Salvianolic acid B in vitro inhibited matrix metalloproteinases-1, -2, and -9 activities

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Objective: Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which as a group can degrade essentially all extracellular matrix components. The proteolytic property of the MMPs is important during wound healing to remove debris and facilitate cell migration. Targeting towards the decreased MMPs activities is a new treatment strategy for healing chronic wounds. *Salvia miltiorrhiza* is a popular Chinese herb that could promote chronic ulcers healing for topical use. Salvianolic acid B (Sal B) is the most abundant bioactive component in *Salvia miltiorrhiza*. The research was designed to explore the inhibitory effects of Sal B on MMP-1, MMP-2 and MMP-9 activities.

Methods: Pure human interstitial collagenase (MMP-1) or gelatinase A (MMP-2) was activated by p-aminophenylmercuric acetate (APMA), and was incubated with Sal B for 1 h. The activities were observed by quenched fluorescent substrate. Gelatinase B (MMP-9) is rich in polymorphonuclear neutrophils (PMN), so the rat PMN was used as a source of MMP-9 for MMPs activity assays. *In vitro* MMP-9 from rats' PMN lysate was incubated with Sal B for 1 h, and its activity was tested by gelatin zymography.

Results: Sal B dose-dependently inhibited the human MMP-1 and MMP-2 activities in the range of 0.002-4 to 0.3 g/L, with 50% inhibiting concentration (IC₅₀) of (0.090 ± 0.015) g/L and (0.080 ± 0.005) g/L respectively. In the range of 0.003 to 0.3 g/L, Sal B could inhibit the MMP-9 activity (*P*<0.01).

Conclusion: The broad-spectrum inhibitory effects of Sal B on MMPs may reveal one of the mechanisms for the effects of *Salvia miltiorrhiza* on chronic wounds.

Keywords: *Salvia miltiorrhiza*; salvioli; matrix metalloproteinases; rats

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丹酚酸 B 在体外对基质金属蛋白酶 1,2 和 9 活性的抑制作用

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目的：基质金属蛋白酶家族（matrix metalloproteinases，MMPs）是一类活性依赖于金属锌离子的蛋白酶，它可以降解绝大多数细胞外基质成分。MMPs 水解蛋白质的特性有利于创伤愈合过程中死坏组织的清除，以及细胞的迁移。降低慢性创面升高型 MMPs 活性可能是治疗慢性皮肤溃疡的新途径。丹参外用可治疗慢性皮肤溃疡，丹酚酸 B（salvianolic acid B，Sal B）是丹参中含量丰富且具有较高生物活性的水溶性成分。本研究主要观察 Sal B 在体外对 MMPs 的活性是否有直接影响。

方法：人 MMP-1 和 MMP-2 被醋酸氨基汞（p-aminophenylmercuric acetate, APMA）激活后，与不同浓度的 Sal B 共同孵育 1 h，通过底物解聚法观察其活性的改变。MMP-9 在中性粒细胞（polymorphonuclear...
neutrophil, PMN)中含量丰富，因此以大鼠腹腔 PMN 作为 MMP-9 的来源。PMN 裂解产物与不同浓度的 Sal B 共同孵育 1 h，通过明胶酶谱法，观察其中 MMP-9 活性的改变。

结果：Sal B 在 0.002 4～0.3 g/L 浓度范围内对 MMP-1 和 MMP-2 的活性有抑制作用，半抑制浓度（IC50）分别为（0.090±0.015）g/L 和（0.080±0.005）g/L，在 0.003～0.3 g/L 浓度范围内对 MMP-9 的活性表现出不同程度的抑制作用（P < 0.01），其抑制作用呈浓度依赖性。

结论：Sal B 可抑制 MMPs 的活性，改善慢性创面高酶活性是丹参治疗慢性皮肤溃疡的作用机制之一。

关键词：丹参；丹参酚；基质金属蛋白酶类；大鼠

Salvianolic acid B (Sal B)，one of the most bioactive water-soluble components of Salea miltiorrhiza，is a commonly used Chinese herb. Salvia miltiorrhiza and its various active components have been widely used in treating cardiovascular and cerebrovascular diseases, and also in treating chronic wounds for its promotion of blood circulation, and anti-inflammatory, anti-oxidant and anti-thrombosis effects[1].

