Pre- and post-operative effects of statin on experimentally induced kidney chronic disease in Wistar rats

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Abstract

Background: Statins are selective competitive inhibitors to Hydroxymethylglutaryl-CoA reductase, an enzyme that catalyzes the initial phase of cholesterol biosynthesis. Statins have hypolipemiant action and pleiotropic effects that may include nephroprotection. The aim of the present study is to analyze pre- and post-operative effects of statin on experimentally induced kidney chronic disease (KCD) in rats.

Method: Forty Wistar male rats were pre-operatively randomized. To Group 1 (n=15), statin was orally administered for ten days pre-operatively, and for fifteen days after 5/6 nephrectomy. Rats of Group 2 (n=15) just received statin post-operatively. Rats of Group 3 (sham, n=10) were just submitted to surgery. Urine and blood samples were collected to analyze serum urea, serum creatinine and proteinuria at three different moments of the experiment. Rats that survived after an observational period of thirty days (n=34) were submitted to ex-vivo excision of the remnant kidneys. Histopathologic analysis was performed by using three stains (hematoxiline-eosine, Gomori tricromium and periodic-acid Shiffer) and four standard indexes (glomerulosclerosis, mesangiolysis, tubulointerstitial and vascular damage indexes).

Results: Statin treated groups presented lower means of urea in postoperative period. Group 2 had the lesser urea means. Statin induced proteinuria in healthy rats prior to nephrectomy. Proteinuria decreased after ten days after surgery in both statin treated groups. Group 2 showed lower mesangiolysis and tubulointerstitial damage scores when compared to other groups.

Conclusion: Use of statin slowed KCD progression in this animal experimentation model.

Introduction

Statins are selective competitive inhibitors to Hydroxymethylglutaryl-CoA reductase, an enzyme that catalyzes the initial phase of cholesterol biosynthesis [1,2]. They have powerful hypolipemic action but also present pleiotropic effects that may include nephroprotection [2].

Statins improve morphology and function on KCD by interfering in vascular complacency, endothelial function, nitrous oxide metabolism and proteinuria [1,3-5]. Several studies [2,6-14] were developed in order to analyze the use of statins on inflammation and fibrosis scenario on cellular and animal models. Some of them have demonstrated that statins diminished renal fibrosis on KCD [10,11,13,14]. Hartner, *et al.* described statin effects on mortality, proteinuria, glomerulosclerosis, macrophage infiltration, podocytes damage and glomerular ostheopœtin expression, but no effects on inflammation and cellular interstitial proliferation [15].

Wide use of statins in hypercholesterolemic patients has suggested that those drugs reduce cardiovascular risk in KCD patients and several clinical trials [16-23]. (HPS [16], CARE [17], PREVEND IT [18], D4 Study [19], AURORA [20,21], ALERT [22], SHARP [23]) had been proposed to study that effect. Relative risk for cardiovascular events were reduced in mild to moderate KCD patients in HPS [16] and CARE [17] studies. Patiens undergoing dialysis may not have their cardiovascular risk reduced by using of statin, as shown by 4D [19] and AURORA [20,21] studies, although other trials [22,24] including SHARP study [23], the larger of them, suggested the contrary.

A systematic revision [5] and two metanalysis [4,25] suggested that statin use reduces proteinuria levels, although PREVED IT study [18] showed no beneficial impact of statins on albuminuria for initial stages of KCD.

Using statins for preventing KCD is a less studied issue. Patti, *et al.* [8] have described lower incidence of contrast induced nephropathy after percutaneous coronary procedures when statins were given pre-operatively.

Subjects and methods

Fourty Wistar male rats (*Rattus norvegicus albinus, Rodentia mammalian*) were submitted to an experimental study using the remnant kidney model (5/6) nephrectomy). Their ages vary from 110 and 180 days, their weights vary from 200 g and 280 g. The rats were kept at Central Vivarium of PUC/PR through observation period under standard conditions – temperature between 20°C and 24°C, controlled lightning (12-hour day/night cycle: 8 am/8 pm) and air relative

humidity between 50 and 60%. *Ad libitum* water and standard feeding for species were offered, except within the twelve-hours preceding surgical procedure, when they did not take anything but potable water to drink.

This study was previously authorized by the Ethical Committee on Animal Experimentation of PUC/PR, where it is registered as number 572, and it followed strictly what Brazilian Law 1794 foresees.

