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Morphological Changes in Major Salivary Glands in Mice Treated With a Choline and Methionine Deficient Diet

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Abstract. *Background/Aim: It has been shown that the methionine-choline deficient (MCD) diet induces hepatocarcinogenesis, but not in extrahepatic organs, such as the testis, and pancreas, although may increase chemical-induced carcinogenesis in the colon, mammary gland, esophagus, and pancreas. Accumulating evidence suggests that salivary glands are very susceptible to stress conditions, such as radiation, hyperglycemia, and exposure to xenobiotics in vivo. This study aimed to analyze the histological changes on the major salivary glands (parotid, submandibular, and sublingual) after MCD diet administration. Materials and Methods: Male Swiss mice were submitted to ad libitum access to the control (AIN-76) or MCD diet for 28 days. The rebound group received the MCD diet for 24 days and the control diet for 10 days. Using the AxioImager A2 microscope, the hematoxylin-eosin (HE) stained specimens (4 mm) were evaluated for tissue degeneration, nuclear hyperchromatism and atrophy. Results: In the parotid gland from the MCD group, tissue degeneration, pyknosis, apoptosis and atrophy were observed, which remained in the rebound group, associated with hyperchromatism. In the submandibular gland from both MCD and rebound groups, severe tissue disorganization was associated with cell pleomorphism, hyperchromatic cells, apoptosis, increased eosinophilia, and inflammatory infiltrate. Finally, in the sublingual gland, there were no histological alterations in the experimental groups compared to the control. Conclusion: MCD can induce pre-neoplastic changes in the mouse parotid and submandibular glands, which are not reversed by a change in the diet.*

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Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease worldwide and has a prevalence of 25% in the adult population (1). It is characterized by excessive fat liver deposition and some aspects of metabolic syndrome (2). It is composed of simple steatosis and non-alcoholic steatohepatitis (NASH). The latter, besides the common accumulation of fat in the hepatocytes, is characterized by severe liver injury including ballooning of the hepatocytes, inflammatory infiltration, and progressive fibrosis (3). The progression of steatosis to NASH is the result of a combination of different mechanisms, such as alteration of lipid metabolism, mitochondrial dysfunction, increased levels of pro-inflammatory cytokines and oxidative stress (4). These hepatocellular lesions, in turn, can lead to cell death by apoptosis or necrosis, activate fibrogenesis, and contribute to the development of hepatocellular carcinoma (HCC) (5).

Since HCC can arise as a consequence of the progression of steatosis, hepatic steatosis-inducing diets may be an alternative to study hepatocarcinogenesis *in vivo* (6). In this context, many animal models have been used to study the pathology of NAFLD (7), however, none of them replicates the full profile of the human disease. The methionine-choline deficient (MCD) diet-fed animals are one of the most used models, and some aspects of the morphological changes are well-characterized (8). It has been described that MCD diet-induced steatosis is reversible for up to 16 weeks, and after is irreversible even if methionine and choline were withdrawn from their diet (8). It has also been described that the MCD diet induces carcinogenesis without the addition of carcinogens in the liver (9), but not in the extrahepatic organs, such as the testis and pancreas (10), although may

increase chemically induced carcinogenesis in the colon, mammary gland, oesophagus, and pancreas (11).

Accumulating evidence suggests that salivary glands are very susceptible to stress conditions, such as radiation injury, hyperglycemia, and continuous exposure to xenobiotics *in vivo* (12-14). However, to the best of our knowledge, no studies have investigated whether, and to what extent, the MCD diet induces changes in the salivary glands. Therefore, the goal of this study was to investigate whether the MCD diet may induce microscopic changes in major salivary glands (parotid, submandibular and sublingual) using an established experimental model of steatosis as far as hepatocarcinogenesis in mice.

Materials and Methods

Animals and experimental design. Six-week-old male Swiss mice were obtained from the Federal University of São Paulo. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals and approved by a local committee. The animals (5 per cage) were kept at a constant temperature of 22˚C±2˚C on a 12 h light-dark cycle with *ad libitum* access to food and water. Throughout the experimental period, the animals were weighed twice a week and feed consumption was evaluated daily. The animals were randomly allocated into three experimental groups according to the diet obtained from Rhoster® (Araçoiaba da Serra, SP, Brazil) The control group received a control diet (AIN-76, RH19522) and the MCD group received a methionine-choline deficient (MCD, RH19524E) diet for 28 days. The rebound group received the MCD diet for 24 days and the control diet for 10 days.

