ACTA ABP BIOCHIMICA POLONICA

Vol. 67, No 4/2020
495–499
https://doi.org/10.18388/abp.2020_5186

Regular paper

Cholesterol (Blood lipid) lowering potential of Rosuvastatin chitosan nanoparticles for atherosclerosis: Preclinical study in rabbit model

Lin Chen#, Caihong Wang# and Yuanchu Wu✉

Department of Cardiology, Affiliated Wujiang Hospital of Nantong University, Suzhou, Jiangsu, China 215200

Atherosclerosis is the condition of narrowing of arteries due to plaque buildup on the artery walls. Aortic valve calcification (AVC) is one of the reasons of atherosclerosis which leads to narrowing at the opening of the aortic valve which is commonly referred as Aortic valve stenosis (AS). The Rosuvastatin-chitosan (ROS-chitosan) nanoparticles were prepared using ionotropic gelation method. Nanoparticulate formulation was optimized by 3 factor, 2 level full factorial design to find the effect of independent variables on particle size and percentage encapsulation efficiency. Particle size, encapsulation efficiency, scanning electron microscopy, *in vitro* drug release of nanoparticles was determined. The adult male rabbit of 4–5 months old were chosen for the study. Hypercholesterolemia was induced in experimental animals by administering diet with Cholesterol and Cholic acid (1.25 % and 0.5%, respectively). Blood lipid profile, interleukin 6 levels and histopathological study was performed. Rosuvastatin was found to be significantly effective in lowering the blood lipid levels. It helps to attenuate atherosclerosis as well as calcification of various valve tissues in experimental animals.

Key words: Rosuvastatin, atherosclerosis, Calcification, lipoprotein, nanoparticles

Received: 13 February, 2020; revised: 01 April, 2020; accepted: 08 April, 2020; available on-line: 22 October, 2020

✉e-mail: benjamin_w@163.com
#These authors contribute to this work equally.

Abbreviations: ANOVA, Analysis of variance; AS, Aortic valve stenosis; AVC, Aortic valve calcification; CS, Chitosan; EE, Encapsulation Efficiency; FESEM, Field Emission Scanning Electron Microscopy; HC, hypercholesterolemia; HPLC, High Performance Liquid Chromatography; IM, Intramuscular; LDL, low density lipoproteins; Lp (a), Lipoprotein (a); MWCO, molecular weight cut off; ROS, Rosuvastatin; ROS-chitosan, Rosuvastatin-chitosan; ROS-CS-NP, Rosuvastatin-Chitosan Naoparticles; TPP, Tripolyphosphate; USP, united States pharmacopoeia; VLDL, very low density lipoproteins

INTRODUCTION

Atherosclerosis is the condition of narrowing of arteries due to plaque buildup on the artery walls. Atherosclerosis starts when endothelium gets damaged which allows the harmful type of cholesterol to build up in the artery wall. Aortic valve calcification (AVC) is a disease condition where calcium deposits are formed on the surface of valve of aorta in the heart which leads to narrowing at the opening of the aortic valve which is commonly referred as Aortic valve stenosis (AS) which leads to the condition of atherosclerosis (Roberts *et al.*, 2005). The AVC can be the early sign of heart disease as well as the indication of atherosclerosis. The condition generally affects older people having age more than 65. When the condition is identified in younger people the reasons underlying were identified as either pre-genital heart defect or other factors like kidney failure. The disease condition usually doesn't cause any problem unless and otherwise thickening and stiffness of valve has occurred. In such cases surgical valve replacement is the only option available (Lindroos *et al.*, 1993). In AVC there is aortic valve thickening with calcification without significant pressure gradient (jet velocity <2 m/sec). The risk factor include increased level of cholesterol, which increases blood pressure. Some epidemiological as well as genetic studies have identified the risk factor and depicted the cellular mechanisms involved in disease progression. One of the outcomes of the finding suggests that progression of disease might be prevented by lipid lowering treatment (Stewart *et al.*, 1997; Aronow *et al.*, 2001). The progression of the disease mostly is confirmed *via* doppler echocardiography which is considered to be a gold standard in diagnosis of the disease. Patient diagnosed with moderate type of aortic stenosis are advised to take the test once in year while people with severe type of disease should do it every six month (Palta *et al.*, 2000). The normal aortic valve opening area is greater than or equal to 2.0 cm^2.

