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Research Article

Vitamin E Does Not Modulate Insulin-Induced Memory Impairment Assessed Using Y Maze Test in Mice

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Abstract
Induction of oxidative stress has been proposed as one of the mechanisms of the mechanisms through which insulin causes its negative effect. Vitamin E is known to counter oxidative stress in different biological systems. This study was aimed at evaluating the effect of insulin administration on long-term visuo-spatial and short-term working memory and brain histology in mice and to examine for any modulatory effects of vitamin E. A set of 24 naïve mice were grouped into four (n = 6) and treated for seven days as follows: Control group received distilled water; Insulin group received insulin (10 I.U./kg/day); Insulin+vitamin E group received insulin (10 I.U./kg/day) + vitamin E (100 mg/kg); Vitamin E group received vitamin E (100 mg/kg). Long-term spatial and short-term working memory was assessed using Y maze at the end of the treatment period. Brain tissue was examined for histological changes. Data was processed using IBM SPSS Statistics version 20.0 with $p < 0.05$ considered as significant. The insulin-treated mice showed reduced preference for the novel arm and performed less number of triads when compared to the controls. The performance of the insulin+vitamin E-treated and vitamin E-treated mice was similar in all the parameters, when compared with the insulin-treated. Normal histology of the cortex and absence of histological lesions were observed. It was concluded that sub-acute insulin treatment impairs long-term visuo-spatial and short-term working memory but does not affect brain histology in mice. Co-treatment with vitamin E does not modulate these insulin effects.

Key Words: vitamin E, insulin, learning and memory, brain histology, diabetes

INTRODUCTION

Insulin, best known for its metabolic actions is fast becoming known as a brain hormone (Ghazemi *et al*., 2013; Blazques *et al*., 2014). Reported findings on the effects of insulin on the brain, particularly on different types of learning and memory have been contradictory - in the positive as well as the negative. Insulin is best known for its beneficial neuroprotective effects on the brain and because of that it has been considered as a potential therapy for brain conditions related to diabetes and other conditions such as neurodegenerative disorders and Alzheimer's disease (Duarte *et al*. 2005; Duarte *et al*., 2012).

On the other hand, some harmful effects of insulin have also been reported. For example, Kamal *et al*. (2012 and 2013) reported that intracerebroventricular insulin administration in rats caused impairment of spatial memory and learning with defects in hippocampal synaptic plasticity. A number of mechanisms have been proposed to explain the negative effects of insulin on the brain, from induction of hypoglycaemia (Kopf and Baratti, 1999) to stimulation of oxidative stress (Choopani *et al*., 2008). It was reported that elevated doses of insulin promoted oxidative stress in humans with type 2 diabetes mellitus and was hypothesized that the oxidative stress could mediate some deleterious effects in the brain (Monnier *et al*. 2011).

Given the involvement of oxidative stress in the insulin effects on learning and memory, we thought that an antioxidant may counter these insulin-induced effects. Vitamin E is a potent, lipid-soluble antioxidant known to effectively counter oxidative stress in various biological systems including brain (Tuzcua and Baydas, 2006; Yarube *et al*., 2010; Yarube and Ayo, 2011). This study aimed to evaluate the effect of insulin administration on long-term visuo-spatial and short-term working memory and brain histology in mice and to examine for any modulatory effects of vitamin E. It was hypothesized that the antioxidant effects of vitamin E will modulate the insulin-induced effects on the brain.

MATERIALS AND METHODS

Animal conditions and materials: Young, 5 - 6 weeks old mice of both sexes, weighing between 18 – 20 g, were used for

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the study. The mice were allowed free access to feed and drinking water during acclimatization and throughout the experimental period. They were maintained under the prevailing natural light-dark cycle (photophase: 6:13 – 18:27). Daily change of drinking water was carried out every morning. Cages were cleaned and saw dust replaced every other day.