Treatment of chronic wounds in the elderly is a major world health problem resulting in distress and disability, and also an increasing burden to health care providers. Chronic wounds encompass a spectrum of diseases and exist in three principal forms: pressure sores, venous ulcers and diabetic ulcers[2]. Matrix metalloproteases (MMPs) are a family of neutral proteases that play a vital role throughout the entire wound healing process. They regulate inflammation, degrade the extracellular matrix (ECM) to facilitate the migration of cells and remodel the new ECM. However, excessive MMP activity contributes to the development of chronic wounds[3]. As inferred from analysis of wound fluids, MMPs accounted for 90% of total proteinase activity in chronic wounds of various etiologies[4].

As to the beneficial effects of Salvia miltiorrhiza on the promotion of chronic ulcer healing and its bioactive components, it needs to explore its possible effects on MMPs activities in vitro and find new therapeutic application for clinical treatment.

1 Materials and methods

1.1 Animals Four normal Wistar rats of both sexes weighing (200 ± 20) g were included. They were purchased from the Experimental Animal Center of Capital University of Medical Sciences, Beijing, China.

1.2 Drugs and reagents Sal B was purchased from the National Institute for the Control of Pharmaceutical & Biological Products in Beijing; 1, 10-phenanthroline and p-aminophenylmercuric acetate (APMA), from Sigma; purified human MMP-1 and MMP-2, from Chemicon; fluorogenic peptide substrate of MMPs (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2) from R&D; glycogen, from Sigma; lower molecular weight protein marker (composition; rabbit phosphorylase B 97.4 kD, bovine serum albumin 66.2 kD, rabbit actin 43 kD, bovine carbonic anhydrase 31 kD, pancreatic trypsin inhibitor 20.1 kD, hen’s egg-white lysozyme 14.4 kD), from Beijing TransGen Biotech.

1.3 MMP-1 and MMP-2 activity assays Purified human MMP-1 and MMP-2 were used in these tests. Both of them were activated by 1 mmol/L APMA in a tetrachloronitrobenzene (TCNB) buffer containing 50 mmol/L Tris, 10 mmol/L CaCl2, 150 mmol/L NaCl, 0.05% Brij35, pH 7.5 at 37 °C for 1 h. The effects of Sal B on MMP-1 and MMP-2 activities were tested in black 96-well plates as follows; activated MMP-1 (10 ng) or MMP-2 (160 ng) was incubated with Sal B for 1 h at 37 °C, in a final volume of 50 μL. Final concentrations of Sal B were 0 (control) 0.002 4, 0.012, 0.06, and 0.3 g/L, and were diluted with TCNB buffer. 1, 10-phenanthroline at 1 mmol/L was used as a positive control for inhibition of MMP-1 and MMP-2 activities. Fifty-microliter fluorogenic peptide substrates (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2) were added at the final concentration of 10 μmol/L in a total of 100 μL reaction mixture. MMP-1 and MMP-2 are capable of cleaving this substrate, causing an increase in fluorescence. Fluorescence was measured at 1 min intervals for 1 h on a fluorescence plate reader (SpectraMax Gemini EM, MD) with λex 320 nm and λem 405 nm. Vi and V0 were the change rates of fluorescence in reaction starting for 10 min with and without Sal B respectively, representing the enzymatic activities. Inhibition ratio (%) = 1−Vi/V0, 50% inhibiting concentration (IC50) was the concentration of Sal B when inhibition ratio was 50%.

