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Melatonin ultrastructural localization in mitochondria of human salivary glands

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Abstract

The hormone melatonin was initially believed to be synthesized exclusively by the pineal gland and the enterochromaffin cells, but nowadays its production and distribution were observed in several other tissues and organs. Among others, the ultrastructural localization of melatonin and its receptors has been reported in human salivary glands. In these glands, the fine localization of melatonin in intracellular organelles, above all in mitochondria, remains to be explored comprehensively. Bioptic samples of parotid and submandibular glands were treated to search for melatonin using the immunogold staining method by transmission electron microscopy. Morphometric analysis was applied to micrographs. The results indicated that, both in parotid and submandibular glands mitochondria, a certain melatonin positivity was present. Within glandular cells, melatonin was less retrieved in mitochondria than in secretory granules; however, its presence in this organelle was clearly evident. Inside striated duct cells, melatonin staining in mitochondria was more prominent than in glandular cells. Our data provide an ultrastructural report on the presence of melatonin in mitochondria of human major salivary glands and represent a fundamental prerequisite for a better understanding of the melatonin role in this organelle.

KEYWORDS
immunogold method, melatonin, mitochondria, salivary glands

1 | INTRODUCTION

In the past years, the source of melatonin into the blood flow was exclusively attributed to the pineal gland. By blood circulation, this lipophilic hormone passively diffuses through the parenchyma of many organs and tissues (Reiter et al., 2015). Nevertheless, these assumptions have been later questioned. Melatonin was found to be synthesized in several non-pineal tissues like retina, Harderian glands, gut, peripheral blood mononuclear cells, testis, bone marrow, skin, cumulus–oocyte complex, thymus, and other organs (Acuña-Castroviejo et al., 2014; Hevia et al., 2008; Reiter et al., 2017; Suofu et al., 2017; Tan et al., 2013; Venegas et al., 2012).

Inside the cell, melatonin was recognized in different districts. In fact, it acts on several organelles, but the main target is considered the mitochondrion. Tan et al. (2013) hypothesized that mitochondria and chloroplasts could be the original sites of melatonin synthesis

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and that high mitochondrial melatonin level promotes protection and function of this organelle.

Melatonin could enter the cells through passive diffusion. However, the existence of a transport mechanism that regulates the diffusion and storage of melatonin inside the cells has been supported by several studies (Hevia et al., 2015; Huo et al., 2017; Isola & Lilliu, 2016; Isola et al., 2013, 2016; Venegas et al., 2012).

Melatonin known actions include not only the regulation of circadian metabolic cycles but also the triggering of immune system, as well as antioxidant defenses, homeostasis, and glucose regulation (Claustrat & Leston, 2015). In several tissues, melatonin was reported to improve the efficiency of the mitochondrial electron transport chain, to enhance adenosine triphosphate (ATP) production (Martin et al., 2002) and to reduce reactive oxygen species (ROS) toxicity, being a direct and indirect ROS scavenger (Acuña-Castroviejo et al., 2001; Reiter et al., 2017). Melatonin was observed to increase, in rat, the activity on mitochondrial respiratory chains Complexes I and IV in a time-dependent manner (Martín et al., 2000).

It is particularly interesting that melatonin levels are variously altered in cancer patients. For instance, in lung, prostate, breast, colon, and stomach cancer, serum levels of melatonin are decreased, while in melanoma, multiple myeloma and oral squamous cell carcinoma are increased (Salarić et al., 2021). Indeed, salivary and/or plasma concentration of this hormone has been suggested to represent a clinical biomarker in different tumors (Farahani et al., 2020; Nijakowski et al., 2022), thus implying its clinical importance.

Recent ultrastructural study of human parotid glands localized melatonin and its receptors MT1 and MT2 principally in acinar secretory granules (Isola & Lilliu, 2016; Isola et al., 2013, 2016, 2019; Loy et al., 2015). Similar data were obtained on human submandibular glands, where melatonin reactivity was lower than that obtained in parotid glands (Isola, Lilliu, et al., 2018b). Evidence for the uptake of melatonin and, similarly, for its synthesis in mitochondria may represent an important step forward in the studies on melatonin role in organs and cells. In this view, we investigated, by transmission electron microscopy (TEM), the melatonin localization in mitochondria of human salivary glands.

