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Cellular pharmacokinetics and pharmacodynamics of dipyridamole in vascular smooth muscle cells

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Abbreviations:
cAMP, cyclic adenosine monophosphate
cGMP, cyclic guanosine monophosphate
EC_{50}, 50% effective concentration
LC_{50}, 50% lethal concentration
LDH, lactate dehydrogenase
MTT, methylthiazoletetrazolium
PDE, phosphodiesterase
PTFE, polytetrafluoroethylene
SMC, smooth muscle cell

ABSTRACT

Hemodialysis arteriovenous grafts are often plagued by stenosis at the vein-graft anastomosis, which is due to the proliferation of venous smooth muscle cells (SMCs). Perivascular delivery of dipyridamole, a potent antiproliferative agent, has been proposed for the prevention of graft stenosis. In order to develop an optimal delivery system for dipyridamole, we examined its pharmacokinetics and pharmacodynamics in human and porcine venous and arterial SMCs *in vitro*. SMCs were incubated with dipyridamole for various durations, and visualized for the uptake and release by fluorescence microscopy, which were further quantified by fluorospectrometry. The antiproliferative effect of dipyridamole was examined by cell counting or the methylthiazoletetrazolium (MTT) dye-reduction assay. Cytotoxicity was examined by the lactate dehydrogenase (LDH)-release assay. The kinetics of dipyridamole transport through the cell membrane was compatible with a passive diffusion mechanism. Dipyridamole inhibited SMC proliferation in a dose-dependent manner and was more effective in venous than arterial cells in both species. The inhibition was completely reversible at 15 μg/ml upon drug removal from the medium. At 25 μg/ml, however, the effect was partially irreversible, which might be attributed to the cytotoxicity of dipyridamole. These data support the need for sustained delivery of dipyridamole to achieve the long-term inhibition of SMC proliferation in the prevention of stenosis since SMCs are continuously stimulated at the anastomosis of hemodialysis arteriovenous grafts.

1. Introduction

Chronic hemodialysis treatment for end-stage renal disease requires routine access to blood vessels that provides high blood flow rates. Arteriovenous polytetrafluoroethylene (PTFE) grafts are the most common form of hemodialysis vascular access in the United States. Unfortunately, the 1-year and 2-year primary patency rates of PTFE grafts are only around 50%

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and 25%, respectively, with the major cause of failure being stenosis followed by thrombosis [1]. The pathology of the stenosis is usually neointimal hyperplasia, which primarily results from the proliferation and migration of vascular smooth muscle cells (SMCs) [2]. Inhibition of SMC proliferation may therefore be a logical strategy to prevent the graft stenosis.

Current strategies to prevent the graft stenosis include drug administration, radiation and gene delivery. Among these strategies drug administration would be relatively simple and easy. Drugs can be applied systemically using oral administration or injection, and locally using the drug-coated graft [3], drug-eluting stent [4], or perivascular drug delivery system [5]. A large number of drugs have been shown to produce antiproliferative effects on various types of vascular SMCs, including dipyridamole [6–9], heparin [10], rapamycin [11], paclitaxel [12], tranilast [13], angiotensin-converting enzyme inhibitors such as perindopril [14], and calcium channel blockers such as nifedipine, verapamil and diltiazem [15].

Dipyridamole, which is well known as an antiplatelet drug, has also been demonstrated to inhibit human and rabbit vascular SMC proliferation *in vitro* [6–9]. The molecular basis for its antiproliferative activity has not been completely elucidated. An important molecular effect of dipyridamole is the inhibition of cyclic nucleotide phosphodiesterase (PDE) activities in platelets and other cells [16,17], however, its specific mechanism of action is still unknown in vascular SMCs. PDEs are responsible for the hydrolysis of intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The inhibition of PDE activities by dipyridamole may result in the accumulation of cAMP and cGMP, thus inhibiting SMC proliferation [18–20]. Systemic administration of dipyridamole, however, has been found to be ineffective in preventing the development of neointimal hyperplasia following interposition venous grafts to the femoral artery in the dog [21] and interposition PTFE grafts in the carotid artery in the goat [22]. In a small prospective randomized study, chronic oral dipyridamole decreased the occlusion rate of PTFE grafts in hemodialysis patients, however, neointimal formation was not determined [23]. In contrast, continuous infusion of dipyridamole into the adventitia for more than 2 weeks was successful in partially inhibiting intimal thickening after balloon injury in the carotid or femoral artery of the rabbit [6]. This latter study suggests that the continuous local delivery of dipyridamole may be efficacious.

Local drug delivery is beneficial to deliver high dosage of drugs in the target area and minimize systemic side effects. Sustained drug release using local drug delivery adds benefits for continuous drug therapy without the need for daily dose administration. In order to optimize the delivery for dipyridamole to achieve continuous inhibition of SMC growth, it is essential to understand its cellular pharmacokinetics and pharmacodynamics in response to various durations of drug exposure. In this study, we performed a detailed *in vitro* transcellular kinetic and pharmacodynamic examination of dipyridamole, in order to facilitate the design of an optimal drug delivery system for the prevention of hemodialysis arteriovenous graft stenosis.

2. Material and methods

2.1. Materials

Two individual cell lines of human saphenous-vein and aortic SMCs, and smooth muscle growth medium-2 bullet kit were purchased from Clonetics (Walkersville, MD). Dipyridamole, fluorescein isothiocyanate (FITC)-conjugated anti-human smooth muscle actin and lactate dehydrogenase (LDH) assay kit (substrate, cofactor, dye and lysis solution) were purchased from Sigma–Aldrich (St. Louis, MO). RPMI medium 1640 was purchased from Invitrogen (Carlsbad, CA). Methylthiazoletetrazolium (MTT) was purchased from Calbiochem (La Jolla, CA). ReGel® was obtained from MacroMed Inc. (Sandy, UT).

