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Synchrotron Based Phase Contrast Tomography of Hyper cholesteromic Rat Liver

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ABSTRACT

X-ray phase contrast imaging technique has been applied for the study of morphological variations in soft tissues. The effect of an antioxidant, α-lipoic acid in reducing hypercholesterolemia in rats is investigated. The experiment was conducted to measure serum lipid profile and diameter of vessels in rat liver, as liver is the most vital organ in hypolipidemic activity studies. ***Methods:*** *Four groups of male Wistar rats, control (Group I), hyperlipidemic (Group II), positive control (Group III) and treated Group IV)) were studied for serum lipid profile and liver vessels with synchrotron X-ray phase tomography. The Group I rats received chow diet, in Group II rats, administration of 20% butter rich diet induced hyperlipidemia. Group III, treated rats received hypolipidemic drug Atorvastatin and Group IV animals received a potent antioxidant DL-α-Lipoic acid. The excised liver tissue immersed in 10% formalin. X-ray phase contrast tomography was performed for comparison of diameter of liver vessels.* ***Results:*** *Among the four group of animals, the diameter of liver vessels was much larger in hypercholesterolemic rat (Group II). The liver vessel diameter comparison with X-ray phase contrast tomography and the lipid profile shows reduction in serum lipids and lipoproteins by ALA treatment.*

Keywords: Phase contrast imaging (PCI), hypercholesterolemic, antioxidant, DL-α-Lipoic acid, volume rendering

INTRODUCTION

Applications of advanced X-ray imaging techniques in medicine research are increasing with the availability of synchrotron-based facilities. In the absorption based X-ray imaging, the contrast is generated by the differences in attenuation within the sample. The absorption coefficient for X-rays is proportional to the fourth power of atomic no. of element (Z^4). Hence, absorption based X-ray images of soft tissues have poor contrast. The technique is therefore not suitable in imaging of weakly absorbing materials like soft tissues, which are composed of low atomic no. elements like carbon, oxygen, nitrogen. To improve the contrast in X-ray absorption images, contrast media is often needed. The X-ray phase contrast imaging based on the X-ray phase modulation caused by the sample overcomes this difficulty and has gained attention in the recent years [1,2]. The X-ray refractive index is $n = 1 - \delta + i\beta$, where δ is the refractive component and β is absorptive component, both of them are wavelength dependent. The contrast in the absorption based images is obtained from changes in β. In the X-ray phase contrast imaging, contrast depends not only on absorption but also on the variations in δ, real part of refractive index. At 10 keV to 100 keV energy range the real part of refractive index (δ) is 1000 times more than β [3-6].

The limitations of absorption based imaging methods to visualize the microstructures of soft tissues have encouraged the applications of phase contrast imaging in the biomedical research [7-9]. This novel X-ray imaging technique based on the phase effects has been applied suitably for imaging of different soft tissues without the contrast media [10-14]. The applications of this technique include imaging of the blood vessels in animals [15-17]. X-ray computed tomography (CT) is an extensively used technique for the structural visualization in three dimensions. The introduction of phase effects into X-ray CT has led to the development in X-ray phase tomography [18]. Phase contrast tomography

and its applications in quantitative studies of tissues are reported [19-21]. The technique has the potential to reveal microstructures of biological tissues [22]. The lab sources are the common and inexpensive source of X-ray but the medical applications of phase contrast imaging demand synchrotron source, which provide strongly collimated and coherent beam [23-25].

The development of a disease is associated with the changes in the microstructure and morphology of the organ. High contrast microstructural visualization of rat liver by phase contrast tomography and the study of microvasculature of liver fibrosis in rats have been reported [26,27]. One of the major health concerns these days is hypercholesterolemia also known as hyperlipidemia, which is one of the major causes of the development of cardiovascular disorders [28]. It evokes damages in various tissues, thus deregulates the cellular functions leading to various pathological conditions [29]. Elevated blood lipid levels, like total cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL), and a decrease in high density lipoprotein (HDL) are directly associated with hyperlipidemia and atherosclerosis [30]. There are various known lipid lowering drugs, such as fibrates, statins, and bile acid sequestrants [31], but due to their side effects and involvement of oxidative stress in hyperlipidemia [32] attention is paid to various antioxidants, which may have a potential role in curing hyperlipidemia.

One of the antioxidant, α-lipoic acid (ALA) is known to directly quench free radicals, inhibits reactive oxygen-generation and regenerates other antioxidants [33]. ALA functions as a cofactor in multi-enzyme complexes, including pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, and branched-chain alpha-ketoacid dehydrogenase. In the present study, we sought to determine whether ALA is able to alter blood lipid levels in hypercholesterolemic animals and it is further supported by technique synchrotron phase tomography. The projection images obtained through tomography are quantified for diameter of liver vessels and compared for animals in different groups.