Insulin (Actrapid, Novo Nordisk A/S, Denmark) was reconstituted 1:3 with deionized water for ease of dosing. A total of 1500 mg food grade vitamin E powder (BulkSuppliments.com, 7511 Easgate Road, Henderson, NV 89011; lot # 20140717) was dissolved in 37.5 ml of dH_2O to form a stock solution. All drugs were stored according to manufacturer's instructions and administered using insulin syringe daily between the hours of 8:00 – 9:00 am.

Experimental design and grouping

A set of 24 naïve mice were grouped into four (n = 6) and treated for seven days (Francis *et al.*, 2008) as follows: Control group: received distilled water (0.2 ml) sub-cutaneously (*s.c.*); Insulin group: received insulin at 10 I.U./kg/day *s.c.* (Sharma *et al.*, 2007); Insulin+vitamin E group: mice received insulin as above, and, in addition, were treated with vitamin E at the dose of 100 mg/kg, intraperitoneally (*i.p.*) (Hasanein and Shahidi, 2010) for 7 days; Vitamin E group: mice were treated with vitamin E at the dose of 100 mg/kg, i.p. Neurobehavioural tests were done 30 minutes after the last treatment.

Assessment of long-term visuo-spatial and short-term working memory using Y maze

The test was conducted as described by Wright *et al.* (2006) and slightly modified. Briefly, the Y-maze consisted of three identical wooden arms (50 cm L, 16 cm W, 32 cm H) with multiple extra-maze cues located around the perimeter of the maze. The maze was rotated between training and testing such that the arms, termed Novel, Start and Other, referred to the position of the arm in the test area and not the actual arm. The investigator stood in white coat in the same position during training and testing. One hour delay was allowed between training and testing.

During training, one arm (Novel) of the Y-maze was blocked with a shutter, allowing the mice to explore the Start and Other arms for 15 min. The shutter that blocked the novel arm was the height of the arms (32 cm), preventing mice from rearing and seeing into the novel arm or viewing the spatial cues, visible only from the novel arm. Following training, mice were returned to their home chambers during the 1 hour delay. After the delay, the shutter was removed, and mice were placed in the same Start arm, and allowed to explore the Y-maze for 5 min. Movements of the mice were recorded using an overhead video camera (Handycam, SONY, Japan) for quantification subsequently. A blind investigator unaware of the treatment groups determined the number of entries made into, and time spent (dwell) by each mouse in the Novel, Start, and Other arm across all five minutes.

An entry was counted, when the forearms of the mouse entered the arm. The number and the sequence of arms entered were also recorded. The dependent variables were activity, defined as the number of arms entered, and percent alternation, calculated as the number of alternations/triads (entries into three different arms consecutively) divided by the total possible alternations (i.e., the total number of arms entered minus 2) and multiplied by 100 (Sarnyai *et al.*, 2000).

Y-maze navigation relies upon a mouse's innate tendency to explore novel environments (Ennaceur and Delacour, 1988). In the present experiments mice that recognized and choose the Novel arm more than the other arms were defined as having intact spatial memory, whereas those that entered the Novel and other arms similarly were considered to have impaired spatial memory.

Histological evaluation of cerebral cortex

At the end of treatment, the mice were sacrificed and brain tissues harvested and prepared for histopathological examination as described by Carleton (1976). Briefly, the tissue samples were fixed in 10% formaldehyde, embedded in paraffin and cut into sections of 5 µm thickness. The tissues were sectioned, and slides were prepared and stained using Haematoxylin and Eosin (H&E). Tissue slides were examined under light microscope (x200 magnification). Four sections from each mouse were selected and examined for histopathological changes. Photomicrographs were prepared from selected sections to demonstrate the findings.

Statistical analyses

All data were collated and analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0. Values were expressed as mean ± S.E.M. General Linear Model-repeated measures ANOVA was used to compare means of time spent in arms and number of entries into arms. One-way ANOVA was used to compare number of triads. Bonferroni test was employed for *post-hoc* multiple comparisons. Values of $P < 0.05$ were considered significant.