2.2. Cell culture

Porcine venous and arterial SMCs were isolated from normal femoral veins and arteries of two Yorkshire cross domestic pigs. Briefly, the femoral vein or artery (5-cm length) was minced into pieces of approximately 1 mm^3 in size. Endothelial cells were detached from the segments by incubating with collagenase A (5 mg/ml) at 37 °C for 10 min. The remaining SMCs were cultured in the culture medium at 37 °C in a humidified 5% CO_2 incubator. The culture medium contains a smooth muscle growth medium supplemented with 10% fetal calf serum, 5 μg/ml insulin, 0.5 ng/ml human recombinant epidermal growth factor (EGF), 2 ng/ml human recombinant fibroblast growth factor (FGF), 50 μg/ml gentamicin and 50 ng/ml amphotericin-B. SMCs were identified by their typical elongated swirling and overlapping morphology under the light microscope, positive staining with FITC-conjugated anti-smooth muscle actin. Cells from passages 3 to 6 were used in the following experiments.

Human venous and arterial SMCs were cultured in the culture medium at 37 °C in a humidified 5% CO_2 incubator. Cells from passages 3 to 7 were used in the following experiments. SMCs were seeded in various densities based on the consideration of incubation duration and assay property.

2.3. Uptake and release

Human venous SMCs were seeded at a density of 1×10^5 cells per well in a 12-well tissue culture plate and cultured for 24 h. Dipyridamole was dissolved in a vehicle of 0.5% (v/v) ethanol in culture medium. For drug uptake measurement, cells were incubated with 5, 15 or 25 μg/ml of dipyridamole at 37 °C for incremental durations. For drug release measurement, cells were first incubated with 5, 15 or 25 μg/ml of dipyridamole at 37 °C for 4 h to allow drug uptake and then incubated in a fresh drug-free medium for incremental durations. At the end of incubation, the cells were washed three times with PBS in the plate. The cell-associated dipyridamole was visualized using fluorescence microscopy (Olympus 1×70 inverted system microscope, Scientific Instrument Company, Aurora, CO). In an alternative experiment, after cells were washed with PBS, intracellular dipyridamole was extracted in a lysis solution (from the LDH assay kit), and quantified by fluorospectrometry (F-4010 spectrophotometer, Hitachi Ltd., Japan) with excita-

tion at 412 nm and emission at 495 nm, using dipyridamole standards at 0–500 ng/ml. The measurement was performed in an ethanol/water medium (15%, v/v) at pH 10.0, provided by adding an ammonium chloride/ammonia buffer solution [24]. Dipyridamole concentration in the remaining medium was also measured to determine the total drug amount in each experiment. Using the mass balance, the recovery rate of intracellular dipyridamole was determined to be 98.7 ± 2.0%.

2.4. Temperature-dependent cellular uptake

Human venous SMCs were seeded at a density of 1×10^5 cells per well in a 12-well plate, cultured for 24 h, and incubated with 5, 15 or 25 μg/ml of dipyridamole at 4, 15, 25 or 37 °C for 4 h. Cellular uptake of dipyridamole was determined using cell lysis and fluorescence measurement.

2.5. Proliferation assay

Human and porcine venous and arterial SMCs were seeded at a density of 2.5×10^4 cells per well in a 12-well plate and cultured for 24 h. To study the effect of intact dipyridamole on SMC proliferation, culture medium was replaced with a fresh medium containing various concentrations of dipyridamole (1–200 μg/ml). The vehicle of 0.5% ethanol in culture medium served as a control. The cells were cultured for 72 h and the proliferation was assessed by cell counting using a hemocytometer after trypsin digestion.

We also tested the effect of the released dipyridamole from a sustained delivery system we have developed previously, by incorporating the drug into poly(lactide-*co*-glycolide) microspheres and combined the products with an injectable and sustained-delivery polymer, ReGel® [25]. A separate *in vitro* release experiment was performed by incubating the mixture of microspheres (containing 4 mg of dipyridamole) and 0.4 ml of ReGel, 0.4 ml of ReGel alone, or drug-free microspheres alone (with same amount to drug-incorporated microspheres) in 15 ml of culture medium at 37 °C for 24 h. The medium was measured for dipyridamole concentration by fluorospectrometry, and diluted to 15 μg/ml of dipyridamole with the medium incubating ReGel alone. Human and porcine venous SMCs were seeded at a density of 2.5×10^4 cells per well in a 12-well plate, cultured for 24 h, and treated with the medium obtained above for 72 h. Cell proliferation was assessed by cell counting.

2.6. LDH-release assay

Human and porcine venous and arterial SMCs were seeded at a density of 2.5×10^3 cells per well in a 96-well plate and cultured for 24 h. After incubation with various concentrations of dipyridamole (1–200 μg/ml) for 72 h, the plate was centrifuged at 1000 rpm for 4 min. The supernatant containing the released LDH from the damaged cells was set aside. The cells remaining in the plate were lyzed to release all intracellular LDH. The LDH released from the damaged cells and from the lyzed cells were separately subjected to the LDH assay. In brief, 50 μl of the mixture of LDH-assay substrate, cofactor and dye solution (1:1:1) was added to each well, and the plate was incubated at room temperature for 30 min, followed by adding 15 μl of 1N HCl to each well. The absorbance at 492 nm (A_{492}) was measured on a plate reader (Multiskan Ascent, Bio Laboratories, Singapore) with the reference wavelength of 690 nm. Percentage of LDH released from the damaged cells was calculated using the following formula:

$$\text{LDH release}\,(\%) = \frac{A_{492}\ \text{of damaged cells}}{A_{492}\ \text{of damaged cells} + A_{492}\ \text{of lyzed cells}} \times 100$$

2.7. Reversibility study

Human venous SMCs were seeded at a density of 1×10^3 cells per well in a 96-well plate, cultured for 24 h, and incubated with 15 or 25 μg/ml of dipyridamole. At various time points up to 10 days, the medium was removed and the cells were further incubated in a fresh drug-free medium up to 16 days. During the experiment, medium was replaced with a fresh drug-containing or drug-free medium every 48 h. At the end of incubation, cell proliferation was assessed by the MTT dye-reduction assay. Briefly, the medium was replaced with 25 μl of 0.5 mg/ml MTT in RPMI and the cells were further incubated for 4 h at 37 °C. The cells were then solubilized in 100 μl of 0.1N HCl and isopropyl alcohol. The absorbance was measured at 540 nm with the reference wavelength of 690 nm.

2.8. Data analysis

Each experiment was performed three to six times on separate days. All values were expressed as mean ± S.D., and compared using one-way or two-way ANOVA with the Tukey test. *P*-value less than 0.05 was considered significant.

