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Research Article

Goat's Hepatic Microcirculation with Age-Related Changes and Anatomical Characterization

Dina MM AlSadek[1], Haitham A Badr[2], Attia AA Moselhy*[3] and Sherif Kh A Mohamed[3]

[1]Department of Histology and Cytology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt
[2]Department of Biochemistry, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt
[3]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt
***Corresponding author:** atiaanatomy@gmail.com

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ABSTRACT

The peculiar features of the hepatosinusoidal blood flow decrease with age, shaped by pseudocapillarisation, represent a key factor in normal aging. The ability to dissect the certain pseudocapillarisation beneficial and detrimental function highlights a target for anti-aging. We performed anatomical and histological examinations of the young and old liver goats and identified ultrastructural changes associated with advanced age in the defenestration and capillarisation of the hepatic sinusoids. Furthermore, the accompanied secretory vascular endothelial growth factor (VEGF) crucial for liver sinusoids was measured at protein expression level. The old liver goats were exhibited constant age changes resulted in age-impaired defenestration with reduced porosity of the hepatic sinusoids, thickness of the endothelium lining, infrequent developmental basement membrane formation, and collagen deposition achieved in the space of Disse. In turn, the release of secretory VEGF was significantly lowered in old goat livers. Overall, these findings suggest that the aging goat livers are impaired because of decline in the fenestration of hepatic sinusoids while the accompanied VEGF release implying a therapeutic anti-aging target. The lobation of the goat liver formed from four lobes, left undivided large ventral located, undivided right, quadrate and caudate lobe. The hepatic artery branched into right, left and middle branches which ramified to each lobes of the liver.

Key words: Pseudocapillarisation, Lipofuscin, Endothelial fenestrate, Anti-aging therapies, Anatomy

INTRODUCTION

The percentage of elderly goat deaths attributed to a variety of liver diseases are growing rapidly in Egypt, in turn accelerates the research of aging and age-related pathologies (AlSadek *et al.*, 2018). The dysfunctions of aged-livers are accompanied by metabolism impairment, microbe interactions, toxins susceptibility and climatic changes that have potential healthcare problems from both medical and economic perspectives. Although a few features have been identified like the consensus is that liver volume and blood supply concomitantly decline with advanced age in goats, the effects of age on liver structure and function remain unresolved. Further conventional histological and immune examinations that connect between young and old livers with prolonged life expectancies are still needed. (Le Couteur *et al*., 2002) described the hypothesis on age in relation to the hepatic microcirculation structure lying between the blood and the liver hepatocyte that act as a barrier to substance pass through them. Partly, the hepatic sinusoidal endothelium is lining by highly specialized liver endothelial cells and separate between the sinusoidal blood and hepatocytes (Le Couteur *et al*., 2002). Hepatic sinusoids in elderly animals differs from other capillaries, due to fenestrae dynamics in the sinusoidal lining cells which lacking a diaphragm and underlying basal lamina (Fraser *et al*., 1995). In the past, little is known about the effect of ageing on liver functions and all liver age-groups studied have been considered to be relatively unaffected by the normal ageing and/or age-related pathologies (Cotreau *et al*., 2005). Recently, appeared ultra-structural morphology changes with aging liver sinusoidal endothelial cells and space of Disse in rats (Le Couteur *et al*., 2001), baboons (Cogger *et al*., 2003), human beings (Mclean *et al*., 2003) and mice (Warren *et al*., 2005 and Ito *et al*., 2007). These changes of the hepatic sinusoidal endothelium were termed early capillarisation capillaries and recently termed as pseudocapillarisation which characterized by defenestration with reduced porosity, thickening of the

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endothelium and deposition of basal lamina and extracellular matrix in the space of Disse. Notably, there was also no loss of integrity of the observed hepatic sinusoidal endothelium lining which suggests preserved filtration capabilities until older age. Several lines of evidence links secretory VEGF to age-related pseudocapillarisation observed in highly vascularized tissues and organs (Risau, 1995). VEGF and its respective tyrosine kinase receptors are crucial for liver sinusoidal endothelium vascularization and functionality (Risau, 1995 and Roberts and Palade 1995). The purpose of present study is to elucidate the pseudocapillarisation of the hepatic sinusoidal lining in elderly goats and contributes to the release of secreted VEGF as therapeutic anti-aging target.

