THE HYPERLIPIDEMIC DIET INDUCES LOCALIZED AMYLOIDOSES IN HAMSTER

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The hyperlipidemic (HL) diet represents an important risk factor for atherosclerosis, coronary heart disease, stroke or hypertension. The amyloidoses are clinical disorders in which misfolded proteins accumulate extracellularly in tissues as insoluble fibrils, impairing the normal organ function. The correlation between hyperlipidemia and localized amyloidosis is not well documented so far. The aim of the present study was to determine if hyperlipidemia induces amyloid accumulation in the kidney, small intestine, pancreas and liver, organs that generate complications of the atherosclerotic disease. The experiments were performed on male Golden Syrian hamsters fed either a standard or a HL diet for up to 9 months. The animals were sacrificed, the collected organs were processed for paraffin embedding and the sections were stained with Congo red and thioflavin S for specific amyloid detection. After 9 months of diet, serum cholesterol and triglycerides levels were significantly increased in HL hamsters compared to control animals. The kidney, small intestine, liver and pancreas sections showed significant amyloid deposits compared to the control hamsters. The study brings another medical warning about the risks of the HL diet, not only in cardiovascular diseases, but also in the development of localized amyloidoses.

Keywords: Amyloidosis; Hamster; Hyperlipidemic diet; Kidney; Liver; Pancreas; Small intestine.

INTRODUCTION

Hyperlipidemia is an established risk factor for cardiovascular diseases at the level of the arterial walls, being a prerequisite for lesion initiation and for the subsequent development of coronary heart disease, stroke, hypertension, etc.

The impact of hyperlipidemia on the generation of amyloidoses – clinical disorders caused by extracellular deposition of abnormally folded proteins[1] – is less documented at present. Systemic amyloidosis, with amyloid deposits in the viscera, blood vessel walls and connective tissues are usually fatal[2]. Local amyloid formation affects certain organs, like the kidney, pancreas, liver, or brain. The factors that determine the organ distribution of amyloid deposits are complex and not well understood, some of the incriminating causes being genetic mutations[3] and aging.

Amyloid deposits induce clinical manifestations, impairing the normal function of the organs: in the brain, a cerebral amyloid deposit induces Alzheimer's disease[4]; in the pancreas, amyloid formation is associated with diabetes[5]; in the myocardium, the amyloid may induces cardiomyopathy, valvular heart disease or arrhythmia[6].

The association between hyperlipidemia and amyloidosis is still poorly understood. For kidney maladies, most of the data report hyperlipidemia as a secondary consequence for the renal disease and not an initiating risk factor. Patients with a nephrotic syndrome[7] develop hyperlipidemia as a consequence of renal insufficiency, or after renal transplantation, reflected in an altered apolipoprotein profile, as well as elevated plasma lipid levels[8]. Studies on experimental animals suggest that hypercholesterolemia induces the initiation and progression of glomerular pathology, feeding cholesterol to various animals inducing the develop-

ment of glomerular injury[9, 10]. Still there are no studies detecting the appearance of amyloidosis as a consequence of hyperlipidemia. The mechanisms whereby lipids may amplify glomerular injury are not completely understood, but may include an interaction with macrophages, alteration in vascular and mesangial functions, changes in the production of mediator substances or alterations in membrane fluidity[11].

Studies made on chronic, high-fat fed mouse model showed that beta-amyloid or its precursor protein expression are enhanced in enterocytes from the mouse small intestine[12]. The mature enterocytes have the highest rate of chylomicron production and one physiologic role of the amyloid was described as being related to the regulation of the lipid metabolism, enhancing the uptake of triglyceride-rich lipoproteins by fat-rich tissues[13]. The conclusion of these studies was that the small intestine amyloidosis is stimulated by high-fat diet.

In the pancreas, the amyloid cytotoxicity is thought to be an early mechanism involved in the disappearance of the insulin-producing beta cells from the Langerhans islets in type 2 diabetes mellitus[5]. It was reported that the transgenic mice expressing the amyloidogenic human islet amyloid polypeptide (hIAPP) in their beta-cells developed islet amyloidosis when fed a breeder chow containing increased amounts of fat[14]. No such effect was obtained in non-transgenic mice, yet.

While the liver is recognized as a major site of amyloid deposits and has an important role in lipid catabolism, hepatic amyloidosis has not been routinely described as a secondary effect of hyperlipidemia.

