# 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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(Received 15 Aug 2016; revised version 19 Jan 2017; accepted 31 Jan 2017)

## 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 test 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 induction of diabetes), the formalin test was performed.

## Surgery procedure and intra-cerebroventricular injection 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 (Dubbuisson, 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 2:** Nociceptive scores (mean±SEM) in different phases in the test and control groups

| Group | Time | | |
|---|---|---|---|
| | First phase | Inter phase | Second phase |
| 1 | $0.06 \pm 0.20^{a}$ | $0.00^{a}$ | $0.00^{a}$ |
| 2 | $2.20 \pm 0.05^{c}$ | $1.73 \pm 0.10^{d}$ | $2.04 \pm 0.02^{c}$ |
| 3 | $0.05 \pm 0.02^{a}$ | $0.01 \pm 0.01^{a}$ | $0.00^{a}$ |
| 4 | $1.26 \pm 0.20^{b}$ | $0.62 \pm 0.16^{b}$ | $1.45 \pm 0.16^{b}$ |
| 5 | $2.05 \pm 0.09^{c}$ | $1.25 \pm 0.05^{c}$ | $1.84 \pm 0.09^{c}$ |
| 6 | $0.1 \pm 0.02^{a}$ | $0.00^{a}$ | $0.00^{a}$ |
| 7 | $2.067 \pm 0.08^{c}$ | $1.64 \pm 0.12^{d}$ | $2.03 \pm 0.02^{c}$ |
| 8 | $0.06 \pm 0.03^{a}$ | $0.00^{a}$ | $0.00^{a}$ |

[a, b, c, d] Different small alphabetic letters show significant differences between groups

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

| Group | Time (min) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |
| 1 | $0.06\pm0.2^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ |
| 2 | $2.20\pm0.05^{c}$ | $1.79\pm0.1^{d}$ | $1.59\pm0.12^{d}$ | $1.86\pm0.11^{cd}$ | $2.01\pm0.10^{c}$ | $2.17\pm0.05^{c}$ | $2.14\pm0.04^{c}$ | $2.11\pm0.04^{c}$ | $2.08\pm0.06^{c}$ | $2.03\pm0.02^{c}$ | $1.98\pm0.01^{bc}$ | $1.98\pm0.02^{c}$ |
| 3 | $0.05\pm0.02^{a}$ | $0\pm0^{a}$ | $0.01\pm0.01^{a}$ | $0.02\pm0.02^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ |
| 4 | $1.26\pm0.2^{b}$ | $0.73\pm0.14^{b}$ | $0.52\pm0.20^{b}$ | $0.74\pm0.22^{b}$ | $0.83\pm0.29^{b}$ | $0.99\pm0.28^{b}$ | $1.42\pm0.21^{b}$ | $1.63\pm0.19^{b}$ | $1.69\pm0.18^{b}$ | $1.78\pm0.13^{b}$ | $1.80\pm0.09^{b}$ | $1.73\pm0.12^{b}$ |
| 5 | $2.05\pm0.09^{c}$ | $1.41\pm0.07^{c}$ | $1.09\pm0.07^{c}$ | $1.63\pm0.12^{c}$ | $1.77\pm0.14^{c}$ | $1.80\pm0.15^{c}$ | $1.88\pm0.12^{c}$ | $1.88\pm0.10^{bc}$ | $1.90\pm0.10^{bc}$ | $1.91\pm0.09^{bc}$ | $1.90\pm0.09^{bc}$ | $1.92\pm0.08^{bc}$ |
| 6 | $0.1\pm0.02^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ |
| 7 | $2.067\pm0.08^{c}$ | $1.69\pm0.12^{d}$ | $1.59\pm0.14^{d}$ | $2.04\pm0.09^{d}$ | $2.19\pm0.06^{c}$ | $2.09\pm0.07^{c}$ | $2.08\pm0.04^{c}$ | $2.01\pm0.04^{c}$ | $1.95\pm0.06^{bc}$ | $2.03\pm0.03^{c}$ | $2.01\pm0.01^{c}$ | $1.96\pm0.05^{c}$ |
| 8 | $0.09\pm0.03^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ | $0.00^{a}$ |

[a, b, c, d] Different small alphabetic letters show significant differences between groups

was 0.06 ± 0.2 in the first phase and no pain was observed in the inter phase 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 2.067 ± 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 (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).

