*Original article*

# Proliferative and oxidative response of hepatocytes (Hep) and hepatic stellate cells (HSC) isolated from rats exposed to ketogenic diet

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## Abstract

Ketogenic diet (KD) is considered in the context of its anti-epileptic effects, but its influence on liver dysfunction has not been elucidated yet. The study was aimed to investigate the activity of hepatocytes (Hep) and hepatic stellate cells (HSC) isolated from rats fed with KD, in respect of NO and superoxide generation by these cells as well as their proliferative activity *in vitro*. We also sought to characterize the plasma FFA profiles in control and ketogenic rats. Hep and HSC were isolated by the collagenase perfusion method and separated by the Percoll gradient centrifugation. After the 4th, 8th and 12th day of incubation, the media were collected for further analysis. NO generation increased within the time of incubation both in Hep and HSC isolated from KD-rats. In HSC group NO production raised significantly from $2.65 \pm 0.07$ $\mu M/10^6$ cells on 4th day of incubation to $5.49 \pm 1.2$ $\mu M/10^6$ cells on 12th day of incubation. In respect to $O_2^-$ generation experimental Hep and HSC provide considerably higher quantities of this free radical. until 12th day of incubation ($2.5 \pm 0.07$ and $3.2 \pm 0.3$ $nM/10^6$ cells, respectively). Although KD exerts anti-proliferative effect on hepatocytes, in respect to HSC it intensifies their proliferative activity. Furthermore, as we estimated on the basis of NO and $O_2^-$ generation both Hep and HSC exposed to KD are the source of free radicals.

**Key worlds**: hepatocytes, hepatic stellate cells, ketogenic diet, free fatty acids

## Introduction

The ability of a ketogenic diet (KD) to produce many metabolic changes makes it a plausible candidate for the modulation of hepatocytes (Heps) and hepatic stellate cells (HSCs) which exerts an important influence on liver structure and function. In a nor-

mal physiological state, quiescent HSC comprise no more than 1.4% of total liver volume and their lipid droplets consisted of 60.5% nonretinoid lipid and 39.5% retinoids as retinyl palmitate (Bobowiec et al. 2013). However, after any insult HSC initiate the production of fibrogenic cytokines, mainly TGF-β 1 and transform into proliferating myofibroblastic feno-

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types. The next step is believed to be excessive production of reactive oxygen species (ROS) by mitochondria and cytochrome P-450 system in the liver. Oxidative stress and upregulation of pro-inflammatory cytokines potentially contributes to inflammatory liver damage, progression of steatohepatitis, fibrogenesis and finally cirrhosis (Browning and Horton 2004, Wobster et al. 2009).

KDs are considered in the context of their anti-epileptic effects and their involvement in alleviation of signs of other serious disorders such as among other neurodegeneration and neuroinflammation, metabolic defects, as well as trauma and ischemia, but some effects involved in liver dysfunction were not precisely studied until now (Dell et al. 2001, Cullingford 2004, Barañano et al. 2008). Because of the high-fat nature of the KD, special emphasis has been given to :lipostat; transcription factors, such as the peroxisome proliferator-activated receptor a (PPARa), that is activated by a range of fatty acid ligands, including essential free fatty acids (FFAs) and fatty acid derivatives. FFAs are major components of biological cell membranes which play important roles in intracellular signalling and as precursors for ligands for nuclear receptors. According to recent hypothesis FFA appear to be the major mediators of excessive hepatic lipid accumulation. When capacity of Heps and HSC to safely store the excess of FFA in form of TG in lipid droplets is exceeded, hepatic injury may occur (Taha et al. 2005, Ricchi et al. 2009).

The lack of studies addressing the influence of KD on Hep and HSC, prompted us to investigate the activity of these cells isolated from rats fed with KD, in respect to NO and superoxide generation as well as their proliferative activity *in vitro*. We also sought to characterize the plasma FFA profiles in control and ketogenic rats.

## Materials and Methods

### Animals and experimental design

Ten-week-old female Wistar rats (total n=6) weighting 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 randomly assigned to one of two groups: control group (K) fed with standard diet (n=3) and experimental group fed with KD (n=3) (Table 1). After 4 weeks of KD administration, from each rat the samples of the blood were collected. All experimental procedures were approved by the Local Ethics Committee on Animal Care at the University of Life Sciences.