The animals were pre-operatively randomized to receive orally administered statin for ten days pre-operatively, then fifteen days after 5/6 nephrectomy (Group 1, n=15), to receive statin for fifteen days post-operatively (Group 2, n=15) or surgery only (Group 3, sham, n=10). All animals received statin 0.2 mg/ml (Cravo & Canela® Compounding Pharmacy, Curitiba, Brazil), 2 mg/kg/day given by orogastric feeding tube.

Urine and blood samples were collected to analyze serum urea, serum creatinine, and proteinuria at three different moments of the experiment: D0 (two days before surgery), D10 and D30 (respectively ten and thirty days after surgery). Group 1 had other samples collected before starting to receive preoperative statin (D0c). Serum urea and creatinine were analyzed by colorimetric method (Bionostic® Laboratory, Curitiba, Brazil). Proteinuria levels were quantitatively assessed by specific kit (ChoiceLine10 Urinalysis®, Roche, Mannheim, Germany).

Anesthesia was induced by intramuscular injection of ketamine 50 mg/kg and xylazine 10 mg/kg. After trichotomy and ventral abdominal antisepsis, the rats were placed in horizontal dorsal decubitus position. A right-side nephrectomy followed by bipolar left-sided partial nephrectomy through midline ventral incision was performed. Per-operatory care included postural maintenance of airway permeability, prevention of bleeding through rigorous hemostasis, prevention of hypothermia and using of peritoneal analgesics.

Rats that survived after an observational period of thirty days after surgery (n=34) were submitted to ex-vivo excision of the remnant kidneys through similar incision. They were then sacrificed using ketamine (DL_{50}).

After that removal, kidneys were fixed with 10% formaldehyde solution, embedded in paraffin and cut into 2-mm sections. Histopathologic analysis was performed using three stains -- hematoxiline-eosine (HE), Gomori tricromium (GT) and periodic-acid Shiffer (PAS) and four standard indexes.

Glomerulosclerosis Index (GSI) [26,27] quantified mesangial matrix accumulation and sclerosis of the glomerular tuft through a score of 0 to 4. Mesangiolysis Score (MS) was used to analyze the presence of mesangiolysis, defined as mesangial matrix attenuation and/or mesangial cells degeneration [28] (Figure 1).

Subsamples of 100 glomeruli per animal were systematically studied through a semiquantitative score of 0 to 4. Tubulointerstitial changes characterized as tubular atrophy and dilatation, interstitial inflammation and fibrosis, were assessed using a semi-quantitative scoring system of 0–5 (Tubulointerstitial Damage Index, TDS [29]), modified by the authors (Figure 2). The Vascular Damage Score [30] analyzed interlobular vessels and small arteries through a semi-quantitative score of 0-4 (Table 1).

The results were expressed by means, medians, minimal and maximal values, and standard deviation. The Friedman test was used for comparing quantitative variables and percentual variations at three
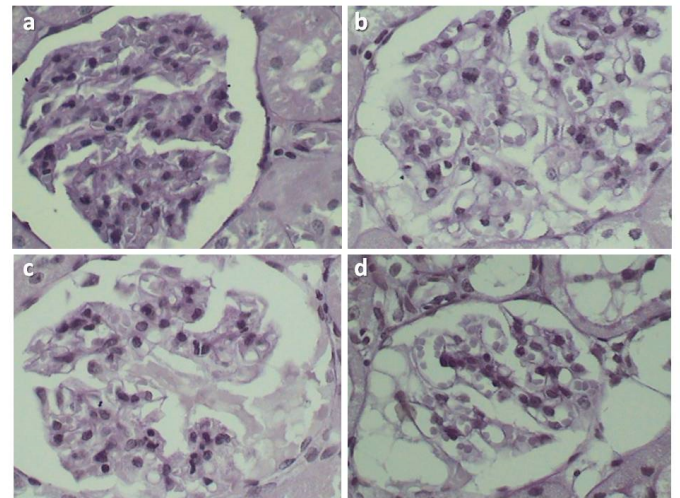