Tissue preparation. After treatment, animals were anaesthetized with urethane (1.3 g/kg; intraperitoneally) and the salivary glands were harvested. The tissue was kept in 10% paraformaldehyde for 24 h. The following day, tissues were rinsed under running tap water for 1 h, dehydrated in a series of ethanol solutions (70, 80, 90, 95, and 100%) for 45 min per solution and cleared twice in xylene for 45 min. Finally, all tissue samples were embedded in paraffin for sectioning. For hematoxylin-eosin (HE) staining, four mm-thick slices were made.

Histopathological analysis. Using the AxioImager A2 microscope (Zeiss, Oberkochen, Germany), the specimen was evaluated for tissue degeneration, nuclear hyperchromatism and atrophy. Such changes were comparatively evaluated in relation to animals belonging to the control group.

Statistical analysis. Data were evaluated for sample normality using the Shapiro-Wilk test. Samples were analyzed using ANOVA repeated measures followed by *posthoc* Tukey. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

Results

Experimental model. Body weight (g) was evaluated twice a week and at the end of the experimental period, compared to the initial body weight, animals in the control group gained weight (9.1±0.7 g) in contrast to the animals in the MCD group that lost weight (–12.0±0.5 g) in the same period. Animals of the rebound group had the same profile as the MCD group up to 24 days, when they received the MCD diet, with weight loss (–10.3±0.6 g) followed by an increase in the body weight (9.4±1.3 g) in the last week that received the control diet, reaching a final weight gain (3.9±0.5 g). The body weight differences among the experimental groups were confirmed by statistical analysis (F=11.66; p=0.004); the MCD group had a lower body weight than the control group (p=0.0276), whereas the body weight of the control group was lower than that of the rebound group (p=0.0001) at the end of experiments. The MCD (p=0.0276) and rebound (p=0.0001) groups had a lower body weight than the control group, whereas the body weight of the rebound was the same as that of the control group at day 28 (Figure 1).

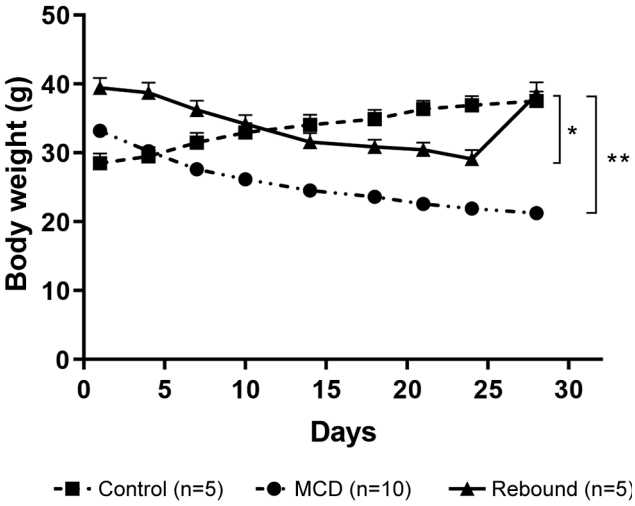

Figure 1. *Body weight (g) of mice submitted to AIN-76 (control group) or methionine-choline deficient diet (MCD group) for 28 days and to MCD-diet for 24 days followed by AIN-76 diet in the last week (rebound group). Data presented the mean±SEM analyzed by ANOVA Repeated Measures, posthoc Tukey. *p=0.0276; **p=0.0001.*

Histopathological analysis.

Parotid gland. Histopathological evaluation pointed out that in the control group, there were no remarkable changes in glandular parenchyma, in acinar cells and ducts (Figure 2A). In the MCD group, however, tissue degeneration was observed in all animals as depicted by the loss of acinar architecture, and the presence of pyknosis, apoptosis and atrophy (Figure 2B). In the rebound group, in which the animals were subjected to the MCD diet followed by a recovery period (control diet) for 10 days, the presence of degenerative changes remained, and was associated with the presence of hyperchromatism (Figure 2C).