In 1976, Japanese scientist identified metabolite which blocks enzyme HMG-CoA reductase leading to decreasing intracellular cholesterol synthesis (Colli *et al.*, 2007). Statins were reported to interfere with secretion of very low density lipoproteins (VLDL) leading to decreasing level of low density lipoproteins (LDL) in blood. Statins helps to slow hemodynamic progression and their use might reduce cardiovascular end points (Colli *et al.*, 2007; Schoen, 1987).

Rosuvastatin (ROS) belongs to the class of "Statins" and often marketed by name Crestor for the treatment of high levels of cholesterol. The effect of the drug is generally dose related on LDL cholesterol whereas high doses may incorporated in therapy in patients with hypercholesterolemia in improving lipid profile (Rajamannan *et al.*, 2005). The drug is found to be inhibitor of HMG-CoA reductase. The effects on chronic heart diseases can be negated by increase in collagen turnover as well as reduced plasma coenzyme Q10 (CoQ10) (Olsson *et al.*, 1999). Lipoprotein (a) is LDL associated with extra protein called as apolipoprotein (a) which is covalently bound to apolipoprotein B-100. Elevated plasma Lp (a) level are major risk factors for CVD (Cardio vascular diseases). Lp (a) mediates CVD through its effect on fibrinolysis, wound healing and atherosclerotic stenosis (Miller *et al.*, 2009). Also plasma Lp (a) levels have been associated with calcific aortic valve disease (Galante *et al.*,

2001; Wen *et al.*, 2009). The aim of this study was to investigate blood lipid lowering potential of Rosuvastatin chitosan nanoparticles to attenuate atherosclerosis as well as calcification of various valve tissues in experimental animals.

MATERIAL AND METHODS

Rosuvastatin was purchased from Jiangsu Hengrui Medicine Co. China. Pluronic F 68, Chitosan (CS) was purchased from Sigma Aldrich, USA. Male Rabbits were obtained from the Department of Cardiology, Affiliated Wujiang Hospital of Nantong University, Suzhou, Jiangsu, China 215200

Animals. All animals were maintained as per norms/guideline of Animal Ethical Committee and Medical Ethical Committee of Department of Cardiology Affiliated Wujiang Hospital of Nantong University, Suzhou, Jiangsu, China 215200. The adult male rabbits of 4–5 months old were chosen for the study. The animals were kept in cages to have free food and water. Before and during experiment, animals were kept on standard diet. The standard temperature and humidity were maintained at the comfort condition.

Statistical analysis. In this investigation 8 run, 3 factors and 2 level fractional factorial design was used for statistical optimization of experimental results. Second order polynomial models were generated using Design-Expert® software (Version: 6.0.8). Preliminary experiments demonstrated that chitosan, surfactant and TPP (Tripolyphosphate) concentration majorly influence the drug loading, particle size, and encapsulation efficiency and drug release form the nanoparticles. Independent variables and different levels are represented in Table 1.

Table 1. Variable and three levels

Independent Variables	Low level (-1)	High level (+1)
A= CS Conc. (mg)	50	100
B= TPP Conc. (%)	0.25	0.50
C=Tween 80 Conc. (%)	0.2	0.5
Dependent Variables		
Y1= EE		

CS, Chitosan; TPP, Tripoly phosphate; EE, Encapsulation efficiency

Preparation of ROS-CS nanoparticles. Ionotropic gelation method was used for preparation of ROS-CS nanoparticles. Chitosan was dissolved in acetic acid solution with continuous stirring at room temperature in order to obtain clear gel. CS was made to dissolve in surfactant (Tween-80; 0.5% v/v) while drug in organic phase of acetone solution is added to aqueous phase drop wise using different concentrations making o/w emulsion under stirring. TPP was added drop wise from syringe with needle at various concentrations in to o/w emulsion under stirring. The system was kept under stirring overnight to evaporate the organic solvent. Separation of nanoparticles was done at 20 000 rpm for 15 minutes at –80°C using cooling centrifuge. The supernatant obtained was used for determination of free ROS from nanoparticles by HPLC (Allemann *et al.*, 1993).

Evaluation of Nanoparticles (Calvo *et al.*, 1997; Mehrotra *et al.*, 2011; Farivar *et al.*, 2003). Field Emission Scanning Electron Microscopy (FESEM). Determination of particle size, shape was done using Zetasizer (Malvern/DTS 4.1) while surface morphology was done by FESEM. The sample was made suspended in water, mixed rapidly. Readings should be noted in triplicate.

