Original Article
Histological characteristics following a long-term nitrate-rich diet in miniature pigs with parotid atrophy

Dengsheng Xia[1*], Xingmin Qu[1*], Simon D Tran[2], Laura L Schmidt[2], Lizheng Qin[1], Chunmei Zhang[1], Xiuyu Cui[3], Dajun Deng[4], Songlin Wang[1]

[1]Salivary Gland Disease Center and Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, Capital Medical University School of Stomatology, Beijing, China; [2]Faculty of Dentistry, McGill University, Montreal, Canada; [3]Institute for Neuroscience, Capital Medical University, Beijing, China; [4]Key Laboratory of Carcinogenesis and Translational Research, Peking University Cancer Hospital and Institute, Beijing, China. *Equal contributors.

Abstract: The aim of this study was to investigate the histological characteristics following a 2-year nitrate-rich diet in miniature pigs with parotid atrophy. Using averages collected data from three time points at 6, 12, and 24 months following the induction of parotid gland atrophy, salivary nitrate levels of the nitrate-diet parotid-atrophied group (17.3±3.9 ng/μl) were close to those of the control group (19.6±5.1 ng/μl). Compared to the control group, the nitrate-diet group had significantly higher nitrate levels in blood ($P < 0.05$) and urine ($P < 0.001$). Histological and electron microscopy analyses showed no abnormalities in the organs of experimental or control animals. No significant differences on apoptosis rate were found in liver and kidney tissues between the standard- and nitrate-diet groups. Therefore, dietary nitrate supplementation could restore salivary nitrate levels. High-dose nitrate loading for 2 years had no observed systemic toxicity in miniature pigs with parotid atrophy.

Keywords: Nitrate supplementation, parotid atrophy, oral cavity, histology, safety

Introduction

Dietary nitrate supplementation reduces blood pressure [1-3], improves brain perfusion [4], and lowers oxygen demand during aerobic exercises [5], among other beneficial functions [6]. Normal nitrate absorption in humans is a multi-step process that culminates when the parotid glands secrete nitrate into saliva [7]. In the first step, 98% of dietary nitrate is absorbed into the blood stream through gastric and duodenal cells. Subsequently, 25% of the absorbed nitrate is secreted by salivary glands, mainly the parotid glands, into the oral cavity [8].

Injuries to the salivary glands, such as a result of radiotherapy or Sjögren's syndrome, reduce nitrate levels in saliva and contribute to oral and gastrointestinal (GI) infections [9]. Salivary gland hypo-function leads to dry mouth, dysphagia, dental caries, candidiasis, oropharyngeal infections, and mucositis [10].

We have previously reported that parotid gland hypo-function decreases salivary nitrate levels in patients [7] and miniature pigs [11], and that salivary nitrates inhibit the growth of several pathogenic bacteria in the oral cavity [11]. Due to the beneficial effects of nitrate [9, 12], we hypothesize that nitrate supplementation prevents bacterial overgrowth in humans. However, nitrate supplementation is conflicting, because nitrites and nitrates are considered to be chemical pre-carcinogens, especially in the GI tract [13-17]. To this date, few studies have focused on whether nitrate supplementation in patients with salivary gland hypo-function is beneficial and safe. Using a parotid-atrophied animal model that our group developed, this study investigated the effects of a nitrate-rich diet on miniature pigs, especially on their body fluids (e.g., saliva, blood, and urine) and tissues of the oral cavity and GI tract.

Materials and methods

Parotid atrophy model

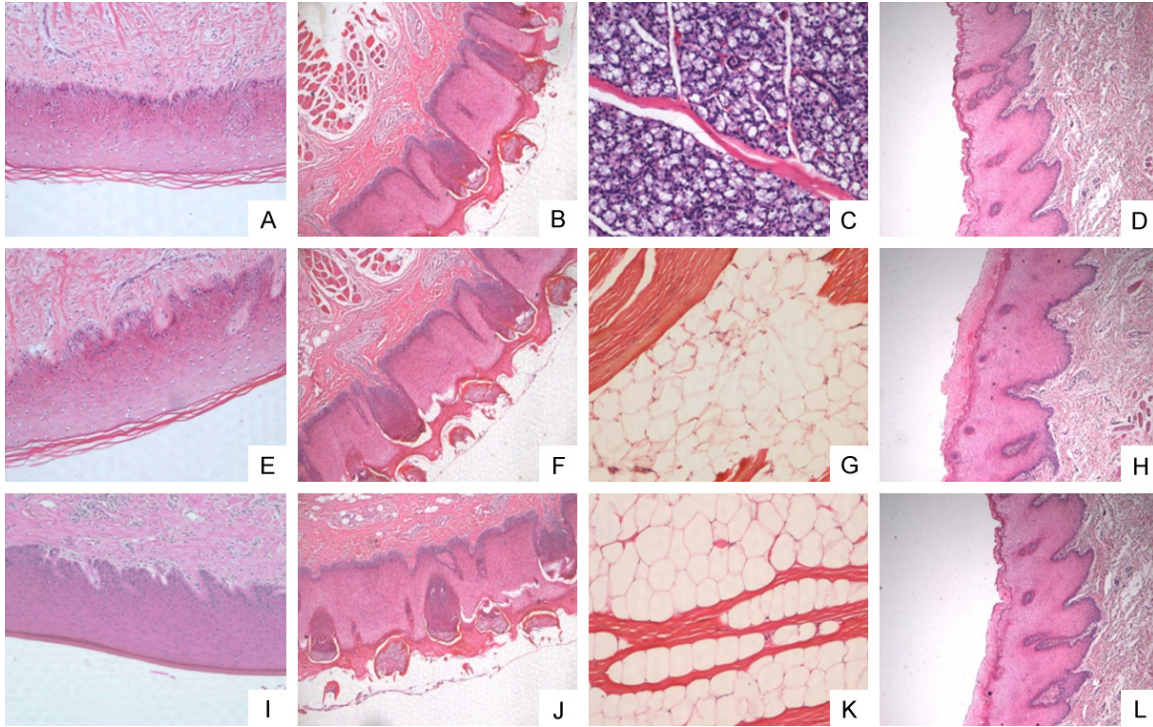Thirteen inbred healthy male miniature pigs were provided by the Chinese Academy of

