



## Hypothesis Paper

## Effects of Intracellular Superoxide Removal at Acupoints with TAT-SOD on Obesity

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## ABSTRACT

TAT-SOD is a recombinant protein of superoxide dismutase fused with TAT peptide. By pure accident, we discovered that topical application of TAT-SOD to acupoints could result in acupuncture-like action. This study aimed to validate the accidental discovery by investigating the effect on simple obesity of the topical application of TAT-SOD to acupoints in comparison with acupuncture. 90 subjects were divided into 3 groups for 12-week treatments. Regular hospital acupuncture treatment was given to Acupuncture Group 3 times a week. TAT-SOD Group were instructed first to locate acupoints and apply 0.1 ml of 5000u SOD/ml TAT-SOD cream in an area of 1 cm<sup>2</sup> to each of the same set of acupoints, which they then conducted at home three times daily. Placebo Group applied the vehicle cream the same manner as TAT-SOD Group. Both TAT-SOD and acupuncture treatments decreased adiposity with overall clinical effective rates of 60.0% and 76.7%, respectively. The placebo group showed no improvement. The results validate that the enzymatic removal of the intracellular superoxide at acupoints could generate acupuncture-like effects, and indicate a possibility of the new method as a simple substitute to acupuncture and an insight of superoxide modulation along meridians for acupuncture mechanism.

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## Introduction

Superoxide is one of the main reactive oxygen species (ROS) in the cell, which act as second messengers in the intracellular signaling pathways involved in activation of proinflammatory responses and mediate degradation of aggrecan and collagen [1]. The cellular defense against the ROS utilizes antioxidant enzymes [2] such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Insufficient intracellular anti-oxidant enzymes, especially SOD can cause overproduction of ROS, leading to apoptosis and necrosis, eventually cell damage [3].

Although a variety of exogenous anti-oxidant enzymes are available to protect against oxidative stresses, the major problem in using these enzymes is that they can not be delivered into cells, thus resulting in their inability to detoxify intracellular ROS [4]. As a promising solution to the problem, several protein segments have been identified as protein transduction domains (PTDs) which can transport exogenous protein into living cells [3,4]. One of PTDs is TAT, an 11 peptide derived from HIV-1 trans-activator of transcription protein [5]. When fused with PTDs by gene-recombinant expression, proteins

can be delivered cross the cell membrane while maintaining their activities [4]. In previous work, we have prepared TAT-green fluorescent fusion protein (TAT-GFP) to study transduction of GFP into cells. It was demonstrated to be efficiently transduced into not only various mammalian cells such as rat hepatocytes [6], HeLa cells [7–9], BEL-7402 cells, SMMC-7721 cells, and L02 cells [8,9], but also into rat dermis by directly smearing on skin [7] and nematode digestive system and coelom and mouse heart, liver, kidney and brain by intraperitoneally injection [10]. Furthermore, TAT-Cu,Zn superoxide dismutase (TAT-SOD) was found to be delivered not only into cells [11–15] but also into mitochondria where superoxide is generated [16], suggesting a great potential of TAT-SOD as an ideal intracellular anti-oxidant solution. It was found that TAT-SOD could increase the viability of mammalian cells irradiated by UV-C [11] and UV-B [17], prevented [14] and protected [15] the skins of guinea pigs damaged by single dose and repeated UV-B radiation.

By pure accident, we discovered that topical application of TAT-SOD to acupoints around the neck instantly relieved fatigue symptoms in the throat resembling the action of acupuncture. Biochemically, the only effect TAT-SOD application can cause is a decreased intracellular superoxide level as TAT-SOD can be delivered across cell membrane to quench superoxide. The same effect can be reasonably expected of acupuncture as needle insertion unavoidably damages cell membrane and cause intracellular superoxide leakage. Of various effects possibly caused by the needle stimulation, it is unclear which triggers the therapeutic results, but it is certain that the only effect that acupuncture can share with TAT-SOD application is the intracellular superoxide removal, though mechanically for the former

**Abbreviations:** ROS, reactive oxygen species; SOD, superoxide dismutase; PTD, protein transduction domain; TAT, transactivator of transcription; TAT-SOD, TAT-Cu,Zn superoxide dismutase; BMI, body mass index; NPY, neuropeptide Y; ST 8, acupoint Toudou; ST 24, acupoint Huaroumen; ST 25, acupoint Tianshu; ST 26, acupoint Tianshu; SP 15, acupoint Daheng; CV 5, acupoint Shimen; CV 10, acupoint Xiawan.

