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Electroacupuncture improves glucose tolerance through cholinergic nerve and nitric oxide synthase effects in rats

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ABSTRACT

The purpose of this investigation was to evaluate the effect and mechanisms of electroacupuncture (EA) at the bilateral Zusanli acupoints (ST-36) on glucose tolerance in normal rats. Intravenous glucose tolerance test (IVGTT) was performed to examine the effects of electroacupuncture (EA) on glucose tolerance in rats. The EA group underwent EA at the ST-36, with settings of 15 Hz, 10 mA, and 60 min; the control group underwent the same treatments, but without EA. Atropine, hemicholinium-3 (HC-3) or NG-nitro-L-arginine methyl ester (L-NAME) were injected into the rats alone or simultaneously and EA was performed to investigate differences in plasma glucose levels compared to the control group. Plasma samples were obtained for assaying plasma glucose and free fatty acid (FFA) levels. Western blot was done to determine the insulin signal protein and nNOS to exam the correlation between EA and improvement in glucose tolerance. The EA group had significantly lower plasma glucose levels compared to the control group. Plasma glucose levels differed significantly between the EA and control groups after the administration of L-NAME, atropine, or HC-3 treatments alone, but there were no significant differences in plasma glucose with combined treatment of L-NAME and atropine or L-NAME and HC-3. EA decreased FFA levels and enhanced insulin signal protein (IRS1) and nNOS activities in skeletal muscle during IVGTT. In summary, EA stimulated cholinergic nerves and nitric oxide synthase for lowering plasma FFA levels to improve glucose tolerance.

Parasympathetic stimulation increases hepatic glycogen content and decreases hepatic glucose release [2]. Small amounts of the neurotransmitter nitric oxide (NO) produced by the neuronal isoform of nitric oxide synthase (nNOS) play a crucial role in a number of physiological functions including neurotransmission [3,12,13]. The nNOS is expressed in rat pancreatic islets and helps to control insulin secretion; after nNOS blockade with NG-nitro-L-arginine methyl ester (L-NAME), the β-cell response to arginine and glucose was dysfunctional [7]. Furthermore, Ach acts on muscarinic receptors and causes release of NO in the liver, which then releases hepatic insulin sensitizing substance (HISS), which sensitizes the skeletal muscle in response to insulin. Use of L-NAME or disruption of any part of this pathway results in immediate insulin resistance [16,17]. Besides, increases in free fatty acid (FFA) flux to skeletal muscle are implicated in the inhibition of muscle glucose uptake [1].

Electroacupuncture (EA) combines traditional needle acupuncture with an electrical current passing through the needles into the acupoints to produce a hypoglycemic response [5]. Researchers also found that EA inhibits sympathetic activity by regulating the expression of NOS in the central nervous system [10]. From these findings, we speculate that EA can modulate different types of NOS to achieve different effects.

The purpose of this investigation was to evaluate the effects and mechanisms of EA to the bilateral Zusanli acupoints (ST-36) on glu-

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cose tolerance. The cholinergic inhibitors and NOS antagonists were applied to explore the mechanisms. The changes in plasma glucose and FFA levels were determined for evaluation. Furthermore, we examined whether EA activated insulin-signaling protein (IRS1) and enhanced nNOS activity to improve glucose tolerance.

Male Wistar rats weighing 320–340 g and 8–10 weeks of age were used in this study. The rats were maintained in the Animal Center of China Medical University, Taichung, Taiwan. Overnight fasting and anesthetized with 40 mg/kg pentobarbital i.p. (MTC Company, Cambridge, Ontario, Canada) was done before all the experiments in this study. Animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and this protocol was approved by an ethics committee at China Medical University. In the laboratory determinations, plasma glucose (mg/dl) levels were determined using a spectrophotometric system (Cobas System, Roche Diagnostics Ltd., Rotkreuz, Switzerland) and commercially available enzymatic kits run in duplicate. The plasma FFA levels (meq/L) were determined spectrophotometrically on the Cobas System (Roche Diagnostics) using a commercially available non-esterified fatty acid kit (Randox Laboratories Ltd., Ardmore, United Kingdom). In the effect of EA on the insulin signaling protein (IRS1) and nNOS, the relative amounts of signal protein (IRS1) and nNOS to β-actin in skeletal muscle were assayed by Western blot as previous method [9].