MATERIALS AND METHODS

Chemicals

DL-α-lipoic acid (ALA) was procured from Himedia Laboratories Pvt. Ltd, Mumbai, India. Atorvastatin tablets were procured from Lupin Pharmaceuticals Limited, India.

Animal preparation

Male albino rats of Wistar strain weighing 180 g to 200 g were kept in polypropylene cages at an ambient temperature. Animals had free access to feed and water. The experiments were performed according to the guidelines of the institutional animal ethics committee (IAEC).

Experimental design

Rats were divided into four groups, each consisting of six animals. Group I served as the control. Group II animals were fed 20% butter in standard chow laboratory diet for 30 days. Group III animals along with butter diet were given anti-hypolipidemic drug, atorvastatin (10 mg/kg) in the form of oral emulsion from 15th day through gavage. Group IV along with butter diet received α-lipoic (25 mg/kg body weight, orally) daily from 15th day. On 30th day, all the animals were anesthetized and decapitated.

Sample collection

Liver was excised immediately and washed three times with ice cold 0.1 M phosphate buffered saline (PBS, 1:9), pH 7.4. The tissue was blotted dry and fixed in the Bouin's solution for phase contrast imaging. Blood was collected by cardiac puncture and was allowed to clot at room temperature followed by centrifugation at 2000 × g for 10 min to obtain serum for lipid profiling.

Lipid profiles estimation

The serum TC, TG, LDL, HDL and VLDL was measured using commercial enzymatic kits (Beacon Diagnostics Pvt. Ltd., India) and were analyzed on UV-1800 Shimadzu spectrophotometer.

Experimental set-up

The phase tomography experiment was carried out at Imaging Beamline (BL-4) at Indus-2 synchrotron radiation source operating at 2.5 GeV energy and current 200 mA. This beamline provides broad white band of electromagnetic

radiation with high intensities and using a double crystal monochromator of Si (111) type, suitable energy in the range of 8 keV to 35 keV can be selected.

The experimental set up for propagation based phase contrast imaging at imaging beamline consists of motorized precision translation-rotation stage and an imaging detector. The sample holder has a centrally fitted chuk for holding the samples. The imaging detector is a high-resolution CCD camera with an active area of 4 k × 2.5 k pixels; each pixel is of 4.5 micron, with Gadox scintillator at its input face coupled to the CCD via fibre-optic [34,35].

The excised whole liver of rat was placed in a plastic vail with a transparent region for beam to pass and the vail was mounted on the rotating sample stage as shown in Figure 1. The sample to detector distance (SDD) optimized was 300 mm for the liver samples of the control, hyper cholesterolemic, atorvastatin and lipoic acid treated rats. The beam energy 16 keV with an exposure time of 600 milliseconds was used to obtain 900 projections with angular increment of 0.2°. Dark field and flat field corrections were performed to limit CCD and beam related artifacts during reconstruction. Two-dimensional tomographic slice images and volume rendered images were produced with the suitable software [36,37]. All acquired images were analyzed using ImageJ [38].

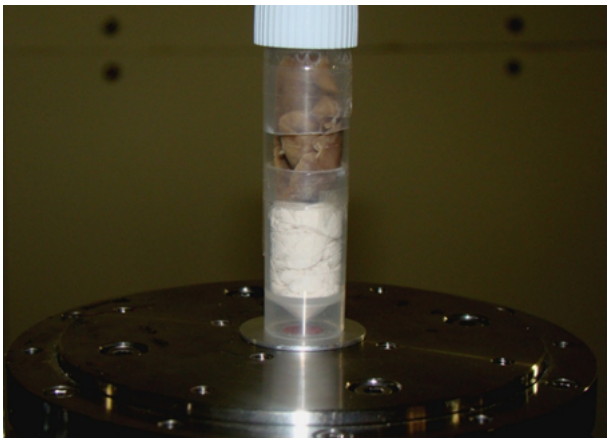

Figure 1 Liver sample of rat mounted in plastic vail with a transparent region for synchrotron beam

Statistical analysis

The results obtained were analyzed by the SPSS software package version 20. The mean ± SE values obtained for the different groups were compared by one-way ANOVA, followed by *post-hoc* Tukey's (HSD) test.

RESULTS AND DISCUSSIONS

Lipid profile parameters

The present study aims at evaluating the structural changes in soft tissues like liver in diseases such as hyperlipidemia, including hyper cholesterolemia and hypertriglyceridemia, which are considered as major risk factors in the development of cardiovascular diseases [39]. High fat diet induced hyperlipidemic rats showed significant increase in serum TC. A significant decrease in TC in ALA treated rats as compared to hypercholesterolemic rats was observed. It is suggested that due to increased transfer of blood cholesterol for bile synthesis bile lead to increased biliary excretion of cholesterol, resulting in lesser cholesterol being incorporated into lipoproteins. Serum TC, TG, LDL and VLDL were significantly increased in hypercholesterolemic animals (Table 1). Oral administration of atorvastatin significantly normalized the levels of TC, TG, LDL and VLDL as compared to hypercholesterolemic animals (Group II).

