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# The influence of the jiangtang fanglong prescriptions to nitric oxide synthase mrna expression of the diabetic animal model of deaf

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## ABSTRACT

Objective : To investigate the effect on Jiangtang Fanglong prescriptions to the diabetic animal model of deaf mRNA expression of nitric oxide. Methods: To select 49 from 60 Wistars, randomly and then divide them into three groups through the modeling way of immdiate diabetic deaf Wistars induced by Streptozotocin (STZ). Group A has 13 Wistars with aminoguanidine, group B has 13 ones with Jiangtang Fanglong prescriptions and Group C has 13 ones with pathology. Wistars in group A are fed with dilute solution reconstituted to a concentration of 3% of the aminoguanidine. Wistars in Group B are fed with JiangtangFanglong Suspension. The remaining 11 Wistars is the control group. The 11 Wistars in control group and 13 ones in Group C can drink water free. All the four groups eat a regular diet of chow for 12 weeks under the room temperature from 20 to 25 degrees Celsius. In sixth week and twelveth week, test the blood sugar, urine sugar and weight. After twelve weeks, observe the bone tissue and xun OsmRNA expression changes by testing the bonemineral density of femur and tibia with dual-energy X-ray absorptionmetry, using histological grade a reverse transcription PCR techniques. Result: The bone mineral density in group C is significantly reduced. Degenerative changes occur in the osseous tissue. The expression of bone iNOsmRNA is increasing. Density in group B was obviously higher, significantly improving the bone histomorphology. the expression of bone iNOsmRNA was decreased, which is similarly to Group A. Conclusion: The abnormal expression of iNOsmRNA in diabetic deafness will inhibit the expression of bone iNOsmRNA and may affect the kidney to advocate bone osteocalcin to insulin regulation. Therefore, speculating that hypoglycemic prevent deafness through kidney raising the function of “ kidney advocate bone “ can have effect on diabetes prevention and control of deafness. This speculation also provide an objective foundation for the scientific hypothesis of diabetes-kidney-bone-ear integration theory. © 2013 Trade Science Inc. - INDIA

## KEYWORDS

Jiangtang fanglong prescriptions;
Diabetes;
Deafness;
Nitric oxide.

## INTRODUCTION

Diabetes mellitus is a kind of whole body chronic metabolic disease with high glucose as the main clinical characteristics, which varied pathogenic factors affected the organism. Because of the relative or absolute lack of varied pathogenic factors, diabetics usually suffer from different levels of glucose and lipid metabolism disorder. The clinic manifestation include metabolic disorders, Diabetic angiopathy, circulatory disturbance and Neurological complications. If the patients maintained in a hyperglycemic state over a long period of time, which will lead to diabetic microangiopathy, they will suffer from hearing loss and tinnitus. With the further research in diabetes, the reports of 4 tinnitus and hearing loss caused by diabetes are increasing[1]. In recent years, nitric oxide (NO) and nitric oxide synthase (NOS)have been found to play an important role in the process of participating and promoting the pathophysiological procedure of diabetes mellitus. a variety of cytokines can affect the activity of nitric oxide synthase (NOS) in bone tissue, inducing to generate high levels of nitric oxide (NO) and affect the normal function and the dynamic balance of osteoclasts and osteoblasts. In patients with type 2 diabetes mellitus (T2DM), the increased nitric oxide accelerates bone absorption and promotes the dissolution of bone, which may be one of the reasons for diabetic osteoporosis[2]. According to the theories —— "kidney governing bones", "kidney opens at the ears", this study tends to copy the diabetes deaf model with Streptozotocin (STZ) and observe the expression of correlation of JiangtangFanglong prescriptions in the prevention and treatment of diabetes deaf in bone mineral density, bone Histomorphometry and the expression of nitric oxide synthetase (iNOS).This also can clarify the pathomechanism of diabetic deafness and the mechanism of prevention and cure of traditional Chinese medicine.