The IVGTT was performed as previous method [5,9]. The fasting normal male rats ($N = 16$) were divided into the experimental group (EG, $N = 8$) and control groups (CG, $N = 8$) randomly. Blood samples were then collected at 15, 30, 60, and 90 min after 1 mg/kg glucose i.v. administration for the measurement of blood glucose. The changes in the plasma glucose levels were then compared between the EG and CG. In the EG, the bilateral Zusanli acupoints located at the anterior tibia muscle near the knees were identified based on previous studies [4,15]. After adjusting the EA apparatus to 15 Hz and 10 mA (Han's Healthronics Likon, Taipei, Taiwan), acupuncture needles (0.5 in., 32 gauge) were inserted 2–5 mm into the muscle layer of the selected acupoints. The positively charged (red pole) clip was connected to the right needle and the negatively charged (black pole) clip was connected to the left needle.

In the exploration of the mechanisms of EA on improving glucose tolerance, fasting normal male rats ($N = 16$) were randomly assigned to the EG ($N = 8$) or the CG ($N = 8$) in each one of the experiments. A single injection of a cholinergic inhibitor, atropine (0.1 mg/kg, i.p.) or HC-3 (0.1 mg/kg, i.p.), or the NOS antagonist, L-NAME (10 mg/kg, i.p.), was administered to all rats in EG and CG. During IVGTT, blood samples were drawn for the measurement of plasma glucose. In the evaluation of combined-agent glucose tolerance blockade, a single injection of atropine (0.1 mg/kg, i.p.) combined with L-NAME (10 mg/kg, i.p.) or HC-3 (0.1 mg/kg, i.p.) combined with L-NAME (10 mg/kg, i.p.) was administered to all rats of both groups. During IVGTT, blood samples were drawn for the measurement of plasma glucose in each group.

In the assay of plasma FFA, fasting normal male rats ($N = 16$) were randomly assigned to EG ($N = 8$) and CG ($N = 8$). A single injection of atropine (0.1 mg/kg, i.p.) combined with L-NAME (10 mg/kg, i.p.) was administered to all rats of both groups. The IVGTT was started in EG and CG. During IVGTT, blood samples were drawn for the measurement of plasma FFA levels in each group. In the study of insulin signal protein (IRS1) and nNOS, fasting normal male rats ($N = 16$) were randomly assigned to EG ($N = 8$) and CG ($N = 8$). At the end of IVGTT, a section of the gastrocnemius muscle was taken from each rat in the both groups for Western blot analysis of insulin signal protein (IRS1), nNOS and β-actin (served as the control).

In this study, all values are expressed as means ± SEMs. The independent-sample Student's t-test was applied to assess differences between the EG and CG. The Student's paired t-test was used to assess differences within the same group before and after treatment. Statistical analyses were performed using SPSS (version 11.5) analytical software. A p-value of less than 0.05 (two-sided) was considered statistically significant.

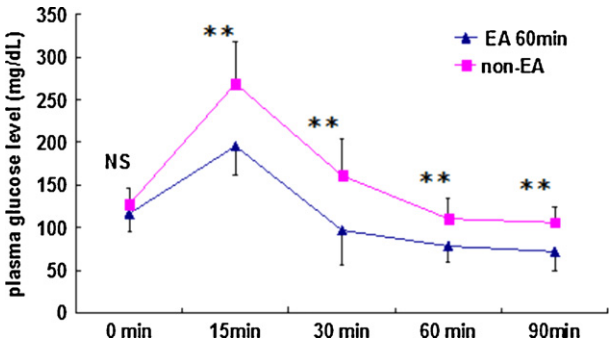

Fig. 1. Effect of EA on glucose tolerance in normal rats during IVGTT. $^{**}p < 0.005$; NS, not significant.

