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CELL AND TISSUE STRUCTURAL MODIFICATIONS IN HIBERNATING DORMICE

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ABSTRACT - Tissues and cells of hibernating mammals undergo striking seasonal modifications of their activity through a quiescence-reactivation cycle. During winter, the temperature drastically decreases, the cell timing greatly slows down, the mitotic index sharply falls, DNA, RNA and protein synthesis are drastically reduced; however, upon arousal, all metabolic and physiological activities are quickly restored at the euthermic levels. The physiological, biochemical and behavioural aspects of hibernation have been extensively studied, but data on the morpho-functional relationships of cell and tissue components during the euthermia-hibernation-arousal cycle are rare. In this review, an overview of cell and tissue structural modifications so far reported in hibernating dormice is given and the possible role in the adaptation to the hypometabolic state as well as in the rapid resumption of activities upon arousal is discussed.

Key words: cell components, dormice, microscopy, hibernation, tissues

RIASSUNTO - ***Modificazioni strutturali di cellule e tessuti in Gliridi ibernanti***. I tessuti e le cellule dei mammiferi ibernanti subiscono profonde modificazioni stagionali della loro attività attraverso un ciclo di quiescenza-riattivazione. Durante l'inverno, la temperatura corporea si abbassa a valori vicini a quelli ambientali, il ciclo cellulare rallenta, l'indice mitotico si riduce notevolmente e la sintesi di DNA, RNA e proteine è drasticamente ridotta. Tuttavia, al risveglio, tutte le attività metaboliche e fisiologiche sono rapidamente ristabilite ai livelli eutermici. Mentre gli aspetti fisiologici, biochimici e comportamentali dell'ibernazione sono stati ampiamenti studiati, i dati sulle relazioni morfo-funzionali dei componenti cellulari e tessutali durante il ciclo eutermia-ibernazione-risveglio sono piuttosto rari. In questo articolo vengono riassunte le attuali conoscenze sulle modificazioni strutturali di cellule e tessuti nei Gliridi ibernanti e viene discusso il loro possibile ruolo nell'adattamento allo stato ipometabolico e nel rapido ripristino delle attività al risveglio.

Parole chiave: componenti cellulari, Gliridi, microscopia, ibernazione, tessuti

1. HIBERNATION AND HIBERNATORS

Mammals have evolved the ability to maintain a high and constant body temperature over a wide range of ambient temperatures using endogenous heat production. However, for many mammals the loss of heat in winter together with the scarcity of food exceed energy supply, so, to survive, these animals have developed a capacity for adaptive hypothermia in which they lower their body temperature to a new regulatory set-point, usually a few degrees above the ambient. This process, generally known as hibernation, may last from a few hours to some weeks and implies a drastic reduction of body temperature, metabolic activity, heart rate, and energy demand as well, thus facilitating survival (Nelson, 1980; Hoffman, 1964; Lyman *et al*., 1982; Wang, 1987; French, 1988; Storey and Storey, 1990; Geiser and Ruf, 1995). Unlike ectotherms (e.g. frogs and snakes), hibernating mammals are able to leave the depressed metabolic state at any time, using endogenously produced heat to restore normal body temperature. In fact, during arousal from hibernation, the substrates are mobilized for energy production, the cardiovascular system is stimulated for tissue perfusion and non-shivering thermogenesis in brown adipose tissue (BAT) starts, thus restoring normal body temperature and all metabolic and physiological functions in a short time (Haywards *et al*., 1965; Wang and Abbots, 1981; Cannon and Nedergaard, 1985; Horwitz *et al*., 1985; Himms-Hagen, 1986).