Figure 1. Mesangiolysis Score (MS). On periodic acid-Schiff stain one can observe: a) MS score 0 – no capillary changes; b) MS score1 – capillary dilatation and/or 25% of capillary convolute; c and d) MS score 2 - capillary dilatation and/or 25% of convolute capillary or capillary aneurisms, or 50% of capillary convolute
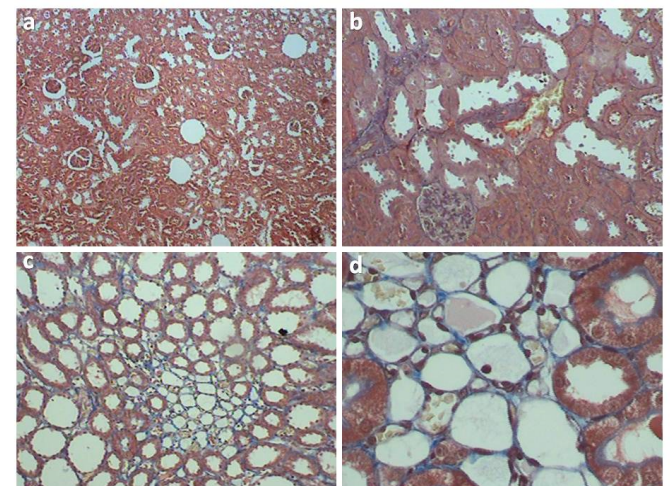

Figure 2. Tubulointerstitial Damage Index (TDI). On Gomori tricromium stain one can observe: a and b) TDI score 1 – normal tubulointerstitial structure; c and d) score 2 – tubulointerstitial damage up to 10% of analyzed area

moments of evaluation. The Wilcoxon signed-rank test was used for comparing quantitative variables at two moments of evaluation when suitable. Kruskal-Wallis one-way analysis of variance was used for comparing percentual variation at two moments between the groups. Significance level was p-value lower than 0.05. Fisher's exact test plus Bonferroni correction was used to evaluate histopathological scores when suitable.

Results

Thirty-four rats survived surgical procedure and observation period (Group 1, n=14, Group 2, n=12, Group 3, n=8). Overall mortality rate was 15%, with 10% morbidity. Five rats died during the experiment. Inferior vena cava bleeding was observed in one case. Post-operative deaths occurred in 4 cases. Non-fatal peroperative bleeding was observed in 4 rats, accidental intestinal loop damage in one, incisional hernia in one. Loose adherences of liver, spleen, pancreas and intestine to kidney poles were common at reoperation procedures.

Table 1. Histopathologic standard indexes

Score	Glomerulosclerosis index	Mesangiolysis score	Tubulointerstitial damage index	Vascular damage score
0	Normal glomerulus	No changes of capillaries	Normal tubulointerstitial structure	No thickening of vascular wall
1	Mesangial expansion or sclerosis up to 25% of the glomerular tuft	Capillary dilatation and/or 25% of capillary convolute	Tubulointerstitial damage up to 10% of analyzed area*	Mild thickening of vascular wall
2	Sclerosis of 25 to 50%	Capillary dilatation and/or 25% of convolute capillary or capillary aneurisms, or 50% of capillary convolute	Tubulointerstitial damage up to 25% of analyzed area	Moderate thickening of vascular wall
3	Sclerosis of 50 to 75% and/or segmental extracapillary fibrosis or proliferation	Capillary aneurysms comprising 50–75% of the capillary convolute	Tubulointerstitial damage 25% to 50% of analyzed area	Severe thickening of vascular wall
4	Global sclerosis, 75% of global extracapillary fibrosis or complete collapse of the glomerular tuft	Capillary aneurysms comprising 75% of the capillary convolute	Tubulointerstitial damage affecting 50% to 75% of analyzed area	Fibrinoid necrosis of the vascular wall
5	-	-	Tubulointerstitial damage in almost the entire area	-

*Veniant, *et al.* described a score from 0 to 4. The authors decided together with the pathologist to include one more rank between scores 0 and 1 (score 1 in the present study), in order to refine analysis of the tubulointerstitial area.

