Neuroprotective effect of melatonin in experimentally induced hypobaric hypoxia

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Abstract
Melatonin (MEL) is an endogenous neurohormone with many biological functions, including a powerful antioxidant effect. The aim of the present study was to determine whether MEL protects the brain tissue from the oxidative stress induced by hypobaric hypoxia (HH) in vivo.

This study was performed on Wistar rats randomly assigned in four groups, according to the pressure conditions and treatment: Group 1: normoxia and placebo; Group 2: HH and placebo; Group 3: normoxia and MEL; and Group 4: HH and MEL. The following aspects were evaluated: cognitive function (space reference and memory), oxidative stress parameters – serum and brain malondialdehyde (MDA) and reduced glutathione (GSH) levels –, and brain tissue macroscopic and microscopic morphological changes. Exposure to oxidative stress results in cognitive dysfunctions and biochemical alterations: significant increase of MDA and reduction of GSH in both serum and brain tissue. The most important morphological changes were observed in Group 2: increased cellularity, loss of pericellular haloes, shrunken neurons with scanty cytoplasm and hyperchromatic, pyknotic or absent nuclei; reactive gliosis, edema and blood-brain barrier alterations could also be observed in some areas. MEL treatment significantly diminished all these effects. Our results suggest that melatonin is a neuroprotective antioxidant both in normoxia and hypobaric hypoxia that can prevent and counteract the deleterious effects of oxidative stress (neuronal death, reactive astrogliosis, memory impairment and cognitive dysfunctions). Dietary supplements containing melatonin might be useful neuroprotective agents for the therapy of hypoxia-induced consequences.

Keywords: melatonin, neuroprotective, oxidative stress, brain tissue.

Introduction
Exposure to high altitudes (i.e., above 1500 m) and low oxygen availability results in hypobaric hypoxia (HH), considered to be a severe adverse environmental condition, causing neurodegeneration and cognitive functions impairment [1].

Millions of people travel every year in such harsh environments, exposing themselves to deleterious effects arising from the oxidative stress i.e., increase in the production of reactive oxygen species (ROS) [2–8].

The important oxidative property of ROS results in an overall damage affecting vital organs [9–12]. Oxidative stress might be associated with complications such as myocardial injury, pulmonary edema, kidney and liver failure and increased mortality [13, 14].

A potential way to combat the effects of oxidative stress caused by hypobaric hypoxia has been reported to be the administration of exogenous antioxidants (polyphenols, vitamin E) demonstrated to protect cellular functions in pharmacological concentrations [15–18].

One of the ubiquitous molecules in the human body, a well known neurohormone with important actions in many directions, is melatonin (MEL). Secreted by the pineal gland, 5-methoxy-N-acetyltryptamine has been regarded as a hormone of darkness, a chronobiotic and chronohypnotic internal sleep facilitator [19–22].

Extensive research has certified the antioxidant action of MEL and its beneficial potential in preserving the nervous tissue from oxidative damage [23–26].

The aim of the present study was to assess the in vivo effect of oxidative stress induced by HH and the neuroprotective role of MEL on the hypoxic brain. We performed biochemical determinations, morphological examinations and behavioral procedures.

Materials and Methods
Reactants
MEL used in this experiment was produced by Cosmopharm/Tishcon Corp (USA). It was administered orally before the exposure to hypoxia, in a single dose: 25 mg/kg body weight and after hypoxia, in a double dose: 50 mg/kg body weight. Lactose was used as a placebo for its inert effects and administered orally at equivalent moments in the same dosage.

Animal groups
The study was performed on 32 male rats (Wistar race, three months old, medium weight 220±10 g), randomly divided into four different experimental equal Groups (1, 2, 3 and 4). The animals were kept in the Physiology Department Animal Facility, in a temperature-controlled room (25°C), on a 10/14 hours light/dark cycle and a standard diet.

All rats were handled in compliance with the Protocol
for Animal Care, elaborated by the Ethics Committee of “Iuliu Hâțieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania, and surgery was performed under sodium pentobarbital anesthesia, all efforts being made to minimize the animals suffering.