## MATERIALS AND METHODS

### Drugs

JiangtangFanglong prescriptions (from research group).The main ingredients consist of Radix Puerariae, Ligusticum chuanxiong Hort, common yam rhizome, poria cocos, dogwood, magnet, etc. These drugs, identified as the authentic Chinese herbal medicine, are bought in LeRenTang, Shijiazhuang and are made into Injectable Suspension.

### Animal

60 Wistars, weight 200-250 g, without contact with noise, pellet feed, free drinking water, natural light illumination, room temperature between 20-220 c, relative humidity between 40-70%.

Animal model Among 60 wistar rats, 49 are chosen randomly, adopting Wang Shili and Yu Demin's streptozoto (STZ)[3,4] to lead to the fast hairstyle diabetic deaf rat. Choose 50 artificial model wistar rats, with abrosia for 12 hours before injecting drug. Each rat is given injections at 55 mg/kg streptozoto (sigma company) on intraperitoneal, then using aseptic sodium critrate to buffer diuent, PH4.2. One week after the injection, the blood sugar and the urine sugar are measured. If the blood sugar exceed 14.2 mol/L, and the urine sugar >+++, this experiment succeed. 2 month later, the diabetes mellitus deaf model appears; (ABR threshold advances obviously), then start the experimental observation.

Grouping and pharmacy 60 wistar rats, 49 are chosen randomly and divided into 3 groups. Group A includes 13 rats (Amino guanidine; Beijing chemical reagent company product), group B includes 13 rats (traditional Chinese medicine group), group C includes 13 ones (pathology group). Group A is diluted by Amino guanidine solution at 13%. Group B is irrigated by 10g/kg.d JiangtangFanglong prescriptions Suspension. The other 11 wistar rats are left for comparing. The Compare group and Group C (pathology group) are fed normally under the temperature 20-25C for 12 weeks, then detect blood sugar and urine sugar on the sixth week and twelfth week.

### ABR detection method

Before the measure, animal are injected pentobarbital sodium (45mg/kg) in enterocoelia for anesthesia, then use Traveler Express E type ABR detector to detect the ABR threshold. Need electrode, the record electrode, sets on the parietal periosteal surface upon the two ears' mid. The contrast electrode upon the same pinna, touching ground electrode on the contrast pinna,

TDH-39P type headphone, which is 2cm far away outer ear crossing give sound. Band-pass filter 3000H2 that detected in the shielding room sound insulation, then recording each group ABR threshold (wave II) and wave changes in the incubation period.

Main instrument; Dual X-ray Absorptiomety (American LUNER company conduct); TL-16R table type high speed freezing centrifuge (Shanghai centri fuge machinery research institute product); ultraviolet spectrophotometer UV1601 (Japanese shimadzu company product); PCR thermal cycler 100type (MJ Reaeher INC company production); American JNJ glucometer, etc.

**Primer**

The gene sequence comes from Gene Bank. Applying professional primer design software, Primer 5.0, composed by Shanghai biology engineering company. The specific sequence is as below.

i NOS

Upstream: 5'–AGGAGCGAGTTGTGGATTGT –3'

Downstream: 5' –GTGAGGTTTTGGCTGAGTGA–3'

Amplification product is 124pb, Tm is 58.8-55.6C,

β – action

Upstream: 5' –GAGACCTTCAAGACCCCAGCC–3'

Downstream: 5' –TCGGGGCATCGGAACCGCTCA–3'

amplified products: 443pb

**Specimen collection**

After fed 12 weeks, animals are fasting for 12 hours and then killed by bloodletting on arteria cruralis. Serum is taken up and put into the minus 70c refrigerator for spare. Peel the animal's thighbone and tibia on ice, and take 3 animal's right thigh bone and tibia in each group and fix them into 3% formaldehyde solution, and then decalcify, HE dye and make pathological section. Use BDM detection to detecting the other right thigh bone and tibia. Put the left femoral into plastic bag and sink it into liquid nitrogen to preserve. iNOS mRNA is to be tested.

