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IMPACT OF WATER TREATMENT METHODS ON SALIVARY GLAND HEALTH: A MOLECULAR AND HISTOPATHOLOGICAL STUDY IN RATS

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ABSTRACT

Saliva produced by the salivary glands is critical for oral health. The respiratory and digestive systems could be negatively affected by trauma to these glands. This study investigates the impact of three water types on salivary gland function at molecular and histopathological levels using twenty-one male albino rats divided into three groups. The control group (Group I) consumed regular tap water, while Group II was given magnetic water, created by passing water through a 14500 Gauss magnetic field. Group III received microwave-heated tap water. Following a 60-day period and overnight fasting, rats were euthanized for salivary gland analysis. Results indicated significant body and salivary gland weight reductions, particularly in the microwave water group, which also showed decreased mRNA expression for MUC7, MUC19, HTN1, HTN3, CST2, and CST4 genes and increased amylase and HDAC3 expression. In contrast, magnetic water rats exhibited increased mRNA expression for MUC7, MUC19, CST2, and CST4 compared to the tap water group. Histopathological analysis revealed abnormal acini architecture and vacuolization in microwave-treated rats, along with significantly stronger BCL-2 expression in the tap and magnetic water groups than in the microwave water group, highlighting microwave-treated water's detrimental effects on salivary glands.

KEYWORDS: Tap water; Magnetic water; Microwaved water; Salivary glands; Epigenetic study

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INTRODUCTION

Saliva is produced by a complex network involving three groups of main salivary glands and hundreds of minor salivary glands. Oral hygiene relies on a wide variety of components and physicochemical qualities. Saliva is essential for chewing and swallowing because it preserves the teeth and the oropharyngeal mucosa and aids speech articulation. In addition, saliva is critical for sustaining a healthy microbiome [1,2]. Xerostomia, dental cavities, and fungal infections are all symptoms of salivary gland dysfunction, which can be caused by several medical conditions and drugs [2,3].

Signaling pathways involving particular mucins coordinate cellular responses, including proliferation, differentiation, death, and production of specialized cellular products [4]. Saliva contains at least five mucins, each in a slightly different ratio. MUC1, MUC4, MUC5B, MUC7, and MUC19 are these genes [5]. The salivary protein histatin (HTN) helps maintain oral homeostasis by preventing dental decay and fungus infections [6]. Human fluids and secretions contain a class of cysteine proteinase inhibitors called type 2 cystatin proteins, which have been hypothesized to have protective effects [7]. HDAC3 is a member of a group of genes with crucial roles in epigenetic processes—the gene HDAC3 genes for a protein with histone deacetylase activity.

Water is the most common element on Earth. It is the foundation of all known forms of life [8]. Because of its importance to all life forms, several methods have been devised to improve drinking water quality through physical and chemical processes [9]. Running regular tap water through a magnetic field yields magnetic water (magnetic tubes). This causes the water's qualities to become exceptionally receptive and dynamic [10]. Microwave ovens have been linked to the production of carcinogenic compounds [11]. Microwave ovens are used regularly by millions of individuals throughout the world. This research aims to examine the impact of magnetic and microwave-treated water on the salivary glands of rats at the molecular and histological levels. From what we can tell, this is the first investigation into how magnetic or microwaved water affects the salivary glands.

MATERIALS AND METHODS

Experimental plan

Twenty-one male albino rats, weighing between 140-160 grams and aged 2.5-3 months, were housed in cages under controlled environmental conditions. The cages provided a 12:12-hour light/dark cycle, maintained a temperature of 21-25°C, and maintained a humidity level of 60%. The rats were fed a pelleted diet consisting of various ingredients such as soybean meal (44%, 11%), sunflower oil (15%), yellow corn (49%), concentrate mixture (45%, 10%), wheat bran (10%), common salt (0.5%), molasses (3%), dicalcium phosphate (0.1%), ground limestone (0.2%), dl-methionine (0.7%), lysine (0.2%), and a mineral-vitamin premix. The diet was provided to the rats at a concentration of 0.3%.