***In-vitro* Dissolution Study using dialysis technique**. The *in-vitro* dialysis technique involves using dialysis membrane of certain molecular weight cut off (MWCO). The suspension of microsphere is kept in dialysis bag which is sealed at both ends and suspended in buffer solution using paddle. The method offers advantages like sample withdrawal, physical separation of microparticles from buffer. The only condition is drug should not bind to dialysis membrane. The USP type XXII (rotating paddle) apparatus was used to perform study. About 100 mg of nanoparticles were kept in apparatus and release was observed. The parameters set were 37±2°C using 1000 ml of phosphate buffer solution at 100 and 200 rpm. About 5 ml of aliquot was removed as sample for testing at intervals of 0, 5, 15, 30, 60, 120 minutes and again replaced with same amount of buffer solution to maintain sink condition. The sample withdrawn was analyzed by UV spectrophotometrically at 244 nm.

Drug Entrapment Efficiency. Non entrapped drug was estimated from supernatant solution after centrifugation. UV spectrophotometer was used for drug content estimation at 244 nm. Encapsulation efficiency was calculated by following formula

$$\% \text{ E.E} = \text{Total drug-free drug/Total drug amount} \times 100$$

Induction of Hypercholesterolemia. The hypercholesterolemia (HC) was induced in experimental animals by administering diet with Cholesterol and Cholic acid (1.25% and 0.5%, respectively.) up to 12 weeks by keeping all experimental conditions same. Blood lipid profile (non fasted condition) and LDL (Low density lipoprotein) cholesterol were estimated before start of the experiment. The total cholesterol level of above 200 mg/dL is considered as high level (Pandey *et al.*, 2006).

Grouping of Animals. After induction of hypercholesterolemia all blood lipid profile of all animals was checked for cholesterol level and animals only with high lipid level were selected for study. Total 50 animals were selected for the study from which 7 animals failed to show hypercholesterolemia while 3 animals gone wild during entire process so dropped out of study. Total 40 numbers of animals were selected for the study. Such selected animals were grouped as following:

- Group I: No hypercholesterolemia; Normal diet with No Rosuvastatin intake (n=10)
- Group II: No hypercholesterolemia; Normal diet with Rosuvastatin intake (n=10)
- Group III: hypercholesterolemia; Normal diet with No Rosuvastatin intake (n=10)
- Group IV: hypercholesterolemia; Normal with Rosuvastatin intake (n=10)

Blood Lipid Profile. Blood lipid profile of experimental animals was measured by anesthetizing animal with Xylamine and Zoletil solution (Obtained from sigma Aldrich USA) (09 mg/kg and 28 mg/kg respectively). 2.0 mL of sample of blood was removed from jugular vein at initial time point and at 2 hrs time point. The sample thus obtained was placed in heparin tube as well as serum separating tube to centrifuge the sample at 5000 rpm for about 5–6 minutes. Enzymatic colorimetric assay was performed (Illum, 1998).

Interleukin 6 levels. The serum II-6 levels in the blood were determined using ELISA kit (Quantikine kit, MN, USA) at interval of 5 and 12 weeks.

Table 2. Formulation of ROS-CS nanoparticles using 2^3Factorial Design

Formulations	Factor			Response
	A	B	C	Y1
F1	+1	+1	+1	90.13
F2	–1	+1	+1	87.34
F3	–1	–1	–1	87.11
F4	+1	–1	–1	94.11
F5	+1	–1	+1	97.98
F6	+1	+1	+1	82.13
F7	–1	–1	-1	69.90
F8	–1	–1	-1	91.11

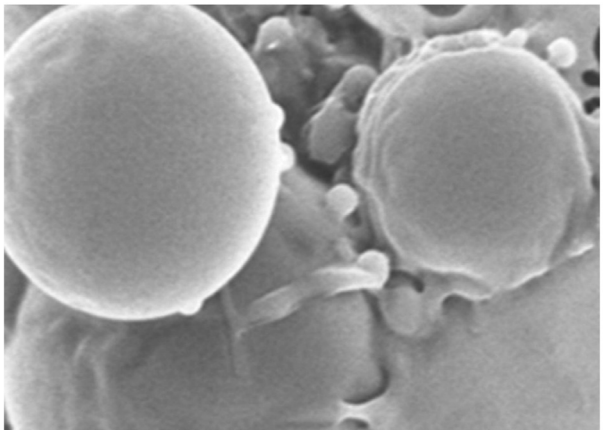

Figure 1. Scanning Electron Microscopy of ROS-CS nanoparticles showing smooth and spherical surface.

