

***Rheum rhabarbarum* extract promotes healing of the incision through relieving inflammation and stimulating angiogenesis**

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Abstract: As one of the most important treatment strategies in clinic, surgery has improved to be more and more efficient and safe. However, the infection risk of incision caused by surgery is still the main concern of patients. In our research, we found extract of *Rheum rhabarbarum* (rhubarb) could be used to diminish this risk through promoting the healing of the incision. Using MTT assay, flow cytometry and clinical statics, we also tried to explore the mechanism of rhubarb's effect. The data showed that rhubarb extract decreased the number of leukocytes and neutrophils and inhibited the growth of bacteria. Moreover, the vascular endothelial cells cultured in medium containing rhubarb extract grow faster than control. The flow cytometry also demonstrates that the ratio of cells in S and G2/M phase increase after treated with rhubarb extract. There after, we hypothesize that rhubarb extract can promote incision healing through relieving inflammation and stimulating angiogenesis.

Keywords: Surgery, incision, rhubarb extract, inflammation, angiogenesis.

INTRODUCTION

Nowadays, surgery is one of the most common and efficient treatments in clinic (den Brok *et al.*, 2006). However, the infection of incision caused by surgery is still one of the dangers that threaten the health of patients. To minimize the risk of infection after surgery, a promising solution is to accelerate the healing of the incision (Li and Zheng, 2016). There are many factors that can affect the incision healing, among which inflammation and angiogenesis are most studied. Inflammation is a normal part of the incision healing to remove contaminating bacteria from the wound (Gosain and DiPietro, 2004, Francois *et al.*, 2007). However, if effective decontamination is absent, inflammation phase will be prolonged because uncompleted bacteria clearance that results in elevation of pro-inflammatory cytokines, such as IL-1 and TNF- α (Edwards, 2004). Moreover, the prolonged inflammation leads to increase of matrix metalloproteases level, which causes the rapid degradation of growth factors that appear at the incision (Menke *et al.*, 2007). Finally, the long term inflammation enters the incision into a chronic state that fails healing.

Angiogenesis is another important factor that affects the incision healing (Gosain and DiPietro, 2004). Due to vascular disruption and oxygen consumption, the early incision is under a very hypoxic condition. Temporary hypoxia after surgery triggers angiogenesis to compensate the lack of oxygen (Bishop 2008, Rodriguez *et al.*, 2008). The proliferation of the vascular endothelial cells is the foundation to form new blood vessels. Normally, the vascular endothelial cells are in the stationary phase, after

the surgery, the primary hypoxia induces the release of cytokines and growth factors that promote vascular endothelial cells into dividing phase (Lancero and Orgill, 2014, Crafts *et al.*, 2015). Nevertheless, if angiogenesis is delayed and the oxygen cannot be restored, healing impairs.

Rhubarb is a kind of medical herb which has been used for more than 1200 years in China. As the traditional Chinese medical books record, rhubarb can be used to stimulate circulation and diminish inflammation (Neyrinck *et al.*, 2016, Chang, 1999), suggesting it may be used to promote healing of incision caused by surgery. Xu *et al.* found rhubarb extract could help rabbit recover from skin wound (XU *et al.*, 2008). However, the use of rhubarb extract in clinic is not reported and the mechanism of how rhubarb promotes wound healing is still not uncovered. In our study, we explored the effect of rhubarb extract on healing of incision caused by surgery and preliminarily studied the mechanism of its function.

MATERIALS AND METHODS

Patients

From November, 2013 to December, 2015, 120 patients were referred to our department to accept appendectomy and all of them were divided into two groups evenly and randomly. Group A including 33 females and 27 males whose age is between 25 and 50 years old (average 37.8 \pm 5.7). In group B, there are 32 females and 58 males whose age are between 23 and 53 years old (average 37.6 \pm 5.8). There are no differences in health index between these two groups ($p > 0.05$).

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Ethical approval

This study is conducted according to the standards of Good Clinical Practice and in compliance with local regulations. Oral and written informed consent forms are obtained from all patients prior to treatment.