Food and water were readily available for the rats. The rats were split into three groups after they had acclimated. Rats in Group I (the control group) were given only plain tap water throughout the study (60 days). The rats in Group II (magnetic-water group) were given magnetic water provided magnetic water derived from regular drinking water by running it through a magnetizer with a 14500 Gauss (G) magnetic field magnetizer (Delta Water Company, Alexandria, Egypt). Rats in Group III (microwave-water group) were given tap water heated in a microwave oven (Sharp Microwave 34 Liters, Stainless steel, 60 Hz, Model: R-77AS) till boiling for 10 minutes in a glass beaker, cooled to room temperature, and then supplemented after the ending of the study. At the end of study, rats were terminally anesthetized with pentobarbital (40 mg/kg) following[12]. Quickly following the salivary glands' extraction, they were cleaned in isotonic sa-

TABLE (1) qRT-PCR primer.

Gene	F	R
MUC7	CGTCCCTAATAAACAGTTA	TATCACAAATTCAACCACA
MUC19	CATCATTCCTGTAGCAGTAGTGAGG	GGTACCCAGGTCTACACCTACTCCG
HTN1	CGCTGATTCACATGAAAAGAGAC	AGGGAAGTATCATGAAACACAGA
HTN3	AATGTTAAGCTGTTTCACTGCTG	CATAATAATGTGCTTGAATTTTATTGC
CST2	GGTCACTTTCTGGGTGGCATA	ACCACACGGCTCAGGTACAAG
CST4	CTAGGTCACTTTCTGGGTGGC	TATGACAGCTGGAGCTCAGC
HDAC3	CGTCCGAAATGTTGC	GAAGTTCCTCACTAATGG
GAPDH	GGTGATGCTGGTGCTGAGTA	GGATGCAGGGATGATGTTCT

line and weighed. After that, we divided the salivary glands in half: a) one half was kept at -80°C for later RNA for gene expression, and b) the other half was fixed in 10% formalin for one day, then immersed in 70% alcohol for further histopathological and immunohistochemistry study.

Delta university's Animal Ethical Committee approved the experiment in conformity with Egyptian regulations on the use of animals in scientific research. All procedures complied with applicable laws. The study strictly adhered to the ARRIVE guidelines.

RT-qPCR in salivary gland tissues

Total RNA was isolated from the salivary gland using the Direct-zolTM RNA MiniPrep Plus kit (Catalog # R2071, ZYMO Research Corp., USA). The purity and concentration of the extracted RNA were measured using the NanoDropTM (ND-1000) spectrophotometer from Thermo Scientific, Waltham, MA, USA. To synthesize cDNA, the SuperScriptTM IV One-Step RT-PCR kit (Catalog Number 12594100; Thermo Scientific, Waltham, MA, USA) was employed after RNA extraction. Following cDNA synthesis, the NanoDropTM (ND-1000) spectrophotometer was used to dilute all the cDNA samples to a consistent concentration. For real-time qPCR procedures, QuantiTectTM SYBR Green qPCR Master Mix, along with the cDNA and gene-specific primers, were utilized. Primer3webTM(version 4.1; [13] was used for the online primer design (Table 1). Quantitative real-time PCR assays were performed using the Step OnePlusTM system (Applied Biosystems, USA). The threshold cycles (Ct) of the genes of interest were standardized to those of the housekeeping gene GAPDH [14].

Histological analysis

The Hematoxylin and eosin staining was performed on the right salivary glands, and anti-B cell lymphoma two immunohistochemistry was performed on the left (Bcl2), for H& E dewaxed section, then rehydrated. After 30 minutes in Ehrlich's hematoxylin, slices were stained for 10 minutes with 1% eosin Y. In preparation slides for Bcl2, we did it following by the manufacturer's instructions. Serial sections in thickness of 4μm were taken. Deparaffinization of the portions was placed in 5% xylene for 5 minutes. Then, the sections were hydrated again in graded alcohol for 3 minutes. Phosphate buffer saline was used to wash the slides before they were treated with the biotinylated antibody for 30 minutes.

