The ability of a ketogenic diet (KD) to produce many metabolic changes makes it a plausible candidate for the modulation of hepatic stellate cells (HSCs) which have an important influence on liver structure and function. According to one group of authors, the KD is a positive regulator of brain excitability and metabolism in some pathological conditions, including pediatric epilepsy, obesity, cardio-vascular disturbances and oxidative stress (1-4). Unfortunately, another group presents adverse consequences of following KD, such as growing fatigue and hepatic growth hormone (GH) resistance lowering perceived effort and an undesirable reaction which resembles nonalcoholic fatty liver disease (NAFLD) (5-7). These opposing examples of KD action create a distinctive, but incompletely defined, cellular, molecular, and integrated metabolic state. In this respect, no studies have addressed KD on HSC, even though these cells are well-known, important contributors to fibrotic liver responses.

In a normal physiological state, quiescence HSC comprise no more 1.4% of total liver volume and their lipid droplets consisted of 60.5% nonretinoid lipid and 39.5% retinoids as retinyl palmitate. The nonretinoid lipid consisted of 58.6% triglycerides, 28.4% cholesteryl ester, 8.7% cholesterol, and 4.4% free fatty acid (8). However, after any insults HSC initiate the production of fibrogenic cytokines, mainly transforming growth factor (TGF)-β1, and transform into myofibroblastic phenotypes. In this new form, they begin to produce extracellular matrix (ECM) components including collagen, α smooth muscle actin (ASMA), desmin, and glial fibrillary acidic protein (GFAP) (9). It is also important that these cells, after activation, amplify disturbances in hepatocytes by secreting cytokines such as TNF and IL-6, chemo-attracting inflammatory cells and acquiring a fibrogenic potential that paves the way to cirrhosis (10).

This double-edged action of HSC prompted us to determine the influence of KD on HSC derived from female ovarectomised rats subjected to the long-term administration of a high-fat, low-carbohydrate ketogenic diet.

On the basis of findings conducted by Oishi et al., KD mimic the metabolic condition of fasting or caloric restriction (11). In such circumstances sex differences in metabolic response have been observed (12, 13). Female sex steroids, most of all estradiol (E2), became more ketogenic than men, resulted in plasma ketones and betahydoxybutyrate elevation and free fatty acids depletion. Since the ketogenic effect was associated with a relative suppression of plasma free fatty acids, this may be indicative of a direct action of E2 on hepatic disposition of free fatty acids and/or the biosynthetic pathways for ketone body production (12, 13).

Taking into account estrogenic influence on ketogenesis as well as different responses of HSC exposed to E2, namely...
activation of HSC with enhancement of fibrogenesis and antifibrogenic actions of E2 due to suppression of HSC proliferation and transformation, we also sought to define action of estrogens on HSC subjected ketogenic influences.

MATERIALS AND METHODS

Animals and experimental design

Ten-weeks-old female Wistar rats (total n=35) weighing 300–350 g were used in this study. Animals were kept in a temperature- and humidity-controlled room with a 12 h light-dark cycle. The rats had 10 days to adapt to the facility, during which they consumed the standard diet. Then they were divided into:

- control group fed with a standard diet, and additionally separated into nonovarectomised (NOVX) (n=5) and ovariectomised (OVX) rats (n=5);
- first experimental group fed with a ketogenic diet (Table 1), separated into NOVX (n=5), OVX (n=5) and OVX rats injected with E2 (5.70 mg/kg b. wt. once a week) (n=5);
- second experimental group fed with standard diet and additionally subjected by thioacetamide (500 mg/l) in drinking water, composed with NOVX (n=5) and NOVX rats injected with E2.

After 4 weeks of KD and TAA administration, from each rat samples of blood and liver tissue were collected. All experimental procedures were approved by the Local Ethics Committee on Animal Care at the University of Life Sciences.

Isolation and culture of hepatic stellate cells

Hepatic stellate cells were isolated as described previously (9, 14). Before laparotomy, animals were anesthetized by a mixture of ketamine (90 mg/kg b. wt) and xylasine (10 mg/kg b. wt) intramuscular administration. The liver was perfused in situ through the portal vein by a Krebs-Ringer solution, a Krebs-Ringer buffer: a) containing EGTA, b) without Ca2+ and a chelating agent, and c) with type IV collagenase. After the perfusion, the liver was transferred to a HAMS-12/DMEM (1:1 v/v) culture medium, filtered through double gauze and digested (1 h; 37°C) with trypsin and 0.004% DNase I. Then, non-parenchymal cells were separated from the supernatant obtained after harvesting the liver was transferred to a HAMS-12/DMEM (1:1 v/v) culture medium, filtered through double gauze and digested (1 h; 37°C) with trypsin and 0.004% DNase I. Then, non-parenchymal cells were separated from the supernatant obtained after harvesting parenchymal cells at 50 × g for 20 min at 4°C. The mixture was decanted through a 70 µm-nylon mesh, followed by a 40 µm-nylon mesh, and then loaded on a discontinuous gradient of Percoll and centrifuged. (2000 rpm, 20 min). The collected cell suspension was washed with an antibiotic-supplemented medium containing 10% foetal calf serum and plated on plastic at 1 × 10⁶ cells per well in 1000 µl of medium in a 24-well plate in a 37°C incubator with 5% CO₂. The viability of the cell was estimated by the trypan-blue exclusion method, and ranged between 75–85%.

