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From Cell Biology to Tissue Engineering

Relationships between variable time, percentage of food restriction and liver histology: which alternative is the best for non-alcoholic fatty liver disease (NAFLD) prevention?

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Summary. The objective of this study was to analyse the hepatic effects of food restriction in an experimental rabbit model. The study comprised 105 rabbits divided into 6 groups. The two control groups were fed *ad libitum* (ADL) during the entire experiment (C1 and C2). The experimental groups were restricted between 42-49 days of age, where the rabbits received 50g (R1) or 65g (R2) of food per rabbit per day. Others were restricted between 35-42 days of age, where the rabbits received 50g (R3) or 65g (R4) of food per rabbit per day. For liver analysis, 5 rabbits per group were slaughtered at the ages of 49, 56, 63, 70 days from the R1, R2 groups and at 42, 49, 70 days from the R3, R4 groups. All animals from the C1 and C2 groups developed steatosis with inflammation. Animals from the R1 and R2 groups developed steatosis without inflammation while in the R3 and R4 groups steatosis was not visible. In C1 and C2 groups we observed mostly fatty deposit accumulations while in the R1, R2, R3 and R4 groups, more PAS-positive material accumulations were visible. Liver steatosis correlated with inflammation development and interstitial tissue growth. These results can be used in clinical praxis as signs of NAFLD progression. Early food restriction had intense effects on liver morphology and it seems promising that similar approaches could be applied as preventive treatment for NAFLD development.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) comprises a wide spectrum of clinical-pathological classifications. Manifested with simple steatosis in the initial stage, it can be associated with other nosological classifications including steatohepatitis (NASH), liver fibrosis and liver cirrhosis. First recognized in 1980, our understanding of this disease has undergone significant change in the interim years (Ludwig et al., 1980). At present, there are in the recent literature, data suggesting a relatively high prevalence of the disease in the European, Chinese and USA population with qualified data estimating the order to be up to 30% of the total population (Blachier et al., 2013; Veena et al., 2014; Fung et al., 2015). One of the major roles for histopathological praxis is to establish the steatosis transition to other nosological units that are causally related to liver cirrhosis. It seems likely that a key factor in progression is the presence of inflammatory infiltrate and liver fibrosis. It is known that many cases are asymptomatic, so the majority of diagnoses remain appointed in the florid stage, with a lower degree of therapeutic response, while others remain undiagnosed. Therefore there is a need to identify indicators that would better aid NAFLD diagnosis, already in the initial stage. These would increase therapeutic options with significantly better prognosis for the patient. Towards this goal, there has been reported in the recent literature

biochemical parameters (Yilmaz, 2012; Fitzpatrick and Dhawan, 2014), ultrasonography (Koplay et al., 2015), and other imaging techniques (Lee et al., 2013; Lee and Park, 2014). However, the main diagnostic tool for NAFLD or NASH remains biopsy (Nalbantoglu and Brunt, 2014; Abd El-Kader and El-Den Ashmawy, 2015) and histological examination represents the standard approach for diagnosing NAFLD, fibrosis, NASH, and eventual liver cirrhosis (Preiss and Sattar, 2008; Brunt, 2011, 2012).

