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PATHOLOGICAL ALTERATIONS IN HEPATOCYTES OF DAIRY COWS WITH A TENDENCY TO EMACIATION AND FATTENING

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ABSTRACT

The aim of this study was to demonstrate the histochemical and histopathological alterations in the livers of cows with a tendency to become emaciated (body condition score — BCS1 and 2) and a tendency to become fattened (BCS4 and 5) in comparison to the cows of average body condition (BCS3) presented as a control. The histochemical analysis (PAS reaction) showed that the influence of emaciation and fattening in our study was manifested by a decreased occurrence of glycogen and a decreased level of the PAS-positive matter in the hepatocytes of dairy cows with BCS1, 2, 4 and 5. An abundant accumulation of lipids in the form of large lipid droplets, liposomes and lipoproteins observed in the hepatocytes of emaciated and fattened (BCS1 and 5) cows may be related to moderate-severe steatosis. These observations suggest a relationship between liver steatosis and the occurrence of lipoproteins in cows with a tendency toward emaciation and fattening.

Key words: body condition score; hepatocyte; liver; steatosis

INTRODUCTION

Several studies indicate that the negative energy balance (NEB) in cows during early lactation may cause excessive mobilization of fatty acids in the liver tissue [7]. In particular, in cows with severe fattening, the cell structure in the liver and their functions are destroyed [22]. These cows are less fertile, have significantly longer time of calving and higher numbers of inseminations per conception compared to cows with moderate fattening [23]. The body condition of dairy cows influences not only ovarian follicle

development [21] but also liver metabolism [23]. Fat in the liver of dairy cows is accumulated in the peri-partum period. According to imposition of lipid droplets in hepatocytes, dairy cows can be classified into two groups: cows with moderate fattening, and cows with heavy fattening [23]. Therefore, the evaluation of the fat content in the liver is relevant to determine the relationship between the body condition and milk performance of dairy cows and their metabolic status.

It is known that the liver plays an important role in the lipid and lipoprotein metabolism and effectively influences metabolic processes throughout the organism. According to Alexander et al. [1], lipoproteins are formed following the fusion of lipid particles of the smooth endoplasmic reticulum (ER) with the apoproteins of rough ER, when B apoprotein and probably other very-low-density lipoprotein (VLDL) apoproteins are bound to the lipid particle to form a nascent lipoprotein. Therefore, it can be assumed that any damage to the hepatocytes (steatosis) is accompanied by changes in the metabolism of lipoproteins [20]. It seems that there are some relationships between glycogen, lipid metabolism in the liver and variable nutrition levels in dairy cows. These changes may affect body condition and health status of cows and also may influence their performance. Liver steatosis negatively affects the production of gonadotropic hormones leads to cystic atresia of the ovarian follicles and thus may cause the deterioration of the fertility of cows.

The aim of this study was to elucidate associations between histopathological/ histochemical findings in the liver of dairy cows and their body condition evaluated by a five-point scale of BCS.

MATERIALS AND METHODS

Biological material

As a source of biological material, the livers were acquired at the slaughtering of Holstein dairy cows (n = 23) at a local abattoir at different times of the post-partum period. The cows were kept under normal feed regimes. The animals were estimated as belonging into certain grades of body condition score (BCS) according to a five-point scale of BCS [8]. Our experimental dairy cows were categorized into four different groups: BCS1 (emaciation; n = 4), BCS2 (tendency towards emaciation; n = 4), BCS3 (optimal body condition status; n = 4), BCS4 (tendency to fattening; n = 7) and BCS5 (fattening; n = 4). The data on these cows were taken from the cow's individual cards on farms and were as follows: average age 6.2 years, 4.1 years, 5.7 years, 5.5 years, 6.36 years; and post-partum period 4.8 ± 0.51 weeks; 4.49 ± 0.59, 5.82 ± 0.62; 10.94 ± 1.37 and 12 ± 2.2 for BCS1, BCS2, BCS3, BCS4 and BCS5, respectively.

