Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 351 (2008) 219–226
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pingyangmycin loaded bovine serum albumin microspheres for chemoembolization therapy—*in vitro* and *in vivo* studies

Changguang Wang [a], Jie Liu [b], Weisan Pan [a], Xinchun Wang [b], Qinghong Gao [c], Shixiang Hou [b,*]

[a] *Department of Pharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 100016, China*
[b] *West China College of Pharmacy, Sichuan University, Chengdu 610041, China*
[c] *West China College of Stomatology, Sichuan University, Chengdu 610041, China*

Received 14 May 2007; received in revised form 13 September 2007; accepted 30 September 2007
Available online 13 October 2007

Abstract

Chemoembolization based on microspheres have been emerged as a novel and promising way for interventional therapy, however, the exact effect and probable mechanism have not been revealed. The purpose of our study was to evaluate the potential of Pingyangmycin loaded bovine serum albumin microspheres (PYM-BSA-MSs) for chemoembolization therapy both *in vitro* and *in vivo*. The effect of PYM-BSA-MSs on cell growth curves and changes of cell morphology and activities measured by MTT assay were carried out in human umbilical vein endothelial ECV-304 cells. The *in vivo* occlusion effect was evaluated in 24 healthy rabbits. Macroscopic examinations and Hematoxylin-Eosin (H-E) staining of cross-section of rabbits' central auricular arteries were employed to observe the apparent and histological changes of arterioles. The results show that the PYM-BSA-MSs could inhibit the proliferation and induce the apoptosis of ECV-304 cells in a time-dependent manner. *In vivo* studies demonstrated that 21 days after artery embolization with the PYM-BSA-MSs, neointimal thickening of arterioles and significant hyperplasia of endothelial cells could be detected, but without completely interruption of blood flow. Compared with plain PYM aqueous solution or BSA-MSs oily suspension, PYM-BSA-MSs showed excellent potential as an alternative to interventional embolization materials.

Keywords: Chemoembolization; Pingyangmycin; Microspheres; ECV-304 cell line; Hematoxylin-Eosin staining

1. Introduction

The incidence of head and neck tumors is nearly 10% of the total incidence of malignant tumors in China (Li, 1993) and most of them belong to "fast flow" vascular malformations. Various methods including sclerosing, embolization, frozen, radionuclide, laser and plastic surgical operation have been applied in the treatment of these tumors (Andrew, 1998; Moon, 1999; Borjesson et al., 2003; Melle et al., 2003; Ibrahim et al., 2005; Isaev et al., 2005). Treatment strategies often depend on the location of the lesion and the size (Lucas et al., 1997) of the tumor, for example, airway lesions are commonly treated by laser ablation (Paiva et al., 1998) and if the lesion is accessible surgically, surgical excision is the gold standard (Low, 2003). However, there are so many challenges for conventional surgery such as high risk, heavy trauma and high tumor relapse rate due to the complex blood supply and anatomic structure in the oral and maxillofacial regions (Wu et al., 2006). And further, the potential of deformation of facial features after surgery also have significant effects on the patients' quality of life (Wang and Zhu, 2004).

Embolizations have been extensively used to occlude vessels in the last few decades (Rafael et al., 2002). Recently, embolization techniques have been employed more broadly with greater precision and convenience (Bendszus et al., 2000; Yamamoto et al., 2003). However, transcatherial arterial embolization (TCAE) was not sufficient because the conventional embolic materials could only occlude arterioles and capillaries, and the lesion can get filled with collateral circulation.

Sclerosants have been found in a wide variety of applications and their earliest use could be dated back to over 100 years for the treatment of varicose veins (Chandler et al., 2000). For

* Corresponding author. Tel.: +86 28 85502809; fax: +86 28 85502809.
E-mail address: chg-wang@hotmail.com (S. Hou).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.09.045

small simple ectasia, a satisfactory effect could be obtained with sclerosants while a longer treatment circle was required for the extensive vascular deformations with low blood flow rate, however, they are incapable in the case of vascular malformations with high blood flow rate.

Chemoembolization, which combines the advantages of therapeutic agents and embolic materials, has become an encouraging therapy for vascular abnormality (George et al., 2001; Liu et al., 2006). However, few reports have been concerned about the combined use of sclerosants and embolic agents in the perfused area. Our purpose of this study was to achieve a microinfarction effect and a maintained therapeutic effect by reduction of blood flow via sclerosis effect and sustained release behavior of the microspheres. This study is a continuation of our previous study and the results were published in this journal (IJP 336 (2007) 361–366). Microspheres with suitable diameter could reduce or even cut off the blood flow and the sclerosant release from microspheres can induce the proliferation and migration of smooth muscle cells and thus thickening of the vessel wall which finally result in the occlusion of the blood vessels.

In the present study, bovine serum albumin (BSA) was chosen as carrier matrix due to its good biocompatibility and little immunogenicity. Pingyangmycin (PYM), which is a new type of cytotoxic glycopeptide antitumor antibiotic and was developed in China in the 1980's (Gao et al., 2007), has been used extensively in the far East for treatment of head and neck tumors with a definite therapeutic effect (Zhao et al., 2004) was selected as a model sclerosant. However, short half-life and lung-toxicity (Li et al., 1995) restrained its further application in clinic. So, in current study, PYM-BSA-MSs were prepared and played dual functions as sclerosant and embolic agent. In a previous study, PYM-gelatin microspheres were employed for embolization therapy (Wu et al., 2006). However, the interaction of microspheres with cell line as well as the *in vivo* fate and therapeutic effect of the PYM loaded vehicles was rarely reported. Thus, in our experiment, MTT assay and cell growth curve were employed to estimate *in vitro* inhibitory effect of PYM-BSA-MSs on ECV304 cell line and central auricular arteries of rabbits was chosen as a model for *in vivo* experiment. These experiments can not only confirm the potential therapeutic efficacy of PYM-BSA-MSs, but also reveal the mechanism of chemoembolization, which might provide some useful references for clinical applications.