In the evaluation of EA effect on glucose tolerance, the IVGTT revealed significantly lower plasma glucose levels in the EG compared to the CG from 15 to 90 min after treatment. The plasma levels of glucose at 15, 30, 60 and 90 min were 195.7 ± 33.8, 96.9 ± 40.3, 78.4 ± 18.3 and 71.8 ± 21.9 mg/dl in the EG, which were markedly lower than the results in the CG ($p < 0.005$) (Fig. 1).

In the blocking effect of EA on glucose tolerance, after injection with L-NAME alone, IVGTT still revealed significantly lower plasma glucose levels in the EG compared to the CG from 15 to 90 min post-treatment (Fig. 2A). After injecting atropine or HC-3 alone, IVGTT revealed significantly lower plasma glucose levels in the EG compared to the CG at only 15 and 30 min post-treatment; no significant differences occurred at 60 and 90 min post-treatment (Fig. 2B and C). In the blocking effect of EA on glucose tolerance by combined antagonists, after injection with L-NAME and atropine or L-NAME and HC-3, IVGTT revealed no significant differences in plasma glucose levels in the EG compared to the CG from 15 to 90 min post-treatment (Fig. 3A and B).

Furthermore, in the impact on plasma FFA levels by combined antagonists, the tendency of FFA levels was lower in the EG compared to the CG. At the end of IVGTT (90 min), the EG exhibited lower plasma FFA levels compared to the CG. The average plasma FFA level of the EG at 90 min was 0.53 ± 0.21 meq/L, which was markedly lower than the level (1.05 ± 0.24 meq/L) in the CG (Fig. 4A). After injection with L-NAME and atropine simultaneously, the effect of EA on plasma FFA levels during IVGTT did not differ significantly at 15, 30, 60 and 90 min (Fig. 4B). In the effect on the molecular signals of insulin signaling protein expression and nNOS, after comparing the relative quantities of insulin signaling protein to β-actin, we found that significantly greater expression of IRS1 protein occurred in the EG (1.29 ± 0.74) compared to the CG (0.25 ± 0.15) (Fig. 5). Additionally, expression of nNOS in the skeletal muscle of the EG was significantly greater (0.64 ± 0.21) than in the CG (0.37 ± 0.07) (Fig. 5).

In a previous study, glucose tolerance and insulin sensitivity were enhanced by EA stimulation at the bilateral Zusanli acupoints in normal male Wistar rats and streptozotocin induced diabetic rats, but endogenous plasma insulin levels did not differ between the EG and CG [5]. In another study, the EA group exhibited lower plasma glucose levels than the non-EA control group in steroid background male Wistar rats (SBRs). The EA group and the non-EA control group exhibited no significant differences in plasma insulin levels in these SBRs [9]. The enhanced glucose tolerance and the improvement in insulin resistance were shown by the decreasing plasma FFA levels and increasing the expression of insulin signal proteins from EA stimulation, and the materials and methods of this study were the same lab condition as the previous two studies