Dipyridamole cellular uptake data were further analyzed for the calculation of uptake rate constant k_u and release rate constant k_r. Assuming a two-compartment model for dipyridamole permeation between the extracellular medium (compartment 1) and the intracellular space (compartment 2), and the first-order kinetics of drug permeation, intracellular dipyridamole concentration C_2 at time t can be expressed as:

$$V_2 \frac{dC_2}{dt} = V_1 C_1 k_u - V_2 C_2 k_r \quad (1)$$

where V_1 and V_2 are the volumes of the extracellular medium and the intracellular space; and C_1 is the dipyridamole concentration in the extracellular medium. Let C_0 represent the drug concentration in the medium at the beginning of incubation. Substitution of V_1C_1 by $(V_1C_0 - V_2C_2)$ gives:

$$V_2 \frac{dC_2}{dt} = (V_1 C_0 - V_2 C_2) k_u - V_2 C_2 k_r \quad (2)$$

By integration of Eq. (2) when $C_2 = 0$ at time 0, C_2 at time t can be expressed as:

$$C_2 = \frac{V_1 C_0 k_u}{V_2 (k_u + k_r)} (1 - e^{-(k_u + k_r)t}) \quad (3)$$

At 4 h when cellular uptake of dipyridamole reached a steady state, intracellular drug concentration $C_{2,SS}$ can be

expressed as:

$$C_{2,ss} = \frac{V_1 C_0 k_u}{V_2(k_u + k_r)} \quad (4)$$

Therefore,

$$\frac{C_2}{C_{2,ss}} = 1 - e^{-(k_u + k_r)t} \quad (5)$$

Dipyridamole cellular uptake data were fitted to Eqs. (4) and (5) for the calculation of k_u and k_r using the non-linear regression function in SigmaPlot (Systat Software, Point Richmond, CA).

Temperature-dependent cellular uptake data were fitted to the following bi-exponential equation using the non-linear regression function in SigmaPlot

$$M = a\, e^{bT} \quad (6)$$

where M is the percentage of intracellular dipyridamole amount at the steady state at temperature T relative to that at 37 °C; a and b are the factors related to the membrane transport properties of dipyridamole. The mechanism of drug

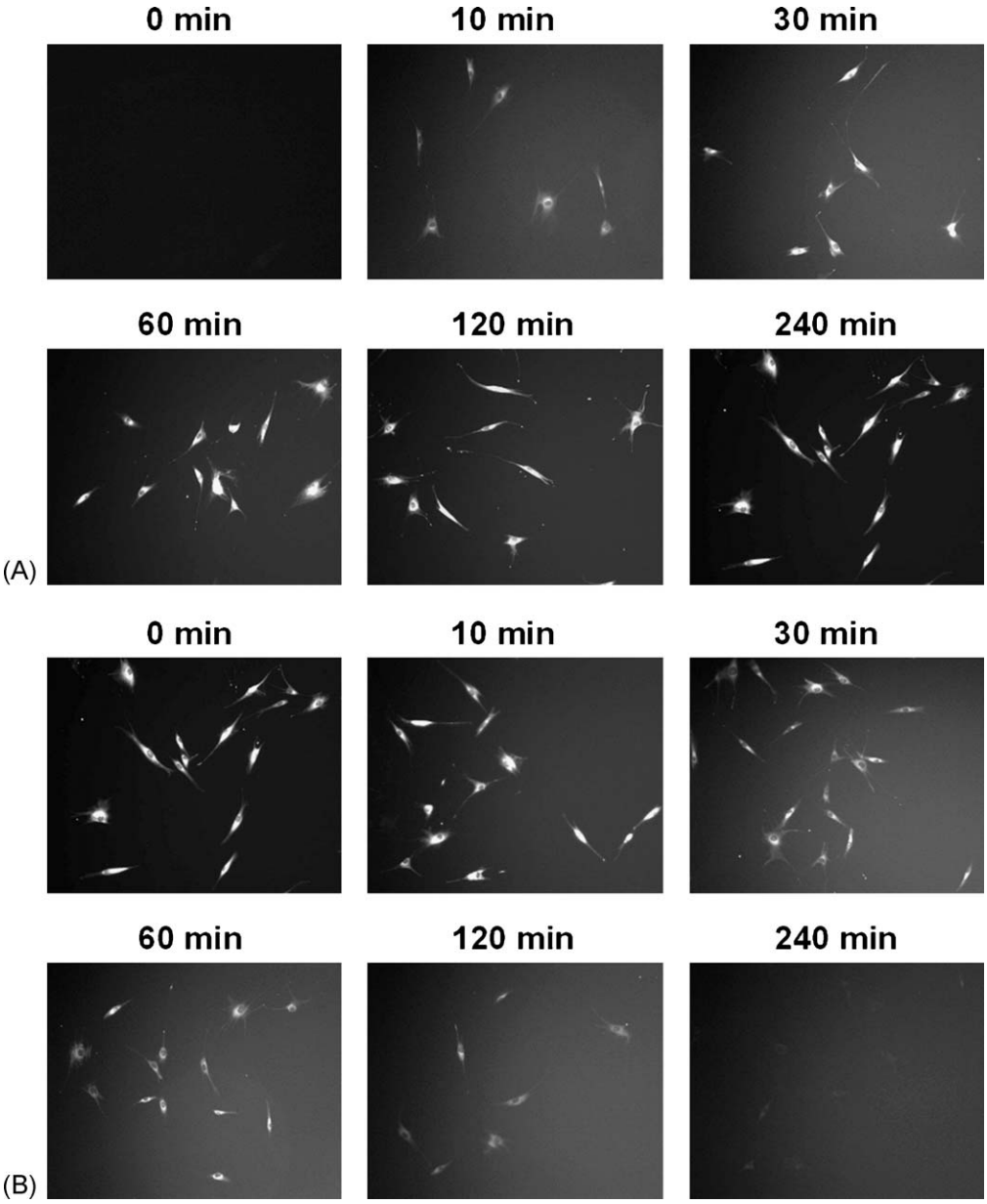

Fig. 1 – Fluorescence images of uptake and release of dipyridamole in human venous SMCs. (A) Uptake. Cells were incubated with 15 μg/ml of dipyridamole at 37 °C for various durations. After cell washing, cell-associated dipyridamole was visualized using fluorescence microscopy. (B) Release. Cells were first incubated with 15 μg/ml of dipyridamole at 37 °C for 4 h and further incubated with a fresh drug-free medium for various durations. After cell washing, cell-associated dipyridamole was visualized using fluorescence microscopy.