2. Materials and methods

2.1. Materials

ECV-304, an immortalized human vascular endothelial cell line, was kindly donated by the Biochemical laboratory, School of Life Science, Sichuan University. PYM was obtained from Taihe Pharmaceutical Co. (Tianjin, China). PYM-BSA-MSs and blank BSA-MSs with an average size of 80 μm were prepared in our laboratory according to the method previously reported by our group (Wang et al., 2007) and sterilized by ^{60}Co radiation. RPMI Medium 1640 was purchased from GIBCO Co. (NY, USA) and fetal calf serum (FBS) was obtained from Hali Bioengineering Co. (Chengdu, China). Sumianxin which consists of Dihydroetorphini, Baodingning, EDTA and Haloperidol was supplied by veterinary institute of Liberation Army supplies University.

2.2. Cell culture

ECV-304 cells were grown in RPMI Medium 1640, supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), in 5% CO_2 at 37 °C in a humidified incubator. Treatments were carried out on 80–90% confluent cells.

2.3. Cell growth curves

Cells were rinsed with Hank's balanced salt solution, trypsinized and planted on a 24-well plate at a density of 2×10^4 cells per well. The plates were replaced with fresh culture medium after 36 h incubation. The cells were cultured in the presence of PYM solutions (37.5 and 150 μg/well), PYM-BSA-MSs (equal to 37.5 and 150 μg PYM/well) and blank BSA-MSs (0.565 mg BSA/well), cells without treatment were employed as control. For each treatment, the medium of four parallel wells was removed; the cells were trypsinizationed and counted after addition of 1 ml of fresh RPMI medium 1640 at predetermined time points (12, 24, 36, 48, 72 h). Cell counts of parallel four wells were made using a light microscope and a hemocytometer.

2.4. MTT assay

Cultured cells were measured at a scheduled time intervals (24, 36, 48, 72 h) using MTT colorimetric assay described previously by Mosmann (Bryant et al., 2000). Cells were cultured in 96-well culture plates at a cell density of 1×10^4 cells/well. At the end of the treatment period in absence or presence of PYM-solution, PYM-BSA-MSs or BSA-MSs, 20 μl of the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/ml) was added to each well. The cells were incubated for further 4 h. Hundred microlitres of DMSO was then added and plates were shaken in a horizontal shaker for 15 min at room temperature. The optical density (OD) was measured at 570 nm wavelength using an ELISA plate reader (DG3022A, Donghua Instruments, Nanjing, China). IC_{50} values which defined as the drug concentrations required to reduce the absorbance by 50% of the control values (Popiolkiewicz et al., 2005) were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel 2003) to the curve formed from the data. The IC_{50} value was obtained from the equation for $y = 50$ (50% value).

2.5. Cell morphology

The alteration of cell morphology of vascular cells after treatment of PYM, BSA-MSs and PYM-BSA-MSs was observed using a light microscope. ECV-304 were plated in 24-well culture plates (2×10^4 cells per well). At con-

fluence, the monolayers were exposed to PYM solutions (150 μg/well), BSA-MSs (0.565 mg/ml BSA/well) or PYM-BSA-MSs (equaled to 0.565 mg/ml BSA, 150 μg/well). Cell culture medium were aspirated and replaced by 0.5 ml of fresh RPMI medium 1640. A control group was also employed in this series of experiment. After 24 h post-treatment, cells were fixed with 10% formaldehyde solution in PBS. Each well was examined with an inverted microscope (TE2000-U, Nikon, Japan) at 200× magnification, a video capture and analysis system was used to acquire three digital images of each group.

2.6. In vivo studies

The experimental animals consisted of 24 healthy Japanese big ear rabbits, half male and half female, weighing 2.0–2.5 kg (Experimental animal center of Sichuan University, Chengdu, China). They were treated as prescribed in the publication 'Guide for the care and the use of the laboratory animals' (NIH Publication No. 92–93, revised 1985) for all animal studies. The animals were housed singly in standard cages, in a controlled breeding room (temperature: 20 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light cycle) for 3 days before experiments and fed with standard laboratory food and water *ad libitum*.

Twenty-four rabbits were divided into four groups using randomized block design (four time durations). The hairs on the auricle lateral of the rabbits were removed by depilatory the day before administration. They were anesthetized *via* intramuscular injection of Sumianxin (0.2 ml/kg) and then placed supine on a board. And the internal branches of the central auricular artery were blocked by bulldog clamp which could prohibit the solution or suspension from entering the inner ear of rabbit. PYM dissolved with physiological saline solutions (5 mg/ml), BSA-MSs suspended with soybean oil (5 mg/ml) and PYM-BSA-MSs suspensions (5 mg/ml) were slowly injected into the proximal part of central auricular arteries (0.26 ml/ear). After administration, the injection site was pressed with gossypium absorbents and unclamped simultaneously. Animal care and treatment were conducted in conformity with the Institutional guidelines of Sichuan University.

2.7. Macroscopic observation

All groups were maintained under normal conditions in the same manner, the changes in color and shape of rabbits' ears after locally administrated of PYM aqueous solution, BSA-MSs oily suspension and PYM-BSA-MSs suspension were visually inspected everyday to assess any change.

