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REGULAR ARTICLE

Long live the liver: immunohistochemical and stereological study of hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells of male and female rats throughout ageing

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Abstract Male/female differences in enzyme activity and gene expression in the liver are known to be attenuated with ageing. Nevertheless, the effect of ageing on liver structure and quantitative cell morphology remains unknown. Male and female Wistar rats aged 2, 6, 12 and 18 months were examined by means of stereological techniques and immunohistochemical tagging of hepatocytes (HEP), liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC) and hepatic stellate cells (HSC) in order to assess the total number and number per gram of these cells throughout life. The mean cell volume of HEP and HSC, the lobular position and the collagen content of the liver were also evaluated with stereological techniques. The number per gram of HSC was similar for both genders and was maintained throughout ageing. The mean volume of HSC was also conserved but differences in the cell body and lobular location were observed. Statistically significant gender differences in HEP were noted in young rats (females had smaller and more binucleated HEP) but were attenuated with ageing. The same occurred for KC and LSEC, since the higher number per gram in young females disappeared in older animals. Liver collagen increased with ageing but only in males. Thus, the numbers of these four cell types are related throughout ageing, with well-defined cell ratios. The shape and lobular position of HSC change with ageing in both males and females. Gender dimorphism in HEP, KC and LSEC of young rat liver disappears with ageing.

Keywords Hepatic stellate cells · Kupffer cells · Hepatocytes · Liver · Ageing

Introduction

Nowadays, it is widely agreed that perfusion is reduced in the aged liver (Schmucker and Sanchez 2011; Loustaud-Ratti et al. 2016). This affects, for instance, the transhepatocellular transport of dyes and IgA (Popper 1985), the diffusion of small lipoproteins (Hilmer et al. 2007), bile salt formation (Le Couteur and McLean 1998; Vollmar et al. 2002) and the clearance of drugs. Differences in microsomes have been reported with ageing, with a decrease in cytochrome-P-450 concentration and NADPH-cytochrome C reductase activity (Van Bezooijen 1984; Popper 1985; Frith et al. 2009). Notably, for rodents, this occurs mainly in the male liver, because enzyme levels in females remain mostly unchanged with ageing (Kitani 1992). In effect, a sort of feminisation of the male liver occurs with ageing, as enzymes more active in younger males usually decline to approach the activity levels seen in the female liver and those less active in males increase up to the female levels (Kitani 1992, 2007). Curiously, this pattern also appears to exist at the level of gene expression (Kwekel et al. 2010). Nevertheless, ageing effects on liver structure are much less clear. The state of the art in this field is characterised by few consistent observations and a lack of correlation between

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structural and functional data (Zeeh 2001). Furthermore, to the best of our knowledge, the liver structure of the aged male and female liver has never been studied in detail by quantitative morphology.

Despite this, work during the last decade has shed some light on liver ageing. Sinusoids in the aged liver have been reported to have fewer *fenestrae*, surrounded by basal lamina and collagen, leading to so-called pseudocapillarisation (Le Couteur et al. 2001, 2008). Furthermore, intralobular collagen has been described to increase with age (Gagliano et al. 2002). Since this collagen is mainly produced by hepatic stellate cells (HSC), at least under pathological conditions, morphological differences might appear with ageing. Only a few studies have explored the role of HSC in liver ageing, either from a qualitative (Enzan et al. 1991) or from a semi-quantitative (Martin et al. 1992; Imai et al. 2000; Vollmar et al. 2002; Warren et al. 2011) perspective and their conclusions are controversial. Moreover, an investigation of gender ageing differences among rats is timely because of the common practice of using males and females interchangeably for experimental studies and in protocols in vitro. For instance, older rats (typically male and/or retired female breeders; Ramadori and Saile 2002; Tacke and Weiskirchen 2012; Friedman 2008) are recommended for the isolation of HSC; however, whether HSC differ in quantitative morphology between genders is unknown.

In order to reveal any eventual ageing differences, a quantitative approach should be applied (e.g., by using stereology), since qualitative morphology alone may overlook important structural changes. Other methods, such as cell isolation, are unable to reveal liver cell ratios, since the cell yield from these methods varies between parenchymal and non-parenchymal cells. Nevertheless, an in-depth knowledge of liver structure (and cell ratios) is important for the bioengineering construction of artificial livers and for in vitro studies, since precise cell ratios are necessary to model paracrine effector mechanisms in co-culture models. Studies in vitro have used ratios of parenchymal and non-parenchymal cells varying from 10:1 to 1:10 have been carried out (Bathia et al. 1999) but might not mirror the in vivo organisation of the aged liver. Furthermore, the true aspects of ageing are known to be difficult to ascertain from a simple comparison between young and old animals, since changes can occur in between, or to obtain from the study of only the male gender (Kitani 1992; Schmucker 2001).

We employed design-based stereological methods to study morphological changes in the rat liver throughout ageing in both males and females. We sought differences in the size of lobules, in the collagen content and in the total number (N) and number per gram (N/g) of hepatocytes (HEP), Kupffer cells (KC), HSC and liver sinusoidal endothelial cells (LSEC). Finally, we examined the cell volume and the position of HSC in liver lobules.

Materials and methods

Animals

We examined male and female Wistar rats ($n = 5$ per group) at young (2 months old), adult (6 months old), middle-aged (12 months old) and old (18 months old), initially bought from Charles-River Laboratories (Barcelona, Spain). Based on the mean lifespan of this strain, these ages corresponded to around 10, 25, 50 and 75 % of their lifespan, respectively (Porta et al. 1980; Sawada and Carlson 1987; Manikonda and Jagota 2012). All the animals had been weaned at 20 days and were kept under standard conditions, receiving water and food (Mucedola 4RF21, Settimo Milanese, Italy) ad libitum. The rats were housed in pairs or individually (old males) in a controlled environment (temperature: 25 °C; 12 h light–dark cycle). Animal management followed European Union Directives (1999/575/CE and 2010/63/UE) for the protection of animals used for scientific purposes and the study was approved by local ethical authorities (ORBEA ICBAS-UP Project 152/2016).

Tissue preparation

Sampling was performed during the morning (from 10:00 to 12:00) to circumvent oscillations in liver function because of circadian rhythmicity (Davidson et al. 2004). In females, daily vaginal cytologies were observed in order to avoid collecting samples on proestrous/oestrous days. Beforehand, animals were deeply anaesthetised with ketamine plus xylazine. Blood was collected and centrifuged to obtain serum for assessing alanine transaminase and aspartate transaminase levels. Transcardiac perfusion was performed with an isosmotic solution. The liver was weighed and its volume determined by Scherle's method, as detailed elsewhere (Marcos et al. 2012). A smooth fractionator sampling scheme was applied (Marcos et al. 2012): half of the paraffin blocks were used for thick sections (30 μm thick) and exhaustively sectioned, whereas the other half was used for thin sections (3 μm thick). Of the thick sections, five sections in every 30 were sampled for immunostaining against: (1) glial fibrillary acidic protein for estimating N and N/g of HSC; (2) ED2 for estimating the N and N/g of KC; (3) E-cadherin, to differentiate mononucleate HEP from binucleated HEP, estimating their percentage and to assess the N and N/g of HEP; (4) von Willebrand factor to estimate the N and N/g of LSEC; (5) glial fibrillary acidic protein and glutamine synthetase (an established marker of centrilobular HEP; Gebhardt and Mecke 1983) to evaluate the lobular distribution of HSC. Thin sections were used for immunostaining against: (1) α-smooth muscle actin to evaluate the existence of activated HSC; (2) glial fibrillary acidic protein to determine the relative volume of HSC (whole cell); (3) glutamine synthetase to

estimate lobular size by measuring the porto-central distance. Thin sections were also used for assessing liver collagen by Sirius red staining. In addition, tiny liver fragments (<0.5 mm^3) were removed from the rat liver for electron microscopy. These were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h and subsequently post-fixed in phosphate-buffered 1 % OsO_4 for another 2 h. After dehydration in ethanol and propylene oxide, the pieces were embedded in epoxy resin. Semithin sections were obtained, stained with methylene blue-azur II and used to quantify volume densities of HSC (cell body). Additionally, ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate and observed in a transmission electron microscope, JEOL 100CXII, at 60 kV.