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

| Lard | 47.5 |
|---|---|
| Butter | 19.95 |
| Corn oil | 11.4 |
| Proteins | 9.5 |
| Fiber | 5.0 |
| Ash | 3.8 |
| Vitamin Mix | 2.09 |
| Dextrose | 0.76 |

### Isolation and culture of hepatocytes and hepatic stellate cells

Hep and HSC were isolated as described previously with minor modifications (de Leeuw et al. 1984, Bobowiec et al. 2013). Before laparotomy animals were anesthetized with the mixture of Ketamine (90 mg/kg b. Wt.) and Xylasine (10 mg/kg b. Wt.), intramuscularly. The liver was perfused *in situ* trough the portal vein by Krebs-Ringer buffer: a) containing EGTA, b) without $Ca^{2+}$ and chelating agent and c) with type IV collagenase. After the perfusion, the liver was transferred to HAMS-12/DMEM (1:1 v/v) culture medium and divided into two parts. To obtain Hep, first part of liver, was centrifuged at 50 *g* for 5 minutes. Then, cells were washed twice with medium and plated on plastic dishes at medium density of $1x10^6$ cells/ml and incubated at 37°C and 5% $CO_2$. Second part of liver was digested (1 h, 37°C) in PBS containing 0.1% collagenase, 0.1% protease E, 0.25% trypsin and 0.004% DNase I. Then, non-parenchymal cells were prepared from the supernatant obtained after harvesting parenchymal cells at 50 x *g* for 20 min at 4°C. The mixture was decanted trough a 70 μm-nylon mesh, followed by a 40 μm- nylon mesh and then loaded on discontinuous gradient of Percoll and centrifuged (2000 rpm, 20 min). Collected cell suspension was washed with antibiotic- supplemented medium containing 10% foetal calf serum and plated on plastic at 250,000 cells per well in 1000 μl of HAMS-12/DMEM (1:1 v/v) medium in a 24-well plate at a 37°C with 5% $CO_2$. The viability of the cells was estimated by the Trypan blue exclusion method and ranged between 75-85%. On 4th and 8th and 12th day of incubation HSC their lysates and the media were collected for analysis.

## Analytical procedures

### Nitric oxide production

Nitric oxide level was determined by Griess reaction. Briefly, 50 µl of supernatant were mixed with 200 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylendiamine dihydrochloride and 2.5% $H_3PO_4$). All tests were done in duplicate. Absorbance at 545 nm was measured with microplate reader (Alab Plate Reader ELISA) after 10 min incubation with Griess reagent and compared with a standard curve prepared with serial dilutions of sodium nitrite in medium without cells. Obtained values were expressed as a concentration of nitrite, the stable product of NO, which accumulates in medium (Wessely-Szponder and Szponder 2010).

### Superoxide anion analysis

Superoxide anion production was measured by the method described previously (Wessely-Szponder and Szponder 2010). Heps and HSC were incubated with 0.1% nitroblue tetrazolium (NBT-Sigma) solution at room temperature for 10 minutes and then absorbance was read at 545 nm. Nanomoles of superoxide produced over the incubation period were calculated using the extinction coefficient 21.1 nmol.

### Cell Proliferation Assay

MTT analysis of cell proliferation was based on the reduction of tetrazolium salt into blue formazan by mitochondrial dehydrogenase of viable cells (Wójcik et al. 2010). Cultures will be pulsed with 15 µl of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) for 3 hours at 37°C and solubilised to dissolve the dark blue crystals overnight. Microplate reader measured the absorbance (OD) of formed blue formazan at the wavelength of 600 nm. The results were expressed as the proliferation index (PI) according to the formula for results of duplicate assays:

### PI= OD of ketogenic cells/ OD of control cells

### Determination of plasma fatty acids profile

Stock solution of the reagents was prepared just prior to each experiment. The 1.9 ml of stock required for each sample included 1.4 ml of methanol, 0.3 ml of toluene, 100 µl of acetyl chloride, and 100 µl of the internal standard. Then, 250 µl of plasma and 1.9 ml of the stock solution were heated at 80°C for 60 min with 100 µl of heptadecanoic acid methyl ester (C17:0) as an internal standard. The tubes were allowed to cool to room temperature. Hexane (1 ml) was added and the tubes were vortexed for 2 min. The upper organic phase was collected and this extraction procedure was repeated in order to optimize lipid extraction. The combined hexane solution was evaporated under nitrogen to dryness, and the dry residue was then redissolved in 25 µl of hexane. For gas chromatography (GC) analysis Capillary Column Chrompack CP-SII 5CB (15 m x 0.25 mm ID x 0.26 µm) was used. Analysis was performed on Hewlett Packard 5890 Gas Chromatograph equipped with flame ionization detector (FID). Temperature program was as follows: 150°C with 0.25 min hold; ramp 35°C/min to 200°C, 8°C/min to 225°C with a 3.2 min hold, and then 70°C/min to 245°C with a 3.0 min hold. Carrier gas was nitrogen 25 ml/min (Masood et al. 2005).

### Statistical analysis

Obtained values were compared using Microsoft Excel and *STSTISTICA.PL* analysis software.