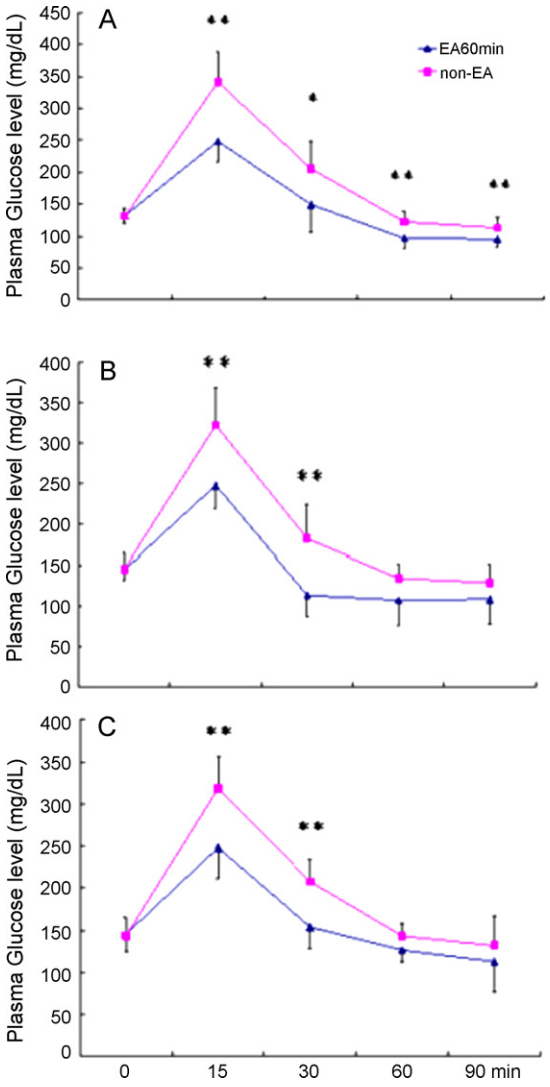

Fig. 2. (A) Effect of EA on glucose tolerance blocked by L-NAME. (B) Effect of EA on glucose tolerance blocked by atropine. (C) Effect of EA on glucose tolerance blocked by HC-3. $^{*}p < 0.05$; $^{**}p < 0.005$.

[5,9]. It is the reason why we did not need to assay the plasma level of insulin in the present study. In a recent study, insulin caused the release of hepatic insulin-sensitizing substance (HISS) from the liver. Hepatic parasympathetic nerves also played a permissive role in the release of HISS [17]. Also, some researchers support that EA acts on parasympathetic nerves [8,18]. Therefore, the stimulating cholinergic nerve and decreasing plasma FFA levels of EA should be considered via enhancing insulin sensitivity to lower down the plasma glucose.

According to the data of this study (Fig. 4), atropine and L-NAME blocked the lowered plasma FFA of EA under IVGTT. Therefore, the effect of cholinergic nerve and nitric oxide synthase simultaneously via lowering plasma FFA to enhance insulin sensitivity can thus be hypothesized. Regarding plasma glucose, the difference of plasma FFA between EA group and the control group only at 90 min is showed in Fig. 4, on the other hand, Fig. 1 shows the difference of plasma glucose from 15 min to 90 min. Though it seems difficult to define that FFA level influences plasma glucose level, the possible reasons are the different hypoglycemic pathways of cholinergic nerve and nitric oxide earlier than the change of plasma FFA via improving glucose tolerance to lower down the plasma glucose [8,16,17].

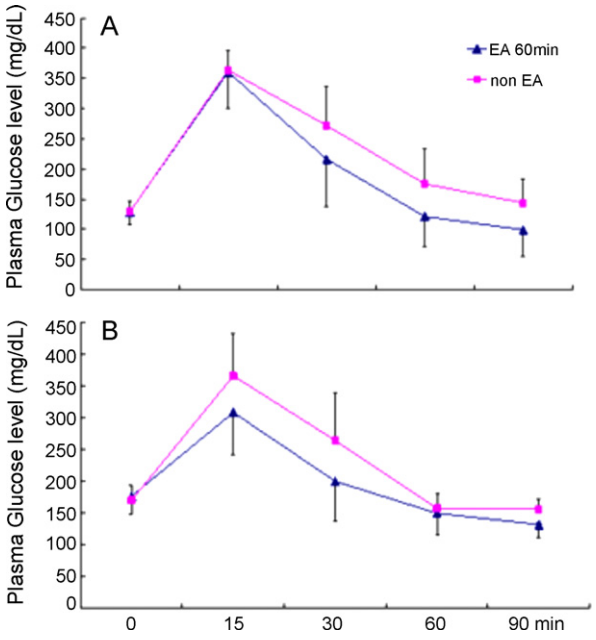

Fig. 3. (A) Effect of EA on glucose tolerance blocked by L-NAME and atropine. (B) Effect of EA on glucose tolerance blocked by L-NAME and HC-3. $p > 0.05$.

