Hypoglycemic Effect of Electroacupuncture Combined with *Antrodia cinnamomea* in Dexamethasone-Induced Insulin-Resistant Rats

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ABSTRACT

Objective: *Antrodia cinnamomea* (AC), a medicinal mushroom indigenous to Taiwan, exerts various pharmacologic activities. This study compared and evaluated the hypoglycemic effect of treatment with electroacupuncture (EA) combined with AC in steroid-induced insulin-resistant (SIIR) rats.

Materials and Methods: Rats were divided into saline, EA, AC, AC+EA, and rosiglitazone (TZD) groups. Plasma-glucose levels were measured in serial blood samples and compared before and after treatment in each group. The levels of signaling proteins—glucose transporter 4, (GLUT4), phosphoinositide 3-kinase (PI3-K), and 5' adenosine monophosphate-activated protein kinase (AMPK)—were analyzed by Western blotting to explore their mechanisms of action.

Results: The AC+EA group had reduced plasma-glucose levels at 30 and 60 minutes in SIIR rats, compared to normal rats, and this was better than the EA, AC, and TZD groups at 60 minutes. Furthermore, the signaling protein (GLUT4, PI3-K, and AMPK) levels were increased significantly.

Conclusions: These findings showed improved hypoglycemic activity and insulin resistance after EA combined with AC treatment. Therefore, the combined therapy might be a more-effective method than the individual therapies that elevates the expression of the signal proteins, as observed in this study.

Keywords: electroacupuncture, *Antrodia cinnamomea*, plasma glucose, dexamethasone, insulin, signal transduction proteins

INTRODUCTION

SEDENTARY LIFESTYLE AND OVEREATING can result in an overabundance of glucose and fatty-acid accumulation within the muscles, adipose tissue, and pancreatic cells. Hyperglycemia is a common outcome of uncontrolled diabetes that leads to severe damage in many parts of the body's system over time, especially the nerves and blood vessels.¹ High doses

of glucocorticoid are the most common cause of drug-induced diabetes mellitus.² The pathophysiology of insulin resistance involves a complex network of insulin-signaling pathways. Activation of the inhibitor κ -B kinase (IKK) pathway increases insulin receptor substrate-1 (IRS-1) serine phosphorylation, leading to suppression of insulin signaling. The suppression of the IKK pathway decreases insulin resistance and ameliorates glucose intolerance, thus, likely activating stress signaling.¹

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A previous study of *Antrodia cinnamomea* (AC) demonstrated that insulin sensitivity was enhanced with AC, indicating its insulin sensitizer function in insulin-resistant animal models, and an optimal dose for further study was determined.³ AC is a medicinal mushroom indigenous to Taiwan and grows only in the inner cavity of *Cinnamomum kanehirae hayata* (Lauraceae). Taiwan aborigines commonly use the fruiting body of AC as a folk medicine to promote health and to treat liver disease, drug and food intoxication, diarrhea, and cancer.⁴ Recently, AC has been found to exert a variety of biologic activities, including antioxidant, hypoglycemic, anticancer,^{5,6} liver protective,^{7,8} anti-inflammatory,^{9,10} and immune-regulatory effects.^{11,12} In addition, many bioactive components of AC have been identified, such as polysaccharides, terpenoids, benzenoids, and nucleic acids.¹³ The extracts of AC mycelia and its components, such as polysaccharides, anthraquinone, and antrocin B, were found to improve diabetes and dyslipidemia in mice fed a high-fat diet.^{12,14,15}

Electroacupuncture (EA) is a modified therapeutic technique for piercing the skin with acupuncture needles while reinforcing them with low-voltage electrical currents.¹⁶ Hence, the therapeutic effect of EA was investigated for the ability to increase expression of insulin-signaling proteins IRS-1 and glucose transporter 4 (GLUT4) in skeletal muscle, which is consistent with an improvement in steroid-induced insulin-resistant (SIIR) rats.³ The insulin receptors, GLUT, and peroxisome proliferator-activated receptors are considered potential targets in the development of new treatment methods for diabetes mellitus. For example, the c-Jun N-terminal kinases (JNK) and IKK pathways are involved in the development of insulin resistance, and these could be therapeutic targets for diabetes.¹

Therefore, this present study explored the effect of combined EA with AC, compared with single-therapy EA, AC, or rosiglitazone (TZD), on the SIIR rat model, and observed the mechanisms of effects on hyperglycemia through the expression of signal proteins. The EA and AC combination has been suggested as a potential therapy for patients with diabetes mellitus.

