rapid Golgi staining:

Mice were anesthetized with ether and sacrificed by cervical decapitation. The brain was shelled out immediately and fixed in the Golgi fixative. The Golgi fixative contained Potassium dichromate (500mg), Chloral hydrate (500mg), Glutaraldehyde (0.8ml), Formaldehyde (0.6ml), Dimethyl sulfoxide (4-6 drops),

Distilled water (10ml). The brain tissue was processed in the Golgi fixative, silver nitrate impregnation, shell embedded with paraffin wax and sections were taken using microtome.

Rapid Golgi staining steps:

1- First day

Mice were anesthetized with either and quickly decapitated. The brain was rapidly shelled out and cut into two halves for better impregnation. Each half was immediately transferred to a dark-colored bottle with Golgi fixative to prevent any photo-chemical reaction and kept in a dark place. Further processing of the brain was continued after 24 hours.

2- Second day

The Golgi fixative was slowly poured out from the bottle and again brain tissue was immersed with the Golgi fixative, prepared on the previous day and stored in a dark place.

3- Third day

Fresh Golgi fixative was prepared on the third day. The brain tissue was rinsed with fresh Golgi fixative and again kept immersed in the bottle containing fresh Golgi fixative and stored in a dark place.

4- Fourth day

The bottles containing brain samples were left undisturbed.

5- Fifth day (Silver impregnation):

Silver nitrate (AgNO3), 1.5% solution, was prepared freshly. The brain tissue was gently poured out onto a Petri dish and carefully rinsed several times with the aqueous 1.5% silver nitrate solution, until the reddish brown color of the potassium dichromate–silver complex disappeared. Finally, brain tissue was placed back in a cleaned colored bottle with AgNO3 solution and kept in a dark place for a minimum of 48 hours for proper silver impregnation.

Tissue embedding:

After 48 hours of impregnation with AgNO3, the brain tissue was removed and immersed in absolute alcohol solution. The silver-chromate deposits were gently cleared using a soft paintbrush. The brain tissue was dehydrated, the tissue blocks were mounted onto a tissue holder and shell embedded with paraffin wax.

Section cutting:

Section cutting was done using a base sled microtome. The horizontal sections of the brain were taken at 120 μ thickness. The tissue surface and the cutting surface of the knife were kept moist by constant application of absolute alcohol with a brush. The section was collected in a Petri dish containing absolute alcohol and dehydrated for 10-15 minutes.

Clearing and mounting:

The sections were lifted gently with the brush, blotted dry, and then transferred to xylene in a Petri dish for clearing. The clear section was mounted on a slide with D.P.X and a cover slip. The slide was air-dried for a week before viewing under the microscope.

Dendritic spine count:

To quantify the dendritic spine, well-stained hippocampal neurons were selected. The dendrites were classified as follows, the main apical dendrite arising from soma designated as main shaft (MS), the branch arising from the main shaft as primary branch (PS), branch arising from the primary branch as secondary branch (SS). In the case of basal dendrites, the dendrite arising from the soma will be designated as primary dendrites (P) from primary as secondary (S), and from secondary as tertiary branch (T). In all types of dendrites, the number of dendritic spines along a length of 100μm (counted in 5 successive segments of 20μm each) was quantified using a calibrated ocular micrometer at high magnification. The spines were counted from the origin of the main shaft, primary and secondary in apical dendrites, and primary, secondary, and tertiary branches of basal dendrites as shown in Fig. 1

In all dendritic quantification, from each animal, 8-10 neurons were selected and the group mean was calculated. A compound microscope with 100X magnification was used to count the dendritic spines both in apical and basal dendrites. After counting, slides were marked with slide markers to avoid repetition errors.

Results

Data obtained from the above experiment was correlated and analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The graphs were plotted using the InStat GraphPad software package. There was a significant decrease in the number of dendritic spines, in the hippocampal CA3 neurons in group (iii) however, there was a significant increase in group (iv) and group (V) which was subjected to restraint stress and treated with CeA leaves and CTR root extracts respectively.

Dendritic spines:

Figure 1 shows the photograph of the dendritic spines of CA3 hippocampal neurons in all four study groups.

