The effect of intra-cerebroventricular injection of insulin on nociception of formalin test in non-diabetic and short-term diabetic rat models

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Summary

Pain is a complex process in the central nervous system (CNS). Several factors can alter the pain threshold and insulin is one of them which is produced by the beta cells of pancreas and capable of crossing blood-brain barrier. The aim of this study was to evaluate the effects of intra-cerebroventricular (ICV) injection of insulin on the pain response to formalin in short-term induced diabetic and non-diabetic rats. Sixty-four Sprague-Dawley male rats (280 ± 30 g) were divided into non-diabetic and diabetic groups. Diabetes was induced with streptozotocin (STZ, 60 mg/kg, i.p) for elimination of peripheral insulin. After proving diabetes, insulin (5 mU/animal, 5 μL) was injected to the left lateral cerebral ventricle while equal volume of normal saline was injected in control groups. After 10 min, formalin test was performed. Present study showed that ICV injection of insulin possessed anti-nociceptive effect in non-diabetic rats in formalin test while in diabetic rats, it did not have this effect and even decreased pain threshold partially.

In conclusion we showed that ICV injection of insulin in non-diabetic rats, in contrast with diabetic rats, has an anti-nociceptive effect in formalin test. In short-term diabetic rats, ICV injection of insulin was not able to reduce pain response and partially decreased pain threshold.

Key words: Formalin test, Insulin, Pain, Short-term diabetes

Introduction

Pain is a phenomenon that everyone experienced it during the life span. There are different pathways to control pain in the central nervous system (CNS) such as descending noradrenergic, serotonergic and dopaminergic systems (Millan, 2002). Pain threshold can alter via some factors such as gender, depression, individual differences, obesity and endocrine changes (Guneli et al., 2010).

Insulin is a polypeptide hormone that is produced from beta cells of the pancreas. In diabetes induced by streptozotocin, the secretion of insulin from beta cells is inhibited due to destruction of the beta cells (Deeds et al., 2011). Today it is well-known that insulin can cross from the blood brain barrier (BBB) through insulin-receptor-mediated active trans-endothelial transport (Banks et al., 2012; Duarte et al., 2012). Previous studies have determined that CNS is able to release insulin (Duarte et al., 2012; Blazquez et al., 2014). Some evidence indicated that pre proinsulin-I mRNA is expressed in the adult rat brain (Zhao, 2001; Zhao, 2002). Insulin-I is primarily expressed in the hippocampus and it is also proved that there are moderate levels of insulin-I in the olfactory bulb, piriform cortex, and the Purkinje cells of the cerebellar cortex (Zhao, 2001; Zhao, 2002).

Insulin receptors are generally distributed in the CNS (Schulingkam, 2000). It has been indicated that insulin has an ameliorative effect in rodents in the pain test (Takeshita and Yamaguchi, 1997; Anuradha et al., 2004). The pain control centers in the brain stem have expressed insulin receptors and up to now no study has been done on the central anti-nociceptive effect of insulin. According to the presence of insulin receptors in the CNS, CNS can be considered as a target of the anti-nociceptive effect of insulin. In this study we aimed to evaluate the analgesic effect of intra-cerebroventricular (ICV) injection of insulin using formalin test in short-term induced diabetic and non-diabetic rats.

Materials and Methods

Ethics

The protocol of this study was approved by Ethics Committee of the School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

Animals

Sixty-four male Sprague-Dawley adult rats (280 ± 30 g) were divided in eight groups (4 groups non-diabetic and 4 groups diabetic). Rats were acclimatized for a week to environmental conditions including feeding with commercial pellets and tap water ad libitum, ambient temperature of 22 ± 2°C and 12/12 h light/dark cycle.
Study design
The animals were divided into eight groups (n=8): 
group 1 (non-diabetic main control group) injected with 
normal saline (5 μL, ICV and 50 μL SC in the left hind 
paw); group 2 (non-diabetic control insulin group) 
 injected with normal saline (5 μL, ICV) and formalin (50 
μL, SC in the left hind paw); group 3 (non-diabetic 
control formalin test group) injected with insulin (5 μL, 
ICV) and normal saline (50 μL, SC in the left hind paw); 
group 4 (non-diabetic control group) injected with insulin (5 
μL, ICV) and formalin (50 μL, SC in the left hind paw); 
group 5 (diabetic control insulin group) injected with 
normal saline (5 μL, ICV) and formalin (50 μL, SC in the 
left hind paw); group 6 (diabetic control formalin test 
group) injected with insulin (5 μL, ICV) and normal 
saline (50 μL, SC in the left hind paw); group 7 (diabetic 
test group) injected with insulin (5 μL, ICV) and 
formalin (50 μL, SC in the left hind paw); group 8 
diabetic main control group injected with normal 
saline (5 μL, ICV and 50 μL SC in the left hind paw).

