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Effect of mucus extract of *Helix aspersa* on scopolamine-induced cognitive impairment and oxidative stress in rat's brain

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Snail extracts are complex multicomponent mixtures comprising antibacterial, antiviral, immunomodulating, antioxidant and anti-inflammatory activities. Oxidative stress along with inflammatory and immune mechanisms are believed to be critical factors in the pathogenesis of neurodegenerative diseases. The aim of this pilot study was to evaluate the effect of snail (*Helix aspersa*) mucus extract on scopolamine-induced cognitive impairment and oxidative stress in rat brain cortex. The scopolamine (Sco) was applied i.p. (2 mg/kg) in male Wistar rats for 11 days, along with oral administration of snail mucus extract (0.5 mL/100 g). On the 1st, 5th and 12th day, the animals were subjected to Step-through behavioral test. On the 12th day, the cortex was isolated and oxidative stress parameters of lipid peroxidation (LP) and total glutathione (GSH), activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were spectrophotometrically assayed. The results of the behavioral test showed a significant improving effect of snail extract on the learning and memory of Sco-treated animals. The Sco treatment provoked increase in LPO, CAT and GPx activities and decrease in tGSH and SOD activity. The application of snail mucus extract led to recovery of oxidative stress parameters close to the control group values. In conclusion, the snail extract demonstrated a protective effect against Sco-model of dementia, probably *via* an antioxidant mechanism. Further research is needed to evaluate the therapeutic potential of *Helix aspersa* mucus for treatment of neurodegenerative disorders.

Keywords*:* snail (*Helix aspersa*) mucus extract, oxidative stress, scopolamine dementia, rats

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of great social importance that has estimated prevalence of 10-30% in the population over the age of 65, with an incidence of 1-3% [1]. According to available forecasts worldwide, based on data from continuous surveys, by 2030 the total number of patients with AD in the world will reach 65.7 million, and by 2050 - 115.4 million people [2].

Oxidative stress is recognized as a common pathway of cellular injury in both acute and chronic neurological diseases [3]. The sources of ROS-mediated damage appear to be multi-faceted in AD, with interactions between abnormal mitochondria, redox transition metals and other factors [4]. Because of brain's high oxygen consumption, along with its abundant lipid content, lipid peroxidation was accepted as the primary mechanism for neuronal degeneration [5]. Reactive oxygen species (ROS) rapidly oxidize cellular lipids, resulting in the formation of numerous lipid peroxidation products, leading eventually to neuronal death. Increased activity of endogenous antioxidant enzymes (i.e. catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase) have also been observed along with β-amyloid deposits in temporal regions (e.g. hippocampus) of the AD brain, reflecting a compensatory mechanism to counter oxidative stress [1].

Nowadays, the used treatments against AD give limited symptomatic improvements and cannot stop the progression of the disease. Since AD is a heterogeneous disorder, the efforts should be directed to examine multimodal strategies, including antioxidant therapy [6]. In the recent years, there are accumulating data about wide varieties of natural antioxidants from different biological sources, such as plants, fungi, bacteria, marine sponges and molluscs with neuroprotective effects [7].

It is known that terrestrial slugs and snails produce mucus which performs a variety of functions and the excreted biological fluid is a rich source of bioactive natural compounds that are used for the treatment of a number of skin ailments like wounds, burns, scars, psoriasis, acne, keratosis, wrinkles, and age and skin damage [8, 9]. In general, these properties are bound to the ability of the organism to counteract free radicals generation.

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The results from the antioxidant screening of *C. aspersum* mucus and its fractions showed that this naturally derived product, specifically the low-molecular weight fractions, possess the properties to counteract the formation of reactive free radicals [10].