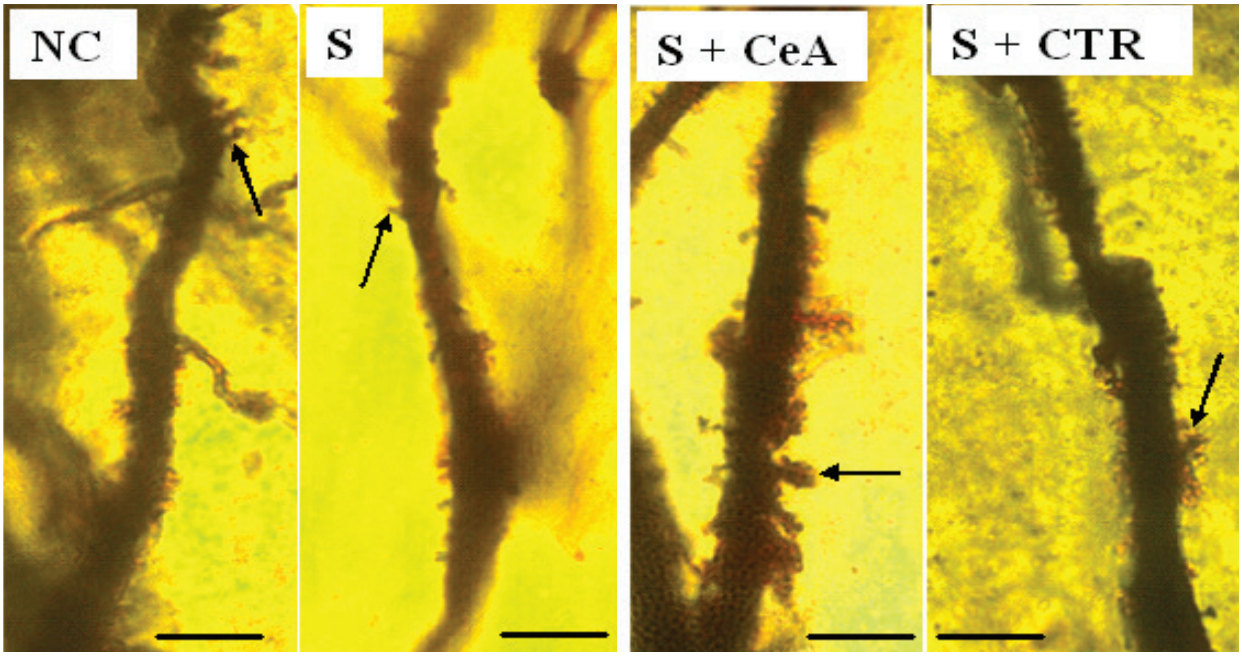

Figure 1. Dendritic spines (arrows) in CA3 hippocampal neurons in the different experimental groups.

Spine density on apical dendrites:

Dendritic spines (100µm length) on the main shaft, primary, and secondary shaft were decreased significantly in the stressed group compared to the normal and saline control group (P<0.01). Dendritic spines are significantly increased in CeA and CTR extract-treated groups compared to the stress group (P< 0.001) (Fig. 2)

Spine density on basal dendrites:

Dendritic spines (100µm length) on the primary, secondary, and tertiary branches were decreased significantly in the stressed group compared to the normal and saline control group (P<0.05-0.001). Dendritic spines are significantly increased in CeA and CTR extract-treated groups compared to the stress group (0.001) (Fig. 3).

