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Is the hepatocyte ultrastructural zonal heterogeneity changed by overnight (16 h) fasting? Morphometric study

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ABSTRACT

Background and objectives: Hepatocyte ultra-structure is influenced by feeding status, circadian rhythm, and zone location. The goal of the present study was to study the effect of overnight fasting on the hepatocyte ultrastructure and the zonal heterogeneity and to discuss the functional correlation.

Methods: A total of 14 male albino rats were divided into two groups: negative control group fed ad libitum and overnight fasting rats for 16 hours. The different subcellular structures of both centrilobular and periportal hepatocytes in both control and fasted groups were compared by transmission electron microscopy. Morphometric analysis of the electron micrographs was also done using imageJ software.

Results: The lysosomes surface density, mitochondrial volume and surface densities were significantly higher in periportal hepatocytes however surface density of smooth endoplasmic reticulum (SER) and peroxisomes were significantly higher in centrilobular hepatocytes of the control group. Fasting caused a significant decrease in the surface density of rough endoplasmic reticulum and glycogen volume density but with significant increase in SER surface density with more mitochondrial fusion and stronger mitochondrial ER contacts, isolation membranes, and autophagosomes. The zonal differences were maintained after fasting. The organelles appeared normal with no signs of degeneration.

Conclusion: The organelles appeared normal with no signs of degeneration and the zonal differences were maintained after fasting. The change in hepatocyte ultrastructure after fasting may be related to autophagy.

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Hepatocytes; liver zonation; ultrastructure; fasting; electron microscopy

Introduction

Hepatocyte ultrastructure is dynamic. Certain factors as feeding status[1], lobular pattern[2], and circadian rhythm[3] can modify the hepatic ultrastructure.

There are many heterogeneous aspects in liver function and metabolism between different hepatic zones. Fatty acid metabolism and gluconeogenesis are performed mainly in periportal hepatocytes, but perivenous hepatocytes have a greater ability for detoxification.[4] This heterogeneity is mirrored into the ultrastructure of hepatocytes in many aspects e.g. mitochondria and rough endoplasmic reticulum (RER) are abundant in periportal zone in contrast to centrilobular zone that shows more smooth endoplasmic reticulum (SER) and peroxisomes.[5] Also, the difference in the chromatin texture between the hepatic zones.[6] Therefore, the metabolic zonation is therefore an active theory.[7] As for the carbohydrate metabolism, gluconeogenesis, and its opposing activity, glycolysis can be done concurrently by the periportal and perivenous hepatocytes, respectively. There are diverse factors modifying metabolic zonation. Presence of biliary salts in periportal zone is a leading factor for bile formation in that zone.[8] Similarly, oxygen and hormone grades through the hepatic lobule affect the expression of metabolic enzymes.[9] Furthermore, the Wnt/β-catenin signaling pathway was reported to play an important role in modulating the liver zonation.[10]

Basal autophagy is responsible for the continuous recycling of cellular contents, thereby eliminating damaged or dysfunctional cellular contents and providing substrates for production of energy and this is induced by starvation. In macroautophagy,

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cytoplasmic content is sequestrated within a double membrane structure, a phagophore (also known as isolation membrane) is formed, which later elongates to form an autophagosome. The autophagosome fuses with a lysosome to form an autolysosome where its content will be degraded.[11]

The effects of starvation on the ultrastructure of hepatocytes of different animals for different periods have been investigated e.g. Bizjak Mali et al.[1] have studied the effect of long-term starvation on ultrastructural changes of hepatocytes. After starvation, glycogen was decreased, dispersed, and failed to pack as in normal hepatocytes. Hepatocytes size was decreased, lipid droplets decreased in number, clustering of peroxisomes occurred with small rounded mitochondria that lost cristae. Lysosomes and autophagosomes increased in number.

Many reports have discussed the zonation of hepatocyte organelles in the feeding state. Others have discussed the effect of different periods of fasting on the hepatocyte ultrastructure, but few reports were there to discuss the effect of fasting on the hepatocyte ultrastructural zonal heterogeneity with morphometry with functional correlation. The main objective of our work was to study the possible change of the ultrastructure of both centrilobular and periportal hepatic zones, after overnight fasting, using transmission electron microscopy. Cell morphometry was done to get quantitative information of cellular fine structure and correlating these morphological data obtained in the present study with the biochemical, physiological, or pathological data from other studies.

Materials and methods

Fourteen male adult albino rats weighing 200 grams were kept in metal cages (two per cage) and lighting was regulated to provide 12 hours of light (7 AM to 7 PM) and 12 hours of darkness (7 PM to 7 AM) and room temperature was maintained between 25°C and −27°C. Rats were fed ad libitum for 5 days, then were divided into two equal groups; seven rats/each group:

***Group I*: (Control group)** rats were fed ad libitum with a basal diet.

***Group II*: (Fasting group)** rats were overnight fasted for 16 hours from 4 PM to 8 AM. From both groups, livers were obtained and processed for electron microscopic (EM) study.[12] Semithin sections 1-μm thick were cut and stained with toludine blue and examined by light microscope for orientation and the blocks were trimmed to select areas containing a central vein, a portal tract, or both. Both centrilobular (those near central veins within three or four cells layers) and periportal (those near portal tract or terminal branch of portal vein within three or four layers of portal vein) hepatocytes were examined in all liver sections. All experiments were conducted in accordance with the NIK Guide for the Care and Use of Laboratory Animals and were approved by our local Committee for Animal Experiments.

Morphometric analysis of electron micrographs

The electron micrographs of both periportal and centrilobular hepatocytes were analyzed for each group. Images were recorded at original magnification of X 14,000 and photographically enlarged at final magnification of X 28,000 and only hepatocytes with clearly defined boundaries were selected for morphometric analysis.