1.4 Preparation of rat polymorphonuclear neutrophil lysozyme Rat polymorphonuclear neutrophils (PMNs) (rich in MMP-9) were used as a source of MMP-9. Rats’ abdominal cavity PMNs were collected by injecting 5 g/kg glycogen intraperitoneally. Six hours later, abdominal cavity was washed with 50 mL cool phosphate buffer saline (PBS). And then the lavage fluid was washed by using PBS for three times. The deposit was suspended in TCNB buffer and frozen-thawed three times in liquid nitrogen and 37 °C water bath. The lysozyme was centrifuged at 12 000 r/min for 15 min. The supernatant was stored at −80 °C.

1.5 MMP-9 activity assay Gelatin zymography was performed to test the activity of MMP-9. The PMN lysate was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition. Twenty-four micro-
gram PMN protein was loaded in each lane and run in parallel with a lower molecular weight marker on concentrate gel (2% SDS-polyacrylamide gel) at 80 V and separate gel (10% SDS-polyacrylamide gel) containing 0.1% gelatin as the substrate at 120 V. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 20 min at room temperature and then the gel was cut into several pieces along lanes, and these pieces were incubated for 20 h at 37 °C with incubation buffer (0.05% Tris-HCl, pH 8.8, 5 mmol/L CaCl₂, 0.02% NaN₃) containing 0 (control), 0.008, 0.03, 0.3 g/L Sal B respectively. The gels were stained (0.25% Coomassie blue, 10% acetic acid and 45% methanol) for 1 h and then destained (7.5% acetic acid and 25% methanol) for 1 h. Proteolytic activity was seen as clear lysis bands of degraded protein on a uniformly blue background.

Positive control gels contained 2 mmol/L 1, 10-phenothioniline in the incubation buffer so as to confirm that the lysis bands were the results of MMPs.

Gels were scanned and analyzed by using Image Master VDS (Amersham). The image of the gel was inverted to reveal dark bands on a white background. The area, and optical density of each band were determined. The relative amounts of the proteinases were determined by multiplying the area of each band by the optical density.

All the sample pH values were monitored before experiment. The final pH of the assay mixtures was around 7.2 to 7.4.

1.6 Statistical analysis All data concerning MMPs activity analysis were reported as x ± s. One-way ANOVA was utilized to test for differences in MMPs activities among the control group and the drug groups. A value of P < 0.05 was considered statistically significant.

2 Results

2.1 Effects of Sal B on activities of MMP-1 and MMP-2 After incubated with 1 mmol/L APMA at 37 °C for 1 h, MMP-1 and MMP-2 were activated. The activities of MMP-1 and MMP-2 were inhibited by 1, 10-phenothioniline completely. In the range of 0.002 to 0.3 g/L, Sal B could dose-dependently inhibited the activities of MMP-1 and MMP-2 (Figure 1 and Figure 2). According to inhibition ratio = (1−Vi/Vo) × 100%, the inhibition ratios of Sal B in different concentrations were obtained (Figure 3, Figure 4 and Table 1). According to the regression curve of inhibition ratios, the values of IC₅₀ of Sal B on MMP-1 and MMP-2 were (0.090 ± 0.015) g/L and (0.080 ± 0.005) g/L respectively.
Table 1 Inhibition ratios of Sal B on the activities of MMP-1 and MMP-2

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Inhibition ratio of MMP-1</th>
<th>Inhibition ratio of MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>0.002 g/L Sal B</td>
<td>33.20 ± 6.56 **</td>
</tr>
<tr>
<td>0.012 g/L Sal B</td>
<td>3</td>
<td>47.23 ± 3.29 **</td>
<td>43.97 ± 2.67 **</td>
</tr>
<tr>
<td>0.06 g/L Sal B</td>
<td>3</td>
<td>61.83 ± 4.70 **</td>
<td>71.27 ± 6.21 **</td>
</tr>
<tr>
<td>0.3 g/L Sal B</td>
<td>3</td>
<td>83.03 ± 15.67 **</td>
<td>90.83 ± 3.09 **</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group.