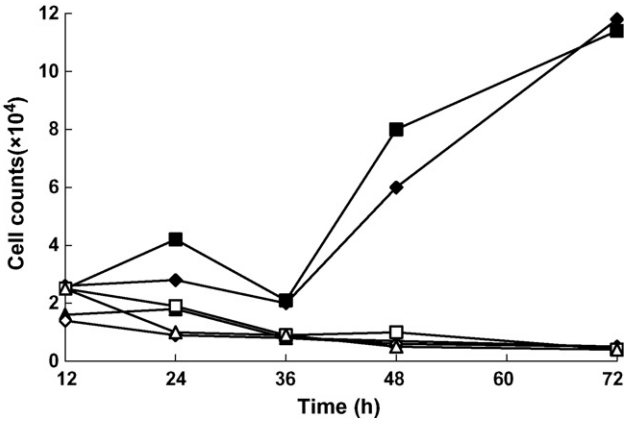

Fig. 1. The growth curve of ECV304 after gives different concentration of PYM ♦ Negative control; ■ Blank BSA-MSs (0.565 mg BSA/well); ▲ PYM-solution (150 μg PYM/well); □ PYM-solution (37.5 μg PYM/well); □ PYM-BSA-MSs (equal to 150 μg PYM/well); □ PYM-BSA-MSs (equal to 37.5 μg PYM/well).

2.8. Histological examination

On days 2, 7, 14 and 21, six animals were sacrificed and both ears were resected from sulcus of posterior auriculae, the central auricular arteries were isolated and adipose tissues were removed, then the arteries were fixed with 10% formalin solution and embedded in paraffin. The sections were stained with Hematoxylin-Eosin (H-E) and examined under a light microscope. The fate of the occluding material was also investigated by the observation of histological sections.

3. Results

3.1. Cytotoxic effects of PYM-BSA-MSs on ECV304 cells

PYM has been reported to reduce the rate or even block the synthesis of DNA, which is supposed to induce the death of cells *in vitro* assays (Umezawa et al., 1966, 1972; Kuo et al., 1998). The cell growth curves of ECV-304 cell after treatment of PYM solution, PYM-BSA-MSs and BSA-MSs were shown in Fig. 1. It indicated that cell growth was significantly inhibited with a time-dependent pattern when exposed to PYM solutions and PYM-BSA-MSs. However, the relationship between concentration and inhibitory effect was unclear. The inhibitory effect of PYM-BSA-MSs was milder than PYM-solution, which might attribute to the slowly release of PYM and the promotion effect of BSA.

Cytotoxicity of PYM-solution and PYM-BSA-MSs measured by MTT assay was expressed as IC50 values determined 24, 36, 48 h after exposure to drugs (Table 1). The result was in

Table 1
Cytotoxicity of PYM-solutions and PYM-BSA-MSs against ECV-304 cells

Formulation[a]	IC50 (μg/ml)			
	24 h	36 h	48 h	72 h
PYM	707.95 ± 122.36	138.04 ± 10.19	34.36 ± 2.76	8.51 ± 2.40
PYM-BSA-MPs	1667.25 ± 102.09	167.49 ± 17.83	46.99 ± 3.01	4.62 ± 0.29

[a] Cytotoxicity determined by MTT assay. Data obtained after treating ECV-304 cells with varying concentrations of PYM-solutions and PYM-BSA-MSs (5–60 μg) for 24, 36, 48, 72 h. Data are means ± S.D. of three replicates.

accordance with the cell count assay, both formulations show a significant decrease of IC50 against the treating time. The result of MTT assay also revealed that degraded BSA played a positive role on proliferation of cells (Fig. 1).

3.2. Morphological changes of endothelial cells

The morphology of ECV-304 cells after exposure to PYM-solution, PYM-BSA-MSs and BSA-MSs were shown in Fig. 2. Compared with negative control cell, no conspicuous changes in morphology and growth state in vascular cells by 24 h could be observed by BSA-MSs without PYM. The cells were tightly tiled like cobblestones. In contrast, a definite morphological change in these cells was caused by either PYM-solution or PYM-BSA-MSs after 24 h of cultivation. The cells were swelling and deformated, however, cell division circle still went on.

3.3. Macroscopic observations

The macroscopic appearance of the rete in the embolized side was different among animals received different treatments. The plexiform nature of the rete was no longer visible after receiving PYM-BSA-MSs, while in the other groups, no obvious thrombs and arterial occlusions could be observed. The time-course study of embolization demonstrated that the thrombogenesis occurred soon (within 1 day) after administration of PYM-BSA-MSs due to large size of MSs which exceeded the diameter of arterioles. Neointimal thickening of arterioles appeared in rabbits embolized with PYM-BSA-MSs during 7–14 days, but there still have blood flow without completely interruption (Fig. 3). On the 21st day, more layers appeared in the wall of central auricular artery and occlusion of vessels occurred simultaneously, however, no tissue necrosis could be observed during the whole course.

3.4. Histologic change of arteriole samples

Fig. 4 shows representative micrographs of cross-sectioned and H-E stained tissues from arteries exposed to one of three experimental conditions. In the arteries received PYM-aqueous solutions, a single layer of endothelial cells protruded from the inner surface of the vessel, and the artery was surrounded by homogeneous internal elastic laminas with several layers of circular smooth muscles in the medial layer. Vascular endothelial cells were slightly swelling 2 days after administration (Fig. 4A), however, no further morphological change was observed during 7–21 days (Fig. 4B–D).

