



## OPEN

SUBJECT AREAS:  
APOPTOSIS  
TOXICOLOGYReceived  
16 October 2013Accepted  
19 March 2014Published  
9 April 2014Correspondence and  
requests for materials  
should be addressed to  
L.Q.Y. (yanlianqi@  
126.com) or J.C.W.  
(jingchengwyz@163.  
com)\* These authors  
contributed equally to  
this work.

# The optimal concentration of topical hydroxycamptothecin in preventing intraarticular scar adhesion

Yuan Liang\*, Yu Sun\*, Xiaolei Li, Lianqi Yan, Jingcheng Wang, Jinlong Hu, Huan Yu, Haixiang Xiao, Hui Chen, Zhongwei Sun, Jun Cai, Xinmin Feng, Chuanzhi Xiong &amp; Jinshan He

Department of Orthopedics, Clinical Medical College of Yangzhou University, Subei People's Hospital, Nantong West Road 98, Yangzhou, 225001, China.

**10-Hydroxycamptothecin could reduce intraarticular adhesion by inhibiting fibroblasts proliferation after knee surgery. However, the ideal concentration of hydroxycamptothecin have not been defined. This study was tried to verify the optimal concentration of 10-hydroxycamptothecin in preventing knee intraarticular adhesion. Sixty rabbits were randomly divided into five groups. Approximately 10 mm × 10 mm of the cortical bone was removed from both sides of the femoral condyle and the underneath cancellous bone was exposed. Various concentrations of hydroxycamptothecin (0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml) or saline were applied to the decorticated areas for 10 minutes. After four weeks, the degree of intraarticular adhesion was assessed by macroscopic evaluation, biochemical analysis of hydroxyproline content and histological evaluation. The results demonstrated that the extent of knee intraarticular adhesion in 1.0 mg/ml group and 2.0 mg/ml hydroxycamptothecin group were significantly lower than those of 0.5 mg/ml group, 0.1 mg/ml hydroxycamptothecin group and control group. Moreover, there was no significant difference between 1.0 mg/ml group and 2.0 mg/ml hydroxycamptothecin group. In conclusion, topical application of 1.0 mg/ml hydroxycamptothecin may be the optimal concentration in reducing intraarticular adhesion after knee surgery in rabbits.**

The formation of intraarticular adhesion is a common complication after total knee arthroplasty or anterior cruciate ligament (ACL) reconstruction<sup>1–3</sup>. Intraarticular adhesion of knee can extremely debilitate for the patients, which often causes the activities of daily living painful and difficult, such as climbing stairs, rising from a chair and tying a shoelace<sup>4,5</sup>.

A lot of treatment strategies have been made to reduce intraarticular adhesion formation after knee surgery. For example, manipulation under anaesthesia, arthroscopic lysis and open debridement are used to relieve arthrofibrotic symptoms<sup>6–8</sup>. Moreover, many materials and pharmaceutical agents have also been used to prevent intraarticular adhesion in experimental and clinical studies. The result of these treatments are controversial and complete prevention of intraarticular adhesion has not yet been achieved<sup>9,10</sup>.

Recently, it has been reported that 10-hydroxycamptothecin(HCPT), an chemotherapeutic drug, could inhibit fibroblasts proliferation and reduce epidural scar adhesion after laminectomy surgery<sup>11–13</sup>. Our previous study showed that topical use of 0.1 mg/ml hydroxycamptothecin could prevent knee intraarticular adhesion by inhibiting fibroblasts proliferation in a rabbit model<sup>14</sup>.

However, the optimal concentrations of topical HCPT in preventing intraarticular adhesion is still unclear. Based on previous study, we established the intraarticular adhesion in rabbits model and determine the optimal concentration of topical HCPT in preventing intraarticular adhesion after knee surgery. This study may be helpful to reduce complications after knee surgery.

## Results

The surgery was well tolerated by all rabbits. There was no any sign of wound infection, cutaneous necrosis and mortality.