Histopathological and histochemical analysis

For the histological analyses of the liver samples (n = 36) from cows, they were fixed in 10 % neutral buffered formalin (Sigma-Aldrich), dehydrated in a rising set of ethanol solutions (70 % and 90 % for 2 hours and 100 % for 1 hour) and embedded into Technovit 7100 resin (Heraeus GmbH, CoKG, Werheim/Ts., Germany) according to the producer's manual. The tissue sections of 1—2 μm in thickness were cut on a Ultracut E (Reichert, Jung, Austria) and two sections obtained from each sample were placed on the standard slides (Bamed, Czech Republic). Afterwards, the first sections were stained with haematoxylin and eosin (HE). The second sections were stained for the detection of glycogen and PAS-positive material according to the PAS-Hotchkiss-McManus methodology (DiaPath Srl., Italy). The samples were described and evaluated from light-microscopy images using a Carl Zeiss AxioScope A1 microscope (Zeiss, Germany). The figures were made using the NIS-Elements AR version 3.0 software (Laboratory Imaging s.r.o., Czech Republic).

Electron microscopy analysis

The liver samples (n = 37) from cows (n = 17) were fixed in the aldehyde mixture (2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate) at 4 °C for one hour and subsequently post-fixed in 1 % OsO_4 in 0.1 M sodium cacodylate for one hour. Thereafter, for the lipoprotein visualization, the samples were soaked in 1 % p-phenylendiamine (Sigma-Aldrich) solution in 70 % acetone for 30 min according to Boshier et al. [4]. After the dehydration in acetone, the samples were embedded into Durcupan ACM (Fluka). Ultrathin sections (90 nm) were cut using a Leica EM UC6 ultramicrotome (MIKRO Ltd., Bratislava, Slovak Republic) and then contrasted with uranyl acetate and lead citrate. The contrasted sections were analyzed under a JEM100 CXII transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

The lipids were visualized following osmium post-fixa-

tion. Lipoproteins (VLDL) were visualized by p-phenylenediamine and localized mostly in the Disse space in the form of lipid particles with a diameter of 80 nm (determined by a scale bar for each electron micrograph) according to [1]. The occurrence of lipid droplets was evaluated from electrongrams subjectively as follows; + rare occurrence, ++ moderate occurrence, +++ abundant occurrence, and ++++ very abundant occurrence. This occurrence of lipid droplets is correlated with the definition of steatosis according to Reid et al. [24], who differentiated four degrees of steatosis based on the amount of hepatic fat as follows; healthy dairy cows ≤ 10 %, moderate steatosis ≤ 20 %, moderately severe steatosis ≤ 30 %, and severe steatosis ≥ 30 %.

RESULTS

Light microscopy and histochemistry

By the evaluation of semi-thin sections of the cow's liver samples, we found sporadic dilatation of hepatic sinuses in cows with BCS2 (Fig. 1A) in contrast to the BCS3 cows, where such dilatation was not revealed (Fig. 1B). These sinuses occurred only occasionally and were filled with erythrocytes. Part of the liver parenchyma, which was composed of radially arranged hepatocytes with regressive changes, was filled with several smaller or larger optically-empty intracytoplasmic vacuoles. In the spaces between adjacent hepatocytes some sinuses contained several cells resembling Kupffer's cells. Normal liver parenchymal tissue with characteristic hepatocytes was observed in BCS3 cows (Fig. 1B).

We have found that the PAS-positive matter is present in the cytoplasm of hepatocytes both in the corpuscular and diffuse form. Histochemically detected PAS-positive material is distributed diffusely (homogenously) in the full range of the hepatic acini. In comparison to the group of BCS3 cows (Fig. 1C), the less PAS-positive matter was found in the hepatocytes of cows with BCS2, 4 and 5 (Table 1, Figure 1D–F).

Electron microscopy analysis of the livers

According to the ultrastructural images of the hepatocytes, the occurrence of glycogen granules in BCS3 cows (Fig. 2A) was higher than in BCS4 cows (Fig. 2B). Ultrastructural images of the hepatocytes demonstrated damages to the organelles in the hepatocytes mainly in cows of BCS1, 2, 4 and 5. In comparison to the cows of BCS3 (moderate condition), characterized by prominently granulated endoplasmic reticulum surrounding numerous mitochondria and lipid droplets (Fig. 2C), the cows with BCS1 and 5 manifested the increased occurrence of swollen mitochondria or mitochondria with significant electron-optic density, smooth endoplasmic reticulum with cytoplasm vacuolization, absence of polysomes and a rise in the number of lysosomes in portobiliary space of hepatocytes, which indicated on initial stages of liver cell degeneration (Fig. 2C–F). Using subjective evaluation of the electrongrams we observed an increased occurrence of lipid droplets and liposomes in the hepatocytes of cows with BCS1 and 5, when compared to the BCS3 cows (Table 1, Fig. 2C–F). In the hepatocytes of cows with BCS1, 2, 4 and 5, the lipofuscin pigments had accumulated (Table 1). Also, in the hepatocytes of the cows of BCS1, 2 and 5 higher accumulations of lipoprotein granules were observed compared to the BCS3 cows (Table 1).