Histopathological Study of aortic valve tissue. Animals were made euthanized by intramuscular (IM) injection of Ketamine + Xylazine at a dose of 35 mg/kg + 5 mg/kg IM and samples of aortic valve tissues were collected and fixed in 100% neutralized buffered formalin and embedded in paraffin. The 3–5 µm thick sections were cutted off and staining was done with hematoxylin and eosin dye. The cell count was done using 400× magnification microscope.

RESULT AND DISCUSSIONS

In vitro characterization of nanoparticles

In recent year CS nanoparticles has proved excellent drug carrier due to biocompatibility, biodegradability, non-toxicity, bioadhesive and absorption enhancing properties. Apart from this antioxidant and antitumor activities of CS has made excellent biomaterial in cancerous treatment (Rosenhek *et al.*, 2004). Table 2 shows batches of ROS nanoparticles using CS in varying concentration and their effect on dependent variables i.e. Encapsulation efficiency (Y1).

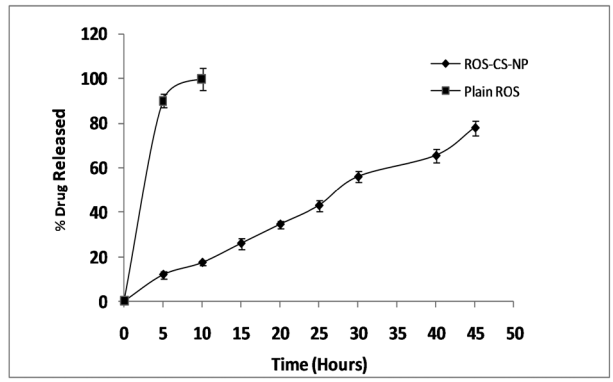

Figure 2. *In-vitro* drug release profile of ROS-CS-NP (Rosuvastatin-Chitosan Naoparticles) compared to plain-ROS showing extended release period up to 48 hrs.

The surface analysis of nanoparticles was studied with the help of FESEM. The nanoparticles showed well-formed spherical shape with smooth surface. Nanoparticles were found free flowing particulate forms which were separated from each other with maintained their size and shape. The surface morphology is shown in Fig. 1.

In- vitro drug release study was conducted in pH 7.4 phosphate buffer saline. Initial burst release of 11.89% followed by gradual sustained release of drug (~88.11%) towards the end of 48 h of study period as shown in Fig. 2 was observed from optimized formulation. This sustained drug release pattern quite essential for such type of cancer treatment.

Higuchi model (r=0.992) was found to be the best fitted diffusion based drug release pattern. Koremeyer-Peppas formula was used to describe the drug release mechanism more precisely.

'n' value of 0.87 in this study suggests a non-fickian mode of drug release and diffusion and erosion being the main mechanism of action. The statistical data of ANOVA for both Encapsulation efficiency and particle size was shown in Table 3.

In vivo study in animal model

Continuous administration of high amount of fat directly leads to hyperlipidemia in humans. The same technique has

Table 3. ANOVA of models for Y1 and Y2

Source	DF	Sum of squares	Mean square	*F* value	*P* value
Model for Y1	3	4015.12	2014.89	412.21	<0.005
A	2	1315.23	1537.90	509.11	0.0003
B	1	189.11	139.11	98.10	<0.005
C	1	915.34	400.21	189.23	0.002
Model for Y2	3	750.32	432.76	190.65	<0.001
A	2	890.65	540.09	487.03	<0.002
B	1	89.43	77.54	30.65	0.0003
C	1	72.47	40.32	87.30	0.0011

Table 4. Tabular representation of changes in clinical pathology and organ weight in animals after induction of hypercholesterolemia.

	Initial	Control	Week 4	Week 8	Week 12
Initial weight (g)	1100	1110	1225	1400	1575
Liver Weight (g)	84.20	84.20	90.35	102.15	110.5
Cholesterol (mg/dL)	117	117.1	121.24	124.12	125.23

been tested in laboratory animals which help to understand relationship between cholesterol metabolism and various diseases. Diet rich in triglycerides (containing cholesterol) has been fed in order to increase the level of cholesterol. Certain changes in clinical pathological data and organ weight were noted after hypercholesterolemia induction and are represented in Table 4. The body weight of the experimental animals was noted as unchanged in initial weeks which found to be lowering after succession of weeks.