There were two surfaces; visceral and parietal; four borders (right, left, dorsal and ventral) and four main lobes (right, left, quadrate and caudate lobe). The latter was divided into caudate and papillary processes (Ashraf, 2001). The hepatic artery was divided into three main branches at Porta hepatis; R. dexter, R. dorsalis dexter and R. sinister (Ashraf, 2001).

MATERIALS AND METHODS

Animals

Young (aged 1-week) and old (24 months) healthy Baladi male goats were used in this study. They were collected from Zagazig abattoir. The livers were taken as soon as possible after slaughter of the animals**.** The age of these animals was determined by dentition (Jaudas and Mobini, 2006).

The present study was performed under the use of experimental animals guidelines followed the protocol approved by the Animal Care and Use Committee of the University of Zagazig.

Anatomical examination

For studying the arterial blood supply, two freshly livers of goat immediately after slaughtering, were injected with red gum milk latex by a through the hepatic artery (Tompestt, 1970). One healthy Baladi goat was used for the topography of the liver of goat. The nomenclature applied in this study was adopted by Nomina Anatomica Veterinaria (2017).

Light microscopy examination

The freshly collected Liver specimens were fixed immediately in 10% buffered neutral formalin then the specimens were processed using the usual histological techniques to be embedded in paraffin wax, sectioned at 5-7 micrometer thick**.** The sections were stained with Harris's haematoxylin and Eosin, Crossmon's trichrome stains and PAS technique.

Electron microscopy examination

Routine methods were used to prepare liver specimens for transmission (TEM) and scanning (SEM) electron microscopy**.** Small pieces of livers tissue were collected immediately after slaughter. These specimens were fixed in 2% glutaraldehyde and processed for ultrastructural study.

VEGF protein expression

Samples were lysed in Triton X-100 lysis buffer (10 mM Tris-HCl [pH 8.0]; 5 mM Ethylenediaminetetraacetic acid; 320 mM Sucrose; 1% Triton X-100; 1 mM PMSF; 2 mM DTT; 1 µg/mL Leupeptin; 1 µg/mL Aprotinin) and then kept on ice for 10 min. After centrifugation steps, the supernatant was obtained and protein concentrations were determined by BCA protein bioassay kit (Pierce). A 50 µg total lysate for each liver sample was separated by SDS-PAGE and transferred onto PVDF membranes (Pierce) following standard procedures. After incubation with primary antibody specific for VEGF (sc-7269) and β-Actin (sc-47778 Santa Cruz)], the blots were incubated with corresponding secondary antibody-horseradish peroxidase (HRP)-conjugate (Santa Cruz) and final signals were detected by ECL system (Pierce). Negative control was included PBS instead of primary antibody.

VEGF binding affinity

For VEGF binding affinity, surface plasmon resonance measurements with (Biosensing Instrument, Tempe, AZ) were employed. Carboxyl groups on the surface of gold-coated SPR chip (20 mm x 20 mm x 125 µm), which is a BK7 glass slide coated with a 45 nm layer of gold over a 5 nm layer of chromium, were rinsed in copious amounts of water and ethanol, dried under a stream of N_2, and placed in 1 mM MUA/ethanol solution for 12 h at 4°C, allowing self-assembled monolayer (SAM) formation according to the supplier's instructions. A 670 nm wavelength laser with a 72.2° incident angle on the bottom of the gold layer through the prism was employed as the light source. The sensing surface was flushed by 150 mM PBS buffer (pH 7.4) at a 150 mL/min flow rate until a stable SPR baseline signal-time curve was acquired. The PBS buffer flow rate was then switched to 50 µL/min optimized binding time and maintained during the whole SPR measurement without altering the instrument settings. Next, a solution of 75 mM EDC and 15 mM NHS in water was injected into the flowing buffer to activate the carboxylic groups at the end of the SAM layer. Following notice difference in shape and amplitude scaling of the distinct curve indicating formation of the activated NHS-ester, 150 µL of Anti-VEGF (sc-7269) at concentration 50 nM per ml in PBS solution was loaded into the system to cover the activated SPR chip. The remaining unoccupied sites were capped with ethanolamine solution to prevent non-specific binding. Lastly, VEGF liver samples (150 µL) were submitted into the system to bind the immobilized Anti-VEGF on the sensor surface. All injections in this study were performed with a 20 µL/min rate. Data collection and control software was performed using the Biosensing Instrument SPR control systems that run on a PC as reported previously (Liu *et al*., 2014).