Using an Olympus microscope with a digital camera (VE-MC5 5.0 MP). The obtained photos were processed with ImageJ (version 1.51r; NIH, Maryland, USA). The color deconvolution plugin

was utilized to measure the stained area percentage. Each slide had five random areas examined.

Data analysis

Statistical analysis was conducted using one-way ANOVA and Duncan's multiple-range tests, performed with SPSS software version 22.0. The results are presented as mean values and standard deviations (SD). Statistical significance was considered at a p-value of less than 0.05, indicating that differences in means were deemed statistically significant.

RESULTS

Body and salivary gland's weight

The results in Figure 1 Show a significant decrease in body weight in a microwave-water group compared with other groups. The groups drinking tap water and those drinking magnetic water did not differ significantly in body weight. In addition, the same results appeared in the weight of salivary glands Figure 2.

Molecular analysis

The results in Figures 3 and 4 refer to a substantial decrease in all studied genes in the microwave water (MUC7, MUC19, HTN1, HTN3, CST2, CST4) except the expression of amylase and HDAC3; they showed an increase in the activity concerning tap water and magnetic water. When linked to tap water, magnetic water has noticeable changes in MUC7, MUC19, CST2, and CST4. There were no significant differences between the other genes (HTN1, HTN3, Amylase, and HDAC3).

Hematoxylin and eosin results

The control group shows normal acini with round basal basophilic round nuclei. Normal shape ducts with a basophilic nucleus and standard shape convoluted granular GCTs. However, GCTs were found as pyramidal in shape with basophilic basal nuclei (fig.5A). In magnetic water, the acini appeared normal as tap water with the normal basophilic basal round nucleus. The ducts and GCTs seemed normal with basophilic basal nuclei (fig.5 B). Finally, the

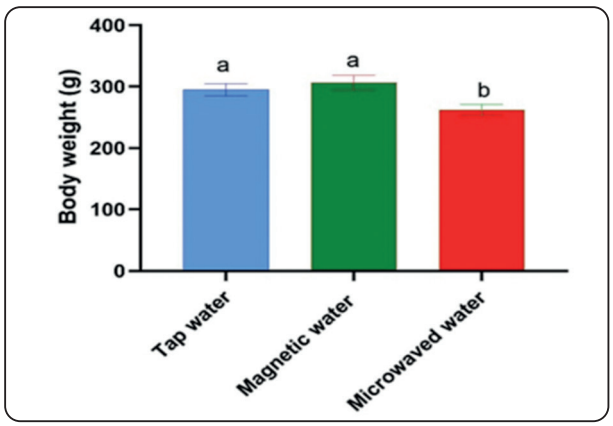

Fig. (1) The impact of microwave and magnetic water on rat body weight. Data are shown as Mean± SEM. a,b Significant differences exist between columns denoted by various letters at $p < 0.05$.

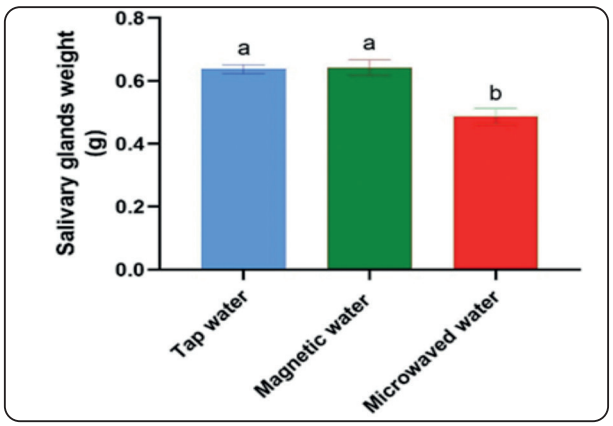

Fig. (2) The impact of microwaved and magnetic water on rat salivary glands weight. Data are shown as Mean± SEM. a,b Significant differences exist between columns denoted by various letters at $p < 0.05$.