Members of at least six mammalian orders are capable of entering hibernation. Some examples are echidna of *Monotremata*, many dasyurids of *Marsupialia*, tenrecs, shrews of the subfamily *Crocidurinae*, hedgehogs of Insectivora, many bats of both suborders *Megachiroptera* and *Microchiroptera*, dwarf and mouse lemurs of *Primates* and the various sciurids, cricetids, heteromyids, murids and zapodids of *Rodentia*. The preponderance of research in hibernation has been carried out using species of the order Rodentia. In the conventional division of rodent in subgroups, the Myomorpha or mouse-like rodents encompass many hibernators such as the hazel dormouse *Muscardinus avellanarius*, the edible dormouse *Glis glis* and the garden dormouse *Eliomys quercinus*. These dormice are common in Europe, they can be easily kept in healthy condition in captivity and hibernate readily. These characteristics make them suitable for hibernation studies, although these species are protected by the law and only a limited number of individuals can be employed upon permission from local authorities.

2. CELL AND TISSUE MODIFICATIONS DURING HIBERNATION

The hibernator tissues and cells undergo striking seasonal modifications of their activity through a quiescence-reactivation cycle. During winter, the temperature drastically decreases, the cell timing greatly slows down, the mitotic index sharply falls, DNA, RNA and protein synthesis are drastically reduced; however, upon arousal, all metabolic and physiological activities are rapidly restored, even if the reacti-

vation time-course can vary in tissues involved in different physiological functions (Kolaeva *et al.*, 1980). This extraordinary capacity of varying the functional activity in hibernators implies a novel regulation of metabolic pathways and molecular and structural adaptation of nuclear and cytoplasmic components to maintain homeostasis (Zancanaro *et al.*, 2000). However, while physiological, biochemical and behavioural aspects of hibernation have been extensively studied, the literature on the morphological features of cell and tissue components during the euthermia-hibernation-arousal cycle is poor. The structural constituents of some key organs have been investigated at light and/or electron microscopy, and morphometrical, cytochemical and immunocytochemical approaches have been frequently coupled to conventional morphological analyses in order to get insight into morpho-functional relationships.

In the following, a summary of the structural modifications described so far on dormouse cells and tissues during the hibernation cycle is reported.

3.THE CELL

3.1 Cell nucleus

Transcriptional activity is severely inhibited by low temperature during hibernation (van Breukelen and Martin, 2002b); however, ultrastructural studies on several tissues (liver, BAT, pancreas, adrenal cortex) of *Muscardinus avellanarius* and *Glis glis* have revealed that the general aspect of cell nuclei does not change significantly between euthermia and hibernation (Zancanaro *et al.*, 1993a; Malatesta *et al.*, 1994a, 1995, 1999). In fact, during hibernation, the cell nuclei contain all the usual structural components: perichromatin fibrils, representing the structural counterpart of pre-mRNA transcription; perichromatin granules, considered storage and/or transport sites of mRNA; interchromatin granules, sites of accumulation and assembly of splicing complexes (review in Fakan, 1994); nucleoli with well recognizable fibrillar centers, dense fibrillar and granular components (Jordan, 1984). However, the cell nuclei of hibernating dormice show several unusual nuclear bodies undetectable during euthermia: a) coiled bodies, constituted by tangled electron-dense threads containing both nucleoplasmic and nucleolar splicing factors (Zancanaro *et al.*, 1993a; Malatesta *et al.*, 1994a, 1999), and occurring also in association with the nucleolus (Malatesta *et al.*, 1994b); b) amorphous bodies, poorly structured electron dense domains containing RNPs and Clock, a transcription factor essential for circadian rhythms (Zancanaro *et al.*, 1993a; Malatesta *et al.*, 1994a, 1999, 2003); c) dense granular bodies, made by strongly electron-dense packed granules and containing several nucleoplasmic RNA processing factors (Tamburini *et al.*, 1996); d) lattice-like bodies, constituted by loosely intertwined fibres and containing large amounts of RNPs (Malatesta *et al.*, 1995); e) bundles of nucleoplasmic fibrils, made of proteinaceous material (Zancanaro *et al.*, 1993a, Malatesta *et al.*, 1994a, 1995, 1999). The distribution of these nuclear bodies is quite heterogeneous: some of

them are common to different tissues (e.g. amorphous bodies occur in liver, pancreas, BAT and adrenal cortex of both dormouse species), others seem to be tissue- and/or species-specific (e.g. lattice-like bodies have been observed only in adrenocortical cells of hazel dormice). In arousing animals only a few, poorly structured coiled bodies have been found (Malatesta *et al.*, 1994a) and *in vitro* studies (Malatesta *et al.*, 2001c) have revealed that, upon arousal, all nuclear bodies rapidly disassemble as fibrous material. On the other hand, a few days after entering hibernation, only rare poorly structured coiled bodies and amorphous bodies occur, demonstrating that these nuclear bodies form during the hibernation bouts (Malatesta *et al.*, 1994a).