Group 1, the control group, received lactose as a placebo and was not exposed to HH, but maintained in normal pressure conditions (719 mmHg). Group 2 received lactose and was exposed to HH. Group 3 received MEL and was maintained in normal pressure conditions. Group 4 received MEL and was exposed to HH.

**Timeline**

The experiment lasted 10 days. From the 1st until the 4th day the rats underwent the water maze test. On the 4th day, 1/2 hour before exposure to hypoxia, MEL/placebo was administered. Then, from the 4th to the 7th day of the experiment, an altitude of 5500 m (375 mmHg) was simulated using the hypobaric chamber where animals in Groups 2 and 4 were kept for 72 hours continuously. On the 7th day, 1/2 hour after exiting the hypobaric chamber, another dose of MEL/placebo was administered. During the last three days, the cognitive functions were tested again. On the 10th day, after the testing, blood samples were taken from the retro-orbital sinus of all rats; then, the rats were sacrificed and the whole brain was removed. Brain from four rats in each group was used for biochemical analysis and from four rats in each group for morphological assessment.

**Behavioral procedures**

Spatial learning and memory were tested using the Morris water navigation task [27–29]. The following two parameters were assessed: (1) the escape latency and (2) the time they spent swimming in quadrant D. Training and memory tests were performed before and after the exposure to HH and MEL administration (from 1st to the 7th day of the experiment, an altitude of 5500 m (375 mmHg) was simulated using the hypobaric chamber where animals in Groups 2 and 4 were kept for 72 hours continuously. On the 7th day, 1/2 hour after exiting the hypobaric chamber, another dose of MEL/placebo was administered. During the last three days, the cognitive functions were tested again. On the 10th day, after the testing, blood samples were taken from the retro-orbital sinus of all rats; then, the rats were sacrificed and the whole brain was removed. Brain from four rats in each group was used for biochemical analysis and from four rats in each group for morphological assessment.

The Morris water maze consisted of a round opaque tank, conventionally divided into four quadrants (A, B, C and D) (Figure 1). A platform was placed in quadrant D, approximately 2 cm below the water level and it was the only region of the maze where the rats could stand without swimming. The container was filled with a solution of water and chalk powder in order to make the medium less transparent preventing the rats from seeing the platform.

![Figure 1 – The Morris water maze and the Weibel graticule.](image)

**Training the animals**

During training, the spatial reference was evaluated, and the escape latency (the time until they got to quadrant D and climbed on the platform) was recorded. A trial began when each rat was released into the maze with the head pointing towards the wall of the tank (to minimize bias) and it was given 60 seconds to find the platform. After climbing on the platform, the rat was allowed to remain there 10 seconds, and then it was removed from the water. Whenever the animal was not able to find the platform in the given time frame, it was gently guided to the platform where it remained for 10 seconds – the same amount of time as the ones which did find the platform on time. For each individual, the time passed until the platform was found, was recorded. Each rat, from each of the four groups, was placed in quadrant A, B and C in this order, in three different trials per day with at least 1/2 hour breaks between them. The platform was maintained in the same location throughout the training period. In each trial, the escape latency (in seconds) was recorded and the mean latency was calculated.

**Memory test**

During the memory test, rats were tested for short-term memory consolidation. This test took place in the 4th and 10th day respectively. It also consisted in three trials wherein the rats were released into the water (in quadrants A, B and C) and allowed to swim for 60 seconds, with the platform removed from its initial position. The time spent in the former platform quadrant (i.e., quadrant D), mainly over the original location of the platform, was measured and the mean was calculated.

**Biochemical determinations: oxidative stress**

We measured the following parameters in brain tissue and serum: malondialdehyde – MDA (as a marker for lipid peroxidation) and reduced glutathione – GSH (component of the endogenous antioxidant system).

Oxidative stress parameters were determined in the Laboratory of Oxidative Stress Study, within the Physiology Department.

After blood centrifugation, the serum was separated. The brain samples were homogenized, using the Polytron device for three minutes, in a phosphate-buffer saline (pH 7.4) added in proportion of 1:4. The resulting suspension was centrifuged for five minutes at 3000×g and 4°C. The level of proteins in the homogenate was determined using the Bradford MM method [30]. Both the serum and the homogenate were maintained at -80°C until laboratory determinations were performed.

