

Review

The research progress of MSCs proliferation and differentiation in bone injury according to the TCM theory

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Chinese have enjoyed not only quick recovery from but also effective daily prophylaxis of bone injuries. According to the Traditional Chinese Medicine (TCM) theory, Shen (or nephridium) is in charge of the bone, which corresponds to teeth, spine, skull, femur and the like. The function of Shen can be divided into Shen Yin (or negative nephridium) and Shen Yang (or positive nephridium). Bone injury is usually accompanied with blood stasis and loss in microenvironment. Therefore, traditional Chinese doctors had chosen tonifying Shen Yin, invigorating Shen Yang, enriching and activating the blood or even combining the above three strategies for injured bone repairing. Nowadays, researchers are reunderstanding TCM drugs and formulas by studying mesenchymal stem cells (MSCs) proliferation and differentiation *in vitro* and *vivo*. This review will introduce some newest articles mainly in China focusing on the growth and differentiation of MSCs, which were treated with a single herb or formulas of those drugs (belonging to the above four classes) to demonstrate the huge potential of TCMs in the future bone-injury-therapeutic production of MSCs.

Key words: MSC, traditional chinese medicine, osteogenic differentiation, bone injury repairing.

INTRODUCTION

Bone damage has been afflicting doctors and patients in both Western and Chinese Medicine. With the help of the Traditional Chinese Medicine (TCM), Chinese fracture patients had enjoyed a quick recovery, fine prognosis, and most importantly, the effective daily prophylaxis. According to the TCM theory, Shen (or nephridium) is in

charge of the bone (Huang Di Nei Jing: Su Wen: Perspicuity of Five Qi), and “Shen produces the marrow” (Huang Di Nei Jing: Su Wen: Summary of Yin and Yang Visualization). Thus Shen usually corresponds to teeth, spine, skull and femur, which contain differentiated osteocytes. The function of Shen can be divided into Shen Yin (or negative nephridium) and Shen Yang (or positive nephridium). In the bone part, Shen Yin acts as the role of moistening, placating cells, nutrition storing, supporting the comfortable environment for osteocytes. And primarily, Shen Yin should be reckoned as the assembly of the cells itself. Shen Yang, correspondingly, can be characterized as the rate or power of Shen to perform its function.

On the other hand, bone injury is usually accompanied with blood stasis and blood loss in microenvironment. Traditional Chinese fracture therapists had always administered blood-activating and stasis-dissolving drugs along with blood enriching drugs. We realize its rationale from the experience of stem cells culture, for blood-

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Abbreviations: TCM, Traditional Chinese medicine; MSC, mesenchymal stem cell; NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; GSB, a TCM drug called Gui-Si-Bu; ALP, alkaline phosphatase; TGF- β , transforming growth factor- β ; BMP-2, bone morphogenetic protein 2; RT-PCR, real-time polymerase chain reaction; DR, *Drynariae Rhizoma*; MHC, major histocompatibility complex; BEAs, blood enrichers and activators; NF, neurofilament; BCBC, bovine cancellous bone complex; CTPM, Chinese-Traditional-Patent-Medicine; DQTMP, a TCM medicine called Dan-Qi-Tong-Mai-Pian.

stagnation-and-loss is detrimental to mesenchymal stem cells in the state of osteogenic differentiation and rapid skeleton regeneration.

Mesenchymal stem cell (MSC) is a useful sort of multipotential stem cell in bone tissue engineering. In China, researchers have turned to TCM theory and drugs in studying MSC proliferation/differentiation for bone repairing. They selected TCM drugs that tonify Shen Yin, invigorate Shan yang, enrich and activate the blood or even combine the above three strategies and determined the effects on MSCs, proving that the TCM was truly valuable in bone regeneration.

TONIFYING SHEN YIN

The ordinary TCMs tonifying Shen Yin include *Tortoise plastron*, *Pulp of Cornus*, *Wolfberry Fruit* and so on. *Tortoise plastron*, a Shen Yin tonifier in clinic, is abundant in fatty acid esters, sterol esters and ketosteroids, as far as we know. And the most effective constituents of tortoise plastron like fatty acid esters were proven promotive for MSCs proliferation in low concentration while restraining of the overgrowth in high concentration (Wang et al., 2007). Zhou's workgroup investigated the effect of *Plastrum testudinis* on MSCs growth using MTT and immunohistochemical detection, finding that the growth rate and morphology of cells in the presence of *P. testudinis* were significantly better than the control team. The promoting effect appeared linearly dependent on the concentration of the effective constituents in the serum (Zhou and Chen, 2005). Qiu's workgroup made a rat model of aplastic anemia and used high dose of *Tortoise plastron* to treat MSCs, resulting in notable improvement of the model team's cellular vitality, which had turned even higher than the healthy control team (Xu and Qiu, 2005).