RESULTS

Assessment of visuo-spatial and working memory using Y maze

***Number of entries into each arm*:** There was an overall significant difference between the groups in the number of entries into individual arms ($F_{(3, 20)} = 5.713$, df = 3, $P = 0.005$, multivariate partial Eta squared= 0.461, n=6) (Figure 1). There was also a significant difference within groups in the number of entries into individual arms (Box's test of equality of covariance matrices - Box's M = 24.108, $P = 0.056$; Wilk's lambda = 0.435, $F_{(3, 20)} = 12.325$, $P = 0.000$, multivariate partial Eta squared = 0.565, n = 6).

Time spent in each arm: The time spent (seconds) in individual arms when compared within groups was significantly different (Pillai's trace = 0.604, $F_{(3, 20)} = 14.475$, $P = 0.001$, multivariate partial Eta squared = 0.604, n = 6) (Figure 2). There was no significant difference in time spent in the arms, when compared between the groups ($F_{(3, 20)} = 2.826$, $P = 0.065$, multivariate partial Eta squared = 0.2984, n = 6). The insulin-treated mice spent significantly less time in the novel arm compared to the untreated controls (p=0.046).

Number of triads and percent alternations performed by the animals: The difference between groups in the number of triads performed by the animals was insignificant ($F_{(3, 20)} = 0.478$, $P = 0.701$) (Figure 3). Similarly, there was no significant difference between groups in percent alternations, performed by the animals ($P = 0.232$, $F_{(3, 20)} = 1.554$, df = 3, n = 6).

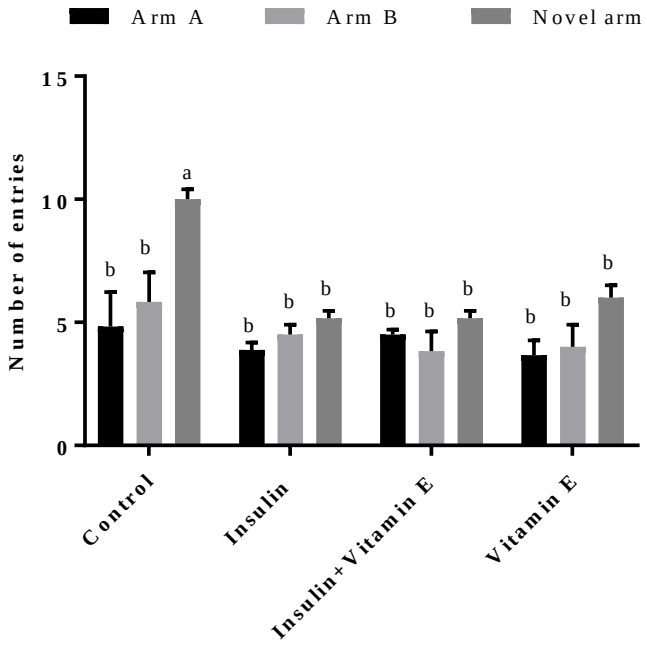

Figure 1:
Number of entries into each arm by control and treated mice during a Y maze task.
[a,b] = Bars with different superscripts (between and within groups) are significantly different (P < 0.05). (Mean ± S.E.M, n = 6)