Immunohistochemistry

The protocol used for thick sections has been previously described (Marcos et al. 2004, 2006; Santos et al. 2009). Briefly, antigen recovery was carried out in a microwave oven (four cycles of 4 min, at 600 W, in buffered citrate 0.01 M, pH 6.0) and a streptavidin–biotin protocol was used (Histostain Plus, Invitrogen, Camarillo, California) for all antibodies, except for LSEC (pre-treatment of tissues with 0.4 % pepsin [Sigma, St. Louis, Mo., USA] in HCl 0.01 M for 30 min). For glial fibrillary acidic protein, we used rabbit polyclonal antibody (Dako, Glostrup, Denmark) diluted at 1:3000, whereas for ED2 and E-cadherin, we employed monoclonal mouse antibodies from Serotec (Oxford, United Kingdom) diluted at 1:100 and from Dako (clone NCH 38) diluted at 1:250. We should mention that ED2 is unanimously recognised as a marker of fully differentiated, long-lived KC (Roskams et al. 2007; Santos et al. 2009). Additionally, we used an antibody against von Willebrand factor (Dako), diluted at 1:3200, in order to tag LSEC. All the slides were incubated for 4 days at 4 °C.

For double-immunohistochemistry, slides were also placed in the microwave oven (this time for three cycles of 4 min at 600 W in buffered citrate). After the blocking of endogenous biotin and peroxidase, the first streptavidin–biotin protocol was followed with antibody against glial fibrillary acidic protein (1:1500 dilution for 4 days at 4 °C). Slides were developed for 2 min in 0.05 % 3,3′-diaminobenzidine (Dako) in TRIS-buffered saline with 0.03 % H_2O_2. Sections were then rinsed in tap-water and dipped in 50 mM glycine buffer (pH 2.2) for 5 min to strip off the antibodies of the first immunoreaction. The second streptavidin–biotin protocol against glutamine synthetase was followed by using rabbit polyclonal antibody (graciously provided by Professor Rolf Gebhardt, University of Leipzig), diluted at 1:4000, for another 4 days at 4 °C. Slides were developed with aminoethylcarbazole (Dako) for 10 to 20 min (final red colour controlled by microscopic observation) and mounted in Aquatex (Dako).

To quantify glial fibrillary acidic protein in thin sections, a streptavidin–biotin protocol was also used (Histostain Plus) but at lower dilutions (1:1200) and with an overnight incubation. The blocking solution, the secondary antibody and the streptavidin–peroxidase complex were all applied for 20 min and colour development in diaminobenzidine was restricted to 2 min. For α-smooth muscle actin immunostaining, slides were placed in a pressure cooker for 3 min in citrate buffer (pH 6.0). After the sections had been rinsed in phosphate-buffered saline, a polymer based immunohistochemical protocol was followed (Novocastra Novolink Polymer; Leica Biosystems, Newcastle, UK). The protein blocking solution was applied for 5 min and α-smooth muscle actin antibody (clone HM45, Dako) diluted at 1:500 was used to cover the sections overnight. The post-primary solution and polymer were both applied for 30 min. Finally, sections were developed for 2 min in diaminobenzidine. For the assessment of the porto-central distance, immunohistochemistry against glutamine synthetase was used (diluted at 1:4000), following the protocol for glial fibrillary acidic protein immunostaining in thin sections.

Positive and negative controls (omission of first antibody and replacement by non-immune serum) were included, both for thin and thick sections and all slides were evaluated blindly (i.e., the observer was unaware of the gender or age of the animal) to avoid eventual observer-related bias.

Histochemistry with Sirius-red

Five thin sections were randomly selected per animal, dewaxed and hydrated. Counterstaining was achieved with celestial blue and haematoxylin, each for 5 min. After the sections had been washed in tap water, Sirius red (Sigma, coloration index 35782) dissolved in picric acid (1 mg/ml) was applied for 1 h at room temperature (Kumar 2005). After being washed in acidified water (1 % acetic acid), the sections were dehydrated in ethanol, cleared in xylene and mounted in DPX.

Morphometrical analysis

In thin sections, the linear distance between a central venule (surrounded by HEP immunostained against glutamine synthetase) and the closest portal tract was measured using CAST-Grid software (version 1.5, Olympus). Both peripheral (closer to Glisson's capsule) and inner lobules were considered for the measurements.

Stereological analysis

A stereology workstation with CAST-Grid software was used (Marcos et al. 2012). At the monitor, a final magnification of ×4750 allowed easy and accurate recognition of all cells.

Throughout the disector height (20 μm), a software-generated counting frame with defined areas (1673, 1267 and 418 μm^2 for HSC, KC/LSEC and HEP, respectively) was used for unbiased counting. For assessment of the position of HSC in liver lobules, systematic uniform random sampling was applied, with HSC being counted only if fields were in the vicinity of the portal tracts or central venules (5-6 HEP around those landmarks were settled upon as boundaries). For the purpose of counting cells, the nucleus was considered as the counting unit (in the case of binucleated HEP, this was predetermined to be the first nucleus in focus) and cells were counted following optical disector rules (Marcos et al. 2012). The total N of cells in the whole liver was estimated by using optical fractionator formulae (Marcos et al. 2004, 2012). Simultaneously, the N/g was determined, because it was useful for comparing animals with different liver weights. The coefficient of error of the number of cells counted was also estimated (Marcos et al. 2004, 2012).

Additionally, the mean cell volume (the so-called number-weighted mean cell volume) of mononucleate and binucleated HEP was estimated by the nucleator method (Gundersen 1988; Marcos et al. 2012). In the latter, the first nucleus with a nucleolus in focus was considered for the measurements (Marcos et al. 2012). In the case of HEP with two nucleoli (or more), two (or more) measurements were performed (Gundersen 1988).

Semithin and glial-fibrillary-acidic-protein-immunostained sections were used, respectively, in order to estimate the relative volume of the cell body and whole cell of HSC. In both cases, point counting (grid with 108 points) was used to estimate the relative volume (Fig. 1). The number-weighted mean volume was then obtained by an indirect approach, through the division of the relative volume by the relative number of HSC per unit of volume (corrected for paraffin shrinkage; Marcos et al. 2012). With regard to the assessment of collagen content, point-counting (grid with 36 points) was also performed by CAST-Grid software. In a preliminary study, we have shown that this test-system allows the easy discrimination of collagen fibres (Marcos et al. 2015).

Statistical analysis

The normality of the data was checked by using Shapiro-Wilk's test. Pearson's correlation analysis was performed to detect linear correlations. After the homogeneity of variances (Levene's test) had been checked, a two-way analysis of variance was performed taking into consideration the effects of gender and ageing. When significant differences existed, multiple comparisons were carried out by using the post-hoc Tukey's test. The statistical significance level was set at $P \leq 0.05$. The software SPSS 18 (IBM, Armonk, USA) was used. Quantitative results are presented with their means and standard deviations.