The result of animals exposed to BSA-MSs oily suspensions showed that endothelial cells were also located along the intima, light edema was found 2 days after injection (Fig. 4E). Some of the endothelial cells were exfoliated from the arterial wall on the 7th day (Fig. 4F) but no significant morphological change was

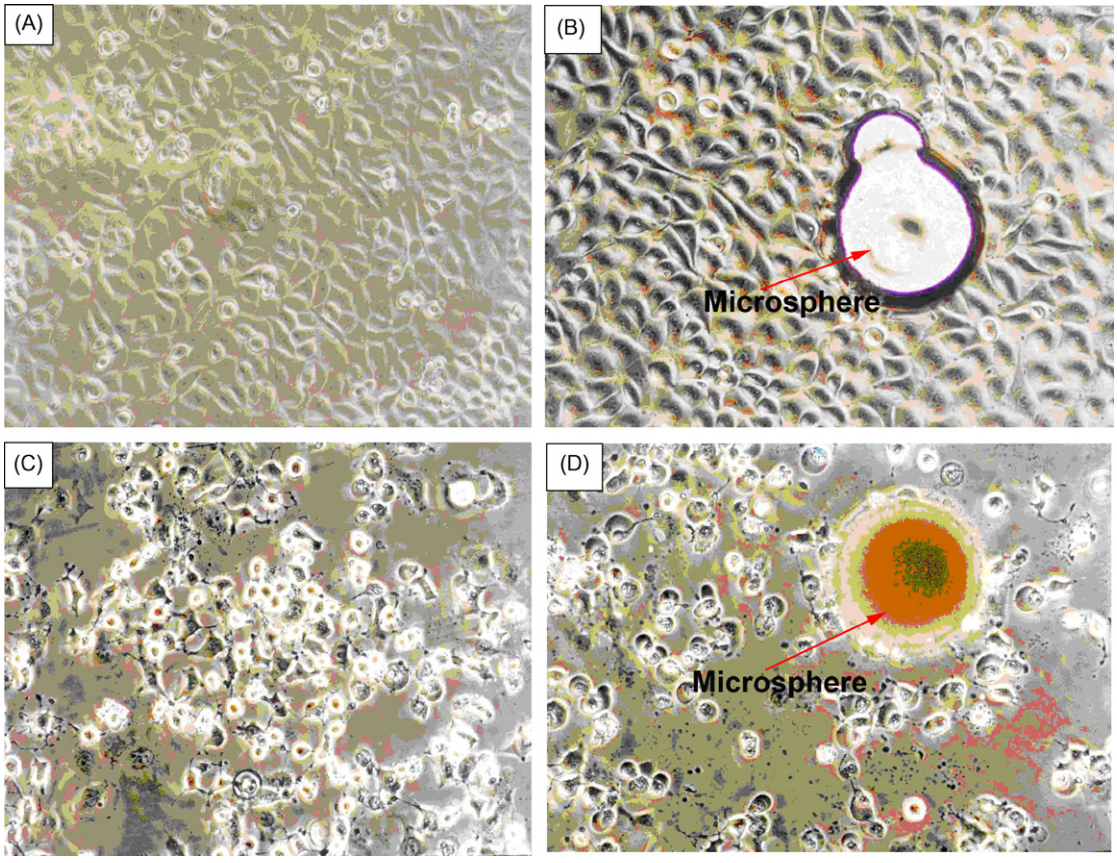

Fig. 2. Cell morphological change of ECV-304 cells 24 h after being exposed to different conditions, original magnification by 200 times:(A). Blank cells;(B). Cells treated with BSA-MSs; (C). Cells treated with PYM; (D). Cells treated with PYM-BSA-MSs.