Steatosis is present in 100% of all NAFLD cases (Kubrusli et al., 2010; Yki-Jarvinen, 2014). There was described a causal relationship with NASH and it was found that in the late stage of diagnosis, therapeutic compositions are limited with significantly worse prognosis for the patient. Therefore early NAFLD diagnosis is of crucial importance for therapy. It has been demonstrated that NAFLD develops especially in obese populations (Wree et al., 2011; Caballero et al., 2012; Fock and Khoo, 2013) and several animal models have been designed to further study NAFLD initiation and progression (Tipoe et al., 2009; Chavez-Tapia et al., 2011; Hebbard and George, 2011; Kanuri and Bergheim, 2013). The main purpose of these experiments was to find additional indicators that would aid early NAFLD diagnosis and to further understand the underlying pathophysiological mechanisms associated with NAFLD transition to other nosological units. Experiments which are focused on preventive effects, although available, are found to a much lesser extent in the literature. We believe that prevention is the best way forward with this disease, thereby minimizing overall health care costs. This is especially true regarding several metabolic diseases, where the risk played by diet, exercise and lifestyle, is significant. Also NAFLD is included to this category. It has been repeatedly demonstrated that one of the preventive measures for developing NAFLD is food restriction (Carvalhana et al., 2012; Nseir et al., 2014). However, no concrete data exist that could clearly refer to the degree of food restriction, including the duration of restrictive feeding, to NAFLD prevention. These results could be useful in clinical practice in patients with risk of NAFLD development. Our experimental studies associating different degrees of food restriction in different time intervals with their influence on liver morphology are therefore justified. Variability in the degree of food restriction, including different time settings between the previous and subsequent *ad libitum* (ADL) intake may prove which properties of food restriction are most effective in the prevention of NAFLD. The results could be used in practice, both in prevention and in treatment of NAFLD. The objective of our work is the histological analysis of the liver under food restriction in different time intervals, including the degree of variability in the percentage food restriction in combination with ADL intake in an experimental rabbit model.

Material and methods

This study was approved by the Ethics Committee of the Institute of Animal Science and the Central Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic and carried out according to the guidelines for applied nutrition experiments in rabbits (Fernandez-Carmona et al., 2005).

Sample animals' characteristics

Liver samples from 105 broiler rabbits kept in experimental conditions were analysed. Briefly, the animals were kept under controlled environmental conditions and housed in standard cages. The experiment with Hyplus broiler rabbits was conducted in the rabbit building of the Institute of Animal Science from a weaning age of 32 to 70 days of age. The rabbits were divided into five groups. The control group was fed *ad libitum* (ADL) during the entire experiment (C1 and C2). The second group was restricted between 42 and 49 days of age, when the rabbits received 50 g of food per rabbit per day (R1) and the next group was also restricted between 42 and 49 days, but the rabbits obtained 65 g per rabbit per day (R2). The fourth group was feed-restricted between 35 and 42 days of age, receiving only 50 g of food per rabbit per day (R3). The last group was also restricted between 35 and 42 days of age, but received 65 g of food per rabbit per day (R4). Before and after restriction, all rabbits were fed ADL with a commercial food mixture. For liver analysis, 5 rabbits

Table 1. The design of the experiment.

Groups	At weaning	At 32 day	At 35 day	At 42 day	At 49 day	At 56 day	At 63 day	At 70 day
C1	Mother milk	ADL	ADL	ADL	ADL	ADL	ADL	ADL
R1	Mother milk	ADL	ADL	R1	ADL	ADL	ADL	ADL
R2	Mother milk	ADL	ADL	R2	ADL	ADL	ADL	ADL
C2	Mother milk	ADL	ADL	ADL	ADL	ADL	ADL	ADL
R3	Mother milk	ADL	R3	ADL	ADL	ADL	ADL	ADL
R4	Mother milk	ADL	R4	ADL	ADL	ADL	ADL	ADL

ADL - *ad libitum*, C1, C2 - control groups, R1 - restriction 50g of food per rabbit/per day, R2 - restriction 65g of food per rabbit/per day, R3 - restriction 50g of food per rabbit/per day, R4 - restriction 65g of food per rabbit/per day.

per group were slaughtered at the ages of 49, 56, 63 and 70 days from the R1 and R2 groups and 5 rabbits per group were slaughtered at the ages of 42, 49 and 70 days from the R3, R4 groups (Table 1).

Sample collection

Liver samples were obtained at necropsy using standard procedures. Two samples, approximately the same size, were taken from the centre of the liver parenchyma. The first set of samples was packed in foil and frozen in liquid nitrogen. These frozen samples were then stored in a freezer box at a temperature of -30°C. The other samples were fixed with a 4% Bouin solution for one day. All the liver samples were collected within 30 minutes after slaughtering.

Sample evaluation and processing

Frozen samples were embedded in Tissue-Tek (Jung Tissue Freezing Medium, Leica Biosystems) and cut into 5 μm-thick slices using a freezing microtome (Leica CM 1850 Cryostat) at –20°C. Special silanized slides were used for mounting (DAKO, Denmark). The sections were then stained for the verification of neutral lipids using Oil Red „0“ (DiaPath Srl., Italy).