In parallel with the change of the nucleoplasmic constituents during hibernation, the nucleolus undergo structural and molecular modifications as well (Malatesta *et al.*, 2000): it becomes irregular in shape, with nucleoplasmic invaginations and dense fibrillar component clumps extending from the surface; in addition, it contains significant amounts of splicing factors usually present in the nucleoplasm only.

Since all the nuclear bodies occurring during hibernation contain molecules involved in mRNA pathways, it is likely that they represent storage/assembly sites of several factors for processing some RNA which could be slowly synthesised during hibernation and rapidly and abundantly released in early arousal, in order to meet the increased metabolic needs of the cell. Similarly, the nucleolar modifications could allow the continuation of important nucleolar functions also in deep hibernation or, alternatively, permit a most efficient reactivation upon arousal.

3.2 Cytoplasmic organelles

The major source of chemical energy in the cell is the mitochondrion, where carbohydrates, fatty acids and amino acids are used to produce ATP. During hibernation, mitochondrial functions are drastically reduced, although not completely arrested. Accordingly, the mitochondria undergo morpho-functional modifications in different tissues of hibernating mammals (Romita and Gatti, 1980; Brustovetsky *et al*., 1989, 1993b; Malatesta *et al.*, 2001a; Hittel and Storey, 2001, 2002; Barger *et al*., 2003; Kabine *et al*., 2003). In particular, in many tissues of hibernating dormice (e.g. liver, pancreas, BAT, adrenal cortex), the mitochondrial size and the inner membrane length significantly increase from euthermia to hibernation. These structural modifications are related to the preferential utilisation of fatty acids instead of carbohydrates as energetic substrate during hibernation (Hoffman, 1964; Lyman *et al.*, 1982; Halestrap and Dunlop, 1986; Halestrap, 1987; Wang, 1987, French, 1988; Loncar *et al*., 1988). In addition, mitochondrial matrix granules, containing inorganic (calcium, phosphorous, sodium, magnesium, chlorine) and organic (lipids, phospholipids, glycoproteins, cytochrome c oxidase) components involved in the regulation of various mitochondrial functions (Bronnikov *et al*., 1990; Brustovetsky *et al*., 1992, 1993a; Jacob *et al.*, 1994), drastically increase in number during

hibernation and decrease upon arousal in many dormouse tissues. This suggests that they represent storage sites of substrates needed for respiratory functions, which would be rapidly utilised upon arousal. Accordingly, such granules are almost absent in BAT mitochondria, where an extensive uncoupling of oxidative phosphorylation occurs with a consequent production of heat instead of ATP (Wang and Abbots, 1981; Cannon and Nedergaard, 1985; Himms-Hagen, 1986).

The protein synthesis rate is drastically reduced during hibernation by both active (e.g. inhibition of the elongation phase) and passive (e.g. low temperature) mechanisms (review in van Breukelen and Martin, 2002a). Accordingly, the proteosynthetic apparatus undergoes important modifications during the euthermia-hibernation-arousal cycle. In many cell types, RER and Golgi complex drastically reduce their size during hibernation and restore their usual (euthermic) features upon arousal (Krupp *et al*., 1977; Frink *et al*., 1977; Malatesta *et al.*, 1998, 2001b, 2002; Popov *et al.*, 1999; Kolomiytseva *et al.*, 2003). This represents a fast and energetically efficient mechanism for responding to drastic changes in metabolic needs, which has been reported also in non-hibernating species under hypometabolic conditions (Jamieson and Palade, 1971; Bendayan *et al.*, 1985).