Cell culture

HUVEC cells (ATCC, CRL-1730) were cultured in the F-12K medium (ATCC, 30-2004) containing 0.1 mg/ml heparin, 0.03-0.05 mg/ml endothelial growth supplement and 10% fetal bovine serum. The cells were cultured in the humidified incubator (37°C, 5% CO₂).

Preparation of rhubarb extract

Rhubarb root was bought from Tongrentang Company. To prepare hydro-alcoholic extract, 250g ground rhubarb root was macerated in two liters of methanol and water (1:1) for four days. Then it was filtered and concentrated under reduced pressure using a rotary apparatus. Remaining contents were transferred into Petri dishes and were put into an oven with 37°C temperature to be dried up. The powdered extract was dissolved in water and used when it was needed. For patients use, 2mg rhubarb extract is added to 1g pharmaceutical ointment.

MTT assay

The MTT assay was used to estimate cell proliferation (Grabinger *et al.*, 2016). Briefly, HUVEC cells were seeded in 96-well plate with 1×10^4 cells/well. After exposure to rhubarb extract, the cells were incubated with MTT at a final concentration of 0.5mg/mL for 4 hours at 37°C. After the removal of the medium, 150mM DMSO solutions were added to dissolve the formosan crystals. The absorbance was read at 570nm using a multi-well scanning spectrophotometer.

Flow cytometry

Flow cytometry was used to analyze cell cycle as described (Darzynkiewicz *et al.*, 1980). Briefly, 1×10^7 HUVEC cells was suspended in 1 ml of 1xPBS (pH 7.4). The cell suspension was added drop-wise to ice-cold 70% ethanol under gentle agitation for fixation. After fixation, cells were centrifuged and re-suspended in 1ml PBS containing 0.5ug/ml RNase and incubated for 30 min at 37°C. Afterwards, cool down the cells on ice to stop the digestion of RNase. Then, add 1.5 ml 0.1mg/ml PI into the tubes and incubate at least 30min in dark environment. Filter the cells and conduct the flow cytometry using a Beckman-Coulter Epics XL flow cytometer.

STATISTICAL ANALYSIS

Results are expressed as the means \pm SD from at least three independent experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Rhubarb extract can promote healing of the incision

To study the effect of rhubarb extract on incision healing, 120 patients referred to our department to accept the appendectomy were involved in our research. To exclude the possibility of differences result from individuals, we divided the patients into two groups, named group A and group B, evenly and randomly. Take group A as the control, all of the patients in group B were suggested to treat the incision with rhubarb extract. As our record showed, the incision healing in group A will take 9.4 ± 2.8 days, however, the healing time for the patients who were suggested to treat the incision with rhubarb extract was shorten to 6.0 ± 1.8 days (fig. 1). The healing time means the time to take out the suture after surgery.

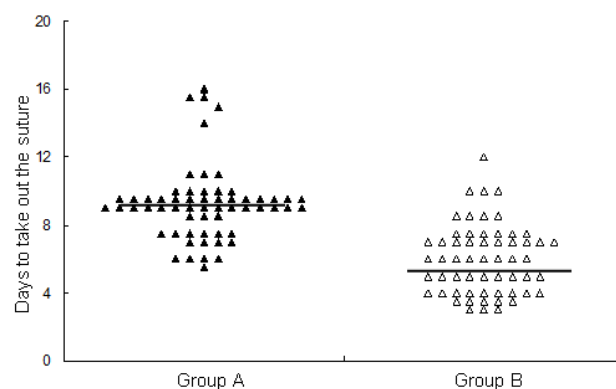


Fig. 1: Rhubarb extract promotes incision healing after appendectomy

120 patients accepted appendectomy were divided into two groups evenly and randomly. The patients in group A did not use the rhubarb extract, and the patients in group B treated the incision two twice every day with ointment containing 1 mg/g rhubarb extract. The lines indicate the average time taken by incision healing in two groups. The difference between group A and B is significant ($p < 0.05$).