Figure 1. Light micrographs of four oral tissues stained with hematoxylin and eosin. A-D represent gingival, glossal tissue, parotid gland, and buccal mucosa, respectively, from control miniature pigs; E-H represent the four tissues from parotid-atrophied miniature pigs; I-L represent the four tissues from parotid-atrophied miniature pigs supplemented with nitrate.

Agricultural Sciences. The study protocol was reviewed and approved by the Animal Care and Use Committee of the Capital Medical University. The miniature pigs were 6 months old at the beginning of the study, and weighed 35-50 kg per capita. The animals, which were housed under conventional conditions with free access to water and food, were randomly divided into three groups: group A (control) with no parotid atrophy and fed a standard diet (n = 3); group B, with parotid atrophy and fed a standard diet (n = 5); and group C, with parotid atrophy and fed a nitrate-rich diet (n = 5). The parotid glands of pigs in groups B and C were bilaterally ablated by retrograde injection containing 1% methyl violet (4 ml per gland) via the Stensen's ducts, thereby destroying the acini of both parotid glands [11]. Control miniature pigs received a retrograde phosphate buffered saline (PBS) injection.

Nitrate supplementation

Animals in groups A and B (parotid-atrophied animals) were fed a standard diet without nitrate supplementation. Parotid-atrophied animals in group C received nitrate supplementation (1 mol/L of potassium nitrate added to their drinking water) for 2 years.

Collection of whole saliva, serum, and urine samples

Biological samples (saliva, serum, and urine) were collected from the animals prior to treatments to determine baseline nitrate levels [16]. Additionally, biological samples were collected at 6, 12, and 24 months to monitor the changes in nitrate and nitrite concentrations. General anesthesia (ketamine/xylazine) was administered during biological sample collection. Saliva samples were collected for 10 min following 0.5 mg/kg pilocarpine administration. During saliva collection, the head of the animal was held down; saliva was collected into 10-ml vials. Blood samples (5 ml) were obtained from the precaval vein. Urine samples were collected from the metabolic cages where the animals were housed in. All biological samples were stored at -70°C until analysis.
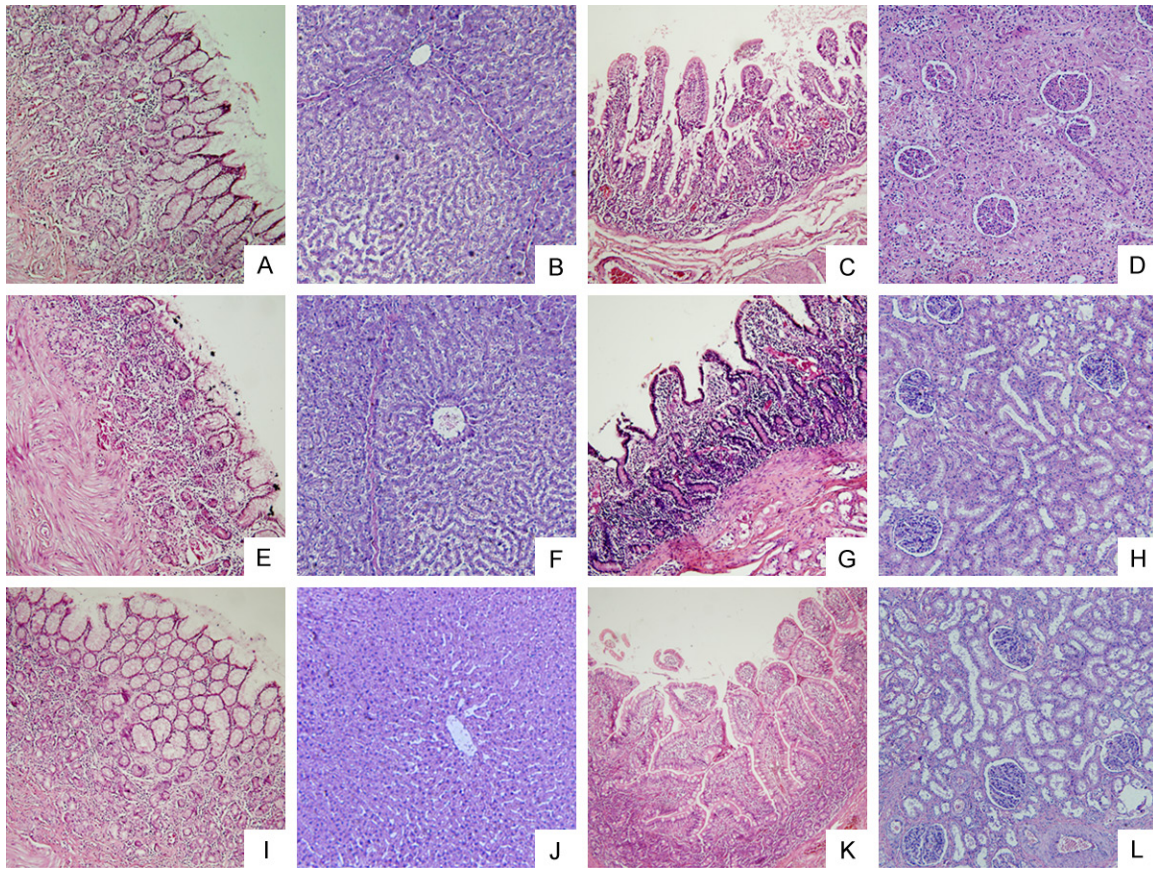
Figure 2. Light micrographs of four organs. A-D represent gastric mucosa, liver, small intestine mucosa, and kidney, respectively, from control miniature pigs; E-H represent the four tissues from parotid-atrophied miniature pigs; I-L represent the four tissues from parotid-atrophied miniature pigs supplemented with nitrate. No histological differences were obtained among control, parotid-atrophied miniature pigs with or without nitrate supplementation.