The surface density (Si) of the mitochondria, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), Golgi apparatus (GA), peroxisome, lysosome, lipid, and nucleus were calculated using ImageJ software v.1.52a with Weibel's multipurpose grid[13] plugin, a method modified by Haslob et al.[14] The grid consisted of series of short lines arranged in rows with the ends of the lines forming an equilateral triangular network (Figure 1(a)). The ends of the lines are used as markers for point counting, those lying over the structure of interest being recorded as (Pi), while intersections of the lines with the surface of the structure were noted as (Ni). The surface area (Si) of the organoids studied can be estimated by placing the Weibel's grid over electron micrograph, counting intersections of membrane profile with the grid line (Ni) and Si was calculated using the Weibel's formula[15] Si was calculated to be equal to twice the number of membrane intersections (2 Ni) divided by the total length of test lines of the grids lying within the hepatocytes cytoplasm. The data were expressed as μ^2/μ^3 of the hepatocyte cytoplasm.

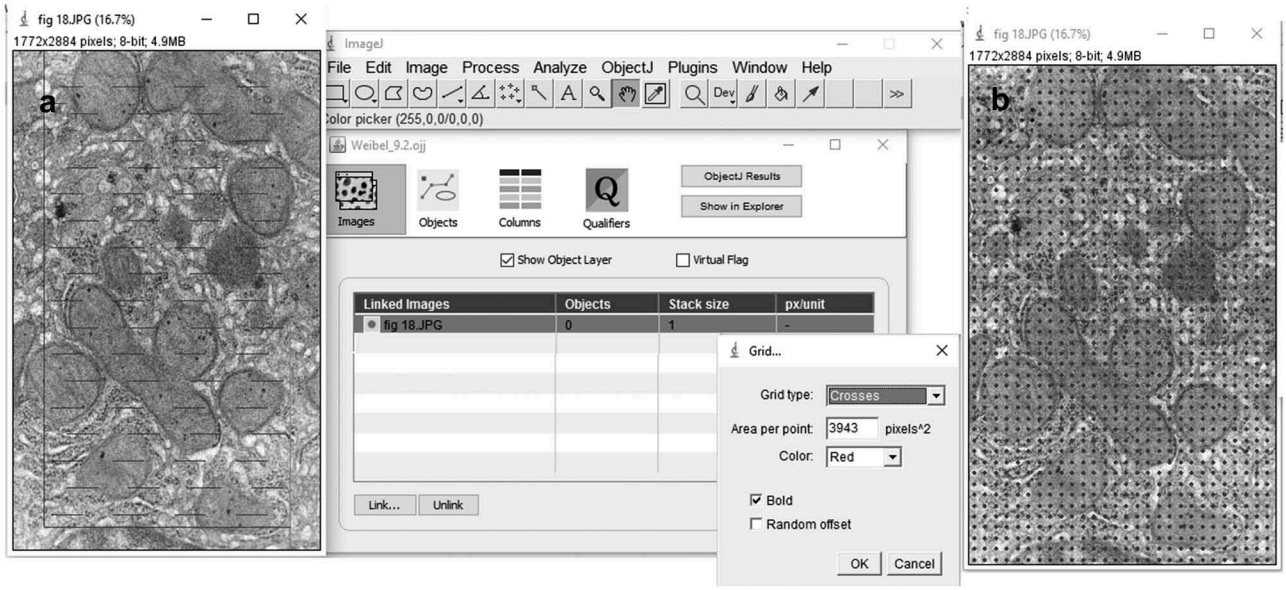

Figure 1. A. Weibel's grid plugin, B. Combined grid plugin added to ImageJ software, superimposed on electron micrographs.

The volume density of the glycogen (Vg) is the volume of glycogen per unit volume of hepatocytes cytoplasm. It was calculated from electron micrographs using ImageJ software with coherent double lattice grid plugin[16] (Figure 1(b)). All the points lying over the glycogen were recorded (Pi). The volume density (Vi) of the glycogen was calculated using the Weibel's formula[15] where Vi equal the number of points overlying the component (Pi) divided on the total number of points of the grid. The volume density of mitochondria was calculated in the same way. The volume density of both mitochondria and glycogen were expressed as μ^3 per μ^3 of the hepatocytes cytoplasm in both centrilobular and periportal zones in all electron micrographs.

The measurements were done; the mean and standard error of the mean were calculated and *statistically analyzed*. The mean was considered significantly different from control mean if the probability of error was less than 0.05.

Results

The EM micrographs of periportal hepatocytes of the control group are shown in Figure 2; EM micrographs of centrilobular hepatocytes of the control group are shown in Figure 3; EM micrographs of periportal hepatocytes of the fasting group are shown in Figure 4; while EM micrographs of centrilobular hepatocytes of the fasting group are shown in Figure 5.

The periportal hepatocytes from the fed group contained a fewer but large elongated mitochondria in comparison with the centrilobular hepatocytes of the fed group which showed a higher number of rounded but small mitochondria, located close to RER and to the nucleus and along the sinusoidal surface. After fasting, mitochondria did not show any sign of degeneration of the cristae in both zones. However, there was fusion between the mitochondria with stronger approximation between mitochondria and the endoplasmic reticulum.

The periportal hepatocytes from the fed group were characterized by large masses of tightly packed glycogen particles in contrast to the centrilobular hepatocytes of the fed group that showed dispersed glycogen particularly in areas adjacent to blood sinusoid. The particles were separated from each other by considerable amount of cytoplasm, which contained tubules and vesicles of SER. After fasting, the density of glycogen masses markedly decreased. However, the pattern of glycogen masses, either packed or dispersed, was maintained in both zones.