Rhubarb extract relieves bacteria caused inflammation

The infection of surgery incision is mainly caused by *Staphylococcus aureus* and *Pseudomonasaeruginosa* (Gordon and Lowy, 2008, Lodise *et al.*, 2007). To explore the inhibition effect of rhubarb extract, both of bacteria are cultured in appropriate medium with or without 300mg/ml rhubarb extract. After being seeded, the OD600 was detected at 2h, 4h, 6h, 8h, 10h and 12h. We found the growth of *Staphylococcus aureus* was inhibited robustly, but slightly on the *Pseudomonasaeruginosa* (fig. 2a,b). This is in accordance with the results that rhubarb extract prefer to inhibiting the growth of Gram-positive bacteria. Because bacteria infection is one of the reasons for inflammation which may delay incision healing, we supposed that rhubarb extract should partly relieve the inflammation by inhibiting the growth of certain bacteria. Thereafter, the leukocytes and neutrophils level in group

A and B were measured, and we found the average leukocytes and neutrophils level in group B was lower than group A (table 1).

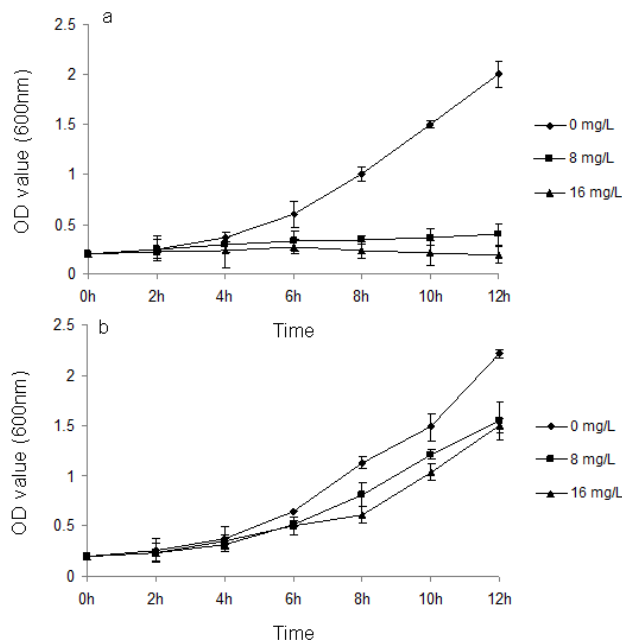


Fig. 2: Rhubarb extract affect growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Staphylococcus aureus (a) and *Pseudomonas aeruginosa* (b) grew in 100ml medium containing 0 mg/L, 8 mg/L and 16mg/L rhubarb extract. Every two hour, 1ml was taken out to read the OD value at 600nm wave length with the spectrophotometer.

Rhubarb extract stimulates proliferation of vascular endothelial cells

During angiogenesis, the proliferation of vascular endothelial cells provides the foundation for new blood vessels formation (Crafts *et al.*, 2015). To check if rhubarb extract can affect the proliferation of vascular endothelial cells, we cultured HUVEC cells in medium containing different concentration of rhubarb extract and analyzed the cell viability by MTT assay. As the concentration of rhubarb extract increased, the cells grew faster, but extremely high concentration lead to cell death (fig. 3a). Furthermore, the flow cytometry results demonstrated that rhubarb extract promoted more cells into S and G2/M phase, which indicated higher proliferation activity (fig. 3b).

a. HUVEC cells were plated into 96-well plates and cultured in F-12K medium containing 0 g/L, 5 g/L and 10 g/L rhubarb extract. Every 24 hour did the MTT assay and read the OD value at 570nm; b. 1×10^7 HUVEC cells were washed and suspended in 100ul PBS, staining the cells with DAPI and analyzed the phase distribution with flow cytometer.

DISCUSSION

The medical property of rhubarb has been recorded in China for many centuries. Combined with other medical herbs, rhubarb can be used to inhibit bacteria infection, diminish inflammation and cure burn wound (Hu *et al.*, 2014). In our study, we confirmed the effect of rhubarb extract to promote the healing of incision after appendectomy. Even though most of the patients who used the rhubarb extract recovered faster from the surgery incision, two diabetes and three obese patients were less influenced. Vincent *et al.* found hyperglycemia could increase the oxidative stress that is responsible to impaired wound healing (Vincent *et al.*, 2004). Woo *et al.* also found the MMP level was almost 60 times higher in chronic wound fluid of diabetes patients (Woo *et al.*, 2007). In obese patients, the increased tension on the edge of the wounds contributes to healing impair (Wilson and Clark, 2004, Anaya and Dellinger, 2006). Further research is still demanding about how diabetes and obesity affect wound healing.