Measurements of nitrate and nitrite concentrations

Nitrate and nitrite concentrations were determined by a high-performance liquid chromatography (HPLC) system (Model 1050, Hewlett-Packard, Waldbronn, Germany) coupled to a Hypersil ODS C-18 column (250×4 mm, 5 mm particle size, Agilent Technologies, Waldbronn, Germany) and an ultraviolet detector (set at 210 nm). The injection volume was 6 ml, the mobile was KH_2PO_4-H_3PO_4 buffer (0.03 mol/L, pH 3.5) at a flow rate of 1.0 ml/min. In the samples, the recovery rate of nitrate (retention time: 2.59 min) and nitrite (retention time: 3.46 min) was 95-98% and 97-100%, respectively. The detection limit for nitrate was 0.8 mg/L, and for nitrite, 0.1 mg/L. To assess the reproducibility of the HPLC system, the initial sample was analyzed twice. Reproducibility was satisfactory; therefore, only one analysis was performed for each of the remaining samples.

Collection and preparation of tissue sections

All animals were sacrificed after 2 years; tissues from the oral cavity (gingiva, buccal mucosa, tongue, parotid, submandibular, and sublingual glands) and from the main GI organs (gastric mucosa, kidney, liver, and small intestine) were harvested. For light microscopy, tissue samples were fixed in 4% neutral formalin and embedded in paraffin. Slices were cut with a 5 µm thickness and stained with hematoxylin and eosin. For transmission electron microscopy (TEM, Philips EM208 s, Eindhoven, The Netherlands), tissues were fixed with 2% paraformaldehyde/2.5% glutaraldehyde for 2 h and rinsed three times (10 min each time) with 0.1% mol/L sodium dimethylarsenate buffer (pH = 7.4). Subsequently, the tissues were fixed with 1% OsO_4 for 2 h, rinsed with boiling water, and subjected to gradient alcohol dehydration by propylene polymerization. After the embedding and polymerization steps, the tissue samples

Table 1. Salivary flow rates (ml/min) at 6, 12, and 24 months in miniature pigs

Time (month)	A (n = 3)	B (n = 5)	C (n = 5)	P value
6	1.38±0.73	0.82±0.26*	0.81±0.31*	< 0.05
12	1.32±0.65	0.72±0.34*	0.74±0.28*	< 0.05
24	1.36±0.74	0.86±0.57*	0.83±0.46*	< 0.05

Group A: pigs with normal parotid glands and fed a standard diet.
Group B: pigs with induced-parotid atrophy and fed a standard diet.
Group C: pigs with induced-parotid atrophy and fed a nitrate-rich diet.

Table 2. Nitrate concentrations (ng/µl) in saliva, serum, and urine samples, averaged for three time points (6, 12, and 24 months)

Group	n	Saliva	Serum	Urine
A	3	19.58±5.14	0.87±0.12	31.07±0.35
B	5	7.01±1.95	2.04±1.24	69.31±13.50
C	5	17.34±3.89*	11.47±1.50*	6,014.90±1,291.60**

Group A: pigs with normal parotid glands and fed a standard diet.
Group B: pigs with induced-parotid atrophy and fed a standard diet.
Group C: pigs with induced-parotid atrophy and fed a nitrate-rich diet.
*P < 0.05; **P < 0.001.

were cut into 1 µm semi-thin sections, and light microscopy analysis was performed following azure-methylene staining. For TEM, 50 nm ultrathin sections were prepared from the semi-thin sections and stained with uranyl acetate and lead citrate.