MATERIALS AND METHODS

Preparation of AC Mycelia

The AC mycelia were cultured in the dark on solid-state cereal medium (provided by author W.-J. H., of Da-Yeh University, Changhua, Taiwan) for 85–90 days at 21°C–23°C. The medium was mainly supplemented with yeast extract and glucose. After this, the cultures were harvested, dried, and ground into powder for subsequent experiments. The final yield from the solid-state culture was ~20% of the

original dry weight of the cereal medium. In addition, the powder was mixed with normal saline to produce oral solutions at a concentration of 500 mg/kg of body weight (BW) and stored at 4°C until needed for analysis.

Animal Models

Wistar rats were purchased from the Bio LASCO Animal Center (Taipei, Taiwan), allowed to acclimatize at the Animal Breeding Center. The rats were housed at 25°C ± 1°C with a relative humidity of 65% ± 5% in a 12-hour light/dark cycle with *ad libitum* access to water and food (general rat chow). The Institutional Animal Care and Use Committees (IACUC), of Da-Yeh University, reviewed the animal research ethics and ethical approval (Number: DYU-100011-R0038) was given.

This study was performed according to previously published research methods.¹⁷ The SIIR rats were injected intraperitoneally (i.p.) with 1 mg/kg of dexamethasone daily for 5 days. It was concluded that insulin resistance was successfully induced when the rats' fasting plasma glucose levels were higher than 150 mg/dL. The SIIR rats were fasted before each test and anesthetized with pentobarbital (40 mg/kg, i.p.), and the experiments were performed at 8:00 AM.³

EA Treatment

The rats in the EA treatment group were administered EA on bilateral *Zusanli* acupoints (ST 36). The electrical current was provided through Han's acupoint nerve stimulator (Healthtronics, Singapore) with a frequency of 15 Hz¹⁸ at a 10 mA-intensity level between 30 and 60 minutes.¹⁹

Experimental Protocol

The rats were randomly divided into the following different groups ($n=6$ per group): (a) saline group: Wistar rats orally administered an equal volume of saline; (b) AC group: Wistar rats orally administered AC (500 mg/kg); (c) EA group: Rats administered normal saline injection and given bilateral *Zusanli* acupoint (ST 36) EA at 15 Hz/10 mA treatments; (d) AC+EA group (AC+EA): Rats administered AC and EA per the previous schedules; (e) TZD group: Rats orally administered rosiglitazone (0.1 mg/kg).³

Assay for the Plasma Glucose Levels

Plasma glucose levels assays were performed with a commercial enzymatic method using a Glucose HK stable liquid reagent (Randox Laboratories Ltd., Crumlin, UK). The percentage of the hypoglycemic activity was calculated as follows: plasma glucose levels at 0 minutes, then plasma glucose levels at:

$$30 \text{ minutes}[(\text{or } 60 \text{ minutes})/\text{plasma glucose levels at } 30 \text{ minutes}(\text{or } 60 \text{ minutes})] \times 100.$$

The optimal group dosage for inducing hypoglycemia was determined and used in subsequent experimental procedures.

Enzyme-Linked Immunosorbent Assay of Plasma Insulin Levels and Resistance Test

We performed enzyme-linked immunosorbent assays (ELISAs) with kits obtained from Mercodia AB (Uppsala, Sweden). First, 25 μ L of serum and 50 μ L of conjugate solution was prepared in each measuring tank and shaken for 2 hours at room temperature. Then, the mixed solution was rinsed 5 times with 350 μ L of buffer, 200 μ L of peroxidase substrate (3,3',5,5'-tetramethylbenzidine) added, and incubated for 15 minutes at room temperature. Finally, a 50- μ L solution (1M H₂SO₄) was added to the reaction and shaken for 5 seconds, then measured with a spectrophotometer (450 nm). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the following formula:

$$[\text{fasting plasma insulin levels}(\mu\text{U/mL}) \times \text{fasting plasma glucose}(\text{mmol/L})] / 22.5.^3$$