As reported above, the primary energy source utilized during hibernation is lipid; this is stored in white adipose tissue during summer and early fall. However, lipid is also accumulated in other tissues, probably because mitochondria need an *in situ* fatty acid reserve to maintain their functions. A relevant increase in the lipid amount occurs in the BAT, where it obviously subserves the tissue thermogenic functions (Haywards *et al*., 1965; Wang and Abbots, 1981; Horwitz *et al*., 1985). Lipid is increased in the liver as well; here, abundant lipid droplets accumulate in hepatocytes in the fall, to progressively decrease during hibernation up to arousal (Malatesta *et al.*, 2002). In parallel, an increase in smooth endoplasmic reticulum takes place during hibernation in many tissues (liver, pancreas, adrenal cortex, kidney) (Zancanaro *et al*., 1997, 1999; Malatesta *et al.*, 1998, 2001b, 2002), probably due to the augmented lipid metabolism. However, some tissues, such as the brain, need carbohydrates for their metabolic functions; moreover, carbohydrates are an important energy source upon arousal (Burlington and Klain, 1967; Klain and Whitten, 1968; Tashima *et al*., 1970; Galster and Morrison, 1975; Riedelsen and Steffen, 1980; Gehnrich and Aprille, 1988). Therefore, hepatic glycogen reserves are slightly used during the hibernating bouts and are depleted upon arousal (Malatesta *et al.*, 2002).

4. Specific tissues

4.1 Liver

The liver is involved in several metabolic and physiological functions, being strategically located between the intestinal tract and the general circulation; it receives, metabolises and transforms most of the products of digestion,

degrades and detoxifies toxic compounds, synthesises many protein components of blood plasma and exerts an important control over the general metabolism. The hepatocytes are, therefore, multifunctional cells which are strongly affected by the drastic changes related to the euthermic-hibernation-arousal cycle. According to the extreme reduction in hepatic protein synthesis (Whitten and Klain, 1968), RER and Golgi complex are quite reduced during hibernation, while the smooth endoplasmic reticulum increases and lipid droplets and glycogen accumulate in the cytoplasm (Malatesta *et al.*, 2002). Upon arousal, all cytoplasmic organelles revert to their euthermic features, lipid droplets and glycogen are depleted and numerous residual bodies appear in the cytoplasm. Interestingly, a significant decrease in hepatocyte size (both cytoplasm and nucleus) occurs during hibernation, probably due not only to the reduction/packaging of cytoplasmic organelles, but also to a loss in fluids which could be needed in some compartments of the organism during the hibernating period, when no water nor food intake occurs.

4.2 Exocrine pancreas

The exocrine pancreas is a gland specialised in the synthesis, storage and regulated secretion of at least twenty different proteins that constitute the digestive enzymes of the pancreatic juice. During hibernation, dormice eat and drink only occasionally (Vogel and Frey, 1995), therefore the pancreas enters a resting phase. In fact, similarly to liver of hibernating animals (Malatesta *et al*., 2002) as well as to exocrine pancreas of starving non-hibernating species (Bendayan, 1985), the pancreatic acinar cells of hibernating dormice show a reduction in size and/or a reorganisation of the cytoplasmic organelles; moreover, the acinar lumina are closed (Malatesta *et al.*, 1998, 2001b). Surprisingly, considerable amounts of zymogen granules containing highly concentrated enzymes are kept in the cell, maybe to have pancreatic juice available for the first meal after arousal. In arousing dormice most of cell components restore their euthermic features, although the acinar lumina remain closed, probably because the full activation of the gland takes place only when the animal starts refeeding.