Apoptosis detection in renal and hepatic cells by TUNEL

Apoptotic cell death was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, using the *in situ* Cell Death Detection Kit, POD (Roche, Mannheim, Germany). Briefly, paraffin sections of kidney and liver tissues were prepared and incubated with protease K working solution at 37°C for 15 min. The reaction mixture was added and incubated at 37°C for 1 h in a humidified atmosphere in the dark. Following a PBS wash, 50 µl Converter-POD was added and incubated for 30 min at 37°C. After a PBS wash, 50 µl DAB substrate was added and incubated for 10 min at 20°C. The sections were dyed with hematine and observed under a light microscope. Brown-labeled TUNEL-positive cells were counted under a 400× magnification. Apoptotic index was calculated as the percentage of TUNEL-positive cells relative to the total number of renal or hepatic cells. The apoptosis area ratio was analyzed using Image-pro plus 6.0 software.

Statistical analyses

Differences among groups were assessed by one-way ANOVA and Duncan's multiple comparisons test using SPSS (V13.0) software. Data were expressed as mean±SD. Statistical significance was set at P < 0.05.

Results

Histo-pathological changes in nitrate-supplemented miniature pigs

The tissues examined included the oral cavity (**Figure 1**) and GI tract (**Figure 2**) of all 13 miniature pigs. None of the sampled tissues or organs showed any abnormalities at both the macroscopic or microscopic levels, with the exception of the atrophied parotid glands. The parotid glands of the control miniature pigs had serous acinar cells with intercalated, striated, and excretory ducts, and adipose cells in the stroma (**Figure 1C**). The parenchymal tissue in the parotid-ablated miniature pigs had acini that were replaced with fat and connective tissue (**Figure 1G** and **1K**), which was due to the induced parotid atrophy procedure. Other tissues from the oral cavity (e.g., gingival, buccal, and glossal tissues; **Figure 1E-L**) had no abnormalities compared to control tissues. Tissues from the oral cavity had stratified squamous epithelium and underlying lamina propria in both the gingival tissue and buccal mucosa (**Figure 1A**, **1B**, **1E**, **1F**, **1I** and **1J**). These structures were also present in glossal mucosa and papillae tissues (**Figure 1D**, **1H** and **1L**). There were no significant histo-pathological changes in the stomach, liver, small intestine, or kidneys (**Figure 2**). Normal histological features were observed in gastric cells, i.e., normal mucous membranes, mucosal substratum, and muscular layers (**Figure 2A**, **2C**, **2E**, **2G**, **2I** and **2K**). The integrity of other structures, such as hepatic lobules, renal corpuscles, and renal tubules remained unchanged in the animals (**Figure 2B**, **2D**, **2F**, **2J**, **2H**, and **2L**). Histological characteristics of all tissues from the nitrate-supplemented group (group C) were normal.

Parotid atrophy in miniature pigs

Following ablation of their parotid glands, miniature pigs consuming either a nitrate-rich or a standard diet, exhibited a decrease in whole saliva flow rates at 6, 12, and 24 months com-
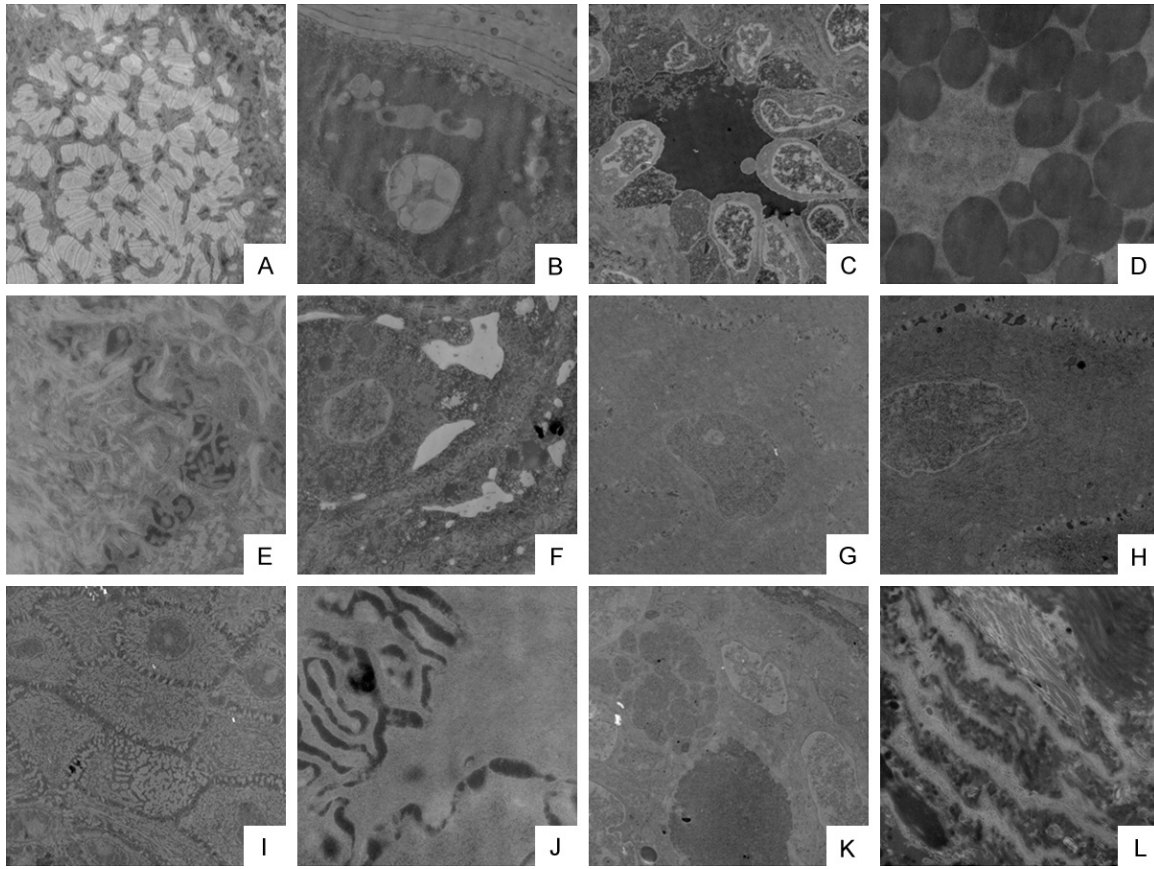
Figure 3. TEM micrographs of oral tissues at 15,000× magnification. A-D represent gingiva, dorsal of tongue, buccal mucosa, and parotid gland, respectively, from control miniature pigs; E-H represent the four tissues from parotid-atrophied miniature pigs; I-L represent the four tissues from parotid-atrophied miniature pigs supplemented with nitrate.