The Bouin fixed samples were processed by standard histological methods using an automated tissue processor (Leica ASP6025, Leica Microsystems, Germany) and then embedded in paraffin blocks using a Leica EG 1150H paraffin embedding station (Leica Microsystems, Germany). From three to five µm-thick slices were cut from each sample using a microtome (Leica RM2255, Leica Microsystems, Germany) and mounted on standard glass slides (Bammed, Czech Republic). The first slices were stained with haematoxylin-eosin (DiaPath Srl., Italy). The second set, for the verification of collagen type I and reticulum, was stained using a Sirius red kit (DiaPath Srl., Italy). The third set, for verification of elastic fibres, was stained using an Acid Orcein kit (DiaPath Srl., Italy). The last sections were stained for the detection of glycogen and PAS-positive material according to the PAS-Hotchkiss-McManus methodology (DiaPath Srl., Italy). The prepared samples were evaluated as light-microscopic images obtained using a Carl Zeiss Axio Scope A1 (Zeiss, Germany) and the Axio Scan.Z1 slide scanner (Zeiss, Germany). The samples were evaluated for steatosis in accordance with Kleiner et al. (2005). For each group the proportions of interstitial tissue to the parenchyma of the liver were measured. For each group the diameter of a total of 500 hepatocytes was measured.

Statistical analysis

The results were evaluated using the SAS program (SAS Institute Inc., Cary, NC, 2003), and the ANOVA method. Three-way analysis of variance with interactions of genotype and feeding was also used.

Results

Description and results of the evaluation of liver samples

These are in the Table 2.

Hepatocyte diameter

The largest average hepatocyte diameter was measured in the restriction 50g of food per rabbit/per day, specifically in R1 (25.08 μm) and R3 (21.14 μm) ($P<0.001$), but at the first measurement after restriction time-point this group also had the smallest average hepatocyte diameter, R1 (19.16 μm) and R3 (14.99 μm) ($P<0.001$). Hepatocytes responded to food restriction with a rapid enlargement of their diameter. At the end of the experiment, visible changes were observed in the R1 and R3 groups compared to the C1 and C2 groups. There is a progression of the average growth of hepatocytes and at the end of the experiment the average hepatocyte diameter was higher in R1, R3 groups compared to the C1 and C2 groups ($P<0.001$), but the differences between R2, R4 groups and C1 and C2 groups was not statistically significant ($P>0.001$). Major differences, correlating with the growth of hepatocyte diameter, were observed in intracytoplasmic material. In C1 and C2 groups we observed mostly fatty deposit accumulations, but in the R1 and R3 group there were more PAS-

Table 2. Results of the evaluations of liver samples.

Marking	At 42 day		At 49 day		At 56 day		At 63 day		At 70 day	
	SG	I	SG	I	SG	I	SG	I	SG	I
C1	NS	NS	G0	ND	G0	ND	G0	ND	G1	D
R1	NS	NS	G0	ND	G0	ND	G0	ND	G1	ND
R2	NS	NS	G0	ND	G0	ND	G0	ND	G1	ND
C2	G0	ND	G1	ND	NS	NS	NS	NS	G1	D
R3	G0	ND	G0	ND	NS	NS	NS	NS	G0	ND
R4	G0	ND	G0	ND	NS	NS	NS	NS	G0	D

C1, C2 - Control groups, R1 - restriction 50g of food per rabbit/per day, R2 - restriction 65g of fed per rabbit/per day, R3 - restriction 50g of food per rabbit/per day, R4 - restriction 65g of food per rabbit/per day. SG - Steatosis grade, I - Inflammation, NS - No sampling, ND - No diagnosed, D - Diagnosed.

positive material accumulations visible. (Tables 2, 3).