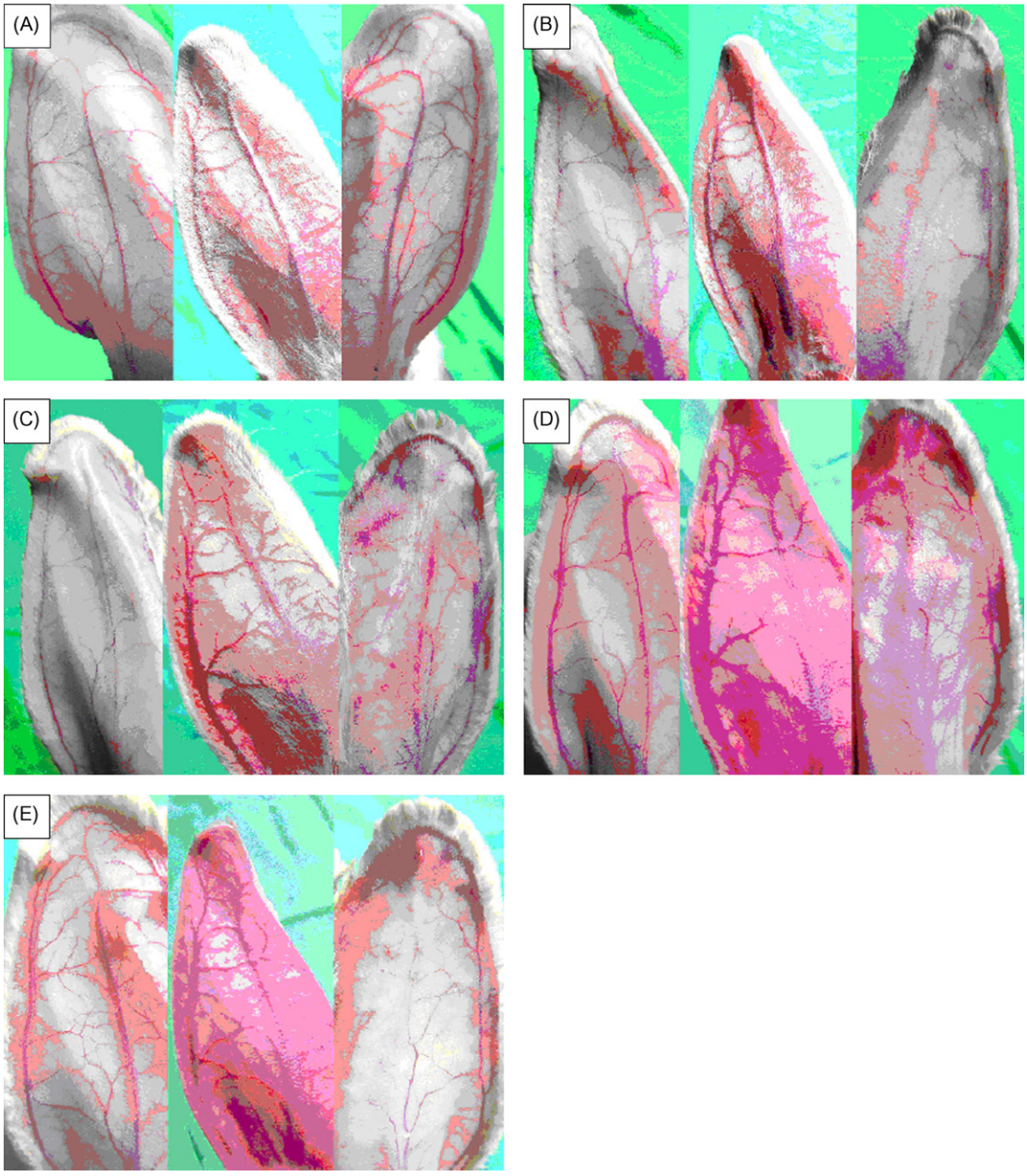

Fig. 3. Macroscopic changes of rete systems of rabbits' ears embolization with different formulations before and after 2, 7, 14 and 21 days (A–E). Left: PYM aqueous solution; middle: BSA-MSs soybean oily suspension; right: PYM-BSA-MSs soybean oily suspension.

observed on 14th day (Fig. 4G). On the 21st day, after treatment, cell proliferation was found on the vessel wall of central artery (Fig. 4H).

In the PYM-BSA-MSs-treated arteries, on the contrary, embolization by MSs can be easily seen in the distal arterioles 2 days after administration (Fig. 4I and M), slight swelling also existed on the 7th day (Fig. 4J). The MSs exhibited as integrities with spherical morphology (Fig. 4N), which could be attributed to the matrix-release pattern of PYM-BSA-MSs (data were published in our previous reports). On the 14th day, in some regions of the arterioles, proliferation of endothelial cells could be observed, which cause the thickening of vessel wall and narrowing of arterial lumen (Fig. 4K). MSs began to be erosed and deformed in the biological fluids which suggested the release and absorption of PYM. Significant hyperplasia of endothelial cells could be detected on the 21st days, and some separated nuclei were seen in the division phase. Thickening of arterial walls with a narrowing of vessel lumina and occlusion of distal arterial lumen were observed (Fig. 4L). The microsphere was seriously deformated and the matrix presented a nearly collapsed feature (Fig. 4P).

4. Discussion

Recently, intratumor injection of PYM offers a simple, effective and safe approach for the therapy of oral and maxillofacial