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and enzymatically in the later. Moreover, reproducible fluorescence lines superimposable to meridian lines were revealed on the frontal interior abdominal wall when intracellular superoxide indicators were injected into living SD-rats tail vein [18], suggesting the substantial implication of superoxide in acupuncture meridian system.

This study aimed to investigate if TAT-SOD application could work like acupuncture in treating simple obesity. Simple obesity is a prevalent, refractory, and serious problem [19]. Acupuncture has been demonstrated to be effective in treating simple obesity in daily clinical practice, especially in China, but no conclusive results have been obtained from well controlled clinical studies [20]. The major reason for the failure to draw conclusion on the efficacy of the acupuncture treatment is the impossibility of an acupuncture placebo due to the destructive nature of needle puncture. It is apparent that this practically effective treatment cannot be scientifically validated and accepted unless its mechanism is elucidated. However, evidence suggests an association between obesity and oxidative stress caused by superoxide production [21]. In light of our accidental discovery of acupuncture-like effect due to the decreased intracellular superoxide resulted from the topical application of TAT-SOD, it is reasonable to expect the same decreased intracellular superoxide level at acupoints in acupuncture as needle insertion unavoidably damages cell membrane and cause intracellular superoxide leakage. If the removal of intracellular superoxide can be confirmed to be the biochemical mechanism that triggers therapeutic effects of acupuncture, not only an effective placebo method can be presented, which will render it possible to validate acupuncture's efficacy, but also our accidental discovery will explicitly indicate a possible association of superoxide implication with a novel biochemical mechanism for mysterious acupuncture.

## Materials and Methods

TAT-SOD was prepared by recombinant expression of a fusion protein of human Cu,Zn-SOD fused with TAT peptide in *E. coli* as follows: Constructs preparation: The nucleic acid sequence encoding TAT-SOD fusion protein was constructed by DNA recombinant technology and inserted into expression vector pGEX-2 T; Cell culture and transfections: *E. coli* (BL21, DH5 $\alpha$ ) cells were transformed with the expression vector pGEX-2 T containing the inserted TAT-SOD; TAT-SOD fusion protein preparation: TAT-SOD was expressed in the *E. coli* by the induction of IPTG and prepared by affinity chromatography to electrophoretically pure for use [13]. TAT-SOD cream was prepared by the homogenization of the isolated TAT-SOD with the vehicle cream. We used baby lotion (Johnson & Johnson, Shanghai, China) as the vehicle cream, which contains water, propylene glycol, myristyl myristate, glyceryl stearate, oleic acid, stearic acid. TAT-SOD in the cream is remarkably stable. SOD activity loss was less than 5.6% when TAT-SOD was stored at room temperature for 6 months [12].

Healthy adults between the ages of 24 and 34 years with simple obesity (body mass index, BMI > 25) were recruited from those referred to Fujian University of Traditional Chinese Medicine Hospital for simple obesity between November 3, 2008, and September 13, 2010. The following subjects were excluded from this study: patients with a history or physical examination suggestive of renal, hepatic, or

cardiovascular disease; pregnant or lactating women; those with secondary obesity; undertaking weight reduction by medication or other measures during the past six months, or with severe organopathy. The ethics committee of Fujian University of Traditional Chinese Medicine provided approval for this study, and all participants gave informed consent for publication of these data.

90 subjects were divided into 3 groups for 12 week treatments. Regular hospital acupuncture treatment was given to Acupuncture Group 3 times a week with prescription of ST24, ST25, ST26, SP15, CV5, CV10 (Table 1). TAT-SOD Group were instructed first to locate acupoints and apply 0.1 ml of 5000u SOD/ml TAT-SOD cream prepared by adding purified TAT-SOD to the vehicle cream in an area of 1 cm<sup>2</sup> to each of the same set of acupoints, which they then conducted at home three times daily. Placebo Group applied the vehicle the same way.