Shen Yin nourishes the tissues which stem from MSCs or similar progenitor cells, which is beneficial to the impaired cell mass recovery and organs regeneration. Even injured neurocytes can be regenerated from MSCs, assisted by complementarity of Shen Yin. That is the reason why some patients with badly damaged spines could finally retrieve sensation after a period of Tonifying-Shen-Yin therapy. For example, another Shen Yin tonifier called *Herba-Gynostemae* was able to induce MSCs to mature into neurocytes. After treatment of cultured MSCs from the vertebral column with *Gynostemae* solution, nestin, neuron 2 specific enolase (NSE), and glial fibrillary acidic protein (GFAP) could be detected by immunocytochemistry method (Dong et al., 2003). Also *Tortoise Plastron* as mentioned above had demonstrated the ability of inducing MSCs to transdifferentiate into neural stem cells, in view of relatively high proportion of nestin positive staining (Zhang et al., 2004). Actually, the premier for *Tortoise Plastron* is high performance of osteogenic inducement. MSCs cultured in the serum containing effective constituents of *Tortoise Plastron*

(using the drug GSB) changed from a spindle shape to a polygonal appearance. Moreover, the alkaline phosphatase (ALP) and von Kossa staining were both positive (Yan et al., 2007). Li's group used the water decoction of *Tortoise plastron* and experimented in the similar method and confirmed that *Tortoise plastron* could effectively promote the expression of ALP, increase osteocalcin level and the dose of calcified deposition (Li et al., 2005). Furthermore, some researchers proved that *Tortoise plastron* can not only promote MSCs propagation but also differentiation into myoblast to some extent (Chen, 2006). Inferentially, other TCMs assisting Shen Yin tonifiers in traditional prescriptions may be also instructive for MSCs differentiation. *Rhizoma Gastrodiae* is a useful herb for "expelling the Wind Demon" and "appeasing Gan (liver)", normally compatible with Shen Yin tonifiers. Dong revealed its capability of committed inducement for MSCs into neuron-like cells. When just induced by *Gastrodiae* for 2 h, MSCs produced cytodendrites and immunohistochemical analysis showed positive of NSE and nestin and negative of GFAP staining (Dong et al., 2004).

INVIGORATING SHEN YANG

Shen Yang is related to the level of cellular metabolism vitality. To invigorate means to raise the grade or proportion of a special differentiation. *Epimedy* is just one Shen Yang invigorator. Some researchers had reported this herb improved the osteogenic differentiation of MSCs for enhancing the expression of TGF- β 1 and BMP-2 (Yang et al., 2007). This enhancement of osteogenic differentiation had also been authenticated by the number of colonies positively stained of ALP, osteocalcin secretion and calcium deposition level (Ma et al., 2004). The osteogenic inducement capability of this herb, as some data show, could reach the similar level of dexamethason (Yang, 2007). *Drynariae Rhizoma* (DR), another important Shen Yang invigorator, is effective in some gynecological diseases such as osteophoresis and bone resorption according to TCM literatures. The mechanism in fracture healing and osteoporosis treatment is due to its property of promoting MSCs proliferation and osteogenic differentiation in large probability (Chen, 2003). Some articles claimed that treatment of osteoclasts with DR extract could prevent intracellular maturation of cathepsin K. It was hence considered that DR is a pro-drug of a potent bone resorption inhibitor (Jeong et al., 2003). Jeong's workgroup had further clarified this role on nontransformed osteoblastic cells (MC3T3-E1) and rat bone marrow cells, showing that DR enhanced the ALP activity and mineralization (Jeong et al., 2004).