During the IVGTT experiments, the two groups of rats exhibited no difference initially, but a better hypoglycemic effect was noted in the EG compared to the CG (Fig. 1). In Fig. 2A, the hypoglycemic effect of EA could not be blocked by L-NAME alone. After injection with atropine or HC-3 alone, IVGTT revealed significant differences in hypoglycemic effects in the EG compared to that of the CG, only at 15 and 30 min post-treatment, and there were no significant differences at 60 and 90 min post-treatment (Fig. 2B and C). We speculate that the EA hypoglycemic effect was only partially blocked by the cholinergic antagonists, atropine blocked on the Ach muscarinic receptor or HC-3 inhibited choline uptake by the

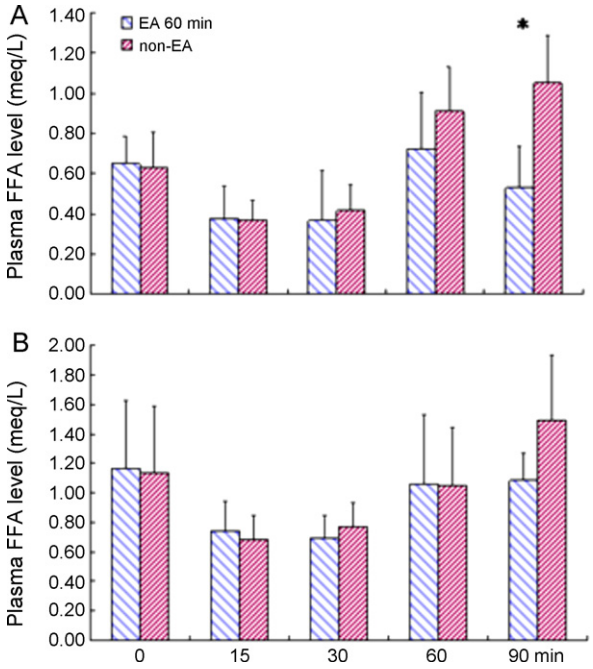

Fig. 4. (A) Effect of EA on plasma FFA levels during IVGTT on normal rats. (B) Effect of EA on plasma FFA levels during IVGTT blocked by combined L-NAME and atropine. $^{*}p < 0.05$.

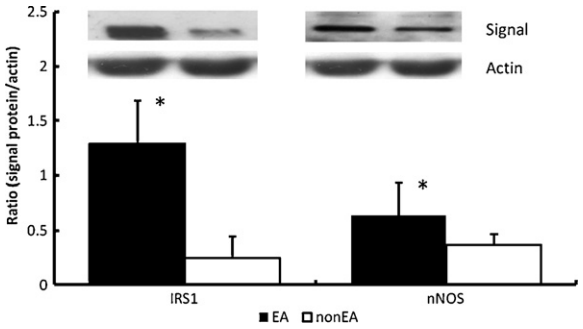

Fig. 5. The bar plot shows the effect of EA on the insulin signaling protein IRS1 and nNOS in normal male Wistar rats. The X-ray films show relative amounts of signal proteins IRS1 and nNOS to β-actin in skeletal muscle assayed by Western blot. $^{*}p < 0.05$.

disruption of synaptically available acetylcholine alone. Therefore, this study did not further explore more detail change of cholinergic system.

The mechanisms of EA were explored using a combined cholinergic inhibitor and NOS antagonist (L-NAME) during IVGTT. There were no significant differences in plasma glucose levels between the EG and CG post-treatment (Fig. 3A and B). The improved glucose tolerance effect of EA was totally blocked by simultaneously giving L-NAME and atropine or L-NAME and HC-3. These results are compatible with previous studies, which showed that insulin sensitivity relative to release of acetylcholine (Ach) in the liver and release of nitric oxide (NO), which was responsible for release of HISS from the liver, enhanced insulin activity in skeletal muscle [6,16]. Acute effects of EA on improving glucose tolerance in normal male Wistar rats could result from combined effects of releasing acetylcholine (Ach) and NO via secretion of HISS to facilitate insulin activity.

