Quantitative ultrastructural changes of hepatocyte constituents in euthermic, hibernating and arousing dormice (*Muscardinus avellanarius*)

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**Abstract.** Hibernating animals represent a suitable model for investigating the structural effects of drastic changes in cell activity under physiological conditions. In this study we investigated by means of electron microscopy and morphometrical analysis the fine structural counterpart of functional rest in hepatocytes of the hibernating dormouse, *Muscardinus avellanarius*, in comparison with arousing and euthermic dormice. Our observations demonstrate that during hibernation several structural constituents of the hepatocyte undergo modifications. In particular, during deep hibernation, the total cell and cytoplasm area significantly reduced, as well as the total and percent glycogen and residual body area, and the Golgi apparatus almost disappeared. Upon arousal, the amount of glycogen was minimal, whereas total cell and cytoplasm area significantly increased towards the euthermic value as well as total and percent residual body area. In comparison with the euthermic condition, the total and percent cell lipid area significantly increased in early hibernation, reduced in deep hibernation and almost disappeared during arousal. Taken together, our findings give quantitative ultrastructural support to the marked reduction found in hepatocyte functional activities during hibernation. Such a reduced activity involves profound rearrangement of the euthermic cell structure, which is rapidly resumed upon arousal.

Keywords: hibernation, hepatocyte, cytoplasmic organelles, cytoplasmic inclusions, ultrastructure, *Muscardinus avellanarius*

**Introduction**

Hibernating animals represent a suitable model for investigating the structural effects of drastic changes in cell activity under physiological conditions. Indeed, hibernators are able to enter, in the cold season, long phases of lethargy characterised by a body temperature approaching ambient temperature and minimum physiological and metabolic rate (for reviews, see Hoffman, 1964; Lyman et al., 1982; Wang, 1987; French, 1988). During arousal, normal body temperature and all metabolic and physiological activities are rapidly resumed, although the time-course of such resumption can change in different tissues, depending on the physiological functions.

Previous studies revealed important modifications of cellular structural components in several tissues—e.g. pancreas, adrenal cortex, kidney, bladder—of the hazel dormouse *Muscardinus avellanarius*, a true hibernator of small size living in the wild, during the euthermia-hibernation-arousal cycle (Malatesta et al., 1995, 1998, 2001a,b; Zancanaro et al., 1997, 1999). Moreover, modifications of hepatocyte nuclear structural constituents of the same dormouse were previously described (Malatesta et al., 1994a,b).
Liver is a highly metabolising organ strategically located between the intestinal tract and the general circulation and plays several metabolic and physiological functions. It receives, metabolises and/or transforms most of the products of digestion; it degrades and detoxifies toxic compounds received from the intestine or from the general circulation; it synthesises many protein components of blood plasma and exercises an important degree of control over the general metabolism. The hepatocyte is, therefore, a multifunction cell type, which is especially well suited to clarify the morphological counterpart of functional rest during the euthermia–hibernation–arousal cycle.

In the present study, we have examined the hepatocytes of hibernating, arousing and euthermic hazel dormice by means of electron microscopy and ultrastructural morphometry.

Materials and methods

Eleven individuals of the hazel dormouse *M. avellanarius* (Gliridae) were used in this study. This dormouse, like many other wild-living animals in Europe, is protected by law and only a limited number of individuals could be taken for the purpose of multiple investigations, with permission from local authorities. Wild-living animals were trapped and maintained in an external animal house and provided with food and bedding material; under such conditions, they spontaneously began to hibernate in November and awoke in March. Five animals were sacrificed during hibernation (two in late November, in early hibernation, and three in January, in deep hibernation), three during arousal (March) and three during the euthermic period (June–July). Dormant animals were taken from the cage and immediately killed by cervical dislocation. Arousing animals were allowed to awake undisturbed apart from placing a thermistor probe on the abdominal dislocation. Euthermic animals were anaesthetised with ether and sacrificed. Samples of the liver were quickly removed and small fragments were fixed by immersion in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.4, at 4 °C for 2 h; washed, post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide at 4 °C for 1 h, dehydrated through graded concentrations of acetone and embedded in Epon-Araldite. Semithin sections (2 μm in thickness) were stained with 1% toluidine blue and observed with a Leitz Orthoplan light microscope. Ultrathin sections were stained with lead citrate and observed with a Zeiss EM 902 electron microscope operating at 80 kV.