Statistical analysis

Data set are expressed as means ± S.E. of at least three independent experiments. ANOVA is used to determine statistical significance by analysis the ratio of two population variances. The observed differences were considered significant when $P \leq 0.05$.

RESULTS

Livers anatomy

Ageing of the liver and pseudocapillarisation plays a fundamental role in health and disease. Liver is the largest body gland, reddish brown in color and rectangular which lies entirely to the right of the median plane. Its longitudinal axis is directed obliquely downward and forward to be corresponded to the concave visceral surface of the diaphragm (Fig. 1 A). Goat liver has two surfaces; parietal and visceral. Parietal surface is convex and related to right visceral surface of the diaphragm, last 2-3 ribs and extended upward and backward to reach the flank region. The visceral surface is concave and having impression for reticulum and omasum and also related to duodenum and pancreas. Just dorsal to the omasal fissure, the portal fissure is lined containing the hepatic vessels, ducts and lymph nodes. There are four borders; dorsal border is short and thick situated below the proximal part of the rib, the ventral, right and left borders are thin. The fixation of goat livers was performed via attachment with the caudal vena cava (coronary ligament), with right kidney (hepatorenal ligament), with sternal part of diaphragm (falciform and round ligaments), with right and left muscular parts of diaphragm (right and left triangular ligaments), with the rumen (hepatogastric), and with duodenum (hepatoduodenal ligament ligament). The lobation of the goat liver formed from four lobes, left undivided large ventral located, undivided right, quadrate and caudate lobe. The caudate lobe has two processes, small papillary process and large, elongated caudate process which projected to cover much of visceral surface of the right lobe and had renal impression (Fig. 1 B and C). The blood supply of the goat livers was followed through hepatic branch of celiac artery and portal vein which poured into caudal vena cava via hepatic veins. The hepatic artery branched into right, left and middle branches which ramified to each lobes of the liver (Fig. 1 D).

Light microscopy

Under approved protocol by the Animal Care Committee, liver samples were collected from two goat groups as follows: one week (n=3) regarded as “young” group and 24 months old (n=3) considered as the “old” group. Binucleated hepatocytes were commonly observed in the older goat. Round, large cells with peripherally located nucleus giving appearance of a signet ring were noted in old ages (Fig. 2 A and B). There's no other age-related differences was seen by light microscopy.

Scanning electron microscopy

Scanning electron microscopy revealed reduced fenestration in the sinusoidal endothelium of the old ages (Fig. 3 A and B).

Transmission electron microscopy

In all specimens, the morphology of the hepatocytes was well preserved with no evidence of autolytic changes or fixation artifacts. In particular, there were no signs of organelle swelling and the structure of the mitochondria and microvilli was intact. With the exception of the increased frequency of binucleated cells in older group (Fig. 4 A), no difference between young and old animals could be detected on the basis of hepatocyte morphology. The hepatic sinusoidal endothelium of goat liver undergoes significant age-related ultrastructural changes. These included an increase in the thickness of sinusoidal endothelium. The number of endothelial fenestrations was significantly decreased (Fig. 4 B and C). The space of Disse showed development of collagen deposits (Fig. 4 D). Old age was associated with deposition of lipofuscin pigment and multinucleated cells in hepatic parenchyma (Fig. 4 E).