Recently, some researchers have confirmed that the *Helix aspersa* mucus contains a large number of natural substances as allantoin and glycolic acid, with beneficial and therapeutic properties for human skin [11]. Moreover, according to Guskov *et al.* [12] the antioxidant potential of the mucus may be due to the allantoin, which has been shown to possess antioxidant properties. Other findings showed that allantoin has therapeutic potential for the cognitive dysfunctions observed in Alzheimer's disease [13]. However, to this date, there are scarce data and no conclusive evidence for the antioxidant potency of the compounds in snail species mucus, and this fact opens a variety of future prospective investigations in this aspect – eventual antioxidant properties in pathological conditions, including scopolamine-induced Alzheimer's type dementia. These facts determined the purpose of our pilot study: to evaluate the effect of snail (*Helix aspersa*) extract on cognitive impairment and oxidative stress in brain cortex of rats with experimental scopolamine-induced dementia.

EXPERIMENTAL

Materials and treatment

The main reagents (glutathione, riboflavin, methionin, 2-thiobarbituric acid, NADP+, reduced and oxidized NADPH) were obtained from Sigma-Aldrich (Germany). Scopolamine was purchased from ACROS Organics (Germany) (as Thiogamma Turbo-Set solution for injection 600 mg, 50 ml). All other used chemical substances were of the highest commercially available purity.

Crude mucus was collected from Bulgarian snail *Helix aspersa* and a fresh extract was purified using patented technology without suffering of any snail. We evaluated the snail extract (SE) effects in an experimental model of neurodegeneration - Alzheimer's disease (AD). AD type dementia was produced by scopolamine treatment (2 mg/kg, i. p., 11 days) of male Wistar rats (180-200 g). The animals were housed at 22°-25°C with free access to food and water, and a natural day/night light cycle. During scopolamine treatment, the animals received also snail extract (SE) (0.5 ml/100 g. p.o.) or saline for controls. The snail extract was administered for 16 days, 5 days before, and 11 days simultaneously with Sco. On the 12th day after last treatment, dynamics of changes in learning and memory performance in animals were evaluated behaviorally by a Step-through test. Immediately after the behavioral test was made and the brain cortex was isolated - dissected by the method of Valzelli and Garattini [14] and was spectrophotometrically assessed for the main oxidative stress markers. In 10% homogenates, centrifuged for 10 min at 3000 rpm, we measured the levels of lipid peroxidation and total glutathione, part of the post-nuclear homogenate was centrifuged for 20 min at 12,000 rpm. The post-mitochondrial supernatant was used for measuring the activities of antioxidant enzymes: catalase, Cu,Zn-superoxide dismutase and glutathione peroxidase.

All experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Behavioral Method

Using passive avoidance (Step-through test) [15] learning and memory performance of the rats were evaluated. Acquisition latency (initial latency (IL) time was measured before all treatments. The retention trial, where the interval between the placement in the illuminated chamber and the entry into the dark chamber was measured on the 12th day as step-through latency (STL). Behavioral observations were carried out from 9 a.m. to 12 a.m.

Analytical methods

Protein content was measured by the method of Lowry *et al.* [16]. Lipid peroxidation (LP) was determined by the amount of thiobarbituric acid reactive substances (TBARs) formed in fresh biological preparations according to Hunter *et al.* [17]. The values were expressed in nmoles malondialdehyde (MDA) per mg protein, with a molar absorption coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Total glutathione (tGSH) levels were measured according to Tietze [18] and were expressed in ng/mg protein, with glutathione oxidized (GSSG) as a reference standard.

Catalase (CAT) activity was determined according to the method described by Aebi [19] and the enzyme activity was expressed as ΔA240/min/mg protein.

Cu,Zn-superoxide dismutase (SOD) activity was determined according to the method of Beauchamp and Fridovich [20], expressed in U/mg protein (one unit of SOD activity is the amount of the enzyme, producing a 50% inhibition of Nitroblue tetrazolium

reduction).

Glutathione peroxidase (GPX) activity was measured by the method of Günzler *et al*. [21], using a molar absorption coefficient of $6.22\times10^{6}M^{-1}cm^{-1,}$ and was expressed in nmoles NADPH oxidized per minute, per mg protein.

Statistical analysis

The results were statistically analysed by one-way ANOVA and Dunnett post-hoc test, with $p<0.05$ accepted as the minimum level of statistical significance of the established differences.