Shen Yang invigorators are also useful in stimulating neuron differentiation. Many TCMs capable of improving the sexual-function can improve the neural excitability, as Shen conserves the reproduction-essence in the TCM theory (Huang Di Nei Jing: Su Wen: The Antediluvian

Puerility). Another Shen Yang invigorator named *Cuscuta Chinensis*, mostly used in treatment of inferior reproductive function and soreness in the waist and knee, was demonstrated to have inducing effect based on neuronal specific enolase (NSE) and GFAP expression of rat MSCs. By RT-PCR, researchers found that the content of mRNA encoding NSE and GFAP was remarkably higher than in the control (Shen, 2006).

Referring to numerous traditional prescriptions, it is appreciable that some other herbs belonging to the Qi Supplements (according to the TCM classifying) can also motivate Shen Yang invigorators playing a role in strengthening the sinew and bone. These TCMs may conduce to the MSC differentiation potentially. *Astragalus* is just one perfect kind. Yu found that its effective components (astragaloside and the like) could induce MSCs for cardiomyocyte-like differentiation, as myocardial specific antibody cTnT and GFAP are positively expressed by immunohistochemistry detection. In particular, the inducement rate was actually as high as in some special inducers (Yu et al., 2007). Similar results had also been found by RT-PCR and α/β -MHC expression examination (Yang et al., 2008; Wang, 2006). Remarkably, *Astragalus* could stimulate MSCs forming the neuron like cells by just 90 min treatment (Dong et al., 2004).

ENRICHING AND ACTIVATING THE BLOOD

Enriching-and-activating-blood strategy is very common in TCM treatment of Bone Traumatology. TCM doctors believe Blood Enrichers and Activators (BEAs) are perfect at improving microcirculation and contributive to ossification and carnification. This theory indicates these herbs may play a part in MSCs differentiation process.

Salvia miltiorrhiza is a front-line drug in bone fracture recovery and gynaecology-haemostasis treatment. Tang (2008) added *S. miltiorrhiza*-parenteral-solution into medium and detected type-II collagen, indicating that rat MSCs were thus cartilagenic induced. Another explanation of *S. miltiorrhiza* improving the damaged bone tissues' growth state is the inducement of vessels formation, which brings about superior blood supply. Fan extracted salvianolic acid from *S. miltiorrhiza* to treat the growing MSCs followed by cells transplantation into animal models of myocardial infarction. Later the capillary density of the infarcted zone and expression of CD34 *in vivo* were examined, the results of which verified this mechanism (Fan et al., 2007a). In addition, *S. miltiorrhiza* has neuron-like cells inducing action (Yu et al., 2004; Yu et al., 2006).

Dipsacus asperoids is another BEA frequently combined with Shen Yang invigorators. This herb was studied for its bone-repairing potency and its effective constituents (*D. asperoids* glucoside). According to MTT staining and ALP activity results, it was proclaimed to stimulate MSCs reproduction and osteogenic differentiation (Wang, 2002;

Feng and Fu, 2004).

Angelica is a very popular drug in blood enriching/activating therapy. Parenteral solution of *Angelica* had been used to induce goat and SD rat MSCs into neurocytes. Induced cells not only presented positive NF and NSE expression but also very high cell viability in growth (Hua et al., 2004; Liu et al., 2003).

Niu-po-zhi-bao pellet, a useful drug working in vascular infarction, contains large quantity of BEAs, including *Hawksbill*, *Amber*, *Dragon's Blood*, *Saffron*, etc. It has been reported that MSCs treated with the solution of this pellet displayed the neuron-like cellular morphology, positive NF and negative GFAP staining, suggesting these drugs collection is suitable for nerve system inducement (Huang et al., 2003).

According to the TCM classification, generalization of BEAs suggests that, for bone repairing, the research on osteogenic and neurogenic differentiation should be both focused.

DRUGS COMBINATION

One most noticeable feature of TCM therapy is drugs combination. From the above three classes, we realize a single herb may induce MSCs differentiation in more than one direction. It is also possible that different drugs in different classes may cause differentiation in one direction. Synergistic effects should be well taken advantage of in MSC-treating area.

Ancient formulas have been studied in laboratories all around the world by current technologies. Gui-Lu-Er-Xian-Jiao decoction is still widely used for "Both Deficiency of Shen Yin and Yang". It has two drugs as the brunt, the *Antler Glue* and *Tortoise Plastron*. Its effect on cartilagenic inducement for rabbit MSCs was significantly higher than those of single inducer (TGF- β 3) (Li et al., 2007).