The liver weight of was found to be increasing with increasing time period as the size of liver increases due to fatty deposits. We can observe significant amount of increase in weight at week 12 than initial week. The cholesterol level in body was increased with each passing week (117 mg/dL to 125.23 mg/dL). After considering all above results the animals with hypercholesterolemia were selected and used in further study. The animals that are grouped according to the diet fed were observed for numerous observations as shown in Table 5.

The blood lipid profile of experimental animals was shown in Fig. 3. The level of calcium, IL II-6, Total cholesterol, Low density lipoprotein (LDL) were estimated using blood collection techniques. From Fig. 3A it can be seen that calcium level (Resting value: 1.86 mg/g) in experimental animals of group I was found to be near to resting value while animals with no HC when treated with ROS, a slight increase in level of calcium was seen. In animals with HC when no ROS is administered maximum increase in level of calcium was seen. The reading values for group IV shows that ROS–NP were found to be effective in maintaining the blood calcium level to near to resting 1.9 mg/g. Figure 3B shows the variations in level of Interleukin II-6 (IL-II 6) levels (resting value: 8.12 mg/dL) in experimental animals group. Animals in group I showed nmajor change in basic value.

While for animals in Group II, ROS had lowered the level of IL-II 6 level to lowest possible which was increased to highest level in Group III where no treatment of ROS for HC animals was given. The animals in Group IV showed IL-II 6 to nearby resting level when treated with ROS-NP. Figure 3C shows level of Total cholesterol (T chol) i.e. cholesterol fractions in plasma, not in whole rabbit body (resting value: 71.56 mg/dL). As expected animals in group I showed no change in T chol level while animals with no HC when treated with ROS-NP showed slight decrease in level of total cholesterol as compared to

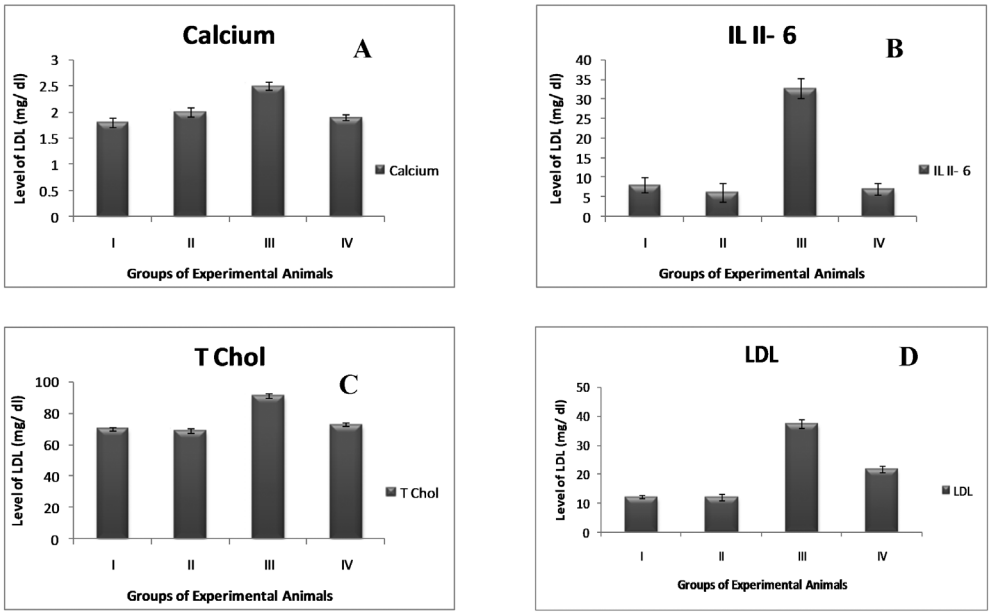

Figure 3. Graphical representation of blood Lipid Profile showing significant increase in level of lipids in experimental group (Group III and IV) with HC without RSV.
IL II-6, Interleukin II -6; T Chol, Total Cholesterol; LDL, Low Density Lipoprotein; HC, Hypercholesterolemia; RSV, Rosuvastatin

Table 5. Summary of data for each animal experimental group.