Western Blot Analysis

For Western blotting, anti-phosphoinositide 3-kinase (PI3-K), anti-GLUT4, and 5' adenosine monophosphate-activated protein kinase (AMPK) antibodies were added in a radioimmunoprecipitation assay lysis buffer, and ground using a homogenizer-pending protein extraction. The appropriate concentration of protein was added to reduce sample buffer (glycerol, β -mercaptoethanol, sodium dodecyl sulfate [SDS], Tris-HCl), and boiled to denature at 95°C for 15 minutes. A total of 90 μ g/mL of protein was separated by 8% SDS-polyacrylamide gel electrophoresis, and the gel transferred to a polyvinyl fluoride (PVDF) membrane at 4°C.

The PVDF membrane was then submerged in 5% nonfat milk to block the nonspecific binding sites in the membrane at 25°C for 1 hour. The membrane was incubated overnight with anti-PI3-K, anti-GLUT4, and AMPK antibodies at 4°C, and washed four times (10 minutes each) with phosphate buffered saline with Tween[®] detergent buffer after the incubation. Finally, the membranes were incubated with goat antirabbit immunoglobulin G-horseradish peroxidase antibodies at 25°C for 1 hour, and specific bands were detected, using an enhanced chemiluminescence kit. The bands were quantified using optical densitometry, while actin bands were used as internal loading controls, and the results were presented as the ratio of signal-to-actin.

Statistical Analysis

Statistical analysis was performed using a Student's *t*-test or one-way analysis of variance, and the results were presented as the mean $\pm$ standard error for each group. Statistical significance was obtained at a *P*-value <0.05.

RESULTS

Effect of Hypoglycemic Activity in Normal Wistar Rats

The different treatments in the experiments were compared to the hypoglycemic activity of normal Wistar rats. The hypoglycemic activity of each group at 30 minutes were as follows: saline group = $11.47 \pm 4.89\%$ (b); AC group = $19.75 \pm 5.46\%$ (b); EA group = $41.36 \pm 9.25\%$ (a); AC+EA group = $41.27 \pm 6.71\%$ (a); and TZD group = $14.92 \pm 3.05\%$ (b), where a and b represented the statistical markers, $a > b$; $P < 0.05$. Furthermore, the hypoglycemic activity of each group at 60 minutes was as follows: saline group = $21.56 \pm 4.01\%$ (C); AC group = $25.25 \pm 3.13\%$ (C); EA group = $45.68 \pm 9.68\%$ (B); AC+EA group = $60.35 \pm 5.60\%$ (A); and TZD group = $20.27 \pm 9.84\%$ (C), where A, B and C represented the statistical markers, $A > B > C$; $P < 0.05$. The plasma glucose levels of the normal Wistar rats treated with

EA, and EA+AC, decreased significantly, compared to the saline, AC, and TZD groups, at the 30 and 60 minutes. In particular, the hypoglycemic activity of the combined therapy (EA+AC) was greater than the signal therapy (EA, AC, TZD) at 60 minutes; $P < 0.05$ (Fig. 1).

Effect of Hypoglycemic Activity in SIIR Rats

In the SIIR group, the hypoglycemic activities at 30 minutes were as follows: saline group = $21.06 \pm 7.87\%$ (b); AC group = $28.97 \pm 8.71\%$ (b); EA group = $51.27 \pm 3.47\%$

FIG. 1. Hypoglycemic activity of different treatments in Wistar rats. The saline group was administered normal saline. In the *Antrodia cinnamomea* (AC) group, Wistar rats were administered oral AC extract (500 mg/kg). The electroacupuncture (EA) group received normal saline and EA at ST 36 (15 Hz/10 mA). The AC+EA group received AC and EA as previously scheduled. The TZD (rosiglitazone) group was administered 0.1 mg/kg of oral rosiglitazone. Statistical analysis was performed using an analysis of variance, $a > b > c$; $A > B > C$; $P < 0.05$. min, minutes.

