Effects of tachykinin neuropeptide NKB and A*β* (25-35) on antioxidant enzymes status in 17*β* estradiol treated aging female rats

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ABSTRACT

Aging is the leading risk factor for neurodegenerative diseases and oxidative stress involved in the pathophysiology of these diseases. These changes increase during menopausal condition in females when the level of estradiol is decreased. The aim of the present study was to determine the effect of tachykinin neuropeptide, Neurokinin B (NKB) and Amyloid beta fragment A*β* (25 - 35) on 17*β* estradiol (E2) treated aging female rat synaptosomes of different age groups. Aging brain functions were assayed by measuring the activities of antioxidant enzymes—superoxide dismutase (SOD) and monoamine oxidase (MAO) with neuropeptides. An *in-vitro* incubation of A*β* (25 - 35) in E2 treated brain synaptosomes showed toxic effects on all the parameters. However, NKB and NKB combined with A*β* (25 - 35) showed stimulating effects in E2 treated rat brain synaptosomes. In the present study, an increase in activity of SOD and decrease in the level of MAO, in the presence of NKB and combined NKB and A*β* in E2 treated brain synaptosomes of aging rats. This study elucidates that treatment of NKB and A*β* with E2 in combination exerts more protective influence than their individual application, against excitotoxicity in age related changes.

Keywords: Aging; Neurokinin B; Amyloid Beta (25 - 35); Estradiol; Antioxidant Enzymes

1. INTRODUCTION

Aging is a time-dependent functional decline, leading to the cell's incapacity to withstand external and internal challenges. With aging, there is a physiological decline and an increase in the prevalence of diseases [1]. It is the consequence of two independent biological processes: the loss of functionality, and the loss of resistance or adaptability to stress. Increase in reactive oxygen species (ROS) production and imbalance in antioxidant defense and repair mechanism leads to cell death during aging and age related neurological disorders [2]. Highly ROS cause cell damage, including lipid peroxidation, inactivation of enzymes, alteration of intracellular oxidation-reduction state and resultant damage in aging brain [3].

The ovarian steroid hormone E2 is one of the most important hormones, and it can protect neurons against A*β* toxicity, oxidative stress and excitotoxicity [4-6]. The effect of E2 is primarily mediated by ERa and ERb which are members of the nuclear receptor superfamily of ligand-activated transcription factors [7]. E2 modulates multiple functions of the brain, via activation of ERa and ERb including development, cognition and memory [8], highlighting its protective effects against neuronal damage [9].

There are significant gender differences in the human brain diseases. For example, females on hormone replacement therapy (HRT) are significantly less likely to suffer from Alzheimer's disease (AD) than men [10]. There has been growing interest in the action and functions of the E2, particularly on whether they are neuron-protective for such age related diseases and neurodegenerative conditions like stroke, Alzheimer's disease (AD) and Parkinson's disease (PD) [11].

Peptides and proteins convert under some conditions from their native soluble state into highly ordered fibrillar aggregates. Such transitions are associated with numerous illnesses such as AD and PD [12]. The major constituents of amyloid plaques are fibrils formed from A*β*. The aggregation pathway involves the formation of intermediate oligomers that elongate to form fibrils.

These oligomers are believed to be the major neurotoxic species [13].

Mammalian tachykinins comprise a family of regulatory peptides including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) [14,15]. They are known to reduce oxidative stress in the brain [16-18]; to reverse the neurotoxic effects of Aβ in neurons and a role in neurodegenerative diseases [19,20].

E2 treatment had beneficial effects on antioxidant enzymes in aging rat tissues as reported earlier by Baquer and colleagues [5]. In the present study, we examine the neuroprotective effect of NKB and with E2 against Aβ (25 - 35) toxicity on the activity of superoxide dismutase and monoamine oxidase in the brain of aging female rats.

2. MATERIALS AND METHODS

2.1. Animals

The present study was conducted on female albino rats of the Wistar strain in different age groups (3, 12 and 24 months). Animals were maintained in the animal house facility of Jawaharlal Nehru University (JNU), New Delhi, India at a constant temperature of 25°C, humidity 55% and 12 h dark and light cycle. The animals were fed standard chow rat feed (Hindustan Leaver Ltd., India) and given tap water until the time of sacrifice. The Institutional Animal Ethics Committee (IAEC) of JNU approved all the animal experiments; all institutional guidelines for care of animals were followed.

