Morphometric Study of Diabetes Related Alterations in Human Parotid Gland and Comparison with Submandibular Gland

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ABSTRACT

Type 2 diabetes mellitus represents one of the principal diseases that afflict the world population and is often associated with malfunction of salivary glands and consequent oral diseases. We recently described significant ultrastructural alterations in the human submandibular gland in diabetic patients without evident oral pathologies. Herein, an analogs morphometrical investigation was focused on the parotid gland in order to evaluate if one of the two glands is more affected by diabetes. Parotid fragments from diabetic and nondiabetic patients were fixed, dehydrated, and processed for light and electron microscopy. Serous cells were randomly photographed and the density and size of several structures involved in the secretory process were examined by morphometry. Scanning electron microscopy images revealed significant changes in the number of apically docked granules and vesicles, suggesting that the last steps in exocytosis are somehow altered in diabetic cells. Other variables analyzed by light and transmission electron microscopy such as the size of acini and secretory granules did not show significant changes, but comparison with previous data obtained with submandibular gland cells demonstrated that the two glands are affected differently. Anat Rec, 00:000–000, 2015. © 2015 Wiley Periodicals, Inc.

Key words: salivary glands; diabetes; electron microscopy; morphometry

INTRODUCTION

Type 2 diabetes mellitus is often accompanied by severe malfunction of heart, vessels, kidney, and nerves. Other frequent complications associated with the diabetic condition are dry mouth and periodontal disease, which cause difficulties, even in common activities such as eating and speaking, and worsen the quality of life. They are generally associated with noninflammatory and non-neoplastic disorders of the salivary glands known as sialosis, which implicate hyposalivation, reduced mouth lubrication and increased susceptibility to infections. A large volume of studies, often conflicting, have documented the diabetes-related changes in salivary flow and composition, as well as the histological alterations of salivary glands, while only a few studies have dealt with the ultrastructural

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aspects (Donath and Seifert, 1975; Ben-Aryeh et al., 1993; Dodds et al., 2000; Satoh and Yoshihara, 2004; Carda et al., 2006; Aydin, 2007; Negrato and Tarzia, 2010; Piras et al., 2010; Mascarenhas et al., 2014). In diabetic subjects without sialosis and/or periodontal disease, salivary flow and composition were reported as normal by some authors, altered by others (Dodds and Dodds, 1997; Mata et al., 2004; Barnes et al., 2014), while salivary gland morphology was not investigated since assumed as normal. Signs of altered secretion appeared when we compared statherin expression in salivary glands of diabetics without sialosis to that in healthy subjects (Isola et al., 2011a,b;). Its significant reduction suggested that diabetic status influences protein secretion even when salivary function is apparently normal. These findings led us to examine more carefully the morphology of acinar cells in an attempt to recognize in which cell compartment the secretory process could be disturbed. In our latest paper, ultrastructural alterations chiefly affecting secretory granules (Lilliu et al., 2015) were revealed by morphometry in diabetic submandibular glands. We subsequently carried out an analogous morphometrical examination on diabetic parotid glands, whose results are shown in this paper. Moreover, we compared parotid and submandibular gland data in order to disclose whether one of the two glands was more affected by diabetes. Only a few studies considered parotid and submandibular glands separately in this regard (Dodds et al., 2000; Isola et al., 2012), while most authors investigated salivary glands without distinction. However, it is not at all obvious that they show equal susceptibility to diabetes-related alterations, given that many peculiarities render each gland unique, both morphologically and functionally (Riva et al., 1990; Denny et al., 1997).

MATERIALS AND METHODS

Fragments of parotid glands were obtained from 20 male patients (mean age 60) undergoing surgery for the removal of cancers from the oral region at the Otorhinolaryngology Clinic, University of Cagliari. All samples were examined for verification of normal histology. The patients were not usual smokers or alcohol consumers, nor were they affected by autoimmune diseases or obesity. No evident sign of oral diseases such as xerostomia, infection, periodontitis, or taste dysfunction was observed. 10 patients were affected by medically diagnosed type 2 diabetes mellitus and the blood glucose level was controlled by diet or medications. The patients were not given medications that could have affected salivary secretion. Informed consent was obtained from all patients, and all procedures were approved by the local Institutional Committee for human experimentation at the ASL 8 (Azienda Sanitaria Locale 8), Cagliari.