Table 1 Effect of ALA on serum lipid profile

Groups	TC	TG	LDL	HDL	VLDL
I	81.16 ± 6.39	27.33 ±1.14	34.33 ± 3.48	37.33 ± 1.40	5.50 ± 0.22
II	135.33 ± 2.02^{c}	67.66 ± 4.29^{c}	76.83 ± 2.66^{c}	33.66 ± 1.33^{NS}	13.33 ± 0.76^{cz}
III	88.50 ± 2.59^{z}	42.00 ± 2.98b^{z}	52.66 ± 4.01b^{z}	34.83 ± 4.25^{NS}	8.33 ± 0.66^{bz}
IV	105.33 ± 2.12^{bz}	53.50 ± 1.60^{cy}	55.83 ± 3.70^{b}y	31.33 ± 2.43^{NS}	10.83 ± 0.40^{cx}

Values in mg/ml are mean ± SE of 6 animals. The data were analyzed by one-way ANOVA, followed by *post-hoc* Tukey's (HSD) test. $^{a}P<0.05$, $^{b}P<0.01$; $^{c}P<0.001$ as compared to control group (Group I). $^{x}P<0.05$, $^{y}P<0.05$; $^{z}P<0.01$ as compared to hypercholesterolemic group (Group II)

Oral administration of ALA significantly decreased TC, TG, LDL and VLDL as compared to Group II. Significant increase in TG in Group II animals may be due to decreased activity of lipoprotein lipase enzyme, which is involved in clearance of plasma lipids into fatty acids and glycerol [40]. Thus, ALA may increase lipoprotein lipase activity, which helps in clearance of LDL from blood serum. LDL is a risk factor and plays a role in development of atherosclerosis [41,42]. Antioxidants, decrease oxidative stress and protect LDL from oxidation, which forms the basic strategy with for the prevention of atherosclerosis and associated cardiovascular disease [43].

The results suggest that high fat diet, which resulted in elevated serum lipids and lipoprotein fractions, was reduced by ALA treatment. This normalcy is evident from serum lipid profiling and confirmed by phase contrast imaging of the liver tissues, suggesting that it would be beneficial in treatment of hyperlipidemic cardiomyopathy.

X-ray phase contrast tomography

Among various modes of X-ray phase contrast imaging techniques, inline phase contrast imaging is the simplest and easiest to implement with a partially coherent source and high-resolution CCD detector. In this study, we have used inline phase contrast tomography to study the morphology and the internal microstructure of rat liver in control (Group I), hypercholesterolemic (Group II), rat administered with atorvastatin (Group III) and lipoic acid (Group IV).

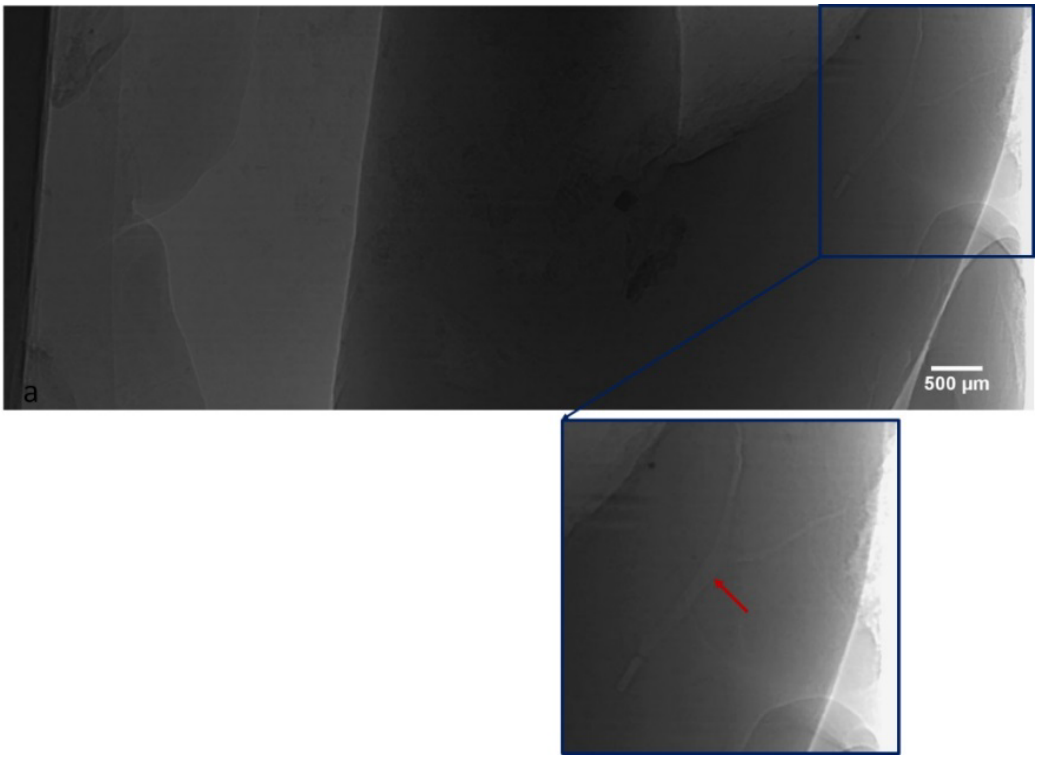

Figure 2a Projection images of a section of liver obtained with synchrotron phase tomography (a) control rat (Group I)