The aim of the present study was to determine if hyperlipidemia induces amyloid accumulation in the kidney, small intestine, liver and pancreas. In the atherosclerotic process the kidney, liver, pancreas and small intestine represent target organs affected by this disease. Our hypothesis was that beside the hyperlipidemia-induced affliction of the mentioned organs, amyloid deposition might be an aggravating factor for their malfunction. Results show that significant amyloid deposits are located in these organs from the HL hamsters.

MATERIALS AND METHODS

Animals and diet

Twenty male Golden Syrian hamsters, kept in standard housing conditions, were divided in two equal groups: (1) HL, fed a fat-diet (1% cholesterol and 15 % butter) for up to 9 months and (2) control, fed standard chow. The standard chow consisted of 22.8% protein, 4.4% fiber, 2.5% fat, and for the hyperlipidemic diet the standard chow was supplemented with 1% cholesterol and 15% commercial butter. Standard granules comprised a mineral mixture (g pulvis/100kg granule) containing: K iodine, 0.02; Cu sulfate, 1.28; Zn sulfate, 3.2; Mn sulfate, 15.5; ferrous sulfate, 20; Mg sulfate, 250; calcium carbonate, 200; potassium chloride, 650; Na chloride, 830; dicalcium phosphate, 1700, and a vitamin mixture (g pulvis/100kg granule) containing: vitamin A, 0.52; D3, 0.02; K3, 0.55; B1, 1; B2, 0.7; B6, 1.22; B12, 0.005; PP, 8; calcium pantothenate, 6. Male hamsters were 6 months old when the diet started and weighing 100-140g. Administration of the diet lasted for 36 weeks. All animal experiments have followed the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985).

Serum parameters

The animals were tested monthly for the lipid parameters in serum. The blood was collected from the retro-orbital plexus and total cholesterol and triglycerides concentration were assayed with commercial kits (Dialab, Austria).

Tissue processing

The hamsters were sacrificed after 9 months of HL diet. They were anesthetized by intraperitoneal injection of 5% chloral hydrate (1 ml/100g weight body). After laparotomy, the blood was washed out by transcardial perfusion through the left ventricle with 300 ml of 0.1M phosphate-buffered saline (PBS) containing 3 mmol/L $CaCl_2$ and the animals were perfusion-fixed with 4% paraformaldehyde in 0,1M phosphate buffer. The tissue specimens from kidney, small intestine, liver and pancreas were further fixed by immersion in 10% formalin for 24 hours at room temperature, washed in tap running water, dehydrated and embedded in paraffin.

Histochemical detection of amyloid

Paraffin sections (7 μm thick) of the tissues were processed for Congo red and thioflavin S histochemical specific staining to detect the presence of amyloid deposits (Sigma Chem. Co., USA). The sections were dewaxed in xylen, dehydrated in absolute alcohol and rehydrated in distilled water.

Congo red

We used the modified Highman's Congo red staining protocol. Shortly, we immersed the sections in Congo red solution (0,5% in 50% alcohol) for 20 min, rinsed in distilled water, rapidly differentiated in alkaline alcohol solution, rinsed in tap running water for 1min, counterstained with Mayer's hematoxylin for 30 sec, dehydrated in increasing concentrations of ethanol and finally mounted in Canada balsam.

Thioflavin S

The hydrated sections were stained in Mayer's hematoxylin for 5 min, washed in tap running water for 5 min, rinsed in distilled water and stained in 1% thioflavin S for 5 min. They were then differentiated in 70% ethanol for 5 min and after washing twice with water, the sections were finally mounted in 90% glycerol in saline.

The sections were examined and photographed under a Nikon Microphot-SA microscope, both in visible and ultraviolet light with B-2A filter cube (excitation/emission wavelengths of 450/520, FITC, green) in epifluorescence system.

RESULTS

In the present study, we report the histochemical detection of significant amyloid deposits in the examined organs of HL hamsters: kidney, small intestine, liver and pancreas, compared with the same organs from control animals. All the hamsters survived to the end of treatment and were sacrificed after 36 weeks. There was only a slight weight gain (~ 10% of the initial weight after 9 months of diet). Blood was collected monthly by retro-orbital puncture from all animals.