Table 2. Results of urea (mg/dl), creatinine (mg/dl) and proteinuria (mg/dl)

Group	n	Urea			Creatinine		Proteinuria	
		Moment	Mean*	p value	Mean**	p value	Mean***	p value
1	14	D0c	38.6	0.245****	0.5	0.367****	52.9	0.008****
		D0	41.7		0.6		265.7	
		D10	67.2	0.001	1.0	0.001	216.4	0.010
		D30	78.7		1.0		136.4	
2	12	D0	37.5		0.5		105.0	
		D10	70.2	0.001	0.8	0.001	301.3	0.002
		D30	65.7		0.9		85.0	
3	8	D0	41.4		0.5		157.5	
		D10	93.1	0.001	0.7	0.001	557.5	0.025
		D30	101.3		0.8		650.0	

Friedman's non-parametric test; p<0.05

*Serum urea mean: Group 1: D10>D0, p<0.001; D30>D0, p<0.001; D30>D10, p=0.080

Group 2: D10>D0, p<0.001; D30>D0, p<0.001; D30>D10, p=0.218

Group 3: D10>D0, p<0.001; D30>D0, p<0.001; D30>D10, p=0.049

** Serum creatinine mean: Group 1: D10>D0, p<0.001; D30>D0, p<0.001; D30=D10, p=0.056

Group 2: D10>D0, p<0.001; D30>D0, p<0.001; D30>D10, p=0.070

Group 3: D10>D0, p=0.001; D30>D0, p<0.001; D30>D10, p=0.195

*** Proteinuria mean: Group 1: D10<D0, p=1.000; D30<D0, p=0.005; D30<D10, p=0.005

Group 2: D10>D0, p<0.001; D30<D0, p=0.545; D30<D10, p<0.001

Group 3: D10>D0, p<0.044; D30>D0, p<0.004; D30>D10, p=0.254

**** The Wilcoxon signed-rank test was used for comparing quantitative variables at two moments of evaluation. Level of significance was p-value <0.05.

Increasing of serum urea levels was observed in all three groups (Table 2). Sham rats (Group 3) presented progressive increasing of urea, confirmed when comparing two samples at a time. Increasing of serum urea levels in the two statin treated groups was precocious and it was not confirmed when we compare D10 and D30 (Group 1, p=0,080; Group 2, p=0,218). Percentual variation analysis presented smaller variations in Group 2 in relation to Group 1 and Group 3).

Serum levels of creatinine increased in all groups nevertheless there was no difference between the groups or increasing levels after D10 for all of them (Tables 2 and 3).

Another blood and urine sample was taken from rats of Group 1, at the day they started to be given statin (pre-treatment sample or D0c). When comparing these samples to D0 samples, it was observed that serum levels of urea and creatinine were not affected by the drug, but proteinuria did, corresponding to a variation mean of 556.7% (p=0,008). Proteinuria decreased postoperatively but increased again until D30, with no significant difference between samples from D0c/ D0 and samples from D30 (Tables 2 and 3).

Group 2 presented precocious increasing of proteinuria, followed by decreasing until the end of the experiment. D0 and D30 levels were equal in this group. Both treated groups showed smaller levels of proteinuria in relation to Group 3 (Tables 2 and 3).

Glomerulosclerosis and vascular lesions were not observed in any group. Mesangiolysis was more evident in Group 3 rats. Smaller MSI scores were found in Group 2 when comparing to Groups 1 and 3 (p<0.001 for both). TDS scores were smaller in Group 2, despite equal results in Groups 1 and 3 do not support statistical analysis (Table 4).

Other histopathologic findings included intraparenchimatous abscesses (n=9, exclusively in treated groups), interstitial infiltrate (n=3, Groups 1 and 3) and intratubular dystrophic micro calcification (Group 1, n=5). Leukocytes were observed in one's rat urine sample (Group 1).

Discussion and Conclusion

Wide using of statin in the few last decades allowed researchers to observe many of its pleiotropic effects and to foresee its various uses in treating a range of diseases – linked or not to lipid metabolism. Many studies pointing to a role of statin in inflammation/fibrosis scenario [2,6-14], nephroprotection evidences [10,11,13,14] and diminished cardiovascular risks in KCD patients in animal models [31] and

Table 3. Percentual variation at two moments between the groups

Variable	Group	n	Urea*	p-value	Creatinine	p-value	Proteinuria**	p-value
D0-D10	1	14	70.2%		134.5%		150.5%	
	2	12	93.3%	0.056	74.9%	0.491	414.3%	0.113
	3	8	127.8%		47.0%		2908.1%	
D30-D10	1	14	21.2%		-5.8%		-31.5%	
	2	12	-5.2%	0.005	23.3%	0.082	-59.3%	0.009
	3	8	11.4%		7.8%		270.4%	
D30-D0	1	14	98.2%		123.7%		180.8%	
	2	12	79.4%	0.052	95.7%	0.260	23.1%	0.003
	3	8	151.9%		54.2%		2703.2%	