In the periportal hepatocytes from the fed group, SER was fewer but more tubular within the small restrict regions of cytosome and was displaced to the periphery of the glycogen masses in contrast to the fed centrilobular hepatocytes showing abundant and

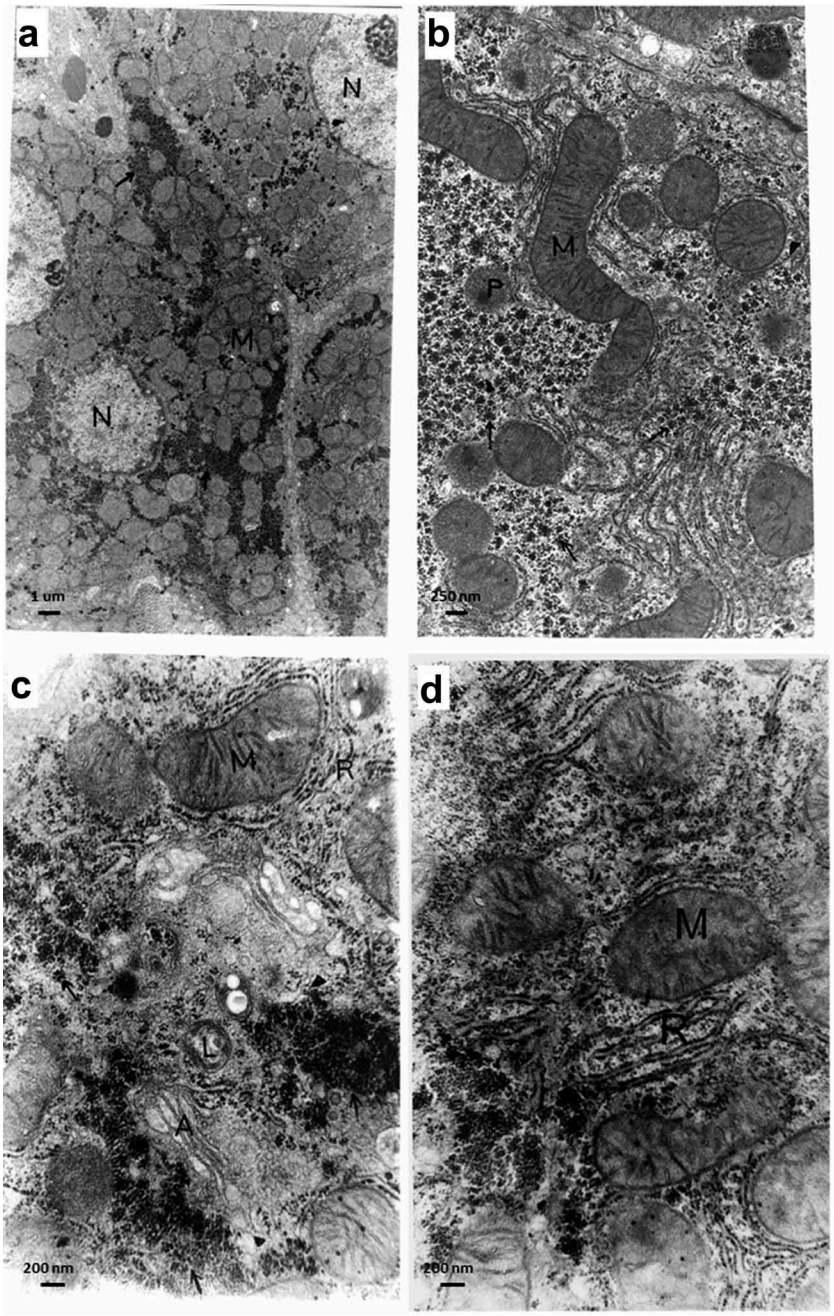

Figure 2. Electron micrographs of periportal hepatocytes from control group: a) (X5000), b) (X20000), c) (X28000), d) (X28000) showing: nucleus (N) in the center of the cell, large and mainly elongated mitochondria (M), large mass of tightly packed glycogen particles (arrows), SER (arrowheads) at the periphery of glycogen masses and peroxisomes (P) located at the periphery of SER and glycogen. Note the presence of Golgi apparatus (A), RER(R) and secondary lysosomes (L).

interspersed SER between individual glycogen particles. After fasting, SER becomes more numerous in both zones, predominantly of vesicular form appeared in many regions of the cytosome of periportal hepatocytes.

In the periportal hepatocytes from the fed group, RER cisternae showed less tendency to occur in stacks. However, in centrilobular hepatocytes, well-formed RER with long parallel cisternae was most commonly found in the perinuclear region, in pericanalicular zone and along the sinusoidal surface and located mainly in the vicinity of the mitochondria.