2 | MATERIALS AND METHODS

Fragments of salivary glands were obtained from 10 male patients, aged around 65 years, undergoing surgery for the removal of tumors in the mouth and in the neck regions at the Otorhinolaryngology Clinic, University of Cagliari. Glandular fragments not compromised by oral pathologies were selected. Gland eligibility was confirmed by light and electron microscopy, based on an accurate macroscopic and histological evaluation. Written informed consent was obtained from each patient. All the procedures were approved by the local Institutional Committee for human experimentation at the ASL 8 (Azienda Sanitaria Locale 8), Cagliari.

2.1 | Transmission electron microscopy

Samples were cut into small pieces and immediately treated for electron microscopy observation. In order to confirm no-pathological morphology by conventional TEM, part of the specimens was fixed in a mixture of glutaraldehyde 1.25% and paraformaldehyde 1% in cacodylate buffer 0.15 M (pH 7.2) for 2 h at room temperature (Casu et al., 2020; Isola et al., 2010; Loy et al., 2014), whereas most of the fragments were fixed for 2 h with a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer to be followed by immunocytochemistry (Isola et al., 2012, 2013; Isola, Lilliu, et al., 2018b). After fixation, fragments were rinsed in cacodylate buffer added with 3.5% sucrose, dehydrated, and embedded in Epon Resin (Glycide Ether 100, Merck). In order to preserve the antigenicity of the tissue, in samples for immunocytochemistry treatment with osmium tetroxide was omitted. Semithin sections (2 μm), from both kind of samples, were stained with toluidine blue and examined by light microscopy to check the histological appearance. Ultrathin sections (80 nm) were collected on nickel grids and processed for immunohistochemical analysis.

2.2 | Immunohistochemical analysis for melatonin localization

Grids were treated with 1% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in phosphate-buffered saline (PBS) solution to block non-specific binding. Then, they were incubated overnight at 4°C with a rabbit polyclonal antibody specific for melatonin (Thermo Fisher Scientific Inc., catalog number PA1-85053) diluted 1:20 in PBS + 1% BSA and 5% NGS. Grids were then incubated for 1 h at room temperature with the secondary antiserum, a goat anti-rabbit IgG conjugated to 15 nm gold particles (GE Healthcare), diluted 1:30 in PBS + 1% BSA. After washing, they were stained with uranyl acetate and bismuth subnitrate and finally observed and photographed with a JEOL JEM 1400 Plus (CeSAR core facilities) and with a JEOL 100S transmission electron microscope. Control sections were incubated with a non-immune serum or without the primary antibody.