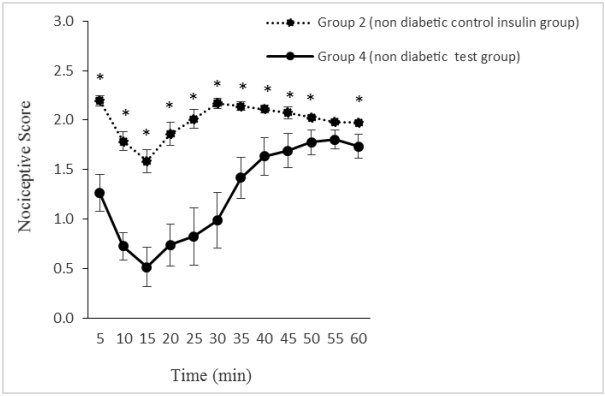


**Fig. 1:** Nociceptive scores in non-diabetic test (group 4) and non-diabetic control insulin (group 2) groups (mean±SEM). * Significant difference between the test and the control (n=8) (P<0.05). There is a significant difference between group 2 and 4 in all 5 min except the 11th 5 min

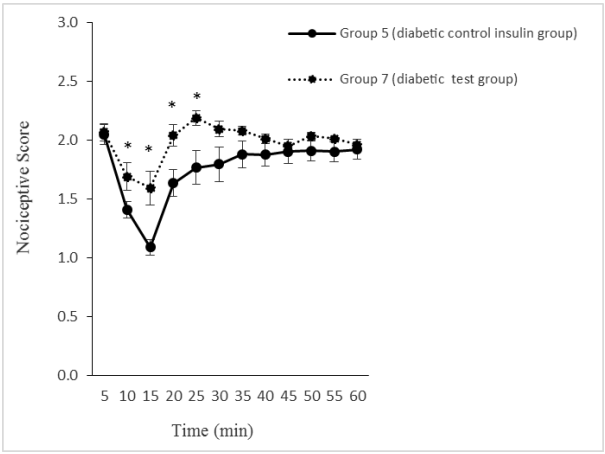


**Fig. 2:** Nociceptive scores in diabetic control insulin (group 5) and diabetic test (group 7) groups (mean±SEM). * Significant difference between the test and the control (n=8) (P<0.05). There is a significant difference between groups 5 and 7 in the second, third, fourth, and fifth 5 min (P<0.05)

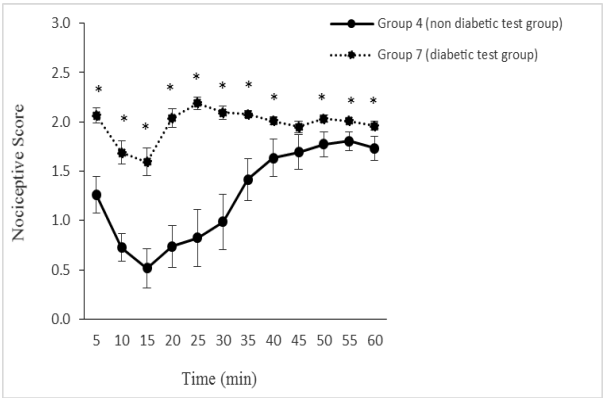


**Fig. 3:** Nociceptive scores in non-diabetic test (group 4) and diabetic test (group 7) groups (mean±SEM). * Significant difference between the test groups (n=8) (P<0.05). There is a significant difference between groups 4 and 7 in all 5 min except the ninth 5 min (P<0.05)

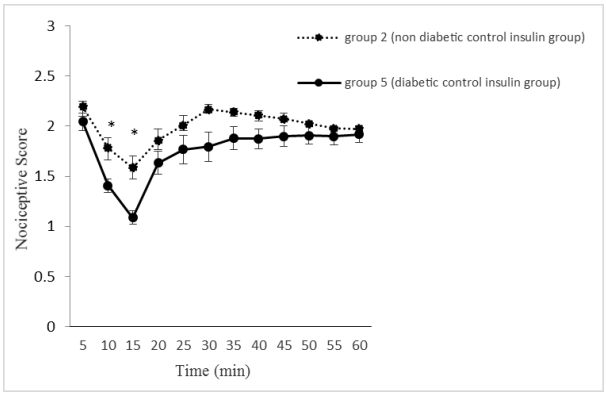


**Fig. 4:** Nociceptive scores in non-diabetic control insulin (group 2) and diabetic control insulin (group 5) groups (mean±SEM). * Significant difference between control groups (n=8) (P<0.05). There is a significant difference between groups 2 and 5 in second and third 5 min (interphase) (P<0.05)

## 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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