4.3 Adrenal cortex

The adrenal gland is a key organ for hibernation as hibernation does not occur in its absence (Jansky *et al.*, 1981). The adrenal cortex secretes mineralcorticoids and glucocorticoids, therefore playing a primary role in the control of hydro-electrolytic balance (mainly plasma concentration of sodium and potassium) and in the regulation of metabolic processes (mainly protein and lipid catabolism for gluconeogenesis). In the hazel dormouse, cells in the adrenal *zona glomerulosa* are enriched in smooth endoplasmic reticulum during hibernation (Zancanaro *et al*, 1997); moreover, mitochondria are larger, containing abundant cristae of tubular type. On the other hand, the *zona fasciculata* and *zona reticularis* do not show major differences in hibernating and euthermic

dormice. This indicates persistent activity (Nussdorfer, 1980) in the *zona glomerulosa* during lethargy. In fact, increased rates of mineralcorticoids secretion would be consistent with an increased need for electrolyte saving during bouts of hibernation, due to the absence of both feeding and salt intake.

4.4 Thyroid

The thyroid is a central endocrine gland undergoing a circannual functional cycle in hibernating dormice, with a peak at the breeding season and a minimum in fall, at the pre-hibernation time (Jallageas *et al*., 1993). This gland is essential for testosterone cycle, but not for entering hibernation bouts (Jallageas and Assenmacher, 1986; Jallageas *et al*., 1992). According to the thyroid functional cycle, both follicular and parafollicular cells show seasonal morphological variations, especially in relation to their secretory activity (Krupp *et al*., 1977; Azzali *et al*., 1990). In hibernating edible dormice, follicular cells show a disorganisation of RER and Golgi complexes and an accumulation of colloid in contrast to euthermic animals, which are characterised by a well developed proteosynthetic apparatus, numerous apical vesicles and a few colloid droplets. On the other hand, the activity of the parafollicular cells appears more intense during hibernation, due to the presence of many secretory granules containing calcitonin. This parafollicular winter activity could be needed to maintain homeostasis of calcium in the hibernating organism, since this element plays a key role in many cellular functions (Denton and McCormack, 1990), such as mitochondrial respiration and muscle contractility (e.g. Bronnikov *et al*., 1990; Brustovetsky *et al*., 1992, 1993a; Horwitz and Ellory, 1995; Yatani *et al*., 2004).

4.5 Kidney and bladder

The kidney and the bladder are involved in excretion of catabolites and toxic compounds. During hibernation, kidney function is strikingly reduced to cessation of glomerular filtration rate and kidney blood flow is kept to a minimum (Zatzman, 1984). The glomerular blood stasis probably provoke the significant increase in size of the corpuscular areas observed in hibernating garden dormice (Milla, 1985). In the same animals, a decrease in size and number of endothelial pores, together with an increase in podocytic processes, smooth endoplasmic reticulum and lysosomes, indicates a reduction in the permeability of glomerular ultrafilter during hibernation (Soria *et al*., 1985; Soria-Milla and Coca Garcia, 1986). In the kidney cortex of the hibernating hazel dormouse, ultrastructural changes of the renal corpuscle are limited to focal enlargement of glomerular endothelial cells and podocytes, while proximal convoluted tubule cells are well preserved and maintain the usual polarity with a hypertrophic apical endocytic apparatus. Interestingly, no major ultrastructural changes take place in the kidney cortex during arousal from hibernation i.e., a period of rapidly resuming body temperature and renal blood flow. This suggests that hibernating animals are able to maintain the fine

structural organization of key organs even during extreme conditions resembling e.g. severe ischemia. The kidney lymphatic system of hibernating dormice displays ultrastructural differences in the lymphatic endothelium, probably related to the drastic reduction in lymph production (Azzali, 1988).

In the urinary apparatus, the bladder is especially stressed during hibernation, because the animal does not urinate for long periods while urine is still produced at low rate in the kidney (Zatzman, 1984). In hibernating hazel dormice, the bladder is extremely distended and filled with urine (Zancanaro *et al.*, 1993b). Unexpectedly, a larger number of fusiform vesicles (typical membrane structures of the urothelium used to extend the luminal plasma membrane) is present during hibernation in the upper layers of urothelial cells in comparison with the relaxed bladder of euthermic individuals. The opposite has been found considering the amount of multivesicular bodies and lysosomes in these cells, thus suggesting that the production of fusiform vesicles is still on the way during hibernation to cope with progressive extension of the luminal surface whereas the turnover of cellular components is reduced.

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