Results

Qualitative findings

All livers displayed normal morphology, with no noticeable differences across animals by optical and electron microscopy. Consistent and reliable marking of HEP was achieved with E-cadherin, allowing a clear distinction between mononucleate and binucleated HEP. For glial fibrillary acidic protein, HSC were immunostained in both periportal and centrilobular areas (Supplemental Fig. 1). The HSC of aged rats had larger and more numerous lipid droplets than young rats; these cells occasionally protruded into sinusoids (Fig. 1). However, no differences were noticeable between HSC of males and females. No staining of HSC with α-smooth muscle actin antibody was observed (Supplemental Fig. 2). Fully differentiated KC also exhibited a stellate appearance with ED2 but with shorter and much thicker cytoplasmic processes. With regard to LSEC, they had a characteristic dark nucleus lying on a thin rim of immunomarking against von Willebrand factor (Fig. 2).

Quantitative findings

The livers of males were heavier ($P < 0.001$) than those of females (15.15 ± 1.6 g versus 9.62 ± 1.2 g, respectively). The liver-to-body weight ratio was highest at the age of 2 months (4.27 % ± 0.7 %, $P < 0.01$) compared with other age groups (2.72 % ± 0.3 %); no gender differences were observed. A strong correlation was observed between liver and body weight ($r = 0.77$, $P < 0.001$). Hepatic transaminase values were within the reference ranges, presenting no statistically significant differences (43.1 ± 4.0 IU/l and 39.1 ± 9.3 IU/l for alanine transaminase and 101.4 ± 17.6 IU/l and 105.2 ± 14.5 IU/l for aspartate transaminase in males and females, respectively).

An average of 62 lobules per rat was analysed by morphometry. The average porto-central distance was 450 ± 17 μm in males and 412 ± 22 μm in females (data corrected for paraffin shrinkage). No statistically significant differences were observed for the size of lobules throughout ageing and between genders (Table 1).

An average of 216 fields per animal was screened to assess liver collagen. The amount of intralobular collagen corresponded to 56 % in males and 46 % in females, without significant differences with ageing. This collagen was moderately correlated with the N of HSC ($r = 0.50$; $P < 0.01$) and with the N of HEP ($r = 0.47$; $P < 0.01$). Liver collagen was influenced by gender ($P < 0.001$) and ageing ($P < 0.01$), namely in males. Indeed, collagen was more abundant in males than in females in adult and old rats (Fig. 3). This was attributable to intralobular collagen, since the collagen around central venules and in portal tracts was maintained throughout ageing and gender (varying between 0.9 and 1.3 % of relative volume).

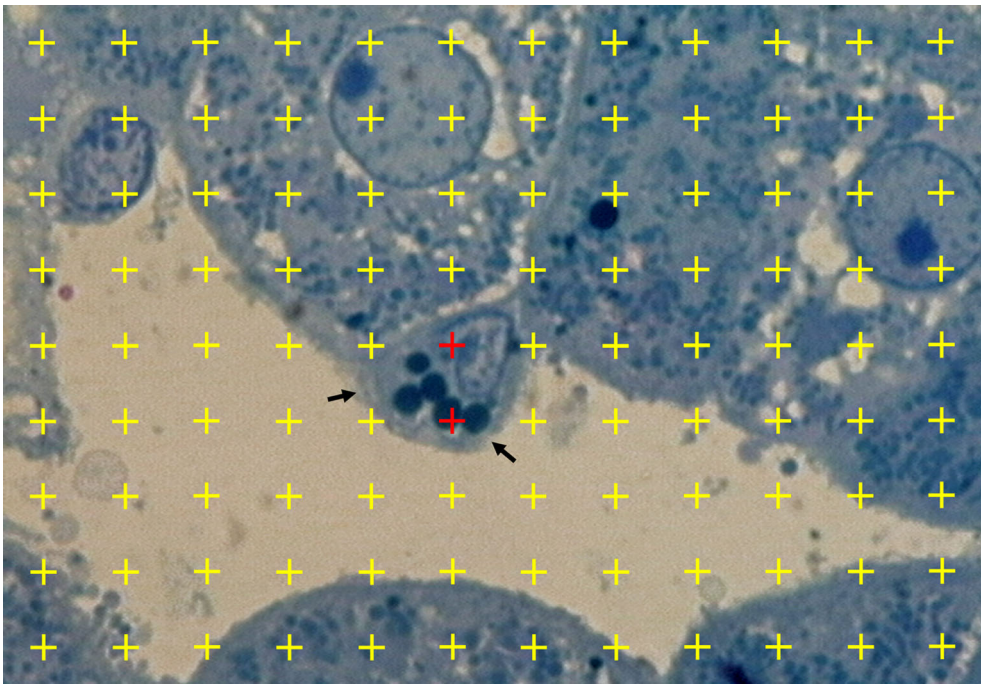

Fig. 1 Semithin section of liver parenchyma of an old rat. A hepatic stellate cell (*arrows*), engorged with lipid droplets, protrudes into a sinusoid. For illustrative purposes, the counting grid (composed of 108 points) used for estimating the relative volume of the cell body of hepatic stellate cells is shown; in this case, two points (*red*) will be counted

From each rat, between 291 and 780 optical disectors were analysed to obtain the N and N/g of HSC, HEP, KC and LSEC and between 150 and 216 fields per rat were assessed to determine the relative volume of HSC (cell body and whole cell). The N of HSC was higher in males ($209 \pm 14 \times 10^6$) than in females ($154 \pm 15 \times 10^6$; $P = 0.016$) but the N/g was similar across genders ($\approx 14.6 \pm 1.3 \times 10^6$; Fig. 4). Neither of these parameters was associated with ageing. A correlation was observed between the N of HSC and (1) liver weight ($r = 0.85$, $P < 0.01$), (2) N of HEP ($r = 0.73$; $P < 0.0001$) and (3) N of KC