Proportion of interstitial tissue to the liver parenchyma

The proportion of interstitial tissue shows different trends in accordance with the restrictions groups. In the R1 group there is a continual growth in the proportion of interstitial tissue up to the end of the experiment. The largest proportion of interstitial tissue at the end of experiment was observed in the C1 group (12.25%), although the differences were not statistically significant (P>0.001). In the C2, R3 and R4 groups, there is continual growth in the proportion of interstitial tissue up to the end of the experiment, with the largest content in the C2 group (10.20%), and this is statistically significant (P<0.001). The connective tissue was almost completely composed of collagen while elastic fibers were either not visible, or were observed very sporadically and rarely.

Discussion

Food restriction is one precautionary way to prevent diseases (Tumova et al., 2012, 2016). It is not exactly

Table 3. Objective results of the evaluations of liver samples.

Marking	At 42 day		At 49 day		At 56 day		At 63 day		At 70 day	
	PIT (%)	HD (μm)	PIT (%)	HD (μm)	PIT (%)	HD (μm)	PIT (%)	HD (μm)	PIT (%)	HD (μm)
C1	NS	NS	9.00	19.18	15.00	21.89	11.25	22.58	12.25	21.04
R1	NS	NS	8.57	19.16	8.70	20.05	9.10	23.38	11.66	25.08
R2	NS	NS	12.25	19.89	15.62	19.22	11.25	22.95	11.66	20.65
C2	7.70	21.12	8.20	19.69	NS	NS	NS	NS	10.20	18.61
R3	3.90	14.99	6.60	19.24	NS	NS	NS	NS	9.55	21.14
R4	5.40	16.20	6.90	17.98	NS	NS	NS	NS	8.75	19.94

C1, C2 - Control groups, R1 - restriction 50g of food per rabbit/per day, R2 - restriction 65g of fed per rabbit/per day, R3 - restriction 50g of food per rabbit/per day, R4 - restriction 65g of food per rabbit/per day. PIT - %-Proportion of interstitial tissue, HD - Hepatocyte diameter, NS - No sampling,