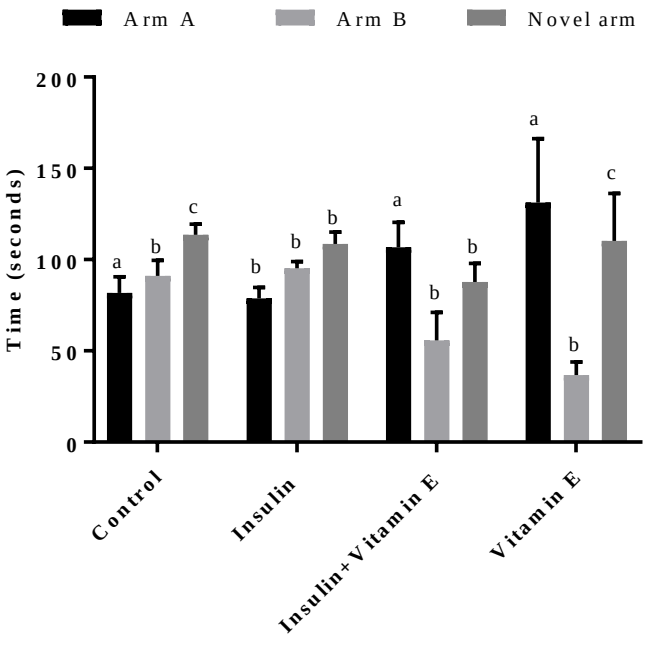

Figure 2:
Time spent in each arm by control and treated mice during a Y maze task
a,b,c = Bars with different superscripts (between and within groups) are significantly different (P < 0.05). (Mean ± S.E.M, n = 6).

Overall result: The insulin-treated mice showed reduced preference to the novel arm, when compared to the untreated controls, indicating memory impairment. This is because they have entered the novel arm significantly less (Figure 1) and spent statistically the same time in all the arms, in contrast to the untreated controls (Figure 2). Again, the number of triads performed by the insulin group was less, when compared to untreated controls, though the difference was not significant (Figure 3). Taken together, the results suggest that insulin impaired memory in the treated mice.

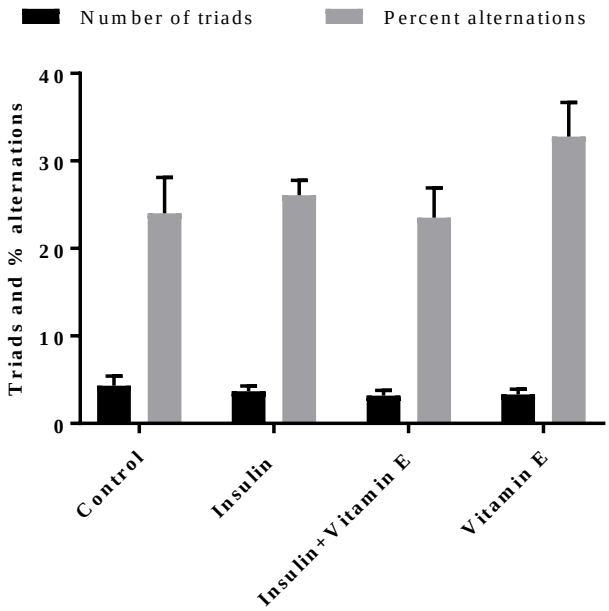

Figure 3:
Number of triads and percent alternations performed by control and treated mice during a Y maze task.
No significant difference between groups (P > 0.05). (Mean ± S.E.M, n = 6)

The performance of the insulin+vitamin E-treated and vitamin E-treated mice was about the same in all the three parameters, when compared with the insulin-treated. This implies that vitamin E did not modulate the insulin-induced impairment on learning and memory. Furthermore, the insulin+vitamin E-treated mice spent significantly less time in the novel arm compared to the insulin-treated animals (Figure 2). This may suggest further impairment of memory, even though the vitamin E-treated mice spent the same time in the novel arm compared to the insulin-treated (Figure 2).

Histological examination of cerebral cortex

Photomicrographs of cerebral cortices of the control, insulin, insulin+vitamin E and vitamin E groups are shown on Plate I. Plate I (A) shows a photomicrograph of cerebral cortex of mouse treated for seven days with distilled water (Control group). Normal histology of the cortex and absence of histological lesions were observed. Neuronal cells, which appear as dots (cross section of axon) as well as glial cells which appear to have distinct cell membrane, cytoplasm and nucleus can be observed. All the other photomicrographs (B-D) appear essentially the same as plate I(A) (normal). This observation implies that neither treatment with insulin, vitamin E or their combination had any significant effect on brain tissue