Table 1. The intensity of occurrence of cell inclusions in the hepatocytes of cows with different BCS

Cells inclusions	Body condition score of cows				
	1	2	3	4	5
PAS-positive material[1]	+	+++	+++	++	+
Glycogen[2]	+	++	+++	+++	++
Lipid droplets[2]	+++	++	+	+++	+++
Liposomes[2]	+++	++	+	++	+++
Lipoprotein[2]	+++	++	+	+	++
Lipofuscin[2]	++	+++	+	++	+++

Occurrence: + — small; ++ — moderate; +++ — abundant
[1] — evaluated by light microscope
[2] — evaluated by electron microscopy

DISCUSSION

The body condition of dairy cows may affect also liver metabolism [23]. Post-partum depression of feed intake and subsequent energy deficiency causes a rapid loss of body mass and the accumulation of intracellular fat in the liver (lipomobilization, fat infiltration of the liver and other organs, and ketosis). The rapid loss of body reserves

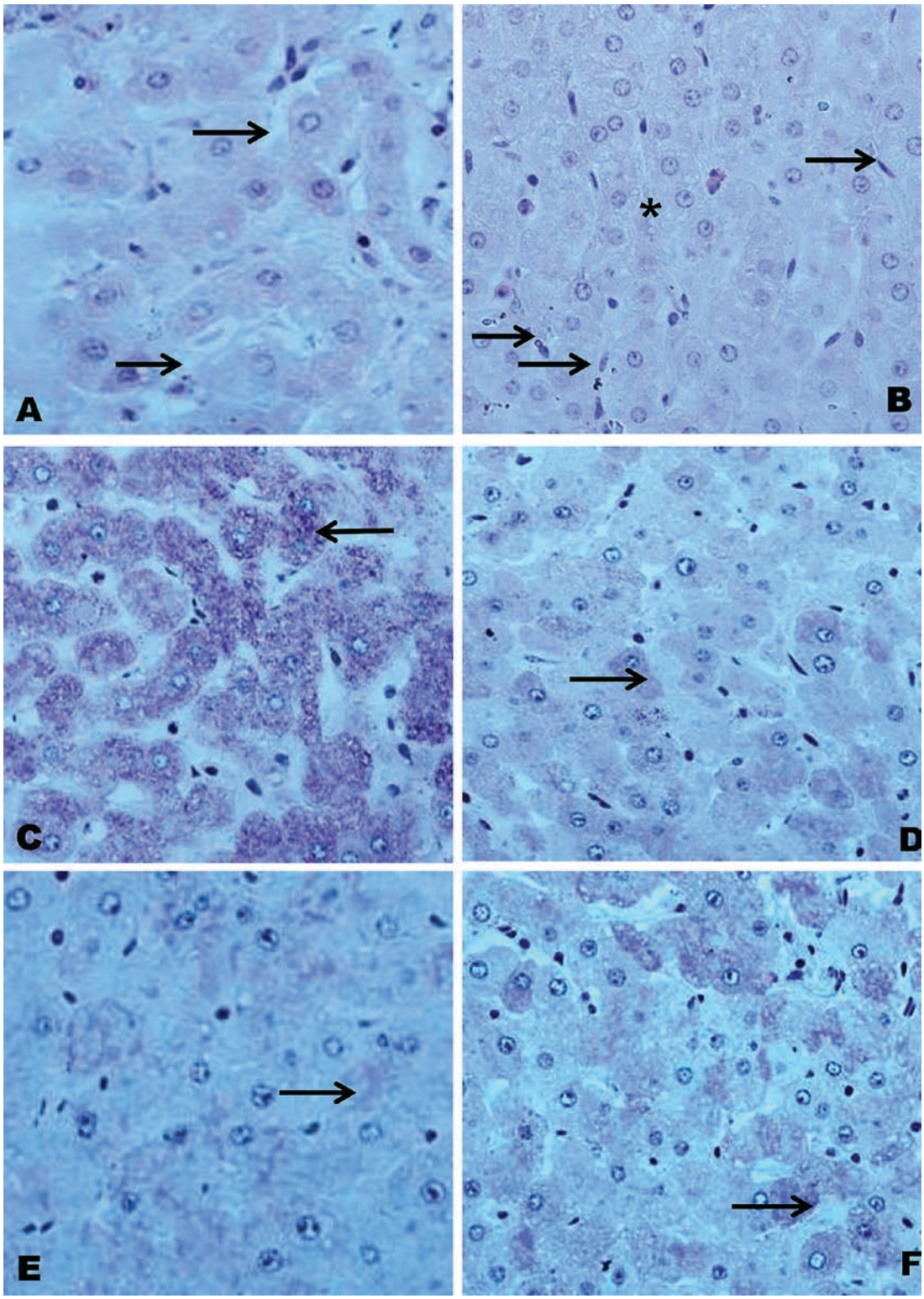

Fig. 1. Microscopical analysis of hepatocytes from dairy cows with different BCS

A (10123P2) — increased occurrence of dilated sinuses (arrows) between radially arranged liver cells of BCS2 cow. **B** (10132P3) — radially arranged hepatocytes of a BCS3 cow with regressive changes in the form of several smaller optically empty intracytoplasmic vacuoles — lipid-like substances (asterisk); there are visible sinuses and Kupffer's cells (arrows) in the spaces between adjacent hepatocytes. **C** (10131P3) — normal parenchyma of the liver of a BCS3 cow with a number of tightly settled PAS-positive hepatocytes in the form of intensive granular reaction (arrow) with the incidence of Kupffer's cells. **D** (10811P) — normal parenchyma of the liver of a BCS4 cow, which consists of numerous similar PAS-positive hepatocytes with a diffuse reaction (arrow). **E** (10142P2) — normal hepatocyte of a BCS2 cow with a moderate diffuse reaction of PAS-positive substances (arrow). **F** (1090P25) — normal parenchyma of the liver of a BCS5 cow, which is composed of a numerous similar in size and shape hepatocytes with more intensive diffuse reaction of PAS-positive substances (arrow). Staining: A, B — haematoxylin-eosin (HE); C—F — PAS-Hotchkiss-McManus methodology. Magn. ×400 (A—F)