2.3 | Morphometric analysis

For semi-quantitative analysis, only serous glandular cells were considered both in parotid and submandibular glands. Fifty-three images of serous cells were randomly collected by TEM. Melatonin positivity was counted in secretory granules and mitochondria of each cell, resulting in a percentage of positive structures out of the total of each category. Labeling density was qualitatively evaluated in each image, giving the following score: +++ high intensity, ++ medium intensity, and + low intensity. Each evaluation was related to a TEM image at magnification of x15000, equivalent to an area of about 11 μm^2. Measurements were performed by two independent observers.
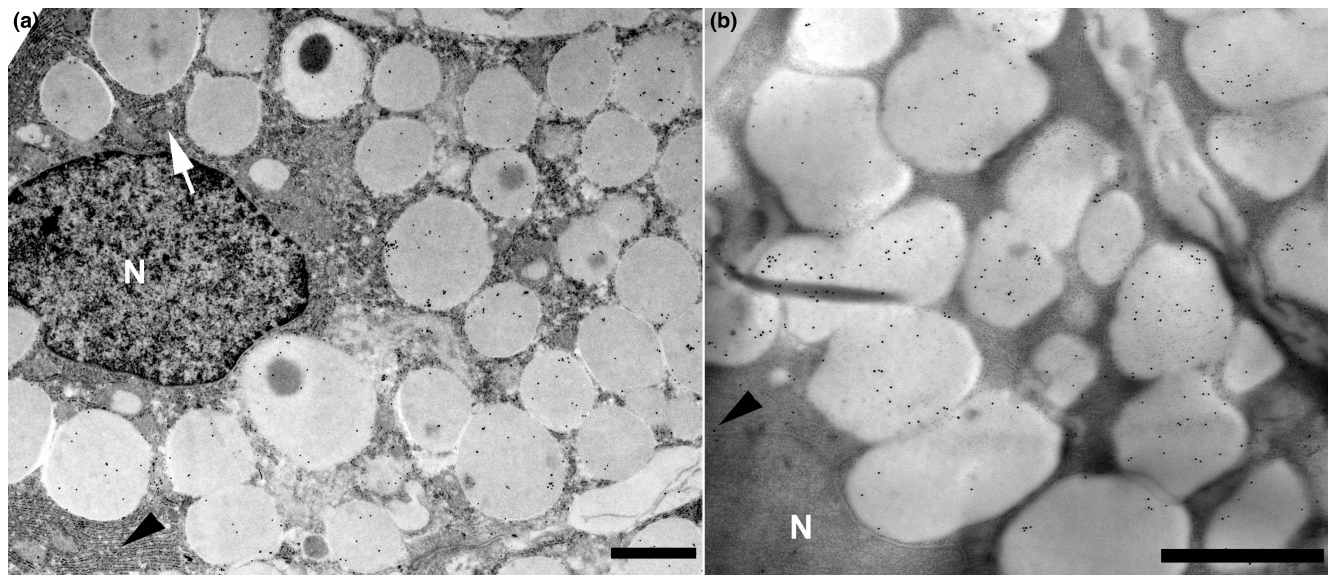
FIGURE 1 Melatonin localization in granules of serous cells in human parotid (a) and submandibular (b) glands. The melatonin localization in mitochondria (arrow) and in rough endoplasmic reticulum (arrowheads) is indicated. N: Nuclei. Bars: 1 μm.

TABLE 1 Percentage of stained granules or mitochondria out of the total of each category of organelles, and related labeling intensity in serous cells of parotid and submandibular glands

	Granules		Mitochondria	
	%	Intensity	%	Intensity
Parotid	88.1	+++	34.1	+
Submandibular	49.5	+/++	29.7	+

Note: Fifty-three images of serous cells were used to perform the semi-quantitative analysis. Labeling score: +, low; ++, medium; +++, high.

3 | RESULTS

By TEM, the immunogold method allowed to detect the presence of melatonin inside several cellular organelles of the two major salivary glands. As previously reported (Isola & Lilliu, 2016; Isola, Lilliu, et al., 2018b), in serous cells of submandibular and parotid glands, most of the intracytoplasmic melatonin staining was localized in secretory granules, principally in their pale peripheral portion but also in the dense core (Figure 1). Sometimes, reactivity was observed in the granule membranes. Melatonin labeling resulted rather intense and frequent in serous cells of parotid, while staining was less prominent and diffused in those of submandibular glands.

A morphometrical semi-quantitative analysis confirmed these data, showing that the labeled granules were more numerous in parotid (88% of the total) than in submandibular (49%) serous cells, in accord to our previous results (Isola & Lilliu, 2016; Isola, Lilliu, et al., 2018b). Moreover, melatonin labeling intensity was higher in parotid than in submandibular glands (Table 1).

Inside serous cells, melatonin reactivity was also observed in rough (Figures 1 and 2), in smooth endoplasmic reticulum, and in small vesicles. Few gold particles were found in nuclei, both in heterochromatin, gathered near the nuclear envelope, and in euchromatin (Figures 1–3).