VEGF protein expression

In our previous study, we applied a FAD approved humanized anti-VEGF monoclonal antibody (bevacizumab) to study the secretory VEGF/VEGFR regulation (Liu *et al*., 2014). To elucidate the release of VEGF as a potent relaxant of the sinusoidal lining and pseudocapillarisation, we measured the secreted VEGF protein levels by Western blots. The primary antibody (sc-7269) used in these VEGF protein blots was very specific to the corresponding VEGF protein in our liver goat samples and only one band base detected in each case (Fig. 5 A). Analysis of the VEGF release by old livers showed significant decreasing by 1.29 ($P<0.001$) fold over young livers (Fig. 5 B).

VEGF binding affinity

In this SPR experiment, we study the affinity of the VEGF and VEGF antibodies binding to demonstrate the key role of VEGF in target pseudocapillarisation and anti-ageing therapy. Secretory VEGF from young or old livers were injected over the sensor surface with immobilized anti-VEGF monoclonal antibody generated by engineering the VEGF binding residues of a murine neutralizing antibody into the IgG framework, and the binding curves were monitored using the BI-SPR software (Fig. 5 C). The maximal mDeg shift monitored at the end of the injection phase is depicted in Figure 5 (C). A surface prepared under the same conditions but without VEGF was used as a control. A modified pseudo-first-order kinetics equation was used to determine the association rate constants $dR/dt = ka[VEGF\ antibody_{conc.}]\ Rmax$ where R is the SPR signal at time t, ka is the association rate constant which indicates the binding affinity between two molecules, $[VEGF\ antibody_{conc.}]$ is the concentration of VEGF antibody, and Rmax is the maximum response of the immobilized ligand (VEGF). The value of (dR/dt) for each sample was determined by calculating the maximum slope of the association curve. As shown in Figure 5 (C), the maximum SPR response of the surface bound VEGF antibody is 1959 mDeg and the Ka for VEGF of old liver is $2.15\pm0.03 \times 10^5\ M^{-1}\ s^{-1}$ (n=3). Similarly, the maximum SPR response of the VEGF antibody surface bound VEGF from young liver is 3006 mDeg and the Ka is $3.29\pm0.09 \times 10^5\ M^{-1}\ s^{-1}$ (n=3). Results of SPR baseline shift from three similar experiments with different VEGF batch from young and old livers are shown in Figure 5 (D). These results indicated that young and old livers expressed different levels of VEGF and the responses obtained for VEGF of young goat livers with higher binding rate and affinity provided a new insight into mapping and quantifying of the VEGF/VEGF-antibody interactions for therapeutic anti-aging targets.