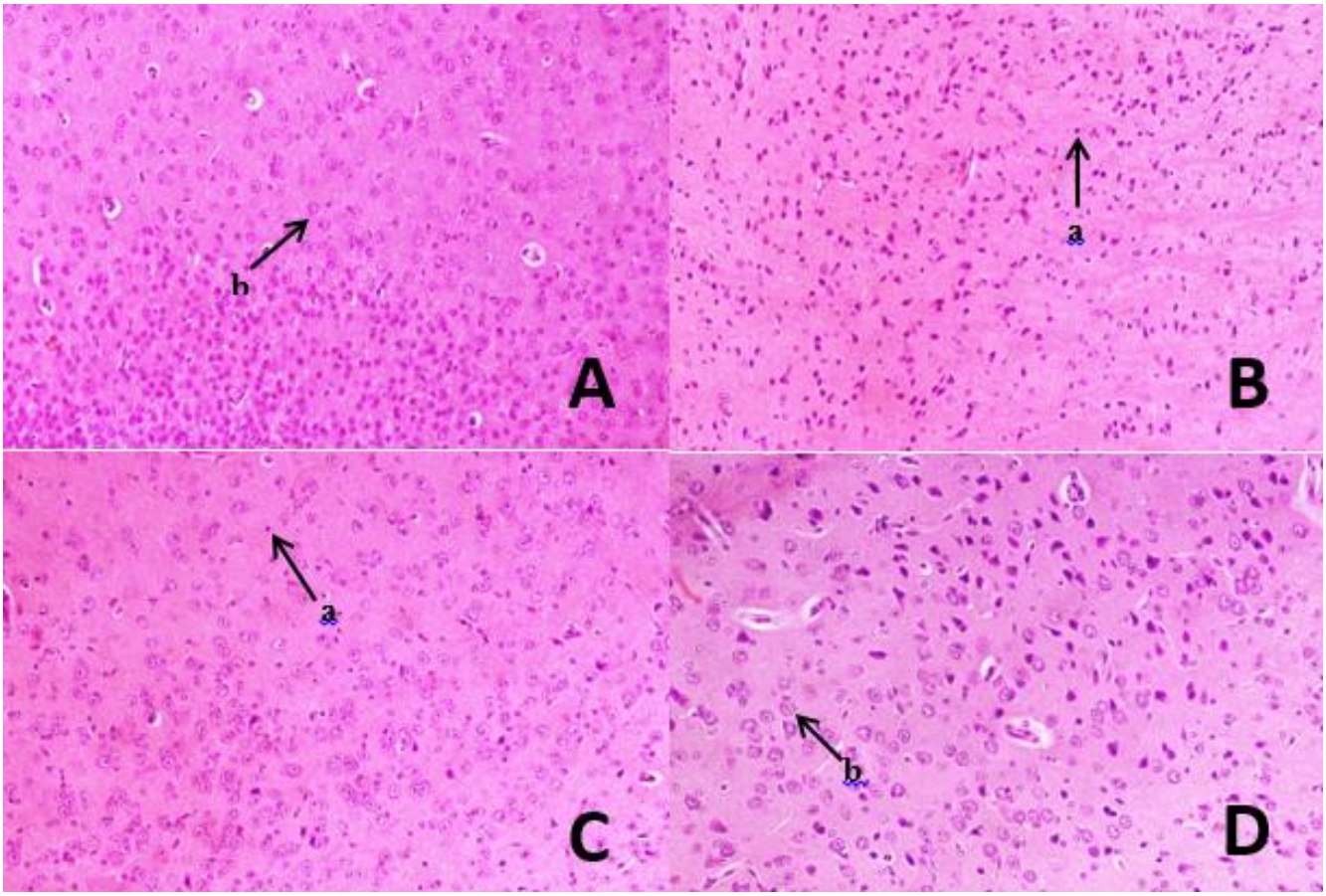

Plate I (A-D):
Photomicrograph of cerebral cortex of a mouse treated for 7 days with water (A), insulin (B), insulin + vitamin E (C) and vitamin E (D) (H & E x 200). Note the normal appearance and distribution of neuronal and glial cells and absence of histopathological lessions. Arrows: a = neurone; b = neuroglia