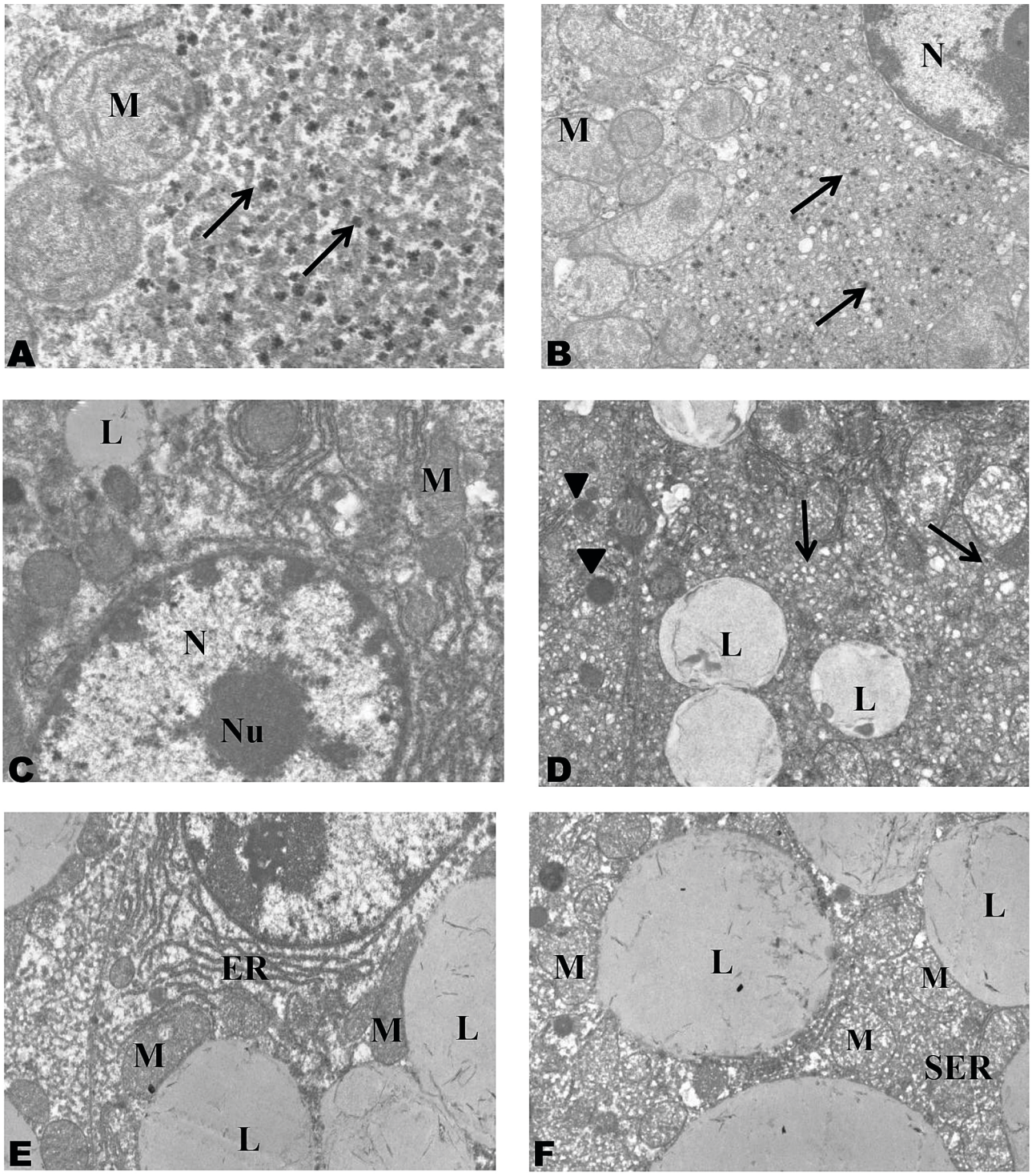

Fig. 2. Ultrastructural image of the liver hepatocytes from dairy cows with different BCS

A (3808) — occurrence of large mitochondria (M); accumulation of large dark granules of glycogen (arrows) in the cytoplasm of the hepatocyte of a BCS3 cow. **B** (3822) — image of the nucleus (N) and mitochondria (M); low incidence of small glycogen granules in the cytoplasm of BCS4 cow's hepatocyte (arrow). **C** (3504) — ultrastructure of the hepatocyte of cows with BCS3; prominently granulated endoplasmic reticulum surrounding numerous mitochondria (M) and lipid droplets (L). The nucleus (N) and nucleolus (Nu) are present in the lower part. **D** (3531) — large lipid droplets in hepatocytes of BCS2 cows; numerous small membrane-enveloped vesicles — liposomes (arrows), large transparent mitochondria (M), several lysosomes in the peripportal space (arrowheads). **E** (3532) — large lipid droplets (L) in most of the hepatocyte of BCS1 cow surrounded by the dark elongated mitochondria (M). The nucleus with nucleolus and granulated endoplasmic reticulum (ER) are present in the upper part. **F** (3507) — the hepatocyte of BCS5 cow with large lipid droplets (L) surrounding numerous mitochondria (M) and smooth endoplasmic reticulum (SER). Staining: A—F uranyl acetate and lead citrate. Magn. ×19 000 (A); ×10000 (C); ×7200 (B, D, F).