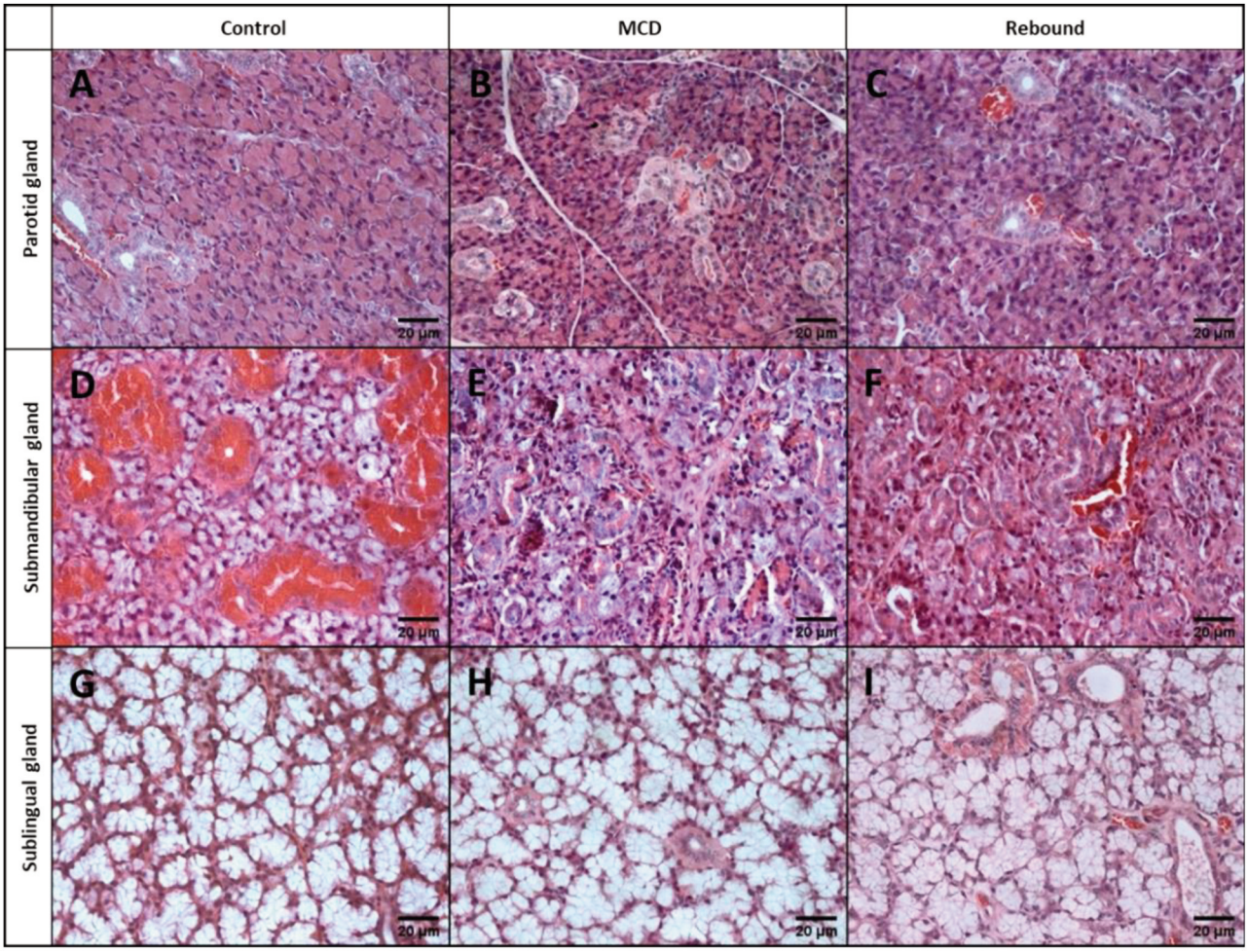

Figure 2. *Representative photomicrography of hematoxylin-eosin stained-major salivary glands from mice fed AIN-76 (control group), methionine-choline deficient diet (MCD group) for 28 days or MCD-diet for 24 days followed by AIN-76 diet for 10 days (rebound group). 40× magnification.*

Submandibular gland. Microscopic analysis indicated that the control group did not present any morphological changes in the glandular parenchyma, and tissue disorganization was absent (Figure 2D). The glandular cells had normal acinar size and architecture and the ducts displayed properly arranged cells. In the MCD group, however, severe tissue disorganization was noticed in all animals as a result of the loss of acinar architecture, which was associated with cell pleomorphism, hyperchromatic cells, apoptosis, increased eosinophilia and inflammatory infiltrate in the glandular parenchyma (Figure 2E). In addition, this was associated with congested and dilated blood vessels. In the rebound group, degenerative changes identical to those detected in the MCD group were observed (Figure 2F).