## Results

As shown in Fig. 1, NO generation increased in control groups of Hep along with the time of incubation and reached the maximal level in 12- days culture ($3.97 \pm 0.6$ µM/$10^6$ cells). In the same conditions, also the HSC produced the highest amount of NO which amounted to $3.25 \pm 0.88$ µM/$10^6$ cells. However, temporary decrease of NO releasing was detected at 8th day of culture. Apparently, NO generation increased within time of incubation both in Hep and HSC isolated from rats fed with KD. Moreover, NO elevation in KD-Hep was not statistically significant. On the other hand, in HSC group NO production raised from $2.65 \pm 0.07$ µM/$10^6$ cells on 4th day of incubation to $5.49 \pm 1.2$ µM/$10^6$ cells (p<0.05) on 12th day of incubation.

Without stimulation with KD concentration of superoxide increased gradually only in Hep culture. The values of $0.9 \pm 0.05$ $O_2^-$ nM/$10^6$ cells, $1.1 \pm 0.2$ $O_2^-$ nM/$10^6$ cells, and $2.8 \pm 0.6$ $O_2$. nM/$10^6$ cells were determined on 4th, 8th and 12th day of culture, respectively (Fig. 2). Generation of $O_2^-$. by control HSC was maintained at the stable level, with minor decrease on 8th day of incubation. The Hep and HSC derived from KD-rats produced similar amount of $O_2^-$ during the

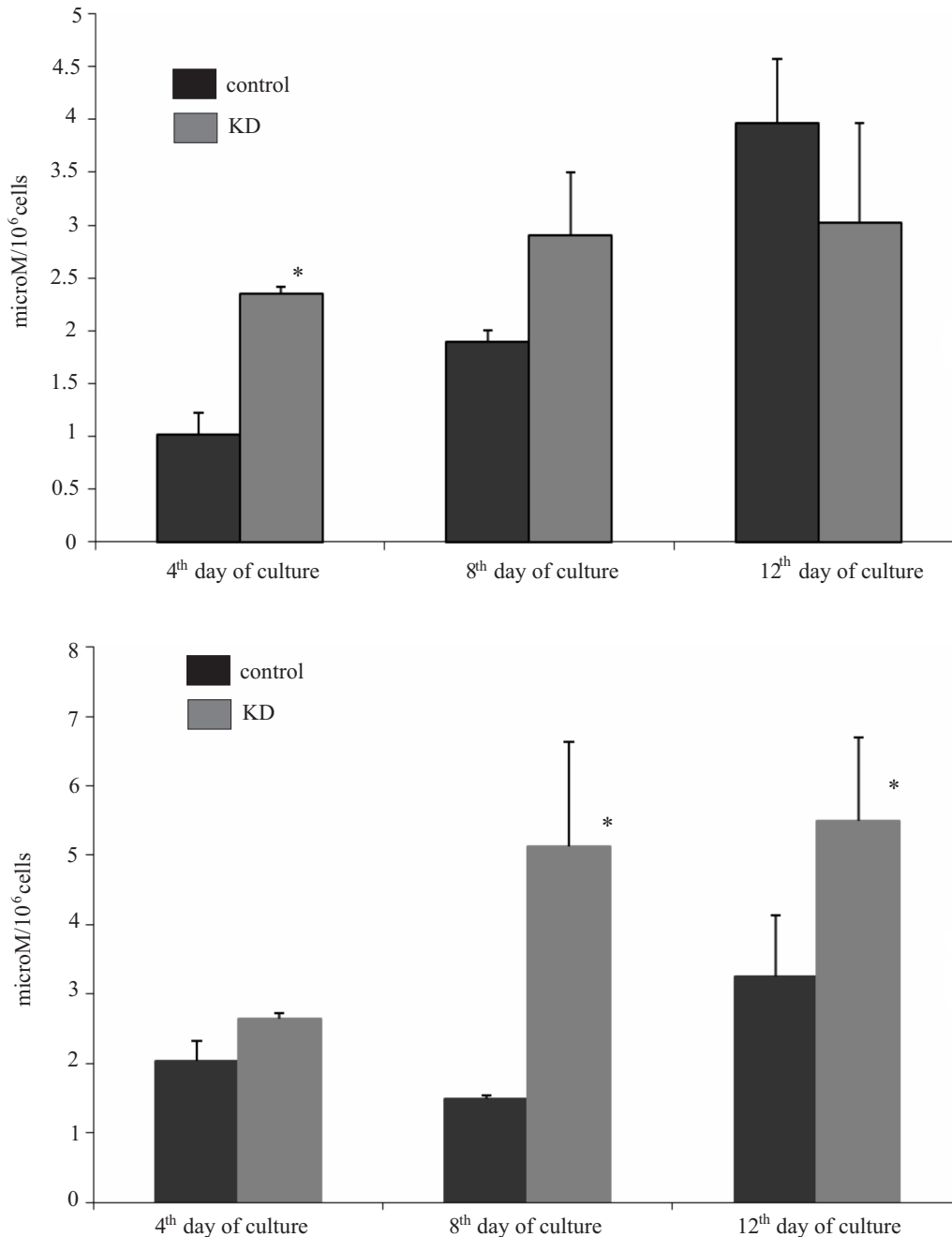Fig. 1. Nitric oxide generation by Hep (above) and HSC (below) isolated from control and ketogenic rats.
* significantly differences at p<0.05 vs. control

first 8th days of culture. At this time the value of superoxide concentration did not exceed $2.0 \pm 0.05$ nM/$10^6$ cells. Both Hep and HSC provide considerably higher quantities of $O_2^-$ until 12th day of incubation ($2.5 \pm 0.07$ and $3.2 \pm 0.3$ nM/$10^6$ cells, respectively).