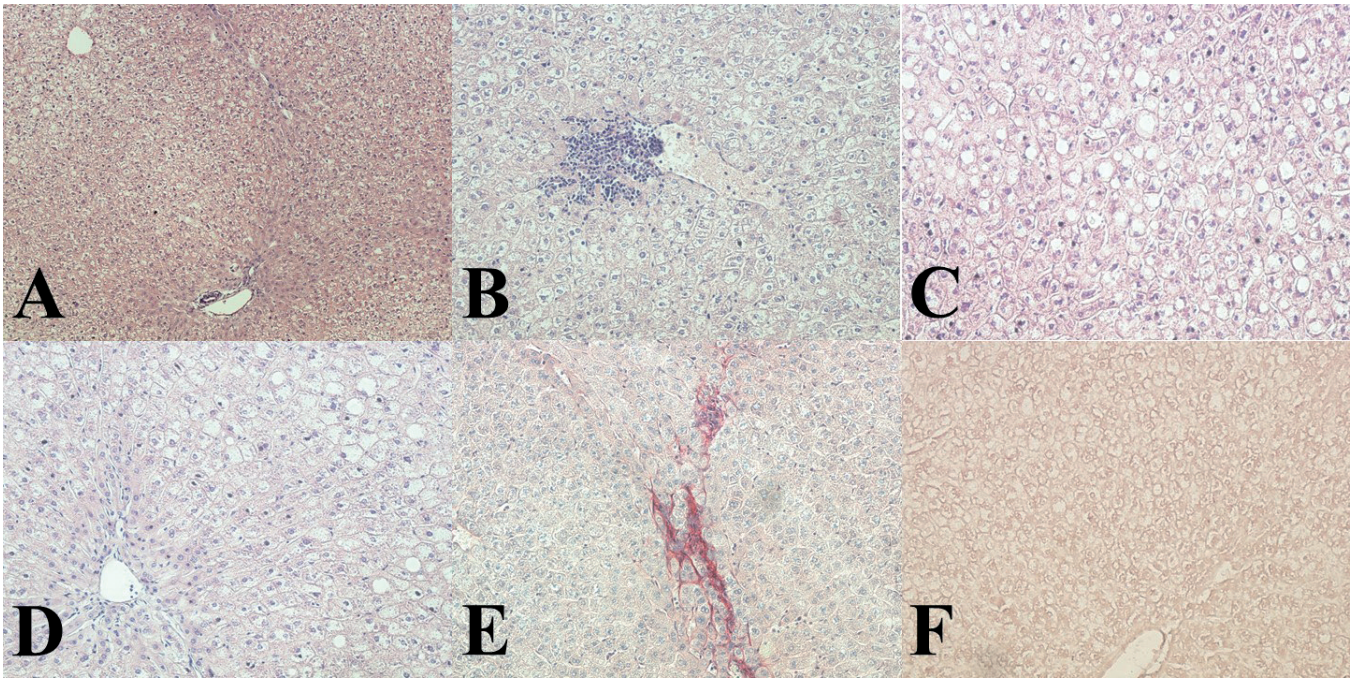

Fig. 1. Microscopic view of the rabbit liver at different time points of the experiment. **A.** Part of liver with well-visible portal space and central canal and classical hepatocytes, which are located close to each other. Legend: C2 group - day 42, HE. **B.** Central view of liver with one inflammatory foci. Legend: C2 group - day 70. **C.** Well-differentiated steatosis with intracytoplasmic vacuoles. Legend: R1 group - day 70, HE. **D.** Central view of liver with vacuolated hepatocytes showing steatosis. Legend: R2 group - day 70, HE. **E.** Liver section with some positive reaction to collagen and reticular fibres between two liver lobules. Legend: C2 group - day 42. **F.** Negative reaction for elastic fibres in central part of liver. Legend: R2 group - day 70, Orcein. x 200

known which alternative is the best for non-alcoholic fatty liver disease (NAFLD) prevention. Within this study, our primary objective was to understand risk factors leading to liver disease, and in particular NAFLD. These hold great promise for preventive medicine. Obesity and liver diseases are significant problems in pediatric and adult medicine, and they are interrelated. With regard to the present increasing trends in passive human lifestyle and obesity in the population, it is assumed that the disease is under-diagnosed and that there are many latent cases. As such, preventive medicine is very important in this field. Previous studies have reported that modifications in lifestyle constitutes a first line of approach for the management of NAFLD (Yang et al., 2014; Ordonez et al., 2015; Rudwill et al., 2015). This includes also nutritional habits. Dietary restriction and fasting is a very old observance, often associated with religious practice. It is mentioned in the Bible and also in the Qur'an. Even in the present-day general public, it is one of the most popular and applied therapeutic practices. We believe that managed restriction needs to be conducted under the supervision of an expert. It is an individual therapy and is not applicable as a general method. The one objective of our experiment was to find if there was some possibility to prevent NAFLD development, by applying restriction. Other studies have asked similar questions, employing histological evaluation of liver biopsies following variable diet (Centis et al., 2013; Jun, 2013). The results of Ayala et al. (2009) show that diet-induced hypercholesterolemia and hypertriglyceridemia are associated with severe impairment of liver function, embodied by fat accumulation, inflammation and hepatocellular ballooning. As shown in Fig. 1A,E, C2 groups initially exhibited normal liver morphology, but by the end of experiment exhibited steatosis with some inflammatory infiltrations and hepatocellular ballooning (Fig. 1B). In the R1 and R2 groups there was also steatosis, but without inflammation (Fig. 1C,D) and (as also observed in the C1 and C2 group) without fibrosis (Fig. 1F). We did not study the ultrastructure of cells, but it is possible that these signs are the results of intense oxidative metabolism in the mitochondria, or lipid metabolism in the endoplasmic reticulum of hepatocytes. These hypotheses were similarly mentioned in other work examining the differences between steatosis and steatohepatitis (Rao and Reddy, 2001; Ahishali et al., 2010; Rector et al., 2010; Lotowska et al., 2014). The results of Pan et al. (2015) document that steatosis leads to inflammation. The mechanism is unknown, but the authors presuppose that hepatocytes released pro-inflammatory cytokines and can activate macrophages when co-cultured *in vitro*. In our experiment we have found that developing steatosis leads to liver