**Determination of MDA**

The MDA content of both serum and brain homogenate was measured by assessing the concentration of thiobarbituric acid reactive substances (TBARS). A solution of 2-thiobarbituric acid and K2HPO4, with a pH of 3 was added to the samples and then heated in a water bath for one hour. After cooling, the reacting product was extracted in n-butanol. MDA was determined spectrophotometrically using a synchronous technique, with excitation at 534 nm and emission at 548 nm. MDA values were conveyed in nmol/mL and nmol/mg protein (homogenate).

**Determination of GSH**

The fluorimetric measurement of GSH was based on the reaction between these particular groups (–SH) and...
ortho-phtalaldehyde. The serum and brain homogenate were first treated with trichloroacetic acid (10%) and centrifuged. To the supernatants, we added sodium phosphate buffer, EDTA (pH 8) and a solution of ortho-phtalaldehyde (1 mg/mL in methanol). After 15 minutes, the fluorescence was read with excitation at 350 nm and emission at 420 nm. GSH values were established using a standard curve and were presented in nmol/mL and nmol/mg protein respectively.

**Morphological assessments**

The histopathological examinations were performed in the Histology Department Laboratory.

The brains were examined macroscopically; hippocampus together with the suprarectal cortex samples were prelevated and microscopically analyzed. Prior to microscopic examination, brain tissue samples were immersed into 10% formaldehyde solution for fixation, and then embedded into paraffin. For Hematoxylin–Eosin (HE) staining and immunohistochemistry, consecutive sections of 4 μm in thickness were prepared according to paraffin-sectioning technique. The specimens were examined using a Nikon YS2-H light microscope at 20× and 40× magnification.

From the macroscopic point of view, the following qualitative aspects were studied: brain volume, color, consistence and the general appearance of cerebral convolutions.

Microscopically, we examined the hippocampus area and the cortex above it, evaluating in each hemisphere the cellular damage. We were interested to assess the cellular morphology (nuclei and cytoplasm) and the aspect of the extracellular matrix (fibrillarity, edema, angiogenesis).

Quantitative measurements (cytomorphometry) implied the determination of cell density and nuclear dimensions. Fifty high-quality images of the most representative fields were taken for each group using a camera connected both to the computer and to the microscope with a magnification value of 20×. Each image was divided in nine quadrants. Cell density was assessed superimposing electronically the counting grid Weibel type 2 (Figure 1) on each quadrant and counting together the profiles of both intact-appearing and degenerating cells which intersected the short lines. Nuclear diameter was calculated by specialized software (Formdet 2001) and presented to the user in the form of a table of values (nine values per image), on which statistical analysis was performed.

Immunohistochemistry (IHC) assessed the different neuronal and glial cells reaction when exposed to oxidative stress. We used 2F11 DAKO clone for neurofilaments (NF) and GF2 DAKO clone for glial fibrillary acidic protein (GFAP).

**Statistical analysis**

The data was analyzed using Analysis Toolpack: Fisher test ($F$-test: two samples for variances), Student $t$-test ($t$-test: paired two samples for means and $t$-test: two samples assuming equal/unequal variances) and Z-test. We considered statistically relevant the data having $p<0.05$.

**Results**

**Assessment of cognitive functions**

**Escape latency (spatial reference)**

At baseline, there was no significant difference among the four groups. After exposure to HH, Group 2 had an increased escape latency ($p=0.0063$). There was no significant difference before and after the treatment in Group 4 ($p=0.4433$). In Group 3, there was a significant decrease in escape latency ($p=0.0009$) after the treatment. After treatment, the escape latency was significantly lower in Group 4 compared to Group 2 ($p=0.0038$).

**Time spent in quadrant D (short-term memory)**

There was no significant difference regarding the values for this parameter in the four groups before any treatment. After exposure to HH, there was a significant decrease in time spent in quadrant D in Group 2 ($p=0.0178$). There was a significant increase in the time spent in quadrant D in Group 3 ($p=0.0282$) and no difference in Group 4 ($p=0.3321$). After the treatment, the time spent in the former platform quadrant was significantly longer in Group 4 compared to Group 2 ($p=0.0368$) (Figure 2).