As shown in Fig. 3 the normal PI of Hep decreased from $0.76 \pm 0.02$ on 4th day to $0.59 \pm 0.01$ on 12th day of incubation. Contrary, proliferative activity of HSC was markedly elevated, especially between 4th and 8th day of incubation ($0.65 \pm 0.01$ and $0.78 \pm 0.04$,

respectively). The HSC derived from KD-rats had a higher proliferative activity than the Hep obtained under the same experimental condition. The PI of KD-HSC ranged between $0.7 \pm 0.03$ and $0.89 \pm 0.04$ on 4th and 12th day of experiment, respectively. Proliferating and activated HSC lost their intracytoplasmic lipid droplets and started to enlarge their cytoplasmic extension (Fig. 4). It should be noticed that values of PI in KD-Hep did not exceed $0.64 \pm 0.02$ on 4th day and decreased along with each day of cell culture.
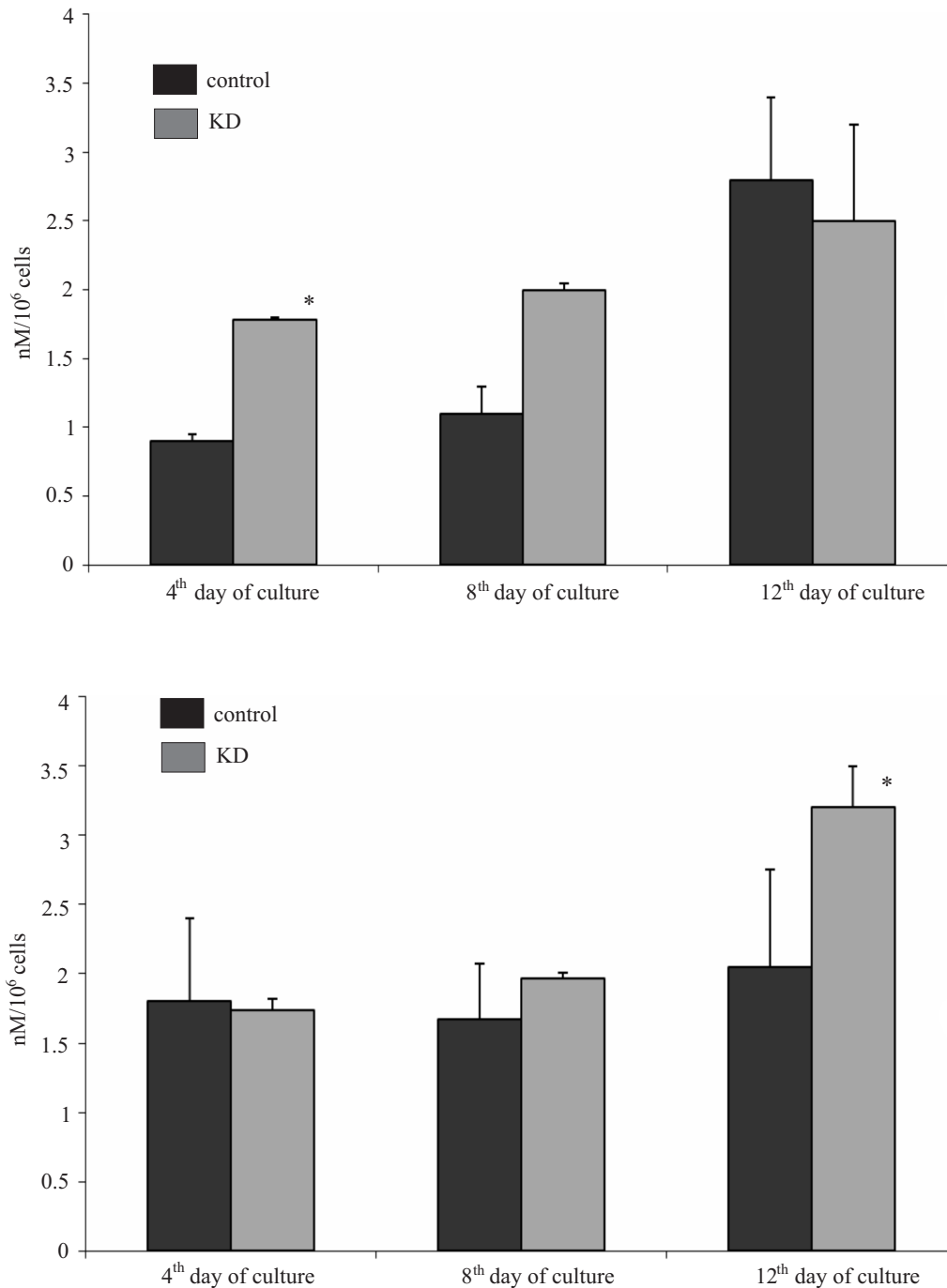
Fig. 2. Superoxide generation by Hep (above) and HSC (below) isolated from control and ketogenic rats.