Serum parameters

During the 36 weeks of hyperlipidemic diet a constant increase of serum cholesterol and triglycerides was observed in the sera isolated from HL hamsters, as compared to control animals that kept the initial mean levels of cholesterol and triglycerides. As shown in Figure 1, a 7 fold increase of the control mean cholesterol level (from 118.28 ± 18.4 to 950.24 ± 317.19 mg/dl) and of the control mean triglycerides value (from 134.8 ± 20.6 to 954.5 ± 450.9 mg/dl) was determined after 36 weeks of fat diet.

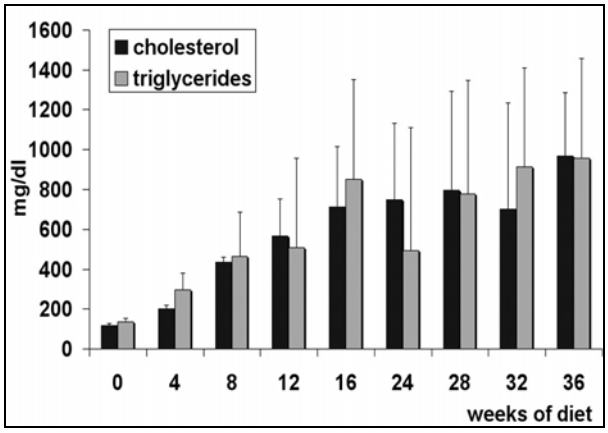

Fig. 1. Serum cholesterol and triglycerides levels during the 36 weeks of HL diet in hamsters. A constant increase of these parameters was observed in HL as compared to control animals. At 36 weeks of fat diet, a 7 fold increase of the control mean cholesterol level (from 118.28 ± 18.4 to 950.24 ± 317.19 mg/dl) and of the control mean triglycerides value (from 134.8 ± 20.6 to 954.5 ± 450.9 mg/dl) was determined.

Histochemical detection of amyloid

Examination of hematoxylin stained paraffin sections from HL hamsters kidneys showed areas occupied by glomeruli and proximal tubules (Fig. 2a). Serial sections from the kidney were stained with Congo red. In Figure 2b the Congo red labeling appeared as a pale pink color in visible light on the glomeruli; examined under UV light, the staining appeared as a strong red signal (Fig. 2c). A more significant fluorescent reaction for the amyloid presence was detected by using thioflavin S labeling (Fig. 2d); the fluorescent signal appeared not only in glomeruli, but also in the proximal tubules; some of the glomeruli had an intense reaction in the Bowman capsule zone as shown in Figure 2e, inset. The kidney of the control hamsters showed no specific reaction for amyloid (Fig. 2f).

The two histochemical labeling applied on the serial sections of the kidney showed that the thioflavin S staining was more sensitive, revealing more labeled areas than the Congo red. Thus we decided to eliminate the Congo red staining and for the other organs to use only the thioflavin S staining.

The Figure 3a represents a paraffin section of the small intestine of HL hamsters stained with hematoxylin. Thioflavin S staining of the section from the same organ showed a significant labeling of the lamina propria of the microvilli (Fig. 3b). In some areas where a few blood vessels were visible (Figure 3c), the vascular wall also appeared labeled. Figure 3d represents the phase contrast image of Figure 3c, and Figure 3e is a mixed image realized by superimposing the visible light and fluorescent images from Figure 3c and Figure 3d, to show better the labeling of the vessel wall. The thioflavin S staining showed no signal in the small intestine sections from control animals (Fig. 3f).

Hematoxylin staining of the liver sections from HL hamster shown in Figure 4a marks an area of the lobular ducts and Thioflavin S labeled a similar area presented in Figure 4b. The liver sections from control animals presented no amyloid deposits (Fig. 4c).

Hematoxylin staining of the pancreas sections from HL hamsters showed a disorganized histological structure of the acini (Figure 4d), suggestive of the damaging effect of hyperlipidemia on this organ. The thioflavin S staining in Figure 4e revealed amyloid deposits in large areas, located mostly extracellular, suggestive of vanished beta cells from the islets. The pancreas of control animals showed no thioflavin S reaction (Fig. 4f).

DISCUSSION

The goal of the present study was to establish a correlation between hyperlipidemia, the main risk factor for atherosclerosis, and the appearance of

localized amyloid deposits in organs known to be affected by this disease. To this purpose, we used male Golden Syrian hamsters as an experimental model[15], which simulates very well characteristics of human atherosclerosis, in terms of modifications of the blood vessels occurring during a saturated fat diet. The elevated levels (7 fold) of serum cholesterol and triglycerides in hamsters fed a HL diet for 9 months set the necessary condition to study the effect of hyperlipidemia in targeted organs in terms of amyloid deposits, namely: kidney, small intestine, liver and pancreas.