Figure 2a and 2d shows the projection images of control, diseased (Group II) and treated (Group III and Group IV) rat liver for the region of interest, obtained using phase contrast imaging. The projection images show visible vessel boundaries marked with red arrows. The projections have overlapping features; thus, clarity of structural features is affected. To overcome this limitation, we have carried out micro-tomography reconstruction to obtain the tomographic slice images. An inline phase image has a complex combination of contrast generated due to attenuation and phase modulation within the sample and requires phase retrieval techniques for quantitative phase image extraction before tomographic reconstruction for 3D imaging if distribution of electron density is sought. However, we have applied phase contrast tomography without phase retrieval by applying filtered back projection to inline phase image which is reasonably good for the study of morphological variation in the sample caused by induced drugs. Inline phase contrast is helpful here for enhancing the contrast at the boundaries of the veins not visible in the absorption contrast images.

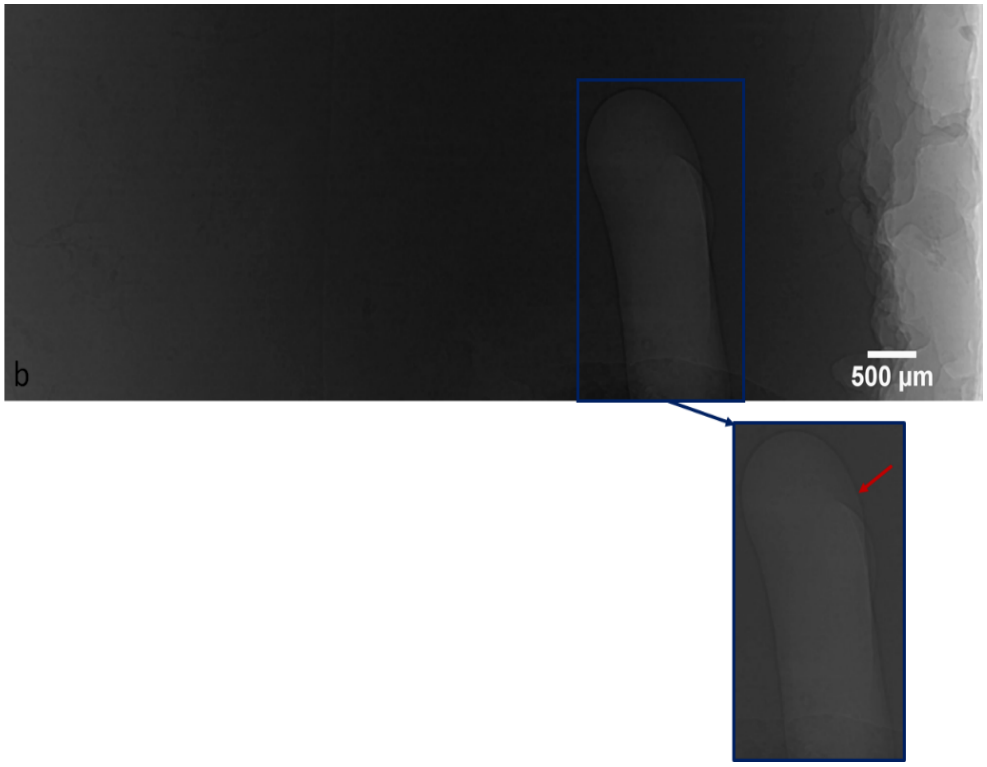

Figure 2b rat fed with cholesterol diet (Group II)