Within glandular cells, melatonin was less retrieved in mitochondria than in secretory granules; however, its presence in this organelle was clearly evident. In parotid and in submandibular serous cells, morphometrical analysis revealed that about one-third of mitochondria was stained (34% and 30% of the total, in parotid and submandibular gland, respectively). In mitochondria, melatonin density was lower than in granules (Table 1). Due to the immunogold technique that avoids osmium, the inner morphology of mitochondria (outer and inner membrane, cristae) is not clearly discernible by TEM. However, low (but evident) reactivity for melatonin was observed both nearby the outer membrane and inside the mitochondria in acini of both glands (Figure 4); in submandibular secretory cells (Figure 2) mitochondrial positivity appears similar to that observed in parotid (Figure 3). In striated ducts of both major salivary glands, mitochondria showed higher reactivity for melatonin than those of secretory cells (Figure 5); moreover, positivity can be observed both inside mitochondria and at their periphery.

4 | DISCUSSION

In the present paper, based on our preliminary observations (Isola, Isola, et al., 2018a), we showed that melatonin was found in mitochondria of salivary glands. Its presence was rather moderate in mitochondria of secreting cells in human parotid and submandibular glands, whereas melatonin concentration was a bit higher in mitochondria of striated ducts in both. With respect to serous cells of the two major salivary glands, melatonin localization was slightly higher in parotid than in submandibular mitochondria. Similarly, labeling in
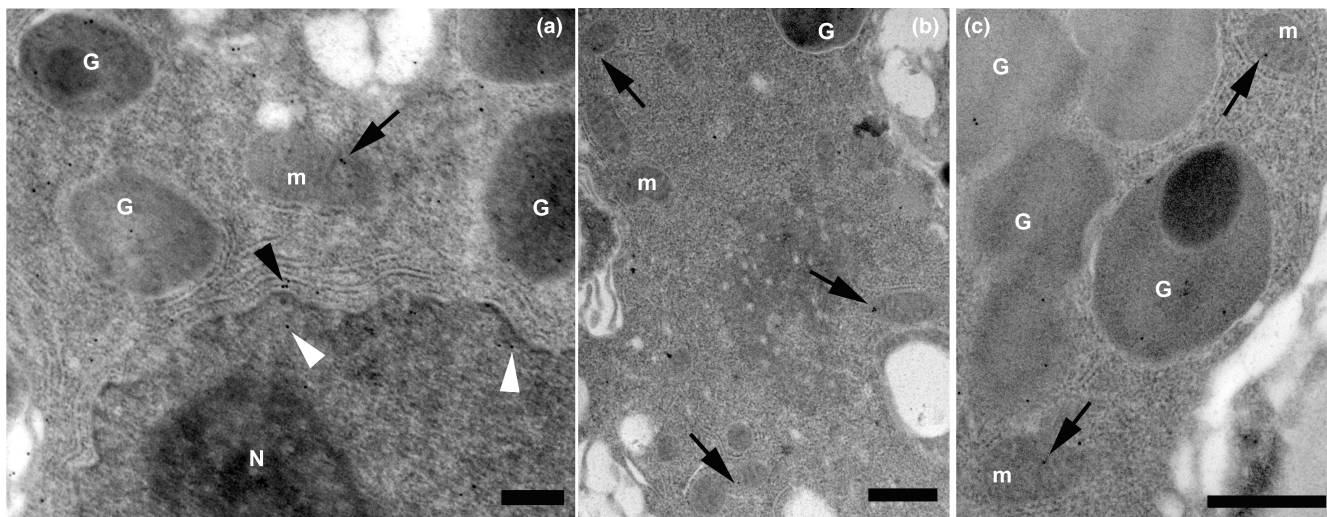
FIGURE 2 Submandibular gland. Serous cells show melatonin localization in the granules (G), in the rough endoplasmic reticulum (black arrowhead) and nuclei (N; white arrowheads). Note that mitochondria (m) show reactivity for the hormone (black arrows). a, b, c: Bars 500 nm.
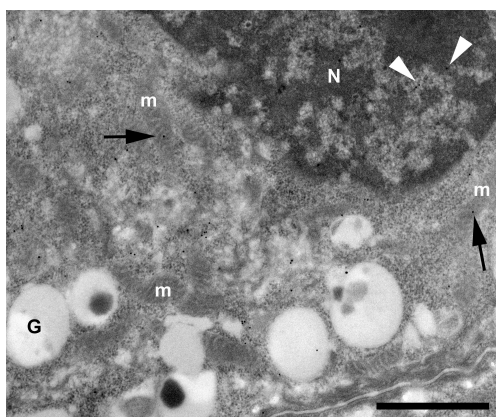