Induction of diabetes
After 24 h of fasting, diabetes was induced by a 
single dose of streptozotocin (60 mg/kg, i.p) (Sigma 
Aldrich Company) dissolved in 0.01 mol/L citric acid 
solution (pH = 4.5) (Gomar A, 2014; Silva, 2010). 
Seventy-two h after diabetes induction, fasting blood 
glucose was measured by a glucometer (ACCU-
CHECK) from tail vein. Animals with fasting blood 
glucose higher than 250 mg/dl were selected 
for the study. The non-diabetic rats received equal volume of 
citrate buffer (pH = 4.5) (Gomar A, 2014; Silva, 2010). 
After proving diabetes (approximately 48-72 h after 
diabetes induction), the formalin test was performed.

Surgery procedure and intra-cerebroventricular 
inejection of insulin
Rats were anesthetized with sodium pentobarbital (50 
mg/kg i.p). Guide cannula was implanted in lateral 
ventricle using stereotactic co-ordinates (AP= -0.8 mm, 
L= +1.5 mm, and DV=-3.6 mm) (Paxinos, 1977). After 
surgery, animals were caged separately for 24 h, then 
placed in the experimental apparatus at the time of 
formalin test. In 4 groups, insulin from bovine pancreas 
(Sigma Aldrich Company) (5 mU/animal, 5 μL) 
was injected in to the left ventricle at the rate of 1 μL/min by 
Hamilton syringe (10 μL) and in the other 4 groups, 
equal volumes of normal saline was used.

Formalin test
After 10 min of ICV injections, 50 μL of 2.5% 
formalin was injected subcutaneously into the plantar 
region of the left hind paw with a 27-gauge needle. 
Normal saline was injected instead of formalin in non-
diabetic, diabetic main control and control formalin test 
groups. The level of nociception was recorded every 15 s 
for 1 h (Dubuisson, 1977).

Histological verification
For verification of placing guide cannula in the lateral 
ventricle, at the end of each test, rats were euthanized 
with an overdose of diethyl ether, the brains were 
harvested and stored in formalin then position of cannula 
tracing was compared with the rat brain atlas (Paxinos, 
1977).

Statistical analysis
The statistic analysis was done by SPSS (version 
16.0). Two-way repeated measure ANOVA were used 
for analysis among the groups. Results are expressed as 
mean±SEM. The significant level was set at P<0.05.

Results
The mean of nociceptive score in each group of 
diabetic and non-diabetic rats were calculated during the 
formalin test. The results are illustrated in Tables 1 and 
2. There was no significant difference between non-
diabetic main control and control formalin test groups (1 
and 3). The mean±SEM of nociceptive score in group 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>First phase</th>
<th>Inter phase</th>
<th>Second phase</th>
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<tr>
<td>1</td>
<td>0.06±0.02</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>2</td>
<td>2.20±0.05</td>
<td>1.79±0.17</td>
<td>1.90±1.12</td>
<td>1.86±0.11</td>
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<tr>
<td>3</td>
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<td>0.00±0.00</td>
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<td>0.00±0.00</td>
</tr>
<tr>
<td>4</td>
<td>1.26±0.10</td>
<td>0.62±0.16</td>
<td>1.45±0.16</td>
<td>1.42±0.12</td>
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<td>5</td>
<td>2.05±0.09</td>
<td>1.64±0.12</td>
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<td>2.06±0.06</td>
</tr>
<tr>
<td>6</td>
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<td>2.09±0.07</td>
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<tr>
<td>7</td>
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<td>1.50±0.14</td>
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<td>2.09±0.09</td>
</tr>
<tr>
<td>8</td>
<td>2.06±0.06</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
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</table>

Table 1: Nociceptive scores (mean±SEM) in the test and control groups

Table 2: Nociceptive scores (mean±SEM) in different phases 
in the test and control groups
was 0.06 ± 0.2 in the first phase and no pain was observed in the interphase and the second phase (score = 0). The mean±SEM of nociceptive score in group 3 was 0.05 ± 0.02 in the first phase, 0.01 ± 0.01 in the interphase and no pain was noticed in the second phase (score = 0). On the other hand, no changes were seen in the animals behavior after SC injection of normal saline in the left hind paw of rats in non-diabetic main control and control formalin test groups (Table 2).