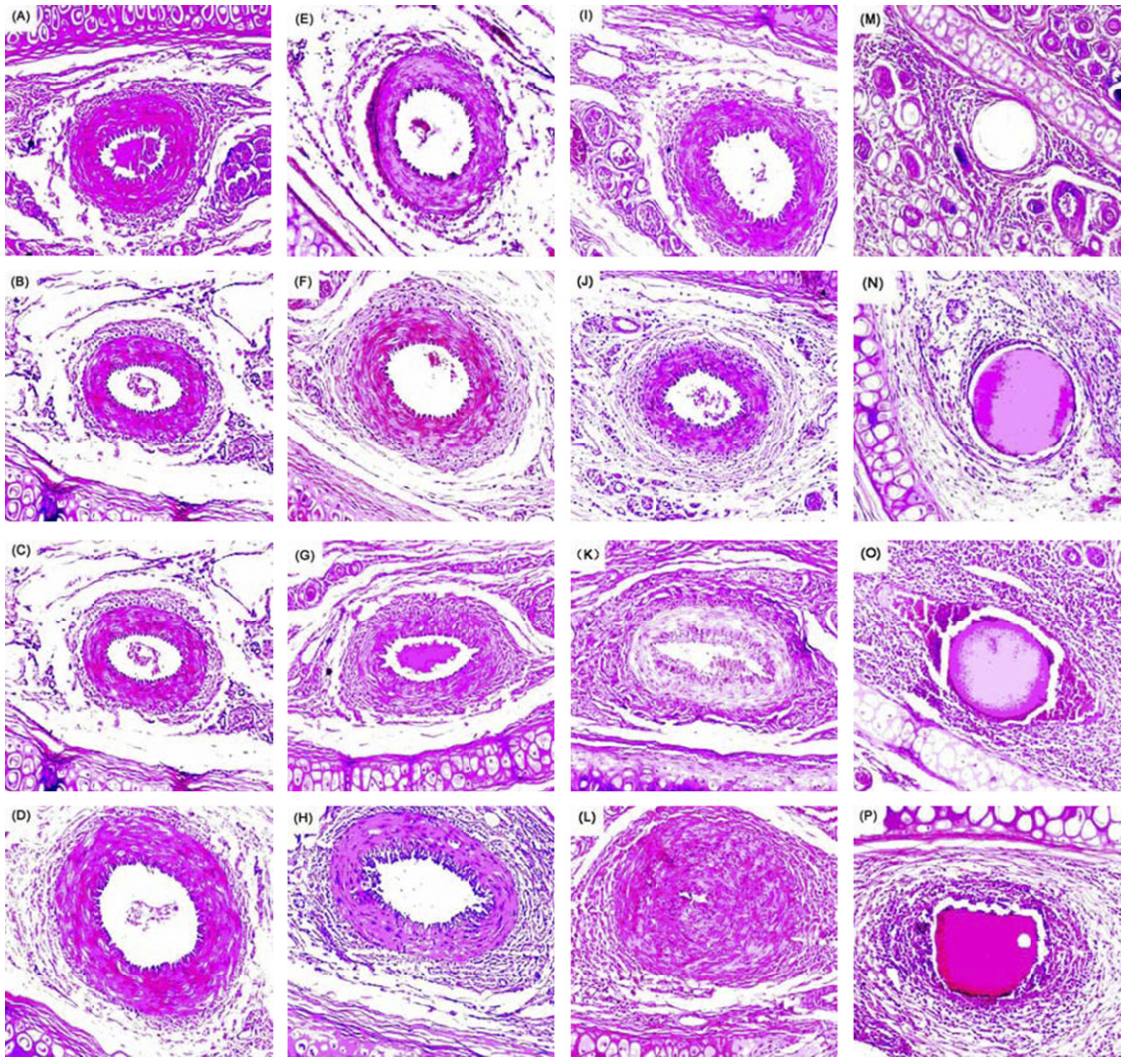

Fig. 4. Histological section of arterioles after injections with 80 μm microspheres and the morphological changes of microspheres (original magnification by 100 times):(A–D) Rabbits received PYM aqueous solutions after 2,7,14 and 21 days; (E–H) Rabbits received BSA-MSs soybean oily suspensions after 2,7,14 and 21 days;(I–L) Rabbits received PYM-BSA-MSs after 2,7,14 and 21 days; (M–P) The morphology of PYM-BSA-MSs 2, 7,14 and 21 days after administration.

tumors (Zheng et al., 2003). However, for those expanded sponginess vascular malformations, longer treatment circles are required because of short residence time of PYM in tumor site and which result in the poor therapeutic effect (Hua et al., 2004; Zheng et al., 2005).

Transcatheter arterial embolization (TCAE) offers a new approach for the therapy of oral and maxillofacial tumors. TCAE has many advantages, including good targeting, high therapeutic effects, and light trauma (Chen, 1998). For TCAE to be successful, three factors must be addressed (Coldwell et al., 1994): embolic agent selection, clinical application, and technical skill. The major embolic agents used include stainless steel coils, polyvinyl alcohol foam, as well as absorbable gelatin pledgets and powder, selection of which should be determined according to the clinical application. In the past, embolization and chemotherapy have been combined to treat malignant tumor, mostly incorporating chemotherapeutics perfused or injected into the tumor. Nowadays, more and more attention has been focused on the study of embolic agents loaded with chemotherapeutic medicine (Bastian et al., 1998).

The combined therapy of PYM and BSA microspheres produced excellent scleritic effects when compared with singlely applied PYM because of the blockage of blood flow. PYM can cause a marked tissue irritation and/or thrombosis with subsequent local inflammation and tissue necrosis. ECV304 originating from human umbilical vein endothelial cells was widely used as a human endothelial cell model for cytotocixity evaluation. The results of MTT assay and cell growth curves showed slight difference. MTT assay is a laboratory test and a standard colorimetric assay for measuring cellular proliferation, it can reflect the number of live cells, and cell count reflects the total number of cells. IC50 decreased significantly with the exposure time, it agreed quite well with some previous reports (Chen et al., 1999; Guo and Wu, 2000; Kong et al., 2003). The IC50 values of PYM are different with various cell lines, for colon carcinoma 26 cells and KB cells, they were 32.46 and 3.94 μg/ml,

respectively (Liu et al., 2000), and it was 8.51 ± 2.40 μg/ml for ECV304 cell line in our study. The blank MSs showed no toxicity effect to cells, whereas, the uncrosslinked and degraded BSA can proliferate the growth of them due to the absorption and metabolism of the cells.

Significant morphology changes in ECV-304 cell can be observed in groups either treated with PYM-BSA-MSs or PYM. Typical injures such as cell edema, large cavities, chromatin margination and nuclear fragmentation occur after 24 h of cultivation. At last, the apoptotic body was formed, which was reported elsewhere (Nelson et al., 1996).

The three essential components of intimal hyperplasia are smooth muscle cell (SMC) migration, proliferation and extracellular matrix production (Powell et al., 1996a,b). In normal human vasculature, the intima is almost completely devoid of SMCs and endothelial cells (ECs) secret a heparinoid material which can prevent the generation of SMCs. Thus, migration of SMC from the vessel into the subintimal space is a critical step in the initiation of the occlusion process. Endothelial denudation has been suggested to contribute to the SMCs proliferative response to vessel injury (Jing, 1997). The inhibitory effect of ECs on proliferation of SMCs would be lost if they are damaged due to platelet adhesion reaction and leukocytic infiltrate (Powell et al., 1996a,b). In present study, ECs were injured by PYM, and they were replaced by the SMCs which went through migration, differentiation and proliferation course and resulted in the intimal hyperplasia.