**Macroscopic evaluation of intraarticular adhesion.** Macroscopic observation showed that no or weak intraarticular fibrous adhesions in HCPT-treated groups were found around the decorticated areas, which can be dissected by manual traction. However, there was no significant difference in 1.0 mg/ml HCPT group and



**Table 1 | The degree of intraarticular adhesion according to the visual scoring system**

Group	Grade			
	0	1	2	3
control				6
HCPT (0.1 mg/ml)		2	4	
HCPT (0.5 mg/ml)	1	4	1	
HCPT (1.0 mg/ml)	3	3		
HCPT (2.0 mg/ml)	4	2		

Six rabbits were selected from each group. The values within the table represent the number of rabbits.

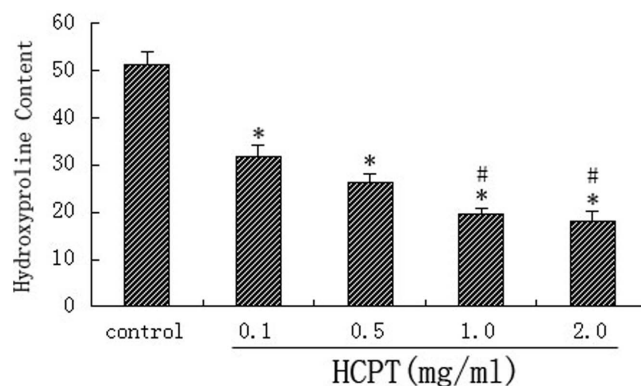
2.0 mg/ml HCPT group. In control group, dense and tenacious fibrous adhesions were observed around the decorticated areas of the femoral condyle, which were difficult to dissect the scar adhesions accompanied with bleeding. The degree of intraarticular adhesions were evaluated according to the visual scoring system (table 1).

**Biochemical analysis of hydroxyproline content.** The hydroxyproline content of intraarticular scar tissues in HCPT-treated groups were significantly less than that in control group ( $p < 0.05$ ). The hydroxyproline content decreased in a dose-dependent manner in HCPT-treated groups. The hydroxyproline content in 1.0 mg/ml HCPT group and 2.0 mg/ml HCPT group were  $19.67 \pm 1.21 \mu\text{g}/\text{mg}$  and  $18.17 \pm 1.94 \mu\text{g}/\text{mg}$ , which were significantly less than those in 0.5 mg/ml HCPT group ( $26.33 \pm 1.75 \mu\text{g}/\text{mg}$ ,  $p < 0.05$ ), 0.1 mg/ml HCPT group ( $31.67 \pm 2.50 \mu\text{g}/\text{mg}$ ,  $p < 0.05$ ) and control group ( $51.33 \pm 2.58 \mu\text{g}/\text{mg}$ ,  $p < 0.05$ ). The hydroxyproline content in 0.5 mg/ml HCPT group was also less than those in 0.1 mg/ml HCPT group ( $p < 0.05$ ) and control group ( $p < 0.05$ ). However, the hydroxyproline content showed no significant difference between 1.0 mg/ml group and 2.0 mg/ml HCPT group ( $p = 0.219$ ). The results of One-Way ANOVA were shown in table 2, the statistical analysis of hydroxyproline contents in the intraarticular scar tissue for each group were shown in the Fig. 1.

#### Effect of HCPT on intraarticular adhesion in histological analysis.

In control group, dense scar adhesions were found around the decorticated areas of femoral condyle, which tethered surrounding soft tissues to the femur. In 0.1 mg/ml and 0.5 mg/ml HCPT-treated groups, mild scar tissues were observed around the decorticated areas compared with those of control group. However, no or loose fibrous adhesion tissue were observed in 1.0 mg/ml 2.0 mg/ml HCPT-treated groups (Fig. 2).