The underlying pathophysiologic mechanisms for the implication of increased lipid levels on the progression of renal disease are not yet fully understood. Beside the studies describing hyperlipidemia as a consequence of different kidney maladies (nephrotic syndrome, allograft dysfunctions, etc.), there are data showing that the oxidative stress and insulin resistance may mediate the lipid-induced renal damage. Cholesterol-feeding of various experimental animals induced the development of glomerular injury[8].

We have chosen the kidney to test two of the histochemical methods for detection of amyloid deposits: Congo red and thioflavin S. Our experiments have shown that the Congo red staining of the amyloid deposits is less sensitive than thioflavin S. There are reports in the literature stating that Congo red staining can be technically difficult, particularly if tissue sections are 5 μm thick. The sensitivity of Congo red staining is substantially lower in many of the familial amyloidoses[1]. Therefore, the absence of Congo red labeling does not eliminate a positive diagnostic. Our data were in good agreement with these reports. Thus, the use of thioflavin S fluorescent staining gave an extra labeling of the kidney structures; the glomeruli, labeled also by Congo red, as well as the proximal tubules were stained with thioflavin S. Some of the glomeruli presented an intense reaction on the Bowman capsule zone. Therefore, we have further used the thioflavin S histo-staining for the detection of amyloid deposits in the other organs examined.

Our data also demonstrate that hyperlipidemia induces formation of the amyloid deposits in the small intestine of the saturated fat-fed hamsters, compared to control animals. These results are in accordance with the study made in the mouse model[12], where the beta-amyloid or its precursor protein expression were enhanced in the enterocytes of the small intestine in response to saturated fat feeding. In addition, we found a significant labeling of the lamina propria of the microvilli. In some areas where a few blood vessels were visible, the vascular wall also appeared labeled.

It is known that hyperlipidemia is a risk factor in diabetes. Data from literature show that fibrillar protein deposits (amyloid) in the pancreatic islets of Langerhans are thought to be involved in the death of the insulin producing beta cells islets in type 2 diabetes mellitus[5]. In our study large extracellular areas were labeled with thioflavin S in the pancreas of HL hamsters. We suggest that these areas were previously occupied by beta-cells islets which disappeared during the high-fat diet. Our data can be correlated with the findings of Hull *et al.*[14] who detected islet amyloid deposition in the pancreas of transgenic mice expressing the amyloidogenic human islet amyloid polypeptide (hIAPP) when fed a breeder chow supplemented with increased amounts of fat. In contrast, non-transgenic mice have adapted to the diet-induced obesity and did not show the same effect. Together, these results suggest that the effect of hyperlipidemia of enhanced islet amyloid formation might play a role in the pathogenesis of the islets lesion in type 2 diabetes in humans.

The amyloid can be deposited in the liver in most forms of systemic amyloidosis, where there are always widespread vascular and sometimes also interstitial amyloid deposits[16]. It was suggested that hepatic amyloidosis can be rather the cause of severe hyperlipidemia than a consequence of it[7]. Our data suggest that hyperlipidemia may induce the amyloid formation in some areas of the liver, namely around the lobular ducts.

The histochemical staining used in this study for detecting amyloid deposits do not identify which type of amyloid is contained in a specific organ; the specific method for identifying the amyloidogenic protein is mass spectrometry or amino acid sequencing of proteins that are extracted from amyloid deposits[1]. These techniques are not available routinely; the most used method in the clinical setting is immunofluorescence or immunohistochemical staining of the tissue by using antibodies that are directed against known amyloidogenic proteins.

In conclusion, our results show that a HL diet that induces systemic hyperlipidemia may generate the formation of localized amyloid deposits in the kidney, small intestine, liver and pancreas. These data represent another warning signal for the induction of more pathogenic consequences of a saturated fat-rich diet.