Morphometric analysis was carried out on the same sections as those used for morphological observations. Sixteen randomly selected hepatocytes from each animal were photographed at a fixed magnification (×6000), taking care that each cell profile contained at least one nucleus. In such a way, i.e. by considering in all animals the perinuclear cytoplasmic region, we could reduce the sampling variability due to the heterogeneous distribution of subcellular components to be measured. The total surface cell area and the areas occupied by the nucleus, lipid droplets, glycogen deposits and residual bodies were then measured semi-automatically by using the computerised image analysis system Image Pro-Plus (Media Cybernetics, Silver Spring, MD, USA). The cytoplasm area, the nucleus-to-cytoplasm (N/C) ratio and the percentage of cytoplasm area occupied by lipid, glycogen and residual bodies were calculated. For each analysed variable, the Kolmogorov–Smirnov two-sample test was performed in order to verify the hypothesis of identical distributions among animals of each group. The data for each variable were then pooled according to the four experimental groups, i.e. euthermia, early hibernation, deep hibernation and arousal, and the mean ± standard error of the mean (SE) values were calculated. Statistical analysis of the results was performed by the non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) test; furthermore, in order to determine which pairs of samples tended to differ, the standard procedure of multiple comparison for the Kruskal–Wallis test was used.

Results

Ultrastructural morphology

In euthermic dormice (Figs 1a & 2a) the hepatocyte showed typical morphology. Cells were polyhedral in shape with round nuclei generally located in the central region of the cell (about 8% of hepatocytes contained two nuclei, regardless of the animal or the seasonal phase). Hepatocyte nuclei of euthermic dormice contained all the structural constituents generally found in mammalian nuclei; the nucleoli (generally one per nucleus) exhibited easily recognisable, intermingled, dense fibrillar and granular components, whereas fibrillar centres were not prominent. In the cytoplasm the rough endoplasmic reticulum (RER) was arranged in irregularly oriented cisternae. The Golgi apparatus were well developed and preferentially located in perinuclear position as well as close to bile canalicula. The smooth endoplasmic reticulum (SER) appeared particularly abundant near glycogen lakes. Mitochondria were distributed in the whole cytoplasm and exhibited ovoid shapes, well-developed cristae and rare electron-dense particles dispersed in their matrix. Glycogen particles were abundant and mostly gathered in lakes; some lipid droplets occurred, sometimes in association with glycogen deposits, and some residual bodies were also present. During hibernation and arousal, hepatocytes showed several ultrastructural modifications when compared with euthermia.

During early hibernation (Fig. 1b), nuclei showed a more irregular shape than in euthermia. The Golgi apparatus was reduced in size and surrounded by numerous small, smooth vesicles (Fig. 3a). The mitochondria showed some electron-dense particles distributed in their matrix (Fig. 3a) and they were frequently observed in association with lipid droplets (not shown). Numerous lipid droplets were found in the cytoplasm, together with large glycogen deposits and a few residual bodies.
Hepatocyte modifications during hibernation

Fig. 1  Hepatocytes from euthermic (a), early hibernating (b), deeply hibernating (c) and arousing (d) dormice. Lipid droplets (L), particularly abundant in early hibernation (b), drastically decrease in deep hibernation (c) and disappear upon arousal (d). Glycogen (G) also progressively decreases from early through deep hibernation to arousal, whereas residual bodies (arrows) accumulate upon arousal (d). ×6600.