PYM-BSA-MSs played dual function during the *in vivo* occlusion course after being injected into the vessel. On the one hand, the PYM absorbed on the surface of MSs would act as a sclerosant as soon as possible and this effect could be maintained when oily medium was applied, on the other hand, thrombosis would began after the embolization of peripheral arterioles and the intimal hyperplasia of arterioles and venules on the distal end could be caused by the sustained release of PYM from the MSs. No tissue necrosis and conspicuous swelling were observed, which was quite different from the occlusion mechanism of PYM-lipiodoi emulsion. Serious and irreversible damages of granulocytes, erythrocytes and endothelial cells can be stimulated by PYM-lipiodoi emulsion, which result in the exposure of endothelial collagens and activation of endogenous thromboxane system. The formed mixed thrombus will block the vessel walls with a manner similar to the traditional sclerosing agents such as sodium morrhuate.

Compared with our previous study, the result is accordance with the pharmacokinetics results. The PYM-BSA-MSs could prolong the MRT of the PYM, and which appeared as the pharmacodynamics results shows in this research. The microspheres is degraded by the enzyme in the blood stream and with the self-erosion effect the PYM were released from the entity smoothly, which prolong the local concentration of the drug and continued the embolic effects.

5. Conclusions

The results presented here indicated that it is possible to chemoembolize central auricular arteries in rabbits with PYM-BSA-MSs and they can suppress the growth of the tumors in a time-dependent fashion. The mechanism of occlusion was *via* the sclerosant and embolization effect, as well as the sustained release of PYM from carriers. In conclusion, compared with other embolization materials, the ready-for-use PYM-BSA-MSs are an excellent alternative to superselective embolization materials for end arteries and treatment of head and neck tumors.

Acknowledgements

We are thankful for the financial support of the Project Funds from Science and Research Council of Zhejiang Province (022219) and the Tackle Key Problems in Science and Technology of Sichuan Province (03SG022-101).

References

Andrew, A.G., 1998. History of cryosurgery. Semin. Surg. Oncol. 14, 99–109.

Bastian, P., Bartkowski, R., Kohler, H., Kissel, T., 1998. Chemo-embolization of experimental liver metastases. Part I: Distribution of biodegradable microspheres of different sizes in an animal model for the locoregional therapy. Eur. J. Pharm. Biopharm. 46, 243–254.

Bendszus, M., Klein, R., Burger, R., Warmuth, M.M., Hofmann, E., Solymosi, L., 2000. Efficacy of trisacryl gelatin microspheres versus polyvinyl alcohol particles in the preoperative embolization of meningiomas. AJNR Am. J. Neuroradiol. 21, 255–261.

Borjesson, P.K., Postema, E.J., Roos, J.C., 2003. Phase I therapy study with 186Re-labeled humanized monoclonal antibody BIWA 4 (Bivatuzumab) in patients with head and neck squamous cell carcinoma. Clin. Cancer Res. 9, 3961–3972.

Bryant, S.J., Nuttleman, C.R., Anseth, K.S., 2000. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts *in vitro*. J. Biomat. Sci-Polym. Ed. 11, 439–537.

Chandler, J.G., Pichot, O., Sessa, C., Schuller-Petrovic, S., Kabnick, L.S., Bergan, J.J., 2000. Treatment of primary venous insufficiency by endovenous saphenous vein obliteration. J. Vasc. Surg. 34, 201–214.

Chen, C.X., 1998. Practical Radiology. People's Health Press, China, Beijing, pp. 1058–1060.

Chen, G., Huang, Q.J., Wang, Y.H., 1999. The *in vitro* inhibitory effect of pingyangmycin on endothelial cells of vascular tumors. Beijing J. Stomatol. 7, 29–30.

Coldwell, D.M., Stokes, K.R., Yakes, W.F., 1994. Embolotherapy: agents, applications, and techniques. Radiographics 14, 623–643.

Gao, Z.B., Ding, P.T., Zhang, L., Shi, J., Yuan, S.Q., Wei, J., Chen, D.W., 2007. Study of a Pingyangmycin delivery system: Zein/Zein-SAIB in situ gels. Int. J. Pharm. 328, 57–64.

George, P., Browman, D., Ian, H., Robert, J.M., Nancy, B., Lisa, Z.H., 2001. Choosing a concomitant chemotherapy and radiotherapy regimen for squamous cell head and neck cancer: a systematic review of the published literature with subgroup analysis. Head Neck 23, 579–589.

Guo, J., Wu, H.J., 2000. Changes of cultured human vein endothelial cells (HVEC) in the presence of therapeutic agents of cavernous hemangioma. West China J. Stomatol. 18, 310–313.

Hua, C.G., Wen, Y.M., Fu, F.H., 2004. The distribution and degradation of albumin microspheres injected into jugular external artery. J. Pract. Stomatol. 20, 100–103.

Ibrahim, Y., Can, C., Yagmur, A., Cenk, C., 2005. Sclerotherapy to a large cervicofacial vascular malformation: a case report with 24 years' follow-up. Head Neck 27, 639–643.

Isaev, P.A., Medvedev, V.S., Pasov, V.V., Semin, D.I., Plichko, V.I., Derbugov, D.N., 2005. Immediate and long-term results of plastic reconstruction of soft tissues of the head and neck in areas previously treated with surgery for tumor and radiation injury. Vopr. Onkol. 51, 662–666.

Jing, Z.P., 1997. The research on the hyperplasy of intima. Chinese J. Exp. Surg. 14, 259–260.

Kong, W.D., Li, Y.H., Zeng, Q.L., Chen, Y., He, X.F., 2003. Effect of pingyangmycin on the growth and cell cycle of human vascular endothelial cells. J. First Mil. Med. Univ. 23, 830–834.