![Figure 2](image-url) – Results of the Morris water maze testing: (A) Escape latency – the time until the animals reached quadrant D. In blue – results before the treatment, and in orange – after the treatment; (B) Time spent in former platform quadrant.
Oxidative stress

Brain MDA levels were significantly higher in Group 2 compared to the control group \( (p=0.0032) \) and Group 4 \( (p=0.0003) \). The Group 3, that received only melatonin, had lower levels of MDA compared to the control group \( (p=0.0083) \), but similar to the ones in Group 4 \( (p=0.1187) \) (Figure 3A).

Serum MDA levels were significantly higher in Group 2 compared to the control group \( (p<0.0001) \). In Group 4, serum MDA levels were significantly lower compared to Group 2 \( (p=0.0001) \), and similar to the ones of the control group \( (p=0.0986) \) There was also an increase in serum MDA levels in Group 3 compared to the control group \( (p=0.0059) \). There is no significant difference between Groups 4 and 3 \( (p=0.1605) \) (Table 1).

Concerning the brain GSH levels, in Group 2, these were significantly lower compared to the control group \( (p=0.0002) \) and to Group 4 \( (p=0.0007) \). There was no statistically significant difference between Group 4 and the control group \( (p=0.0504) \). The 3rd group had higher values than both the control group \( (p=0.0158) \) and Group 4 \( (p=0.0001) \) (Figure 3B).

In Group 2, serum GSH levels were significantly reduced compared to the control group \( (p=0.0094) \). In Group 4, serum GSH levels were significantly higher compared to the control group \( (p=0.0012) \), to Group 2 \( (p=0.0007) \) and Group 3 \( (p=0.0007) \). There were no statistical differences between Group 3 and the control group \( (p=0.0973) \) (Table 1).

Moreover, the GSH level in brain tissue was significantly lower compared to the serum.

Morphological assessment

Macroskopically, there were no noticeable differences between the Groups 1, 3 and 4. Group 2, exposed to HH, presented mild modifications regarding the consistency (which was softer) and an enlargement of the gap between the cerebral convolutions.

Microscopically, the following aspects were recorded:

Cellular morphology

In Groups 1 and 3 (normoxia groups), pyramidal neurons kept their large, vesicular nuclei with round and clear nucleoli; the architecture was maintained, the nervous cells being regularly disposed in three to four layers (Figure 4).

In contrast, widespread neuronal damage was observed in the HH Group 2; the neurons were shrunk, with an eosinophilic cytoplasm; the nuclei were smaller, hyperchromatic or weakly stained and in some cases, pyknotic or even absent. The pericellular haloes were generally diminished. The cell architecture was destroyed, the nervous cells being loosely distributed (Figure 5A).

In Group 4, MEL dramatically reduced the effect of insult, so that little differences were seen between Groups 1, 3 and 4.

![Figure 3](image-url) – Biochemical determinations in brain: (A) MDA; (B) GSH.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum MDA [nmol/mL]</th>
<th>Serum GSH [nmol/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control)</td>
<td>0.829</td>
<td>18.857</td>
</tr>
<tr>
<td>Group 2 (HH)</td>
<td>3.003*</td>
<td>8.519*</td>
</tr>
<tr>
<td>Group 3 (MEL)</td>
<td>1.308*</td>
<td>12.976</td>
</tr>
<tr>
<td>Group 4 (HH+MEL)</td>
<td>1.117**</td>
<td>51.287**</td>
</tr>
</tbody>
</table>

*Significant compared to Group 1. **Significant compared to Group 2.

![Figure 4](image-url) – Group 1, normal aspect of the hippocampus and suprajacent brain (HE staining, ob. 20×).
**Aspect of neuropil**

In HE staining, the neuropil from the Group 2 presented areas with a more dense texture than the rest of the groups, intermingled with areas with a loose texture.

**Presence of cerebral edema**

In Group 2, on the histological sections there were fields with cerebral edema represented by the loose texture areas and expanded pericellular haloes (Figure 5B). Groups 3 and 4 did not exhibit signs of edema.