2.2. Hormone Administration

Subcutaneous injections of E2 (0.1 μg/g body weight) were given daily for one month, to the aged rats (12 and 24 months old; n = 8 for each group). E2 was dissolved in propylene glycol in appropriate concentrations [21]. Control animals received an equal volume of vehicle. There was no treatment on the day of the sacrifice. Animals of all the groups were sacrificed and brains were isolated for further study.

2.3. Preparation of Synaptosomes

The animals from control and E2 treated groups were sacrificed by cervical dislocation. The whole brain was excised and washed in ice-cold saline (0.9% NaCl). Tissue homogenates were prepared as described by Mayanil *et al.* 1982 [22]. Tissues were soaked, dried on blotting paper and weighed, minced and homogenized in nine volumes of homogenizing buffer containing 0.25 M sucrose, 0.02 M triethanolamine (pH 7.4) and 0.12mM dithiothreitol. The pellet obtained after centrifugation at 12,000 (rpm) containing synaptosomes and mitochondria were taken for the present study. The whole procedure was carried out at 4°C.

2.4. Treatment of Synaptosomes with NKB and Aβ (25 - 35)

Each sample containing ~100 μg protein of isolated rat brain synaptosome was incubated with NKB, Aβ (25 - 35) and NKB + Aβ (25 - 35) in microfuge tubes at 37°C for 60 min in a shaking water bath with 0.1, 1 and 5 μM concentration of each of the peptides. All incubations were performed in four combinations; control (without any peptide), Aβ (25 - 35), NKB and NKB + Aβ (25 - 35) in three age groups of control and E2 treated rats at three peptide concentrations.

2.5. Measurement of Superoxide Dismutase (SOD) Activity

Synaptosomes prepared from rat brains of different age groups were treated with Triton X-100, and Mn-SOD activity was determined by the pyrogallol autoxidation. Pyrogallol is known to autoxidize rapidly in alkaline solution and the reaction has been employed for the removal of oxygen from gases [23]. The SOD activity was measured in the synaptosomes isolated from 3, 12 and 24-month-old control rat (without E2 treatment), and E2 treated rat brain synaptosomes in the presence and absence of Aβ (25 - 35), NKB and combined NKB and Aβ (25 - 35). The activity of SOD activity expressed as units per mg protein per minute. One unit of the enzyme activity defined as the amount of enzyme that causes half-maximal inhibition of pyrogallol autoxidation.

2.6. Measurement of Monoamine Oxidase (MAO) Activity

The MAO activity was measured in the synaptosomes isolated from 3, 12 and 24-month-old control rats (without E2 treatment), and E2 treated rat brain synaptosomes in the presence and absence of Aβ (25 - 35), NKB and combined NKB and Aβ (25 - 35). MAO activity was assayed according to the method of Catravas *et al.* 1977 [24] with minor modifications. The amount of the reaction product *i.e.* 4-hydroxyquinoline formed was determined spectrophotometrically at 330 nm. One unit of the enzyme defined as 1 μmol of 4-hydroxyquinoline produced/mg protein/min at 37°C.

2.7. Protein Estimation

Protein was estimated in the synaptosomes by the method of Bradford, 1976 [25] using bovine serum albumin (BSA) as standard.

2.8. Statistical Analysis

Data have been presented as mean ± standard error of mean (SEM). The data were analyzed using one way ANOVA to test for differences between different treat-

ments at different age groups. Differences between the means of the individual groups were assessed by Dunnett's multiple comparisons test. A value of $p < 0.05$ was considered to be statistically significant.

2.9. Chemicals

All substrates, standards, NKB and Aβ (25 - 35) peptide fragment were purchased from Sigma Chemicals Company, USA. All other chemicals were of analytical grade and purchased from SRL and Qualigens, India.

3. RESULTS

3.1. Effect of E2 on General Physiological Parameters in Aging

The changes in general parameters like body weight, brain weight and protein concentration in brain synaptosomes from different rat age groups namely 3, 12 and 24 months, are presented in **Table 1**. In this study, we observed an increase in body weight and brain weight in 12 and 24-month-old control rats when compared to 3-month young rats (Result stated in the **Table 1**) but weight decreased significantly with E2 treatment when compared with respective controls. However, protein content increased significantly in rat brain synaptosomes of E2 treated rats. It was observed that protein content increased by 49% ($p < 0.001$) in 12-month-old control rat brain synaptosomes and 66% ($p < 0.05$) in E2 treated 12-month-old rat brain synaptosomes. Protein content was calculated as mg per gm of whole brain.