In rats assigned to KD group plasma concentration of each of analyzed fatty acids (myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), monounsaturates: linoleic acid (C18:2ω6), arachidonic acid (C 20:4ω6), and polyunsaturates: α linolenic acid (C 18:3ω3)) was higher than in control rats fed with standard diet (Fig. 5, 6). It was noteworthy that in rats fed with standard diet palmitic acid dominated among all analyzed plasma fatty acids, but in KD rats almost equal levels of linoleic and palmitic acids occurred in the plasma. In respect of palmitic, stearic, oleic, and linoleic acids, the plasma level was significantly higher (p<0.05) than in control and reached 50.0±4.0 µg/ml, 10±3.2 µg/ml, 30.75±10 µg/ml, and 49.2±3.0 µg/ml, respectively.
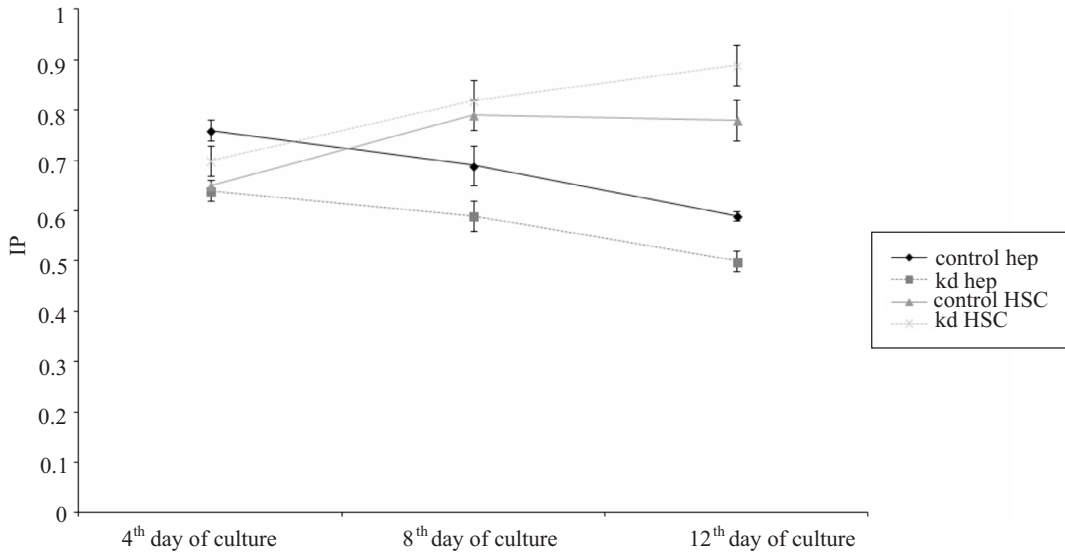
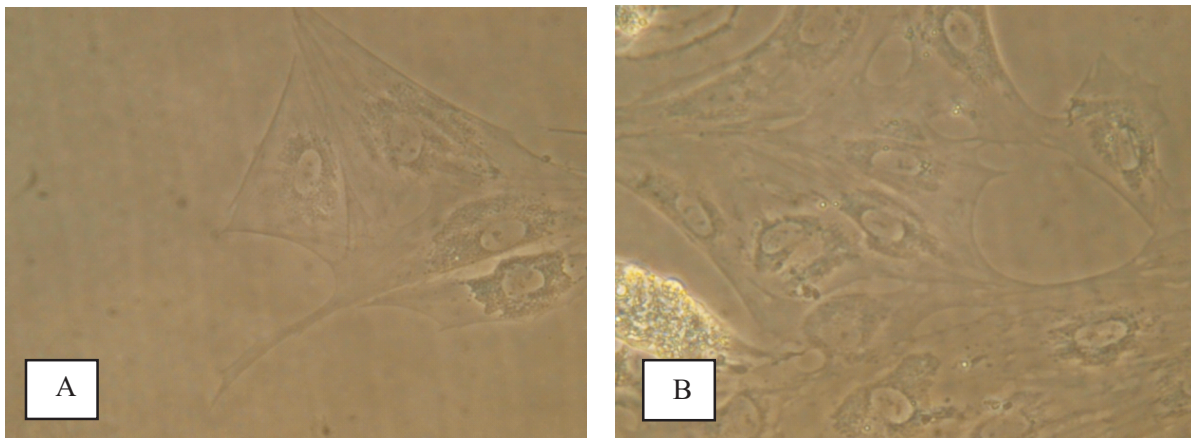Fig. 3. Influence of KD on proliferative activity of Hep and HSC *in vitro*; (mean ± SD).