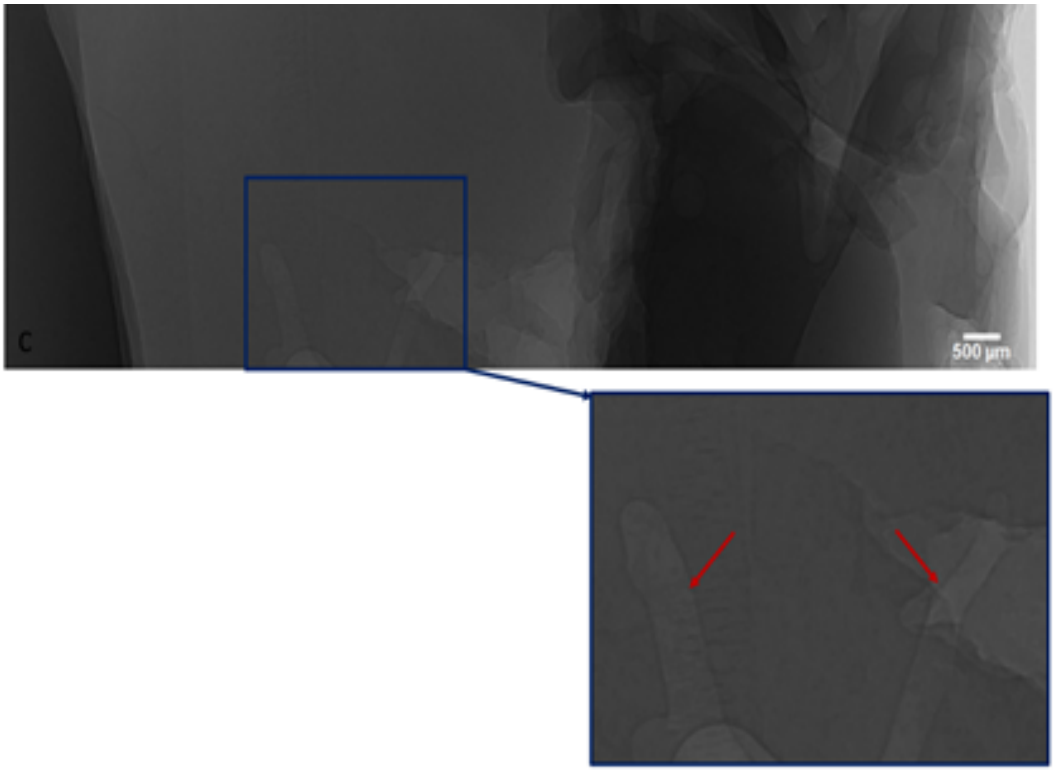

Figure 2c rat administered with Atorvastatin (Group III)

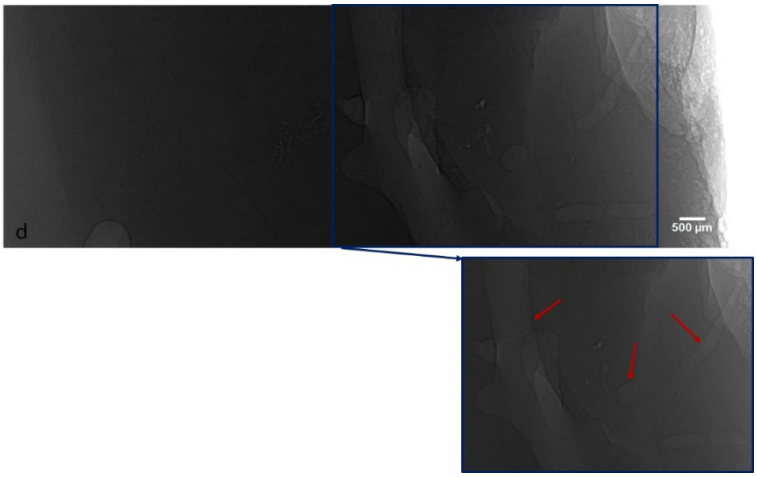

Figure 2d rat administered with α-Lipoic acid (Group IV). The arrows in red indicate the vessels in the liver

The local structures of veins distribution can be seen in reconstructed tomographic slices of all the samples under investigation as shown in figures (Figure 3a and 3d). The vessels in the liver tissues of all the types were observed as voids in the X-ray phase tomographic slices. In order to visualize in 3D volume rendering was applied to the stack of tomography reconstructed slice images of the liver samples. The two-dimensional projection images were transformed into a volume dataset in three dimensions. The vessels were quantified from the tomographic slices of liver after segmentation in Image J.