After fasting, cisternae of RER in hepatocytes of both zones were markedly decreased and usually occurred as single parallel elements very closely associated with mitochondria and numerous areas along

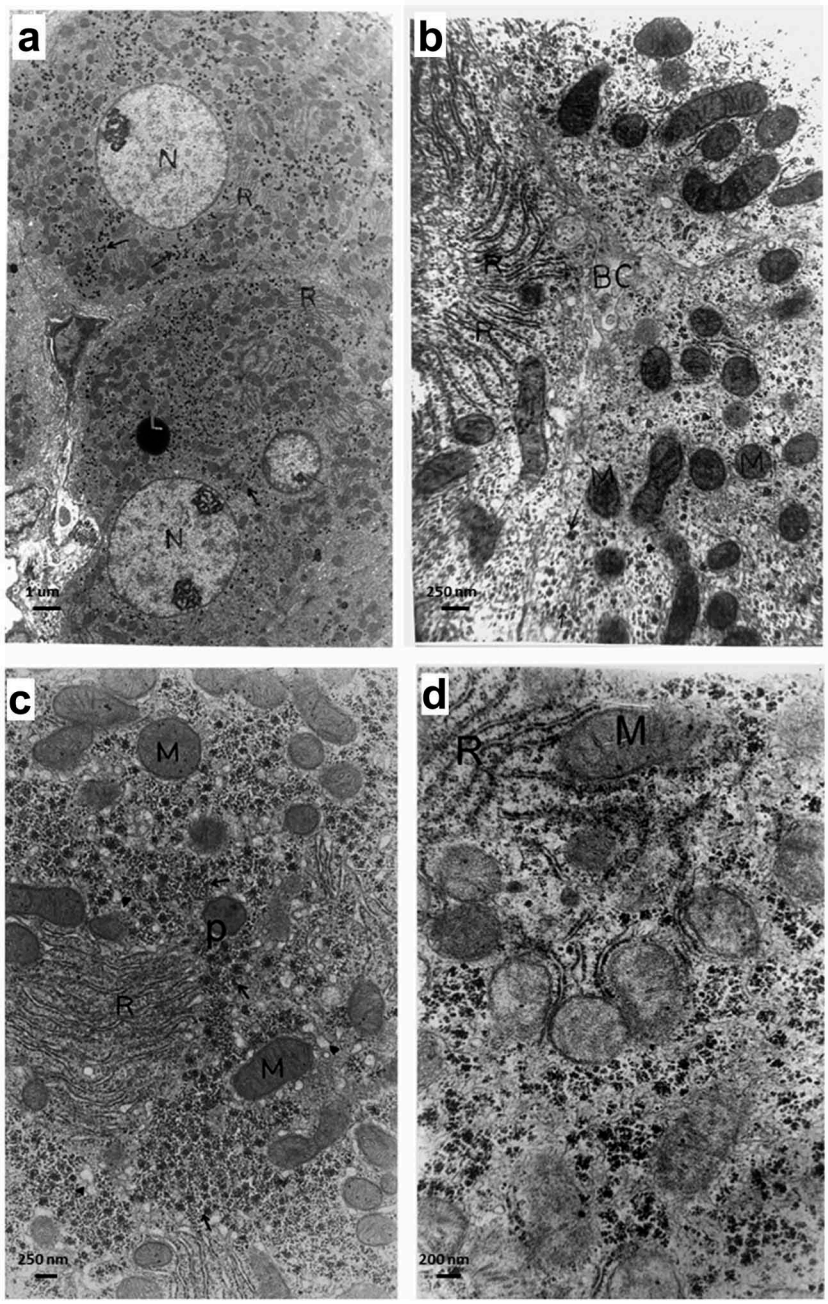

Figure 3. Electron micrographs of centrilobular hepatocytes of control group (fed ad libitum): a) (X4000), b) (X16000), c) (X20000), d) (X28000), Showing: glycogen particles (arrows) found in dispersed form and vesicles and tubules of SER (arrowheads) between glycogen particles and lipid droplets (L).Note the presence of nucleus (N) in the center of hepatocytes, Small and mainly rounded mitochondria (M), and bile canaliculus (BC), RER(R) with long parallel cisternae located mainly in vicinity of mitochondria and Golgi apparatus (A), peroxisomes (P) located at the periphery of SER and glycogen.

the margin of the cell adjacent to sinusoids and scattered throughout the cytosome were devoid of RER. The terminal ends of RER cisternae near the regions of SER showed dilatation and fewer attached polysomal ribosomes than other region of RER.

In the periportal hepatocytes from the fed group, GA were larger and more numerous than that of fed centrilobular hepatocytes where the GA were observed adjacent to the bile canaliculus and occasionally near the space of Disse and the nucleus. After fasting, GA appeared as that of fed group in both zones.

In the periportal hepatocytes from the fed group, lysosomes were larger and more numerous than that