post-partum, besides increased occurrence of metabolic disorders, subsequently disturbs the optimal blood concentration of glucose, insulin and IGF-I [13]. The histopathological images of the livers of emaciated cows (BCS1 and 2) was characterized by; the increase in the diameter of hepatocytes, decline in the volume of rough endoplasmic reticulum, and the number of mitochondria [22]. Starvation in cows results in a restriction of secretory properties of the hepatocytes, as it was found by Cakala and Bieniek [7]. In the liver of starving rats the number of ribosomes decreases [9]. It is generally known, that the content of proteins and phospholipids in the liver of starving animal falls, but the fat level rises, which was also confirmed in our study.

In cows with a tendency to fatten (BCS4) and fattened cows (BCS5), ketones and residual products of lipomobilisation bring the damages to the cell organelles of hepatocytes, especially mitochondria and endoplasmic reticulum and inhibit protein synthesis. The unused fatty acid residues in the hepatocytes generate the triglycerides and very-low-density lipoproteins, which represent a very effective system of endogenous triglyceride transport in the organism. However, when VLDLs are insufficiently produced or released, then triglycerides are accumulated in the hepatocytes resulting in the incidence of steatosis [10], [15]. The pathogenesis of steatosis depends on specific metabolic influences, resulting from the character of existing liver disease, as well as from other individual factors [15], [19], [30].