There was significant difference between non-diabetic control insulin with groups 2 and 4 in all 5 min except for the 11th 5 min (Table 1). In group 2, the mean±SEM of nociceptive score in the first, inter and second phases was 2.20 ± 0.05, 1.73 ± 0.10 and 2.04 ± 0.02, respectively. In group 4, the mean±SEM of nociceptive score in the first, inter and second phases was 1.26 ± 0.20, 0.62 ± 0.16 and 1.45 ± 0.16, respectively. Therefore, ICV injection of insulin in non-diabetic rats reduced nociceptive scores (P<0.05) (Tables 1 and 2, Fig. 1).

There was no significant difference between diabetic control formalin test and main control groups (6 and 8). The mean±SEM of nociceptive score in group 6 was 0.1 ± 0.02 in the first phase and was not observed any pain in inter and second phases (score = 0). The mean±SEM of nociceptive score in group 8 was 0.06 ± 0.03 in the first phase and there was not observed any pain in inter and second phases (score = 0) (Table 2).

Significant differences in the second, third, fourth, and fifth 5 min were observed between diabetic control insulin and test groups (5 and 7) (P<0.05) (Table 1). In group 5, the mean±SEM of nociceptive score in the first, inter and second phases was 2.05 ± 0.09, 1.25 ± 0.05 and 1.84 ± 0.09, respectively. In group 7, the mean±SEM of nociceptive score in the first, inter and second phases was 2.06 ± 0.08, 1.64 ± 0.12 and 2.03 ± 0.02, respectively. The highest mean of nociceptive score was seen in the third 5 min. Meanwhile, the mean±SEM of nociceptive score in the third 5 min in groups 5 and 7 was 1.09 ± 0.07 and 1.59 ± 0.14, respectively (Tables 1 and 2, Fig. 2).

There was no significant difference between non-diabetic control formalin test and diabetic main control groups (3 and 8) (Table 1).

Comparison between non-diabetic and diabetic test groups (4 and 7) demonstrated a significant difference in all 5 min except the ninth 5 min (P<0.05). The highest mean of nociceptive score was seen in the fourth and fifth 5 min, and the mean of nociceptive score in the fourth 5 min in group 4 and group 7 were 0.74 ± 0.22 and 2.04 ± 0.09 and in fifth 5 min in group 4 and group 7 were 0.83 ± 0.29 and 2.19 ± 0.06, respectively (Table 1, Fig. 3).

A significant difference was observed between non-diabetic control insulin and diabetic control insulin groups (2 and 5) in the second and third 5 min (interphase) (P<0.05). The mean of nociceptive score in the second 5 min in group 2 and group 5 were 1.79 ± 0.1 and 1.41 ± 0.07, and in the third 5 min they were 1.59 ± 0.12 and 1.09 ± 0.07, respectively (Table 1, Fig. 4).

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**Fig. 1:** Nociceptive scores in non-diabetic test (group 4) and non-diabetic control insulin (group 2) groups (mean±SEM). *Significant difference between the test and the control (n=8) (P<0.05). There is a significant difference between group 2 and 4 in all 5 min except the 11th 5 min.

**Fig. 2:** Nociceptive scores in diabetic control insulin (group 5) and diabetic test (group 7) groups (mean±SEM). *Significant difference between the test and the control (n=8) (P<0.05). There is a significant difference between groups 5 and 7 in the second, third, fourth, and fifth 5 min (P<0.05).

**Fig. 3:** Nociceptive scores in non-diabetic test (group 4) and diabetic test (group 7) groups (mean±SEM). Significant difference between the test groups (n=8) (P<0.05). There is a significant difference between groups 4 and 7 in all 5 min except the ninth 5 min (P<0.05).
Fig. 4: Nociceptive scores in non-diabetic control insulin (group 2) and diabetic control insulin (group 5) groups (mean±SEM). 7 Significant difference between control groups (n=8) (P<0.05). There is a significant difference between groups 2 and 5 in second and third 5 min (interphase) (P<0.05)

Discussion

The aim of this study was to investigate the effect of ICV injection of insulin on pain behavior using formalin test in diabetic and non-diabetic rats. Results of this study demonstrated that in comparison with diabetic rats, the ICV injection of insulin in non-diabetic rats possessed an anti-nociceptive effect in formalin test.