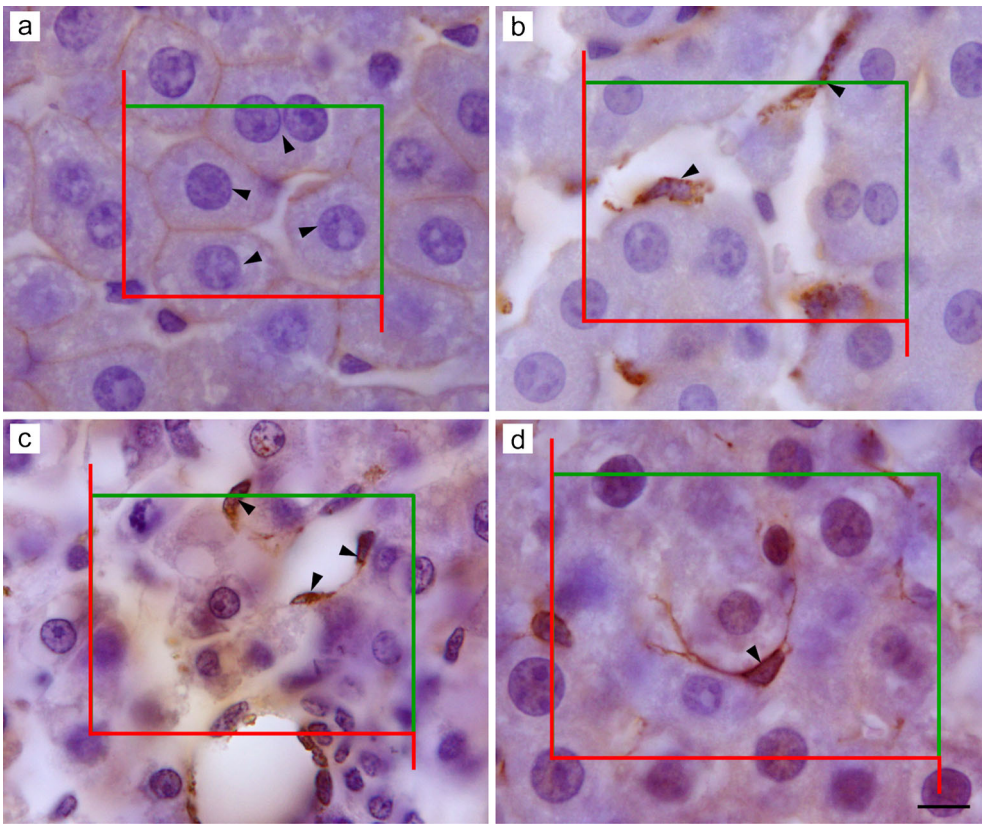

Fig. 2 Thick liver sections (30 μm) immunostained against: (**a**) E-cadherin to highlight the borders of mono- and binucleated hepatocytes; (**b**) ED-2 to tag fully differentiated Kupffer cells; (**c**) von Willebrand factor to depict liver sinusoidal endothelial cells; (**d**) glial fibrillary acidic protein to mark hepatic stellate cells. The counting grid is shown for illustrative purposes, being larger (**d**) for less abundant cells and smaller for the most numerous (**a**). Cells were counted if the nucleus was inside the counting grid or touched the inclusion lines (*green*) but not the exclusion lines (*red*). Cells counted in this example are indicated with *arrowheads*. *Bar* 9 μm

Table 1 Porto-central distance in male and female rats throughout ageing. Values are presented as means ± standard deviations (μm) and are corrected for paraffin shrinkage

Gender	Young	Adult	Middle-aged	Old
Males	463 ± 27	452 ± 14	427 ± 21	454 ± 28
Females	442 ± 12	398 ± 15	414 ± 25	394 ± 18

(r = 0.53, $P < 0.01$). Because of the increased volume of lipid droplets present in older HSC, the number-weighted mean volume of the cell body increased with ageing (101 ± 33 μm^3 to 576 ± 104 μm^3; $P < 0.01$) but the volume of the whole cell was relatively stable, varying from 593 ± 134 μm^3 to 796 ± 192 μm^3 (Fig. 5). The distribution of HSC was not influenced by gender; however, an ageing pattern was observed ($P < 0.001$): in younger animals, HSC were more frequently located pericentrally (56.5 ± 4.9 %), whereas in older animals, these cells were more abundant in a periportal location (61.2 ± 6.7 %).

The N of HEP did not vary with ageing (≈2.0 ± 0.3 × 10^9), in contrast with the N/g of HEP (159 ± 33 × 10^6 in young and 184 ± 26 × 10^6 in old; $P < 0.05$; Fig. 6). The N of HEP was statistically correlated with (1) body weight and (2) liver weight (r = 0.60; $P < 0.0001$, for both) and (3) N of KC (r = 0.5, $P < 0.01$), whereas the N/g of HEP was correlated with the N/g of KC and N/g of binucleated HEP (r = 0.94, $P < 0.001$ and r = 0.75, $P = 0.02$, respectively). Gender differences were observed in the N/g of HEP but these were restricted to younger animals (136 ± 11 × 10^6 in males and 183 ± 39 × 10^6 in females; $P = 0.016$). Similarly, gender differences existed for binucleated HEP and for their percentages, which were higher in females (25 ± 4 % in young males and 34 ± 5 % in young females) but these differences were attenuated with ageing (27 ± 5 % and 31 ± 4 % in old males and females, respectively; Fig. 6). The percentage of binucleated HEP was negatively correlated with the body weight (r = −0.81,

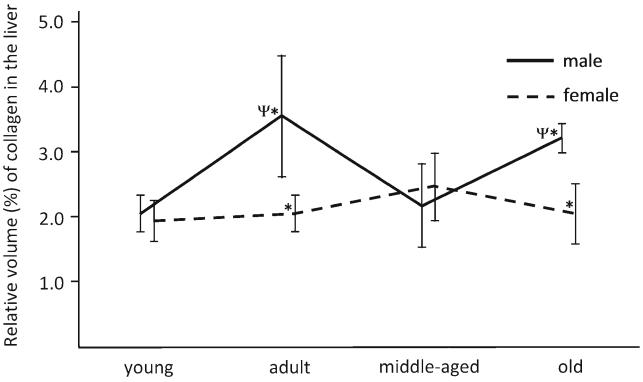

Fig. 3 Relative volume of liver collagen in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders. $^{\Psi}$Statistically significant differences of young animals

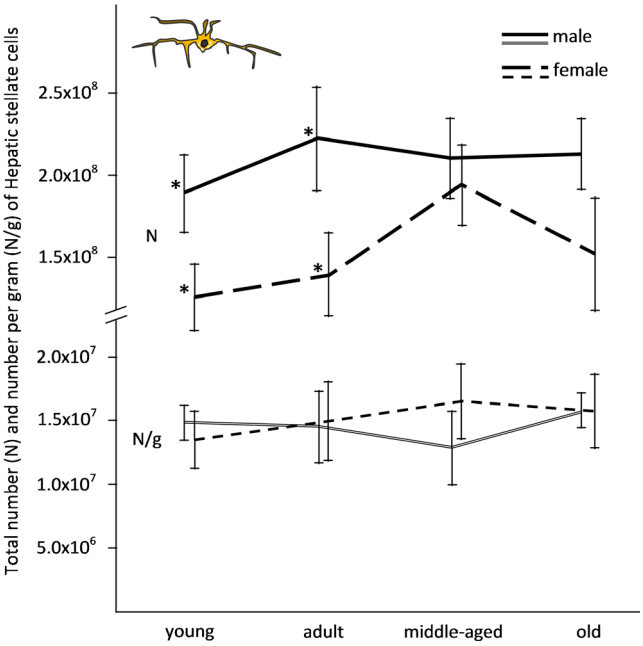

Fig. 4 Total number (*N*) and number per gram (*N/g*) of hepatic stellate cells in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders

$P = 0.015$). For the number-weighted mean cell volume, no statistically significant difference was observed with ageing and gender (except between mononuclear HEP in young males and females; 5861 ± 369 μm^3 and 4915 ± 293 μm^3, respectively; $P < 0.01$; Fig. 7). On average, the volume of binucleated HEP was 7177 ± 752 μm^3, being 31 to 59 % greater than that of mononucleate HEP ($P < 0.001$).

The N of fully differentiated KC was moderately correlated with liver weight (r = 0.67, $P < 0.001$). Overall, this parameter was stable with ageing (≈286 ± 58 × 10^6), as was the N/g of

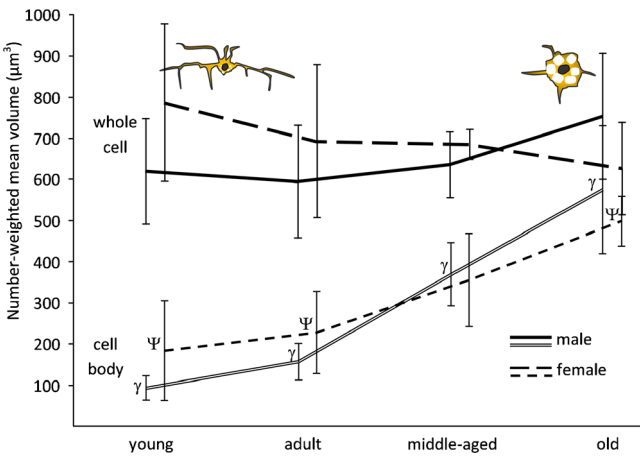

Fig. 5 Number-weighted mean volume of hepatic stellate cells in male and female young, adult, middle-aged and old rats. *Darker lines* refer to the volume of whole cell and *clearer lines* to the cell body. Data are expressed as means ± standard deviations. $^{\Psi, \gamma}$Statistically significant differences of old animals