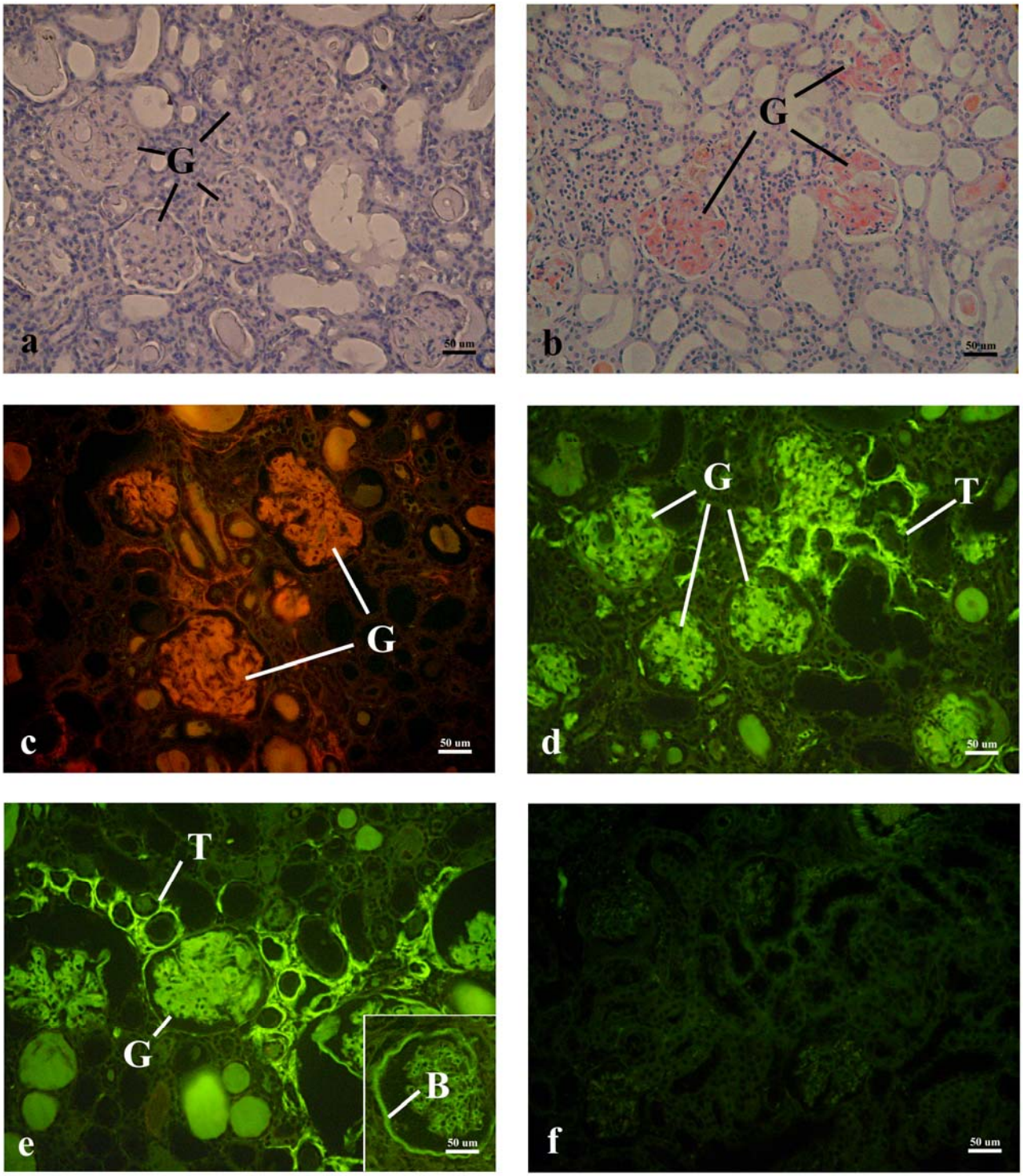

Fig. 2. Amyloid specific staining on paraffin kidney sections from HL hamsters:

a. Hematoxylin staining showing areas with glomeruli (G); b. Congo red staining, examined in bright field, showed the pink signal on glomeruli; c. Congo red staining, examined in fluorescence, displayed as a strong red signal; d. Thioflavin S staining, examined in fluorescence, showed the labeling of glomeruli and the proximal tubules (T) ; e. Detailed labeling of glomeruli and proximal tubules; some specimens showed a marked staining of the Bowman capsule (inset, B); f. Thioflavin S staining on kidney section from control hamsters showed no specific labeling.