FIGURE 3 Parotid gland. Melatonin reactivity (arrows) in mitochondria (m) of acinar cells and in the nucleus (N; arrowheads). G, granules. Bar: 1 μm.

secretory granules showed more reactivity in parotid than in submandibular ones (Isola & Lilliu, 2016; Isola, Lilliu, et al., 2018b). Based on the retrieval of melatonin, our previous work suggested that the parotid gland is the most involved in the release of this hormone in saliva (Isola & Lilliu, 2016). The present study revealed that the two types of salivary glands have different production/import of melatonin in mitochondria and in secretory granules of serous cells.

It was suggested that melatonin enters cells through the glucose transporters GLUT1 and GLUT4 (Hevia et al., 2015). Moreover, some authors have considered the possibility that either of the human oligopeptide transporters PEPT 1 and 2 could be involved in melatonin transport inside the cells (Huo et al., 2017; Reiter et al., 2017). Melatonin could also be internalized in mitochondria through PEPT 1 and PEPT 2 receptors (Reiter et al., 2017, Reiter, Sharma, Rosales-Corral, et al., 2021b; Mayo et al., 2017), localized on the mitochondrial membranes. Despite the difficulty to visualize the inner

morphology of the mitochondria, we observed melatonin localization in the border of this organelle (related to outer membrane) and in the inner part of it (associated both to the inner membrane and the matrix). The presence of melatonin on the outer (and probably on the inner) membrane could be ascribed to the above-mentioned mechanism of melatonin transport via PEPT receptors. Moreover, as it has been reported that melatonin might interact both with electron chain complexes (located on inner membrane) and with antioxidant enzymes (resident in the matrix) (Reiter et al., 2017), it could be reasonably possible that melatonin could be in both.

In salivary glands mitochondria, melatonin import seems the most feasible hypothesis, but we cannot exclude the possibility that in this site it is also produced. As the enzymes for its production have been localized in ductal cells of salivary glands (Shimozuma et al., 2011), we are going to immunolocalize them in mitochondria of glandular cells.

Regarding melatonin levels in cellular compartments, studies carried out on pinealectomized rat cerebral cortex showed that they were higher in mitochondria, followed by membranes, nuclei, and cytosol (Venegas et al., 2012). In rat brain and liver, mitochondrial melatonin levels are more abundant than those detected in plasma (Martín et al., 2000). Rats treated with agomelatine (an MT1 and MT2 agonist) showed a reduced ROS production in the brain, corroborating the hypothesis that melatonin acts on mitochondria also via the MT1 and MT2 receptors (Chanmanee et al., 2022).