	Group I	Group II	Group III	Group IV
Tchol (mg/dL)	70.12 ± 1.16	68.92 ±1.43	91.37 ± 2.12	21.78 ±1.24
LDL (mg/dL)	12.23 ± 2.12	12.14 ± 1.09	37.39±1.21	21.78 ±0.99
IL II-6 (mg/dL)	8.12 ±0.98	6.21 ±1.09	32.12 ±0.87	6.98 ± 1.34
Calcium Level (mg/g)	1.69 ± 2.14	1.89 ± 2.17	2.47 ± 2.13	1.90 ±0.76

Tchol, Total Cholesterol; LDL, Low Density Lipoprotein Cholesterol; IL II-6, Interleukin 6

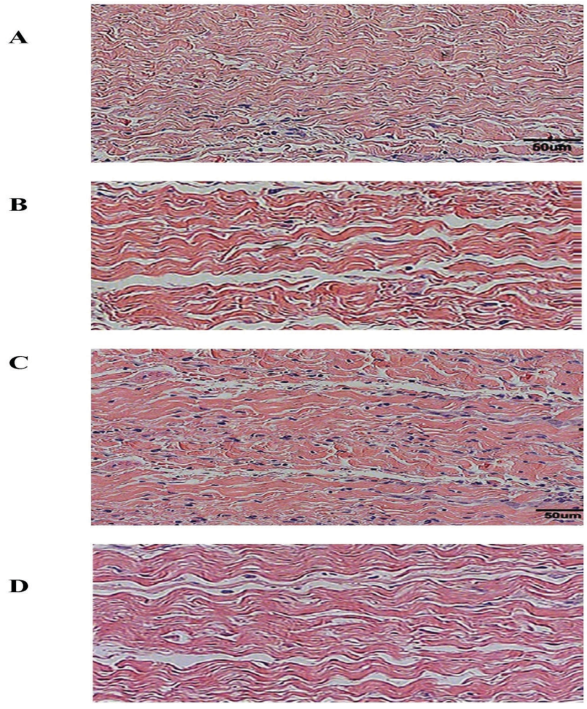

Figure 4. Histopathological Analysis of aortic valve for cellular infiltration study showing Microscopic Images of Group 1 (A), Group II (B), Group III (C), Group IV (D).

those in Group III where high level of total cholesterol were observed as HC animals were not treated with ROS-NP. The normal or resting value for T chol was observed for animals in Group IV which were HC and treated with ROS-NP. Figure 3D briefs about level of Low density Lipoprotein (LDL) in experimental animals (Resting value: 11.33 mg/dL). The animals that are having HC and were treated with ROS NP, only showed the value near to resting value while those with no HC and treated with ROS NP showed lower level of LDL. The results obtained here highlight the affectivity of ROS-NP in lowering the LDL in animals with Hypercholesterolemia (Rosenhek *et al.*, 2004). Rosuvastatin loaded chitosan nanoparticles were found to be significantly effective in lowering the blood lipid levels as compare to pure Rosuvastatin. It helps to attenuate calcification of various valve tissues in experimental animals.

Histopathological study reveals that cellular infiltration has markedly reduced in animals with HC. Microscopic examination was done and results were presented in Fig. 4.

CONCLUSION

In this research work hypercholesterolemia was induced in experimental animals by administering diet with Cholesterol and Cholic acid (1.25% and 0.5%, respectively). Blood lipid profile, interleukin 6 levels and histopathological study was performed. High level of cholesterol is one of the major risk factor for aortic valve calcification. Statin i.e. Rosuvastatin loaded chitosan nanoparticles were found to be significantly effective in lowering the blood lipid levels as compare to pure Rosuvastatin. It helps to attenuate calcification of various valve tissues in experimental animals.

Conflict of interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

REFERENCES

Allemann E, Gurny R, Deolker E (1993) Drug loaded nanoparticles: preparation methods and drug targeting issues. *Eur. J. Pharm. Biopharm.* **39**: 173–191

Aronow WS, Ahn C, Kronzon I, Goldman ME (2001) Association of coronary risk factors and use of statins with progression of mild valvular aortic stenosis in older persons. *Am. J. Cardiol.* **88**: 693–695. https://doi.org/10.1016/s0002-9149(01)01821-5

Calvo P, Remu˜n´an-L´opez C, Vila-Jato J L, Alonso M J (1997) Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *J. Appl. Polym. Sci.* **63**: 125–132. https://doi.org/10.1002/(SICI)1097-4628(19970103)63:1<125::AID-APP13>3.0.CO;2-4