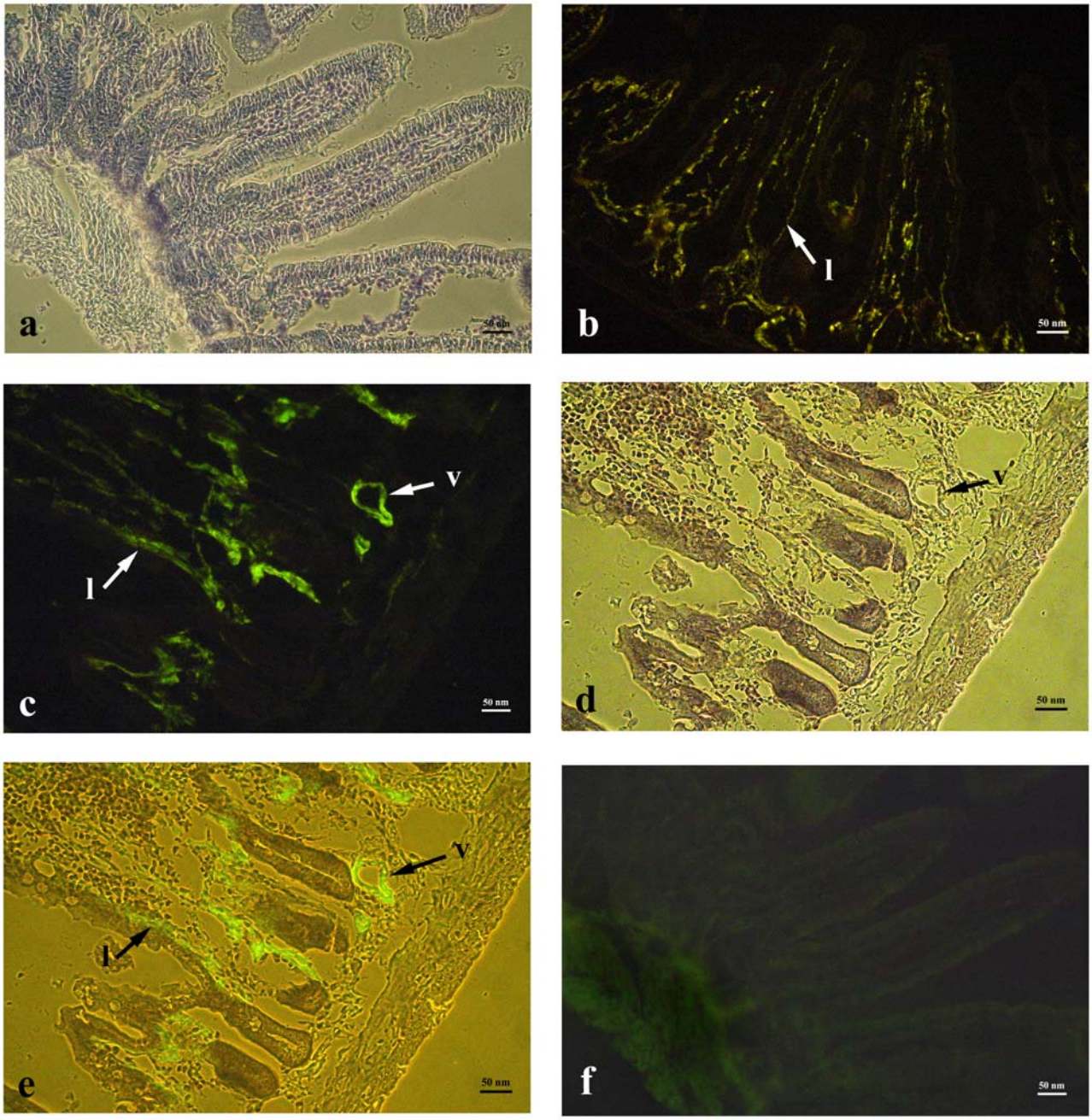

Fig. 3. Amyloid specific thioflavin S staining on paraffin sections from the small intestine of HL hamsters:

a. Hematoxylin staining showing an area of microvilli; b. Thioflavin S staining labeled the lamina propria of the microvillus (l, arrow); c. Amyloid staining appeared also on small vessel wall (v, arrow); d. Phase contrast image of the bright field of c; e. Superimposed images from c and d; f. Thioflavin S staining showed no signal on the small intestine sections from control animals.