For light microscopy (LM) and transmission electron microscopy (TEM) the samples were cut into small pieces (around 2 mm^3) and fixed for 2 h at room temperature with a mixture of paraformaldehyde (1%) and glutaraldehyde (1.25%) in 0.1M of cacodylate buffer (pH 7.2). The pieces were then rinsed in cacodylate buffer plus 3.5% sucrose, fixed with OsO_4 (2%), dehydrated, and embedded in Epon resin (Glycide Ether 100; Merck, Darmstadt, Germany). Randomly chosen tissue blocks from each patient were cut into semithin (1 μm) and ultrathin (90–100 nm) sections with a diamond knife. The semithin sections were stained with toluidine blue and examined with a Leica DMR microscope equipped with a CCD camera (Leica DC 300). The ultrathin sections were collected on copper grids, stained with uranyl acetate, and bismuth subnitrate, and examined with a JEOL 100S transmission electron microscope.

For high-resolution scanning electron microscope (HRSEM), the samples were cut into small pieces (around 1 mm^3) and fixed for 15 min with a mixture of paraformaldehyde (0.2%) and glutaraldehyde (0.25%). After postfixation with a mixture of OsO_4 and $K_3[Fe(CN)_6]$, the samples were exposed to our variant of the osmium maceration method in order to visualize the cytoplasmic side of intercellular canaliculi (Riva et al., 2007). The samples were then examined with a Hitachi S4000 FEG HRSEM at 15–20 kV.

Morphometric and Statistical Analysis

The tissue sections were randomly photographed using an unbiased approach. LM, TEM, and HRSEM images (15 for each patient) were subjected to morphometric evaluation. In LM images the area of serous acini and the density of intracellular lipid droplets were measured. In TEM images, the area and the density of secretory granules and the thickness of basal lamina, including both the lamina lucida and the lamina densa, were measured. In HRSEM images, the mean density (number per μm^2) of microvilli, microbuds and protrusions were measured. All measurements were made using the Image Pro Plus software (Media Cybernetics). Data were analyzed by Student's t test and Mann–Whitney test. $P < 0.05$ represents statistically significant differences between diabetic (D) and nondiabetic (ND) subjects.

In order to compare parotid and submandibular gland data, the morphometrical values recently published (Lilliu et al., 2015) were used and added with the basal lamina thickness calculation. The data concerning the two glands were summarized in Table 1.

RESULTS

Light Microscopy

In LM images of human parotid (Fig. 1) no appreciable differences in morphology were observed upon mere observation of diabetic (b) and nondiabetic (a) samples. The serous acini showed in both groups the typical pyramidal cells arranged around a small lumen. Morphometric analysis did not show statistically significant differences regarding the mean area of serous acini, i.e., 388.3 μm^2 (ND) and 369.9 μm^2 (D), respectively. The density of the intracellular lipid droplets was equally unchanged, being 4.8/100 μm^2 in both samples (Fig. 1, graphs c and d).

Transmission Electron Microscopy

In TEM images, the ultrastructural features of acinar cells were apparently similar in nondiabetic (a) and diabetic samples (b) (Fig. 2). These observations were confirmed by morphometrical data, since no significant difference was recorded for the variables measured. The area of secretory granules was 0.573 μm^2 for ND and 0.574 μm^2 for D; their density (number of granules/100 μm^2) was around 58 granules/100 μm^2 for each group (Fig. 2, graphs c and d). The thickness of the basal lamina in parotid acini

TABLE 1. Mean values (±SEM) of the variables analyzed in diabetic and nondiabetic submandibular[a] and parotid glands