transport through the lipid bilayer of the cell membrane can be indicated indirectly by Q_{10}, temperature coefficient, which is defined as the factor by which the intracellular drug concentration at the steady state (C_{ss}) increases as the temperature is raised by 10 °C. A larger Q_{10} suggests a larger activation energy involved [26,27]. Thus Q_{10} can be an indicator of the diffusion mechanism [28]. Low temperature dependence with a low Q_{10} value (<2) is suggestive of a passive diffusion mechanism. High temperature dependence with a high Q_{10} value (>6) suggests a facilitated diffusion or active transport process. Moderate Q_{10} between 2 and 6 cannot directly indicate the diffusion mechanism [29]. Q_{10} can be calculated as:

$$Q_{10} = e^{10b} \quad (7)$$

The concentrations of dipyridamole which produce 50% of the maximal antiproliferative effect (EC_{50}) and induce 50% of the maximal lethal (toxic) effect (LC_{50}) were obtained by fitting the concentration-effect data to the following sigmoid E_{max} or Hill equation using the non-linear regression function in SigmaPlot.

$$E = \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n} \quad (8)$$

where E is the percentage of antiproliferative or toxic effect at concentration C relative to the maximal antiproliferative or toxic effect E_{max} (1 0 0); EC_{50} is the drug concentration which produces 50% of the maximal antiproliferative or toxic effect; n is the shape factor. Therapeutic index is calculated as the ratio of LC_{50} to EC_{50}.

3. Results

3.1. Cellular uptake and release of dipyridamole

Uptake and release of dipyridamole in human venous SMCs were visualized by fluorescence microscopy (Fig. 1A and B) and quantified by measuring fluorescence intensity of the intracellular dipyridamole at various time points after cell lysis (Fig. 2). Uptake reached a plateau in 120 min. More than 90% of the drug was eliminated from the cells within 120 min after the drug was removed from the extracellular medium. Both uptake rate constant k_u and release rate constant k_r showed no significant differences among the three drug concentrations tested (Table 1). The concentration-independent kinetics was compatible with a passive diffusion mechanism for dipyridamole transport through the cell membrane.

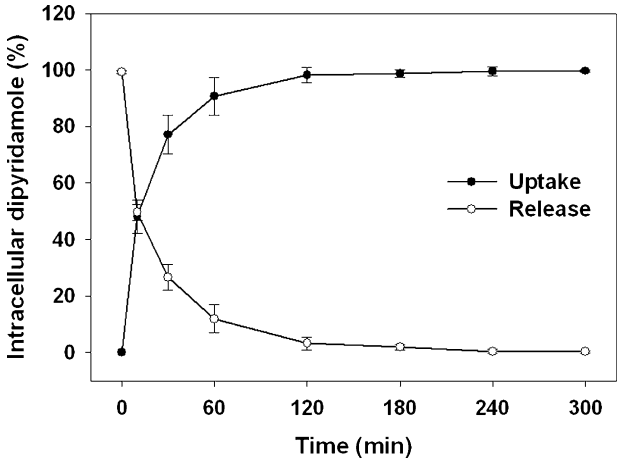

Fig. 2 – Kinetics of uptake and release of dipyridamole in human venous SMCs. Cells were incubated with 15 μg/ml of dipyridamole at 37 °C for various durations and washed as described in Fig. 1. The cells were lyzed and cell-associated dipyridamole was quantified by fluorospectrometry. Each data point represents the mean ± S.D. of three experiments.

3.2. Mechanisms of dipyridamole cellular uptake

Whether cellular uptake of dipyridamole was primarily driven by passive or active transport mechanisms was assessed by the temperature dependence of cellular uptake. The uptake reached a steady state in 2 h at 37 °C and 3 h at 4 °C, which persisted for several hours thereafter when the drug was maintained in the extracellular medium. Therefore, the temperature-dependence study was performed using a constant incubation time of 4 h to ensure equilibrium of drug uptake. Percentage of intracellular drug concentration at the steady state (C_{ss}) following incubation of the cells with 15 μg/ml of dipyridamole at tested temperature relative to that at 37 °C is shown in Fig. 3. C_{ss} was temperature-dependent and increased with the increase of temperature. The calculated Q_{10} were below 2 at all drug concentrations tested (Table 1), which was consistent with a passive diffusion model of transport.

3.3. Effect of dipyridamole on SMC proliferation

The antiproliferative effect of dipyridamole in human and porcine venous and arterial SMCs was assessed by cell

Table 1 – Pharmacokinetic parameters for uptake and release of dipyridamole in human venous SMCs

$C_{initial}$ (μg/ml)	k_u (min^{-1})	k_r (min^{-1})	Q_{10}
5	0.000177 ± 0.000023	0.0587 ± 0.0075	1.27 ± 0.17
15	0.000165 ± 0.000010	0.0547 ± 0.0032	1.31 ± 0.19
25	0.000158 ± 0.000027	0.0525 ± 0.0089	1.33 ± 0.24

$C_{initial}$: initial dipyridamole concentration in the medium; k_u: uptake rate constant; k_r, release rate constant; Q_{10}, the factor by which the intracellular drug concentration at steady state increases as the temperature is raised by 10 °C. Each value represents the mean ± S.D. of three experiments.