3.2. Superoxide Dismutase (SOD)

Changes in SOD activity were measured in rat brain synaptosomes, in different age groups with and without E2 treatment, at different concentration Aβ (25 - 35), NKB and NKB + Aβ (25 - 35). The Results are shown in **Figures 1(a)-(c)**.

3.2.1. Effect of E2 and Varying Concentrations of Aβ (25 - 35) on SOD Activity

The SOD activity was found to be reduced in the synaptosomes of control (without E2) rat brain incubated with different concentrations of Aβ (25 - 35). However, decrease in the activity was less in E2 treated rat synaptosomes. Incubation of 5 μM of Aβ (25 - 35) in E2 treated 12 and 24-month-old rat brain synaptosomes showed less decrease as compared to incubation of 0.1 μM and 1 μM of Aβ (25 - 35) in 12 and 24-month control rats ($p < 0.05$ and $p < 0.05$). The results are shown in **Figure 1(a)**.

3.2.2. Effect of E2 and Varying Concentrations of NKB on SOD Activity

The activity of SOD was observed to increase in all control age groups with NKB incubation, whereas this increase in enzyme activity was more significant in E2 treated rats, as compared to age matched control groups. Increase in SOD activity was highly significant in E2 treated group of 12 and 24-month-old rat using a dose of 5 μM of NKB namely 57% and 72% ($p < 0.01$ and $p < 0.01$) respectively. The results are shown in **Figure 2(b)**.

3.2.3. Effect of E2 and Varying Concentrations of Combined NKB and Aβ (25 - 35) on SOD Activity

The SOD activity in control synaptosomes was observed to increase when treated with a combination of NKB and Aβ (25 - 35), but this increase was more significant in synaptosomes of E2 treated 12 and 24-month aging rats. The combined dose of NKB and Aβ (25 - 35) at 5 μM concentration in 24-month E2 treated rats showed a significantly raised SOD activity as compared to matched control ($p < 0.01$). The results are shown in **Figure 1(c)**.

3.3. Monoamine Oxidase (MAO)

Changes in MAO activity were measured in rat brain

Table 1. Body weight, Brain tissue weight and synaptosomal protein of 3, 12 and 24-month control (C) and estradiol (E2) treated female rats.

Age/Treatment	Body Weight	Brain Weight	Brain Weight/100gm Body Weight	Protein (mg/gm)
3-month (C)	202 ± 14.37	1.65 ± 0.05	0.81 ± 0.11	10.48 ± 0.55
12-month (C)	323 ± 19.46[$]	2.05 ± 0.07[#]	0.63 ± 0.09	15.65 ± 0.35[$]
12-month (E2)	267 ± 10.44[c]	1.86 ± 0.10	0.57 ± 0.07	17.48 ± 0.61[c]
24-month (C)	380 ± 13.32[$]	1.94 ± 0.13	0.51 ± 0.10	13.54 ± 0.72[#]
24-month (E2)	321 ± 13.82[c]	1.80 ± 0.13	0.56 ± 0.12[a]	16.64 ± 0.70[b]