No diet advice was given. Anthropometrics determined included body weight, BMI, waist circumference, hip circumference and waist-hip ratio. As shown in Table 2, the curative effect was evaluated as no improvement (BMI decrease less than 2), moderate (BMI decrease from 2 to 4) or marked (BMI decrease more than 4) improvement.

Data are reported as means (SEM). All statistical analyses were carried out using the Microsoft Excel version 2003 software. Results in TAT-SOD group and acupuncture group were compared using two-sample *t*-test.

## Results

Baseline assessment, changes in anthropometrics are presented in Table 2. No drop out nor adverse events occurred throughout the study.

Subjects from both of acupuncture and TAT-SOD groups reported a decreased appetite during the treatment, indicating both methods may share the same mechanism of appetite suppression.

Both TAT-SOD and acupuncture groups demonstrated decreases in weight (4.5 kg, 5.1 kg), BMI (1.8, 2.1), waist circumference (3.7 cm, 5.2 cm), hip circumference (2.5 cm, 3.6 cm), waist-hip ratio (0.01, 0.02). The placebo group showed no decrease.

Both TAT-SOD and acupuncture treatments decreased adiposity with overall clinical effective rates of 60.0% (18/30) and 76.7% (23/30), respectively. The placebo group showed no improvement.

## Discussion

Both treatments reduced adiposity more effectively than reported results [22] by acupuncture possibly because younger patients were tested in this study. The major criticism about the reported works on the acupuncture treatment of simple obesity is that most trials are descriptive in nature, and therefore, its efficacy in a rigorous scientific sense is not conclusive. However, like numerous reported works, the readily repeatable treatment efficacy as is demonstrated in our work testified a practical value of the treatment as an effective popular clinical practice. Verifying the efficacy in a manner as rigorous as for other modern treatments can be impossible without knowing what exactly to be controlled in the test since there is no any clue

**Table 1**  
Acupoints and their anatomical positions.

Points	location
ST 8: Touwei	On the head, 12 mm directly superior to the anterior hairline at the corner of the forehead, 112 mm lateral to the anterior median line.
ST 24: Huaroumen	On the upper abdomen, 25 mm superior to the centre of the umbilicus, 50 mm lateral to the anterior median line.
ST 25: Tianshu	On the upper abdomen, 50 mm lateral to the centre of the umbilicus.
ST 26: Wailing	On the lower abdomen, 25 mm inferior to the centre of the umbilicus, 50 mm lateral to the anterior median line.
SP 15: Daheng	On the upper abdomen, 100 mm lateral to the centre of the umbilicus.
CV 5: Shimen	On the lower abdomen and on the anterior midline, 50 mm below the centre of the umbilicus.
CV 10: Xiawan	On the upper abdomen and on the anterior midline, 50 mm above the centre of the umbilicus.

**Table 2**

Characteristics of subjects in acupuncture, TAT-SOD and placebo groups, mean (SEM).

Parameter/treatment	Acupuncture	TAT-SOD	Placebo	P value*
<i>Recommended Management Interventions</i>	Clinical acupuncture three times a week	Topical application of TAT-SOD cream at acupoints three times a day	Topical application of placebo cream at acupoints three times a day	NA
<i>Baseline Assessment</i>				
No.	30	30	30	NA
Age (y), mean (SEM)	30.6 (1.6)	31.0 (1.6)	29.6 (2.0)	0.24
Sex (M/F)	5/25	3/27	3/27	NA
Weight (kg)	72.8 (7.4)	73.9 (10.0)	71.6 (4.1)	0.24
Body mass index (kg/m <sup>2</sup> )	29.4 (2.6)	29.5 (3.5)	29.7 (2.7)	0.81
Waist circumference (cm)	87.9 (3.6)	86.7 (4.5)	86.2 (4.5)	0.99
Hip circumference (cm)	94.8 (4.7)	93.9 (5.9)	94.1 (2.8)	0.87
Waist-hip ratio	0.93 (0.05)	0.92 (0.06)	0.92 (0.05)	0.99
<i>Changes in adiposity between baseline and 12 weeks</i>				
Weight (kg)	−5.1 (0.7)	−4.5 (1.0)	0.1 (1.1)	<0.001
Body mass index (kg/m <sup>2</sup> )	−2.1 (0.1)	−1.8 (0.3)	0.0 (0.5)	<0.001
Waist circumference (cm)	−5.2 (0.7)	−3.7 (1.3)	0.0 (0.8)	<0.001
Hip circumference (cm)	−3.6 (0.5)	−2.5 (1.0)	0.1 (0.4)	<0.001
Waist-hip ratio	−0.02 (0.01)	−0.01 (0.01)	0.00 (0.01)	0.99
<i>Therapeutic response, No. (percentage)</i>				
None (BMI decrease less than 2)	7 (23.3%)	12 (40.0%)	30 (100.0%)	NA
Moderate (BMI decrease 2–4)	12 (40.0%)	13 (43.3%)	0 (0.0%)	NA
Marked (BMI decrease more than 4)	11 (36.7%)	5 (16.7%)	0 (0.0%)	NA