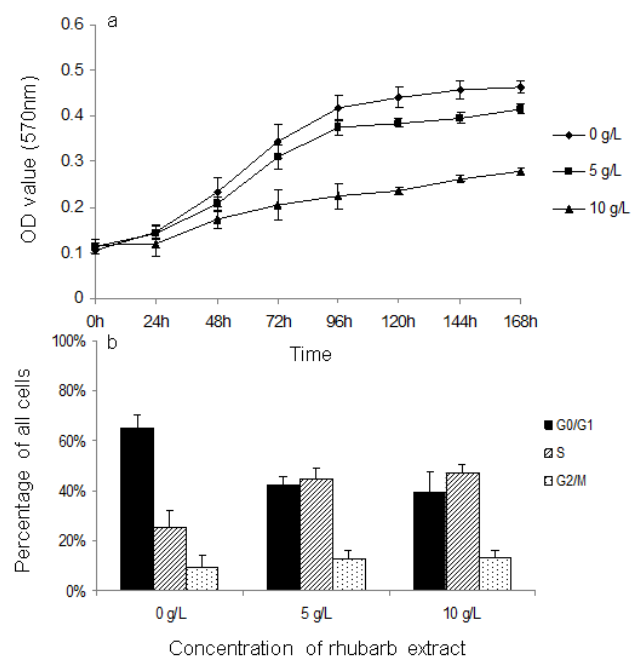


Fig. 3: Rhubarb extract stimulates vascular endothelial proliferation.

The rhubarb extract inhibit the growth of *Staphylococcus aureus* is accordance with the results that Gram-positive bacteria is more vulnerable rhubarb (Khan *et al.*, 2012). To further establish this selectivity, we still need to do some experiments with different Gram-positive bacteria. Moreover, the mechanism of how rhubarb inhibit bacteria growth is not clear yet. The clinical data demonstrated the inflammation relief function of rhubarb extract. Even though this inflammation diminishing effect may caused by inhibiting survival of infectious bacteria, it is possible rhubarb can regulate the release of cytokines that participate in inflammation process.

Table 1: The effect of rhubarb extract on inflammation

	Group A (n=60)	Group B (n=60)	T	P
Luekocytes (x10 ⁹ /L)	11.3±1.7	7.4±2.3	1.3567	<0.05
Netrophiles/lymphocytes (%)	74.1±8.9	58.3±7.2	1.5576	<0.05
PCT (ug/L)	0.54±0.07	0.39±0.02	2.1782	<0.05

Another important role of rhubarb extract is to stimulate the angiogenesis which is critical for incision healing. We verified that rhubarb extract can promote vascular endothelial cells into S and G2/M phase, which indicates active cell proliferation. But the mechanism of how rhubarb regulates the cell proliferation is still mysterious. VEGF is an endothelial cell-specific mitogen and chemotactic agent that is involved in angiogenesis, it has been shown to active many growth and proliferation related pathways, including PKC pathway, MAPK/ERK pathway and PI3K/AKT pathway (Fujio and Walsh, 1999, Hoshi *et al.*, 2002). With the HUVEC cells cultured in medium containing rhubarb extract, we found increased AKT activity (data not shown). The further study will be carried out to confirm the how rhubarb extract regulate PI3K/AKT pathway.

One of the disadvantages of most medical herbs is that the exact functional ingredients are not clear. In spite the effect of rhubarb extract on wound healing was described, the ingredient that is responsible to this effect is unknown. It was reported that five free anthraquinones, including rhein, emodin, aloemodin, chrysophanol and physcion, accounted for its medicinal properties (Ye *et al.*, 2007). To fig. out the pharmacological mechanism, it is necessary to determine the function of all chemicals in rhubarb extract. In summery, even though there are many future works to do our work approved the assumption that rhubarb extract can relieve inflammation and stimulate angiogenesis, which accounts partly for its role of promoting incision healing.

CONCLUSION

Rhubarb extract can promote incision healing through relieving inflammation and stimulating angiogenesis.

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