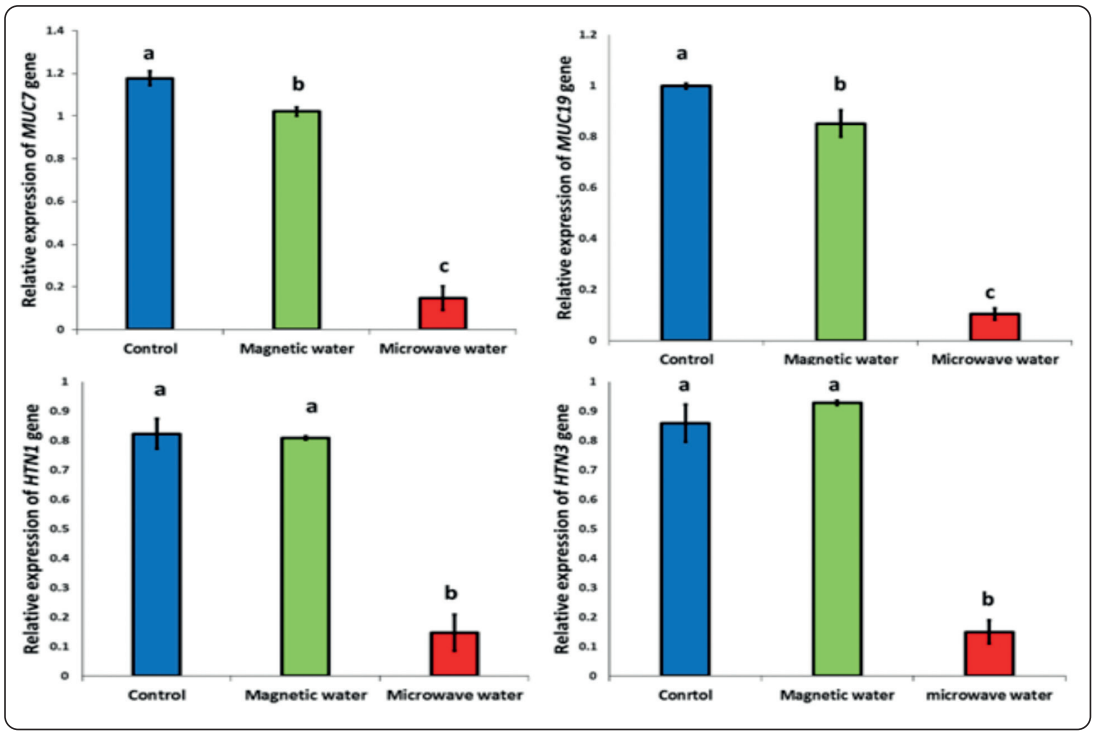

Fig. (3) The effects of tap water, magnetic water, and microwave water treatments on the levels of MUC7, MUC19, HTN1, and HTN3 in rat Salivary glands, Data are shown as Mean± SEM. a,b,c Significant differences exist between columns denoted by various.