pared to miniature pigs in the control group (**Table 1**). Typical parotid atrophy was revealed in the histology (**Figure 1G**, **1K**), while normal parotid histology was found in the control group (**Figure 1C**). These results indicated that a successful parotid-atrophied animal model had been established. Salivary flow rates of both parotid-atrophied groups (groups B and C) were the lowest at 12 months (0.72±0.34 ml/min versus 1.32±0.65 ml/min in the control group, $P < 0.05$). At 24 months, salivary flow rates in both parotid-atrophied groups increased slightly (0.83±0.46 ml/min and 0.86±0.57 ml/min).

Nitrate levels in parotid-atrophied miniature pigs fed a nitrate-rich diet

To monitor nitrate levels, biological samples were collected at 6, 12, and 24 months. **Table 2** shows the average nitrate concentrations at these time points. The concentration of nitrate in the saliva of the nitrate-supplemented minia-ture pigs (group C) was not statistically different to that of the control group (17.34±3.89 ng/µl versus 19.58±5.14 ng/µl, $P > 0.05$). Compared to the control group, the nitrate-supplemented group had approximately 200× higher nitrate levels in their urine (6014.90±1291.60 ng/µl versus 31.07±0.35 ng/µl, $P < 0.001$) and 13× higher nitrate levels in their blood (11.47±1.50 ng/µl versus 0.87±0.12 ng/µl, $P < 0.05$).

Cyto-pathological changes in nitrate-supplemented miniature pigs

As expected, acinar cells of the miniature pigs with chemically-ablated parotid glands were replaced by collagenous tissue, degenerated fibrocytes, adipocytes, and striated muscle cells, which are indicators of successful parotid-atrophy induction (**Figure 3H** and **3L**). The parotid glands of control miniature pigs were normal and contained a large number of acinar cells with secretory granules, regular ribo-
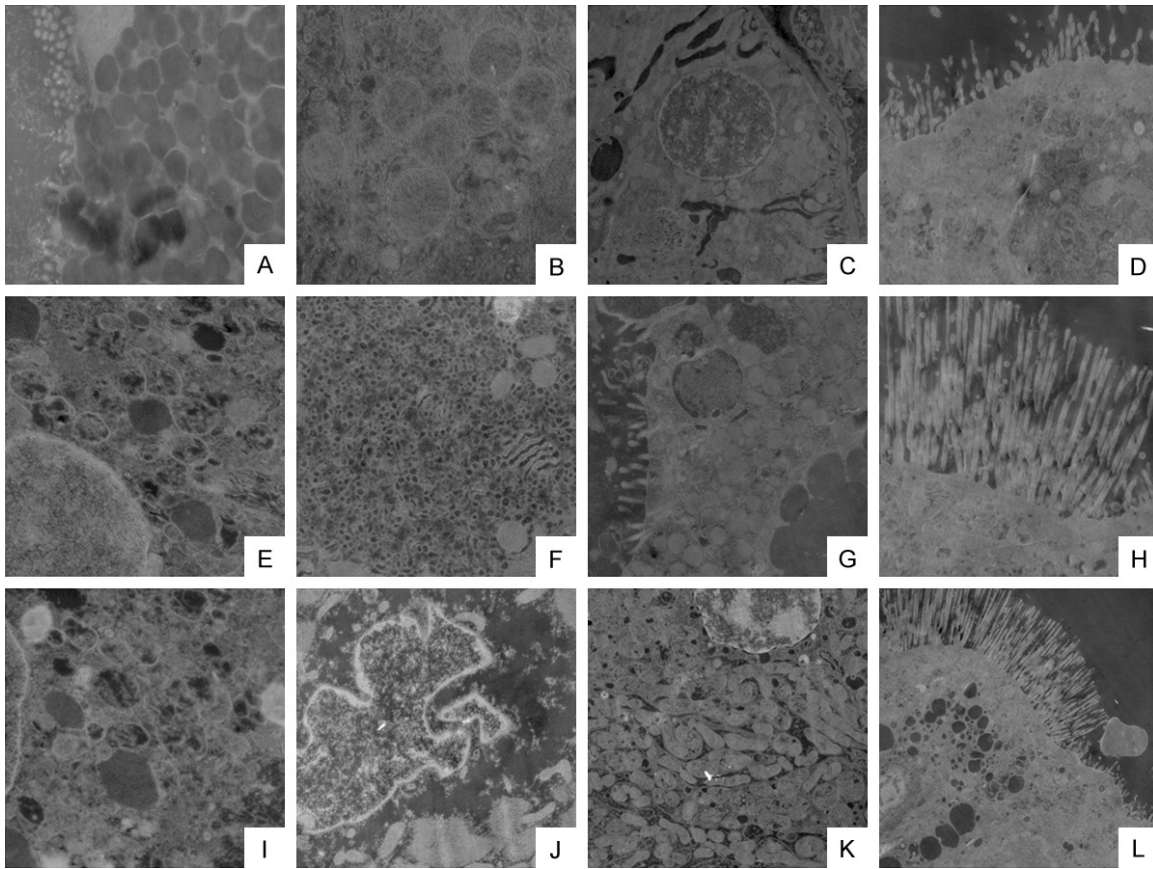
Figure 4. TEM micrographs of four other organs at 15,000× magnification. A-D represent the gastric mucosa, liver, kidney, and small intestinal mucosa, respectively, from control miniature pigs; E-H represent the four tissues from parotid-atrophied miniature pigs; I-L represent the four tissues from parotid-atrophied miniature pigs supplemented with nitrate.

