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Effect of Insulin on Visuo-Spatial Memory and Histology of Cerebral Cortex in the Presence or Absence of Nitric Oxide Inhibition

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Summary: Insulin has emerged from its traditional 'peripheral' glucose-lowering function to become increasingly regarded as a brain hormone that controls a wide range of functions including learning and memory. Insulin action on learning and memory is linked to nitric oxide (NO) signalling, but its effects on memory and histology of cerebral cortex in conditions of varied NO availability is unclear. This research sought to determine the effect of insulin on visuo-spatial learning, memory and histology of cerebral cortex during NO deficiency. Twenty-four mice weighing 21-23 g, were divided into four groups (n = 6) and treated daily for seven days with 0.2 ml distilled water subcutaneously (*s.c.*) (control), 10 I.U/kg insulin *s.c.*, 10 I.U/kg insulin + 50 mg/kg L-NAME intraperitoneally (*i.p.*), and 50 mg/kg *i.p.* L-NAME *s.c.*, respectively. The 3-day MWM paradigm was used to assess memory. Brain tissue was examined for histological changes. There was no significant difference between day 1 and day 2 latencies for all the groups. The mice in all (but L-NAME) groups spent more time in the target quadrant, and the difference was significant within but not between groups. There was significant reduction in number of platform site crossings (4.83 ± 0.5, 0.67 ± 0.3, 0.50 ± 0.3 and 0.50 ± 0.3 for control, insulin, insulin+L-NAME and L-NAME groups, respectively) in all the groups compared to control. Normal histology of the cortex and absence of histological lesions were observed in brain slides of control and treatment groups. It was concluded that insulin administration impairs visuo-spatial memory to a greater extent in the presence of NO block, and to a lesser extent in the absence of NO block. Nitric oxide has a role in insulin-induced memory impairment. Insulin administration in the presence or absence of NO block had no effect on histology of cortex.

Keywords*:* Insulin, learning and memory, cortical histology, nitric oxide

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INTRODUCTION

The effect of insulin on various brain functions has been a subject of intensive research since the late 1970s when the presence of insulin in brain was first detected by Havrankova *et al.* (1978) in humans. Insulin has emerged from its traditional 'peripheral' glucose-lowering function to become increasingly regarded as a brain hormone that controls a wide range of functions from regulation of trophic functions in the brain (Blázquez *et al.*, 2014; Leopold, 2004), to central regulation of peripheral metabolic functions(Kleinridders, 2014), and more complex higher functions of the nervous system such as learning and memory (Chambers *et al.*, 2015; Kim and Feldman, 2015).Insulin action is linked to nitric oxide (NO) signalling in the brain (Vincent *et al.*, 2003; Paul and Ekambaram, 2011).Molecular interactions between *N*-methyl-D-aspartate receptors (NMDARs) and neuronal nitric oxide synthases(nNOS) provide explanation to the relationship between NO and memory. Indeed, antagonist of NMDARs (Xu*et al.*, 2001) and other substances that decrease brain NO level (Yamada *et al.*, 1996) have been used to demonstrate the role of NO in learning and memory. Furthermore, insulin was also reported to directly interact with both NMDARs and Alpha-amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid receptors (AMPARs) to modulate learning and memory (van der Heide *et al.*, 2006; Huang *et al.*, 2004). However, it is not known how insulin affects memory and histology of cerebral cortex in conditions of varied NO availability *in vivo*. This research sought to determine the effect of insulin on visuo-spatial learning, memory and histology of cerebral cortex during NO deficiency. The synthesis of NO from L-arginine occurs in various mammalian tissues. The enzymes responsible for the synthesis are known as NO synthases (NOS). L-Arginine is converted first to

N^{ω}-hydroxyarginine, a reaction requiring one O_2 and one NADPH and the presence of BH_4. N^{ω}-hydroxyarginine is then oxidised to form citruline and NO. Of the three NOS isoforms, nNOS constitutes the predominant source of NO in neurons and localizes to synaptic spines (Zhou and Zhu, 2009). L-NAME hydrochloride – a non-selective nitric oxide synthase (NOS) inhibitor (irreversible inhibition on nNOS isoform) (Víteček et al., 2012), was used in this study as NO inhibitor to create NO deficiency.

Spatial cognition involves acquisition and utilization of general and specific information related to objects and events in spatial environments. It is believed that an event is fixed in the memory by modifications of the neuronal networks, based on long-term potentiation (LTP) and long-term depression (LTD) (Feldman 2009). The LTP process occurs when the presynaptic neurons excite the post-synaptic ones in a repetitive and prolonged manner, and then the depolarization of the post-synaptic neuron is reinforced and maintained for a long time. Accordingly, there is a significant increase in Ca^{2+} input, and the metabolic activities for this cation are prolonged, and as a result the memory is consolidated (Blázquez *et al.*, 2014).