During deep hibernation (Fig. 1c), nuclei were more irregular in shape than those of euthermic dormice, and coiled bodies and amorphous bodies (Fig. 2d & e) occurred in the nucleoplasm, while the nucleoli showed nucleoplasmic invaginations. The RER cisternae assumed a parallel pattern, and many clusters of round vesicles of SER appeared in the cytoplasm (Fig. 2b). The Golgi apparatus was not recognisable.

The mitochondria contained numerous electron-dense particles and were often found to surround lipid droplets (Fig. 2b & c). Some small lipid droplets, small deposits of glycogen and a few residual bodies were also observed.

Upon arousal, some nuclei appeared to return to their euthermic roundish shape (Fig. 1d), while others displayed irregular shapes (Fig. 3b). RER still showing the parallel
Hepatocytes from euthermic (a) and deeply hibernating (b–e) dormice. During euthermia (a), RER is arranged in irregularly oriented cisternae (thin arrows), the Golgi apparatus (arrow) is well developed and mitochondria (M) contain only a few electron-dense particles. During deep hibernation (b & c), RER cisternae assume a parallel pattern (asterisk), clusters of smooth vesicles (V) appear in the cytoplasm and mitochondria (M), which often surround lipid droplets (L), contain numerous electron-dense particles. Moreover, hepatocyte nuclei contain coiled bodies (d) and amorphous bodies (e). (a) × 47 000; (b) × 28 000; (c) × 18 000; (d–e) × 33 000.

Hepatocyte from an early hibernating dormouse (a). The Golgi apparatus (arrow) appears reduced and the mitochondria (M) show electron-dense particles dispersed in their matrix. Hepatocyte from an arousing dormouse (b). Note the irregularly shaped nuclei. (a) × 22 000; (b) × 8000.

The histograms show the mean values ± SE of the indicated cellular variables in four groups of hazel dormice along the year cycle. In each histogram, values identified with common symbols (*, †) are not significantly different from one another (Kruskal–Wallis test).
Fig. 5 The histograms show the mean values ± SE of the indicated cytoplasmic constituents in four groups of hazel dormice along the year cycle. In each histogram, values identified with common symbols (∗, ○) are not significantly different from one another (Kruskal–Wallis test).

The arrangement observed in deep hibernation and RER in the form of less regularly oriented cisternae coexisted in the same cell. The morphology of the Golgi apparatus was similar to euthermia; the mitochondrial particles were less frequent and less electron-dense (not shown). The clusters of round vesicles of SER observed during deep hibernation were only occasionally found. Residual bodies were abundant in the cytoplasm, whereas lipid droplets and glycogen were hardly detectable.

Morphometry
Analysis of rough morphometric data by means of the Kolmogorov–Smirnov test verified the hypothesis of identical distributions among animals of each group for each analysed variable. The mean ± SE values of variables measured in euthermic, early hibernating, deeply hibernating and arousing dormice are shown in Figures 4 and 5. The Kruskal–Wallis test showed significant differences among the four groups for all the considered variables (P < 0.05).

The standard procedure of multiple comparison for the Kruskal–Wallis test showed that the total cellular and cytoplasmic area significantly decreased during deep hibernation (Fig. 6), as well as the total and percent glycogen and residual body area. During the process of arousal, total cell and cytoplasmic area significantly increased in comparison with deep hibernation and there was a striking increase of the total and percent residual body area. Instead, glycogen remained at a minimum during arousal. The total and percent cell area occupied by lipid droplets showed a significant increase in the early hibernation period, decreased in deep hibernation and decreased further during arousal. Between group comparison of the nucleus areas showed a significant decrease of this...
hepatocyte modifications during hibernation

Fig. 6 Light micrographs of liver from euthermic (a) and deeply hibernating (b) dormice. Note the reduction in size of hepatocytes during hibernation. ×400.

variable in early hibernation and a clear increase towards the euthermic value during arousal; such an increase revealed significant when the N/C ratio was considered.