**Effect of HCPT on intraarticular collagen density.** In Masson's trichrome staining, collagen density of intararticular adhesion tissue in HCPT-treated groups was coincidence with hematoxylin and eosin staining. The collagen density of intararticular tissue in control group was dense. However, the collagen density was weak in 1.0 mg/ml and 2.0 mg/ml HCPT-treated groups, which revealed significant decrease compared with those in 0.5 mg/ml and 0.1 mg/ml HCPT-treated groups. Moreover, the collagen density was moderate in 0.5 mg/ml HCPT-treated group, which was also revealed decrease compared with that in 0.1 mg/ml HCPT group (Fig. 3).



**Figure 1 | Hydroxyproline contents in intraarticular scar tissue in HCTP-treated groups and control group.** Hydroxyproline content was expressed as  $\mu\text{g}/\text{mg}$ . \* $p < 0.05$ , compared with the hydroxyproline content in control group. # $p < 0.05$ , compared with the hydroxyproline content in 2.0 mg/ml group and 1.0 mg/ml HCPT group.

## Discussion

This study demonstrated that topical applied HCPT could reduce collagen synthesis and prevent intraarticular scar adhesion in rabbit models after knee surgery. 1.0 mg/ml and 2.0 mg/ml HCPT-treated groups had better effect in preventing intraarticular scar adhesion compared with other HCPT-treated groups and control group. However, there was no significant difference between 1.0 mg/ml HCPT group and 2.0 mg/ml HCPT group. Moreover, the higher of the concentration of HCPT, the more possibility of the potential toxicity for the wound healing, articular cartilage and survival. Considering the preventive effect and potential toxicity of HCPT, 1.0 mg/ml HCPT maybe the optimal concentration in preventing intraarticular scar adhesion in rabbit models.

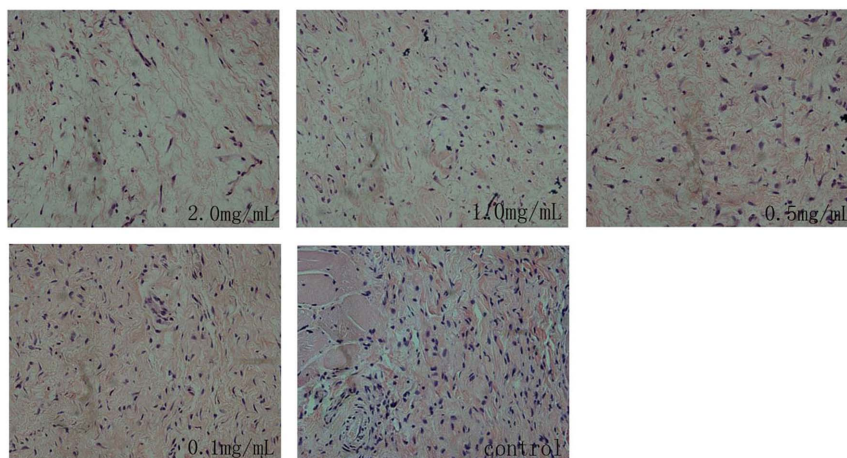
Though the pathophysiological mechanism of knee intraarticular adhesion still remain unclear, many studies have reported that some cytokines and cells were essential in the formation of scar adhesion. The fibroblast was assumed to play an extremely important role on intraarticular scar adhesion. After being activated by some cytokines, such as fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- $\beta$ ), the fibroblasts proliferate, synthesize collagen and form intraarticular scar adhesion<sup>6,18–20</sup>.

Camptothecin (CPT) is one of the camptothecin analogues that is isolated from extracts of *Camptotheca acuminata*. CPT is the inhibitor of topoisomerase 1 and can convert topoisomerase 1 into a cellular poison by inhibiting the religation step of the DNA nicking-closing reaction, thereby trap topoisomerase 1 in a covalent complex with DNA. Then the stable topoisomerase1-CTP-DNA complex can result in the cytotoxic lesions<sup>21,22</sup>. CPTs exhibit a broad spectrum of anti-tumor activity against a panel of solid tumors in animal models. Among natural CPTs, 10-hydroxycamptothecin (HCPT) has been shown to be more active and less toxic in treating malignant tumor in vitro and in vivo<sup>23–27</sup>.