It has been shown that peripheral insulin can pass the blood brain barrier (Gerozissis, 2003; Banks et al., 2012). Insulin receptors are widely distributed in the CNS (Schulingkam, 2000; Plum et al., 2005; Laron, 2009). Therefore, the CNS can be an interesting site for anti-nociceptive effect of insulin. So far, researchers have done some studies on the analgesic effect of insulin (Gordon and Meldrum, 1970; Ohkubo et al., 1991; Gupta et al., 1992; Anuradha et al., 2004). It has been determined that serotonin, dopamine and opioidergic receptors, as well as NMDA receptor, potassium and calcium channels play an important role in analgesic effects of insulin (Anuradha et al., 2004). Takeshita et al. showed that ICV injection of insulin attenuated the second phase of formalin-induced nociception in mice. The possible cause of the above-mentioned result was the activation of central dopaminergic, serotonergic and opioidergic pathways (Takeshita and Yamaguchi, 1997). The ICV injection of insulin in diabetic mice dose dependently decreased pain response in the second phase of the formalin test. It has been shown that the analgesic effect of insulin in diabetic mice was less than that observed in non-diabetic mice which may be due to the alteration of pain control pathways such as dopaminergic pathways (Ohkubo et al., 1991; Takeshita and Yamaguchi, 1997). Our data indicated that, ICV injection of insulin in non-diabetic rats, unlike diabetic rats, possessed anti-nociceptive effect. So, central administration of insulin possesses analgesic effects in non-diabetic rats but not in diabetic rats. This difference may be related to the model of diabetes induction in our study (short-term model of diabetes) in comparison with aforementioned published studies that used chronic model of diabetes. More studies should be conducted to clarify the underlying mechanisms of these alterations.

The aim of short-term induction of diabetes in our study was to minimize the effects of the peripheral insulin.

Diabetes mellitus is a chronic metabolic disorder that is associated with neurological complications including alterations in nociception behavior (Rutledge et al., 2002). Previous studies have reported that hyperglycemia increased the pain sensitivity (Lee et al., 1990; Ibironke, 2004).

Diabetic hyperalgesia and allodynia in diabetic rats was observed two weeks after induction of diabetes suggesting that diabetic neuropathy developed 2 weeks after induction (Courteix et al., 1994; Gheibi et al., 2009). Courteix et al. (1993) suggested that 4 weeks after the induction of diabetes, the intensity of pain of the formalin test in diabetic rats is higher than in the non-diabetic ones. In the present study, we used short-term model of diabetes. Our data showed that, formalin injection in hind paw in diabetic control insulin group caused less pain response when compared to non-diabetic control insulin group (P<0.05; Fig. 3; Tables 1 and 2). It is known that opioids are involved in the mechanism of pain relief due to hyperglycemia, because naloxone eliminates its analgesic effect (Kolta et al., 1986; Akunne and Soliman, 1987). It has been reported that in the diabetic state, endogenous opiates are released acutely along with ACTH, in response to cell hypoglycemia. Raz et al. (1988) suggested that in diabetes, the pain threshold is maintained due to the compensatory secretion of endogenous opioids. Hyperglycemia in diabetes, changes the function of hypothalamic-pituitary and endogenous opioid system in a way that the acute release of opioid with ACTH occurs (Milan, 1985). Diabetes-induced hyperglycemia can increase the level of brain’s serotonin (Kolta et al., 1986) and then increase the pain threshold (Iversen, 1977; Sewell, 1977; Taber and Latranyi, 1981). Researchers have created tonic pain using monosodium urate (msu). They reported that 5-HT microinjection in to the nucleus raphe magnus, produced significant analgesic effects. So, neural transmission mediated by 5-HT in the nucleus raphe magnus plays an antinociceptive action (Inase et al., 1987).

In conclusion we showed that ICV injection of insulin in non-diabetic rats, in contrast with diabetic rats, has an anti-nociceptive effect in formalin test. In short-term diabetic rats, ICV injection of insulin was not able to reduce pain response and only partially decreased pain threshold.

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