Abbreviation: NA, not applicable.

\*Two-sample t-test comparing treatment means of TAT-SOD versus placebo.

about what exactly happened immediately after a need is punctured into acupoints.

The efficacy of the obesity treatment with the topical application of TAT-SOD cream instead of needle stimulation, is somewhat lower than that by acupuncture (see Table 2). It may be attributed to less complete intracellular superoxide removal due to acupoint mislocation, unoptimized dosage and formula, and the inaccessibility of acupoints beneath multi-layers of tissues for TAT-SOD.

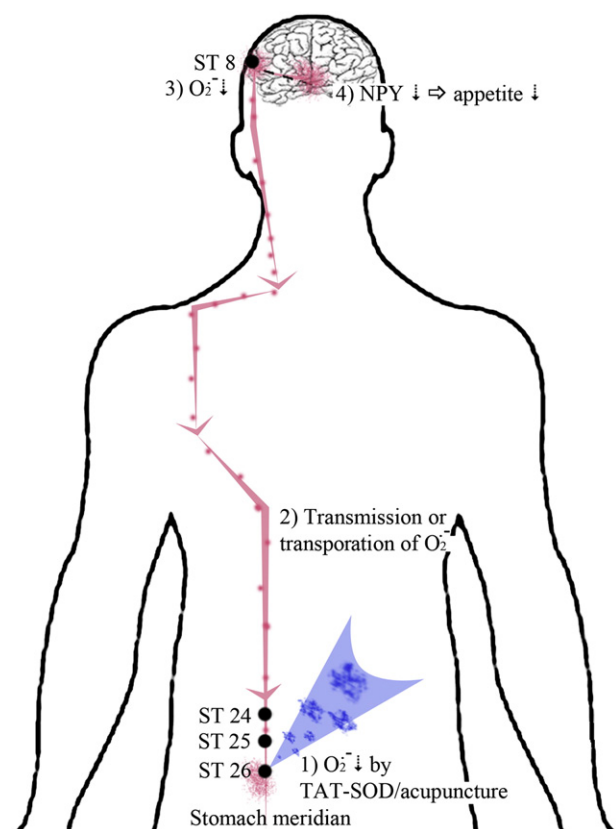
Much more investigation is necessary before a novel regiment of TAT-SOD treatment of simple obesity can be established, the current results clearly suggest a great possibility of replacing a mysterious and cumbersome treatment of acupuncture with an intracellular superoxide scavenging treatment at acupoints no more difficult than applying skin care products. The patient acceptance of an easy topical application can be extremely different from the puncture of a needle into body.

It is reported [23] that acupuncture or electrical stimulation in specific frequencies applied to acupoints can facilitate the release of specific neuropeptides in the central nervous system (CNS), such as neuropeptide Y (NPY) in appetite modulation [24]. In this clinical study, subjects receiving both acupuncture and TAT-SOD treatment reported a feeling of loss in appetite. It indicates that TAT-SOD treatment may possibly work by the same mechanism as acupuncture for weight loss through appetite modulation, which needs to be confirmed by monitoring NPY change patterns after TAT-SOD application and comparison with those of acupuncture treatment. As to how acupuncture or electrical stimulation at acupoints around abdominal should facilitate the release of NPY in the central nervous system, it is a complete mystery.