MATERIALS AND METHODS

Animals, Groupings and Treatments

Young mice of both sexes, weighing between 21 – 23 g, were used for the study. They were kept in large cages under the prevailing natural light-dark cycle (photophase: 6:36 – 18:30) and allowed free access to feed and drinking water during acclimatization and throughout the experimental period. At the end of the experiments, the mice were put to sleep by chloroform inhalation in a closed chamber, and then quickly sacrificed by cervical transection.

Insulin (Actrapid, Novo Nordisk A/S, Denmark) and L-NAME hydrochloride stored according to manufacturer's instructions, were administered using insulin syringe daily between the hours of 8:00 – 9:00 am.

The mice were divided into four groups (n = 6) and treated with 0.2 ml distilled water subcutaneously (*s.c.*) (control), 10 I.U/kg insulin*s.c.*, 10 I.U/kg insulin + 50 mg/kg L-NAME intraperitoneally (*i.p.*), and 50 mg/kg *i.p.* L-NAME *s.c.*, respectively.

Neurobehavioural tests were done 30 minutes after the last injection, following a total duration of seven days daily administration (Francis *et al.*, 2008). The 3-day MWM test was started on the fifth day and ended on the seventh day of treatment.

At the end of the neurobehavioural experiments, the mice were sacrificed and brain tissues harvested and prepared for histological examination. Three sections from each brain were selected and examined for histological changes. Photomicrographs were prepared from selected sections to demonstrate the findings.

Assessment of long-term visuo-spatial learning and memory using Morris water maze

The Morris water maze, also known as Morris water navigation task, is a behavioural procedure, widely used to study spatial learning and memory (Hooge and Deyn, 2001). It is an open circular pool that is filled with water. The animal must search in order to locate an escape platform that is submerged below the water surface and placed in a fixed location. Two principal axes of the maze were designated, each line bisecting the maze perpendicular to one another to create an imaginary '+'. The end of each line demarcates four cardinal points: North (N), South (S), East (E) and West (W) making four quadrants. The diameter of the pool was 133 cm. The circular pool was filled with water to a depth of about 14 cm at room temperature ($25 – 27^0$C). Escape platform was a sealed cylindrical container with a height of 13 cm and diameter of 5 cm. It was submerged 0.5 cm below the water surface in the middle of the target quadrant (quadrant 4 in this study).

Each animal was placed in a desired start position in the maze, facing the tank wall. A timer was started the moment the animal was released. The timer was stopped when the animal reached the platform. A trial limit 2 min per trial was adopted. Animals not finding the platform within this time limit were placed on the platform or guided to it (Cain *et al.*, 1996). Each animal was left on the platform for 20 s. The animal was placed in the maze at new start position until it had four trials in a day with inter-trial interval of 15 minutes. On the subsequent day, the same trials were repeated. Then on the third day the platform was removed and each animal underwent only one trial for 90 seconds, known as probe trial. The time spent in the quadrant where platform was removed was taken as an index of cognition. The water was changed after trial each day of the experiment. An overhead video camera (Handycam, SONY, Japan) recorded movement of the mice for later quantification, and the investigator stood in white coat in the same position during training or testing.

Histological evaluation

At the end of treatment, the mice were sacrificed and brain tissues harvested and prepared for histological 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). Three sections from each brain were selected and examined for histological changes. Photomicrographs were prepared from selected sections to demonstrate the findings.

Statistical Analysis

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 day 1 and day 2 latencies, and time spent in quadrants. One-way ANOVA was used to compare mean values of number of platform crossings. Bonferroni test was employed for *post-hoc* multiple comparisons. Values of $P < 0.05$ were considered significant.

RESULTS

Assessment of visuo-spatial memory using Morris water maze

Latency (time taken) to locate escape platform

There was no significant difference between day 1 and day 2 latencies (seconds) for all the groups ($F_{(1, 22)}$= 2.85, P = 0.107, Wilks' lambda = 0.875, n = 6, partial Eta squared = 0.125) (Figure 1). The difference in latencies between the groups was not significant ($F_{(3, 22)}$= 1.130, P = 0.361, multivariate partial Eta squared = 0.145, n = 6). There was an insignificant increase in day 2 latencies for the mice insulin+L-NAME and L-NAME groups. This result suggests that insulin and the other treatments did not affect the ability of the mice to learn.