The livers of cows with BCS1 assessed in our study were, in most cases, similar to those showed in Fig. 2 E, F. In particular, numerous large lipid droplets in several cases occupied a one-third to half of the volume of the hepatocyte cytoplasm. These structures are surrounded by larger or smaller mitochondria with cristae and by granular endoplasmic reticulum with significantly decreased volume. These observations correspond to the findings in [24] about the proteosynthesis in the hepatocytes. Accumulation of lipids is a more common form of morphological alterations in the liver [6]. Fat accumulation is referred to as steatosis when more than 50 % of the hepatocytes contain microvesicular or macrovesicular forms and when the accumulation of the fat is of a diffuse character [15], [17].

The presence of lipids in hepatocytes of cows with a tendency toward emaciation, but also in fattened cows, appeared very often in the form of lipid droplets, liposomes and lipoproteins. Reid et al. [23], using a stereological method of histological analysis of biopsied liver samples, determined the grades of hepatic steatosis on the basis of percentage of fat as follows: healthy liver — less than 10 %; moderate steatosis — less than 20 %; moderately severe – less than 30 % and severe steatosis — over 30 %. Our subjective estimation on the basis of lipid droplet occurrence in the hepatocytes of cows with BCS1and 5 as abundant (+++) may be, according to [24], equivalent to a fat content of less than 30 %, which is moderately severe steatosis.

Cholesterol, as a precursor of steroid hormones [16], affects not only the metabolism of lipids but also the reproductive hormones. Steatosis of hepatocytes of emaciated (BCS1 and 2) or fattened dairy cows (BCS4 and 5) negatively affects the formation of gonadotropic hormones and prevents ovulation, which gives rise to cystic atresia of ovarian follicles and finally the formation of ovarian follicular cysts [25]. Therefore, the incidence of steatosis may adversely affect the fertility parameters of dairy cows. The effects of the cow's body condition on the fertility of dairy cows has been demonstrated in several reports [21], [27], [28]. In particular, cultured ovarian granulosa cells from the BCS2 cows manifested increased estradiol secretion [28]. This increased estradiol may inhibit the FSH production, which leads to elevated prolactin level, suppressing ovulation and causing follicular cyst formation. These authors also observed elevated zinc concentration in the blood plasma of BCS2 cows. This trace element is present in every cells of the body. Under the influence of free radicals the intracellular zinc is increased and can be available for various signalling and regulatory cascades, which activates kinases responsible for cellular differentiation, cell survival and apoptosis [12], [29]. Zinc ions enter the cells via the ZIP1 cell transporters, which can be induced by testosterone and prolactin [12]. High levels of zinc ions subsequently suppress terminal oxidation and maintain the cells at a low level of oxidation [11]. We assume that this molecular mechanism relates also to zinc in the hepatocytes, and its increased level may interact on the disorders of lipid or lipoprotein metabolism.

Lipofuscin, as an endogenous lipopigment, is produced in hepatocytes from lipids or lipoproteins probably by their peroxidation [3]. Lipofuscin is deposited in lysosomes [5]. Jean et al. [14] observed a high frequency of simultaneous incidence of steatosis and lipofuscin, especially with fibrotic changes in the liver. Lipofuscin granules visible in

the cytoplasm of hepatocytes are associated with the liver of old age [2], [26]. However, Mak et al. [18] have found that lipofuscin is not specific to the aging liver. It should be considered that the regenerative ability of the liver is well-known and very often even profound alterations, revealed on the level of electron microscopy, have not been confirmed by the functional analyses. The alterations that we found in the livers of cows, besides changes in the BCS status, were not manifested clinically.

CONCLUSIONS

Our observations indicate that in cows with emaciation (BCS1 and 2) or with fattening (BCS4 and 5), the occurrence of glycogen and a PAS-positive material was less apparent. In the hepatocytes of such cows, an intensive accumulation of lipids and lipoproteins was observed. These results suggest that histopathological and histochemical evaluation in combination with ultrastructural studies of the hepatocytes can be useful for the assessment of the body condition status in relation to the metabolism of lipids in cattle. The accumulation of lipids, observed in our study, destroys hepatocyte organelles, and these changes may influence biochemical blood parameters, which could be used for the diagnostics of hepatic steatosis.

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