MT1 receptor was retrieved on the outer membrane of brain mitochondria, and it seems that melatonin acts on this receptor protecting cells from apoptosis through inhibition of calcium-induced cytochrome c release (Reiter, Sharma, Rosales-Corral, et al., 2021b; Suofu et al., 2017). We cannot rule out the possibility that, in our observations, mitochondria melatonin was bound also to mitochondrial MT1 receptors. In fact, in human parotid glands, we reported that
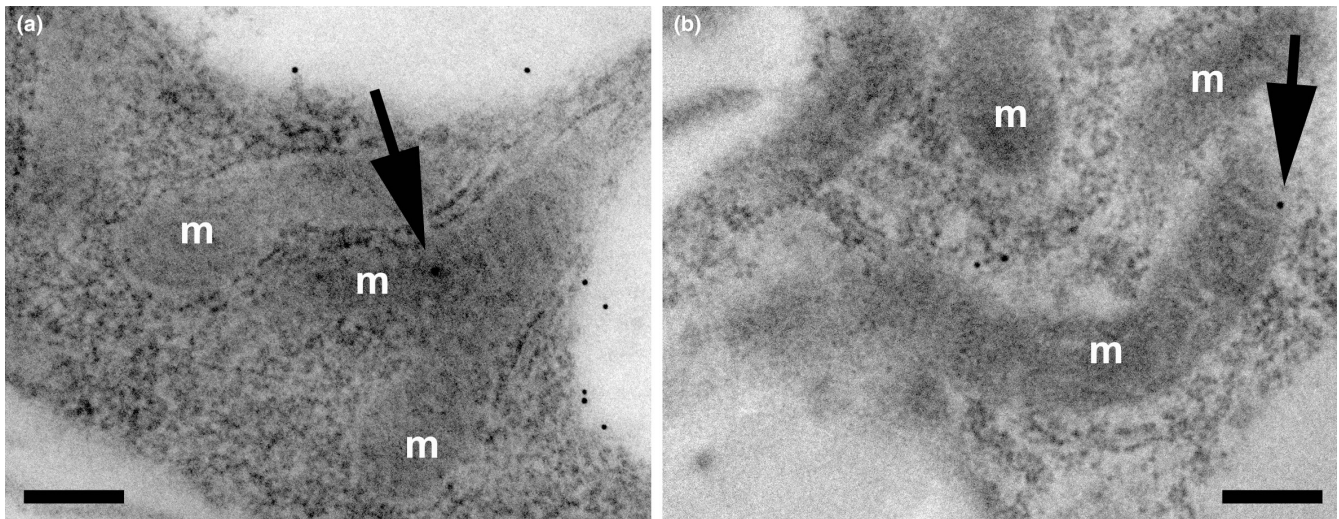
FIGURE 4 TEM. Parotid glands. High magnification of mitochondria (m), labeled for melatonin detection (arrows). These organelles showed reactivity for melatonin in their central part (a) or at their periphery (b). (a, b) Bars 250 nm.
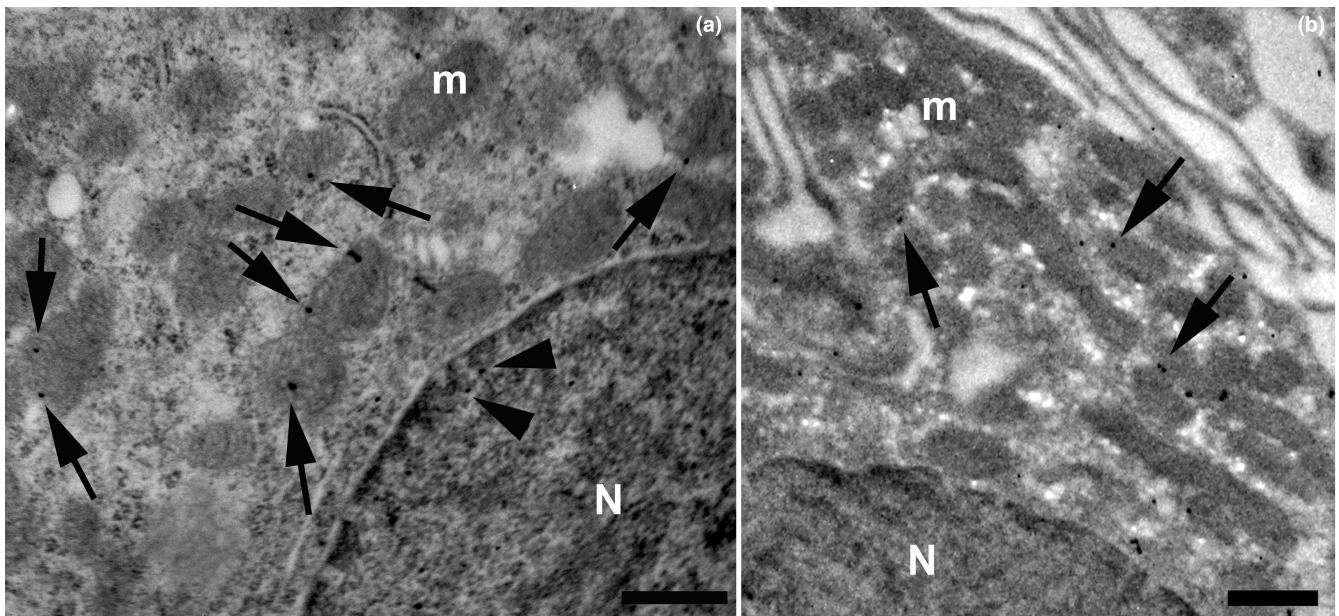