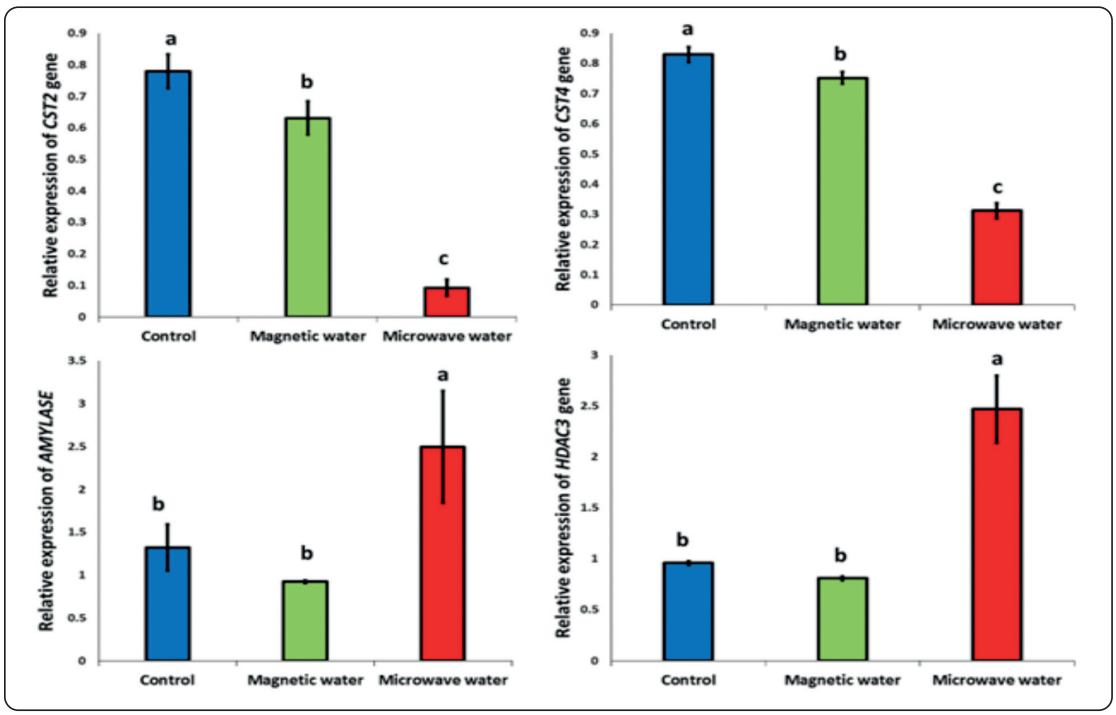

Fig. (4) The impact of tap water, magnetic water, and microwave water treatments on the CST2, CST4, Amylase, and HDAC3 in rat Salivary glands, Data are shown as Mean± SEM. a,b,c Significant differences exist between columns denoted by various letters at $p < 0.05$.

microwave group showed abnormal architecture of acini with some vacuolization in serous acini. There was some faint staining and pyknotic basophilic nucleus. The intralobular ducts and GCTs showed vacuolization, destruction, and a light staining nucleus (fig.5 C).

Immunohistochemistry results

Tap water shows a positive anti-Bcl2 reaction to the submandibular salivary gland acini (fig.5 D). Magnetic water also showed a strong positive anti-Bcl2 response to the submandibular salivary gland acini (fig.5 E). On the other hand, microwave water shows a mild positive anti-Bcl2 reaction to the submandibular salivary gland acini (fig.5 F).

In this experiment, we measured the percentage area of Bcl2 expression to submandibular salivary gland acini. An expansion in the area gave the highest value, and a low area gave the lowest value. So, the control group was insignificant (45.8502± 4.628) ($p= 0.5808$) with the magnetic water group (47.6678± 2.897). On the contrary, the microwave group was highly significant (37.1798± 4.306) ($p< 0.0001$) with the magnetic water and substantial ($p= 0.002$) with tap water (fig. 5G).