The extraction and purity identification of the Total RNA: After taking out femur from minus 70 c refrigerator emove soft tissue and extract the Total RNA by sosulfuric cyanic guanidine. Take a little femur cancellous bone and put them into Guanidine thiocyanate liquid (4mol/L Guanidine Thiocyanate,25mmol/L Sodium Citrate,0.5%SLS,0.1mol/Lβ-ME) by 10ml/g. Add 1/10 of the size of 2.0mol/L NaAc (ph4.0), the same size Phenol,1/5 size of chloroform/ isoamyl alcohol hen mixing and take ice baths for 15 minutes.4C. 12000 r/min centrifuge for 20 minutes. The upper layer aqueous phase is separated. Put the same volume isopropyl alcohol, mix and -20C puting an hour. 4C,12000r/min is centrifuged for 15 minutes. Put Two times volume of 70% Ethanol to Washing precipitation. 4C, 12000r/min is centrifuged for 15 minutes, the supernatant was discarded then air drying nd put 10μSterile three steamed water to dissolve. Take 1μ1RNA liquor esting the purity and Integrity of the RNA by 1.5 Agarose gel. Observe and take photos under the ultraviolet lamp. Take 1μ1RNA liquor after dilution is test for it"s density between 260nm and 280nm, Calculate the content of RNA and the ratio of OD 260nm/OD280nm for assuring the purity. Select OD260nm/OD280nm which ratio is more over than 1.8 of the RNA sample for reverse transcription-polymerase chain (RT-PRC) reaction.

**RT-PRC reaction**

operate as the instruction Access QuickTMRT-PCR System kit. Use 2μg total RNA for RT-PCR action. Adopt two pairs of primer in the same system to conduct. 50μ1 action system include :2×AccessQuickTm Master Mix25μ1.20pmol Primer target gene he positive and negative chain each 2μ1.20pmolβ-actinPrimerÿThe positive and negative chain each2μ1 NA liquid total content is 2μg according its density. The tri-distilled water handled by DEPC reach up to 50μ1, then add1μ1(5U)AMV Reverse Transcriptase and blend them. the reaction condition is 42(' 30min, 95(' 3min;94c45s,57c45s,30 times circularizations ; Then 72c6min, preserving at 0°C after reaction.

**RT-PCR production' electrophoretic analysis**

Take PCR production 10μl to proceed 1.5% agarose gel electrophoresis. After that, observe and take photos under the ultraviolet lamp, and scan photos by

area of gray scale use density to stand for it's expression quantity. Calculate the the relation of expression of target gene. The relative expression quantity of target gene :target gene' density /β-actin density of gene.

Statistical approach:Adopt SPSS11.0 statistic software to analyze and process the result of experiment, measurement data is represented by mean±standard deviation (X±S). The comparison among groups is tested by t.

## RESULT

The influences on The bone mineral density, blood glucose, body weight in GroupA (aminoguanidines ) and group B (Jiangtang Fanglong prescriptions group): TABLE 1 is the comparison between group C (pathological group) and normal group (control group). Bone Mineral Density, Tendons and bone mineral density, blood glucose and body weight change significantly, with P<0.01. Model is copied successfully. Group A, Group B is compared with group C separately. the Bone Mineral Density and the tibia bone density all rise up he blood glucose all decline, body weight all add (P<0.01). Group A and Group B have no distinct difference between them.

The changes in blood glucose and body weight among groups before and after the experiment: the rats in group B put on weight after taking the medicine (p<0.05) and their blood glucose declined (P<0.01); rats in group A doesn't show much difference after taking medicine and their Blood glucose declined (p<0.01). rats in Group C lose weight (p<0.05), but there is no obvious change on blood glucose. See TABLE 2.