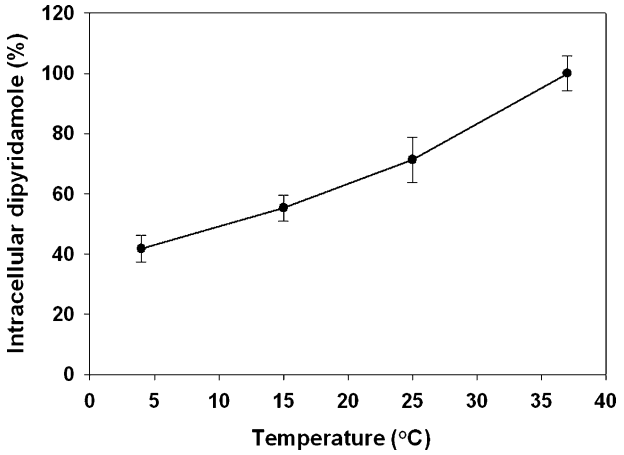

Fig. 3 – Temperature dependence of dipyridamole uptake in human venous SMCs. Cells were incubated with 15 μg/ml of dipyridamole at 4, 15, 25 or 37 °C for 4 h. After cell lysis, cell-associated dipyridamole was quantified by fluorospectrometry. Data were expressed as the percentage of intracellular drug concentration at the steady state at tested temperature relative to that at 37 °C. Each data point represents the mean ± S.D. of three experiments.

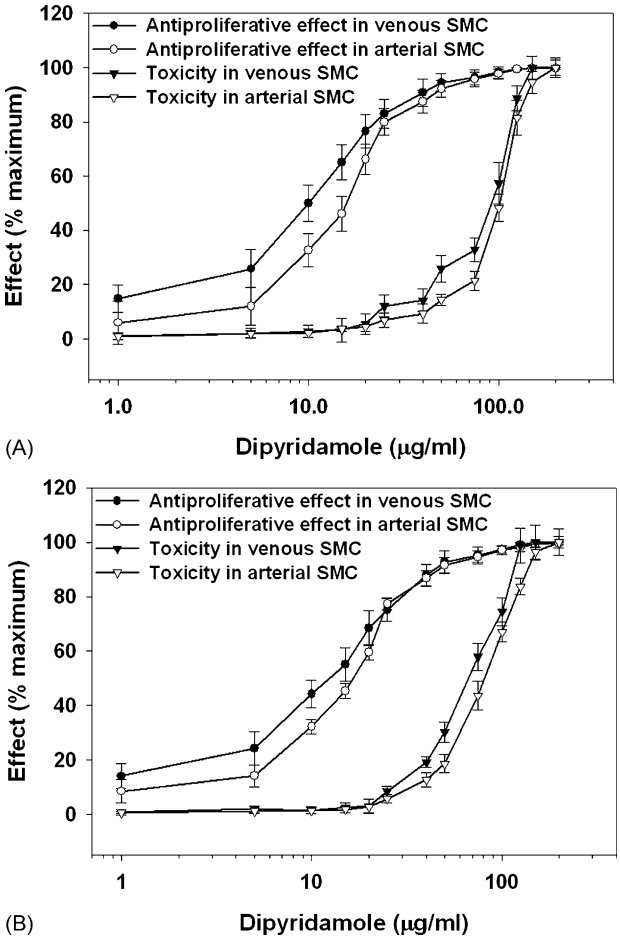

Fig. 4 – Antiproliferative effect and toxicity of dipyridamole in: (A) human venous and arterial SMCs and (B) porcine venous and arterial SMCs. Cells were incubated with 1–200 μg/ml of dipyridamole for 72 h. Antiproliferative effect was assessed by cell counting and toxicity was determined by the LDH-release assay. Each data point represents the mean ± S.D. of six experiments.

counting. Proliferation of cells treated with dipyridamole was attenuated in a dose-dependent manner (Fig. 4A and B). The EC_{50} in human and porcine arterial SMCs were significantly higher (1.5 and 1.2 times, respectively) than those in the corresponding venous cells ($P < 0.05$). The EC_{50} in porcine venous and arterial SMCs were significantly higher (1.3 and 1.1 times, respectively) than those in the corresponding human cells ($P < 0.05$) (Table 2).

Effect of the released dipyridamole from microspheres/ReGel was next examined. The released drug inhibited the proliferation of human and porcine venous SMCs, with the potency similar to the intact drug (Fig. 5). ReGel and drug-free microspheres had no significant effect on cell proliferation.

3.4. *Cytotoxicity of dipyridamole*

Cytotoxicity induced by dipyridamole was assessed by the release of intracellular LDH, normalized by the total cellular LDH. As shown in Fig. 4A and B, LDH release remained similar to the control up to 20 μg/ml of dipyridamole. At concentration higher than 25 μg/ml, increased LDH release which was significantly higher than the control ($P < 0.05$) was observed. Dipyridamole concentration which induces 50% of maximal LDH release, LC_{50}, were significantly higher (1.3 times) in human and porcine arterial SMCs than those in the corresponding venous cell ($P < 0.05$). The LC_{50} in human venous and arterial SMCs were significantly higher (1.3 times) than those in the corresponding porcine cells ($P < 0.05$). Therapeutic

Table 2 – Pharmacodynamic parameters for antiproliferative effect and toxicity of dipyridamole in human and porcine venous and arterial SMCs

Parameters	Human venous SMC	Human arterial SMC	Porcine venous SMC	Porcine arterial SMC
EC_{50} (μg/ml)	9.8 ± 0.5	14.6 ± 0.5*	12.4 ± 0.7	15.3 ± 0.5*
LC_{50} (μg/ml)	83.8 ± 3.0	101.9 ± 2.8*	66.4 ± 1.5	81.3 ± 1.7*
Therapeutic index	8.6 ± 0.7	7.0 ± 0.4*	5.4 ± 0.4	5.3 ± 0.3

EC_{50}, concentration of dipyridamole which produces 50% of maximal antiproliferative effect; LC_{50}, concentration of dipyridamole which induces 50% of maximal toxicity; therapeutic index, ratio of LC_{50} to EC_{50}. Each value represents the mean ± SD of six experiments.
* $P < 0.05$ vs. corresponding value in venous SMCs of the same species.