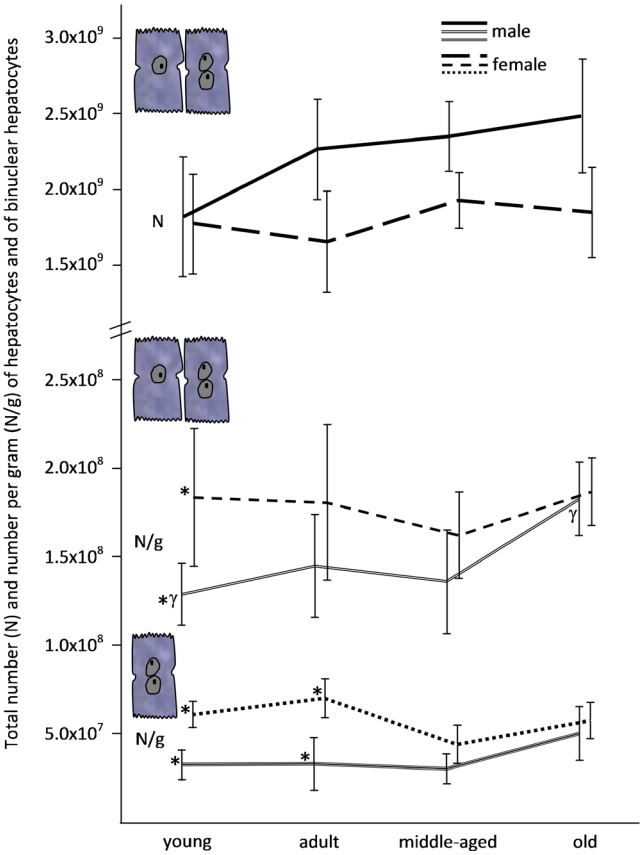

Fig. 6 Total number (*N*) and number per gram (*N/g*) of hepatocytes (mononucleate and binucleated) and of binucleated hepatocytes in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders. ᵞStatistically significant differences between age groups

KC ($\approx 23 \pm 3 \times 10^6$); however, gender differences were observed in young animals ($19 \pm 3 \times 10^6$ and $30 \pm 6 \times 10^6$ in males and females, respectively; $P = 0.016$; Fig. 8). Likewise, the N of LSEC was also stable with ageing and

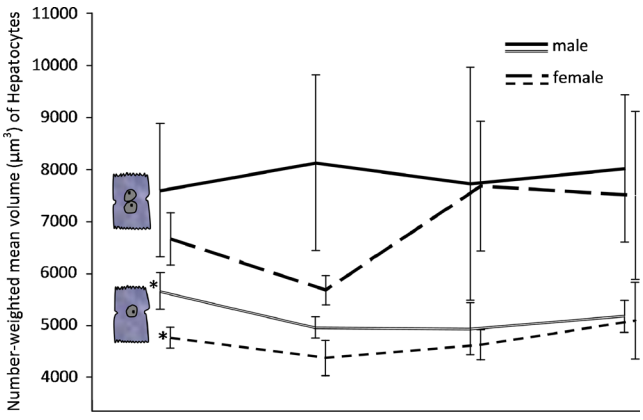

Fig. 7 Number-weighted mean volume of mononucleate and binucleated hepatocytes in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders

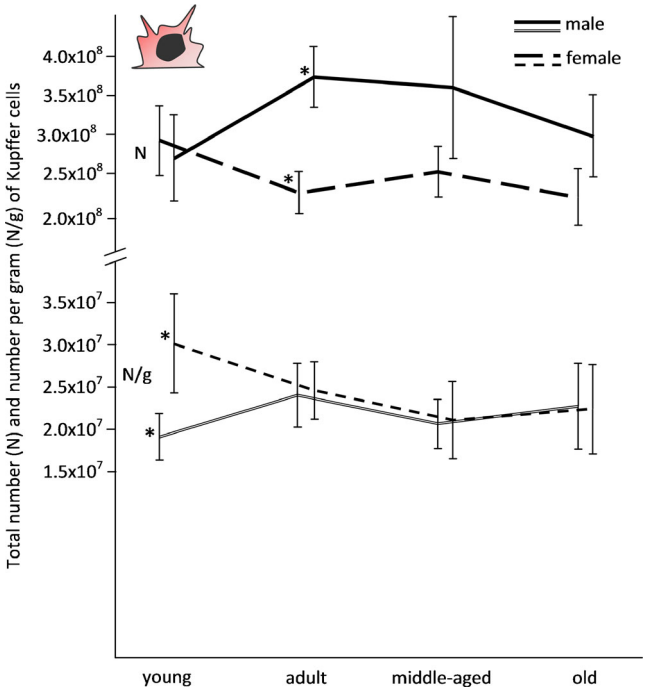

Fig. 8 Total number (*N*) and number per gram (*N/g*) of Kupffer cells in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders

gender ($\approx 802 \pm 25 \times 10^6$). Nevertheless, gender differences existed for the N/g ($P < 0.001$) and these occurred mostly in young animals ($40 \pm 4 \times 10^6$ and $95 \pm 11 \times 10^6$ in males and females, respectively; $P < 0.0001$; Fig. 9).

By estimating the N of HEP, HSC, KC and LSEC in the same set of animals, we were able to estimate the ratios among these cell types in the male and female rat liver (Fig. 10). Overall, the percentage of HEP, LSEC, KC and HSC in the rat liver was 60, 21, 8.7 and 5.7 %, respectively in males and 58, 25, 7.9 and 4.9 %, respectively in females, in the various age groups.

Notably, the coefficient of error for estimations of the N was always below the recommended threshold of 10 % (Marcos et al. 2012), being comprised of between 3.9 % and 6.8 %. Therefore, the sampling procedure was responsible for up to 24 % of the total observed variance, i.e., the variance attributable to the methodological procedures was much less important than the biological variability.

Discussion

To the best of our knowledge, this is the first study of liver ageing using quantitative morphology that addresses differences in both males and females. Wistar rats were used in this study because, on the one hand, this is one of the most common stock of animals used in liver research and, on the other hand, this strain has few age-related liver

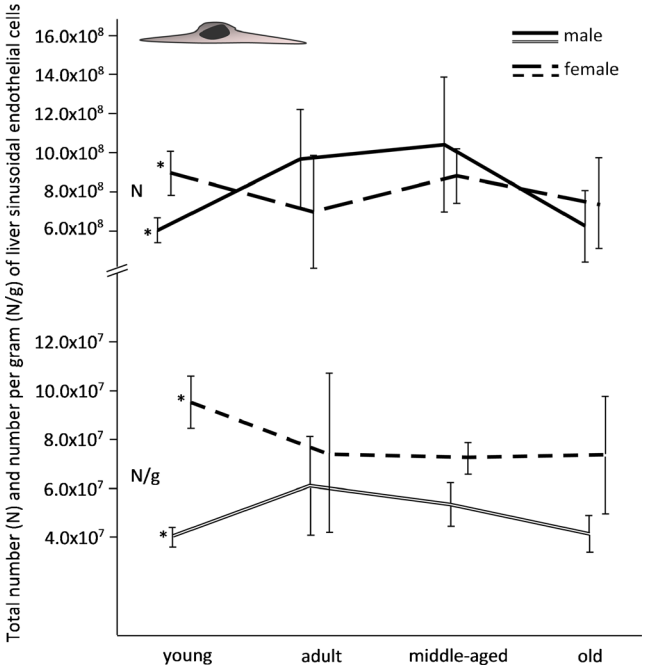

Fig. 9 Total number (*N*) and number per gram (*N/g*) of liver sinusoidal endothelial cells in male and female young, adult, middle-aged and old rats. Data are expressed as means ± standard deviations. *Statistically significant differences between genders

lesions; the only lesions consistently reported are altered cell foci, which tend to occur at a later age, around 2.5 years (Van Bezooijen 1984). Wistar rats therefore differ from other strains used in gerontology research, such as Fischer or Sprague–Dawley rats. Fischer strains have altered cell foci at an earlier age than Wistar and may present focal chronic hepatitis and bile duct hyperplasia (Van Bezooijen 1984). Sprague–Dawley rats have been described as exhibiting periportal inflammation, sinusoidal enlargement, fatty change and eosinophilic foci on reaching older ages (Van Bezooijen 1984; Sakai et al. 1997). With regard to sporadic tumours, aged Wistar rats have an increased incidence of pituitary adeno(carcino)ma, mammary adeno(carcino)ma (in females) and Leydig cell tumor (in males), although hepatocellular neoplasms are reported to be rare (Eiben and Bomhard 1999). The major drawback of using the Wistar strain in liver ageing studies, however, is that animals have to be aged in-house, since suppliers have a limited number of available rats (females over 6 months cannot be bought from existing companies).