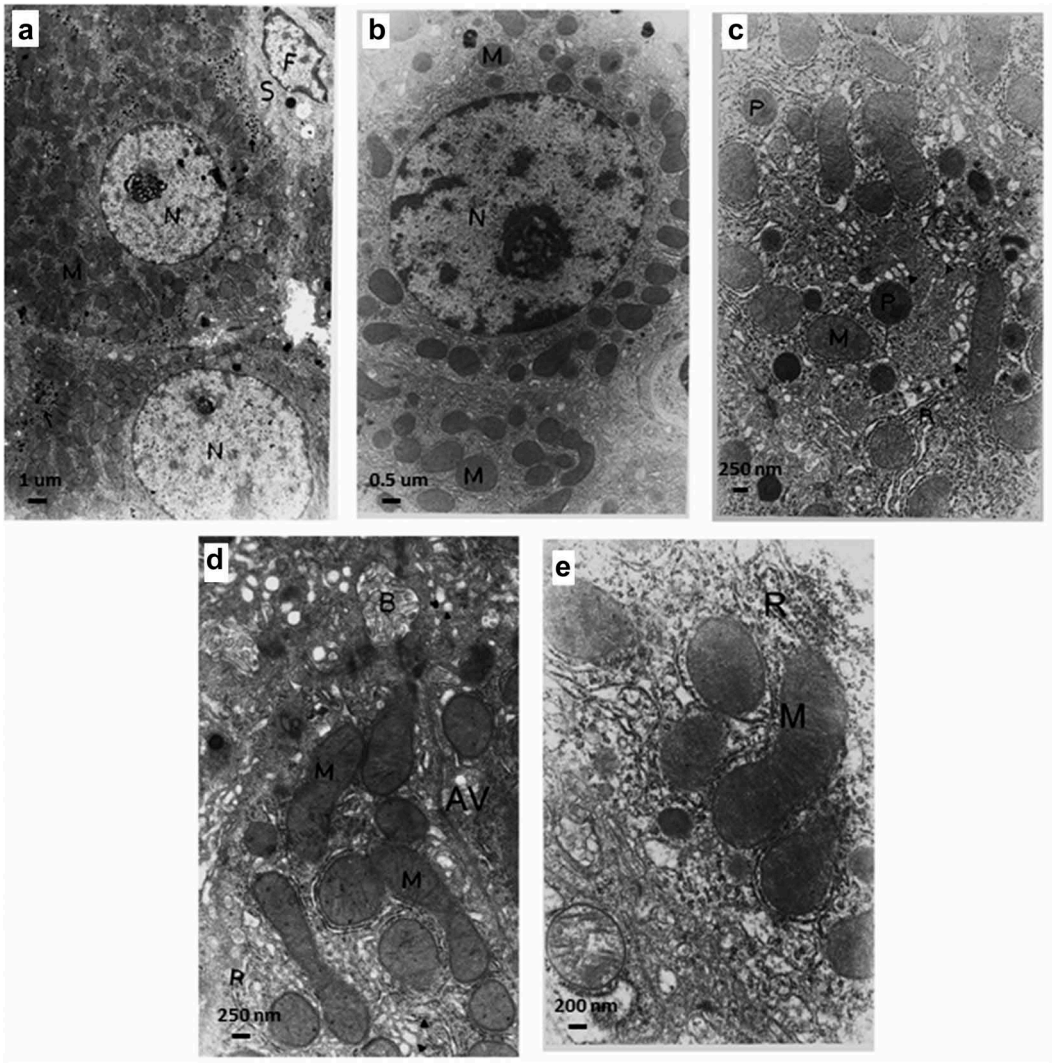

Figure 4. Electron micrograph of periportal hepatocyte from 16 hours fasted rats: a) (X5000), b) (X10000), c) (X16000), d) (X20000), e) (X28000), showing: glycogen particles (arrows) are decreased and absent in some fields, mitochondria (M) showing fusion, elongation and strong contact with RER, with no signs of degeneration, normal nucleus (N) and blood sinusoids (S) contains fibroblast (F), increase of SER (arrowheads), decrease of RER(R), terminal ends of RER cisternae near the regions of SER showed dilatation and fewer attached polysomal ribosomes. Note the presence of peroxisomes (P), Bile canaliculus (B) and autophagic vacuoles (AV).

of fed centrilobular hepatocytes. Lysosomes were observed adjacent to the bile canaliculus. After fasting, the lysosomal appearance did not change in the hepatocytes of both zones, however more autophagosomes and isolation membranes were more obvious mainly in periportal hepatocytes.

In the periportal hepatocytes from the fed group, there was a few number of peroxisomes located at the periphery of SER and glycogen areas in contrast to the fed centrilobular hepatocytes which showed large and numerous peroxisomes, predominantly located within areas of the cell containing SER and glycogen. After fasting, peroxisome appearance did not show obvious change in both zones.

In the hepatocytes of both zones, from the fed group, few lipid droplets were seen scattered in the cytoplasm. After fasting, there was no apparent change in lipid content.

In the hepatocytes of both zones, from the fed group, the nucleus appeared rounded in the center of the cell, limited by double membrane and in nucleoplasm, a nucleolus and chromatin granules appeared. After fasting, the nucleus did not show any sign of degeneration in both zones.