Comparing TAT-SOD treatments with acupuncture, it is incredible that similar efficacies should have been achieved by two methods which are different in every possible aspect. The only similarity considerable may be the removal of the intracellular superoxide at acupoints. It is not unreasonable to postulate that one of the immediate consequences of the needle puncture in acupoints is mechanical destruction to cellular membrane structure which would lead to a leakage of the intracellular compositions including superoxide anion. On the other hand, the only change which can be possibly caused by

the topical application of TAT-SOD at an acupoint is the enzymatic quenching of the intracellular superoxide on the spot. It is apparent that the decrease in the intracellular superoxide level at acupoints is likely the cause for the effect of the both treatments, or at the very least, it is an immediate happening after a needle is puncture into acupoints. On the other hand, it is known that changes of NPY mRNA level were correlated with an increased SOD mRNA levels in hypothalamus [25] and oxidative stress of hypothalamus is related to NPY secretion [26]. Is it possible that the decrease in acupoint's intracellular superoxide level result in the decrease in superoxide level in hypothalamus, which leads to a lower level of NPY mRNA expression and hence a suppression of appetite?

In traditional Chinese medicine theory, meridians connect visceral organs including brain with acupoints located along the meridian lines. In our previous work, meridian lines on rat's frontal abdominal were revealed by tail vein injection of dichlorofluorescein diacetate, an intracellular indicator [18]. It strongly indicates a possibility that the meridian system, which is considered to be channels for energy, Qi, can be a novel channel for superoxide. The fact that topical application of TAT-SOD at acupoints can achieve similar efficacy as acupuncture reconfirms the first observation about the implications of superoxide in the meridian system, and the concept of meridians as a novel superoxide channel in turn may provide an plausible link between the decrease in acupoint's intracellular superoxide level and the decrease in superoxide level in hypothalamus. Acupoints to which acupuncture stimulation and TAT-SOD were applied in this study were ST 24, 25 and 26 in Stomach meridian, which extends to ST8 located on 12 mm superior to the anterior hairline at the corner of the forehead, 112 mm lateral to the anterior median line. It is unknown how acupoints on the outer head surface are connected internal brains, both in traditional Chinese medicine theory and modern anatomy, but it is not impossible that Stomach meridian which ends at ST8 on head connect to some part of the brain, for example, hypothalamus. It is a formidable task to elucidate how superoxide is conducted, transported or transmitted along the meridian line but it is apparent that the removal of intracellular superoxide by either acupuncture or TAT-SOD could possibly have caused removal of superoxide in either hypothalamus or brain as a whole that the meridian



**Fig. 1.** Model of scavenge the intracellular superoxide locally at the acupoints leading to a lower level of neuropeptide Y through a meridian line.  $O_2^-$ , superoxide; NPY, neuropeptide Y; ST 8, acupoint Touwei; ST 24, acupoint Huaroumen; ST 25, acupoint Tianshu; ST 26, acupoint Wailing.

connected in the far end, leading to an increase in NPY secretion and a suppressed appetite. A proposed mechanism for treatment of simple obesity by acupuncture or TAT-SOD is illustrated in Fig. 1. Once it is confirmed that the superoxide removal in acupoints can result in a decrease in superoxide content in hypothalamus or brain, and the mechanism for superoxide transmission along the meridians is elucidated, not will only be the principles for acupuncture treatment of obesity established, and improved treatments available, but also a complete picture of meridians as a novel scientific system revealed.

## Conclusions

In this study a novel acupoint therapy of the intracellular superoxide scavenging by TAT-SOD at acupoints was demonstrated to significantly reduced adiposity with an overall efficacy comparable to acupuncture. TAT-SOD treatment may provide a novel and convenient alternative to acupuncture. Moreover, the removal of intracellular superoxide at acupuncture may be an important process in the mechanism of acupuncture.

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## List of Abbreviations

ROS reactive oxygen species  
SOD superoxide dismutase  
CAT catalase  
GPx glutathione peroxidase  
PTDs protein transduction domains  
TAT trans-activator of transcription  
TAT-SOD TAT-Cu,Zn superoxide dismutase  
BMI body mass index  
CNS central nervous system  
NPY neuropeptide Y  
ST8 acupoint Touwei  
ST24 acupoint Huaroumen  
ST25 acupoint Tianshu  
ST26 acupoint Tianshu  
SP15 acupoint Daheng  
CV5 acupoint Shimen  
CV10 acupoint Xiawan

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