Overall, this study has highlighted statistically significant correlations between the numbers of the various liver cell types in the rat liver. This is a new finding that supports the existence of a morphofunctional organisation, with well-defined ratios of parenchymal and non-parenchymal cells being maintained throughout ageing (Fig. 10). These ratios have been previously hypothesized by Rojkind et al. (2011) but have never been determined throughout ageing in males and females. Based on the volume estimation of mononucleate and binucleated HEP and their relative abundance, a theoretical porto-central cell cord composed of 17 ± 1 HEP in males and 16 ± 1 HEP in females was estimated. Another major conclusion that can be drawn from this study is that the ageing process attenuated most of the cytological differences in liver cells. The

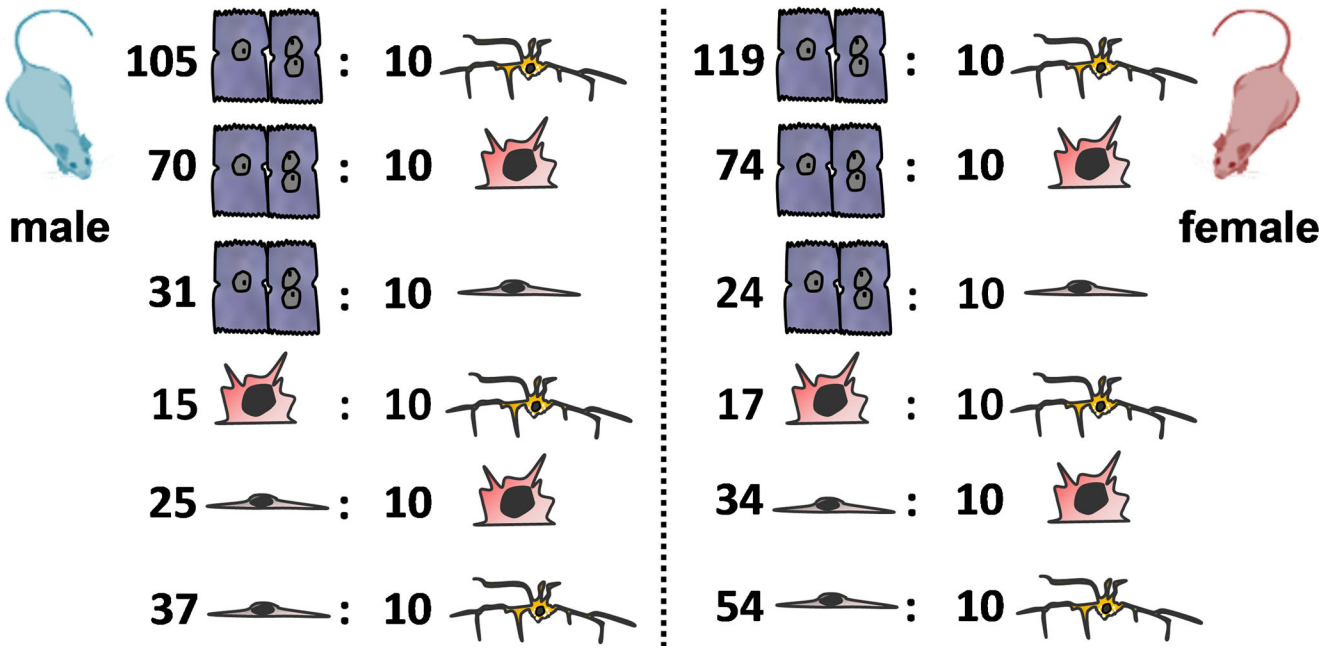

Fig. 10 Estimated cell ratios of hepatocytes, Kupffer cells, hepatic stellate cells and liver sinusoidal endothelial cells in male and female rats (considering all age groups)

gender dimorphism that existed in young animals for HEP, KC and LSEC but not for HSC, disappeared at older ages. Such a trend has never been revealed by morphology but it actually follows known patterns for enzymatic activities (Kitani 2007) and gene expression (Kwekel et al. 2010). An exhaustive micro-array gene expression study with rats has highlighted that most gene differences decrease by middle-age and are completely abolished in old animals (Kwekel et al. 2010).

Our initial hypothesis that morphological differences for HSC might exist with ageing is not supported by our data, since no differences were observed in the number or mean volume of HSC. So far, only HSC "numbers per area" have been determined with ageing; however, the studies have reached controversial conclusions. According to Cogger and Le Couteur (2009) and Warren et al. (2011), HSC double their number with ageing, whereas Enzan et al. (1991) reported no significant differences and Vollmar et al. (2002) suggested that the number of HSC might decrease up to 30 % with ageing. However, two-dimensional counts ("numbers per area") are known to be biased and may not reveal the three-dimensional reality of biological tissues (Mandarim-de-Lacerda 2003; Marcos et al. 2012). The larger cell body of an aged HSC might be prone to an overestimation bias in two-dimensional counts. The shift from a centrilobular to a periportal predominance of the HSC with ageing is another new finding. Quiescent HSC have been reported to be able to move slowly through the space of Disse (Senoo et al. 2007; Friedman 2008), being dynamic in the changeable 3D structure of the sinusoids (Sato et al. 2003; Senoo et al. 2007). It has long been known that, the matrix composition differs along the porto-central axis (Reid et al. 1992; Roskams et al. 2007; Senoo et al. 2010). An increase of factors that drive HSC migration, e.g., transforming growth factor-β1 (Yang et al. 2013), possibly occurs with ageing. Notably, aged HSC are not activated, being negative for α-smooth muscle actin antibody, as previously described (Warren et al. 2011). However, their shape changes with ageing. Classical and more recent studies have reported that HSC in aged animals appear swollen, having significantly more and larger lipid droplets than in young animals (Enzan et al. 1991; Warren et al. 2011). We confirmed this finding, since a significant increase in the number-weighted mean volume of the cell body occurs with ageing. Based on such differences, some authors have concluded that aged HSC are larger than young HSC (Vollmar et al. 2002; Schmucker 2005); however, our study suggests that, based on the number-weighted mean volume of HSC, the cells actually change from a small cell body with long and thin extensions in youngsters to a large cell body with thicker and much shorter extensions in older rats. Indeed, this large cell body justifies the recommendation of using old animals for isolating HSC (Ramadori and Saile 2002; Tacke and Weiskirchen 2012; Friedman 2008); as the cells contain more lipid droplets, they can be better separated by gradient centrifugation. Moreover, the larger cell body of HSC should also contribute to blood flow reduction in the sinusoids of older animals (Vollmar et al. 2002; Warren et al. 2011) because it protrudes into sinusoids, as has previously been reported (Warren et al. 2011) and HSC shift to periportal areas. We can hypothesize that the shorter processes of aged HSC should surround and control the blood flow of fewer sinusoids than in youngsters in whom HSC encircle more than two sinusoids (Friedman 2008).