FIG. 2. Hypoglycemic activity of different treatments in steroid-induced insulin-resistant (SIIR) rats. The saline group was administered normal saline. In the *Antrodia cinnamomea* (AC) group, Wistar rats were administered oral AC extract (500 mg/kg). The electroacupuncture (EA) group received normal saline and EA at ST 36 (15 Hz/10 mA). The AC+EA group received AC and EA as previously scheduled. The TZD (rosiglitazone) group was administered oral rosiglitazone (0.1 mg/kg). Statistical analysis was performed using an analysis of variance, $a > b > c$; $A > B > C$; $P < 0.05$. min, minutes.

(a); AC+EA group = $52.30 \pm 4.25\%$ (a); and TZD group = $25.96 \pm 4.07\%$ (b), where $a > b$; $P < 0.05$. Furthermore, the hypoglycemic activities at 60 minutes were as follows: saline group = $26.21 \pm 14.90\%$ (C); AC group = $30.06 \pm 11.30\%$ (C); EA group = $44.98 \pm 4.11\%$ (B); AC+EA group = $54.63 \pm 8.12\%$ (A); and TZD group = $36.01 \pm 6.55\%$ (C), where $A > B > C$; $P < 0.05$. The plasma glucose levels of SIIR rats treated with EA, and EA+AC, decreased significantly, compared to the saline, AC, and TZD groups at 30 and 60 minutes. In particular, the hypoglycemic activity of the combined therapy (EA+AC) was greater than the signal therapy (EA, AC, TZD) at 60 minutes; $P < 0.05$ (Fig. 2).

Comparison of Plasma Insulin and HOMA-IR in Normal Wistar Rats

The plasma insulin levels of normal Wistar rats did not change significantly in each group after 60 minutes. Furthermore, the HOMA-IR index in each group was significantly attenuated at 60 minutes only for the AC, EA, or AC+EA treatments. Specifically, the AC+EA group had more significant change than the saline group; $*P < 0.05$ vs 0 minutes according to the Student's *t*-test (Table 1).

Comparison of Plasma Insulin and HOMA-IR in SIIR Rats

The plasma insulin levels of SIIR rats did not change significantly in each group after 60 minutes, except for in the TZD group. Furthermore, the HOMA-IR index in each group of SIIR rats was significantly attenuated at 30 and 60 minutes, except in the saline group. The drop in percentage at 60 minutes of the AC, EA, AC+EA and TZD groups was more than that observed for the saline group. Specifically, the drop in the percentage of the AC+EA group (69%) was a more significant change than in the saline (13.6%) group and this was approximately equal to the positive control TZD (61.9%) group (Table 1).

Impact of Signaling Proteins on SIIR Rats

After administering different treatments in the SIIR rats, the gastrocnemius muscle was taken, and the insulin-signaling proteins, for example, GLUT-4 (Fig. 3A), were detected by Western blot. Each treatment group (AC, EA, AC+EA, or TZD) had significantly elevated insulin-signaling proteins, compared with the saline group. The level of the signal protein PI3-K is shown in Figure 3B. The AC and AC+EA groups had