**TABLE 1: Rat bone mineral density ,blood sugar, weight comparation (x±S) among different groups**

| group | case number | Shin bone density(g/cm2)) | femur density(g/cm2) | blood glucose (mmol/L) | weight(g) |
|---|---|---|---|---|---|
| Normal group | 11 | 0.218±0.017* | 0.241±0.026* | 4.44±0,58* | 298.67±33.23* |
| Group C | 13 | 0.182±0.015 | 0.196±0.016 | 22.35±5.60 | 20377±27.08 |
| Group A | 13 | 0.209±0.016* | 0.227±0.017* | 15.21±4.77* | 249.85±35.37* |
| Group B | 13 | 0.212±0.017* | 0.232±0.03* | 16.05±4.26* | 268.38±28.39* |

**notice;compare with group C□P<0.05**

**TABLE 2 : The comparison of changes on weight and blood glucose of rates in every group(x±s)**

| Groups | | case number | weight(g) | blood glucose(mmol/L) |
|---|---|---|---|---|
| A | befor the trial | 13 | 230.25±38.12 | 23.17±6.65 |
| | After the trial | 13 | 20.83±36.25 | 16.71±4.70** |
| B | befor the trial | 13 | 247.3±24.51 | 22.35±6.88 |
| | After the trial | 13 | 268.48±27.35* | 17.08±4.28** |
| C | befor the trial | 13 | 227.59±36.51 | 19.07±54.29 |
| | After the trial | 13 | 204.69±27.09* | 23.39±5.10 |

**Note: compared to the result before the treatment in the same groups*p<0.05;**p<0.01**

HE staining: normal rats' femur and tibia's bone trabecular is rich and bony wall is thick. Femoral SuiDuan mainly composed by vertical arranged bone trabecular. They are connected with transverse bone trabecular. On the surface of bone trabecular, we can see osteopath which is arranged by team. In group C, bone trabecular is rare and thin, medullary cavity is enlarged and bone trabecular is disappeared in several parts. we can see more fat granule in the medullary cavity and rare OB on bone trabecular's surface.compared with group C, in group A and B there are more bone trabecular and more thick wall nce enlarged medullary cavity recoverd and fat granule reduced.

Total RNA result:A260/A280 the ratio is between 1.8 to 2.0. 1.5% agarose gel electrophoresis shows: 18S and 28S are very clear. The lightness and width of 28S are twice than the 18S. 5S stripe is very dark and without macromolecular stripe. it shows that total RNA is degradation and protein pollution.

Comparison of iNOSmRNA 's expression in every bone tissue:in normal contrast group OP, iNOSmRNA has no expression or can not be detected. iNOSmRNA in group C is high expression.however n group B and AG iNOSmRNA 's expression quantity is obviously than group C (p<0.01). compared with group A roup B han no statistical differences.(p>0.05). See TABLE 3:

**TABLE 3 : iNOSmRNA expression grayscale value comparison of rates' femur in every groups(X±S)**

| Groups | case number | iNOSmRNA | |
|---|---|---|---|
| Normal group | 11* | | |
| C | 13 | 1.0498 | 0.0836* |
| B | 13 | 0.4276 | 0.0113Δ |
| A | 13 | 0.5328 | 0.0625Δ |