Whether hyperplasia and/or hypertrophy of HEP occur with ageing remains controversial. The porto-central distance estimated in this study is in accordance with previous reports for rats (Wagenaar et al. 1994; Ruijter et al. 2004; Warren et al. 2008) and no ageing differences in this axis have been found. During the ageing process, only the enlargement of previously existing lobules is thought to occur (Vollmar et al. 2002). However, as the N/g of HEP increases by 35 % between young and old males and as the porto-central axis is maintained, two hypotheses may be proposed: lobules grow in height and/or new lobules might be formed. Taking into account that the relative volume of collagen within portal tracts and central venules is maintained throughout ageing, we susggest that an increase in height (i.e., taller lobules) is probably more important. This increased height, bearing an increased number of primary classical lobules at the base (liver surface), would justify the greater number of superficial lobules reported in the rat (Papp et al. 2009).

Another controversy surrounding HEP relates to the increase of binucleated HEP with ageing (Wheatley 1972; Schmucker 1998). Polyploidy is unanimously agreed to increase in the aged liver and some authors have also reported an upsurge in binuclearity (Popper 1985; Schmucker 1998; Malarkey et al. 2005). Even so, in our set of rats, we found no statistically significant ageing differences in the N of binucleated HEP or in their percentage. This is in agreement with previous studies, based on different methodologies in mouse and rat (Epstein 1967; Wheatley 1972; Faggioli et al. 2011). Notably, the percentage of binucleated HEP is negatively correlated with body and liver weight. This has previously been reported in the rat (Vinogradov et al. 2001), with very similar figures ($r \approx -0.57$).

The N of fully differentiated KC has never been evaluated throughout ageing to the best of our knowledge. Hilmer et al. (2007) reported a three-fold increase in "numbers per area" of KC with ageing by using thin paraffin sections stained with haematoxylin-eosin. Technical problems may explain the differences from our study, such as the use of haematoxylin-eosin, which is undesirable for

quantification not only because of uncontrolled bias in the counts but also because size differences (larger KC in old animals) may lead to overestimations when using "numbers per area". Other studies have suggested that the volume of KC increases with ageing (Martin et al. 1992, 1994), probably because of an accumulation of non-functional material in the cytoplasm (Martin et al. 1994).

Classic and more recent studies have addressed the structure of LSEC throughout ageing. Whereas the reduction in the number of *fenestrae* (ageing defenestration) in LSEC is nowadays well documented in various species (Le Couteur et al. 2001, 2008; Cogger and Le Couteur 2009), the N and N/g values have been sparsely detailed. Nevertheless, the number and percentage of LSEC have been proposed to be constant throughout ageing (De Leeuw et al. 1990) and the numbers of sinusoids have been reported to remain fairly unchanged during the lifespan of rats (Vollmar et al. 2002), results that are consistent with our findings. The value for N/g of LSEC has been determined to be approximately 20×10^6 (De Leeuw et al. 1990) and these cells appear to comprise 20 % of all liver cells (Malarkey et al. 2005). According to our findings, the N/g of LSEC appears higher (49×10^6 and 79×10^6 in males and females respectively) and these cells comprise 21 – 26 % of all liver cells.

The liver has much less collagen than most other organs, namely only ≈ 5 % (Roskams et al. 2007; Friedman 2008), as we have observed herein. Two previous studies followed the collagen content throughout ageing but only in males and noted that it increases with ageing (Porta et al. 1981; Gagliano et al. 2002). Curiously, both studies documented a collagen peak in 6-month-old animals, as we have observed. The reason for such an increase in collagen is unknown but it may represent a remodelling activity with the formation of new lobules, as is believed to occur during liver growth (Roskams et al. 2007). At this age and in older animals, metalloproteinase activity has been shown to be significantly reduced (Gagliano et al. 2002). We should emphasise that (intralobular) collagen cannot be solely related to HSC, since the expression of collagen I has been attributed to LSEC, HSC and HEP (Roskams et al. 2007). In this vein, we observed a significant correlation between collagen content and the two last-mentioned cell types, suggesting that both HSC and HEP are important for collagen synthesis in the normal organ.

In conclusion, this study contributes to knowledge about the process of liver ageing and highlights that liver structure is well preserved throughout life. Herein, we provided defined ratios between cells, ratios that will be relevant for the bioengineering construction of artificial livers and for in vitro studies, namely for co-culture models. Except for collagen content, male/female differences are attenuated by ageing and, thus, no major cytological or structural changes in normal liver cells should be able to compromise the long life of the liver.

References

Bathia SN, Balis UJ, Yarmush ML, Toner M (1999) Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. FASEB J 13:1883–1990

Bezooijen Van CFA (1984) Influence of age-related changes in rodent liver morphology and physiology on drug metabolism—a review. Mech Ageing Dev 25:1–22

Cogger VC, Le Couteur DG (2009) Fenestrations in the liver sinusoidal endothelial cell. In: Arias IM, Alter HJ, Boyer JL, Cohen DE, Fausto N, Shafritz DA, Wolkoff AW (eds) The liver: biology and pathobiology, 5th edn. Wiley, Chichester, pp 389–406

Davidson AJ, Castañón-Cervantes O, Stephan FK (2004) Daily oscillations in liver function: diurnal vs circadian rhythmicity. Liver Int 24: 179–186

De Leeuw AM, Brouwer A, Knook DL (1990) Sinusoidal endothelial cells of the liver: fine structure and function in relation to age. J Electron Microsc Tech 14:218–236

Eiben R, Bomhard EM (1999) Trends in mortality, body weights and tumor incidences of Wistar rats over 20 years. Exp Toxicol Pathol 51:523–536

Enzan H, Saibara T, Ueda H, Onishi S, Yamamoto Y, Okada T, Hara H (1991) Ultrastructural observation of Ito cells in the aged rats. In: Wisse E, Knook DL, McCuskey RS (eds) Cells of the hepatic sinusoid. Kupffer Cell Foundation, Leiden, pp 226–229

Epstein CJ (1967) Cell size, nuclear content, and the development of polyploidy in the mammalian liver. Proc Natl Acad Sci U S A 57: 327–334

Faggioli F, Vezzoni P, Montagna C (2011) Single-cell analysis of ploidy and centrosomes underscores the peculiarity of normal hepatocytes. PLoS One 6:e26080

Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 88:125–172

Frith J, Jones D, Newton JL (2009) Chronic liver disease in an ageing population. Age Ageing 38:11–18

Gagliano N, Arosio B, Grizzi F, Masson S, Tagliabue J, Dioguardi N, Vergani C, Annoni G (2002) Reduced collagenolytic activity of matrix metalloproteinases and development of liver fibrosis in the aging rat. Mech Ageing Dev 123:413–425

Gebhardt R, Mecke D (1983) Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. EMBO J 2:567–570

Gundersen HJ (1988) The nucleator. J Microsc 151:3–21

Hilmer SN, Cogger VC, Le Couteur DG (2007) Basal activity of Kupffer cells increases with old age. J Gerontol Ser A Biol Med Sci 62:973–978

Imai K, Sato M, Kojima N, Miura M, Sato T, Sugiyama T, Enomoto K, Senoo H (2000) Storage of lipid droplets in and production of extracellular matrix by hepatic stellate cells (vitamin A-storing cells) in Long-Evans cinnamon-like colored (LEC) rats. Anat Rec 258:338–348

Kitani K (1992) Liver and aging. Gastroenterol Jpn 27:276–285

Kitani K (2007) What really declines with age? The Hayflick lecture for 2006 35th American Aging Association. Age 29:1–14