Fig. 4. Phase-contrast micrograph of HSC (A-B) isolated from ketogenic diet- rats, cultured on plastic dishes for 4th (A) and 12th (B) days. At the end of cell incubation (12th day) culture of HSC reach confluency and compose flat monolayer. Original magnification 400x.
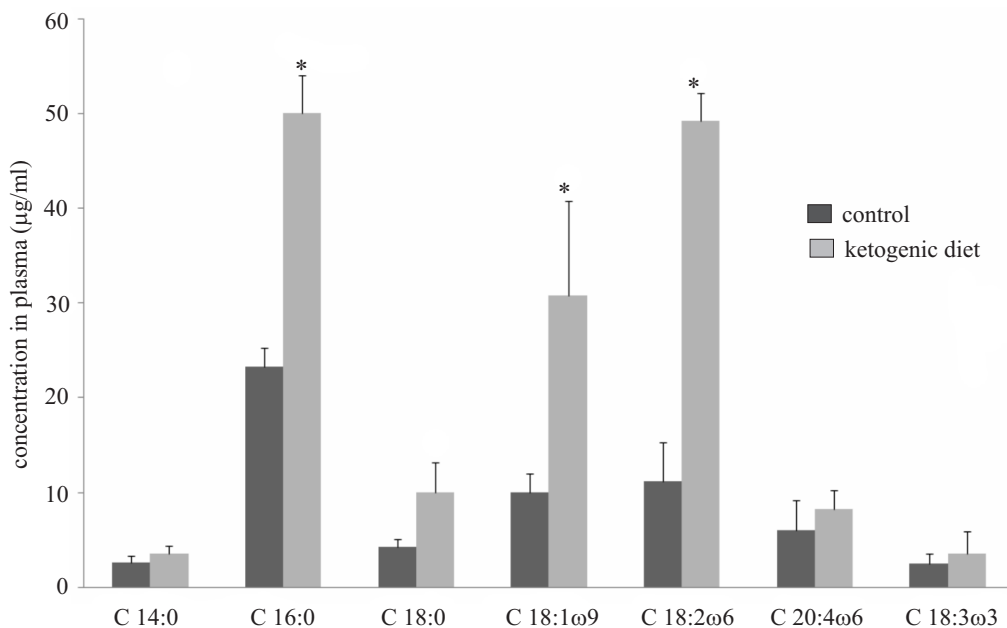


Fig. 5. Gas chromatograms of FFA of rat plasma: A-from rat consuming control diet B-from rat consuming KD. 1 – C14:0; 2 – C16:0; IS-internal standard C17:0; 3 – C18:0; 4 – C18:1ω9; 5 – C18:2ω6; 6 – C 20:4ω6; 7 – C 18:3ω3.
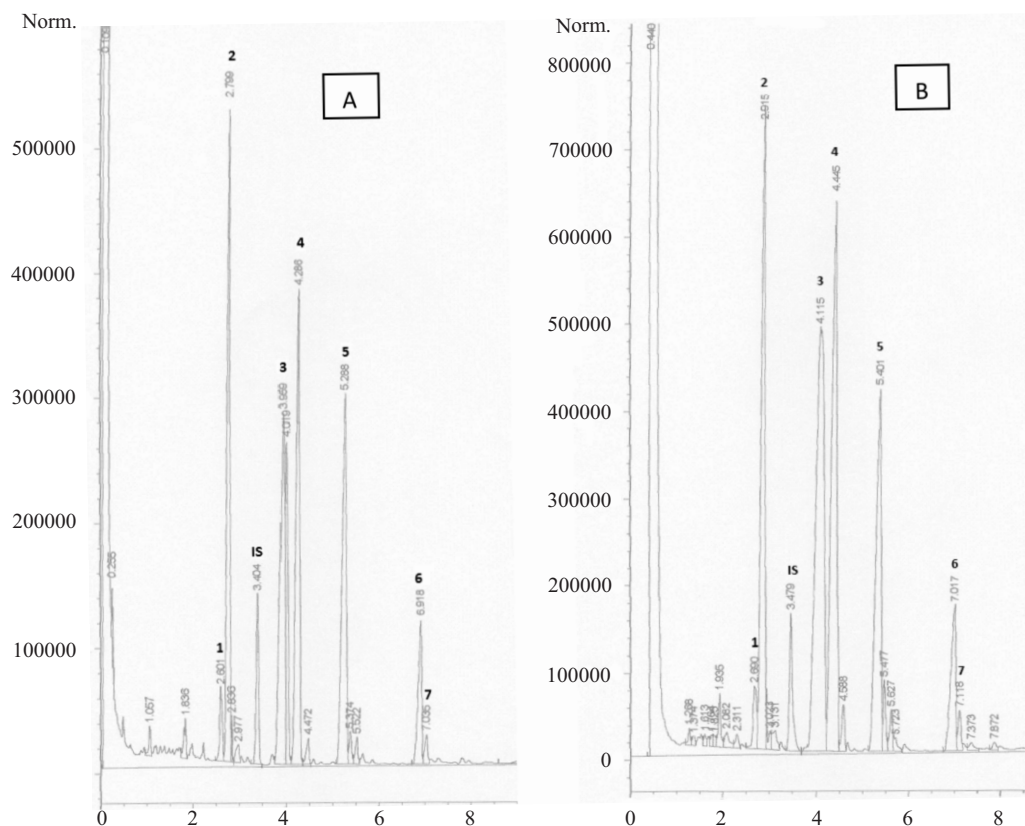
Fig. 6. Composition of FFA in plasma of rats consuming control diet (n=3) or KD (n=3). Data represent the mean ± SD of each group; * significantly differences at p<0.05 vs. control.
(C14:0) myristic acid; (C16:0) palmitic acid; (C18:0) stearic acid; (C18:1ω9) oleic acid; (C18:2ω6) linoleic acid; (C 20:4ω6) arachidonic acid; (C 18:3ω3) h linolenic acid.