### 2.2 Effects of Sal B on the activity of MMP-9

Gelatin zymography analytic result displayed that there were clear lysis bands of degraded protein on a uniformly blue background, while there were no lysis bands in the positive control gel, which confirmed that the lysis bands were the results of MMPs (Figure 5). According to the lower molecular weight protein marker, MMP-9 (92 kD) was identified. In the concentration of 0.003, 0.03, 0.3 g/L, Sal B could inhibit the activity of MMP-9, presenting dose dependence (P < 0.01) (Table 2).

![Figure 5 The lysis bands of MMP-9](image)

Table 2 Effects of Sal B on the activity of MMP-9

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Relative activity of MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>1.124 ± 0.145 9</td>
</tr>
<tr>
<td>0.003 g/L Sal B</td>
<td>4</td>
<td>0.862 ± 0.189 2 **</td>
</tr>
<tr>
<td>0.03 g/L Sal B</td>
<td>4</td>
<td>0.516 ± 0.151 6 **</td>
</tr>
<tr>
<td>0.3 g/L Sal B</td>
<td>4</td>
<td>0.283 ± 0.125 3 **</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group.

### 3 Discussion

MMPs are a family of zinc-dependent endopeptidases, which as a group can degrade essentially all ECM components. So far, more than 20 members of the human MMP family have been identified. Based on their structure and substrate specificity, they can be divided into subgroups of collagenases, stromelysins, stromelysin-like MMPs, gelatinases, membrane-type MMPs (MT-MMPs), and other MMPs. MMPs are produced by several different types of cells in skin including keratinocytes, fibroblasts, endothelial cells, neutrophils, and macrophages. MMP activity is specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs).

Cutaneous wound repair can be divided into a series of overlapping phases including formation of fibrin clot, inflammatory response, granulation tissue formation incorporating re-epithelialisation and angiogenesis and finally, matrix formation and remodelling. MMPs are an important part of the inflammatory stage of wound healing, but they become destructive to the wound matrix when a prolonged inflammatory stage predominates a stalled chronic wound. The proteolytic property of the MMPs is important during wound healing to remove debris and facilitate cell migration. However, excessive accumulation and activation of MMPs can suppress cell proliferation and angiogenesis due to destruction of growth factors and matrix proteins that provide necessary substrates for cell migration and integrity of the tissue.

It has been consistently reported that compared with acute wounds, the levels of MMP-1, MMP-2 and MMP-9 were increased, while the levels of TIMP-1 was decreased in chronic wounds. Lobmann et al. reported that the concentrations of MMP-1, MMP-2, MMP-8 and MMP-9 were increased in biopsies of diabetic foot ulcers, especially MMP-1 was 65-fold higher than traumatic wounds, and the expression of TIMP-2 was reduced in diabetic wounds compared with lesions of non-diabetic patients. Because of the over expression and activation of MMPs and the deficient level of TIMPs, MMPs were not balanced by an equal amount of TIMPs, which led to wound healing failure. New treatment strategies for healing chronic wounds could be targeted towards the recovery of the balance between MMPs and TIMPs.

*Salvia miltiorrhiza* is a popular Chinese herb that has been widely used for treatment of cardiovascular and cerebrovascular diseases, and also for treatment of chronic wounds in China. Lots of experimental and clinical studies had confirmed that *Salvia miltiorrhiza* could promote chronic ulcer healing obviously. *Salvia miltiorrhiza* extracts contain lipid-soluble diterpene quinones (tanshinones) and water-soluble phenolic acid derivatives such as salvianolic acid A, B, and lithospermic acid B. Sal B is the most abundant and bioactive component of salvianolic acid in *Salvia miltiorrhiza*, which has excellent antioxidant properties. In our studies, we want to see if Sal B also has inhibitory effects on MMPs activities which is beneficial for chronic wound closure.