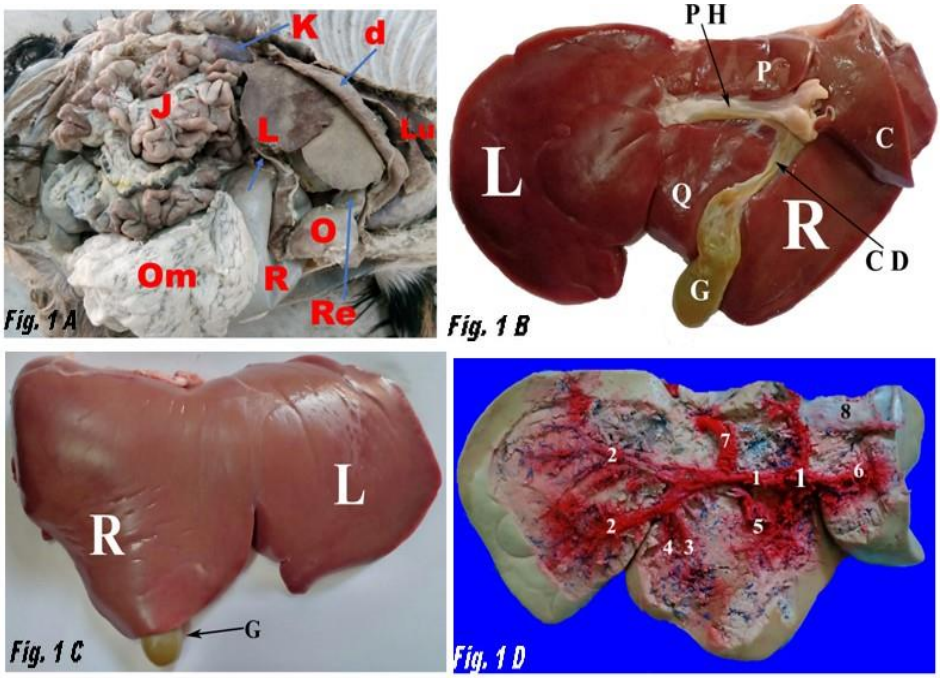

Fig. 1 A: A photomacrograph of the right abdominal topography of the goat showing; Hepar (L), Ren (K), Omasum (O), Reticulum (Re), Diaphragma (d), duodenum (du), Jejunum (J), Rumen (R), Omentum (Om) and pulmo (Lu). **B.** A photomacrograph of the visceral surface of the goat liver shows: (L), Lobus hepatis sinister (Q), Lobus quadratus (G), Vesica fellea (C D), Ductus cysticus (R), Lobus hepatis dexter (C), Processus caudatus (P), Processus papillaris (P H) Porta hepatis. **C.** A photomacrograph of the parietal surface of the goat liver shows: (L), Lobus hepatis sinister (R), Lobus hepatis dexter (G), Vesica fellea. **D.** A photomacrograph of the arterial blood supply of the goat liver shows: (1), Arteria hepatica (2), Rami sinister (3), Arteria cystic (4), Ramus lobi quadrati (5), Ramus dexter (6), branch of caudate process (7), branch of papillary process (8), Vena cava caudalis.

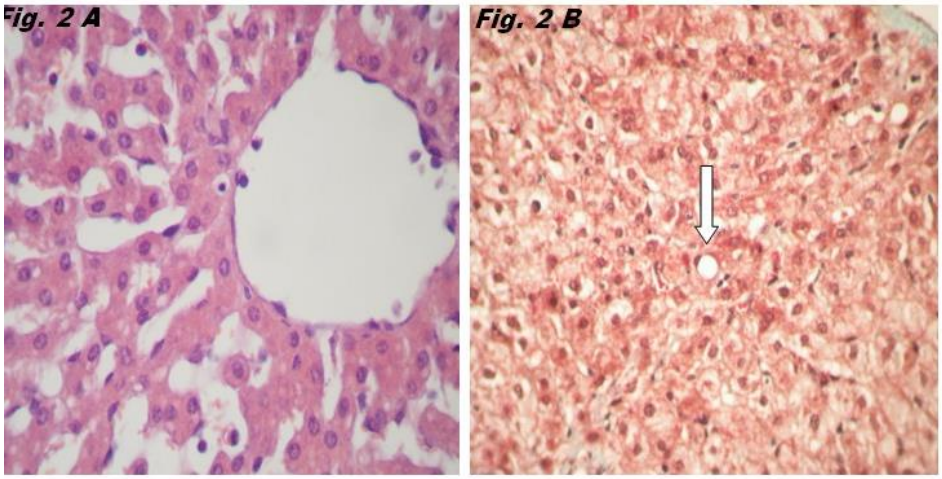

Fig. 2: Liver from young (A) and old (B) goat. In the old liver, there is large signet shaped perisinusoidal cells (arrow).