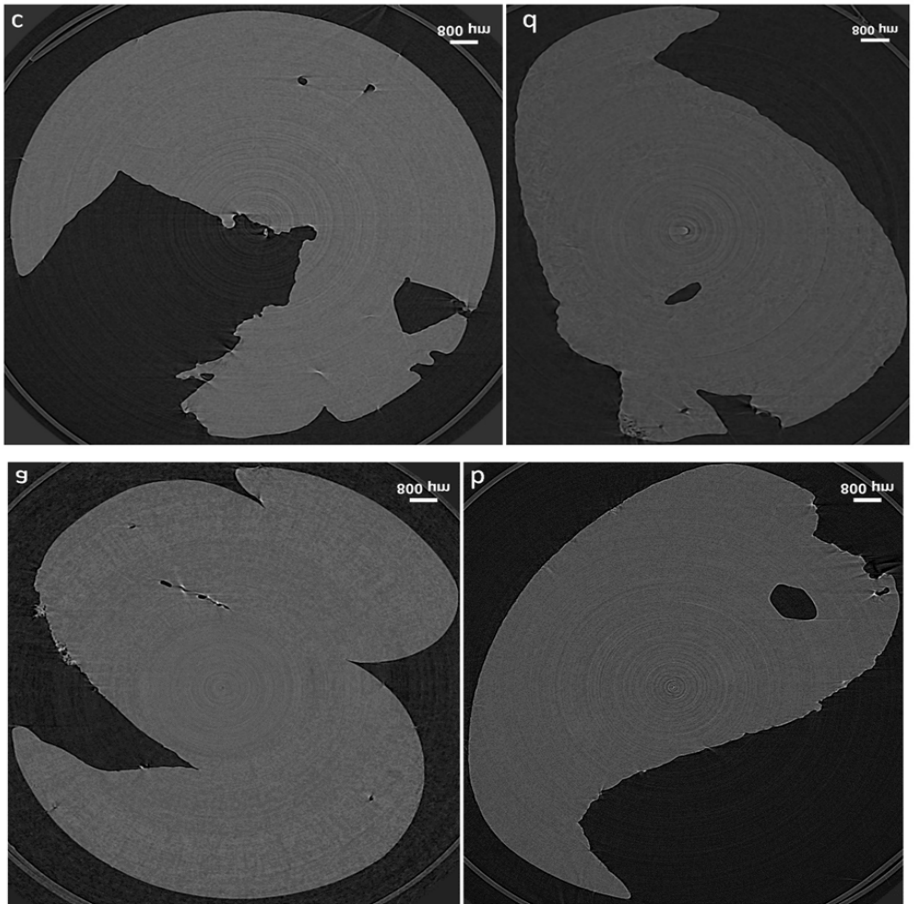

Figure 3a and 3b The tomographic slices show the vessel cross-sections in liver of rat as voids

Table 2 shows the vessel diameters for rat liver in Group I to Group IV. The vessels with smallest diameter 41.48 micron and largest is 241.49 micron are imaged in the control rat. In Figure 4a and 4b the volume rendering of section of liver using phase contrast tomography of the control rat liver depicts the vessels.

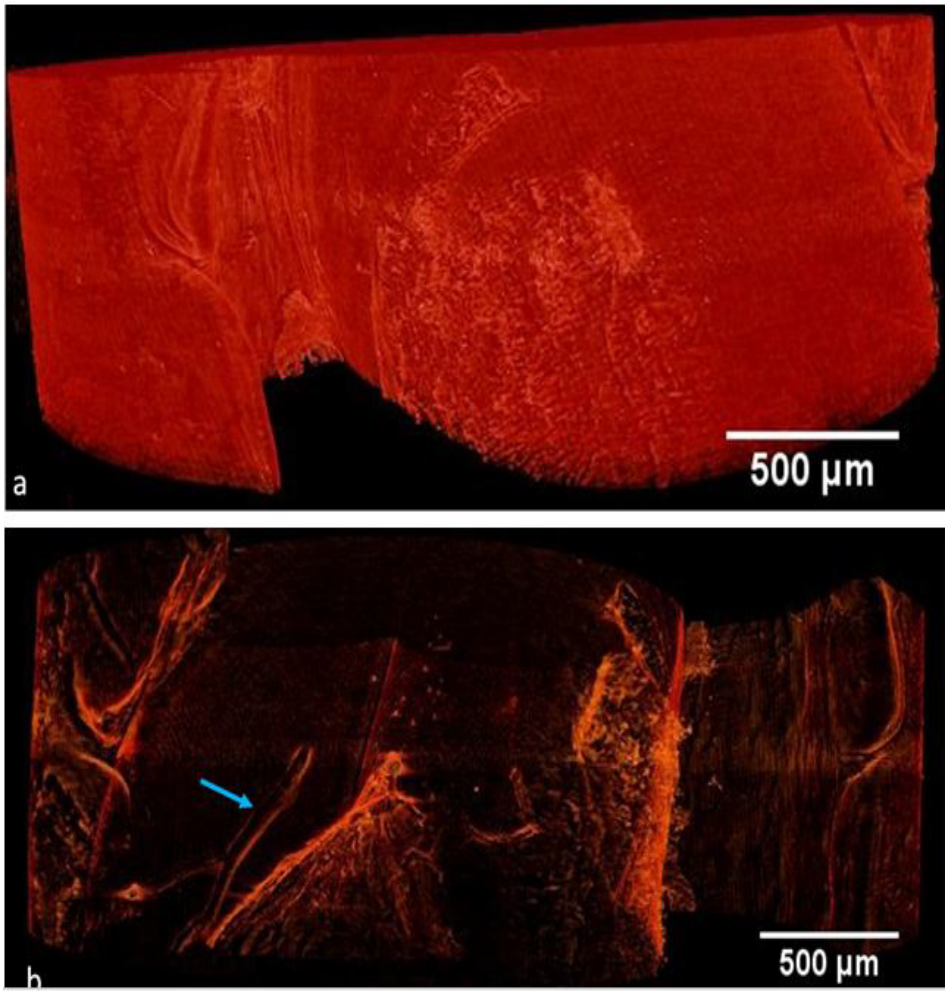

Figure 4a and 4b Volume rendered images shows vessels in liver control rat (Group I) with visible liver vessels marked with arrow in blue