Kuo, W.T., Yu, C.C., Lin, S.C., Ming, Y.C., 1998. Cytotoxic effect of pingyangmycin on cultured KB cells. Oral Oncol. 34, 219–223.

Li, H.S., Wei, S.L., Lu, W., 1995. Studies on gelatin microspheres-in-oil emulsion of pingyangmycin. Acta Pharmacol. Sin. 30, 390–394.

Li, S.L., 1993. Oncology of Head and Neck. Tianjin Technology Press, China, pp. 126–128.

Liu, X.H., Paul, W.H.S., Li, Q., Chan, L.W., 2006. Novel polymeric microspheres containing norcantharidin for chemoembolization. J. Control Release 116, 35–41.

Liu, X.Y., Liu, X.J., Li, Y., Wang, W.G., Zhen, Y.S., 2000. Antitumor effects of pingyangmycin conjugated with Fab' fragment of monoclonal antibody. Acta Pharm. Sinica. 35, 649–653.

Low, D.W., 2003. Management of adult facial vascular anomalies. Facial Plast. Surg. 19, 113–130.

Lucas, C.P., Zabramski, J.M., Spetzler, R.F., Jacobowitz, R., 1997. Treatment for intracranial dural arteriovenous malformations: a meta-analysis from the English language literature. Neurosur. 40, 1119–1132.

Melle, C., Ernst, G., Schimmel, B., Bleul, A., Koscielny, S., Wiesner, A., Bogumil, R., Möller, U., Osterloh, D., Halbhuber, K.J., Eggeling, F.V., 2003. Biomarker discovery and identification in laser microdissected head and neck squamous cell carcinoma with ProteinChip® technology, two-dimensional gel electrophoresis, tandem mass spectrometry, and immunohistochemistry. Mol. Cell Proteomic. 2, 443–452.

Moon, H.H., 1999. Caraniofacial arteriovenous malformation preoperative embolization with direct puncture and injection of *N*-butyl cyanoaerylate. Radiol. 211, 661–667.

Nelson, P.R., Yamamura, S., Kent, K.C., 1996. Extracellular matrix proteins are potent agonists of human smooth muscle cell migration. Vasc. Surg. 24, 25–33.

Paiva, M.B., Blackwell, K.E., Saxton, R.E., Calcaterra, T.C., Ward, P.H., Soudant, J., Castro, D.J., 1998. Palliative laser therapy for recurrent head and neck cancer: a phase II clinical study. Laryngoscope 108, 1277–1283.

Popiolkiewicz, J., Polkowski, K., Skierski, J., Mazurek, A., 2005. *In vitro* toxicity evaluation in the development of new anticancer drugs-genistein glycosides. Cancer Lett. 229, 67–75.

Powell, R.J., Carruth, J.A., Basson, M.D., Bloodgood, R., Sumpio, B.E., 1996a. Matrix-specific effect of endothelial control of smooth muscle cell migration. J. Vasc. Surg. 24, 51–57.

Powell, R.J., Cronenwett, J.L., Fillinger, M.F., Wagner, R.J., Sampson, L.N., 1996b. Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. Ann. Vasc. Surg. 10, 4–10.

Rafael, M.N., Jacob, B., Marcelo, D., Micha, P., Dov, L., 2002. Selective embolization_ for head and neck—vasculopathies. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 93, 127–131.

Umezawa, H., Maeda, K., Takeuchi, T., Okami, Y., 1966. New antibiotics, bleomycin A and B. J. Antibiot. 19, 200–209.

Umezawa, H., Takeuchi, T., Hori, S., Sawa, T., Ishizuka, M., 1972. Studies on the mechanism of antitumor effect of bleomycin on squamous cell carcinoma. J. Antibiot. 25, 409–420.

Wang, C.G., Jie, L., Qing, H.G., Yue, Q.B., Liang, C.G., Xin, C.W., Shi, X.H., 2007. Preparation and characterization of Pingyangmycin-loaded bovine serum albumin microspheres for embolization therapy. Int. J. Pharm. 336, 361–366.

Wang, X.L., Zhu, S.R., 2004. The therapeutic study on children with oral and maxillofacial vascular tumor. J. Oral Sci. Res. 20, 3–5.

Wu, H., Zhang, Z.X., Wu, D.C., Yu, K.T., Li, X.Y., 2006. Preparation and drug release characteristics of Pingyangmycin gelatin microspheres for embolization therapy. J. Biomed. Mater. Res. B 78, 56–62.

Yamamoto, T., Hayakawa, K., Tabata, Y., Shimizu, Y., Ikada, Y., 2003. Transcatheter arterial embolization using poly-L-lactic acid microspheres. Radiat. Med. 21, 150–154.

Zhao, J.H., Zhang, W.F., Zhao, Y.F., 2004. Sclerotherapy of oral and facial venous malformations with use of pingyangmycin and/or sodium morrhuate. Int. J. Oral Maxill. Surg. 33, 463–466.

Zheng, G.J., Gao, Q.H., Zhou, L., Wang, X.Y., Wen, Y.M., Wang, C.M., 2005. The effect of Pingyangmycin-Album in microsphere on the growth of vascular endothelial cells. J. Pract. Stomatol. 21, 248–251.

Zheng, J.W., Chen, C.J., Zhang, Z.Y., 2003. Intralesional injection of Pingyangmycin for hemangiomas and vascular malformations in oral and maxillofacial region: a systematic review of the Chinese literature. China J. Oral Maxill. Surg. 1, 102–105.