somes, mitochondria, and lysosomes (**Figure 3D**). Both experimental and control animals exhibited healthy oral epithelial tissues. There were desmosomal connections between epithelial cells of the gingiva, tongue, and buccal mucosa (**Figure 3A-C**, **3E-G** and **3I-K**). The healthy status of the oral cavity was further confirmed by the presence of structured organelles in all examined oral tissues. The organelles exhibited nuclei with normal shapes, clear membrane outlines, abundant autosomes, and normal amounts of microfilaments in the cytoplasm (**Figure 3C**, **3G** and **3K**). All sampled oral tissues contained large amounts of desmosomes aggregated under the gingival epithelium, complete basement membranes, lower connective tissue, and bulging epithelial cytoplasm (**Figure 3A**, **3E** and **3I**).

In accordance with light microscopy observations, electron microscopy analysis of several

GI tissues revealed no significant differences in cytology between parotid-atrophied and control animals. Similarly, there were no significant differences in cytology between the standard-diet and nitrate-supplemented groups. The GI tracts of all animals had normal tissues, including abundant mucous secretions below the gastric mucosa, short-length microvilli, and gastric glandular cells with visible secretory granules and rough endoplasmic reticula (**Figure 4A**, **4E** and **4I**). Specifically, indicators of fully-functioning tissue, e.g., the presence of epithelial brush-border and mucous granules, were observed in the small intestinal sections (**Figure 4D**, **4H** and **4L**). Animal liver cells had clear nuclei and mitochondrial cristae structures, abundant rough endoplasmic reticula, and normal polysomes (**Figure 4B**, **4F** and **4J**). Renal physiological function was maintained in all animals as evidenced by the presence of normal renal glomeruli capillary loops, podo-
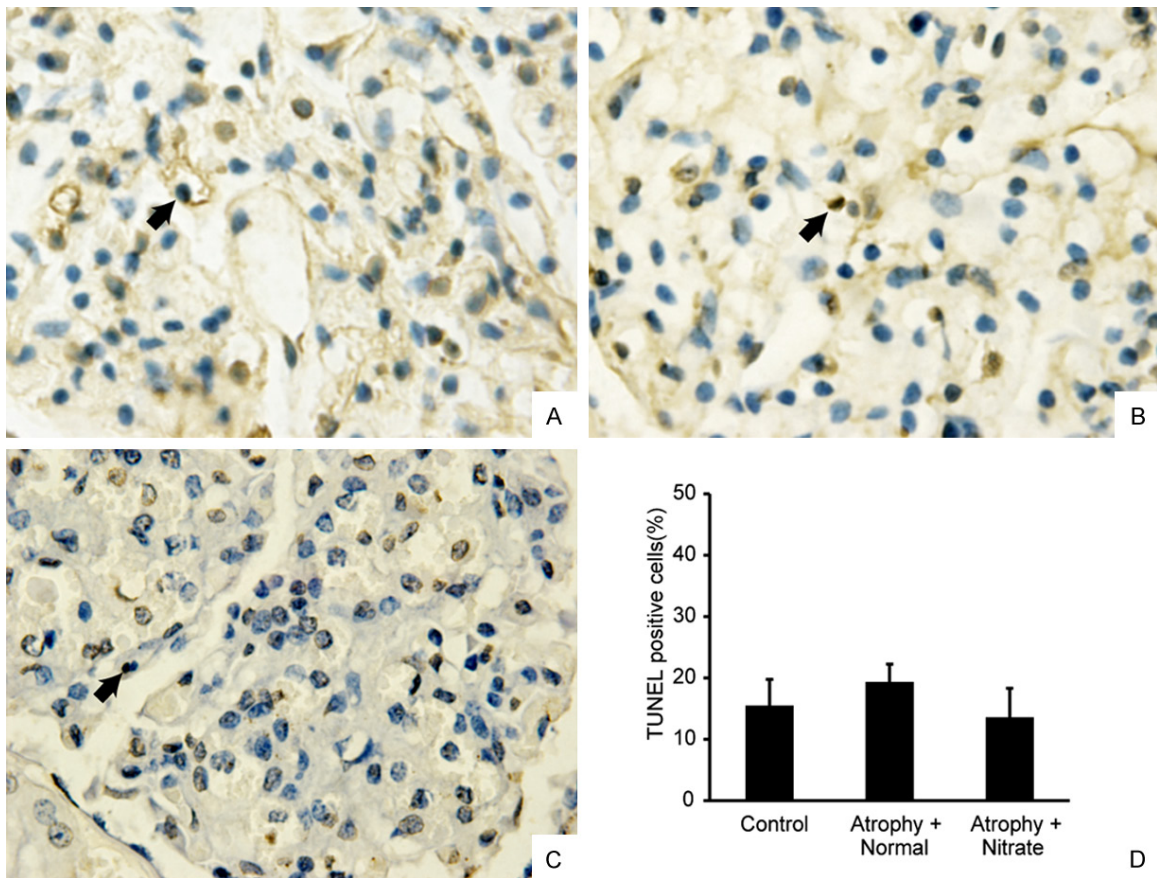
Figure 5. Effects of nitrate supplementation on renal apoptosis. Representative photomicrographs of the TUNEL in situ assay corresponding to control (A), parotid-atrophied group (B), and parotid-atrophied group with nitrate supplementation (C). Arrows indicate the TUNEL-positive cells. Bars represent percentages of TUNEL-positive nuclei (D).