Plasma FFA is a key factor that influences insulin sensitivity. In normal male Wistar rats during IVGTT, the plasma FFA trend lower from 0 to 30 min, then undergo steady elevation from 30 to 90 min. When the plasma glucose levels increased during IVGTT, plasma FFA levels decreased. One possible reason is that the lipolytic effect of fat tissue is inhibited in order to enhance the hypoglycemic effect of endogenous insulin. Furthermore, there was a marked decrease in FFA levels below the normal range at 90 min in the EG compared to that of the CG during IVGTT (Fig. 4A). This tendency was compatible with previous data, which showed a marked decrease in FFA levels at 60 min in the EA group of the SBRs during insulin challenge test (ICT) than in the SBRs without EA [9]. After injection with L-NAME and atropine simultaneously, the plasma FFA lowering effect of EA at 90 min during IVGTT disappeared. The plasma FFA levels during IVGTT did not differ between the two groups (Fig. 4B). The EA effect of lowering plasma FFA level at 90 min into the IVGTT was blocked by combined L-NAME and atropine. We hypothesis that the action of EA is through the cholinergic nerve and NO release via lowering the plasma FFA to improve glucose tolerance.

After comparing the relative amounts of insulin signaling protein and nNOS to β-actin, we found that amount of IRS1 and nNOS was significantly higher in the EG than in the CG (Fig. 5). Therefore, it is reasonable to suppose that EA increases insulin activity in normal male Wistar rats through increased the expression of IRS1 and increased nNOS activity (Fig. 5). A previous study showed that all major isoforms of NOS, including a muscle-specific splice variant of neuronal-type NOS are expressed in skeletal muscles of all mammals [19]. The results of EA stimulation were similar to the hypothesis that release of low concentrations of NO through myocyte nNOS could contribute to insulin sensitization through cyclic guanosine monophosphate (cGMP)-dependent nonhemodynamic mechanisms [20] and by nNOS-derived NO, which could be involved in hepatic blood circulation and hepatobiliary activities to improve glucose tolerance [14]. Additionally, these findings were compatible with previous studies, which showed that skeletal muscle functions regulated by NO derived from nNOS included autoregulation of blood flow and glucose homeostasis [20]. Interestingly, this phenomenon was similar to an another observation, which showed that the stimulatory effect of lower concentrations of NO released from its donors (GSNO and SNAP) enhanced basal and insulin-stimulated glucose uptake in normal and type 2 diabetic rats [11]. In summary, insulin activity was enhanced by EA at normal male Wistar rats via increased activity of nNOS and up-regulation of insulin signaling protein IRS1 expression in the skeletal muscle of rats. Therefore, it is reasonable to suppose that the action of EA improves glucose tolerance of normal male Wistar rats through incremental IRS1 and nNOS activities.

Finally, we conclude that 15 Hz EA at the Zusanli acupoints significantly improves glucose tolerance of normal male Wistar rats during IVGTT. The action of EA has the combined effect of cholinergic nerve and increased nNOS activity via lower plasma FFA concentrations to improve glucose tolerance. Furthermore, improvement of insulin activity was achieved by up-regulating IRS1 expression in the skeletal muscle of rat. Thus, EA should be considered a method of alternative therapy for improving glucose tolerance.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

