Hypothesis Paper

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

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A B S T R A C T

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 cm² 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 aggregan 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 across 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 LO2 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 intraperitoneal 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–Cu,Zn superoxide dismutase (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
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 nucelic 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, DH5α) 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 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 cm² 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>
<thead>
<tr>
<th>Points</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST 8: Touwei</td>
<td>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.</td>
</tr>
<tr>
<td>ST 24: Huaroumen</td>
<td>On the upper abdomen, 25 mm superior to the centre of the umbilicus, 50 mm lateral to the anterior median line.</td>
</tr>
<tr>
<td>ST 25: Tianshu</td>
<td>On the upper abdomen, 50 mm lateral to the centre of the umbilicus.</td>
</tr>
<tr>
<td>ST 26: Wailing</td>
<td>On the lower abdomen, 25 mm inferior to the centre of the umbilicus, 50 mm lateral to the anterior median line.</td>
</tr>
<tr>
<td>SP 13: Daheng</td>
<td>On the upper abdomen, 100 mm lateral to the centre of the umbilicus.</td>
</tr>
<tr>
<td>CV 5: Shenmen</td>
<td>On the lower abdomen and on the anterior midline, 50 mm below the centre of the umbilicus.</td>
</tr>
<tr>
<td>CV 10: Xiawan</td>
<td>On the upper abdomen and on the anterior midline, 50 mm above the centre of the umbilicus.</td>
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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 regimen 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 TAT-SOD could possibly have caused removal of superoxide in either hypothalamus or brain as a whole that the meridian system, and the concept of meridians as 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 inter alia, 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...
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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  
CN5 central nervous system  
NPY neuropeptide Y  
ST8 acupuncture Touwei  
ST24 acupuncture Huaroumen  
ST25 acupuncture Tianshu  
ST26 acupuncture Tianshu  
SP15 acupuncture Daheng  
CV5 acupuncture Shimen  
CV10 acupuncture Xilwan