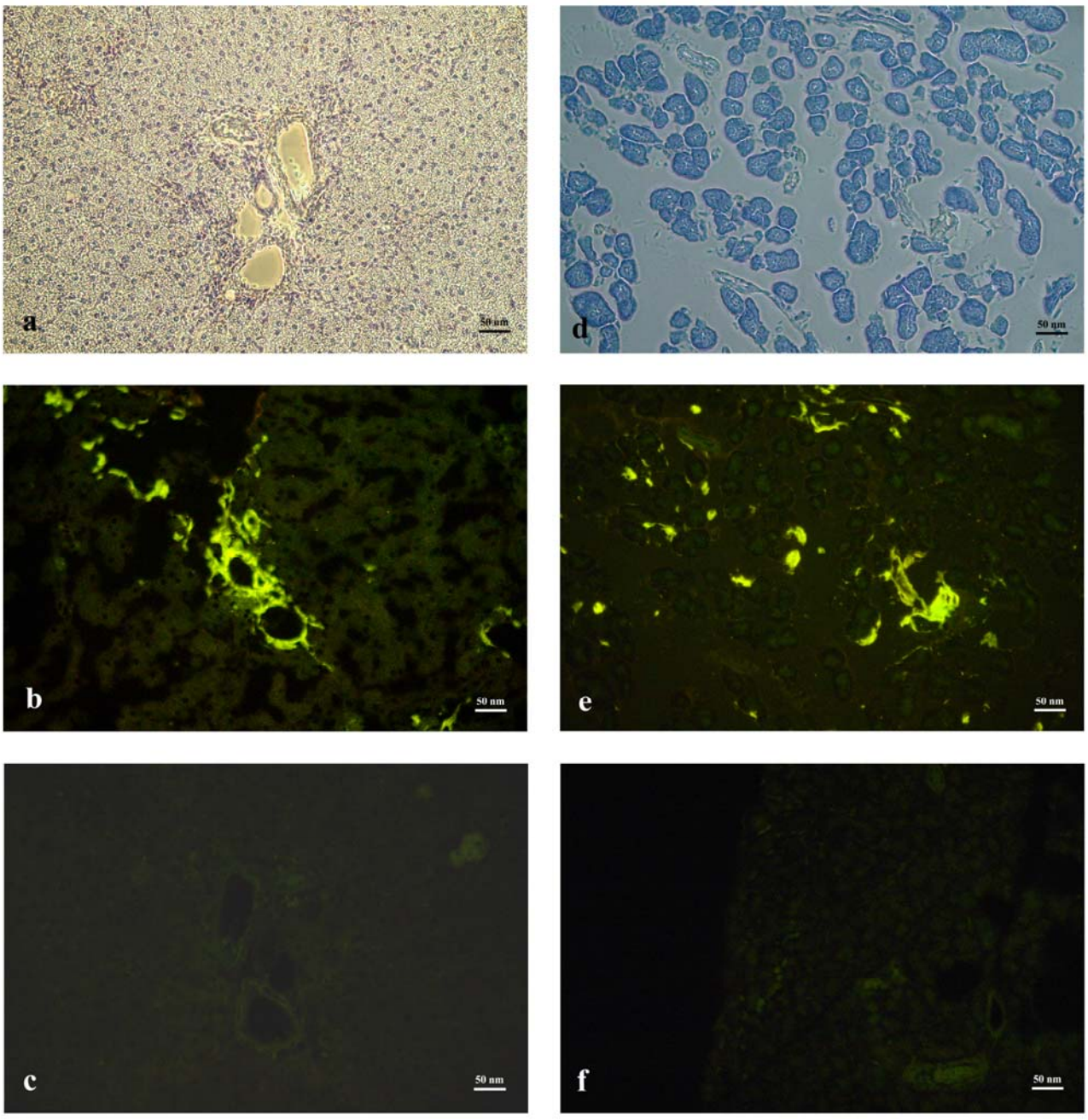

Fig. 4. Amyloid detection by specific thioflavin S staining on paraffin sections from the liver (a,b,c) and pancreas (d, e, f) of HL hamsters:

a. Hematoxylin staining on liver section showing the area of lobular channels; b. Thioflavin S staining labeled areas around the lobular channels; c. No positive labeling on liver sections from control animals; d. Hematoxylin staining on pancreas section showing a disorganized pattern of the histological structure; e. The thioflavin S staining labeled some interstitial areas on pancreas section; f. No positive reaction on pancreas sections from control animals.

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