[1] G. Boden, Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver, Curr. Diab. Rep. 6 (2006) 177–181.
[2] P.J. Boyle, S.B. Liggett, S.D. Shah, P.E. Cryer, Direct muscarinic cholinergic inhibition of hepatic glucose production in humans, J. Clin. Invest. 82 (1988) 445–449.
[3] H. Bult, G.E. Boeckxstaens, P.A. Pelckmans, F.H. Jordaens, Y.M. Van Maercke, A.G. Herman, Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter, Nature 345 (1990) 346–347.
[4] S.L. Chang, J.G. Lin, C.L. Hsieh, J.T. Cheng, Comparision of hypoglycemic effect in different acupoints response to 2 Hz electroacupuncture, J. Chin. Med. 13 (2002) 111–117.
[5] S.L. Chang, K.J. Lin, R.T. Lin, P.H. Hung, J.G. Lin, J.T. Cheng, Enhanced insulin sensitivity using electroacupuncture on bilateral Zusanli acupoints (ST 36) in rats, Life Sci. 79 (2006) 967–971.
[6] M.P. Guarino, N.C. Correia, W.W. Lautt, M.P. Macedo, Insulin sensitivity is mediated by the activation of the ACh/NO/cGMP pathway in rat liver, Am. J. Physiol. Gastrointest. Liver Physiol. 287 (2004) G527–G532.
[7] A.D. Lajoix, H. Reggio, T. Chardes, S. Peraldi-Roux, F. Tribillac, M. Roye, S. Dietz, C. Broca, M. Manteghetti, G. Ribes, C.B. Wollheim, R. Gross, A neuronal isoform of nitric oxide synthase expressed in pancreatic beta-cells controls insulin secretion, Diabetes 50 (2001) 1311–1323.
[8] Y.C. Lee, T.M. Li, C.Y. Tzeng, Y.I. Chen, W.J. Ho, J.G. Lin, S.L. Chang, Electroacupuncture at the Zusanli (ST-36) acupoint induces a hypoglycemic effect by stimulating the cholinergic nerve in a rat model of streptozotocine-induced insulin-dependent diabetes mellitus, eCAM (2010), neq068.
[9] R.T. Lin, C.Y. Tzeng, Y.C. Lee, W.J. Ho, J.T. Cheng, J.G. Lin, S.L. Chang, Acute effect of electroacupuncture at the Zusanli acupoints on decreasing insulin resistance as shown by lowering plasma free fatty acid levels in steroid-background male rats, BMC Complement. Altern. Med. 9 (2009) 26.
[10] S.X. Ma, J. Ma, G. Moise, X.Y. Li, Responses of neuronal nitric oxide synthase expression in the brainstem to electroacupuncture Zusanli (ST 36) in rats, Brain Res. 1037 (2005) 70–77.
[11] D. McGrowder, D. Ragoobirsingh, P. Brown, Acute effects of exogenous nitric oxide on glucose uptake in skeletal muscle of normoglycaemic and diabetic rats, Med. Sci. Monit. 12 (2006) BR28–35.

[12] S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43 (1991) 109–142.
[13] R.M. Palmer, D.D. Rees, D.S. Ashton, S. Moncada, L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation, Biochem. Biophys. Res. Commun. 153 (1988) 1251–1256.
[14] D. Patrice, M. Andre, Obesity-linked insulin resistance: is nitric oxide the missing link? Can. J. Diab. 28 (2004) 59–60.
[15] V.V. Romita, K. Yashpal, C.W. Hui-Chan, J.L. Henry, Intense peripheral electrical stimulation evokes brief and persistent inhibition of the nociceptive tail withdrawal reflex in the rat, Brain Res. 761 (1997) 192–202.
[16] P. Sadri, W.W. Lautt, Blockade of nitric oxide production in the liver causes insulin resistance, Proc. West. Pharmacol. Soc. 41 (1998) 37–38.
[17] P. Sadri, D.J. Legare, S. Takayama, W.W. Lautt, Increased incidence of hepatic insulin-sensitizing substance (HISS)-dependent insulin resistance in female rats prenatally exposed to ethanol, Can. J. Physiol. Pharmacol. 83 (2005) 383–387.
[18] R. Shimoju-Kobayashi, H. Maruyama, M. Yoneda, M. Kurosawa, Responses of hepatic glucose output to electro-acupuncture stimulation of the hindlimb in anaesthetized rats, Auton. Neurosci. 115 (2004) 7–14.
[19] J.S. Stamler, G. Meissner, Physiology of nitric oxide in skeletal muscle, Physiol. Rev. 81 (2001) 209–237.
[20] M.E. Young, B. Leighton, Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle, Biochem. J. 329 (Pt 1) (1998) 73–79.