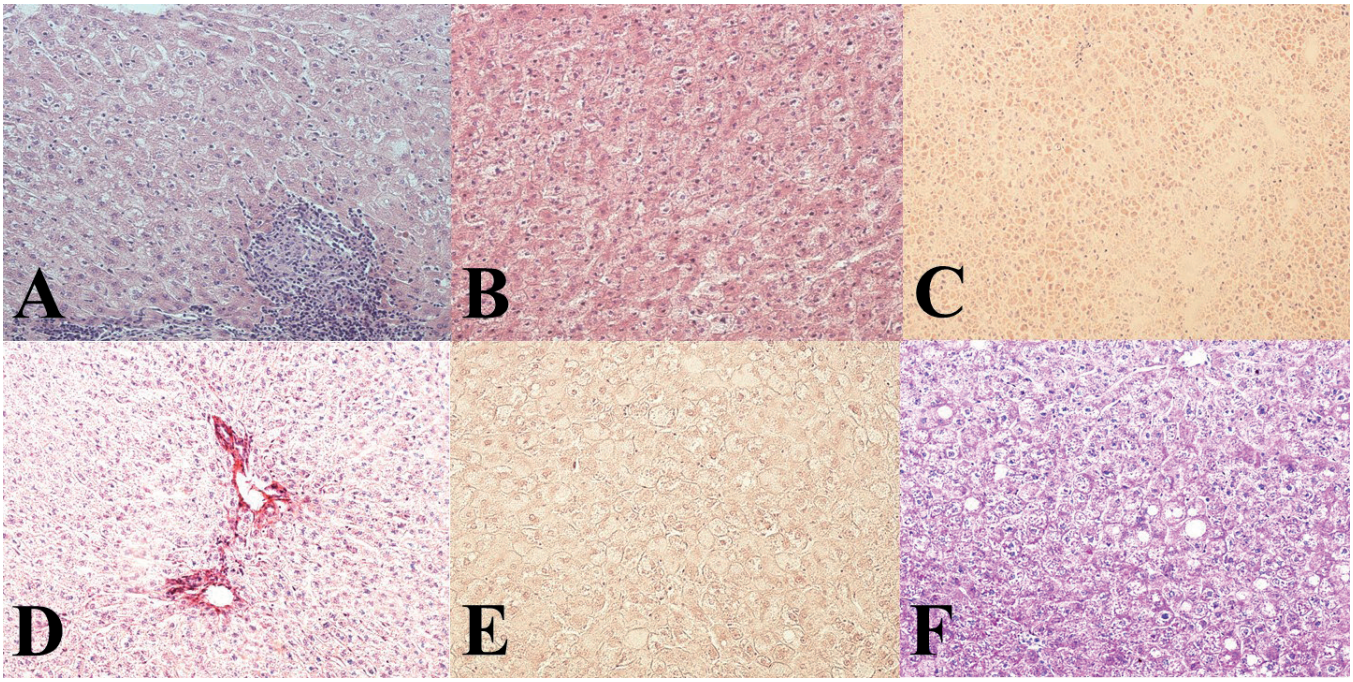

Fig. 2. Microscopic view rabbit liver at different times of experiment. **A.** Liver section with inflammation and lipid saturation of some hepatocytes. Legend: C1 group - day 70, HE. **B.** Liver section with glycogen saturation within hepatocytes. Legend: R3 - day 49, HE. **C.** Central view of liver demonstrating the presence of lipid droplets in hepatocytes. Legend: C2 group - day 70, Oil Red "0". **D.** View of the portal space with minimum interstitial tissue. Legend: C1 group - day 49 , SR. **E.** Negative reaction for elastic fibres in central part of liver. Legend: C2 group - day 49, Orcein. **F.** Section of liver with positivity to glycogen. Also observed are some hepatocytes with intracytoplasmic vacuoles exhibiting negative reaction. Legend: C2 group - day 49, PAS. x 200