		Submandibular		Parotid	
Parameter	Microscope	ND	*D*	ND	*D*
Area of serous acini (μm^2)	LM	397.8 ± 21.73	470.9 ± 29.97[b]	388.3 ± 21.47	369.9 ± 30.30
Number of lipid droplets/100 μm^2	LM	3.698 ± 1.72	4.932 ± 0.94	4.840 ± 0.77	4.831 ± 0.63
Area of secretory granules (μm^2)	TEM	0.343 ± 0.011	0.429 ± 0.015[d]	0.573 ± 0.013	0.574 ± 0.014
Number of secretory granules/100 μm^2	TEM	83 ± 4.9	61 ± 7.4	58.5 ± 8	58.7 ± 9
Thickness of basal lamina (μm)	TEM	0.127 ± 0.021	0.164 ± 0.006	0.073 ± 0.015	0.118 ± 0.019
Number of microvilli/μm^2	HRSEM	29.67 ± 1.499	19.6 ± 1.519[d]	27.05 ± 1.716	20.83 ± 1.368[c]
Number of microbuds/μm^2	HRSEM	2.632 ± 0.273	3.594 ± 0.290[b]	4.402 ± 0.513	7.785 ± 0.937[c]
Number of protrusions/μm^2	HRSEM	0.152 ± 0.036	0.535 ± 0.067[d]	0.191 ± 0.070	0.701 ± 0.104[d]

[a]Data previously described (Lilliu et al., 2015), except for basal lamina.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