Previous studies showed that HCPT could inhibiting fibroblast proliferation and reduce scar formation after laminectomy. Recently, we found that topical applied 0.1 mg/ml HCPT could reduce knee intraarticular adhesion in rabbit models<sup>11,14,28</sup>. However, there is no report about the optimal concentrations of

**Table 2 | The result of One-Way ANOVA as follows**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4299.2	4	1074.8	253.093	0.000
Within Groups	106.167	25	4.247		
Total	4405.367	29			



**Figure 2** | Histological view of the intraarticular adhesion issues in the decorticated areas treated with HCPT (2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml, 0.1 mg/ml) and saline. Note that the loose scar tissues were found in the decorticated areas treated with 1.0 mg/ml group and 2.0 mg/ml HCPT group. Dense scar tissue was found in the decorticated areas treated with saline. The sections were stained with hematoxylin-eosin(200 $\times$ ).

HCPT in preventing knee intraarticular adhesion in rabbit models. The present study were to evaluate the optimal concentrations of topical applied HCPT in inhibiting collagen synthesis and preventing intraarticular scar adhesion.

In the study, we found that all HCPT-treated groups could reduce intraarticular fibrosis by reducing hydroxyproline content and collagen synthesis. Hydroxyproline is the permanent component of the collagen and accounts for 13.4% of the amino acid in collagen tissue<sup>29</sup>. The hydroxyproline content could reflect the formation of collagen and indicate the amount and consistency of scar tissue<sup>30</sup>. However, topical application of 1.0 mg/ml HCPT group and 2.0 mg/ml HCPT group had better effect compared with other groups in reducing intraarticular scar adhesion. Moreover, there is no significantly adverse effect in the signs of wound healing and infections. Considering the preventive effect and potential toxicity of HCPT, we recommend that topical application of 1.0 mg/ml HCPT maybe the optimal concentration in preventing knee intraarticular scar adhesion in rabbit models.

In this study, we only evaluated the effect of different concentrations of HCPT on reducing intraarticular scar adhesion by morphology and histology. However, as an anti-tumor agent, HCPT could inhibit various cells proliferation and affect wound healing. As an scientific and rigorous experiment, we need more experiments to

verify its specific mechanism, safety and potential toxicity in preventing intraarticular scar adhesion before medical application.

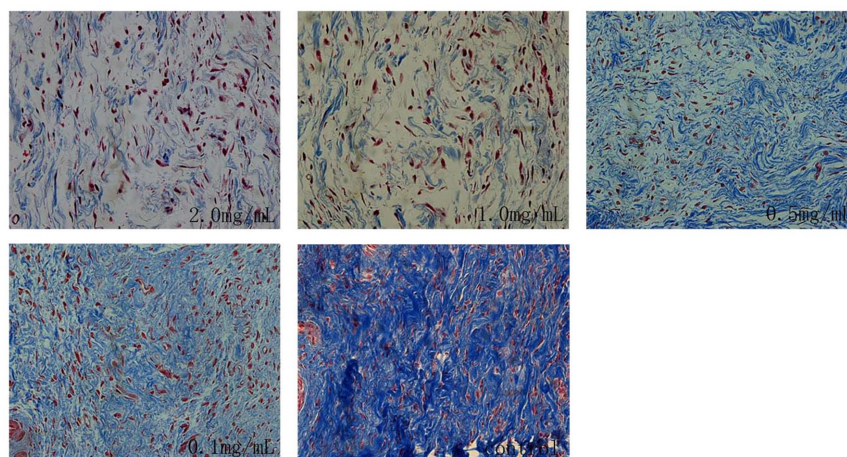
## Methods

**Ethics statement.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocol were performed in accordance with the approved guidelines and were approved by the Ethics Committee of Clinical Medical College of Yangzhou University.

**Animals.** Sixty mature male New Zealand white rabbits, weighing 2.0 to 3.0 kg, were used for this study. They were purchased from the experimental animal center of Yangzhou university, China. The rabbits were randomly and equally divided into five groups: 0.1 mg/ml group; 0.5 mg/ml group; 1.0 mg/ml group; 2.0 mg/ml group or control (saline) group. Before the experiment, the rabbits were acclimated to the condition of laboratory for 1 week.