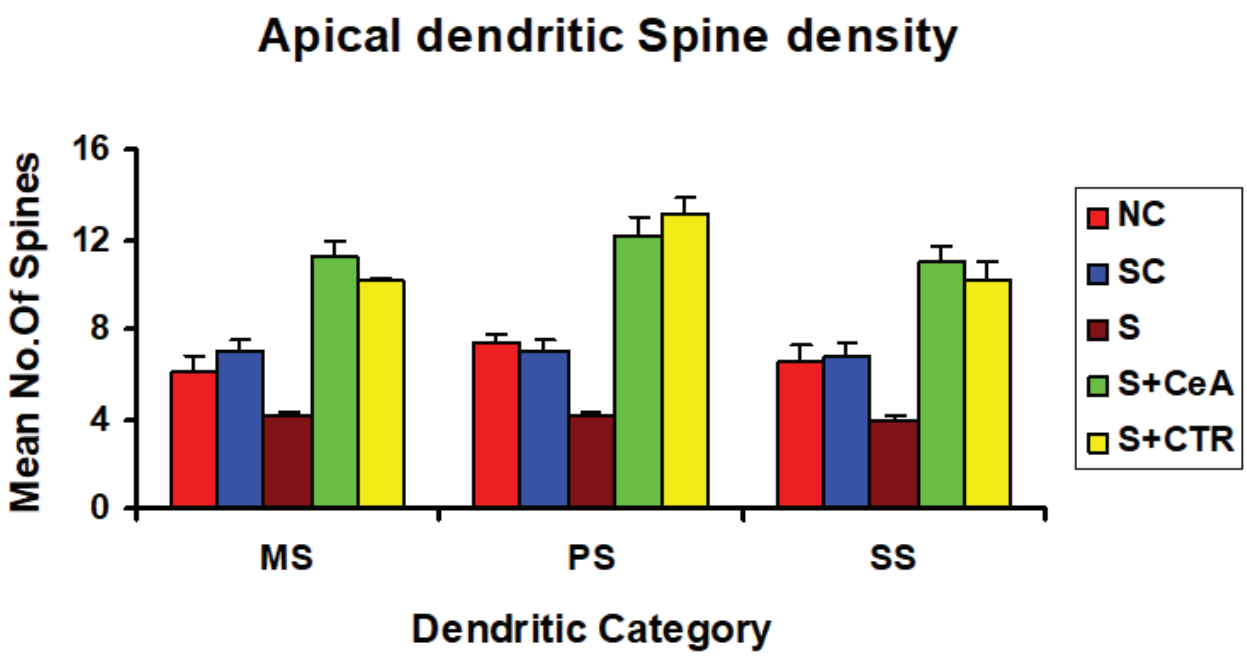

Figure 2. Apical dendritic spine density in CA3 hippocampal neurons in the different experimental groups. Note there is a decrease in dendritic spine density in the stressed group, which was increased in *Centella asiatica* leaves extract and *Clitoria ternatea* root extract treated groups. NC vs S- ** P<0.01; S vs CeA - ### P<0.001; S vs CTR- $$$ P<0.001 (One way ANOVA, Bonferroni's Test).