FIGURE 5 Striated ductal cells. (a) Submandibular gland. (b) Parotid gland. Melatonin localization (black arrows) was evident. Arrowheads: Nuclear localization. M: Mitochondria. N: Nuclei. Bars 1 μm.

the MT1 receptors are localized close to and in the mitochondria (Isola et al., 2013).

It is well known that mitochondria have a central role in metabolism to produce ATP via oxidative phosphorylation (OXPHOS) (Martin et al., 2002). Further properties are ascribed to melatonin in mitochondria: preventing ROS production, directly or by increasing superoxide dismutase 2 and sirtuins expression, inhibiting the mitochondrial permeability transition pore, and activating uncoupling proteins (Reiter et al., 2017; Reiter, Sharma, Zuccari, et al., 2021a; Tan et al., 2016). Moreover, mitochondrial biogenesis and dynamics were preserved and regulated by melatonin (Tan et al., 2016).

From this perspective, the role of this hormone in the mitochondrial physiology could be fundamental for clinical application. For instance, PEPT 1 and PEPT 2 were observed in the mitochondrial membrane of human cancer cell lines and the anticancer effects of melatonin were found to be mediated via the mitochondrial apoptotic pathway (Huo et al., 2017). In ischemia–reperfusion injury, melatonin improved mitochondrial biogenesis, fusion, and cell viability among injured HT22 cells (Nasoni et al., 2021). Moreover, melatonin prevents cell hyperpermeability in blood–retinal barrier and apoptosis of retinal pigmented cells (Doğanlar et al., 2021). Mitochondrial melatonin in cancer cells was related to glucose metabolism; indeed, its synthesis is possible only when pyruvate is internalized

into mitochondria and converted to acetyl coenzyme A, as this is necessary for conversion of serotonin to N-acetylserotonin, the precursor of melatonin. In hypoxia or in cancer and other pathologies, pyruvate is converted to lactate and does not enter mitochondria. Thus, melatonin synthesis in mitochondria could be a sign of a good homeostasis of the cell.

In our study, immunohistochemical localization of the hormone was highly observed in striated ducts cells. This type of cells is characterized by numerous mitochondria located among the long foldings of the basal membrane. They provide ATP for active transport/reabsorption of ions modifying saliva composition. A high expression of the melatonin synthesizing enzyme, arylalkylamine N-acetyltransferase, was found in rat salivary striated ducts (Shimozuma et al., 2011), representing the groundwork for our results. It would be worthy to point out that striated ducts possess high intrinsic peroxidase activity, as its inactivation for immunohistochemistry is rather troublesome (unpublished results; Isola et al., 2013). In this view, a higher melatonin content in striated ducts would particularly shelter them from the more probable ROS damage and confirms the antioxidant role of this hormone in salivary glands, too.

The limits of the present study are that immunocytochemical studies give semi-quantitative results; thus, for a more accurate esteem of mitochondrial melatonin and granules' content fractioned centrifugation to isolate them and other kind of assays (e.g., radio or enzyme-linked immunoassay) should be performed.

Due to the above-mentioned limits and the fact that immuncytochemical technique by electron microscopy is rather time-consuming, we hardly foresee that it can be applied to obtain a clinical diagnosis. Furthermore, studies would need to find a correlation between melatonin presence in salivary glands and a possible protection on the development of selected tumors.

5 | CONCLUSIONS

In conclusion, melatonin was found in parotid and submandibular secretory cells. In both, it was more retrieved in granules than in mitochondria. Mitochondrial melatonin intensity was similar in both glandular cells and its physiological significance might be connected to its antioxidant activity or to its action on mitochondrial efficiency. The correlation between melatonin mitochondrial occurrence and salivary glands' protection from tumors still needs to be elucidated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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