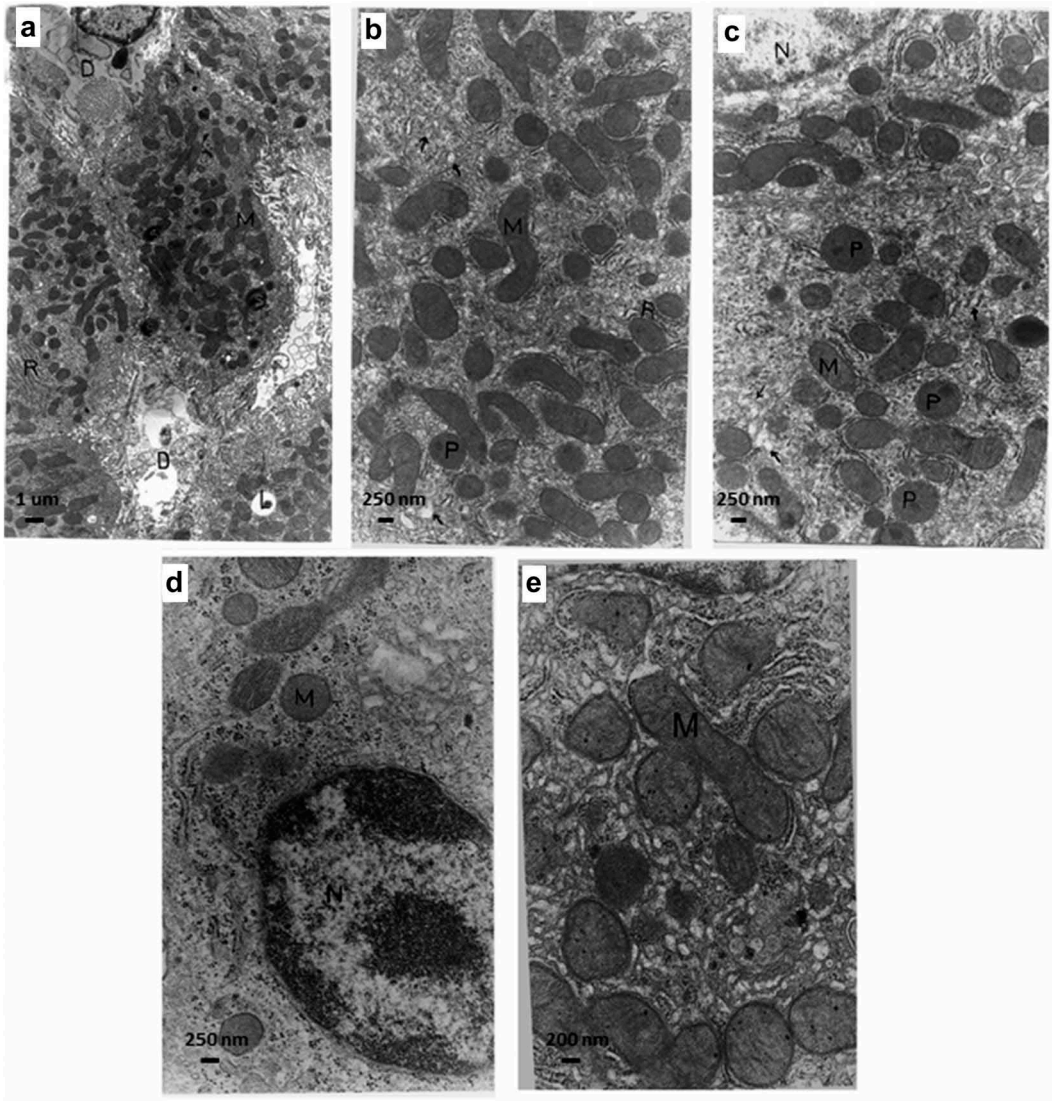

Figure 5. Electron micrographs of centrilobular hepatocytes from 16 hours fasted rat: a) (X5000), b) (X16000), c) (X16000), d) (X20000), e) (X28000), showing mitochondria (M) with no signs of degeneration, cisternae of RER(R) markedly decreased, secondary lysosomes (S), lipid droplets (L) and blood sinusoids (D), absence of glycogen particles in this field, SER (arrows) markedly increased, predominantly of vesicular type, RER(R) is less numerous, terminal ends of RER cisternae near the regions of SER showed dilatation and fewer attached polysomal ribosomes, peroxisome (P) and normally appearing nucleus (N).

Generally, the organelles were normal with no signs of degeneration after overnight fasting.

Morphometric analysis

The results are shown in Table 1, Figure 6, on electron micrographs, photographically enlarged at final magnification of X 28,000.

Statistical analysis of the morphometric results of centrilobular and periportal hepatocytes revealed that there were zonal differences in control group, and these differences were mostly maintained after fasting. The lysosomes surface density, mitochondrial volume, and surface density were significantly higher and the volume density of glycogen and surface densities of RER, GA, and nucleus were non-significantly higher in periportal-fed hepatocytes than in centrilobular hepatocytes, in contrast to the significantly higher surface density of SER and peroxisomes and non-significantly higher surface density of lipids in centrilobular-fed hepatocytes when compared to periportal hepatocytes.

Statistical analysis of the morphometric results of fasted group compared with those of fed group in hepatocytes of both zones revealed that there

Table 1. Comparison between morphometric results of subcellular components of centrilobular and periportal hepatocytes of both control and fasted group.

	Fed Hepatocytes		Fasted hepatocytes			
	Periportal	Centrilobular	Periportal	Centrilobular		
Subcellular structures	Mean ±SD	Mean ±SD	Mean± SD	Mean± SD	P1	P2
Mitochondrial volume density (μ^3/μ^3)	0.255 ± 0.04	0.143 ± 0.06	0.253 ± 0.16	0.142 ± 0.01	*	
Mitochondria surface density (μ2/μ3)	2.56 ± 0.03	1.6 ± 0.1	2.45 ± 0.02	1.48 ± 0. 3	*	
Glycogen volume density (μ^3/μ^3)	0.39 ± 0.05	0.34 ± 0.04	0.005 ± 0.003	0.003 ± 0.002		*
Surface density (μ2/μ3) of RER	2.6 ± 0.08	2.3 ± 0.4	0.86 ± 0.03	0.21 ± 0.09		*
Surface density (μ2/μ3) of SER	0.86 ± 0.09	1.05 ± 0.3	2.05 ± 0.03	3.35 ± 0.13	*	*
Surface density (μ2/μ3) of G. a	0.065 ± 0.03	0.06 ± 0.01	0.055 ± 0.02	0.05 ± 0.01		
Surface density (μ2/μ3) of lysosomes	0.05 ± .002	0.03 ± 0.001	0.06 ± .002	0.045 ± 0.03	*	
Surface density (μ2/μ3) of peroxisomes	0.044 ± 0.001	0.065 ± 0.012	0.043 ± .001	0.056 ± 0.12	*	
Surface density (μ2/μ3) of lipids	0.004 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.006 ± 0.002		
Surface density (μ2/μ3) of nucleus	0.98 ± 0.02	0.96 ± 0.01	0.92 ± 0.03	0.94 ± 0.01		