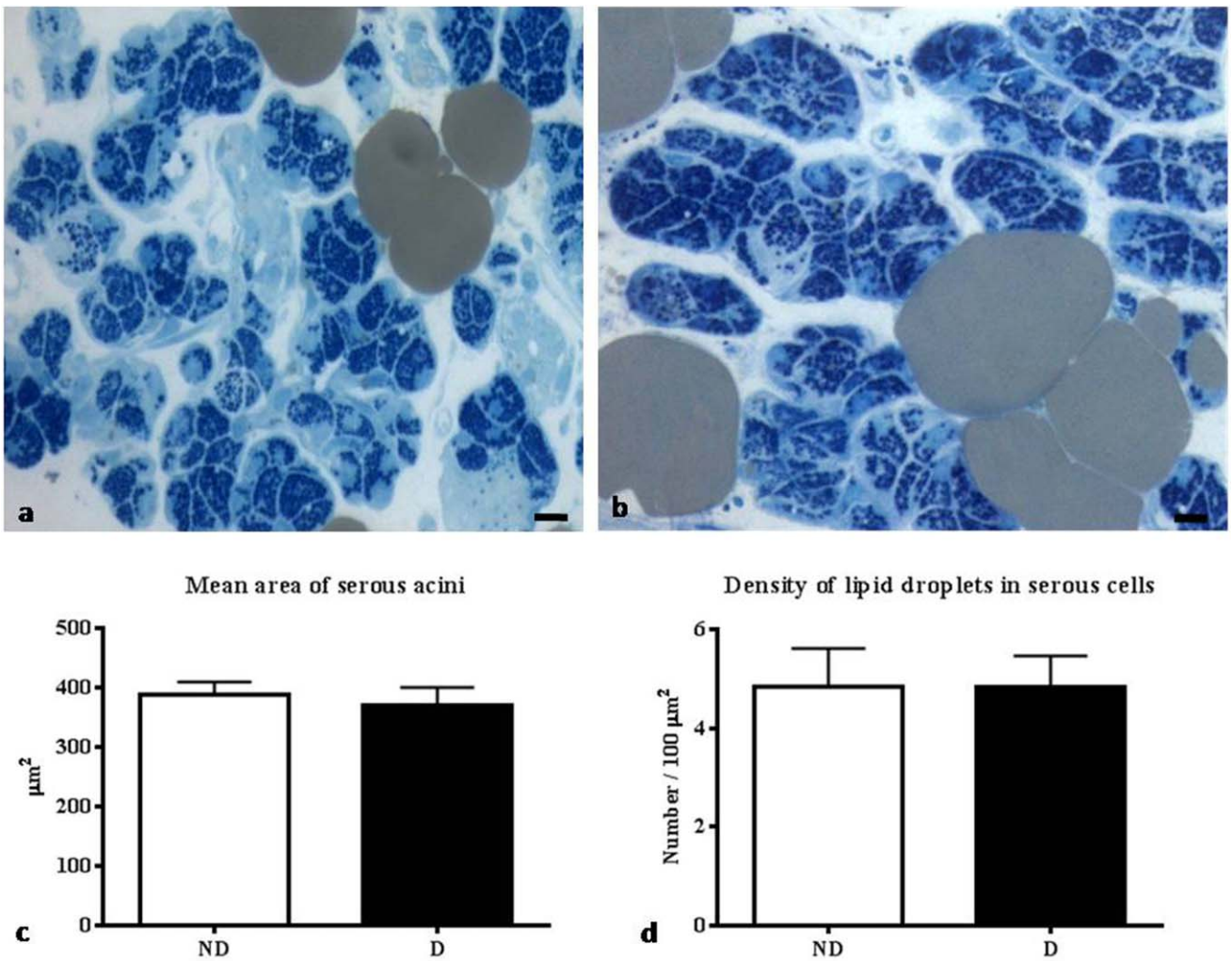

Fig. 1. LM micrographs of parotid glands stained with toluidine blue. Both nondiabetic (a) and diabetic (b) glands show an apparently normal parenchyma. *Bar* 10 μm. Morphometrical evaluation: the mean area of acini (c) and the density (number per 100 μm^2) of intracellular lipid droplets (d) did not show significant differences between diabetic and nondiabetic samples.