TABLE 1. CHANGES IN PLASMA INSULIN AND HOMA-IR INDEX

Group		Plasma insulin levels ($\mu\text{g/mL}$)			HOMA-IR index			
Time (min)		0	30	60	0	30	60	0–60 change %
Wistar	Saline	0.27 ± 0.01	0.29 ± 0.08	0.25 ± 0.03	1.86 ± 0.36	1.83 ± 0.62	1.35 ± 0.28	27.3 ± 1.2 , B
	AC	0.26 ± 0.01	0.26 ± 0.01	0.23 ± 0.01	1.58 ± 0.04	$1.30 \pm 0.04^*$	$1.07 \pm 0.03^*$	32.4 ± 4.7 , B
	EA	0.25 ± 0.01	0.22 ± 0.00	0.23 ± 0.01	1.71 ± 0.05	$0.79 \pm 0.01^*$	$0.68 \pm 0.02^*$	69.1 ± 9.1 , A
	AC+EA	0.26 ± 0.02	0.25 ± 0.03	0.23 ± 0.00	1.90 ± 0.23	1.08 ± 0.07	$0.58 \pm 0.01^*$	68.9 ± 3.5 , A
	TZD	0.54 ± 0.21	0.35 ± 0.13	0.27 ± 0.01	3.48 ± 0.90	2.21 ± 1.12	2.21 ± 0.23	30.1 ± 4.8 , B
SIIR	Saline	0.25 ± 0.01	0.21 ± 0.00	0.24 ± 0.02	1.54 ± 0.14	1.11 ± 0.04	1.32 ± 0.05	13.6 ± 0.7 , c
	AC	0.17 ± 0.07	0.15 ± 0.05	0.17 ± 0.07	1.37 ± 0.07	$0.87 \pm 0.07^*$	$0.97 \pm 0.07^*$	28.5 ± 1.1 , b
	EA	0.26 ± 1.40	0.23 ± 1.41	0.20 ± 1.05	8.65 ± 0.01	$2.81 \pm 0.01^*$	$2.49 \pm 0.00^*$	59.6 ± 0.5 , a
	AC+EA	1.00 ± 0.28	0.54 ± 0.7	0.62 ± 0.29	6.79 ± 2.16	$1.81 \pm 0.15^*$	$1.72 \pm 0.94^*$	69.0 ± 2.3 , a
	TZD	0.29 ± 0.00	$0.16 \pm 0.01^*$	$0.18 \pm 0.00^*$	13.50 ± 0.03	$3.14 \pm 0.03^*$	$3.36 \pm 0.02^*$	61.9 ± 0.1 , a

Saline group: Wistar rats were administered normal saline orally; AC group: Wistar rats were administered extract of AC (500 mg/kg) orally; EA group: received normal saline and EA (15 Hz/10 mA) bilateral ST 36 acupoints; AC+EA group: received AC and EA as previously scheduled; TZD group: received orally administered rosiglitazone (0.1 mg/kg); $*P < 0.05$ versus 0 min per a Student's *t*-test. 0–60 change %: $*P < 0.05$.

Significant differences were determined by least significant difference *post hoc* test following an analysis of variance, $A > B$ or $a > b > c$; $P < 0.05$. HOMA-IR, homeostatic model assessment of insulin resistance; min, minutes; AC, *Antrodia cinnamomea*; EA, electroacupuncture; TZD, rosiglitazone.

FIG. 3. The impact of electroacupuncture (EA) combined with *Antrodia cinnamomea* (AC; 500 mg/kg) on the signaling proteins of steroid-induced insulin-resistant (SIIR) rats. (A) the expression levels of glucose transporter 4 (GLUT4), (B) the expression levels of phosphoinositide 3-kinase (PI3-K), (C) the expression levels of 5' adenosine monophosphate-activated protein kinase (AMPK). In the saline group, Wistar rats were administered normal saline. In the AC group, Wistar rats were administered oral AC extract (500 mg/kg). The EA group received normal saline and EA at ST 36 (15 Hz/10 mA). The AC+EA group received AC and EA as previously scheduled. The TZD (rosiglitazone) group received rosiglitazone (0.1 mg/kg); * $P < 0.05$ versus saline. A Student's *t*-test was used to perform group analysis.

significantly higher protein levels than the saline group. The level of the signal protein AMPK is presented in Figure 3C, where the EA and AC+EA groups had significantly higher AMPK protein than the saline group ($p < 0.05$). Significantly increased levels of insulin-signaling proteins (GLUT-4, AMPK, and PI3-K) were observed in the combined therapy group (AC+EA). In contrast, this was not observed for the single therapy (EA, AC, or TZD) same time.