In chronic wounds, MMP-1 was expressed in keratinocytes bordering, and MMP-2 was seen abun-
dant in dermal fibroblast and endothelial cell beneath. MMP-9 and MMP-12 were found in neutrophils and macrophages that infiltrated in the ulcer bed \( ^{31, 33} \). The rat neutrophils were used as a source of MMP-9 for MMPs activity assays. Compared with the control group, Sal B directly inhibited the activities of MMP-1 and MMP-2 in the range of 0.002 4 to 0.3 g/L and the activity of MMP-9 in the range of 0.003 to 0.3 g/L, which indicated Sal B could inhibit not only the interstitial collagenase activity, but also gelatin. In the wound healing process, fibroblasts were the main repair cells. Studies suggested that in the range of 1 to 10 mol/L (0.000 719 to 0.007 19 g/L) Sal B had no toxicity on NIH/3T3 fibroblasts \( ^{34} \). All these results suggested that the inhibitory effects of Sal B on MMPs might have clinical significance in the treatment of chronic wounds.

REFERENCES


19. Wu HN, Sun H. Study on the clinical therapeutic effect of composite Salvia Injection matched with Western medi-
“循证医学与中医药临床实践”专题研讨会

征文暨第一轮会议通知

近年来，中西医结合领域内循证医学的理论和实践取得了诸多进展，为促进中西医结合临床实践与科研中的进一步应用，中西医结合学会循证医学专业委员会拟于 2009 年 5 月在广州举办“循证医学与中医药临床实践”专题研讨会。会议日期定于 2009 年 5 月 23 日，会期 2 天。会议邀请了国内外著名的循证医学专家和中西医结合领域的专家作主题发言和专题报告。会议将介绍国内外循证医学的最新进展，侧重于中医药临床实践的特点对循证医学带来的启示、挑战和应对策略。

欢迎各位专家积极投稿。征文及会议有关事宜通知如下。

1 会议组织
中国中西医结合学会和循证医学专业委员会共同主办，广东省中医院承办。

2 会议时间
2009 年 5 月 23～24 日。报到时间为 2009 年 5 月 22 日全天。

3 会议内容
专题讲座和会议学术交流。

4 研讨方式
采取大会与专题会议相结合、讲座与讨论相结合及圆桌会议的形式。

5 征文要求
(1) 循证医学的进展与展望；循证医学发展的前沿动态；循证医学在中医、中西医结合领域的应用与展望。(2) 符合中医、中西医结合内在的循证医学实践与探索；循证医学与中西医结合内在的联系与比较；中医、中西医结合的临床研究、临床证据的评价与应用、临床证据的评价与应用。(3) 中西医结合临床研究中的应用与实践、个案报告、病例报告等的规范研究，基于循证医学原则的专家共识的制定。(4) 如何将现有的证据应用于中医、中西医结合的临床决策中，针灸等非药物疗法及中西医结合学科领域的循证医学临床实践。(5) 中医、中西医结合临床的循证医学教育；循证医学教育在中医、中西医结合临床医生、护理人员、科研人员、管理人员、医学生中的开展与传播。(6) 其他与循证医学有关的内容。

6 征文要求
(1) 论文内容真实，可靠，具有科学性、先进性、实用性，未公开发表过。(2) 文字总字数 3000 字左右，文稿附中文摘要、关键词。(3) 论文投稿，以电子邮件发送。(4) 来稿请注明作者姓名、工作单位、通讯方式、邮政编码和联系电话。是否同意参加大会交流。是否同意中国知网（CNKI）的电子版刊登、检索使用、传播等。(5) 征文截稿日期：2009 年 4 月 24 日。

7 继续教育
授予参会者国家级继续教育学分 6 分。

8 会议地点及费用
具体会议地点及费用请见第二轮会议通知。电话：020-81887217-31225；传真：020-81874903；联系人：冯小波 (13825012658)、老秀荣 (13682212103)；E-mail：yangxinbom@163.com，loayr@yahoo.com.cn；地址：广州市大德路 111 号广东省中医院科研部；邮政编码：510120。

中国中西医结合学会循证医学专业委员会
2008 年 11 月 25 日