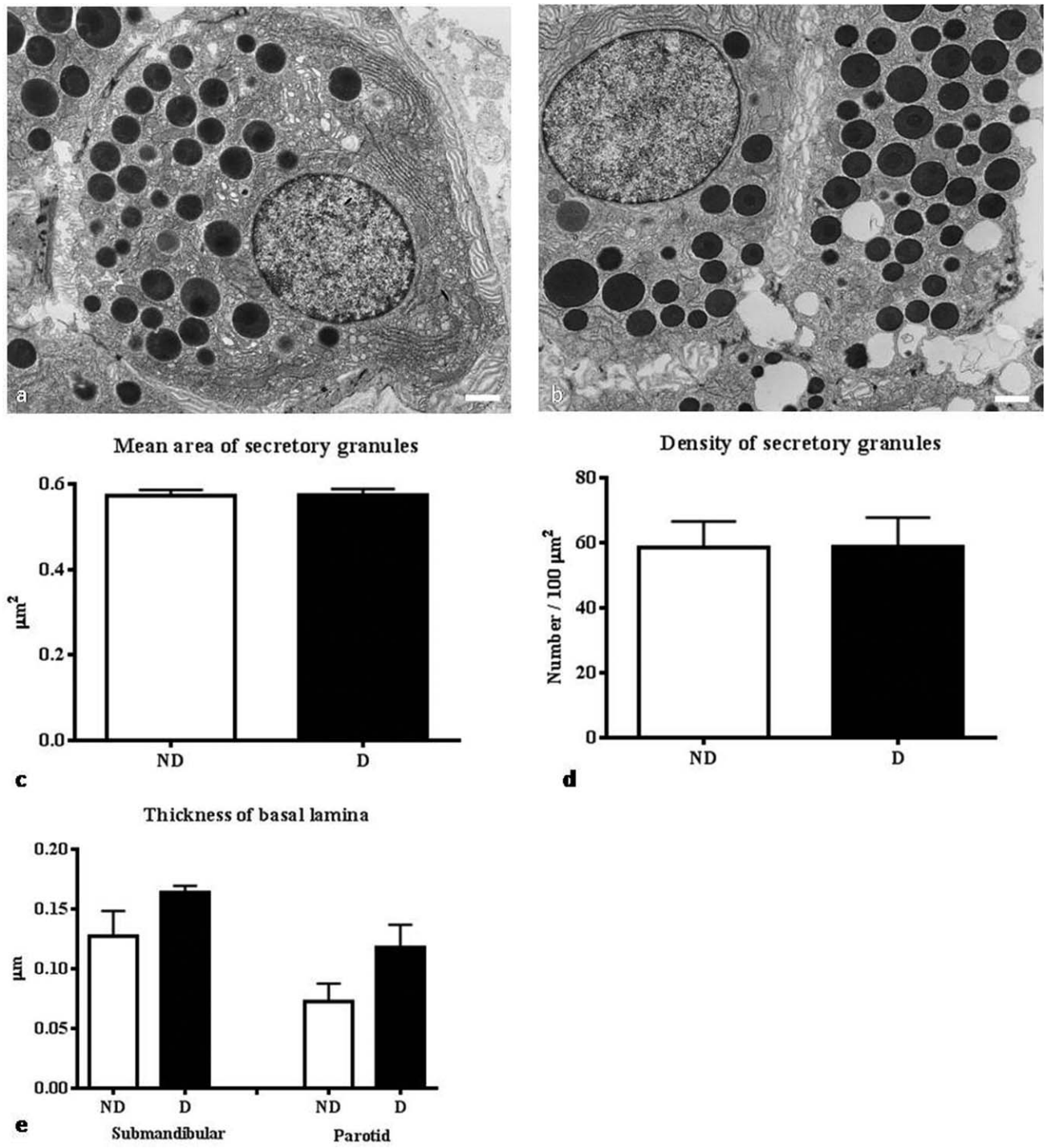

Fig. 2. TEM images of parotid acinar cells from nondiabetic (a) and diabetic samples (b). Cell morphology showed an apparently normal aspect in both groups. *Bar* 1 μm. Morphometrical evaluation: the mean area of secretory granules (c) and their density (number per 100 μm^2) (d) were not significantly different in diabetics with respect to nondiabetics; basal lamina thickness (e) measured in parotid and submandibular glands did not show any significant differences either.

was 0.118 μm in *D* and 0.073 μm in ND without significant difference, and, in submandibular acini 0.127 μm for ND and 0.164 μm for D (Fig. 2, graph e).

High-Resolution Scanning Electron Microscopy

In HRSEM images (Fig. 3), the cytoplasmic face of intercellular canaliculi communicating with the lumen