Friedman's non-parametric test; $p < 0.05$

*Serum urea percentual variation:

D30 – D10: Group 1 x Group 2 p=0.001; Group 1 x Group 3 p=0.299; Group 2 x Group 3 p=0.035

** Proteinuria percentual variation:

D30 – D10: Group 1 x Group 2 p=0.629; Group 1 x Group 3 p=0.006; Group 2 x Group 3 p=0.002

D30 – D0: Group 1 x Group 2 p=0.306; Group 1 x Group 3 p=0.000; Group 2 x Group 3 p=0.05

*** Kruskal-Wallis one-way analysis of variance was used for comparing percentual variation at two moments between the groups.

Table 4. Histopathological results

Histopathologic index	Score	Group 1	Group 2	Group 3	p-value
Mesangiolysis Score**	0	7	11	0	
	1	6	1	3	
	2	1	0	5	<0.001
	3	0	0	0	
	4	0	0	0	
Tubulointerstitial Damage Index	0	0	0	0	
	1	0	12	0	
	2	14	0	8	
	3	0	0	0	- -
	4	0	0	0	
	5	0	0	0	

*Glomerulosclerosis and vascular damage were not observed

**Mesangiolysis Score: Group 1 versus Group 2, p=0.012; Group 1 versus Group 3, p<0.001; Group 2 versus Group 3, p<0.001.

***Given that Groups 1 and 3 have equal results, statistical analysis was not applied.

clinical trials [16-23] have stimulated us on studying that drug effects on experimentally induced KCD in rats.

Remnant kidney model was capable of induce KCD in sham rats (Group 3). Sample collection was easily achieved, but experience from our pilot study contraindicates it to be done at the very same day of 5/6 nephrectomy because of high mortality. Peripheric blood sampling has lesser hemodynamic impact on rats than cardiac blood sampling. Preoperative care should include hypothermia and bleeding prevention as rats and mice presents many systemic alterations as reflex of renal ablation [32-34].

Increasing of serum levels of urea and creatinine is inherent to the model and it was achieved in Group 3 (sham). Using of statin decreased serum urea levels in both treated groups (1 and 2), more evidently in the group that received statin both pre and post-operatively.

Serum levels of creatinine precociously increased in all three groups, with no difference between them, suggesting that statin use did not change creatinine levels in this study.

Previous papers [1,3,4] showed that statins promote decreasing of proteinuria levels and they suggest three mechanisms for that: lesser glomerular protein filtration, blockage of filtered protein endocytosis and inhibition of inflammatory response. Douglas, *et al.* [4] studied fifteen clinical trials about statin in a metaanalysis, concluding that they are beneficial to patients with pathologic albuminuria. ALERT study [22], for instance, showed that fluvastatin did not have impact on proteinuria levels. Strippoli, *et al.* [25] showed that the reduction of proteinuria levels do not correspond to increasing in glomerular filtration rates, so the researchers question if proteinuria could have any clinical impact like bigger free-dialysis time.

Previous studies also state that almost all statins could promote proteinuria in patients that do not have KCD [4,5]. That effect is dose-dependent and directly proportional to the power of the particular statin, and it is more common with rosuvastatin, a statin that is highly concentrated and excreted by the kidneys. Statin induced proteinuria has tubular pattern, mostly low molecular weight protein, and it represents a transient state of higher tubular reabsorption [5]. In our statin pre-treated group (Group 1), levels of proteinuria decreased precociously after surgery, to increase again until D30 although there was no difference between initial and final levels.

Absence of glomerulosclerosis and vascular lesions in our sample, including Group 3, may be explained by the short observation period. Some papers only achieved histopathologic alterations after greater periods of time as ten weeks [35-37]. Santos, *et al.* [34], however, when comparing various models of inducing KCD in Wistar rats, observed full remnant kidney recovery in only eight weeks. More studies are needed to correctly evaluate progression of KCD in Wistar rats and proper windows of observation.

Mesangiolysis signs precedes glomerulosclerosis and were more evident in our sham group. The group that was treated post-operatively presented smaller scores of MSI when comparing to sham (p=0,012), so as smaller TDS scores, despite of subjectiveness of the last analysis. Histopathologic findings demonstrate that Group 2 presented lower scores of glomerular and tubular damages. The association of statin and post-operatory kidney abscesses was not reported until the moment of publication of this article.

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