Chan KL, Teo K, Dumesnil JG, Ni A, Tam J (2010) Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (astronomer) trial. Circulation AHA **19**: 306–314. https://doi.org/10.1161/CIRCULATIONAHA.109.900027. Epub 2010 Jan 4

Colli A, Gherli T, Mestres CA, Pomar JL (2007) Degeneration of native and tissue prosthetic valve in aortic position: do statins play an effective role in prevention? *Int. J. Cardiol.* **116**: 144–152

Farivar RS, Cohn LH (2003) Hypercholesterolemia is a risk factor for bioprosthetic valve calcification and explantation. *J. Thorac. Cardiovasc. Surg.* **126**: 969–975. https://doi.org/10.1016/s0022-5223(03)00708-6

Galante A, Pietroiust IA, Vellini M (2001) C-reactive protein is increase in patients with degenerative aortic valvular stenosis. *J. Am. Coll. Cardiol.* **34**: 1078–1081. https://doi.org/10.1016/s0735-1097(01)01484-x

Illum L (1998). Chitosan and its use as a pharmaceutical excipient. *Pharm. Res.* **15**: 1326–1331. https://doi.org/10.1023/A:1011929016601

Lindroos M, Kupari M, Heikkila J, Tilvis R (1993) Prevalence of aortic valve abnormalities in the elderly: an echocardiographic study of a random population sample. *J. Am. Coll. Cardiol.* **21**: 1220–1225. https://doi.org/10.1016/0735-1097(93)90249-z

Mehrotra A, Nagarwal R C, Pandit J K (2011) Lomustine loaded chitosan nanoparticles: characterization and *in-vitro* cytotoxicity on human lung cancer cell line L132. Chem Pharm Bull **59**: 315–320. https://doi.org/10.1248/cpb.59.315

Miller JD, Weiss RM, Serrano KM, Brooks RM, Berry CJ, Zimmerman K, Young SG, Heistad DD (2009) Lowering plasma cholesterol levels halts progression of aortic valve disease in mice. *Circulation* **119**: 2693–2701. https://doi.org/10.1161/CIRCULATIONAHA.108.834614

Olsson M, Thyberg J, Nilsson J (1999) Presence of oxidized low density lipoprotein in nonrheumatic stenotic aortic valves. *Arterioscler. Thromb. Vasc. Biol.* **19**: 1218–1222. https://doi.org/10.1161/01.ATV.19.5.1218

Palta S, Pai AM, Gill KS, Pai RG (2000) New insights into the progression of aortic stenosis: implications for secondary prevention. *Circulation* **101**: 2497–2502. https://doi.org/10.1161/01.cir.101.21.2497

Pandey R, Khuller G K. (2006) Oral nanoparticle-based antituberculosis drug delivery to the brain in an experimental model. *J. Antimicrob. Chemother.* **57**: 1146–1152. https://doi.org/10.2174/156720181666619020114481

Rajamannan NM, Subramaniam M, Caira F, Stock Sr, Spelsberg TC (2005) Atorvastatin inhibits hypercholesterolemia-induced calcification in the aortic valves *via* the Lrp5 receptor pathway. *Circulation* **112** (Suppl): I-229–I-234. https://doi.org/10.1161/01.CIRCULATIONAHA.104.5243

Roberts WC, Ko JM (2005) Frequency by decades of unicuspid, bicuspid, and tricuspid aortic valves in adults having isolated aortic valve replacement for aortic stenosis, with or without associated aortic regurgitation. *Circulation* **111**: 920–925. https://doi.org/10.1161/01.CIR.0000155623.48408.C5

Rosenhek R, Rader F, Loho N (2004) Statins but not angiotensin-converting enzyme inhibitorsmdelay progression of aortic stenosis. *Circulation* **110**: 1291–1295. https://doi.org/10.1161/01.CIR.0000140723.15274.53

Schoen FJ (1987) Cardiac valve prostheses: review of clinical status and contemporary biomaterials issues. *J. Biomed. Mater. Res.* **21** (A1 Suppl): 91–117

Stewart BF, Siscovick D, Lind BK (1997) Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. *J. Am. Coll. Cardiol.* **29**: 630–634. https://doi.org/10.1016/s0735-1097(96)00563-3

Wen L D, Shan S N, Ying L X, Zi R X, Cheng L F (2009) Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. *Carbohydrate Polymers* **75**: 385–389. https://doi.org/10.1016/j.carbpol.2008.07.039