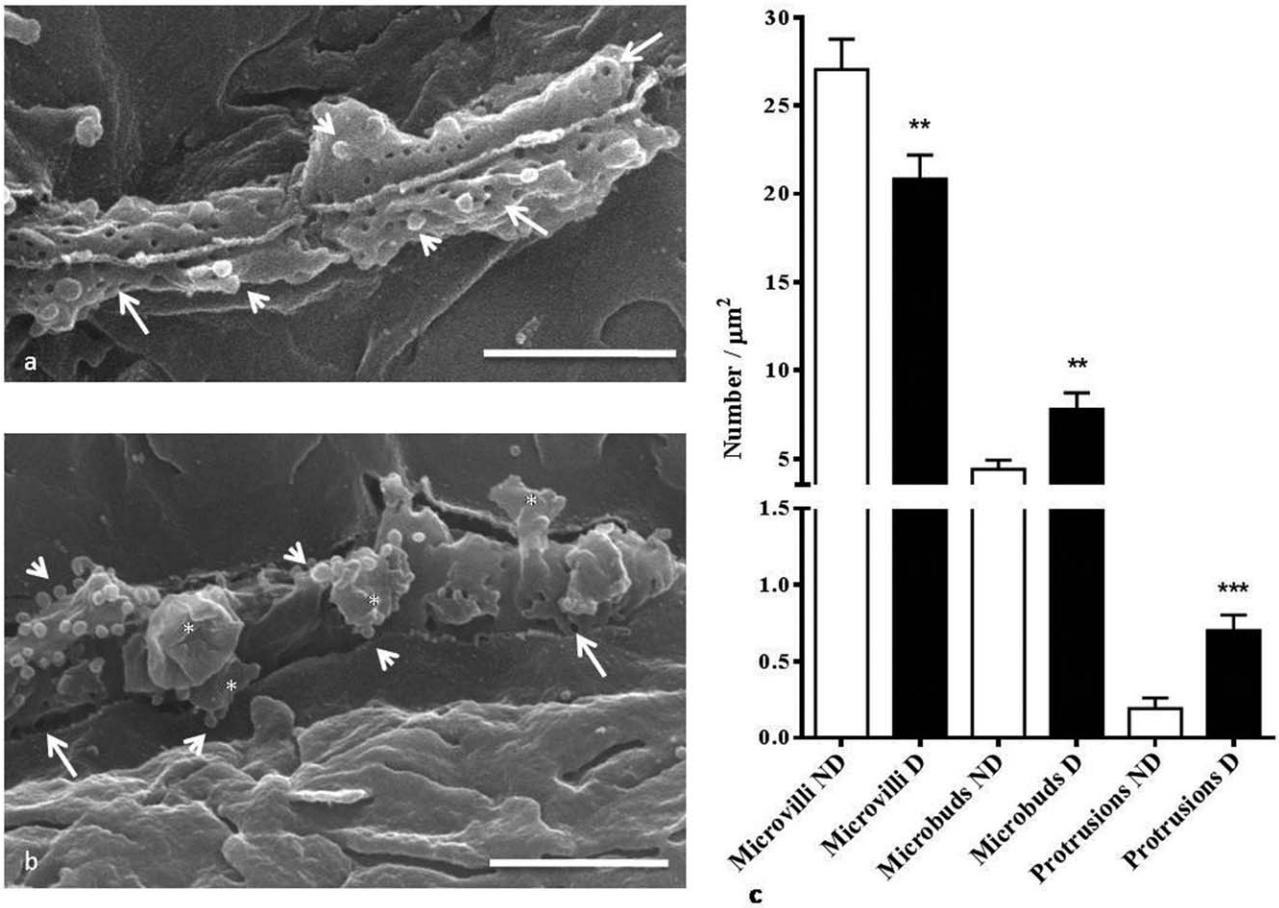

Fig. 3. HRSEM images of parotid intercellular canaliculus membranes of nondiabetics (a) and diabetics (b). The holes (arrows), corresponding to the base of the microvilli, the microbuds (arrowheads) reflecting recycling membranes, and/or vesicle secretion, and the protrusions (asterisks) reflecting docked granules, were observed in all samples. *Bar* 1 µm. Morphometrical evaluation: the density of the microbuds and of the protrusions was significantly increased, while that of the microvilli was significantly decreased in diabetic samples (c). $^{**}P < 0.01$; $^{***}P < 0.001$.

was examined, particularly in the aspects related to the secretory activity. The holes, corresponding to the base of the microvilli, appeared reduced in number in diabetic (b) compared to nondiabetic (a) samples. Otherwise, small and large roundish structures, corresponding to microbuds involved in membrane recycling, and to protrusions, corresponding to the docked granules, respectively, appeared increased in number in the diabetic samples.

Morphometric calculation (c) in the diabetic samples demonstrated a significant increase in the density of the microbuds (ND: 4.402 vs. *D*: 7.85) and of the protrusions (ND: 0.191 vs. *D*: 0.701), while the density of the microvilli was significantly reduced (ND: 27.05 vs. *D*: 20.85).

DISCUSSION

The present results confirm that diabetic status affects salivary gland morphology, even in the absence of sialosis, hyposalivation, or oral pathologies and when glycemia is well controlled. Since morphology reflects physiology, morphological changes suggest that functional alterations occur in diabetic salivary glands, even when clinical signs are not apparent.

Our observations substantiate literature data only partially, because reports on salivary glands in controlled diabetes are rather scarce, often contradictory, and chiefly based on chemical analysis of saliva (Fisher et al., 1987; Dodds and Dodds, 1997; Chavez et al., 2001; Bernardi et al., 2007; Barnes et al., 2014). From a morphological point of view, severe alterations in human salivary glands were described as effects of diabetes, but nearly all reports concerned cases of evident sialosis (Donath and Seifert, 1975; Islas Andrade et al., 1992; Satoh and Yoshihara, 2004; Merlo et al., 2010). Many morphological alterations in salivary glands were also described in rodents (Cutler et al., 1979; Hand and Weiss 1984; Reuterving et al., 1987; Anderson et al., 1990; Szczepanski et al., 1998), in which the effects of this disease were found to be attenuated but not abolished by insulin treatment (Chan et al., 1993; Caldeira et al., 2005). However, given the diversity between human and rodent salivary glands, and given the multiplicity of diabetogenic substances, we believe that these

data must be considered with caution when evaluating human data.

The present results corroborate our recent data on the submandibular gland, revealing ultrastructural changes by morphometry (Lilliu et al., 2015). Calculations on parotid images revealed that some parameters were significantly changed (number of microvilli, microbuds, and protrusions), while others remained unvaried (acinar size, number, and size of secretory granules, etc).