## Discussion

Obtained data depict considerable deviations in measuring parameters of rats consuming ketogenic diet. In the course of experiment we estimated that KD acts on liver cells dependently on their type. Namely, HSC activation leads to increased NO production, whereas, in KD-HEP culture we observed increase of NO generation only after 4 and 8 days of culture in comparison with control cells. The study of He et al. (2010) indicated that inducible nitric oxide synthase (iNOS) and NO production significantly increased in the liver tissue of rats after lipid infusion. However, in the mentioned above study whole liver tissue was examined without differentiation of the hepatic cells types. Similar effect was detected in high-fat diet-fed mice (Fujmoro et al. 2005). The augmentation of NO in HSC could be explained by activation of oxidative stress pathway by elevated level of plasma FFA, which causes increased mitochondrial uncoupling and oxidation leading to the increased production of highly reactive oxygen and nitrogen molecular species in liver (He et al. 2010). On the other hand, Soardo et al. (2011) detec-

ted decrease of NO generation in cultured human hepatocytes stimulated with a mixture of FFA for 7 days. As estimated is some other studies it could be associated with apoptosis of hepatocytes (Ricchi et al. 2009, Wobser et al. 2009).

We estimated increase of superoxide anion production by hepatocytes under the influence of KD up to 12th day of culture. In HSC-KD the significant elevation of superoxide anion generation was noted only after 12 days of culture. Reactive oxygen species can originate both from hepatocytes and HSC and their generation can be enhanced by some molecules and signals including FFA (Lee and Friedman 2011). ROS, which are generated during oxidative stress, are considered as factors that both initiate and perpetuate fibrosis. Also Soardo et al. (2011) observed that oxidative stress is activated by FFA in cultured human hepatocytes, thus intensifying ROS generation.

In the present study we estimated decrease of PI at all time points of HEP-KD and control HEP cultures, what can reflect apoptosis of hepatocytes in both cultures. The similar results were obtained in report by Ricchi et al. (2009), which indicated that

apoptosis of cultured hepatocytes was caused by FFA. It was estimated previously that FFA promote the process of liver fibrogenesis as they induce hepatocyte apoptosis and act profibrogenically. This effect may be attributed to the increased expression of genes encoding profibrogenic proteins and apoptosis-related molecules. This response appeared to be restricted only to hepatocytes, while the opposite applies to HSC, as the activation of HSC results in production of collagen and fibrogenesis in chronic liver injury (Bechmann et al. 2009). We demonstrated the proliferative response in HSC culture isolated from rats fed with KD. Described effect persisted along with time of HSC-KD culture in comparison with control group. The mitogenic effect of FFA on HSC different from the effect on Hep was reported previously by Wobster et al. (2009). Their report on a palmitate-induced in vitro fatty liver model confirmed enhanced HSC proliferation and resistance to apoptosis.