inflammation. Subsequently the developing steatosis in the C1 and C2 groups was causing inflammation. These results are in accordance with liver pathology progression. In one nutritional study it was declared that fatty liver rabbits showed a positive correlation with progression of liver fibrosis (Lu et al., 2014). Another interesting study has shown that liver regeneration is associated with progenitor cells producing chemokines to attract various kinds of inflammatory cells to the liver (Jou et al., 2008). Comparing our results, there is an influence on the growing proportion of interstitial tissue and the degree of inflammation present. Our results show that after restriction there are signs of interstitial tissue growth. The results of Woo et al. (2013) show that in mice, fasting leads to expression of fibroblast growth factor 21 (Fgf-21) in the liver, stimulating gluconeogenesis, fatty acid oxidation and ketogenesis, as an adaptive response to fasting. We have not measured the activity of Fgf-21, but it seems likely that it is a result of interstitial tissue growth. In regions with lower interstitial tissue content inflammation was not diagnosed, but as the content of interstitial tissue grows there is also inflammations. Some studies showing the positive effect of fasting on liver are oriented on the reduction of liver fat, but do not make any histological measurements of hepatocytes, or interstitial tissue (Marina et al., 2014; Yu et al., 2014). In the study of Moller et al. (2008) it is stated that intrahepatic lipid content increases in healthy male subjects during fasting, which demonstrates that the liver actively responds to fasting. If we look at our results from the R1 and R2 cohort, it is observed that after two weeks the average hepatocyte size was higher than in the C1 group, as well as PAS-positive material accumulation followed by lipid droplet accumulation. This represents the reaction of liver to food restriction. The results from the C1, C2, ADL cohort are liver steatosis and liver inflammation formation (Fig. 2A) with visible steatosis development (Fig. 2C), while in the experimental groups there are signs of PAS-positive material saturation in hepatocytes (Fig. 2B) and, at the end of the experiment, signs of steatosis development (Fig. 2F). In summary, the C1 and C2 groups at the end of the experiment displayed visible steatosis as compared to the R1, R2 and R3 groups. In the figure 3 you can find detailed views on how this pathology developed. At the beginning of the experiment there is normal liver morphology in C2 group (Fig. 3D) with some hepatocellular ballooning in C1 group (Fig. 3A). Continuing ADL food intake, there are signs of cytoplasmic material accumulation (Fig. 3B) with