Each value is a mean ± SEM of five or more separate values from two or three experiments. The comparison of experimental values is with the control values. Stastical significane: [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$ comparising age matched control verses E2 treatment, [$]$p < 0.001$, [#]$p < 0.01$ verses 3 months.
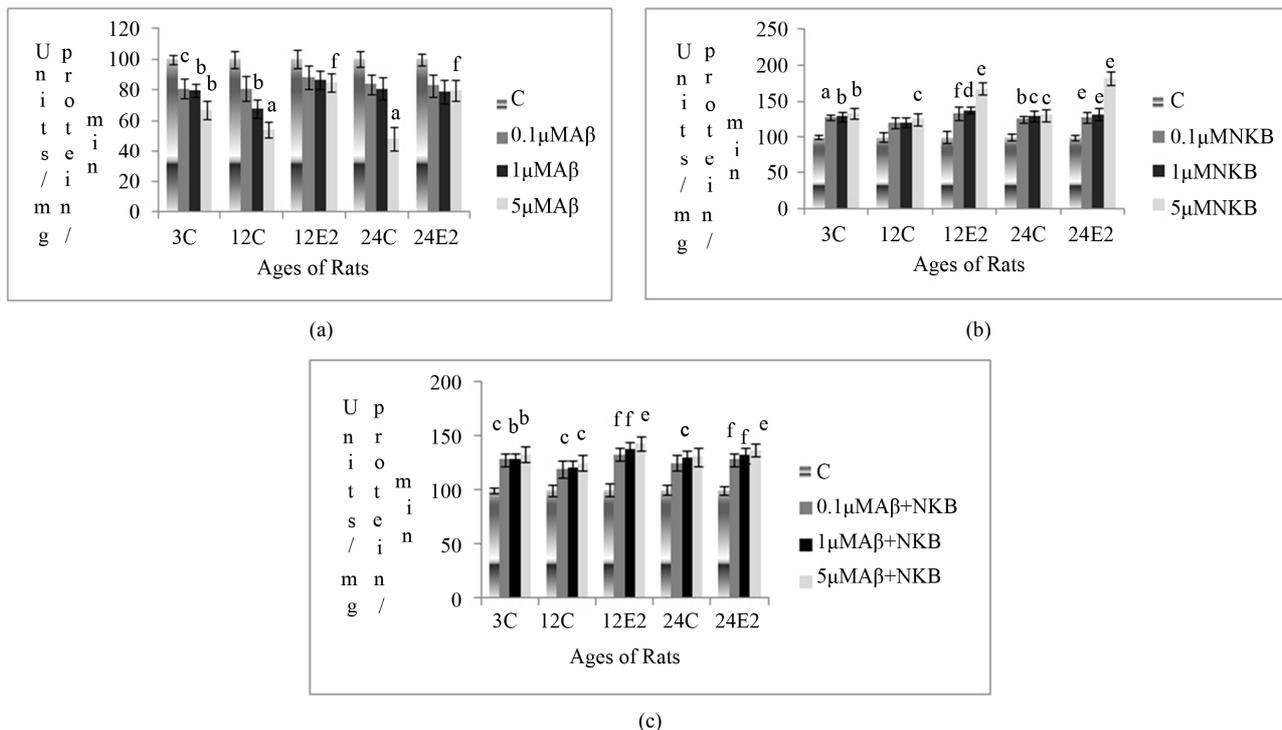
Figure 1. Percentage changes in the SOD activity in synaptosomes of 3, 12 and 24-month control (C) and estradiol (E2) treated aging female rats in presence of (a) Aβ (25 - 35) (b) NKB and (c) NKB + Aβ (25 - 35). Peptide concentrations are 0.1, 1.0 and 5.0 µM. Statistical significance: [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$ comparing age matched control (untreated) versus peptide treated; [d]$p < 0.001$, [e]$p < 0.01$, [f]$p < 0.05$ comparing E2 treated versus peptide treated.
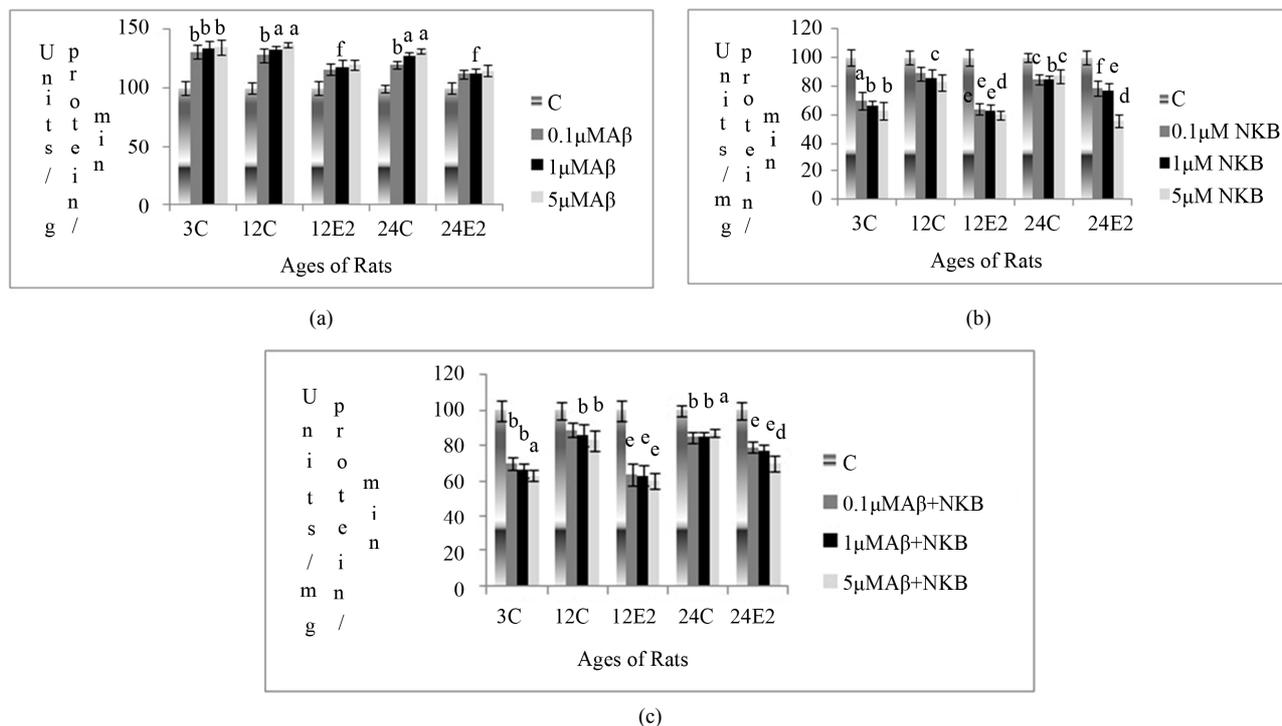