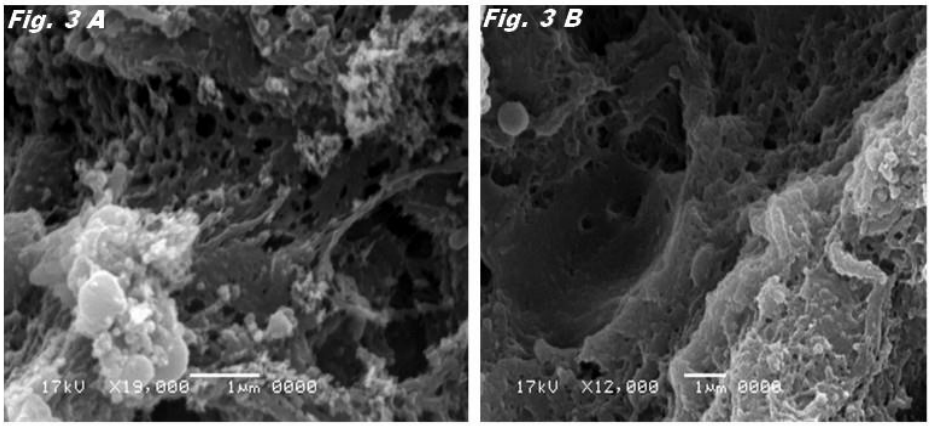

Fig. 3: Scanning electron micrographs of the sinusoids from the livers of young and goat. The endothelium of the sinusoids from young liver show fenestrated sieve plates (A). In old liver there was a noticeable decrease of fenestration (B).

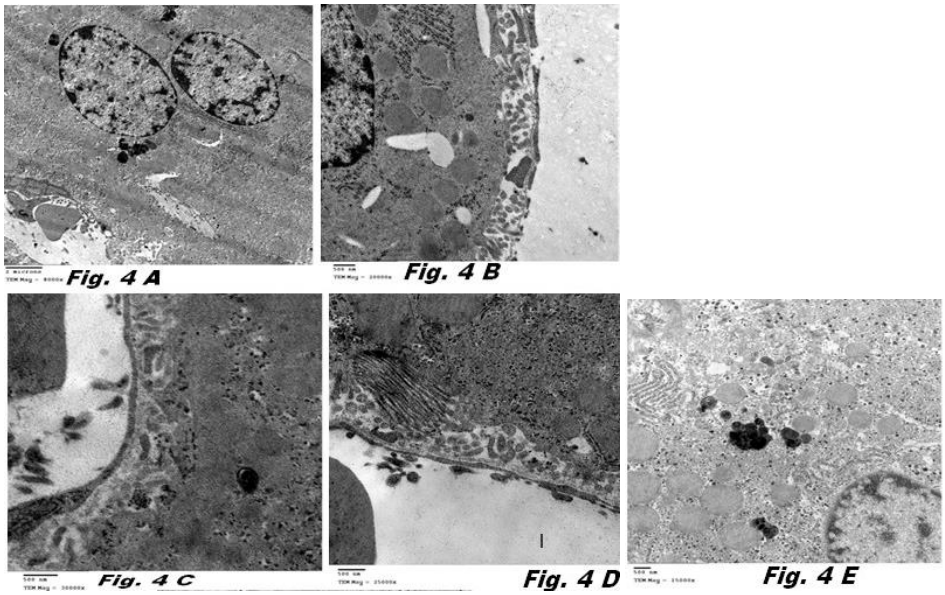

Fig. 4: Electron micrograph of a liver from old goat showing binucleated cell (A). Transmission electron micrograph of the liver sinusoidal endothelial cell (B): In the young liver, the endothelium is thin and perforated with fenestrations. C: In the old liver, the endothelium is thickened and defenestrated. Space of Disse with microvilli. (D) Transmission electron micrograph of the liver sinusoidal endothelial cell of old goat showed collagen fibrils deposition in the space of Disse. (E) Transmission electron micrograph of the hepatocyte of old goat showing accumulation of lipofuscin pigment.