Sublingual gland. Microscopic analysis indicated that the control group did not present any morphological changes in the glandular parenchyma, with the presence of mucous acini and ducts in the glandular parenchyma (Figure 2G). The glandular cells had ordinary appearance with regular acinar size and properly arranged cells in the ducts (Figure 2G). The MCD group (Figure 2H) and the rebound group (Figure 2I) they did not differ from the control group.

Discussion

This study aimed to evaluate the impact of the MCD diet on major salivary glands (parotid, submandibular and sublingual) based on the evaluation of microscopic changes in mice. To the best of our knowledge, this question has not been addressed so far.

MCD diet is a well-established model for hepatic steatosis and steatohepatitis (15) and long-term feeding can induce HCC (9). The lack of methionine reduces synthesis and the levels of S-adenosylmethionine (SAM) and glutathione (GSH) (16). SAM acts on the methylation of proteins,

histones, and DNA (17), whereas GSH is a well-known antioxidant agent (18). The absence of choline alters phosphatidylcholine synthesis, which is required for VLDL (very low-density lipoprotein) secretion (19). Thus, methionine and choline deficiency lead to hepatocellular injury and steatosis (16), which can trigger inflammation, oxidative stress, endoplasmic reticulum stress, DNA damage, hepatocyte proliferation, and chromosomal aberrations, increasing the risk of hepatocarcinogenesis (20). Also, methionine-deficiency is mainly responsible for weight loss in the MCD model (16) and it occurs due to hypermetabolism, as a consequence of a higher body energy expenditure (21).

It has been established that dietary and nutritional components play a crucial role in experimental carcinogenesis, which has been used to further understanding of human tumor biology, as well as for the elucidation of mechanisms of carcinogenicity (11, 22). This is due to the fact that nutritional status interferes with toxicity, antioxidant status, and proliferative activity inasmuch as carcinogenic chemicals found in the environment. Some studies have demonstrated that chronic deficiency in major dietary methyl group donors, such as methionine, choline, folic acid, and vitamin B12 contributes to liver cancer in rodents (23).

Taking into consideration the multi-step process of chemical carcinogenesis, a growing number of studies have demonstrated that MCD may act as a promoter agent (10, 24). Recently, other authors have hypothesized that MCD acts as a complete carcinogen able to induce liver tumorigenesis in the absence of any exogenous carcinogen exposure (25). In fact, all diets namely ''lipogenic methyl-deficient diets'', *i.e.,* "choline-deficient", "methionine-choline-deficient", "methionine-choline-folic acid-deficient" trigger molecular changes in liver cells in a similar manner (26). Therefore, the MCD model of hepatocarcinogenesis is one of the most relevant experimental models for studying the etiopathogenesis of human liver carcinogenesis (27). The experimental model mimics human liver carcinogenesis (28).

In particular, the sequence of pathological and molecular events is remarkably similar to the development of human hepatocellular carcinomas, as well as nonalcoholic fatty liver disease/non-alcoholic steatosis. It is important to stress that this is a major risk factor for developing hepatocellular carcinomas in developed and developing countries (28, 29). In glandular tissues, some authors have also demonstrated that MCD can induce pancreatitis in rodents (30, 31). Therefore, it would be interesting to know whether, and to what extent, MCD can induce microscopic changes in major salivary glands, particularly because there are no previous reports. Our histopathological results demonstrated that the MCD diet can induce microscopic abnormalities in the parotid and submandibular glands as depicted by the presence of hyperchromatic cells, increased eosinophilia, apoptosis, and the presence of pleomorphism. The rebound process was not sufficient to reverse tissue injury when exposure was terminated. These changes in the parotid and submandibular parenchyma in the MCD and rebound groups are clearly indicative of neoplastic transformation. In the sublingual gland, no remarkable changes were noticed in the MCD and rebound groups. Taken as a whole, our results are consistent with the notion that the MCD can induce neoplastic transformation in the parotid and submandibular glands of mice. Moreover, the time of exposure adopted in this study (24 days) was enough to capture the initiation-promotion stages of chemical carcinogenesis.

In conclusion, the MCD can induce pre-neoplastic changes in the parotid and submandibular glands of mice. However, further studies are necessary to more accurately elucidate the end-points for studying chemical carcinogenesis in parotid and submandibular glands.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

MS Thomaz and MR Nagaoka performed the experiments. JN Santos and DA Ribeiro helped in the histopathological analysis. MR. Nagaoka and DA Ribeiro designed the experiments. MS Thomaz, JN Santos, DA Ribeiro and MR Nagaoka wrote the article.

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