Apart from the combination of Shen Yin and Yang replenishers, the more universal cooperation is Shen Yang invigorators united with blood treating drugs. Many popular Chinese-Traditional-Patent-Medicines (CTPMs) are made according to this idea. Zeng et al. (2008a, b) employed an aqueous solution of a group of Shen Yang invigorators and BEAs (including *Cortex Eucommiae*, *Rehmanniate*, *Fructus Psoralea*, *Salvia Miltiorrhiza* and *Achyranthes* etc) to treat and examine the MSCs growth status by detecting the expressed CD44 and analyzing the effect of different drugs concentrations on MSCs growth. The proliferation speed of treated MSCs reached a significantly higher level compared with the normal control. Moreover, the Shen Yang invigorator *Rhizoma Drynariae* and blood Enricher *Radix-Rehmanniae* combination was advantageous to high level of type II collagen production, suggesting chondrocytes could be differentiated by administration of these herbs (Gao, 2007).

The union of Shen Yin tonifiers and BEAs is also an

effective strategy for high propagation vitality. *Shengji Liquor*, a common TCM oral solution containing the effective components of *Tortoise Plastron*, *Angelica Sinensis* and the like, was found favorable to MSCs proliferation based on the MTT and lactate dehydrogenase evaluation (Zhang et al., 2007). A secret formula Xiao-Bi-Ling rich in Shen Yin tonifiers and blood activators from Hubei College of TCM was claimed propitious for cartilaginous differentiation from MSCs according to toluidine blue and type II collagen staining (Wu, 2004).

Fan et al. (2007b) chose SD rats for compound drugs intragastric administration. He selected a formula including both Shen Yin tonifiers (*Tortoise Plastron*, *Medlar* etc) and Shen Yang invigorators (*Eucommia*, *Drynariae* etc), as well as BEAs (*Angelica Sinensis*, *Safflower*, *Citrus Aurantium*, *Peach Kernel*, *Radix Rehmanniae* etc). The later detection of MTT staining and ALP activity for MSCs from femoral marrow of the rats (intragastric administration group and the control group) indicated that MSCs had been induced to osteogenic differentiation (Fan et al., 2007b). These researchers had also utilized the same formula to induce and transplant MSCs of rabbits into the bone-injury animal model, supported by Antigen-extracted Bovine Cancellous Bone Complex (BCBC, as porous carriers). ALP activity and cartilage amount of the experimental group were significantly higher than that of the control group with just grafted pure BCBCs (Sun et al., 2008). This is a good clinical identification of TCM influencing MSCs for bone repairing. The similar transplantation and results on SD rats further proved it (Fan et al., 2007c). Another formula of concern or CTPM is Dan-Qi-Tong-Mai-Pian (DQTMP), containing *Astragalus* and *Salvia* as the main drugs. The hMSCs treated by DQTMP were found stimulative for c-IAP-1/2 expression while restricting for the caspase-3 activation of human endothelial cells, and thereby inhibited apoptosis processes (Li et al., 2008). That underlined the mechanism of how Shen reinforcers and BEAs ameliorate the microenvironment of broken bone tissues.

CONCLUSION AND PROSPECT

Among different classes for MSCs proliferation and osteogenic differentiation, Shen Yin tonifiers are reported more constructive than Shen Yang invigorators in osteoporosis treatment, and the later superior to BEAs (Zhao et al., 2002; Shao et al., 2006). However, the exact effectiveness lies on what constitution of drugs we use and what indexes we are concerned about. There are still large amount of interesting questions to pursue. For example, why do two drugs classified sharply differently have exactly the same inducement direction and similar growth-promoting effect for MSCs? How to understand that an assisting drug in TCM theory, which shows positive effect alone *in vitro* in MSC culture, cannot achieve the significant action when decocted separately

for patients (human/animals)? Further, the research above had used either the decoction or single component in some parenteral solution to treat the growing MSCs. Should we turn to build the gastrointestinal simulation where those drugs can be pre-treated and then extract the post-reaction components for the following MSC inducement? However, numerous herbs belonging to Shen Yin tonifiers, Shen Yang invigorators or BEAs offer us opportunities for screening stem-cell-inducing drugs. The large scale bioreactors for MSC amplification and directional inducing production in the future might create a demand for a single or combination of the TCMs as a routine technique.

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