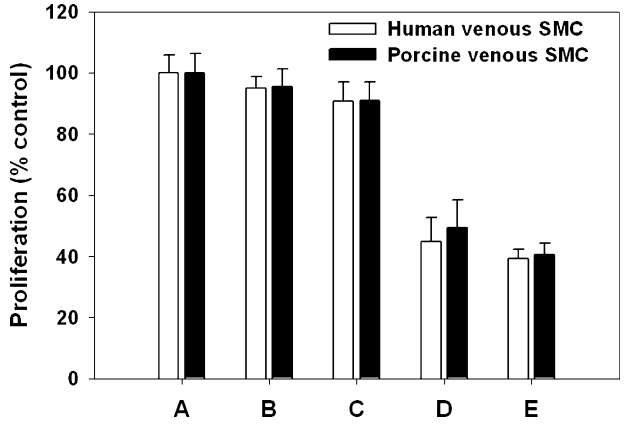

Fig. 5 – Proliferation of human and porcine venous SMCs treated with: (A) control; (B) ReGel; (C) drug-free microspheres; (D) intact dipyridamole at 15 μg/ml and (E) released dipyridamole from microspheres/ReGel at 15 μg/ml. A separate *in vitro* release experiment was performed by incubating dipyridamole-incorporated microspheres, ReGel, or drug-free microspheres in culture medium. After 24 h the medium was sampled, diluted and added to the cell culture. Cell proliferation was assessed by cell counting. Each data point represents the mean ± S.D. of three experiments.

index (LC_{50}/EC_{50}), was significantly higher in human venous than arterial cells, but was not significantly different between porcine venous and arterial cells (Table 2).

3.5. *Reversibility of antiproliferative effect of dipyridamole*

Reversibility of antiproliferative effect of dipyridamole was tested by assessing the cell proliferation when dipyridamole was removed from the culture medium using the MTT assay. Human venous SMCs without dipyridamole treatment (control) continued to grow up to 18 days. When cells were exposed to 15 μg/ml of dipyridamole, the growth rate was markedly diminished. Upon removal of the drug, the growth rate increased, albeit still at a slower rate for the initial 2 days. Thereafter, the growth rate further increased to, or even slightly exceeded, that of the control (Fig. 6A). In contrast, when cells were exposed to 25 μg/ml of dipyridamole for 6 days or longer, the cells resumed their growth poorly even after drug removal (Fig. 6B). In fact, there appeared to be complete inhibition of cell growth during and after exposure to 25 μg/ml of dipyridamole for 10 days.

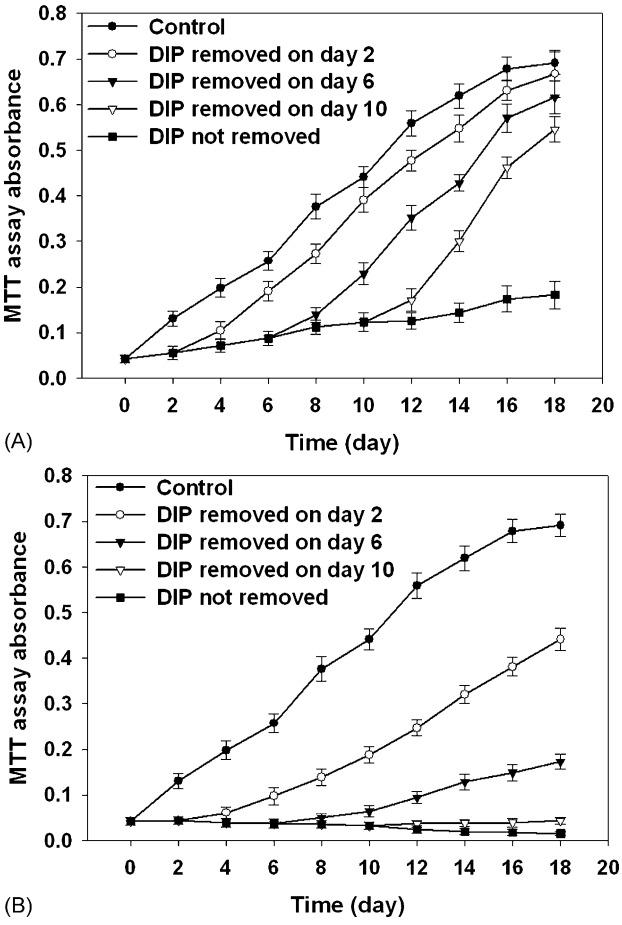

Fig. 6 – Reversibility of antiproliferative effect of dipyridamole at: (A) 15 μg/ml and (B) 25 μg/ml. Human venous SMCs were incubated with dipyridamole for various durations up to 10 days. The medium was replaced with a fresh drug-free medium at 2, 6 and 10 days and the cells were further incubated. Cell proliferation was assessed by the MTT assay. Each data point represents the mean ± S.D. of three experiments. DIP, dipyridamole.

4. Discussion

Stenosis in hemodialysis arteriovenous grafts develops more often at the vein-graft anastomosis than the artery-graft anastomosis [30]. An understanding of the response of venous SMCs to antiproliferative agents is important for designing strategies to prevent the graft stenosis. Despite the well-established effects of many antiproliferative agents on human and animal arterial SMCs, there is a paucity of data on their effects in human venous SMCs. Different properties of SMCs derived from arteries and veins have been reported [9,31,32]. In addition, kinetics of dipyridamole transport across the cytoplasmic membrane and the resulting time-dependent pharmacodynamics at the cellular level has not been carefully investigated. Understanding these properties is necessary to design an optimal drug delivery system. Since the porcine model of arteriovenous graft stenosis has been a well-established model for the study of interventional strategies [33–35], porcine venous and arterial SMCs were also examined in the present study. This may facilitate the understanding of any potential inter-species difference in drug responses.

We have previously found that dipyridamole significantly increased cAMP and cGMP levels in human venous and arterial SMCs with transient peak concentrations of both cyclic nucleotides at 15–30 min; and the levels of both cyclic nucleotides were strongly corresponded with inhibition of cell proliferation [36]. In this study, we evaluated pharmacodynamics of dipyridamole by examining the antiproliferative activity as well as the toxicity. EC_{50} of dipyridamole in

inhibiting SMC proliferation were determined by cell counting, which showed slight differences from those determined by the MTT dye-reduction assay in our previous study [9]. Since the MTT assay only assesses the mitochondrial activity, regardless of the cell number and whether the cells are actively proliferating or not, cell counting can be a better method for quantitative assessment of cell proliferation. Based on EC_{50} and LC_{50} (Table 2), venous SMCs were more susceptible to the antiproliferative effect, and less susceptible to the toxicity of dipyridamole than arterial SMCs. And the differences were preserved between species. These differences are not readily explained, but may be a result of differences in the cellular constituents which are the targets of action of the drug.