**Reagents.** HCPT was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animal model.** The animal model of intraarticular adhesion was developed in the knee of New Zealand White rabbits according to previous study<sup>6,15</sup>. After general anesthesia by intravenous administration of 2% pentobarbital (1.5 ml/kg), the femoral condyle of the left femur was exposed through a medial parapatellar approach. The cortical bone on both sides of the condyle about 10 mm  $\times$  10 mm was removed with a dental burr, and the underlying cancellous bone was exposed. The articular cartilage was left intact.



**Figure 3** | The collagen density of intararticular adhesion tissue in HCPT-treated groups and control group. The collagen tissues show blue in the section with Masson's trichrome staining under the light microscope(200 $\times$ ). HCPT could reduce collagen synthesis and fibrosis. The density of collagen tissue in 1.0 and 2.0 mg/ml HCPT-treated groups revealed significant decrease compared with those in 0.5 mg/ml HCPT group, 0.1 mg/ml HCPT group and control group.



After satisfactory hemostasis, the decorticated areas of femoral condyle were covered with cotton pads soaked with various concentrations of HCPT or saline. The surrounding tissues were covered by wet gauzes to avoid getting in touch with the agent. After 10 min, the cotton pads were removed and then the decorticated areas of femoral condyle were irrigated immediately. After the procedure, the articular capsule and skin were closed with silk sutures, and the knee was immobilized in the fully flexed position with a Kirschner wire for 4 weeks. Cefazolin sodium (50 mg/kg) was administered intramuscularly to prevent infection from the postoperative 3 days.

**Macroscopic evaluation.** Four weeks after the surgery, six rabbits were randomly selected from each group and sacrificed with overdose of pentobarbital. The knee was opened through original incision. Gross observation of the intraarticular adhesion was done by three professional pathologists who were blinded to the treatment groups according to the visual scoring system<sup>16</sup>: Grade 1: no adhesions; Grade 2: weak, mild, filmy adhesions that can be easily dissected by minimal manual traction; Grade 3: moderate adhesions that can be dissected by manual traction; and Grade 4: dense and firmly fibrous adhesions that must be surgically removed.

**Biochemical analysis of hydroxyproline content.** After macroscopic evaluation, about 20 mg scar tissue was harvested from the decorticated areas of the knee joint from each rabbit for hydroxyproline content analysis. The hydroxyproline content in scar tissue was determined according to previous method<sup>6,17</sup>. The samples were lyophilized, ground and hydrolyzed with 6 mol/l HCl at 130 °C for 12 h separately. Then they were neutralized with 2.5 N NaOH on the indication of methyl red. 1 ml chloramine T was added to the hydrolyzed samples and hydroxyproline standards of four known concentrations. After incubation for 20 min at room temperature, 1 ml hydroxyproline developer ( $\beta$ -dimethylaminobenzaldehyde solution) was added to the samples and the standards. The absorbance of the solution was measured at 558 nm wavelength, and the levels of hydroxyproline per milligram of scar tissue were calculated using the standard curve constructed by a serial concentrations of commercial hydroxyproline.

**Histological analysis.** Six remaining rabbits in each group were killed by intravenous injection of lethal dose of pentobarbital for histological analysis. The connective tissue involved in fibrotic adhesive scar formation around joints were excised. Then the samples were fixed in 4% paraform 24 hours, and embedded in paraffin. Eight successive transversal sections of four-micrometer were obtained.

Four odd sections of each group were stained with hematoxylin and eosin (H & E) and the intraarticular scar adhesions were evaluated under the light microscope with the magnification  $\times 200$ . Four even sections of each group were stained with Masson's trichrome and the collagen density of intararticular adhesion tissue was evaluated at  $200\times$  magnification. All sections for an individual case were counted blinded to the group assignment.