Figure 2. Percentage changes in the MAO activity in synaptosomes of 3, 12 and 24-month control (C) and estradiol (E2) treated aging female rats in presence of (a) Aβ (25 - 35) (b) NKB and (c) NKB + Aβ (25 - 35). Peptide concentrations are 0.1, 1.0 and 5.0 µM. Statistical significance: [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$ comparing age matched control (untreated) versus peptide treated; [d]$p < 0.001$, [e]$p < 0.01$, [f]$p < 0.05$ comparing E2 treated versus peptide treated.

synaptosomes, in different age groups with and without E2 treatment, at different concentration Aβ (25 - 35), NKB and NKB + Aβ (25 - 35). Results are shown in **Figures 2(a)-(c)**.

3.3.1. Effect of E2 and Varying Concentrations of Aβ (25 - 35) on MAO Activity

The MAO activity was found to be increased in the synaptosomes of control rats (E2 untreated), when incubated with different concentrations of Aβ (25 - 35). However, this increase in the activity was less in E2 treated rat brain synaptosomes. In the synaptosomes of E2 treated 24-month rats, the enzyme activity was increased with incubation of Aβ (25 - 35) at 1 µM concentration ($p < 0.05$). Results are shown in **Figure 2(a)**.

3.3.2. Effect of E2 and Varying Concentrations of NKB on MAO Activity

The MAO activity decreased in the synaptosomes of control rats of different age groups with different concentrations of NKB. However, there was less marked decrease in enzyme activity, in E2 treated aging rats with different concentrations of NKB incubation. The activity of MAO decreased by 64% in 24-month E2 treated rats, when incubated with 5 µM concentration of NKB ($p < 0.001$). Results are shown in **Figure 2(b)**.

3.2.3. Effect of E2 and Varying Concentrations of Combined NKB and Aβ (25 - 35) on SOD Activity

The SOD activity in control synaptosomes was observed to increase when treated with a combination of NKB and Aβ (25 - 35), but this increase was more significant in synaptosomes of E2 treated 12 and 24-month aging rats. The combined dose of NKB and Aβ (25 - 35) at 5 µM concentration in 24-month E2 treated rats showed a significantly raised SOD activity as compared to matched control ($p < 0.01$). The results are shown in **Figure 1(c)**.

3.3. Monoamine Oxidase (MAO)

Changes in MAO activity were measured in rat brain synaptosomes, in different age groups with and without E2 treatment, at different concentration Aβ (25 - 35), NKB and NKB + Aβ (25 - 35). Results are shown in **Figures 2(a)-(c)**.

3.3.1. Effect of E2 and Varying Concentrations of Aβ (25 - 35) on MAO Activity

The MAO activity was found to be increased in the synaptosomes of control rats (E2 untreated), when incubated with different concentrations of Aβ (25 - 35). However, this increase in the activity was less in E2

treated rat brain synaptosomes. In the synaptosomes of E2 treated 24-month rats, the enzyme activity was increased with incubation of Aβ (25 - 35) at 1 µM concentration ($p < 0.05$). Results are shown in **Figure 2(a)**.

3.3.2. Effect of E2 and Varying Concentrations of NKB on MAO Activity

The MAO activity decreased in the synaptosomes of control rats of different age groups with different concentrations of NKB. However, there was less marked decrease in enzyme activity, in E2 treated aging rats with different concentrations of NKB incubation. The activity of MAO decreased by 64% in 24-month E2 treated rats, when incubated with 5 µM concentration of NKB ($p < 0.001$). Results are shown in **Figure 2(b)**.