Perhaps the most important findings of this study were the fast cellular uptake of dipyridamole (Figs. 1A and 2), the short residence time inside the cells (Figs. 1B and 2) and the reversibility of the antiproliferative effect of low-dose dipyridamole in SMCs (Fig. 6A). Dipyridamole is a small weakly hydrophobic molecule, with a molecular weight of 504.6 Da and the octanol–water partition coefficient of 2.74 (KowWin ALOGPS 2.1 software, Syracuse Research, Denver, CO). These properties are favorable to a passive diffusion transport across the cytoplasmic membrane. The values of k_u, k_r and Q_{10} obtained from our pharmacokinetic analysis (Table 1) were compatible with a passive diffusion mechanism.

The reversibility of the antiproliferative effect of dipyridamole in human venous SMCs was dependent upon the dose and duration of exposure. At 15 μg/ml, which was modestly higher than the EC_{50} (9.8 μg/ml, Table 2), dipyridamole did not induce significant cytotoxicity (Fig. 4A). The antiproliferative effect was completely reversible, even for drug exposure for as long as 10 days (Fig. 6A). At concentration of 25 μg/ml or higher, however, cytotoxicity was observed (Fig. 4A), and the antiproliferative effect of dipyridamole was not completely reversible. After exposure to dipyridamole at 25 μg/ml for 6 days or longer, cell proliferation was markedly impaired even upon removal of the drug (Fig. 6B).

With the conventional dose of 200 mg per day, the maximum plasma concentration of dipyridamole was achieved at approximately 3 μM (1.5 μg/ml) [37], which is in order of magnitude lower than the EC_{50} obtained in our study (Table 2). Although cell culture conditions are undoubtedly different from the *in vivo* environment, these data suggest that the systemic administration of dipyridamole is unlikely to provide adequate plasma levels to inhibit SMC proliferation. Indeed, oral administration of dipyridamole has been found to be ineffective in preventing the formation of neointimal hyperplasia in various animal models [21,22]. In addition, hemodialysis arteriovenous grafts are subjected continuously and indefinitely to stimuli for SMC proliferation, such as the flow disturbance at the anastomosis and the later repeated needle puncture for chronic hemodialysis. Therefore, the chronic delivery of drug to the target site can be a preferable approach. Local drug delivery may achieve sufficient local concentration with minimal systemic side effect. The continuous infusion of dipyridamole to the adventitia using an implanted catheter has been shown to attenuate intimal thickening after balloon injury in a rabbit model [6]. While the result is intriguing, this approach is impractical for clinical application in hemodialysis patients.

Alternatively, we have recently explored a sustained-release system, ReGel®, for perivascular delivery of dipyridamole [25]. ReGel is a polymeric gel that, because of its thermosensitive nature, can be injected into the body to provide a sustained-release drug depot, and facilitate drug re-dosing by percutaneous injection at regular intervals [5,38]. While ReGel can solubilize and stabilize a large variety of drugs, the relatively weak hydrophobicity of dipyridamole may diminish the sustained-release pattern. In order to prolong the release, we have incorporated dipyridamole into poly(lactide-*co*-glycolide) microspheres and combined the products with ReGel [25]. This formulation was found to decrease the initial burst and prolong the drug release. In the present study we showed that the antiproliferative potency was retained in the released dipyridamole from the delivery system (Fig. 5). Our recent study using an *in vitro* model has further demonstrated that dipyridamole on the perivascular surface could readily penetrate the walls of native veins and arteries as well as PTFE grafts. These results suggest dipyridamole delivered by microspheres/ReGel can be a potential candidate for the prevention of arteriovenous PTFE graft stenosis.

In summary, our present observations are compatible with the following sequence of events. Dipyridamole enters the cells by passive diffusion and exerts its antiproliferative effect rapidly by, for example, inhibiting cyclic nucleotide PDE activities. Upon removal of the drug from the extracellular environment, the drug rapidly diffuses out of the cells and the antiproliferative effect dissipates over approximately 2 days as the proliferation functions (e.g. PDE activities) are gradually and fully restored. Since the stenosis develops progressively following the placement of arteriovenous grafts [33–35], the transient antiproliferative effect of dipyridamole would imply that the continuous controlled exposure to a relatively low concentration of drug is necessary for the long-term pharmacological inhibition of SMC proliferation without significant cytotoxicity.

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REFERENCES

[1] Schwab SJ. Vascular access for hemodialysis. Kidney Int 1999;55:2078–90.
[2] Swedberg SH, Brown BG, Sigley R, Wight TN, Gordon D, Nicholls SC. Intimal fibromuscular hyperplasia at the venous anastomosis of PTFE grafts in hemodialysis patients. Clinical, immunocytochemical, light and electron microscopic assessment. Circulation 1989;80: 1726–36.