Discussion

The ultrastructural and morphometric data presented herein demonstrate that:

1. key cellular constituents of the hepatocyte undergo important structural modifications along the euthermia–hibernation–arousal phases of the year cycle, and
2. quantitative changes take place in cell size variables as well as the amount of lipid, glycogen and residual bodies.

In particular, ultrastructural modifications were obvious in the Golgi apparatus and the RER: the Golgi apparatus reduces its size in early hibernation and is unrecognisable in deep hibernation, and RER cisternae progressively assume a parallel pattern; upon arousal, both RER cisternae and Golgi apparatus quickly start restoring their euthermic aspect. Previous studies in pancreatic acinar cells of the same dormice (Malatesta et al., 1998, 2001b) demonstrated consistent RER and Golgi reorganisation during hibernation and arousal, similar to that found in different tissues of hibernating mammals (Fink et al., 1977; Reme & Young, 1977; Popov et al., 1999) and in hepatocytes where protein synthesis alterations had been experimentally induced (e.g. Reaven et al., 1993; Sturgess et al., 1975). RER and Golgi reorganisation is probably a fast and energetically efficient response to drastic modifications in protein synthesis demand (Malatesta et al., 1998, 2001b). Accordingly, protein synthesis was reduced by about two-third in the liver of hibernating ground squirrels (Whitten & Klain, 1968).

The modifications of hepatocyte nuclear structural constituents during the hibernation cycle have been discussed in previous studies (Malatesta et al., 1994a,b, 1999, 2000, 2001c). Briefly, during hibernation coiled bodies and amorphous bodies appear in the nucleoplasm, while the nucleoli become irregular in shape because of nucleoplasmic invaginations. Such nuclear structural modifications have been described not only in hepatocytes but also in other cell types of hazel and edible dormice, suggesting that they represent usual features in tissues of hibernating mammals: coiled bodies and amorphous bodies are presumed to be storage/recycling sites of nucleoplasmic and nucleolar splicing factors to be used rapidly upon arousal (Malatesta et al., 1994a,b, 1999, 2001c), while nucleolar architectural alterations are probably related to peculiar functional relationships established between nucleolus and nucleoplasm during deep hibernation (Malatesta et al., 2000).

A further structural change found in hepatocytes of hibernating dormice is an increase in electron-dense particles of the mitochondrial matrix, which disappears upon arousal. In hibernating dormice, similar particles were found increased in mitochondria of different tissues (Malatesta et al., 2001a). These particles contain several inorganic and organic components and their presence seems to be related to the metabolic state of the cell, since they disappear after strong metabolic activation (see Jacob et al., 1994). Therefore, we hypothesised that electron-dense particles in the hepatocyte represent storage sites of substances needed in large amounts during arousal to rapidly fully resume respiratory functions in mitochondria.