DISCUSSION

Insulin signalling is part of and necessary for normal memory functioning as demonstrated in lower animals (Lin *et al.*, 2010), insects (Chambers *et al.*, 2015), rodents (Liu *et al.*, 2013) and humans (Bloemer *et al.*, 2014; De Felice *et al.*, 2014; Kim and Feldman, 2015). However, insulin may also play a disruptive role in memory process. This study has reported impairment in long-term visuo-spatial and short-term working memory following sub-acute treatment with insulin. The preference for the novel arm tests long-term visuo-spatial memory while the alternations are a demonstration of short term working memory. These results corroborate the findings of Kamal *et al.* (2012) and (2013) who showed impairment of spatial memory with defects in synaptic plasticity.

Although this study did not investigate the mechanism of the insulin-induced effects, reports of previous studies have provided some insights, in addition to the one proposed by Kamal *et al*. (2013). For example, Kopf and Baratti (1999) reported impaired memory retention in mice following intraperitoneal insulin injection, which they attributed to hypoglycaemia, rather than direct effect of insulin on the CNS. Another mechanism of the insulin-induced memory impairment could be connected to insulin's ability to increase the level of nitric oxide (NO) in the brain as reported previously (Facchini *et al.*, 2000; Choopani *et al.*, 2008; Paul and Ekambaram, 2011; Yarube, 2016). NO at higher concentrations can interact with superoxide anion leading to formation of the powerful oxidant species peroxynitrite, resulting in cell damage and altered neuronal physiological function (Knowles and Moncada, 1994; Lipton 1999). Despite the evidence of the negative effect of insulin, there are some studies reporting a neuroprotective effect of insulin against oxidative stress (Duarte *et al.*, 2005).

This study has reported lack of modulatory role of vitamin E in the memory impairment caused by insulin. Patockova *et al.* (2003) and Agrawal *et al.* (2009) previously reported that insulin, an inducer of NO production, causes lipid peroxidation and increases in oxidative stress in the brain. Vitamin E - a lipid-soluble vitamin is known to decrease oxidative stress *in vivo* (Yarube, 2011). Vitamin E has been reported to improve memory and learning in diabetic rats (Tuczu and Baydas, 2006) and non-diabetic humans (Masaki *et al.*, 2000). Because of its antioxidant properties, it was conceived that vitamin E could modulate the effects of insulin. However, the absence of this modulatory effect could be due to relatively short duration of administration or the dose of the vitamin used. It is also possible that different mechanisms, other than oxidative stress may be responsible for the memory impairment caused by insulin treatment.

Brain slides of control mice showed normal histology of the cortices, which was consistent with the normal performance of the control animals during the neurobehavioural experiments. Similarly, normal histology of the cerebral cortex was observed in all treated animals. This finding indicates that treatment with insulin and vitamin E

separately or in combination did not cause any significant change to the cortex detectable by histological examination. The histological findings obtained in the present study disagree with the significant changes reported in this and other previous studies (Kamal *et al.*, 2012; Kamal *et al.*, 2013; Kim and Feldman, 2015; Yarube *et al*., 2016) study at the systemic levels (learning and memory). The results however, did not exclude changes at the cellular levels such as apoptosis, which may be detectable using the appropriate laboratory methods. The absence of changes reported here may be explained by the relatively short duration of treatment, which did not allow enough time for histological changes to manifest. It is conceivable, that, chronic studies could reveal changes at the histological level detectable by simple microscopy.

In conclusion, our data demonstrate that sub-acute insulin treatment impairs long-term visuo-spatial and short-term working memory but does not affect brain histology in mice. Co-treatment with vitamin E does not modulate these insulin effects.

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