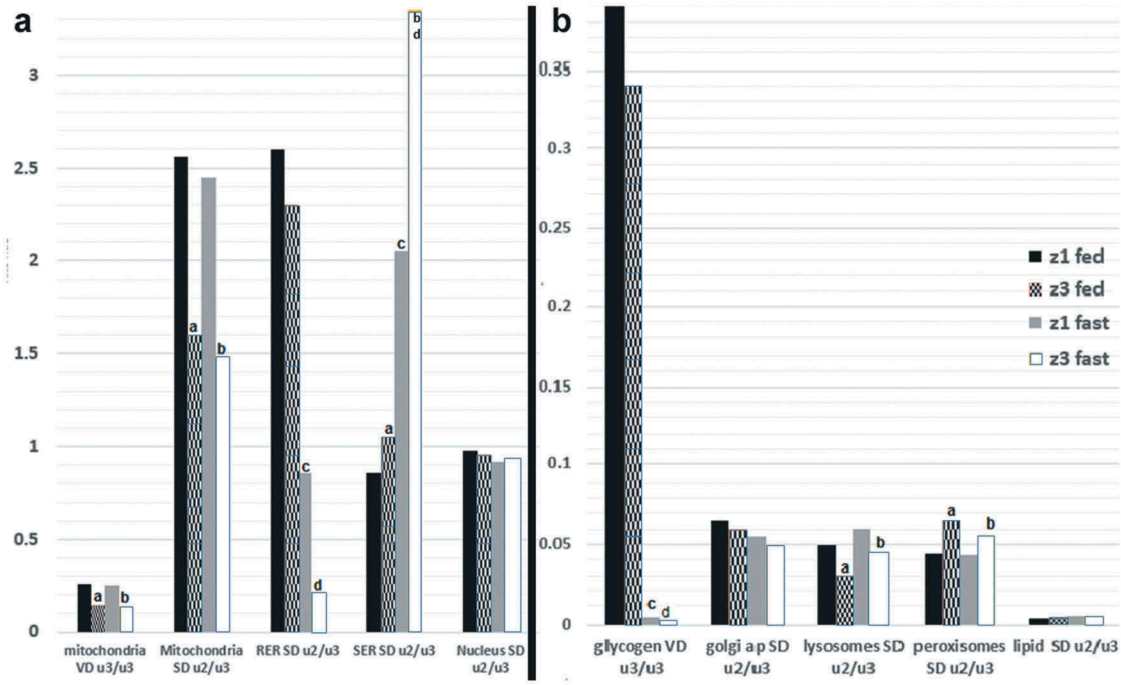

Figure 6. A: comparison of morphometric analysis between the means of volume density (VD) of mitochondria, surface densities (SD) of mitochondria, RER, SER and Nucleus, B: comparison of morphometric analysis between the means of volume density (VD) of glycogen, surface densities (SD) of Golgi apparatus, lysosomes, peroxisomes and lipid droplets in zone 1 (Z1) & zone 3 (Z3) hepatocytes in fed and fasted state. a means significant difference of z3 fed in comparison to z1 fed, b means significant difference of z3 fasted in comparison to z1 fasted, c means significant difference of z1 fasted in comparison to z1 fed and d means significant difference of z3 fasted in comparison to z3 fed.

was a significant decrease of volume density of glycogen and surface density of RER and non-significant decrease in GA, nucleus, peroxisomes, and mitochondrial surface densities and mitochondrial volume density with significant increase of SER and non-significant increase in lysosomes and lipid surface densities in both zones.

The zonal pattern of organelle distribution was maintained after fasting in most of organelles except for non-significantly higher surface density of the nucleus in centrilobular hepatocytes and no difference in lipid surface density between the zones.

Discussion

In the present work, both feeding status of the animal and zonal pattern of hepatocytes were studied. Both ultra-structural and morphometric analysis were done for perfect results. The hepatocytes of rats fed ad libitum revealed morphologic and

quantitative differences depending on the position of hepatocytes within the hepatic lobules. The zonal differences were roughly maintained after overnight fasting.

Our finding of mitochondria with significantly higher volume and surface densities in periportal hepatocytes are parallel with the fact that the large mitochondria and high levels of cytochrome oxidase and succinic dehydrogenase found in periportal cells show that these cells are the main providers for oxidative respiration. The periportal zone, into which the arterial and portal blood first drain, receives more sufficient oxygen and nutrient supply in comparison with the centrilobular zone. The periportal zone has been considered as the prime area of protein metabolism, glycogen synthesis, and glycogenolysis.[5]

The decrease in mitochondrial volume and surface densities with fasting can be explained by a recent report showing that mice exposed to overnight fasting underwent a shift in the bioenergetics of hepatic mitochondria with a decrease in the respiratory ability and oxidative phosphorylation. This might be a metabolic inhibition to conserve energy.[17]

We also found that the volume and surface densities of mitochondria vary similar to those of RER. This may be due to that the outer mitochondrial membrane is considered as a developed and specialized form of ER. After fasting, our finding of stronger contact between ER and mitochondria may be related to the claim that RER provides the mitochondria with important components essential for the elongation, curvature, and closure of the isolation membranes. According to cellular needs or stresses, mitochondria-associated membranes (MAMs) allow the exchange between mitochondria and ER of calcium, lipids, and reactive oxygen species (ROS), permitting adaptations of both bioenergetics and cellular fate. MAMs might also act as a hub of hormonal signaling in many insulin-sensitive tissues, indicating a specific role of MAMs in the control of glucose metabolism. Upon metabolic energy intake, MAMs thickness and length changed, i.e., increased after fasting but decreased in over-nutrition.[18]

It must also be noted that food deprivation is a strong stimulator for autophagy and mitophagy; however, our finding of mitochondrial fusion upon fasting may be linked to the claim that mitochondrial tubulation protects mitochondria from autophagic degradation, which could allow mitochondria to maximize energy production and supply autophagosomal membranes during starvation in a process that depends on the attachment of mitochondria to the ER.[19]