**Note:Compared with the normal group*p<0.05,contrast with group C p<0.01**

## DISCUSSION

Nitric Oxide (NO) has complicated physiological function and plays a dual role in pathological and physiological conditions. its function in the pathogenesis of type 2 diabetes mellitus has been a hot point in medical research. In normal condition, as a kind of signal transduction molecules between or in the cells, NO plays an extensive role in adjusting nerve ardiovascular, lungs and immune system's physiological function. However, Under pathologic conditions, because of its free radical features, NO's productive will exert biological effects to injure histiocytic and plays a great role under type 2's initiating mechanism of diabetes (insulin resistance and secondary effects)and islet B cells function obstacle. it has become an important media of various factors[5] caused by diabetes. resent researches have shown that it has a close relation between type 2 diabetes mellitus islet B cells function obstacle and pathogenesis. IL-1 shows through inducting islet NOS that reactive NO can reduce the contains of islet B cells gluccokinase mRNA and protein synthesis. in order to reduce the glucose decomposition using leed by islet B cells.the mechanism is not clear, the possible reasonmaydue to that NO could make transcription factor's important thiol groups react S-nitrosoation[6]. Kwon and his group[7] show that TNF-A alone or together with other endogenous cytokines lead the islet in macrophages to release interleukins (IL)-1 which can be interacted directly in B cells inducing the expression of Inos and the excessive production of NO. NO can be transmitted by the way of inactivating the enzymes existing in the mitochondria and reducing glycolysis, especially by reducing insulin signal stimulated by insulin. The transmission of the aliveness of the key signal molecules—p70s6 kinas, p70s6k leads to the metabolic inhibition of insulin B cells which restrain s the transmission of insulin and other increasing factors related to B cells and proliferation leading to the resist against insulin and then failure on islet in cell function. The function of NO and NOS plays an important role in the bone metabolism. To maintain the normal function of bone cells needs a certain concentration of the existence of CNOS[8]. A basic concentration of NO is necessary to maintain the absorption function of OC to absorb. And the proliferation of OB and the normal play of the bone matrix secretion must rely on proper concentration of the NO. When cytokines introduce excessive iNOS, it can lead to the excessive production of NO overtops 10 times in normal. High concentration of NO can inhibit the bone absorption of OC[2] and inhibit the proliferation of OB facilitating the function of dissolving the bone[9]. In addition, NO can inhibit the proliferation of the aliveness of the OB alkaline phosphates then stop the interaction of PG and OB and inhibit the production of BGP and finally inhibit the bone mineralization[10]. The osteocalcin can improve the insulin secretion level, insulin sensitivity and stop fat piling up and make the type 2 diabetes osteocalcin and the blood sugar level negatively correlated with each other[11,12]. Notifying kidney can promote the proliferation of bone cells and differentiations of ostencalcin[13]. According to the theory of "kidney advocates bones" and "kidney begins at the ears", together with our previous work, we put forward the scientific hypothesis of the theory of "diabetes –kidney-bones-ears". This article is based on the observation of the mRNA of hypoglycemic perverting deafness method against the diabetes deaf animal's model.

JiangtangFanglong prescriptions mainly consists of Kudzu root, Salvia miltiorrhiza, Rhizomes ligustici wallichii, Rehmannia glutinosa, Chinese yam rhizome, Poria cocos, Alisma odestone and so on. Rhizomes ligustici wallichii, together with Salvia miltiorrhiza, pass the twelve meridians, owning the function of invigorating the circulation of blood. Kudzu root can send up the lucid yang and upward the medicines. Rehmannia glutinosa is gentle sweet and rich in the blood, making the body fluid. Chinese yam rhizomes notify spleen and heip to digest. Poria cocos can wet the spleen permeability. Alisma make the dull kidney clear. The root bark o the peony tree makes the liver fire cool. Dogwood nourishes liver and kidney. Lodestone is heavy in qual-

ity and it can strengthen the kidney. Its main ingredient, the four iron oxide has the saying “kidney empty, the deafness are available.” in the book Ben yan righteousness. The method had the function of nourishing the kidney, invigorating the circulation of blood, improving SOD, reducing the blood sugar, fat and fibrinogens, improving NIDDM and so on.

This article makes the view thrown right upon that the method had an evident function together with aninoguanidine in increasing the bone mineral density and reducing the blood sugar and putting on weight. The change in BMD bone morphology and INOSMRNA is almost the same. It shows that INOSMRNA may be one of the courses of diabetes affecting “kidney advocates bone”. The aninoguanidine is the selective inhibitors which can inhibit the aliveness and expression of INOS. The method together with the INOS and MRNA makes the NO and peroxynitriee, ONO reduced and then have the effect of antioxidant. Compared with aninoguanidine, the method has an obvious advantage in improving bone mineral density which accordant with the function of antimony in Chinese medicine and provide evidence for notifying kidney and preventing diabetes.

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