Time spent in each quadrant

There was a significant difference in the time (seconds) spent per quadrant within groups (Wilks' lambda = 0.498, $F_{(3, 20)}$= 6.055, P = 0.005, multivariate partial Eta squared = 0.502, n = 6) (Figure 2). There was no statistically significant difference between groups in terms of the time spent per quadrant ($F_{(3, 20)}$= 0.420, p = 0.741, multivariate partial Eta squared = 0.059, n = 6). The mice in all (but L-NAME) groups spent more time in the target quadrant, and the difference was significant within (P = 0.005) but not between (P = 0.741) groups. This indicates normal memory function and suggests that insulin treatment alone or in combination with L-NAME, did not impair or improve memory in the animals. The decrease in time spent in the target quadrant by L-NAME-treated mice suggests impairment of memory in these animals in the presence of NO block.

Number of platform site crossings

There was a significant difference between the groups in the number of times the animals crossed the platform site (F = 32.023, P = 0.001, n = 6) (Figure 3). There was significant reduction in number of platform site crossings in all the groups compared to control. All the three groups differed with control, but not with one another. This result indicates impairment of memory due to all the treatments.

Taken together, the results of Morris water maze test suggest that treatment with insulin, insulin+L-NAME and L-NAME impaired memory in the treated mice.

Effect of insulin administration on histology of cerebral cortex

Figure 4(A) shows photomicrographs of cerebral cortex of mouse treated for seven days with distilled water (control group). Normal histology of the cortex and absence of histopathological 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. Photomicrographs of cerebral cortices of mouse treated for seven days with insulin,

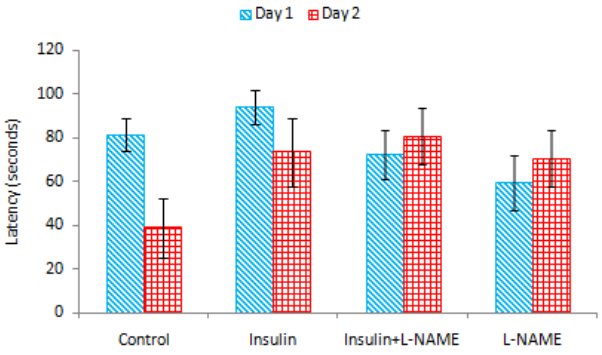

Figure 1: Latency (seconds) to locate escape platform for control and treated mice during a 2-day training session in Morris water maze task. (Mean ± S.E.M, n = 6).

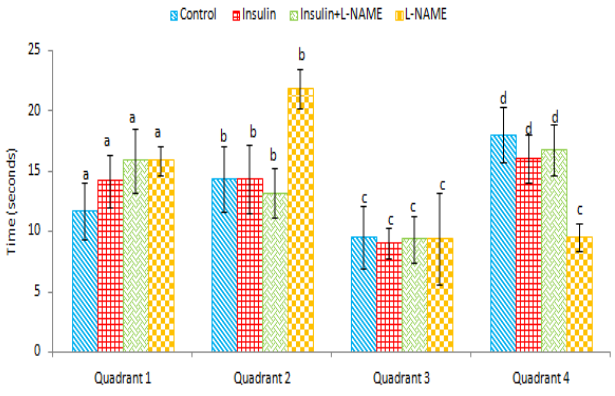

Figure 2: Time (seconds) spent in quadrants by control and treated mice during a 1-day probe trial in Morris water maze task (quadrant 4 contained the removed platform). $^{a,b,c,\ d}$ = Bars with different superscripts (within a group) are significantly (P< 0.05) different. (Mean ± S.E.M, n = 6)

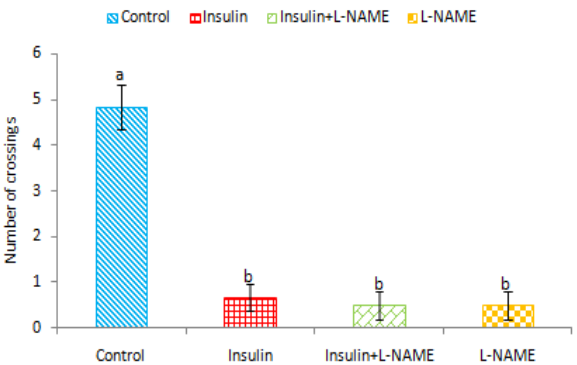

Figure 3: Number of platform crossings by control and treated mice during 1-day probe trial in Morris water maze task. ^{b} = Bars with different superscripts are significantly (P< 0.05) different. (Mean ± S.E.M, n = 6)