RESULTS AND DISCUSSION

After finishing the treatment of rats with scopolamine and snail extract, both the changes in the cognitive functions of the animals and the biochemical parameters were evaluated in all groups.

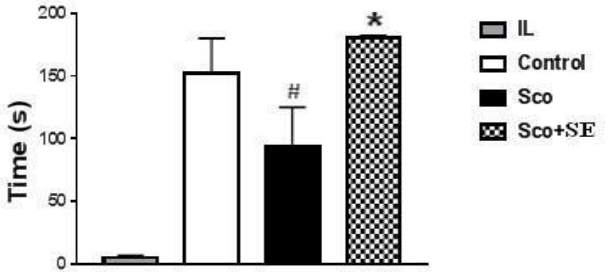

Fig. 1. Effects of SE on step-through latency (STL) in a single-trial passive avoidance test in rats; IL - initial-latency, *p< 0.05 Sco group *vs* Sco+SE group, # p< 0.05 Sco group *vs* control group.

The obtained results demonstrated statistically reliable changes in learning and memory in the Sco group – about 35% memory impairment compared to controls. Sco-SE group demonstrated significant improving effect on the learning and memory of animals treated simultaneously with Sco. SE recovered significantly (by 48%) the memory loss, in comparison to Sco-treated group.

The treatment with SE of Sco-animals also showed significant beneficiary effects upon all measured biochemical parameters. Significantly increased levels of LP were observed in the cerebral cortex of Sco-treated animals in comparison to the control rats (Fig. 2).

Scopolamine administration led to elevation of TBARs content in rat brain cortex by 25%. SE reduced the Sco-induced elevation of TBARs restoring to control levels.

As a result of lipid peroxidation, a great variety of aldehydes can be produced - malondialdehyde (MDA) is the most important indicator and excellent index of lipid peroxidation [22]. Our results correlated with several studies that reported elevated levels of LP in different brain regions (cortex, hippocampus, etc.) in AD [23, 24].

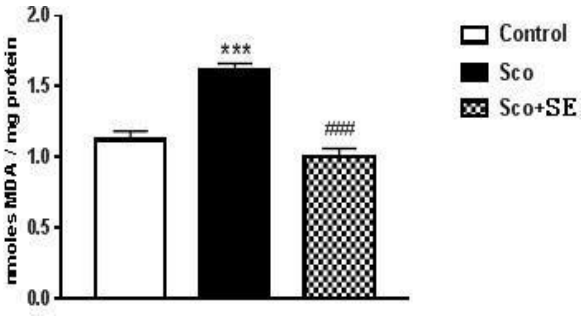

Fig. 2. Effects of SE on lipid peroxidation in rat brain cortex Data are expressed as the mean ± SEM, *p< 0.001 *vs* control group, # p< 0.05 *vs* Sco group

The treatment of the animals with Sco in comparison to the control group led to a decrease of tGSH levels in cortex by about 30% (Fig. 3).

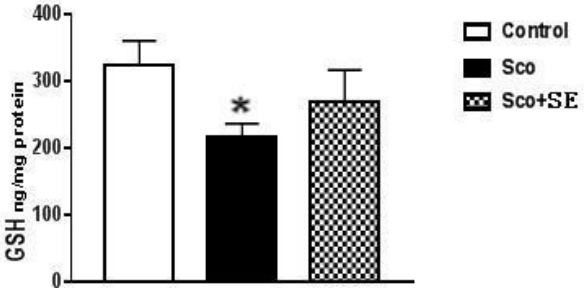

Fig. 3. Effects of SE on tGSH in brain cortex. Data are expressed as mean ± SEM *p< 0.05 *vs* control group.

The SE partly restored the Sco-induced reduction of tGSH levels in cortex and we indicated a preventing positive effect. In the literature, many scientists assessed and described the considerable role of GSH in AD onset and progression. AD-associated reductions in GSH levels have been documented *in vivo* in animal models of AD [25].