After the 4th and 8th day of incubation HSC, their lysates, and the media for further analysis were collected.

Plasma E2 analysis

Steroid hormones (especially 17β-oestradiol) were extracted from the culture medium with dichloromethane and analysed by high performance liquid chromatography (HPLC, Beckman Gold System USA) with UV detection (220 nm). The mobile phase orthophosphoric acid acetonitrile (1:2 v/v) was pumped at a flow rate of 0.8 ml/min (15). The plasma concentration of E2 was calculated according to the following formula: Cp = Cc/As × Ap, Cc – sample concentration of E2, Cc – standard concentration; Ap – standard peak area; As – sample of E2 peak area.

Assessment of liver tissue collagen

Using formalin-fixed liver sections, the Masson trichrome staining was performed to assess changes in collagen deposition and fibrosis (16, 17).

Quantification of transforming growth factor-β1, collagen type I and a smooth muscle actin

The medium concentration of TGF-β1 and collagen type I, as well as the ASMA level in cell lysates, were measured by an enzyme-linked immunosorbent assay (ELISA) kits (USCN, Life Sciences Inc.; Causbio Biotech Co. Ltd., Chondrex, Inc.) according to the manufacturer’s instructions. The absorbance of each well was monitored by a Microplate reader (Alab Plate Reader ELISA).

Cell proliferation assay

MTT analysis-assessment of cell proliferation was based on the reduction of tetrazolium salt into blue formazan by mitochondrial dehydrogenase of viable cells (18). Cultures were pulsed with 15 µl of the MTT (3-[4,5-dimethylthiazol-2-yl]–2,5-diaryl tetrazolium bromide) for 3 hours at 37°C and solubilized with 0.01% SDS to dissolve the dark blue crystals overnight. The microplate reader (Alab Plate Reader ELISA) measured the optical density (OD) of formed blue formazan at the wavelength of 600 nm. The results were expressed as the proliferation index (PI).

Retinol assay

Culture medium (1 ml) was denatured with ethanol. The samples were extracted with hexane, and retinol and β-carotene in the extract were determined spectrophotometrically at A325 and A453 (19). The medium concentration of retinol was calculated according to the following formula: retinol (µM) = A325 – β-carotene conc. × 0.00017/0.00182.

Table 1. Constituents of the ketogenic diet (%).

<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Lard</td>
<td>47.5</td>
<td></td>
</tr>
<tr>
<td>Butter</td>
<td>19.95</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Fiber</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>0.76</td>
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</tbody>
</table>

Table 2. Plasma E2 concentration. HPLC analysis revealed significantly higher values of E2 only in rats treatment with this estrogen.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control OVX</th>
<th>KD</th>
<th>KD OVX</th>
<th>KD OVX + E2</th>
<th>TAA</th>
<th>TAA + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 (pg/ml)</td>
<td>59.5±2.4</td>
<td>10.3±1.7</td>
<td>67.2±7.9</td>
<td>18.4±5.7</td>
<td>154.0±10.8</td>
<td>60.5±8.7</td>
<td>170.5±10.7</td>
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a,b,c - values signed with different letter differ significantly at P≤0.05.
RESULTS

During 4 weeks of ingesting KD, the rats accumulated a collagen in the liver, which was localized primarily in the portal region of the liver lobus (Fig. 1). Apart from this matrix response, distinct macrovesicular steatosis has been appeared prominently. As shown in Fig. 1C, these abnormalities almost completely disappeared in the liver derived from rats additionally treated with E2. Moreover, it should be noticed that KD caused a deposition at almost the same level of collagen as TAA, a well known fibrogenic agent.

As shown Fig. 2C, developing of long control HSC extensions and loss of lipid vacuoles is clearly observed till 8th day of incubation. In comparison to this, cellular extension of HSC obtained from KD and TAA rats appeared much earlier, namely at 4th day of culture. At the end of cell incubation (8th day) culture of these cells reach confluency and compose flat monolayer. Supposing cells derived from KD-rats loss of lipid droplets after 4th day of incubation, the HSC obtained from TAA-rats lost lipid contents already after isolation and acquire more myofibroblastic shape.