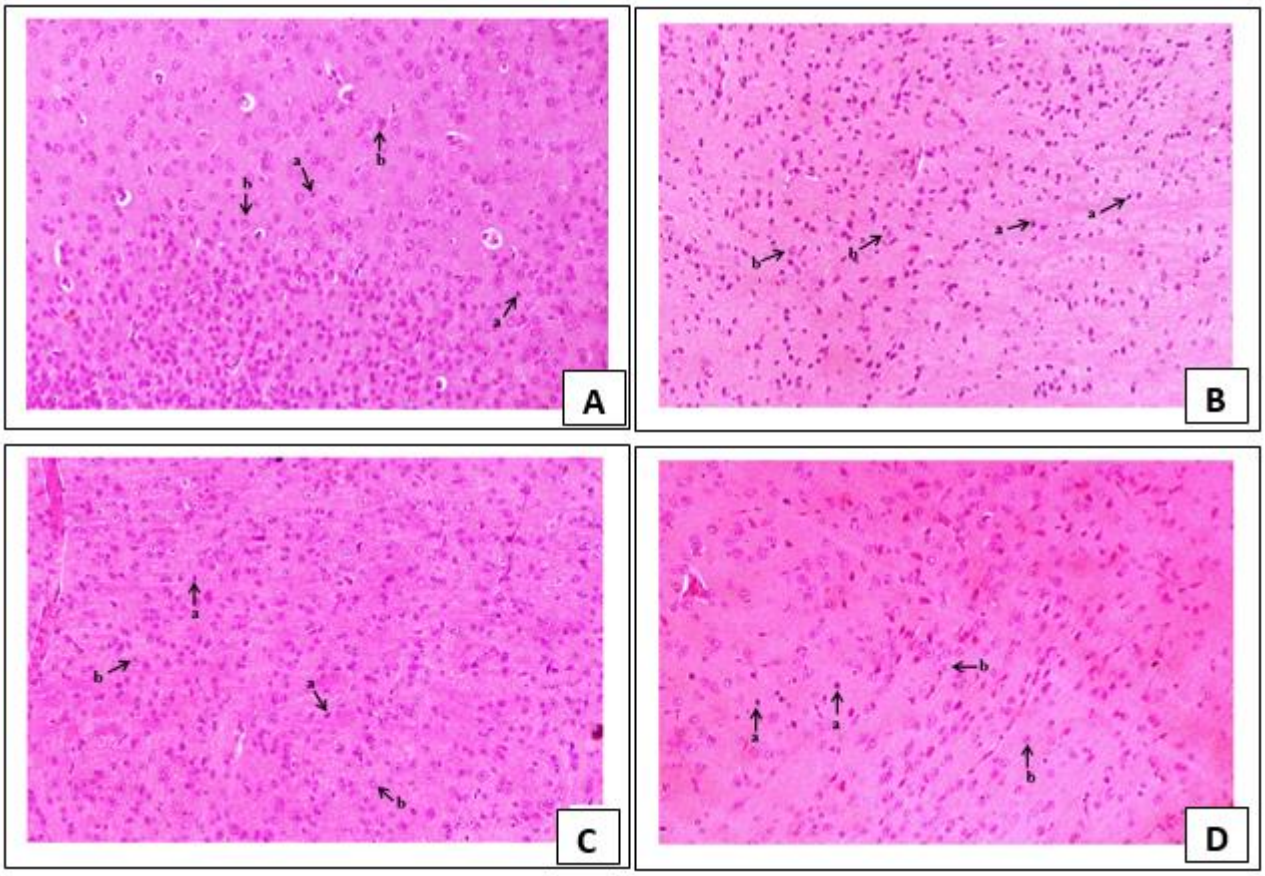

Figure 4: Photomicrograph of cerebral cortex of a mouse treated for 7 days with distilled water (A), insulin (B), insulin+L-NAME (C) and L-NAME (D) (H & E x 200). a = neuron (a cross section of axon which appears as dot) b = neuroglia (note the cell membrane, cytoplasm and nucleus at the centre) Note the generally normal histology of the cortex in all the micrographs (and mild hyperemia on figure 4D).

insulin+L-NAME and L-NAME are presented on figure 4B, 4C and 4D, respectively. The observations on these micrographs were similar to those on Figure 4A (control), except for mild hyperemia in those treated with L-NAME (figure 4D). These findings indicate that treatment with insulin, L-NAME and vitamin E separately or in combination did not cause significant change in the cortex.