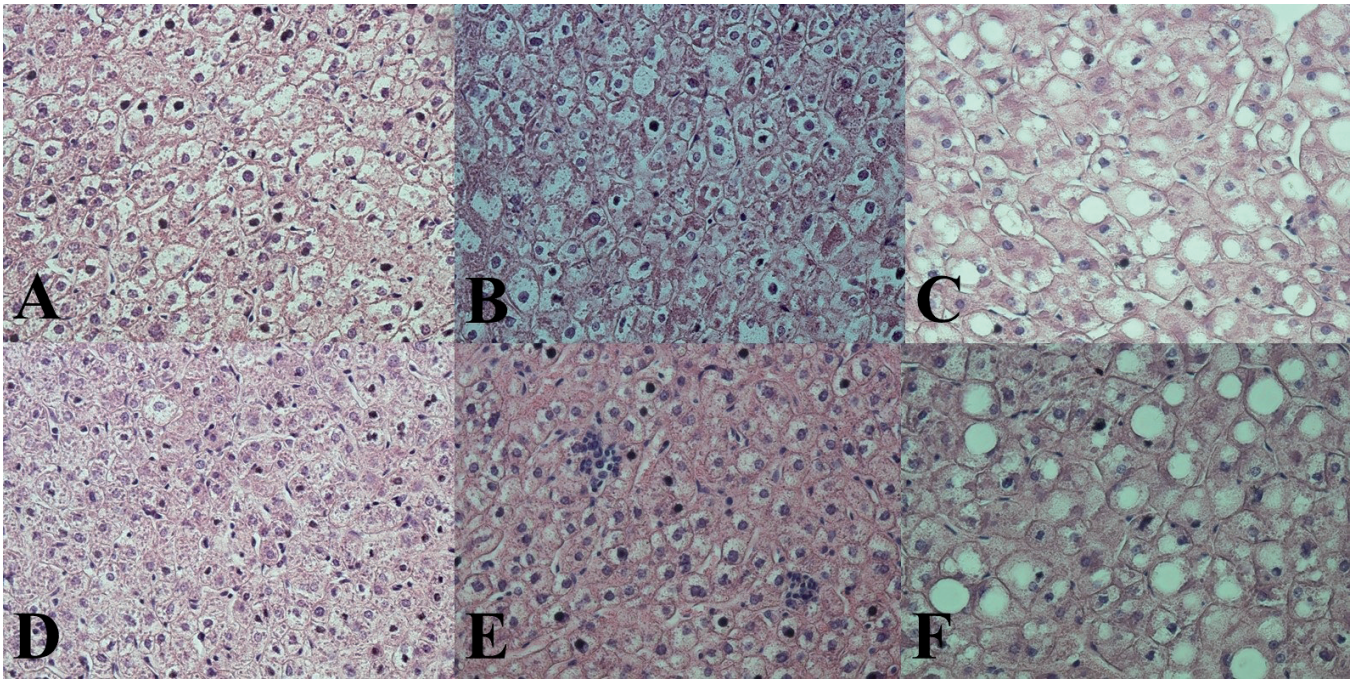

Fig. 3. Detailed microscopic view of the control group at different times of experiment. **A.** A central part of liver with visible hepatocellular ballooning manifested as optic pale cytoplasm with one oval nucleus. Legend: C1 group - day 49, HE. **B.** This is a detailed view of a liver with some regressive hepatocytes, like hepatocellular ballooning and visible dense intracytoplasmic material. Legend: C1 group - day 56, HE. **C.** Visible liver steatosis with vacuolated hepatocytes, which are visible as empty spaces imitating lipid droplets. Legend: C1 group - day 70, HE. **D.** Relatively normally organised liver, with many ovoid hepatocytes, which are localised in trabecular pattern, containing one nucleolus with a little dark chromatin. Legend: C2 group - day 42, HE. **E.** A view into the central part of liver with many similar hepatocytes with glycogen material. Please look see two solid infiltrative lymphocyte knots. Legend: C2 group - day 49, HE. **F.** This is a part of liver with well visible steatosis. Please see the vacuolated hepatocytes. Legend: C2 group - day 70. x 400

lymphocyte knots (Fig. 3E). At the end of the experiment, there is steatosis visible in C1 and also C2 groups (Fig. 3C,F). The content of interstitial tissue grows slowly (Fig. 2D), but there are no signs of elastic fibre change (Fig. 2E). It is possible that for different periods it is necessary to make more or less intensive food restriction. The same view is supported by the results from the R3 and R4 groups, compared to the C2 group. These results are not comparable with the literature because of the lack of data. There exist some correlations between NAFLD and atherosclerosis development and it seems that these are results of interstitial tissue growth in a later stage of liver steatosis (Kim et al., 2014; Madan et al., 2015). In one interesting study from Sanchez-Polo et al. (2015) dealing with the association between the histologic findings of atherosclerosis and those of NAFLD in chickens, it was shown that standard diet and atorvastatin therapy can positively affect both arterial and hepatic lesions, influencing the regression of the changes. The results of Gan and Wats (2008) show, that the accumulation of intrahepatic lipids observed with fasting may explain exacerbations of steatohepatitis. We have found that liver steatosis correlated with inflammation development and with interstitial tissue growth. These results can be used in clinical praxis as signs of NAFLD progression.

Conclusion

Our results show that after a period of food restriction there are, in hepatocytes, visible saturation with PAS-positive material, hepatocellular ballooning, lymphocyte knots and after that occurs, liver steatosis, with or without inflammation. Conversely, in the C1 and C2 groups there is hepatocellular ballooning with steatosis, and steatosis with or without inflammation. This is accompanied by an increase in interstitial tissue. These results are usable for clinical praxis as signs of NAFLD progression and they are indicators for NASH development, which can be used in bioptic practice. The results document that non controlled ADL food intake is associated with NAFLD development and early food restriction has intense effects on liver morphology, and it seems likely that it could be applied as a preventive measure for NAFLD.

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