**Statistical analysis.** All of the statistical analyses were performed using the SPSS software (version 15.0). The results of the data were expressed as mean  $\pm$  standard deviation values. The single factor analysis of variance and q-test were used to calculate the significance in hydroxyproline content. Statistically significant differences were considered when  $p < 0.05$ .

1. Strum, G. M. *et al.* Acute anterior cruciate ligament reconstruction. Analysis of complications. *Clin Orthop Relat Res.* 184–189 (1990).
2. Yercan, H. S. *et al.* Stiffness after total knee arthroplasty: prevalence, management and outcomes. *Knee.* 13, 111–117 (2006).
3. Aglietti, P., Buzzi, R., De Felice, R., Paolini, G. & Zaccherotti, G. Results of surgical treatment of arthrofibrosis after ACL reconstruction. *Knee Surg Sports Traumatol Arthrosc.* 3, 83–88 (1995).
4. Laubenthal, K. N., Smidt, G. L. & Kettelkamp, D. B. A quantitative analysis of knee motion during activities of daily living. *Phys Ther.* 52, 34–43 (1972).
5. Esler, C. N., Lock, K., Harper, W. M. & Gregg, P. J. Manipulation of total knee replacements. Is the flexion gained retained? *J Bone Joint Surg Br.* 81, 27–29 (1999).
6. Fukui, N., Tashiro, T., Hiraoka, H., Oda, H. & Nakamura, K. Adhesion formation can be reduced by the suppression of transforming growth factor-beta1 activity. *J Orthop Res.* 18, 212–219 (2000).
7. Brunelli, G., Longinotti, C., Bertazzo, C., Pavesio, A. & Pressato, D. Adhesion reduction after knee surgery in a rabbit model by Hyaloglide, a hyaluronan derivative gel. *J Orthop Res.* 23, 1377–1382 (2005).
8. Kim, D. H., Gill, T. J. & Millett, P. J. Arthroscopic treatment of the arthrofibrotic knee. *Arthroscopy.* 20, 187–194 (2004).
9. Millett, P. J., Williams, R. J., 3rd & Wickiewicz, T. L. Open debridement and soft tissue release as a salvage procedure for the severely arthrofibrotic knee. *Am J Sports Med.* 27, 552–561 (1999).
10. Babis, G. C., Trousdale, R. T., Pagnano, M. W. & Morrey, B. F. Poor outcomes of isolated tibial insert exchange and arthrolysis for the management of stiffness following total knee arthroplasty. *J Bone Joint Surg Am.* 83-A, 1534–1536 (2001).
11. Sun, Y. *et al.* The effect of 10-hydroxycamptothecin in preventing fibroblast proliferation and epidural scar adhesion after laminectomy in rats. *Eur J Pharmacol.* 593, 44–48 (2008).
12. Tang, W., Zhang, Y., Qian, C., Yuan, Z. & Du, J. Induction and mechanism of apoptosis by hydroxycamptothecin in human Tenon's capsule fibroblasts. *Invest Ophthalmol Vis Sci.* 53, 4874–4880 (2012).