DISCUSSION

The overall result of MWM task showed that treatment with insulin alone or in combination with L-NAME (NO inhibition) impaired memory. This result corroborates that of Kopf and Baratti (1995, 1996 and 1999) who reported impaired memory retention in mice following intraperitoneal insulin injection (but without NO inhibition).In the mice treated with insulin only, the level of NO is expected to rise because insulin is known to increase NO level in the brain (Paul and Ekambaram, 2011). Nitric oxide level is expected to decrease in the mice treated with Insulin+L-NAME(co-administration) and L-NAME (only)when compared to insulin (only). But more decrease in NO availability is expected in the latter more than the former, because in the former, the NO inhibition by L-NAME is partly counteracted by the effect of insulin (increase NO level). This is because in the case of the former, the decrease NO would be partly compensated by some increase in NO level due to insulin. Insulin alone decreased the latency to locate the escape platform in day 2 (the same effect was observed in the control animals), and this represents normal process of learning, corroborating previous reports (Yarube *et al.*, 2016 (a)). However, in the presence of L-NAME (NO inhibition), insulin increased the latency which may suggest some impairment of learning (although the difference was not statistically significant). Similar effect was observed when L-NAME was given alone. So, there is tendency towards more impairment during NO inhibition. It can therefore be concluded that availability of NO at a certain level is necessary for normal learning function in mice.

Time spent in the target quadrant was significantly increased by insulin alone indicating that the animals retained the memory of the escape platform. This suggests normal memory function and agrees with previous findings (Yarube *et al.*, 2016(b)). In the presence of NO inhibition (group 2 mice), insulin (still maintaining some NO availability) also increased the time spent in the target quadrant. However, L-NMAE alone (more decrease in NO availability) significantly decreased this parameter suggesting impairment of memory. This result agrees with the previous one to further suggest that NO is required for normal memory function in mice. It was demonstrated previously that insulin signalling is involved in normal memory

process in *Caenorhabditi selegans* (Lin *et al.*, 2010), *drosophila* (Chambers *et al.*, 2015), *Xenopus* tadpoles (Chiu *et al.*, 2008), rats (Liu *et al.*, 2013) and humans (De Felice*et al.*, 2014). The results of the present study further suggests that NO may play an important role in the insulin-induced effect on visuo-spatial learning and memory, and this is in agreement with previous findings (Trovati *et al.*, 1997; Choopani *et al.*, 2008).

Number of platform site crossing is arguably a more accurate measure of memory compared to time spent in the target quadrant, because crossing the platform site may indicate more accurate memory of the position of the platform than being in any other location in the target quadrant. All the treatments resulted in decrease number of platform site crossing, which indicates memory impairment. Impairment of memory observed during more or less NO deficiency (L-NAME only and insulin+L-NAME treatments, respectively) is consistent with the other results of other measured parameters stated above. The increase in NO level by insulin may lead to its excess in the brain leading to many possibilities including memory impairment, as expressed by decreased number of platform site crossing reported in this study. NO is a ubiquitous molecule with many actions such as induction of oxidative stress (Lipton, 1999) and participation is cellular cell signalling processes in the brain (Mustafa, 2009; Steinert, 2010). NO has been found to influence the release of various neurotransmitters from different brain regions (Guix*et al.*, 2005), and thus, modulate various CNS functions. Although NO appears to be necessary for normal learning and memory function, its excess can result into memory impairment. Indeed, NO production might lead to either neurotoxicity or neuroprotection depending on the location and level of NO production (Lipton, 1999). 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 function (Lipton, 1999).

Apart from the structures in the limbic system (hippocampus, amygdala), neuronal circuits in the cortex of the medial temporal lobe and in the prefrontal cortex are involved in memory function (Sokolowski and Corbin, 2012). In the present study, brain slides of control mice showed normal histology of the cortices, which was consistent with the normal performance of the control animals during the neuro-behavioural experiments. However, there was absence of histological changes in the cortex due to the different treatments despite the observed impairment of memory reported herewith. The histological findings in the cortex do not support the memory changes observed during the MWM test in this study. Absence of histological changes however, does not exclude other changes at the cellular and molecular levels, which may be detectable using the appropriate laboratory methods, and which this work did not seek to examine due to limitations in facilities. This may serve as a rationale for further studies. The absence of histological changes reported here may be explained by the relatively short duration of treatment, which did not allow enough time for changes to manifest. It is conceivable, that, chronic studies could reveal changes at the histological level detectable by simple microscopy.

The results of this study suggest that insulin administration impairs visuo-spatial memory to a greater extent in the presence of NO block, and to a lesser extent in the absence of NO block. Nitric oxide has a role in insulin-induced memory impairment. Insulin administration in the presence or absence of NO block had no effect on histology of cortex.

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