**Aspect of the capillaries**

In Group 2, alteration of the circulation could be identified, consisting in the enlargement of the capillaries. Furthermore, the number of capillaries per microscopic field was increased compared to the other groups (Figure 5C).

![Image](A) ![Image](B) ![Image](C)

**Figure 5** – Group 2: (A) Shrunken neurons; (B) Cerebral edema; (C) Increased number of capillaries (HE staining, ob. 20×).

**Cellular density and the nuclear diameter**

There were no significant differences between Groups 3 and 1, 3 and 4, and 4 and 1.

In Group 2, exposed to hypoxia, the apparent cellular density growth was obvious, the number of cell profiles intersected on the Weibel graticule being greatly increased, compared to Groups 1 ($p<0.0001$) and 4 ($p=0.0001$).

The nuclear diameter was significantly decreased ($p<0.0001$) in Group 2. Degenerating nervous cells under HH-induced oxidative stress were confirmed due to their pyknotic nucleus (Table 2).

**Table 2 – Cytomorphometric results**

<table>
<thead>
<tr>
<th>Diameter of nuclei [μm]</th>
<th>No. of nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control)</td>
<td>7.002 5.389</td>
</tr>
<tr>
<td>Group 2 (HH)</td>
<td>4.244* 7.505*</td>
</tr>
<tr>
<td>Group 3 (MEL)</td>
<td>6.82 5.031</td>
</tr>
<tr>
<td>Group 4 (HH+MEL)</td>
<td>7.412** 5.592**</td>
</tr>
</tbody>
</table>

*Significant compared to Group 1. **Significant compared to Group 2.

IHC enabled us to accurately label neurons versus astrocytes and observe their changes under the oxidative stress caused by hypoxia (amount of NF in the neurons, level of GFAP expressed in astrocytes).

IHC revealed, in Group 2, a pronounced fibrillar aspect and an increased affinity of the neuropil for the dye, compared to Groups 1 and 4. The number of the NF found in the neuronal processes was significantly increased (Figure 6).

Astrocytes exhibited hypertrophy and an increase in the GFAP expression, compared to Groups 1 and 4 (Figures 7 and 8).

In Groups 3 and 4, the distribution of the NF and the expression of the GFAP were similar to the control group.

**Discussion**

At high altitudes (over 1500 m) there is a low atmospheric pressure and a low partial pressure of oxygen which leads to hypoxia. Normally, at sea-level oxygen partial pressure, there is a balance between the oxidant and the endogenous antioxidant systems. During reoxygenation after exposure to hypoxia, the organism’s enzymatic systems are overwhelmed, the balance is being disrupted and free radicals are generated, this phenomena defining oxidative stress. Free radicals are molecules that have an odd electron (usually organic molecules have an even number of electrons), thus a high chemical reactivity, gaining the name of ROS.

HH is an appropriate model to investigate the pathophysiology of people traveling at high altitudes and the possible ways to counteract these changes; therefore, the present study aimed to analyze the effects of oxidative stress induced in rats exposed to HH and the protective role of MEL.

In early 90s, numerous researchers, among whom West JB (1990), Regard M et al. (1991), and Kramer AF et al. (1993) reported that exposure to HH at high altitudes results in cognitive dysfunctions in climbers and mountaineers [31–33]. Their observations were confirmed by more recent studies on aircraft travelers and pilots [3, 7, 34]. This was the cornerstone for numerous studies performed on humans and animals investigating the changes induced by HH, their patho-
genetic mechanisms and the possibilities to prevent and minimize these deleterious effects.

Central nervous system is especially vulnerable to ROS damage because of brain’s high oxygen consumption, its abundant lipid content, and the relative paucity of antioxidant enzymes comparing to other tissues [23]. Moreover, brain has a high ratio of membrane surface to cytoplasm and extended axonal morphology prone to injury.

The reaction of membrane lipoproteins and polyunsaturated fatty acids with ROS produces many oxygenated compounds, mainly aldehydes, such as malondialdehyde (MDA). These resulting products react with the thiobarbituric acid, thus being called “thiobarbituric acid reactive substances” or TBARS. Measurement of the TBARS concentration (expressed by MDA level) has been proved a good assessment of the lipid peroxidation. This method is used by several authors [Park J et al. (2007), Farias JG et al. (2012) and many others] for more than ten years [13, 35].