Table 2 Comparison of diameter of vessels (in micrometers) in rat liver

Groups	Smallest vessel imaged in liver	Largest vessel imaged in liver
I	41.48	241.49
II	1044.3	1263.02
III	99.9	526.82
IV	77.42	711.05

The reconstructed tomography slice (Figure 3b) of Group II rat liver fed with high cholesterol diet shows a vessel expansion. The three-dimensional volume rendered image of the Group II rat liver (Figure 5a and 5b) shows microvascular fatty deposition. The largest diameter observed for the hypercholesterolemic rat is 1263.02 micron.

For the atorvastatin treated rat liver (Group III) the smallest and largest vessel diameter is 99.9 and 526.82 micron respectively obtained from the quantification of tomographic slices (Figure 6a and 6b). Similarly, the vessel diameter for lipoic acid treated rat liver (Group IV) is 77.42 micron and 711.05 micron from the tomographic slices in Figure 3d, Figure 7a and 7b shows volume visualization of vessel microstructure in the two treatments.

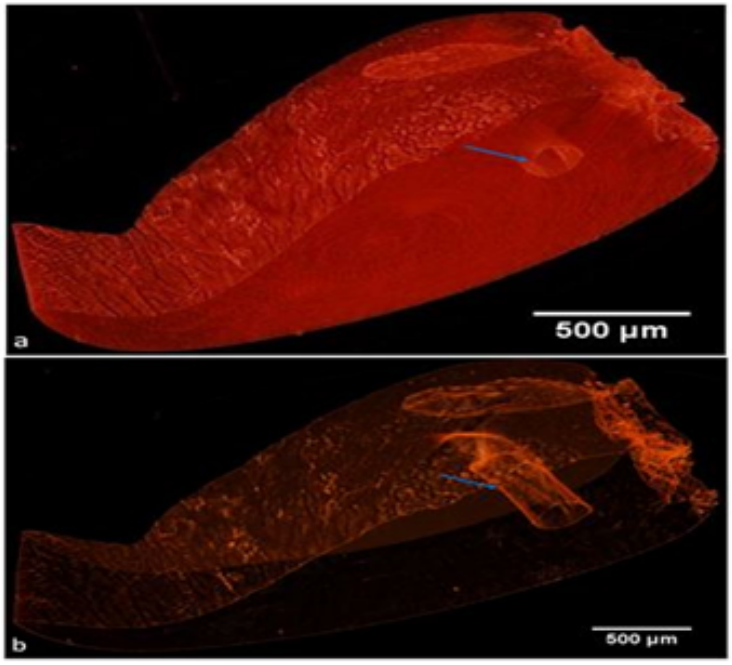

Figure 5a and 5b hypercholesterolemic rat liver (Group II) with visible vessel cross-section with an expansion in vessel due to fat deposition in rat fed with cholesterol diet

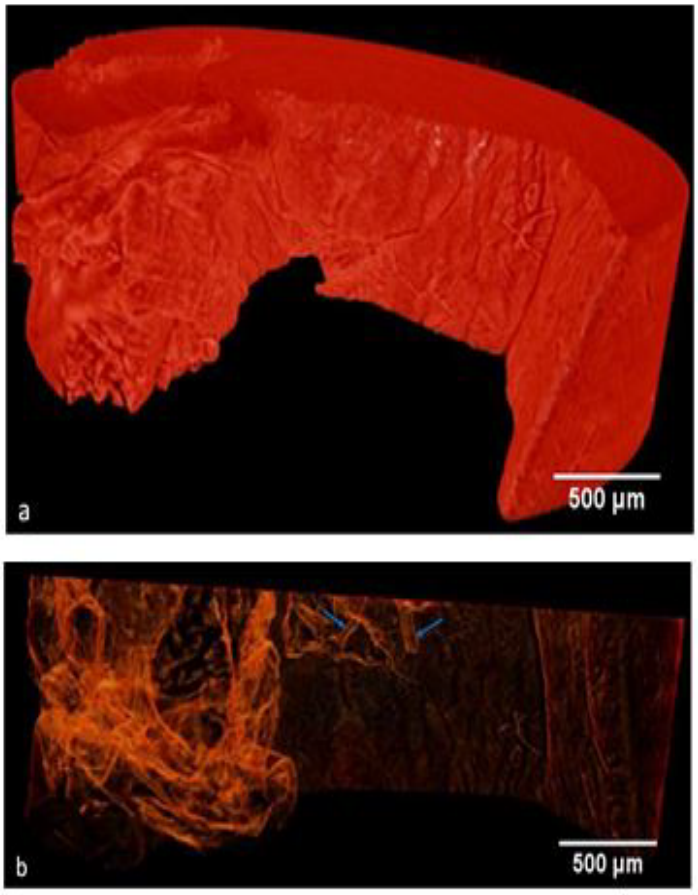

Figure 6a and 6b Volume rendered images of rat liver administered with atorvastatin (Group III) shows microvessels with no fat deposition and vessel expansion