The medium retinol concentration released by the HSC as derived from control and control OVX rats was significantly (p≤0.05) elevated in the 8th day of incubation and averaged 105.8±14.1 and 100.2±5.58 µM, respectively (Fig. 3). The HSC obtained from KD- and KDOVX-rats released considerably more retinol on both the 4th and the 8th day of incubation. This occurrence was markedly (p≤0.05) suppressed under the...
influence of E₂ administration. Importantly, there were any significant enhancement of retinol loss from cells obtained from TAA and TAA+E₂ condition.

The HSC derived from KD-rats released remarkably more TGF-β₁ than the cells obtained from animals fed with a standard diet (Fig. 4). It should be underlined that the ovariectomy of KD-rats markedly intensified the secretion of this fibrogenic cytokine by the HSC, noticed especially on the 8th day of incubation (201.33±17.15 pg/ml). However, in the HCS of rats exposed to E₂, the TGF-β₁ concentration was lower and did not exceed 137±24.39 pg/ml. A similar inhibitory effect of E₂ was found in the culture of the HSC obtained from TAA-treated rats.

There was a negative relationship between the medium concentration of TGF-β₁ and the proliferative activity of the HSC, which remained insignificant after the 4th day of culture but became very clear after the 8th day of culture (r = –0.16, p≤0.733 and r= –0.7, p≤0.075, respectively), (Fig. 5).

In respect to the collagen type I, the HSC obtained from ovariectomised KD-rats released an augmented and notable amount of the ECM protein after the 8th day of culture (1.83±0.14 U/ml) (Fig. 6). At the same time, the HCS of TAA rats released the highest concentration of collagen (2.09±0.17 U/ml). It must be emphasized that the estrogens produced a markedly diminished amount of this ECM protein only in the HSC obtained from the KD-rats. As shown in Fig. 7, the HSC of nonovarectomised and ovariectomised control rats displayed negligible amounts of ASMA which did not exceed 0.02±0.002 and 0.07±0.006 pg/mg protein, respectively. Higher quantities of ASMA appeared in the KD rats with significant augmentation after the 8th day of culture (1.41±0.3 pg/mg protein). At this time of cell incubation, the influence of E₂ did not markedly decrease the amount of ASMA. Only after the 4th day of cell culture, the ASMA medium concentration dropped (p≤0.05) to 0.54±0.07 pg/mg protein. A similar ASMA response of the HSC to E₂ under influence of TAA was noted.
After both the 4th and the 8th day of culture, negative values of the correlation coefficient between analysed proteins and E2 were found (Fig. 8). The value of the correlation coefficient was preserved after the 8th day of incubation in respect to collagen type I, but this was not the case for ASMA.

**DISCUSSION**

We found for the first time that prolonged feeding of rats with KD provokes an activation of HSC which start to produce extracellular matrix components. Results obtained by other
authors clearly showed that no ketone bodies but changes in the tissue content of polyunsaturates create the background of multidirectional influences of this diet (20). After the mobilization of polyunsaturates from adipose tissues, their distribution is qualitatively selective with a preferential direction of more useful arachidonate and docosahexaenoic acids to the brain, but less desirable components such as eicosatrienoic and docosapentainoic acids are abundantly taken up by the liver. Therefore, we hypothesize that under the influence of KD, most healthy polyunsaturates are directed into the brain and the more deleterious forms are trafficked to the liver, and consequently they may be responsible for the activation of HSC. Also Duvnjak et al. described the significance of PUFA in the development of liver fibrosis (21). As mentioned by authors, deficiency of the n-3 series long-chain polyunsaturated fatty acids (LCPUFA) and subsequent increased n-6/n-3 fatty acids ratio leads to higher hepatic uptake of free fatty acids and decrease of hepatic β-oxidation process. The next step is believed to be excessive production of reactive oxygen species (ROS) by mitochondria and cytochrome P-450 system in the liver. Subsequently, ROS trough lipid peroxydation, pro-inflammatory cytokine and Fas-ligand induction promote progression to steatohepatitis, fibrosis and finally cirrhosis.

In our experiment, HSC activated by KD, aside from the remodeling of ECM proteins, started to release cytokine TGF-β1. In relation to control group, KD-derived HSC produce and secrete much more TGF-β1, which is regarded as a key factor not only in tissue repair, but also in the development of fibrogenic disorders (22, 23). Increased levels of the mRNA of this mitogenic and fibrogenic cytokine have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis in rats induced by CCl4, by TAA, or caused by schistosomiasis (24, 25). In such condition plasma level of TGF-β1 markedly exceeded control value 8.9±0.8 ng/ml (22, 23). Acting via TGFRI and TGFRII receptors present on the cell surface, TGF-β1 not only stimulate the HSC for proliferation and matrix protein synthesis, but also maintain activated HSC alive (23). Our results, especially the high negative relationship between released TGF-β1 and the proliferative activity of HSC obtained in the 8th day of culture seem to confirm the stimulating effect of this cytokine, particularly in the late phase of cell activation. Additionally, we found that concomitant with an elevation of TGF-β1 concentration, a higher amount of collagen type I accumulated in the culture medium.