Evaluation of HRSEM images demonstrated significant variations at the level of the acinar cell surfaces, exactly corresponding to those described in the diabetic submandibular gland. Such variations regard the number of structures involved in the secretory process: protrusions, microbuds, and microvilli. Protrusions are interpreted as those secretory granules attached to the luminal plasmalemma (docked granules), awaiting completion of exocytosis. Actually, the last events in exocytosis are still not defined, and various theories await validation. According to the most accepted hypothesis, secretory granules undergo swelling and fusion with the luminal membrane; then they open, release their content, and their membrane is incorporated into the plasmalemma (Chiang et al., 2014). The pedunculated aspect of the protrusions seen by us is in line with this theory, since the peduncle would represent the granule opening channel.

An increased number of docked granules and microbuds, interpreted as endocytotic vesicles involved in membrane retrieval, was recorded in response to stimulation with secretagogue drugs and hormones (Testa Riva et al., 2006; Loy et al., 2012, 2014), but in that case it was associated with a dramatic reduction in secretory granules: clearly it was interpreted as a sign of increased secretion and cell emptying.

In our diabetic samples, no sign of cell degranulation was observed, and a lot of docked granules and vesicles can hardly be interpreted as a sign of increased secretion, given that diabetes does not enhance but rather reduces secretion (Yavuzyilmaz et al., 1996; Lasisi and Fasanmade 2012; Barnes et al., 2014). A plausible hypothesis is that docked granules simply stay longer attached to the plasmalemma, or that their membrane takes much longer to regain its original shape after granule emptying. The same explanation could be proposed for the augmented microbuds, if we imagine them as docked exocytotic vesicles generated by budding from the granules and representative of constitutive-like or minor regulated secretion. Obviously, we cannot rule out the possibility that apical microbuds at least partly represent endocytotic vesicles. A longer attachment of granules and vesicles to the apical membrane could be indicative of disturbances in the last phases of the secretory process. The modified number of microvilli might also point to difficulties in adapting the plasmalemmal shape to the functional requests.

Possible causes of such disturbances in exocytosis could be alterations in membrane composition and in cytoskeletal arrangement, which have been documented in some diabetic tissues (Limaye and Sivakami, 2003; Nashida et al., 2013; Trikash et al., 2015). Secretion, just like many cell activities, strictly depends on membrane proteins, such as those regulating calcium homeostasis. In several cell types, diabetes leads to malfunctioning of calcium transporting proteins with consequent inadequate calcium concentration, oxidative stress, and considerable functional damage (Fedirko et al., 2006; Zarain-Herzberg et al., 2014). Therefore, it appears reasonable to assume that disorders in the cell calcium balance due to membrane defects cause disturbances in the secretory process.

Surprisingly, acinar size and granule area remain unvaried in diabetic parotid samples, whereas both parameters were significantly increased in the submandibular gland (Lilliu et al., 2015). Acinar hypertrophy, which is frequently associated with sialosis (Donath and Seifert, 1975; Satoh and Yoshihara, 2004), probably reflects the impairment of glucose metabolism. In diabetes, malfunction of sodium/glucose cotransporters as well as the altered expression of aquaporins could be responsible of cell enlargement (Wright et al., 2011; Delporte, 2014). The finding of cell and granule swelling in the submandibular gland only suggests that it responds with more intensity than the parotid gland to circulating substances and signals associated with the diabetic status. It is generally thought that the two main salivary glands suffer in a similar way from the effects of diabetes, but it is also known that they differ from each other in their susceptibility to other pathological processes. For example, sialolithiasis affects more frequently the submandibular than the parotid gland (Huoh and Eisele, 2011), while neoplasies involve more frequently parotid glands (Nagler and Laufer, 1997); radiation damage chiefly involves parotid glands (Jeong et al., 2013), while xerostomia is more associated with submandibular dysfunction (Güne et al., 2010). Moreover, anatomical features, such as innervation and vascularization, histological, and histochemical properties, embryonic origin, genetic, and metabolic aspects distinguish parotid from submandibular glands (Riva et al., 1990; Tandler and Phillips, 1993; Denny et al., 1997) so that a different susceptibility to diabetic changes should not surprise.

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