The results of morphometrical analysis add new, quantitative information to structural observations. First, significant changes in hepatocyte cell size variables (total cell, cytoplasm and nucleus area) were found along the hibernation cycle. This change probably reflects the drastic decrease in metabolic activities in the hepatocyte also associated with reduction and/or packaging of cytoplasmic organelles, as suggested by the disappearance of the Golgi apparatus. A reduction in the
volume of hyaloplasm may also take place. The preservation of the hydro-electrolytic balance represents in fact an essen-
tial condition to ensure the survival of animals that neither
eat nor drink for long periods of time and fluids may be
particularly needed in some compartments of the organism
during hibernation. During arousal, the rapid resumption of
metabolic activities would lead to the restoration of normal
size. Similarly, pancreatic acinar cells of the same animals
decrease in size in deep hibernation and return to their euther-
mic size upon arousal (Malatesta et al., 1998, 2001b). With
regard to nuclear size modifications, it is known that larger
nuclei correspond to higher cellular activities and vice versa
(Hildebrand, 1980). Moreover, the brown adipocyte, a cell
type ready to start up thermogenesis in the very early phase
of the arousal process, shows larger nuclei in hibernation
than in euthermia (Zancanaro et al., 1993a). The fluctuations
of the N/C ratio indicate that the cytoplasmic areas change
more extensively than the nuclear ones, possibly because of
the marked quantitative variations of cytoplasmic inclusions
during the euthermia-hibernation-arousal cycle. Indeed, the
amount of lipid and glycogen in the hepatocyte significantly
changed along the hibernation cycle in both absolute and
percent value; specifically, lipid increased from euthermia to
early hibernation and decreased (together with glycogen
stores) during deep hibernation; both variables further de-
creased to a negligible amount upon arousal. An explanation
for such changes may be that, in hibernating hazel dormice,
lipid represents the main energy source during lethargy simi-
lar to most hibernators (see reviews in Hoffman, 1964; Lyman
et al., 1982; Wang, 1987; French, 1988); this explanation is
supported by the large increase in body weight of the hazel
dormouse before entering hibernation and by the absence of
the food in the nest during winter (Vogel & Frey, 1995; Vogel,
1997). It is, therefore, expected that hepatocytes accumulate
lipid droplets before entering hibernation and that this stock
progressively decreases during winter. Accordingly, the close
association of hepatocyte mitochondria with lipid droplets
during hibernation probably facilitates the transport of
triglycerides to the sites of utilisation. However, some organs,
such as brain, need carbohydrates for their metabolic func-
tions (see Musacchia, 1984). In this view, hepatic glycogen
could represent a glucose reserve, although a further carbo-
hydrate source during hibernation is provided by gluconeo-
genesis from lipids and proteins (Burlington & Kälin, 1967;
Whitten & Kälin, 1968; Riedels & Steffen, 1980). Quantifi-
tive data about hepatic glycogen in hibernating animals are
quite contradictory: in some species the amount of glycogen
does not change during lethargy, whereas in other species it
significantly decreases (see review in Musacchia, 1984). Our
results indicate that the hazel dormouse belongs to the latter
group. However, since the glycogen does not accumulate be-
fore entering hibernation, it may be inferred that this metabo-
lite represents a minor energy source for this species. In some
hibernators the hepatic glycogen content falls upon arousal
(Saarikoski & Suomalainen, 1971), when carbohydrates be-
come an important energy source (Burlington & Kälin, 1967).
It could be, therefore, hypothesised that in hazel dormice
glycogen would be mainly needed for the arousals that peri-
odically interrupt hibernation (French, 1985; Vogel, 1997).
Finally, we showed significant changes in the amount of
hepatocyte residual bodies along the hibernation cycle. In
particular, the absolute and percent cell area occupied by
residual bodies significantly decreased during hibernation in
comparison with euthermia, and significantly increased to
highest value upon arousal. The decrease of residual bodies
during hibernation could be related to the general, regulated
shutdown of cellular functions during lethargy, which drasti-
cally reduces lysosomal activity also; this is probably not the
case in cultured quiescent cells, where it has been reported
that residual bodies accumulate (Roeder et al., 1989). The
striking increase of residual bodies upon arousal could be re-
lated to the extreme metabolic effort made by hepatocytes
during this brief but critical phase when euthermic activities
are rapidly resumed. Moreover, it should be noted that our
animals were sacrificed before completing the arousal pro-
cess, which could finally lead to extrusion of ‘waste’ material
as soon as the euthermic metabolic functions are restored in
full. Accordingly, numerous residual bodies have been found
in hepatocytes of hibernating Chiroptera just after arousal
(Romita & Gatti, 1980).

In conclusion, the observational and quantitative data pre-
sented here demonstrate that the hibernating condition is as-
associated with substantial adaptive structural modifications in
the hepatocyte of M. avellanarius.

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