3.3.3. Effect of E2 and Varying Concentrations of Combined NKB and Aβ (25 - 35) on MAO Activity

The MAO activity was found to be decreased in the synaptosomes isolated from E2 treated rats, when incubated with combined NKB and Aβ (25 - 35). The synaptosomes isolated from E2 treated 24-month rats showed a decrease in the MAO enzyme activity with an incubation of 5 µM of the combined NKB and Aβ (25 - 35) as compared to age matched control ($p < 0.001$). Results are shown in **Figure 2(c)**.

4. DISCUSSION

In this study, we observed the increased body weight and brain weight in controls (E2 untreated), but these parameters decreased significantly in E2 treated rats. The above finding could be due to the deposition of adipose tissue during normal aging; however this accumulation reduced due to anti-obesity action of E2 in treated groups. The protein concentrations in E2 treated rats slightly increased when compared with age matched controls. This observation is supported by the earlier findings of Kumar *et al.* 2011 [5]. Earlier data from a previous study showed decreased levels of E2 at 12 and 24 months as compared to the 3-month rats [26].

Further the activity of SOD was assessed in the presence of different concentrations of Aβ (25 - 35), NKB and combined treatment of NKB and Aβ (25 - 35) in various age groups of untreated and E2 treated rat brain synaptosomes. It was observed that the activity of SOD increased in brain synaptosomes of all the age groups with an incubation of NKB and combined NKB and Aβ, as compared with that of age matched control synaptosomes. This observation is also supported by the results of Mantha *et al.* 2006 [17]. In the current study, at different concentrations of Aβ (25 - 35) treatment, a decrease in SOD activity was observed in the synaptosomes of control rats (E2 untreated) of different age

groups The reduced activity of SOD with Aβ (25 - 35) treatment may be due to increased oxidative stress produced by the amyloids in the brain. These findings are supported by an earlier study in the literature [27]. In the present study, the treatment of Aβ (25 - 35) in E2 treated and untreated synaptosomes showed a decrease in SOD activity although the percentage decrease in the activity was comparatively less in E2 treated group. This result shows that E2 plays a neuromodulatory and neuroprotective role against oxidative stress produced by Aβ (25 - 35). However, the SOD activity at different concentrations of NKB in E2 treated rat brain synaptosomes showed more significant increase as compared to their individual application. As per this result, E2 showed increased protective action in the presence of NKB. This increased protection may be due to the antioxidant property of the NKB whereby it reduces the free radical production as well as increased SOD synthesis in the synaptosomes. The combination therapy of NKB and Aβ (25 - 35) again on E2 treated and untreated group showed a rise in enzyme activity, although SOD activity increased significantly in E2 treated group. These results show that NKB along with E2 reversed the action of Aβ (25 - 35), which is neurotoxic and causes degeneration by free radical generation.

We have studied the MAO activity in different concentrations of Aβ (25 - 35), NKB and combined treatment of NKB and Aβ (25 - 35) in various age groups of untreated and E2 treated rats brain synaptosomes. The MAO activity in brain synaptosomes, incubated with Aβ (25 - 35), resulted in an elevation of its activity in a dose dependent manner. This observation has been supported by Song *et al.* 2000 [28]. When we studied the MAO activity in E2 treated rats synaptosomes incubated with Aβ (25 - 35), insignificant increase was observed compared to that of untreated E2 rats. These observations indicate that Aβ (25 - 35) showed less toxicity in the presence of E2 as compared to without E2 treated rats synaptosomes. It was observed that MAO activity in E2 treated synaptosomes, with an increase in the concentrations of NKB showed a sharp decrease as compared with that of the age matched untreated controls. Combined application of NKB and Aβ (25 - 35) reversed the amyloid induced increase in MAO activity, in rats of different age groups. Present results obtained with E2 and NKB suggest their role as an antioxidant and free radical scavenger agent against Aβ (25 - 35) induced deleterious effects in synaptosomes. By reducing the oxidative stress, observed in aging and by amyloid toxicity in the brain, NKB with E2 may contribute to the control of the aging process.

5. CONCLUSION

This study demonstrated that the combination treat-ment of NKB and E2 have significantly positive effects as compared to their individual application, and exerts its antagonism and protective influence against excitotoxicity and aging related changes. This suggests that interaction between peptides and estradiol may exist. Therefore, it can be concluded that neuroprotection by estrogen with neurokinin B holds immense promise for applying new strategies for control of age related neurodegenerative diseases.

6. ACKNOWLEDGEMENTS

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