Significantly, we discovered that a positive correlation between cytokine and the collagen amount was more enhanced when HSC were derived from ovariectomised rats fed with KD. Regulatory actions of TGF-β1 on collagen production by activated HSC has been described by many authors, included Oltenau et al. TGF-β1, derived from both paracrine and autocrine sources, is the most potent fibrogenic cytokine in the liver (17). Once activated, TGF-β1 leads to induction of collagen production and transdifferentiated the quiescent HSC to myofibroblast. Many authors, including Shimizu et al., suggested that endogenous as well as exogenous 17β-estradiol might participate in protecting against hepatic fibrosis (26, 27). First of all, this is associated with a reduced expression of type I procollagen and a tissue inhibitor of metalloproteinase I in the liver (26). The present study showed that E2 diminished KD-induced transformation of HSC to myofibroblast-like cells apparently by the inhibition of TGF-β1 release and low collagen and ASMA production by HSC. The complete pathway by which E2 inhibits HSC activation is still not clear (26-28). It is presumed that ERs of HSCs mediate the fibrosupressive effect of E2. However, several lines of evidence do not support this hypothesis. In this regard, high levels of ERα but low or no levels of ERβ expression were observed in normal and fibrotic livers and also in quiescent and activated HSC from both male and female rats. Results obtained by Schendzielorz et al. revealed that in response to E2, hepatic ERα is substantially more efficient in activating transcriptions than ERβ (29). However, in in vitro studies with liver cell expressing both ER subtypes, ERβ dampens ERα activity, thereby changing of tissue sensitivity under exposure to the different E2 concentrations. Furthermore, many authors indicate that the physiological values of E2 are unable to suppress hepatic fibrosis in rats following the administration of fibrogenic agents (26, 27). We showed that in TAA poisoning, the rats exposure to E2 only temporarily inhibits collagen and ASMA releasing by HSC. During prolonged culture, expression of these ECM proteins, especially ASMA (being a typical myofibroblast marker), continuously rose. As shown some by authors most of total hepatic lipids, reached in rats 51.2±2.8 mg/g wet liver, are stored in the HSC in nonretinoid and retinoid form (8). Percentage distribution of retinoids in rat liver cell indicated that 80–90% is localized in
stellate cells. In our experiment, KD augmented the retinol disappearance by HSC isolated from both ovarectomised and nonovarectomised rats. Many authors suggest that the loss of lipid droplets with retinyl esters is a hallmark of HSC activation and its transformation from quiescent to more fibrogenic phenotypes (9, 14). The disappearance of lipid droplets from HSC may be due to energetic resetting from glucose fuel into increased oxidation of fatty acids resulting from growing insulin resistance. Furthermore, it is regarded that lipids are shifting into skeletal muscle they serve as an energetic fuel in return for glucose under insulin resistance (30). Another possible way for the movement of lipid droplets from HSC is connected with the expression of adipose differentiation-related proteins (ADRP). These proteins are responsible for lipid droplets formation, stabilization, and lipolysis (31). Imai et al. postulated that in mice which were given ADRP-antisense oligonucleotides and fed with a high fat diet, down-regulation of ADRP seems to enhance HSC activation, ameliorate hepatic steatosis, and improve insulin action (32). Oppositely, up-regulation of ADRP caused not only higher lipid contents accumulated in HSC, but also decreased the expression level of ASMA, collagen type I, and MMP-2 mRNA. Finally, KD is able to induce morphological and functional changes in HSC, revealed as transdifferentiation into myofibroblastic like cells. HSC derived from rats deprived of ovary, revealed as transdifferentiation into more retinol changes in HSC, revealed as transdifferentiation into myofibroblastic like cells. HSC derived from rats deprived of ovaries responded to KD in more exaggerated ways. Considerably greater level of ECM remodeling, more retinol disappearance, and the releasing of TGF-$\beta$1 appeared. However, the preservation of E$_2$ in ovarectomised rats didn’t substantially alter the activation of HSC. Since in this approach only healthy rats were used, our observations couldn’t be strictly referred to a clinical state in which KD is employed.

Conflict of interests: None decared.

REFERENCES

20. Taha AY, Ryan MA, Cunane SC. Despite transient ketosis, the classic high-fat ketogenic diet induces marked changes in fatty acid metabolism in rats. Metabolism 2005; 54: 1127-1132.
28. Liu QH, Li DG, Huang X, Zong CH, Xu QF, Lu HM. Suppressive effects of 17$\beta$-estradiol on hepatic fibrosis in...


Received: April 19, 2012
Accepted: January 30, 2013

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