Cognitive impairment is accompanied with alterations in antioxidant enzymes in different brain regions and several laboratories have reported changes in the activities of SOD, CAT and GPx [26-28].

In our findings, there was an activation of antioxidant enzymes CAT and GPx in the co-group in cortex and decreased activity of SOD (Table 1).

Changes of CAT activity were observed - increase in the Sco-group about 50% compared to the control group. Evaluated CAT activity in the Sco-SE group was reduced by SE in cortex and values were lower by about 27% than those of healthy animals.

From the detected values of antioxidant enzymes' activities in cortex, this of the SOD was strongly pronounced – a significant decrease by about 50% in Sco-group compared to control group.

Table 1. Effects of snail (*Helix aspersa*) extract on the activities of antioxidant enzymes catalase, superoxide dismutase and glutathion peroxidase in cortex (controls, sco-treated and sco+snail in rats (mean±SEM).

Cortex	CAT [dA240/min/mg]	SOD [U/mg protein]	GPx [U/mg protein]
Controls	0.073 ± 0.02	10.11 ± 1.243	9.35 ± 2.025
Sco	0.110 ± 0.02	5.00 ± 0.986	13.84 ± 2.967
Sco + SE	0.056 ± 0.03	6.14 ± 2.276	10.86 ± 0.885

Differences were observed in Sco-SE treated animals – a slight increase in cortex by about 15% compared to that of Sco-treated. The obtained results for GRx activity demonstrated the following changes in the cortex – increased in Sco-treated rats by about 35% compared to control group. Snail extract had positive effect in cortex of SE-treated animals, recovering control values (statistically unproven). Our recent results support and extend numerous previous findings of enhanced oxidative stress in AD and also confirmed the ability of scopolamine to produce similar to AD-type dementia accompanied by oxidative stress. The changes of oxidative stress markers significantly correlated with reports about investigations in human post-mortem frontal cortex from individuals characterized as mild cognitive impairment, mild/moderate Alzheimer disease, and late-stage Alzheimer disease [28]. Tissue fraction had significant declines in antioxidants (glutathione, glutathione peroxidase, catalase, and superoxide dismutase) and levels of LPO. This effect was established by many other studies in the literature [29, 30]. It is well established that the neuroprotective effects of antioxidant compounds involved their radical scavenging and metal chelating activity and/or the regulation of antioxidant enzymes [31]. Moreover, molecular biology studies suggested that natural antioxidants were able to modulate the expression of genes that encode for apoptosis-related molecules [32]. There are different types of antioxidants, each of which has a slightly different role and provides guidance around their potential benefits in relation to dementia. This makes it difficult to examine 'antioxidants' as a general and single aspect in dementia risk. The molecular mechanisms of neuronal degeneration remain largely unknown and effective therapies are not currently available [33]. Trying to explain the proposed mechanisms of action, underlying the neuroprotective effects of the natural exogenous antioxidants, they are now being looked upon as persuasive therapeutics against neuronal loss, as they have capability to combat by neutralizing free radicals.

Unfortunately, for now, the outcomes of many clinical trials with different antioxidants demonstrated minimal therapeutic effects or have shown conflicting results, but there are lots of antioxidant supplements on the market containing bioactive compounds, claiming to have undeniable positive health benefits. Further investigations will bring us more clearly about the beneficial role of snail *(Helix aspersa)* extract and to clarify the mechanisms of neurodegenerative disorders, affecting learning and memory processes.

CONCLUSION

From our study, it could be assumed that the activation of antioxidant enzymes in response to Sco-induced OS is a cellular protective mechanism [34]. Although the obtained results could not provide clear understanding of the mechanism of action of the SE, whether acting directly or indirectly, it could be hypothesized that SE is able to affect positively the impaired brain cognitive functions. Owing to the obtained evidence, our suggestion is that snail extract acts as antioxidant and stimulates important stress-response pathways in cell affecting endogenous cellular antioxidant levels and diminishing the neurodegenerative processes and could help develop drugs for demented patients for controlling and manipulating memory, and may represent one of a new generation of bioactive drugs for improving brain functions.

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