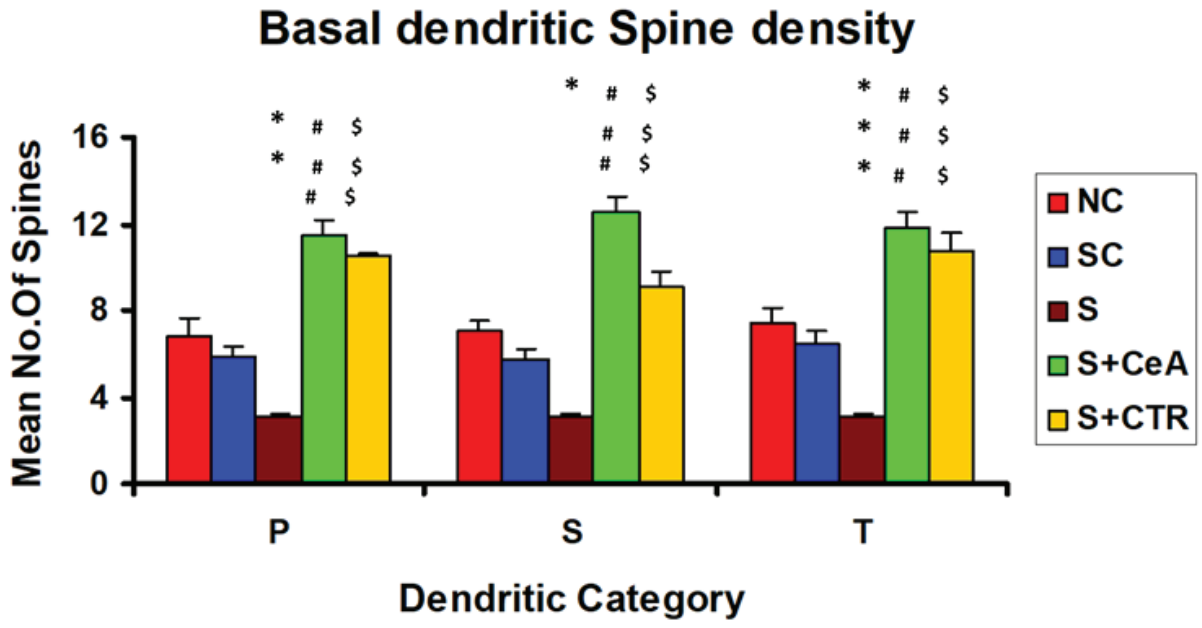

Figure 3. Basal dendritic spine density in CA3 hippocampal neurons in the different experimental groups. Note there is a decrease in dendritic spine density in the stressed group, which was increased in Centella asiatica leaves extract and Clitoria ternatea root extract treated groups. NC vs S- ** P<0.01; S vs CeA - ### P<0.001; S vs CTR- $$$ P<0.001 (One way ANOVA, Bonferroni's Test).

Discussion

It is clear from the study that there was a marked decrease in the number of dendritic spine density in stressed mice. However similar study by Sunanda *et al.* showed a decrease in the number of dendritic branches but an increase in the dendritic spine density of hippocampal CA3 neurons, to compensate for the dendritic atrophy induced by the restraint stress for 21 days. But in our study mice were subjected to restraint stress for a longer period i.e. for 6 weeks, which increased the level of glucocorticoids for a longer duration resulting in atrophy of dendritic arborization[20.21], and also dendritic spine density.

Glutamic acid is the most important excitatory neurotransmitter of the hippocampal neurons[22]. Restraint stress can lead to increased outflow of glutamate in the prefrontal cortex, and hippocampus[23]. Increased levels of glutamate result in dendritic atrophy as a result of excitotoxicity[24]. In addition to glucocorticoids, corticotrophin-releasing hormone might mediate stress-induced dendritic regression[25]. Mice lacking $CRFR_1$, showed profuse branching and dendritic length in hippocampal neurons[26].

An increase in the number of dendritic spines observed in Group (iv) and Group (v) indicates the neuroprotective effect of CeA and CTR[27,28]. The formation of new spines could be because of unusual axon terminal sprouting followed by rearrangement of synapses on the existing dendrites after atrophy. Nora EG *et al.*, in their experiment, reported that several constituents of *Centella asiatica* can increase synaptogenesis and arborization in isolated hippocampal neurons (In vitro)[29] but in our study, it was oral intubation of the CeA and CTR extract (In vivo) and it increased the dendritic arborization and dendritic spine density in CA3 hippocampal neurons.

The exact mechanism by which CeA and CTR extracts improve the dendritic spine density requires explanation. The spine formation and dendritic arborization involves a variety of biological pathways and processes like transcription factors, receptor-ligand interactions, various signaling pathways, cytoskeletal elements, Golgi outposts, and endosomes contribute to the organization of dendrites in neurons[30].

CeA down-regulates histone H3K9me3 which improves memory, promotes spine formation, and increases BDNF levels in the hippocampus[31]. Histone H3K9 can turn the genes on by getting acetylated and silencing them easily when methylated. CeA significantly upregulated the level of activated ERK1/2 and Akt suggesting their involvement in the neurite outgrowth-promoting activity[32,33] but the exact molecular mechanisms are still restricted.

An increase in dendritic spine density in Group (v) by CTR root extract has not been reported. We are the first to report this property of *Clitoria ternatea* root extract increasing the dendritic spine density of hippocampal CA3 neurons in restraint stress mice. The exact mechanism of how it increases the dendritic spine density needs to be investigated.

Conclusions

From the results of our present study, we conclude that oral intubation of both *Centella asiatica* and *Clitoria ternatea* plant extracts in restraint-stressed

albino mice significantly increased the dendritic spine density in hippocampal CA3 pyramidal neurons. The increase in dendritic spine density was comparatively more in the group treated with *Centella asiatica* than in *Clitoria ternatea* group except in the apical dendrites primary branch.

Significance of the study:

This study proves the efficiency of these plant extracts as neurostimulants and neuroprotective nature, protecting the neurons against stress-induced neuronal atrophy.

They can also be used to prevent age-related, drug-induced or spontaneous neurodegeneration.

Limitations

- Biochemical assays (Neurotransmitters, protein PSD95, synaptophysin) and further molecular level investigations are required to tell the exact pathway how the active components of these plant extracts cause the changes in the spine density of hippocampal CA3 neurons.
- Stress related behavioural tests also can be done to confirm the results.

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