13. Yang, J., Ni, B., Liu, J., Zhu, L. & Zhou, W. Application of liposome-encapsulated hydroxycamptothecin in the prevention of epidural scar formation in New Zealand white rabbits. *Spine J.* 11, 218–223 (2011).
14. Li, X. *et al.* Comparison of the effects of mitomycin C and 10-hydroxycamptothecin on an experimental intraarticular adhesion model in rabbits. *Eur J Pharmacol.* 703, 42–45 (2013).
15. Wang, J. *et al.* A comparative study of the preventive effects of mitomycin C and chitosan on intraarticular adhesion after knee surgery in rabbits. *Cell Biochem Biophys.* 62, 101–105 (2012).
16. Rothkopf, D. M., Webb, S., Szabo, R. M., Gelberman, R. H. & May, J. W., Jr. An experimental model for the study of canine flexor tendon adhesions. *J Hand Surg Am.* 16, 694–700 (1991).
17. Woessner, J. F., Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys.* 93, 440–447 (1961).
18. Fukui, N., Nakajima, K., Tashiro, T., Oda, H. & Nakamura, K. Neutralization of fibroblast growth factor-2 reduces intraarticular adhesions. *Clin Orthop Relat Res.* 250–258 (2001).
19. Fukui, N. *et al.* Suppression of fibrous adhesion by proteoglycan decorin. *J Orthop Res.* 19, 456–462 (2001).
20. Yan, L. *et al.* The effect of mitomycin C in reducing intraarticular adhesion after knee surgery in rabbits. *Eur J Pharmacol.* 643, 1–5 (2010).
21. Urasaki, Y., Takebayashi, Y. & Pommier, Y. Activity of a novel camptothecin analogue, homocamptothecin, in camptothecin-resistant cell lines with topoisomerase I alterations. *Cancer Res.* 60, 6577–6580 (2000).
22. Yin, X. *et al.* Hydroxycamptothecin induces apoptosis of human tenon's capsule fibroblasts by activating the PERK signaling pathway. *Invest Ophthalmol Vis Sci.* 54, 4749–4758 (2013).
23. Zhang, R. *et al.* Preclinical pharmacology of the natural product anticancer agent 10-hydroxycamptothecin, an inhibitor of topoisomerase I. *Cancer Chemother Pharmacol.* 41, 257–267 (1998).
24. Ping, Y. H. *et al.* Anticancer effects of low-dose 10-hydroxycamptothecin in human colon cancer. *Oncol Rep.* 15, 1273–1279 (2006).
25. Hu, W., Zhang, C., Fang, Y. & Lou, C. Anticancer properties of 10-hydroxycamptothecin in a murine melanoma pulmonary metastasis model in vitro and in vivo. *Toxicol In Vitro.* 25, 513–520 (2011).
26. Fu, Y. R., Yi, Z. J., Yan, Y. R. & Qiu, Z. Y. Hydroxycamptothecin-induced apoptosis in hepatoma SMMC-7721 cells and the role of mitochondrial pathway. *Mitochondrion.* 6, 211–217 (2006).
27. Zhang, R. *et al.* Antitumor activity and pharmacokinetics following oral administration of natural product DNA topoisomerase I inhibitors 10-hydroxycamptothecin and camptothecin in SCID mice bearing human breast cancer xenografts. *Int J Oncol.* 10, 1147–1156 (1997).
28. Zhu, L. *et al.* Hydroxycamptothecin liposomes inhibit collagen secretion and induce fibroblast apoptosis in a postlaminectomy rabbit model. *Eur J Orthop Surg Traumatol.* 23, S85–91 (2013).
29. Edwards, C. A. & O'Brien, W. D., Jr. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. *Clin Chim Acta.* 104, 161–167 (1980).
30. Zhang, C. *et al.* An Experimental Novel Study: Angelica sinensis Prevents Epidural Fibrosis in Laminectomy Rats via Downregulation of Hydroxyproline, IL-6, and TGF- $\beta$  1. *Evid Based Complement Alternat Med.* 2013, 291814; doi:10.1155/2013/291814 (2013).

## Acknowledgments

Funding was provided by the National Nature Science Foundation of China (Grants#81271994, 81371971 and 81301550); Jiangsu Province Health Department Foundation (H201250); Nature Science Foundation (BK2011433). The authors would like to express severely appreciation to all workers of Pathology laboratory of Yangzhou University.

## Author contributions

This study was conceived and designed by W.J.C., Y.L.Q., L.Y. and S.Y.; H.J.L., Y.H., X.H.X., C.H. and S.Z.W. performed the experiments. C.J., F.X.M., X.C.Z. and H.J.S. analysed the data. All authors discussed the results. L.Y. and S.Y. wrote the paper, and all other authors commented on the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Liang, Y. *et al.* The optimal concentration of topical hydroxycamptothecin in preventing intraarticular scar adhesion. *Sci. Rep.* 4, 4621; DOI:10.1038/srep04621 (2014).



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. The images in this article are included in the article's Creative Commons license, unless indicated otherwise in the image credit; if the image is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the image. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>