cytes, renal tubules, and renal tubular epithelial cells. Mitochondria-rich perinuclear material with clear cristae was observed in kidney sections of all animals (**Figure 4C**, **4G** and **4K**).

Apoptotic changes in nitrate-supplemented miniature pigs

As shown in **Figure 5**, the apoptosis rate (%) of renal tissues in control animals, parotid-atrophied animals fed a standard diet, and parotid-atrophied animals fed a nitrate-rich diet were 0.15±0.04, 0.19±0.03, 0.14±0.05, respectively ($P > 0.05$). Similarly, there were no significant changes in apoptosis rate in hepatic tissues among the three groups ($P > 0.05$, **Figure 6**).

Discussion

In the present study, we evaluated the effects of nitrate supplementation on the nitrate levels of body fluids. We also evaluated the histological effects of nitrate supplementation on the oral cavity and other GI organs. To investigate the effects of nitrate supplementation, we atrophied parotid glands in miniature pigs. This animal model would simulate patients with salivary gland hypo-function. Five animals in this study were administered a high concentration of nitrate. Nitrate levels were measured in animal body fluids (blood, urine, and saliva) prior to, during, and following nitrate supplementation. Examinations of the oral tissues and GI organs were performed after 2 years. Therefore, the long-term effects of nitrate supplementation were assessed.