DISCUSSION

One of the most common human diseases, diabetes mellitus, is caused by defects in insulin signaling. Insulin resistance is a chronic increase in insulin secretion, which overstimulates target cells in the liver and other parts of the body, leading to a chronic elevation in plasma glucose levels, which stimulates the pancreas to secrete more insulin. The two important downstream-signaling pathways are the Ras-Map kinase pathway and the insulin receptor-mediated cascade. Human bodies spend considerable effort maintaining plasma glucose levels within a narrow range.²⁰

In this study, the rats' plasma-glucose levels after high-dose steroid administration were significantly higher than those of normal rats. The rats injected with 1 mg/kg of dexamethasone for 5 days had significantly higher fasting plasma-glucose levels, compared to the nontreated rats. The SIIR rats treated with a chronic dose of dexamethasone could be a useful model to explore new therapies for addressing insulin resistance.² The current study's results showed that EA, and AC+EA treatment improved the hypoglycemic effect significantly by reducing the plasma-glucose levels in the normal Wistar and SIIR rat models. This result was consistent with previous studies that showed the trend of hypoglycemia in rat models after treatment with 15-Hz EA at bilateral ST 36 acupoints that confirmed the hypoglycemic effect.^{18,21}

In the current authors' previous study, the experimental doses were determined in preliminary tests by administering various doses to SIIR rats to establish small, medium, and high doses (100, 200, and 500 mg/kg) appropriate for rats. This previous study showed that the optimal oral dose to achieve a hypoglycemic effect was 200 mg/kg AC.³ A higher dose of AC (500 mg/kg) was chosen for the current study for 2 reasons: (1) A single dose of AC (500 mg/kg) had not been previously used to explore the mechanism of action. (2) The purpose of the current study was to identify a more-effective method other than EA or TZD signal therapy in diabetes treatment by elevating the expression of GLUT4, PI3-K, and AMPK signaling proteins.

The hypoglycemic pathway is speculated to be mediated by the phosphorylation of AMPK and the activation of PI3-K. The GLUT4 in the cell is activated and transferred to the cell membrane as a channel for glucose entry into the cell.

Notably, the role of PI3-K is to activate a variety of signaling pathways. PI3-K provides the binding sites for Pleckstrin homology domain-containing signaling proteins, such as protein kinase B (PKB) and protein 3-phosphoinositide-dependent protein kinase-1. PKB regulates glucose uptake by GLUT4, which resides in the intracellular membrane vesicles tethered to the *cis*-Golgi network, through the cleaving of TUG in response to insulin. This allows the GLUT4 vesicles to fuse with the plasma membrane.²⁰ Therefore, these 3 signaling proteins correlate with each other. The expression of the insulin signaling proteins, GLUT4, PI3-K, and AMPK, in insulin-resistant rats before and after each treatment was measured by Western blot. This study showed that the signal proteins (GLUT4, PI3-K, and AMPK) were enhanced better to perform their functions in the AC+EA treatment group. This seems better than single therapy at 60 minutes, increasing these 3 proteins than with the single therapy at the same time. Previous studies have indicated that AC stimulates AMPK and GLUT4 expression.³

Further exploration of the mechanism revealed that stimulation of synaptic vesicles could increase the release of acetylcholine, leading to an increase in insulin activity. The hypoglycemic mechanism is regulated through parasympathetic-nerve involvement.²¹ This study confirmed further that EA or AC+EA could reduce insulin resistance by lowering plasma-glucose levels. Studies have indicated that AC stimulates β -cells in the pancreas by peroxisome proliferator-activated receptor- γ , increases insulin secretion, restores the pancreas to its function, and reduces the symptoms of diabetes, leading to reduced complications.²² The change in HOMA-IR in SIIR rats was decreased by the AC+EA treatment, which was almost equal to the positive control TZD treatment, indicating an reduction of the insulin resistance induced by the combined therapy in 60 minutes. In addition, the results showed that the combination therapy might be better than the signal therapy (AC, EA, or TZD) according to the expression of signal-transduction proteins in this study. Further randomized controlled studies are needed to confirm the clinical hypoglycemic effect of the AC+EA treatment.

CONCLUSIONS

This study demonstrated that SIIR rats had significantly reduced plasma glucose levels and insulin resistance by the EA combined with AC treatment. Likewise, the therapeutic effect was better than the EA, AC, or TZD therapies. The performance of signal proteins, GLUT4, AMPK, and PI3-K might be an important part of mechanisms in this EA combined with AC therapy to reduce hypoglycemic activity and insulin resistance. More research is needed to determine EA+AC's future role as a therapy for insulin resistance.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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