![Figure 6 – IHC NF (ob. 20×): (A) Group 2; (B) Group 4.](image)

![Figure 7 – IHC GFAP: (A) Group 1 (ob. 20×); (B) Group 1 (ob. 40×); (C) Group 2 (ob. 40×).](image)

![Figure 8 – IHC GFAP (ob. 20×): (A) Astrocytic chart in Group 4; (B) Reactive gliosis in Group 2.](image)

Our results indicated that exposure to HH led to an increase in lipid peroxidation (Group 2 compared to the control group) in both brain tissue ($p<0.05$) and serum ($p<0.05$). The same results (higher MDA levels in serum and brain) were obtained by Maiti P et al. (2006), Baitharu I et al. (2012), and Sharma NK et al. (2011), who used experimental rat models to demonstrate that chronic exposure to HH induced an increase in ROS production and lipid peroxidation [36–38].

As we stated before, the antioxidant systems of the brain are highly different than the ones found in the rest of the body. Our determinations showed that the level of endogenous antioxidants in the brain homogenate were significantly lower than the ones is serum. This was also stated by Park J et al. (2007) and Maiti P et al. (2006) [35, 36].

Reduced glutathione (GSH) is one of the most important antioxidants in animal cells. Most of the cellular GSH is present in the cytosol, but it is also present in the mitochondria, nucleus and peroxisomes. GSH is able to donate an electron to reactive species, rendering them neutral. Two molecules of GSH that have lost electrons combine to GSSG. Consequently, lower amounts of GSH mean increased ROS i.e., oxidative stress.

In our study, the oxidative stress induced by 72 hours exposure to an equivalent altitude of 5500 m decreased the levels of GSH in both brain and serum ($p<0.05$). The same results were obtained by Maiti P et al. (2006), who also used a 72 hours exposure to HH and noticed low levels of GSH, glutathione peroxidase, glutathione reductase, superoxide dismutase [36]. Das SK et al. (2007) reported a decrease in levels of brain GSH after four weeks of oxidative stress due to alcohol consumption [39]. Magalhães J et al. (2005) showed a decrease of GSH in mouse skeletal muscle after 48 hours of HH at an equivalent altitude of 8500 m [40].

We chose to perform the histopathological examination on hippocampus and suprajacent cortex, knowing that these brain areas are the site of cognitive processes (mainly special reference and memory) and they are also extremely vulnerable to insults associated with lower oxygen supply [1, 41, 42].
Our study revealed important morphological changes in Group 2 (the one exposed to HH), this being consistent with the alterations of the biochemical markers.

As cellular morphology demonstrated, in Group 2 features of acute degeneration (loss of pericellular haloes, condensation of the cell body, fragmentation of the process arborisation, eosinophilic cytoplasm, pyknotic or absent nuclei) indicated that those neurons were extensively deteriorated or dying. These results were consistent with the previous studies on the HH injury in brain of Maiti P et al. (2008), Li MM et al. (2011) [41, 43], shrunken eosinophilic neurons representing the hallmark of clinical hypoxic-ischemic encephalopathy [44].

Furthermore, in Group 2 there were fields with cerebral edema. Signs of an impairment in the blood-brain barrier (BBB) (enlargement of the capillaries) and of a mild angiogenesis, represented by an increase capillary density, were also present. These results coincide with those of the previous studies on the HH injury in brain of Maiti P et al. (2008), Li MM et al. (2011) [41, 43], shrunken eosinophilic neurons representing the hallmark of clinical hypoxic-ischemic encephalopathy [44].