There were two main findings in this study. Firstly, normal salivary nitrate levels were re-established in miniature pigs with salivary gland hypo-function while on a nitrate-rich diet. Secondly, a nitrate-rich diet increased nitrate levels in both blood and urine of miniature pigs, with no histo-pathological irregularities in the
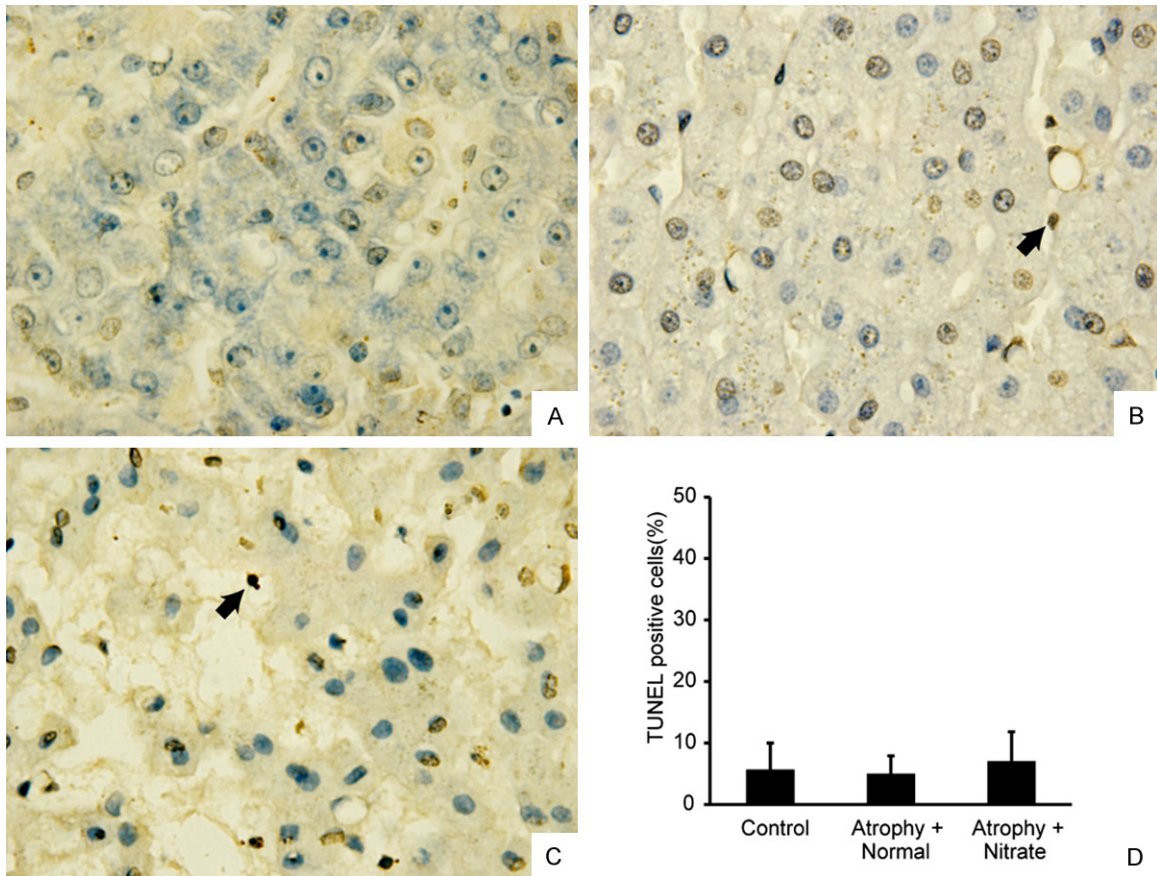
Figure 6. Effects of nitrate supplementation on hepatic apoptosis. Representative photomicrographs of the TUNEL in situ assay corresponding to control (A), parotid-atrophied group (B), and parotid-atrophied group with nitrate supplementation (C). Arrows indicate the TUNEL-positive cells. Bars represent percentages of TUNEL-positive nuclei (D).

oral cavity, GI tract, liver, or kidney after a 2-year period. The higher urine and blood nitrate levels in the nitrate-rich group compared to the control group can be explained by the lower sialin expression levels in kidney and liver cells. We previously reported that the protein sialin is an effective electrogenic co-transporter that mediates the influx of nitrate into salivary cells [12]. This transport mechanism may be better regulated for salivary nitrate levels than for blood or urine nitrate levels [18].

Nitrates are involved in multiple signaling pathways through their conversion to nitric oxide [19]. NO plays key roles in the regulation of cardiovascular function, cellular energetics, immune function, and neurotransmission [18]. This intrinsic property has enabled nitrates to become prominent compounds in multiple secretory fluids, including those of the parotid glands [7]. Historically, nitrates have been used as low-dose antiseptic agents in the food indus-

try with no serious medical implications. Given their potent cellular signaling properties, and their failure to cause any side effects when added to foods, clinicians have hypothesized that nitrate applications in dental medicine could be beneficial. Investigators have historically experimented with nitrate chemistry by adding different concentrations of nitrate to in vitro cultured saliva from patients with dental caries or periodontal diseases. Their findings revealed that nitrite acidification inhibits and destroys dental caries-causing bacteria [20, 21]. Previously we reported that nitrite acidification inhibits and destroys specific types of pathogenic bacteria such as Streptococcus mutans, Lactobacilli spp., and Blastomyces albicans. Nitrate production is beneficial in the GI tract, especially in gastric cells where nitrites from saliva are either chemically converted into NO or acidified [22, 23]. The NO content in the stomach largely depends on the nitrate content

in saliva [24, 25]. Salivary nitrate and nitrite increase blood flow into the stomach mucosa, thereby increasing its thickness and preventing gastric ulcer formation [24]. In light of these studies, it is plausible that nitrates can be used in patients to effectively inhibit pathogenic bacterial growth in the oral cavity and GI tract. There are a few caveats to this hypothesis, however, including the fact that nitrates have been recognized as chemical preludes in the formation of malignant tumors in the GI tract [13, 14, 17]. Additionally, nitrate concentrations > 50 mg/L in drinking water have been shown to cause methemoglobinemia [26] and thyromegaly [27].

The results of this study indicate that nitrate supplementation was effective in maintaining nitrate levels in the oral cavity of parotid-atrophied miniature pigs. We also demonstrated that the long-term administration of a nitrate-rich diet did not result in any morphological changes in the oral cavity or GI organs. The exemplified efficacy and safety of a nitrate supplement leads us to suggest that nitrate use could be justified to alleviate the debilitating circumstances that occur following parotid atrophy.

In conclusion, dietary nitrate supplementation (at 1 mol/L) for 2 years caused no systemic toxicity in pigs; its use appears to be safe in miniature pigs with atrophied parotid glands. A nitrate-rich diet could restore salivary nitrate levels in miniature pigs with parotid atrophy.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Songlin Wang, Salivary Gland Disease Center and Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, Capital Medical University School of Stomatology, 4 Tian Tan Xi Li, Dongcheng District, Beijing 100050, China. Tel: +86 10 83911708; Fax: +86 10 83911708; E-mail: slwang@ccmu.edu.cn

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