Our study on rats fed with KD revealed that plasma concentration of analyzed fatty acids (myristic, palmitic, stearic, oleic, linoleic, arachidonic and α linolenic acid) increased in comparison with control rats fed with standard diet. FFA appear to be the major mediators of excessive hepatic lipid accumulation. The rate of hepatic FFA uptake is not regulated and therefore, is proportional to plasma FFA concentrations. In humans with non-alcoholic fatty liver disease circulating FFA are commonly elevated and their plasma levels correlate with disease severity. Also in rats the intake of high- fat diet is related to lipid peroxidation and non- alcoholic fatty liver disease (Dhibi et al. 2011). The prominent new mechanisms of liver dysfunctions, especially liver fibrosis, have been pointed out recently, with impact on activation of HSC (Lee and Friedman 2011). The term "activation" refers to the conversion of HSC from quiescent to highly proliferative and fibrogenic cells and is a key event of liver fibrogenesis, while HSC are the primary effector cells in regenerative process (Wobser et al. 2009).

## Conclusion

Proliferative response to KD depends on kind of liver cells and time of their incubation. Although KD exerts anti-proliferative effect on hepatocytes, in respect to HSC it intensifies their proliferative activity. Furthermore, as we estimated (on the basis of NO and $O_2^-$ generation) both Hep and HSC exposed to KD are the source of free radicals.

## References

Aleryani SL, Cluette-Brown JE, Khan ZA, Hasaba H, de Heredia LL, Laposata M (**2005**) Fatty acid methyl esters are detectable in the plasma and their presence correlates with liver dysfunction. Clin Chim Acta 359: 141-149.

Baranano KW, Hartman AL (**2008**) The ketogenic diet: uses in epilepsy and other neurologic illnesses. Curr Treat Options Neurol 10: 410-419.

Bechmann LP Zahn D, Gieseler RK, Fingas CD, Marquitan G, Jochum C, Gerken G, Friedman SL, Canbay A (**2009**) Resveratrol amplifies profibrogenic effects of free fatty acids on human hepatic stellate cells. Hepatol Res 39: 601-608.

Bobowiec R, Wojcik M, Jaworska-Adamu J, Tusinska E (**2013**) Fibrogenic response of hepatic stellate cells in ovariectomised rats exposed to ketogenic diet. J Physiol Pharmacol 64: 19-26.

Browning JD, Horton JD (**2004**) Molecular mediators of hepatic steatosis and liver injury. J Clin Invest 114: 147-152.

Cullingford TE (**2004**) The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. Prostaglandins Leukot Essent Fatty Acids 70: 253-264.

De Leeuw AM, McCarthy SP, Geerts A, Knook DL (**1984**) Purified rat liver fat-storing cells in culture divide and contain collagen. Hepatology 4: 392-403.

Dell CA, Likhodii SS, Musa K, Ryan MA, Burnham WM, Cunnane SC (**2001**) Lipid and fatty acid profiles in rats consuming different high-fat ketogenic diets. Lipids 36: 373-378.

Dhibi M, Brahmi F, Mnari A, Houas Z, Chargui I, Bchir L, Gazzah N, Alsaif MA, Hammami M (**2011**) The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. Nutr Metab 8: 65.

Fujimoto M, Shimizu N, Kunii K, Martyn JA, Ueki K, Kaneki M (**2005**) A role for iNOS in fasting hyperglycemia and impaired insulin signaling in the liver of obese diabetic mice. Diabetes 54: 1340-1348.

He B, Zhao S, Zhang W, Li Y, Lu Y, Han P (**2010**) Salicylate prevents hepatic oxidative stress activation caused by short-term elevation of free fatty acids in vivo. Diabetes Res Clin Pract 89: 150-156.

Lee UE, Friedman SI (**2011**) Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol 25: 195-206.

Masood A, Stark KD, Salem N (**2005**) A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J Lipid Res 46: 2299-2305.

Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P (**2008**) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J Gastroenterol Hepatol 24: 830-840.

Soardo G, Donnini D, Domenis L, Catena C, De Silvestri D, Cappello D, Dibenedetto A, Carnelutti A, Bonasia V, Pagano C, Sechi LA (**2011**) Oxidative stress is activated by free fatty acids in cultured human hepatocytes. Metab Syndr Relat Disord 9: 397-401.

Taha AY, Ryan MA, Cunnane SC (**2005**) Despite transient ketosis, the classic high-fat ketogenic diet induces marked changes in fatty acid metabolism in rats. Metabolism 54: 1127-1132.

Wessely-Szponder J, Szponder T (**2010**) Comparison of the effects of two anaesthetic combinations in rabbits on some neutrophil functions in vitro. World Rabbit Sci 18: 169-177.

Wobser H, Dorn C, Weiss T, Amann T, Bollheimer C, Buttner R, Scholmerich J, Hellerbrand C (**2009**) Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells. Cell Res 19: 996-1005.

Wójcik M, Kosior-Korzecka U, Bobowiec R (**2010**) Cytoprotective action of 17β-oestradiol against iron-induced hepatic oxidative stress in vitro. Bull Vet Inst Pulawy 54: 259-263.