Little is known about the impact of oxidative stress on NF expression [47, 48]. Our results indicated an intense IHC reactivity, probably attributed to the aberrant NF accumulations that might result in neuronal and axonal damage. Numerous studies demonstrated that in patients with amyotrophic lateral sclerosis, these accumulations are a hallmark for pathological lesions, but they are also present in other neurological diseases, such as diabetic neuropathy, Alzheimer’s disease and Parkinson disease [49–51]. Since the neurons dye in prolonged hypoxia, their number is depleted. Therefore, the high cellular density found in Group 2, was possibly due to the numeric augmentation of glial cells. GFAP is the most commonly used marker to examine the distribution of astrocytes and their hypertrophy in response to neural degeneration or injury [52]. The cellular density, the hypertrophy of astrocytes cellular body, the proliferation and development of their prolongations (otherwise responsible for the fibrillar aspect of neuropil) and the overexpression of the GFAP are signs of reactive astrogliosis. Recent evidence support that this phenomenon is related with elevated oxidative stress [26, 53, 54]. Up to a point, reactive astrocytes can protect the neurons against oxidative stress, but in prolonged hypoxia, they are surpassed [52]. Consequently, these deleterious effects lead to memory impairment and cognitive dysfunctions [1, 13, 33].

The behavioral procedures that we performed in the present study using the Morris water maze task demonstrated that in Group 2 there was a significant increase in escape latency (p<0.05), meaning that animals exposed to HH needed more time to get to the platform. There also was an impairment of the short-term memory after HH exposure, suggested by a significant decrease in time spent in the former platform quadrant by rats in Group 2 (p=0.05). The same results were reported by numerous other researchers: Baitharu I et al. (2012), Jini V et al. (2013), Hralová M et al. (2013), who used HH experimental models on rodents [1, 37, 55].

On the other hand, Zhu XH et al. (2010) demonstrated that intermittent hypoxia (four hours/day for 14 days in rats) had opposite effects, promoting neurogenesis in the hippocampus and improving depression symptoms in adult rats [56]. Costa DC et al. (2013) also exposed rats to HH, three hours/day for six days, and showed that it induced neuroprotection; they measured MDA levels in brain, which indicated that there was no oxidative stress [57].

To minimize the neurological consequences of hypoxic injury, neuroprotective strategies are required. In this context, there is growing interest in the neuroprotective potential of melatonin in hypoxia [1].

There is evidence that the excretion of MEL is naturally increased during ascent to high altitudes which brings the idea that it is actually useful for the organism in the given stress conditions [58]. If MEL is present, there is less damage done to lipids and the levels of GSH may remain constant during increased oxidative stress.

This ability is proved by recent work, demonstrating that MEL is a free radical scavenger, donating electrons to the reactive species (especially the peroxyl and hydroxyl radicals), similar to the action of GSH [22, 23, 53, 59].

Moreover, it has been established that MEL is also an indirect antioxidant since it can stimulate the gene expression and also regulate the activity of enzymes in the antioxidant systems [59, 61].

The particular positive effect that MEL has on brain tissue was explained by the fact that it crosses the BBB very easily, thus gaining access to subcellular compartments of the neurons [19, 20, 62].

We demonstrated that MEL administration during exposure to HH reduced MDA levels (Group 4 compared to Group 2), in brain tissue (p<0.05) and in serum (p<0.05), suggesting that it plays a protective role against lipid peroxidation caused by hypoxic oxidative stress. The same effect of MEL administration was reported by numerous other authors: Carloni S et al. (2008), Hamada F et al. (2010), Watanabe K et al. (2012), and Alonso-Alconada D et al. (2013) [24, 25, 63, 64].

In our study, MEL administered before and after exposure to hypoxia protected the endogenous antioxidant system, proven by high levels of GSH in brain and serum in Group 4 (p<0.05). The same protection was observed by Baydas G et al. (2006) who induced oxidative stress by hyperhomocysteinemia (HhC) in rats. HhC reduced the activity of glutathione peroxidase that significantly increased the ROS damage; the applied MEL treatment balanced this effect [26].

In the present study, in normoxia, MEL raised the GSH level in the brain but not in the serum. In the same conditions, Swiderska-Kolacz G et al. (2006) showed that MEL augments the level of GSH and the activity of glutathione peroxidase in liver and kidney [65].

Additionally, in Group 4, the deterioration of neurons was attenuated; they were less shrunken and showed a
relatively normal appearance and architecture, corresponding to their situation in hippocampus. Also, there were no important changes concerning the cerebral edema, the capillary density or the reaction of glial cells compared to Groups 1 and 3, which is a sign of the protective effect of MEL.