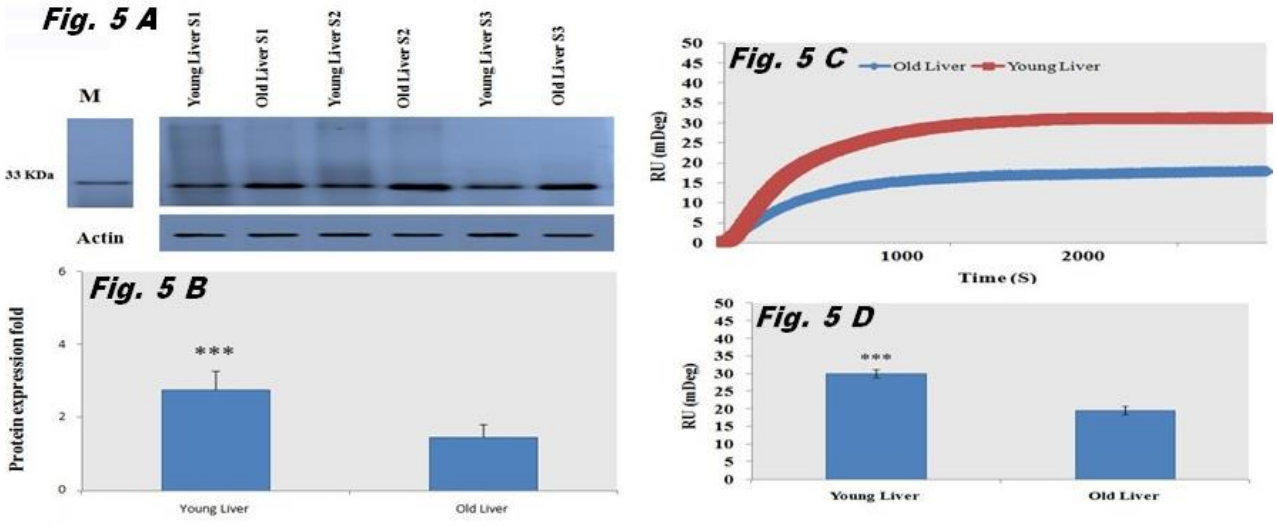

Fig. 5: (A) Western blot detection of VEGF. The protein expression levels of VEGF were analyzed by Western blot. The results were normalized to β-actin expression. For negative control Western blot experiments, PBS was used instead of primary antibody. Each antibody resulted in a single band corresponding to the molecular weight, shown on the right. Negative controls did not result in any bands. (B) The quantitative analysis of protein expression levels of VEGF. (C) SPR sensorgram of VEGF from young (red line) and old (blue line) goat livers bound to immobilized VEGF antibody. (D) A typical increase in SPR response corresponding to VEGF of young liver compared to old liver. The data are representative of three independent experiments with S.D. indicated by error bars. P values are determined by two-tailed student t-test and <0.05, <0.01 and <0.001 are indicated by one, two and three asterisks, respectively.

DISCUSSION

Dynamic changes in normal aging are thought to be associated with hepatic pseudocapillarisation environment due to vascular endothelial dysfunction and the decline in liver blood flow (Le Couteur *et al*., 2001). The basic lobation of the goat liver was similar to that reported by (Getty, 1975) in goat and Mohammed and Mustafa (2017) in indigenous Gazelle. The ramification of the hepatic artery was correlated with (Getty, 1975) in goat and Mohammed and Mustafa (2017) in indigenous Gazelle. In accordance with Mohammed and Mustafa (2017) the current work showed that the gallbladder was narrow, elongated blind pear shape with dark green color attached to the cystic fossa on the visceral face of the liver flanked by the right and quadrate lobes. In the contrast (Siddig *et al*., 2014) in camel and Osman (2008) in pig the hepatic artery has different distribution as right and left only. At the light microscopic examination level there were no major changes in the livers of the old goat. Harris's haematoxylin and Eosin, Crossmon's trichrome stains and PAS technique showed no major changes. This is slightly