Our finding of abundant glycogen in the periportal hepatocytes in fed rats might be due to the availability of the maximal level of the enzymes of gluconeogenesis and that the most of liver glycogen accumulating during feeding after starvation was reported to be made of the gluconeogenic precursors. It was also shown that upon fasting, the glycogen was depleted from the periportal hepatocytes much more rapidly than in perivenous hepatocytes.[5]

It had been proved that after food deprivation, when blood glucose level decreases, the hepatocytes respond by forming SER, which participates in glycogenolysis, the hepatocytes are stimulated to break down their glycogen stores and release of glucose. Furthermore, the biochemical studies reported the presence of glucose 6-phosphatase in SER. While glycogenolysis occurs in glycogen masses connected to ER membrane, glycogen mass might be also joined with phagophores to form autophagosomes. Omegasomes, parts of the ER membrane, are the sites for formation of phagophore. Therefore, ER, the phagophore, and the glycogen masses form a triangle in which degradation of glycogen occurs.[20]

Although the glycogen content and its variation, correlated to SER, are larger in periportal zone, our results showed that the volume and surface densities of SER are significantly higher in centrilobular hepatocytes. This indicates that the variation in the SER is not only related to glycogen metabolism but also related to other functions, especially in centrilobular zone as lipogenesis and drug detoxification. Centrilobular hepatocytes share in cytochrome p450-dependent drug metabolism and NADPH-related hydroxylating systems.[21]

Our finding of RER abundance in the periportal hepatocytes confirms that protein synthesis is most active in that zone. Many proteins formed in the hepatocyte RER are used within the cells or secreted. The rate of protein synthesis could be correlated with the amount of RER present.[5]

Our results show the inverse relationship between surface densities of hepatocyte RER and that of SER. It is widely thought that the SER is derived from the RER. The increase in SER is

partially related to the way in which ribosomes detach from RER membranes. This is supported by the increase in free polysomes coinciding with the SER increase and RER decrease and that the cisternal end of RER is often connected to tubular SER. Furthermore, these ribosomes may be lost by starvation-induced autophagy.[22]

The abundance of GA in periportal hepatocytes of the control rat is parallel with the report that these cells are the main contributors to bile salt secretion.[23] Under starved conditions, the hepatocytes are responsible for the redistribution of lipid brought from adipose tissue after lipolysis to peripheral organs by VLDLs synthesis and secretion. Very low density lipoprotein (VLDL) assembly is started in the endoplasmic reticulum and particles are thought to be further lipidated and modulated in the GA.[24]

Our finding of higher abundance of lysosomes in periportal zone may be because periportal hepatocytes need to clear and digest the materials taken from the blood stream more often than perivenous hepatocytes.[5]

Our data about the lysosomal increase after fasting are parallel to the reports showing that, after short-term fasting, the hepatocytes showed an increasing number of primary lysosomes. All this was associated with intensification of the autophagy processes, significantly higher numbers of autophagic secondary lysosomes. The increase in the number of both primary and secondary lysosomes and their activation appear as an adaptation response to conditions switching over to a forced internal nutrition.[25]

However, other reports found that immunohistochemical staining for LC3; an autophagy marker was mainly distributed in perivenous zone. It was assumed that low rates of autophagy occur in periportal areas and constitutive high levels of autophagy pericentrally in well-nourished conditions.[26]

The peroxisomes abundance in centrilobular hepatocytes indicates a higher biodegradative capacity. Peroxisomes contain a fatty acyl Co-A oxidizing, an important enzyme for lipid metabolism. Thus, it is interesting to find that the daily mean surface densities of lipid droplets are also higher in centrilobular than in periportal hepatocytes.[27] An example of the peroxisomal changes observed in the liver after treatment of rats with a hypolipidemic drug was the marked proliferation of centrilobular peroxisomes, which formed clusters.[22]

Peroxisomes are sources of ROS. ROS production and removal is zonated. This zonation is regulated by the periportal predominance of mitochondria and centrilobular predominance of peroxisomes, and zonal differences in GSH production and content. The pericentral zonation of peroxiredoxins, thioredoxins, and glutaredoxins can be linked to their association with peroxisomes. These mechanisms may be important in nonalcoholic fatty liver disease, especially during the development of nonalcoholic steatohepatitis and, because of this zonation, the appearance of the first signs of the disease was in the centrilobular zone.[4]

The abundance of lipid droplets in centrilobular zone is related to the fact that Lipid metabolism is zoned there. Lipid metabolism is mainly active in centrilobular hepatocytes.[27] Furthermore, lipid metabolism can be differentially regulated by disconnecting periportal lipogenesis and lipophagy from pericentral breakdown by peroxisomes.[2]

P-hydroxybutyric dehydrogenase and esterases are concentrated in centrilobular hepatocytes. These cells also contain a higher number of microbodies that might have a role in lipid metabolism.[23]

Our data of the slight increase in lipid surface density after fasting are parallel to the report showing that fasting increased the hepatic cholesterol and phospholipid levels. It was thought that the main cause could be the reduction of protein and water in addition to triacylglycerol.[28]

Summary

Generally, after overnight fasting, the organelles were normal with no signs of degeneration. The zonal differences were maintained. The change in hepatocyte ultrastructure after fasting may be related to autophagy.

The period of fasting used was relatively short and is somewhat longer than overnight fasting associated with sleeping. It would be assumed that more dramatic changes would be found with longer periods of fasting or markedly decreased nutrition provided.

Conflict of interest

No potential conflict of interest was reported by the author.

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