The number of surviving neurons showing a well-preserved architecture in Group 4 was similar to that observed in the control group. The signs of neuronal damage and the amount of NF were significantly reduced in Group 4, comparative with Group 2, the results being concordant with recent evidence [23, 52]. Using different experimental models Mink RB and Johnston JA (2000), Hamada F et al. (2010) and Alonso-Alconada D et al. (2013) proved that MEL had a remarkable capacity to reduce infarct volume or inhibit neuronal cell death in different mammalian species after ischemic stroke [25, 47, 63]. Signs of reactive astrogliosis were absent in Group 4, suggesting that MEL has the ability to reduce it via inhibiting the oxidative stress. Baydas G et al. (2006) was the first to report that administration of MEL significantly reduced the reactive gliosis in both brain and retina [26]. Moreover, no noticeable BBB alteration was observed. The same results were also presented in other studies, demonstrating that melatonin prevents astrocytes reactivation [53, 54] and can also maintain better BBB function [66].

Additionally, the assessment of the two parameters: escape latency and time spent in the target quadrant in Groups 3 and 4 suggested that MEL administration had a beneficial effect on the rodents’ spatial reference and memory.

Rats in Group 4 showed the same significant improvement in spatial reference and short-term memory when compared to Group 2 (p<0.05). These findings demonstrated that MEL administration before and after exposure to HHI prevented and counteracted the alteration of the cognitive functions.

Animals in Group 3 were able to find the platform in a shorter time frame, with a significant decrease in the escape latency (p<0.05) and also spent more time in the former location of the platform. These results indicate that MEL treatment in normal conditions improved animals’ performances.

MEL’s positive impact on rats’ cognitive functions is based on the decrease of the damage of the brain tissue, especially of the hippocampus cells, done by the oxidative stress. These results were consistent with those reported by Wang Z et al. (2013), who demonstrated that MEL had important functional benefits improving learning and memory performance in the Morris water test; the authors also associated these effects with the attenuation of cell death in the hippocampus [67].

In studies published this year, Liu XJ et al. (2013) and Corrales A et al. (2013) demonstrated the neuroprotective role of MEL in Alzheimer’s disease and Down syndrome; the authors suggested that chronic MEL supplementation may be an effective treatment strategy for preventing the progressive cognitive deterioration [68, 69]. Their research project reflected the up to day trend about the important role played by oxidative stress in various neurological disorders, such as epilepsy, Alzheimer’s disease, Parkinson’s disease, stroke, cerebral ischemia, multiple sclerosis, Huntington’s chorea, etc. [70–72].

Furthermore, melatonin’s action should be researched also in other oxidative stress inducing situations like pregnancy, diabetes mellitus, aging or others [73].

Besides melatonin, various other substances and plant extracts are under research for their protective roles against behavioral dysfunctions caused by hypoxia. Barthwal K et al. (2007) reported that supplementation with acetyl-L-carnitine improved working memory deficits, reduced oxidative stress and inhibited hypoxic neuronal apoptosis [74]. Hralová M et al. (2013) and Jain V et al. (2013) used enriched environment and erythropoietin to prevent cognitive dysfunctions in rats exposed to hypoxia [1, 55]. Hota SK et al. (2009) treated rats with Bacopa monniera leaf extract and Baitharu I et al. (2013) used Withania somnifera root extract in order to obtain an amelioration of memory in rats after exposure to hypoxia [75, 76]. Almli LM et al., verified that intracerebroventricular pretreatment with neurotrphin, a specific brain-derived neurotrophic factor (BDNF), resulted in significant protection against both hypoxia/ischemia induced histological injury and spatial memory impairments [77].

In this context, further studies could be conducted to compare melatonin’s protective benefits with the effects of described above antioxidant molecules, trying to understand the exact mechanisms that lead to their properties.

Conclusions

Briefly, our results suggest that melatonin, due to its high efficacy and low toxicity, is a neuroprotective antioxidant both in normoxia and hypobaric hypoxia, preventing and counteracting the deleterious effects of oxidative stress (neuronal death, reactive astrogliosis, memory impairment and cognitive dysfunctions). Dietary supplements containing melatonin might be useful neuroprotective agents for the therapy of hypoxia-induced consequences.

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References


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