Kumar RK (2005) Morphological methods for assessment of fibrosis. In: Varga J, Brenner DA, Phan SH (eds) Fibrosis research, methods and protocols. Humana Press, Totowa, pp 179–207

Kwekel JC, Desai VG, Moland CL, Branham WS, Fuscoe JC (2010) Age and sex dependent changes in the liver gene expression during the life cycle of the rat. BMC Genomics 11:675

Le Couteur DG, McLean AJ (1998) The aging liver. Drug clearance and an oxygen diffusion barrier hypothesis. Clin Pharmacokinet 34: 359–373

Le Couteur DG, Cogger VC, Markus AM, Harvey PJ, Yin ZL, Ansselin AD, McLean AJ (2001) Pseudocapillarization and associated energy limitation in the aged rat liver. Hepatology 33:537–543

Le Couteur DG, Warren A, Cogger VC, Smedsrød B, Sørensen KK, De Cabo R, Fraser R, McCuskey RS (2008) Old age and the hepatic sinusoid. Anat Rec 291:672–683

Loustaud-Ratti V, Jacques J, Debette-Gratien M, Carrier P (2016) Hepatitis B and elders: an underestimated issue. Hepatol Res 46: 22–28

Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR (2005) New insights into functional aspects of liver morphology. Toxicol Pathol 33:27–34

Mandarim-de-Lacerda CA (2003) Stereological tools in biomedical research. An Acad Bras Cienc 75:469–486

Manikonda PK, Jagota A (2012) Melatonin administration differentially affects age-induced alterations in daily rhythms of lipid peroxidation and oxidant enzymes in male rat liver. Biogerontology 13:511–524

Marcos R, Monteiro RAF, Rocha E (2004) Estimation of the number of stellate cells in a liver with the smooth fractionator. J Microsc 215: 174–182

Marcos R, Monteiro RAF, Rocha E (2006) Design-based stereological estimation of hepatocyte number, by combining the smooth optical fractionator and immunocytochemistry with anticarcinoembryonic antigen polyclonal antibodies. Liver Int 26:116–124

Marcos R, Monteiro RAF, Rocha E (2012) The use of design-based stereology to evaluate volumes and numbers in the liver: a review with practical guidelines. J Anat 220:303–317

Marcos R, Bragança B, Fontes-Sousa AP (2015) Image analysis or stereology: which to choose for quantifying fibrosis? J Histochem Cytochem 63:734–736

Martin G, Sewell RB, Yeomans ND, Smallwood RA (1992) Ageing has no effect on the volume density of hepatocytes, reticulo-endothelial cells or the extracellular space in livers of female Sprague–Dawley rats. Clin Exp Pharmacol Physiol 19:537–539

Martin G, Sewell RB, Yeomans ND, Morgan DJ, Smallwood RA (1994) Hepatic Kupffer cell function: the efficiency of uptake and intracellular degradation of 14C-labelled mitochondria is reduced in aged rats. Mech Ageing Dev 73:157–168

Papp V, Dezsö K, László V, Nagy P, Paku S (2009) Architectural changes during regenerative and ontogenic liver growth in the rat. Liver Transpl 15:177–183

Popper H (1985) Coming of age. Hepatology 5:1224–1226

Porta E, Joun N, Nitta R (1980) Effects of the type on dietary fat at two levels of vitamin E in Wistar male rats during development and aging. I. Life span, serum biochemical parameters and pathological changes. Mech Ageing Dev 13:1–39

Porta E, Keopuhiwa L, Joun N, Nitta R (1981) Effects of the type on dietary fat at two levels of vitamin E in Wistar male rats during development and aging. III. Biochemical and morphometric parameters of the liver. Mech Ageing Dev 15:297–335

Ramadori G, Saile B (2002) Mesenchymal cells in the liver—one cell or two? Liver 22:283–294

Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA (1992) Extracellular matrix gradients in the space of Disse: relevance to liver biology. Hepatology 15:1198–1203

Rojkind M, Philips GG, Diehl AE (2011) Microarchitecture of the liver: a jigsaw puzzle. J Hepatol 54:187–188

Roskams T, Desmet VJ, Verslype C (2007) Development, structure and function of the liver. In: Burt A, Portmann B, Ferrell L (eds) MacSween's pathology of the liver, 5th edn. Churchill Livingstone, Edinburgh, pp 1–73

Ruijter JM, Gieling RG, Markman MM, Hagoort J, Lamers WH (2004) Stereological measurement of porto-central gradients in gene expression in mouse liver. Hepatology 39:343–352

Sakai Y, Zhong RR, Garcia B, Zhu L, Wall WJ (1997) Assessment of the longevity of the liver using a rat transplant model. Hepatology 25: 421–425

Santos M, Marcos R, Santos N, Malhão F, Monteiro RAF, Rocha E (2009) An unbiased stereological study on subpopulations of rat liver macrophages and on their numerical relation with the hepatocytes and stellate cells. J Anat 214:744–751

Sato M, Suzuki S, Senoo H (2003) Hepatic stellate cells: unique characteristics in cell biology and phenotype. Cell Struct Funct 28:105–112

Sawada M, Carlson J (1987) Changes in the superoxide radical and lipid peroxide formation in the brain, heart and liver during the lifetime of the rat. Mech Ageing Dev 41:125–137

Schmucker DL (1998) Aging and the liver: an update. J Gerontol 53:315–320

Schmucker DL (2001) Liver function and phase I drug metabolism in the elderly: a paradox. Drugs Aging 18:837–851

Schmucker DL (2005) Age-related changes in liver structure and function: implications for disease? Exp Gerontol 40:650–659

Schmucker DL, Sanchez H (2011) Liver regeneration and aging: a current perspective. Curr Gerontol Geriatr Res 2011:526379

Senoo H, Kojima N, Sato M (2007) Vitamin A-storing cells (stellate cells). Vitam Horm 75:131–159

Senoo H, Yoshikawa K, Morii M, Miura M, Imai K, Mezaki Y (2010) Hepatic stellate cell (vitamin A-storing cell) and its relative—past, present and future. Cell Biol Int 34:1247–1272

Tacke F, Weiskirchen R (2012) Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques. Expert Rev Gastroenterol Hepatol 6:67–80

Vinogradov AE, Anatskaya OV, Kudryavtsev BN (2001) Relationship of hepatocyte ploidy levels with body size and growth rate in mammals. Genome 44:350–360

Vollmar B, Pradarutti S, Richter S, Menger MD (2002) In vivo quantification of ageing changes in the rat liver from early juvenile to senescent life. Liver 22:330–341

Wagenaar GT, Moorman AF, Chamuleau RA, Deutz NE, De Gier C, De Boer PA, Verbeek FJ, Lamers WH (1994) Vascular branching pattern and zonation of gene expression in the mammalian liver. A comparative study in rat, mouse, cynomolgus monkey, and pig. Anat Rec 239:441–452

Warren A, Chaberek S, Ostrowski K, Cogger VC, Hilmer SN, McCuskey RS, Fraser R, Le Couteur DG (2008) Effects of old age on vascular complexity and dispersion of the hepatic sinusoidal network. Microcirculation 15:191–202

Warren A, Cogger VC, Fraser R, DeLeve LD, McCuskey RS, LeCouteur DG (2011) The effects of old age on hepatic stellate cells. Curr Gerontol Geriatr Res 2011:439835

Wheatley DN (1972) Binucleation in the mammalian liver. Exp Cell Res 74:455–465

Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, Xu L, Eng F, Afdhal N, Kalluri R (2013) Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. Gastroenterology 124:147–159

Zeeh J (2001) The aging liver: consequences for drug treatment in old age. Arch Gerontol Geriatr 32:255–263