The results show vessels in the region of the liver imaged for control (Group I), hypercholesterolemic (Group II), rat administered with atorvastatin (Group III) and lipoic acid (Group IV) rat without any contrast agent. The phase contrast tomographic imaging using synchrotron X-ray source with high coherence and high intensity allows improvement in the image contrast due to refraction at the edges. This improved contrast in X-ray images not only facilitates their 2D and 3D visualization and qualitative observation in veins diameters but also allows their quantitative measurements with application of simple image analysis.

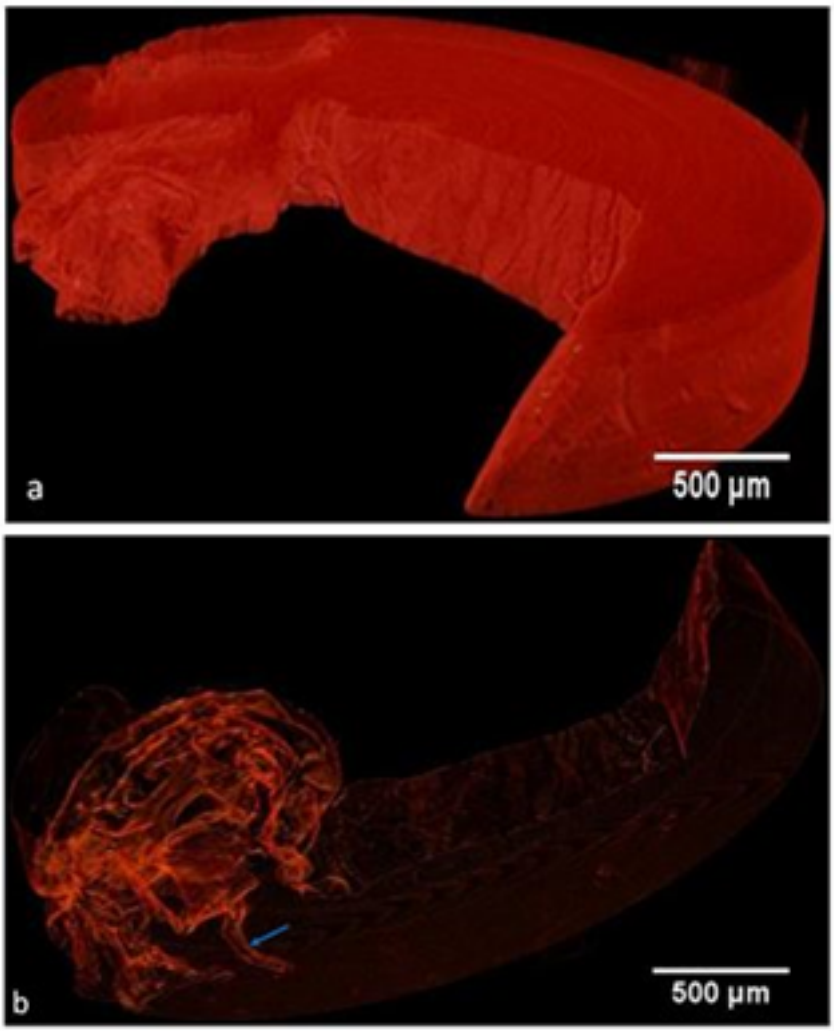

Figure 7a and 7b Volume rendered images rat liver administered with lipoic acid (Group IV) shows microvessels with no fat deposition and vessel expansion (Table 1)

CONCLUSIONS

X-ray phase-contrast tomography using synchrotron based propagation phase contrast imaging set-up could offer improved detection and visualization of drug induced physiological variation in soft tissue samples. The changes in the morphology of the blood vessels could be investigated in the volume of the sample. Phase contrast tomography using synchrotron radiation allows investigate the effect of α-Lipoic acid treatment in the microstructure of hypercholesterolemic rat qualitatively and quantitatively. The high sensitivity of phase contrast imaging technique led to the visualization of the microvessels in the rat liver of all the groups. The quantitative differences in vessel diameter among the control, diseased and treated were revealed by the phase tomography technique. The vessel diameter was found largest for the hypercholesterolemic animals receiving fatty diet as compared to the ones administered with antioxidants. This effect is also confirmed by the serum lipid profiles of the specimens which show significant improved levels in the ALA and atorvastatin treated rats as compared to hypercholesterolemic rats.

ACKNOWLEDGEMENT

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Conflict of Interest

There are no conflicts of interest.

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