different than previously reported aging studies using other models species including human beings where there were some age-related changes detected with these methods (Cogger *et al*., 2003). However, our overall finding indicate that ageing animals is associated primarily with ultrastructural-architecture changes in the endothelial cells rather than with the types of changes in the sub-endothelial matrix that are commonly seen in liver fibrosis and/or cirrhosis. The large round hepatocells with peripheral nuclei that were observed by Cogger *et al*., (2003) in the old non-human primates were also found in aged goat. In animal models like old baboons as well as similar old mice studies these cells appeared to be fat-engorged cells lining the sinusoids, possibly representing hepatic stellate cells on the basis of their pre-location and fat-engorgement (Warren *et al*., 2005). These cells are in concert with the observations reported by Vollmar *et al*., (2002) of age-related increase in stellate cell-associated areas of ultraviolet vitamin A-autofluorescence, which correlated with increasing tissue concentration of vitamin A metabolites. At the ultrastructural level, there were marked changes in the hepatic sinusoidal endothelium in the aged goat. These contributed to increase in the endothelium-thickness; defenestrate dynamics and patchy basal-lamina formulation. These findings are consistent with those reported in elderly aging rats (Le Couteur *et al*., 2001), humans (McLean *et al*., 2003), and non-human primates (Cogger *et al*., 2003) that have been defined as 'age-related pseudocapillarisation'. Thus, we agreed that elderly age is likely to be associated in all animals with a substantial increase in the thickness of the hepato-sinusoidal endothelium and perhaps more importantly a substantial reduction in the porosity of the endothelium. Where, the reduction in porosity is accompanied secondarily to a reduction in the numbers and diameters of fenestrations. These findings are in agreement with those reported in old aged-rats (Le Couteur *et al*., 2001), human (Mclean *et al*., 2003), non-human primates (Cogger *et al*., 2003), mice (warren *et al*., 2005 & Y. Ito *et al*., 2007). These age-research and related structural changes in liver sinusoidal endothelial cells and in space of Disse have been termed "pseudocapillarisation" by LeCouteur (LeCouteur *et al*., 2002). The developmental infrequent basal lamina and arrangement of collagen-deposition in the space of Disse are well established to narrow the hepatosinusoids lumen and impaired sinusoidal perfusion. Functionally, this pseudocapillarisation in the liver also is thought to minimize blood-parenchymal exchange. Hilmer *et al*. (2005) have shown reduced diffusion of small lipoprotein structures in old aging of rat livers. One of the most fenestrate functions appears to be related to the transfer of chylomicrons remnants across the observed-endothelium for subsequent hepatic circulation metabolism (Fraser *et al*., 1995). This transfer of lipoproteins is impaired in old aging (Hilmer *et al.*, 2005). The great functional implications of such age-related liver changes in the endothelial structure are of interesting application. The loss of fenestrations may contribute to the impaired metabolism and clearance of lipoproteins that biologically traverse through the fenestrations when binding to the LDL receptors on the hepatocyte membranes (Le Couteur *et al*., 2002). The aged-pseudocapillarisation may also have implications for anti-aging drug design and oxygen-metabolites transfer across the hepatic endothelial membranes (Le Couteur *et al*., 2005). In the old goat livers, the sinusoidal architecture showed persistence in particulate substrates and transfer of the vascular endothelial pattern. Previously, we have addressed the importance of VEGF/VEGFR regulation and molecular role in endothelial cell model (Liu *et al*., 2014). Here, we proposed that optimizing the releasing of VEGF could refine the age-related changes of liver sinusoids. Taken together, these finding indicates that pseudocapillarisation opening the door for a widespread anti-ageing animal prevention of liver-related diseases.

In conclusion, ageing in elderly goat is associated with fine ultrastructure changes in the hepatic sinusoidal endothelium that are similar to those documented in other animal models. The old liver goat pseudocapillarisation displayed loss of endothelial fenestrations, thickness of the endothelium lining, infrequent of developed basal lamina membranes, collagen deposition achieved in the space of Disse, and moreover indicate that the associated release of VEGF in younger subjects as a prove model for the study of age-related changes and developing anti-ageing prevention therapies.

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