[3] Lumsden AB, Chen C, Coyle KA, Ofenloch JC, Wang JH, Yasuda HK, et al. Nonporous silicone polymer coating of expanded polytetrafluoroethylene grafts reduces graft neointimal hyperplasia in dog and baboon models. J Vasc Surg 1996;24:825–33.
[4] Rotmans JI, Pattynama PM, Verhagen HJ, Hino I, Velema E, Pasterkamp G, et al. Sirolimus-eluting stents to abolish intimal hyperplasia and improve flow in porcine arteriovenous grafts: a 4-week follow-up study. Circulation 2005;111:1537–42.
[5] Masaki T, Rathi R, Zentner G, Leypoldt JK, Mohammad SF, Burns GL, et al. Inhibition of neointimal hyperplasia in vascular grafts by sustained perivascular delivery of paclitaxel. Kidney Int 2004;66:2061–9.
[6] Singh JP, Rothfuss KJ, Wiernicki TR, Lacefield WB, Kurtz WL, Brown RF, et al. Dipyridamole directly inhibits vascular smooth muscle cell proliferation in vitro and in vivo: implications in the treatment of restenosis after angioplasty. J Am Coll Cardiol 1994;23:665–71.
[7] Himmelfarb J, Couper L. Dipyridamole inhibits PDGF- and bFGF-induced vascular smooth muscle cell proliferation. Kidney Int 1997;52:1671–7.
[8] Masaki T, Kamerath CD, Kim SJ, Leypoldt JK, Mohammad SF, Cheung AK. In vitro pharmacological inhibition of human vascular smooth muscle cell proliferation for the prevention of hemodialysis vascular access stenosis. Blood Purif 2004;22:307–12.
[9] Kim SJ, Masaki T, Leypoldt JK, Kamerath CD, Mohammad SF, Cheung AK. Arterial and venous smooth-muscle cells differ in their responses to antiproliferative drugs. J Lab Clin Med 2004;144:156–62.
[10] Edelman ER, Adams DH, Karnovsky MJ. Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. Proc Natl Acad Sci USA 1990;87:3773–7.
[11] Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ Res 1995;76:412–7.
[12] Sollott SJ, Cheng L, Pauly RR, Jenkins GM, Monticone RE, Kuzuya M, et al. Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. J Clin Invest 1995;95:1869–76.
[13] Kusama H, Kikuchi S, Tazawa S, Katsuno K, Baba Y, Zhai Y-L, et al. Tranilast inhibits the proliferation of human coronary smooth muscle cell through the activation of $p21^{waf1}$. Atherosclerosis 1999;143:307–13.
[14] Wong J, Rauhoft C, Dilley RJ, Agrotis A, Jennings GL, Bobik A. Angiotensin-converting enzyme inhibition abolishes medial smooth muscle PDGF-AB biosynthesis and attenuates cell proliferation in injured carotid arteries: relationships to neointima formation. Circulation 1997;96:1631–40.
[15] Block LH, Emmons LR, Vogt E, Sachinidis A, Vetter W, Hoppe J. Ca^{2+}-channel blockers inhibit the action of recombinant platelet-derived growth factor in vascular smooth muscle cells. Proc Natl Acad Sci USA 1989;86:2388–92.
[16] Harker LA, Kadatz RA. Mechanism of action of dipyridamole. Thromb Res Suppl 1983;4:39–46.
[17] Gillespie PG, Beavo JA. Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948. Mol Pharmacol 1989;36:773–81.
[18] Yu SM, Hung LM, Lin CC. cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. Circulation 1997;95:1269–77.
[19] Fukumoto S, Koyama H, Hosoi M, Yamakawa K, Tanaka S, Morii H, et al. Distinct role of cAMP and cGMP in the cell cycle control of vascular smooth muscle. Circ Res 1999;85:985–91.
[20] Hayashi S, Morishita R, Matsushita H, Nakagami H, Taniyama Y, Nakamura T, et al. Cyclic AMP inhibited proliferation of human aortic vascular smooth muscle cells, accompanied by induction of p53 and p21. Hypertension 2000;35:237–43.
[21] Brody WR, Brown JW, Reitz BA, Fry DL, Michaelis LL. Effects of dipyridamole and methylprednisolone on intimal thickening in vein grafts. J Thorac Cardiovasc Surg 1977;73:601–4.
[22] Rainwater LM, Plate G, Gloviczki P, Bahn RC, Hollier LH, Kaye MP. Morphologic quantitation of pseudointima and effects of antiplatelet drugs on vascular prostheses in goats. Am J Surg 1984;148:195–202.
[23] Sreedhara R, Himmelfarb J, Lazarus JM, Hakim RM. Anti-platelet therapy in graft thrombosis: results of a prospective, randomized, double-blind study. Kidney Int 1994;45:1477–83.
[24] Murillo Pulgarin JA, Alanon Molina A, Fernandez Lopez P. Direct determination of dipyridamole in serum. Anal Biochem 1997;245:8–16.
[25] Zhu W, Masaki T, Bae YH, Rathi R, Cheung AK, Kern SE. Development of a sustained-release system for perivascular delivery of dipyridamole. J Biomed Mater Res Part B Appl Biomater 2006;77B:135–43.
[26] Stein WD. The movement of molecules across cell membranes New York/London: Academic Press; 1967.
[27] Hille B. Ionic channels of excitable membranes Sunderland: Sinauer Associates; 1984.
[28] Christensen HN. Biological transport Reading: Benjamin; 1975.
[29] Lane P, Vichi P, Bain DL, Tritton TR. Temperature dependence studies of adriamycin uptake and cytotoxicity. Cancer Res 1987;47:4038–42.
[30] Kanterman RY, Vesely TM, Pilgram TK, Guy BW, Windus DW, Picus D. Dialysis access grafts: anatomic location of venous stenosis and results of angioplasty. Radiology 1995;195:135–9.
[31] Bishop-Bailey D, Pepper JR, Larkin SW, Mitchell JA. Differential induction of cyclooxygenase-2 in human arterial and venous smooth muscle: role of endogenous prostanoids. Arterioscler Thromb Vasc Biol 1998;18:1655–61.
[32] Kim SJ, Masaki T, Rowley R, Leypoldt JK, Mohammad SF, Cheung AC. Different responses by cultured aortic and venous smooth muscle cells to gamma radiation. Kidney Int 2005;68:371–7.
[33] Johnson MS, McLennan G, Lalka SG, Whitfield RM, Dreesen RG. The porcine hemodialysis access model. J Vasc Interv Radiol 2001;12:969–77.
[34] Kelly BS, Heffelfinger SC, Whiting JF, Miller MA, Reaves A, Armstrong J, et al. Aggressive venous neointimal hyperplasia in a pig model of arteriovenous graft stenosis. Kidney Int 2002;62:2272–80.
[35] Rotmans JI, Velema E, Verhagen HJ, Blankensteijn JD, Kastelein JJ, de Kleijn DP, et al. Rapid, arteriovenous graft failure due to intimal hyperplasia: a porcine, bilateral, carotid arteriovenous graft model. J Surg Res 2003;113:161–71.
[36] Zhuplatov SB, Masaki T, Blumenthal DK, Cheung AK. Cyclic nucleotide-dependent protein kinases mediate the antiproliferative effects of dipyridamole. J Am Soc Nephrol 2005;16:156A [Abstract].
[37] Rajah SM, Crow MJ, Penny AF, Ahmad R, Watson DA. The effect of dipyridamole on platelet function: correlation with blood levels in man. Br J Clin Pharmac 1977;4:129–33.
[38] Zentner GM, Rathi R, Shih C, McRea JC, Seo M-H, Oh H, et al. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. J Controll Rel 2001;72:203–15.