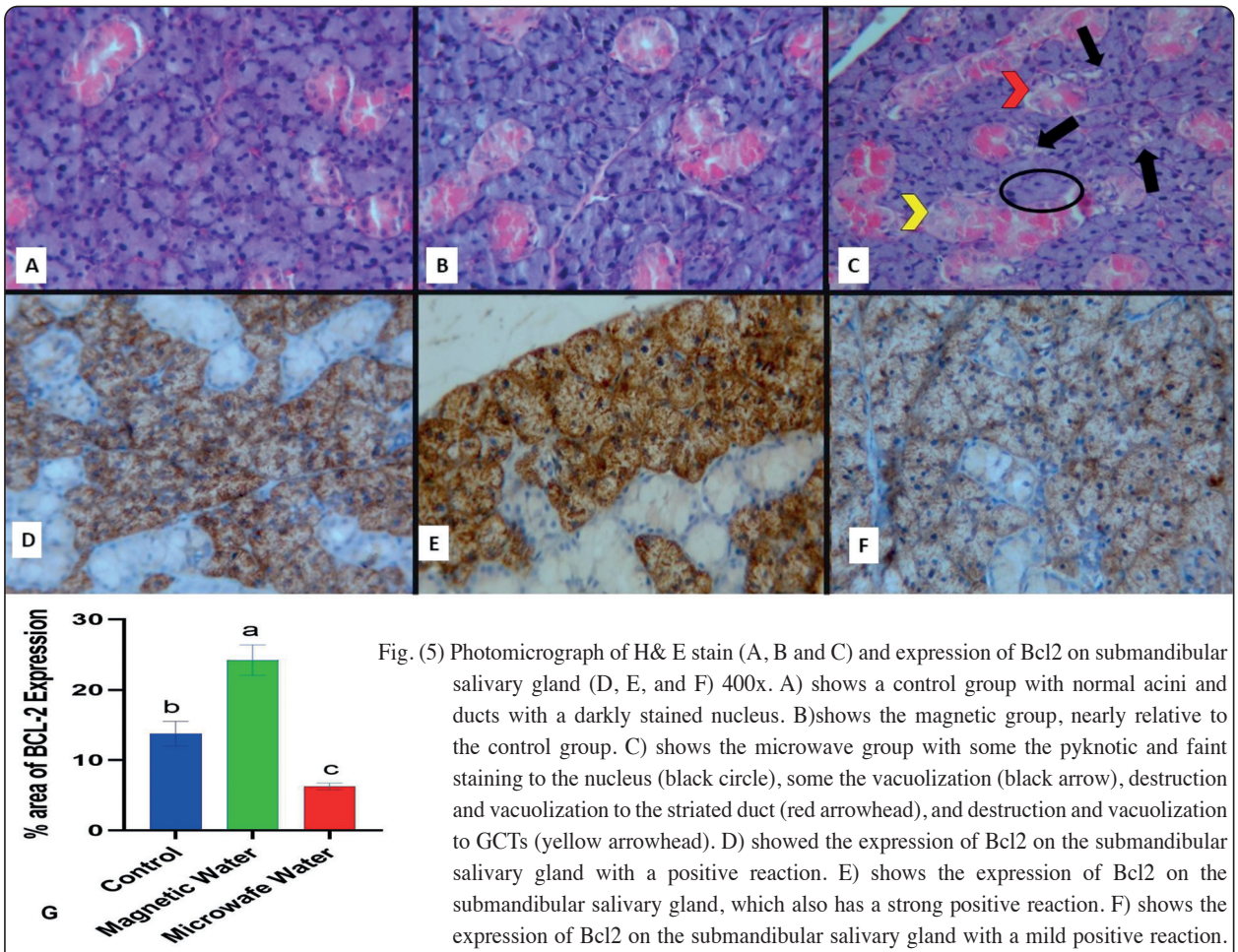

Fig. (5) Photomicrograph of H& E stain (A, B and C) and expression of Bcl2 on submandibular salivary gland (D, E, and F) 400x. A) shows a control group with normal acini and ducts with a darkly stained nucleus. B)shows the magnetic group, nearly relative to the control group. C) shows the microwave group with some the pyknotic and faint staining to the nucleus (black circle), some the vacuolization (black arrow), destruction and vacuolization to the striated duct (red arrowhead), and destruction and vacuolization to GCTs (yellow arrowhead). D) showed the expression of Bcl2 on the submandibular salivary gland with a positive reaction. E) shows the expression of Bcl2 on the submandibular salivary gland, which also has a strong positive reaction. F) shows the expression of Bcl2 on the submandibular salivary gland with a mild positive reaction. G). Area % of Bcl2 expression. Data are shown as Mean± SEM. a,b,c Significant differences exist between columns denoted by various letters at $p < 0.05$.

DISCUSSION

Global Salivary glands produce saliva, which is necessary to maintain oral homeostasis. Damage to these glands may impact a person's oral health and other systems, including the digestive system[15]. In our study, compared with other groups, there was a considerable reduction in the body and salivary glands' weights in the microwave-water group. Weight differences between the tap-water and magnetic-water groups were insignificant. It is well-known that gland size is another element influencing saliva flow rates.[16]. The decrease in the salivary glands' weight reflects the reduction in gene expression in most studied genes.

We looked for changes in gene expression associated with the glands' function to examine the impact of three water treatments on salivary gland activity at the molecular level (MUC7, MUC19, HTN1, HTN3, CST2, CST4, Amylase, and HDAC3). We found a substantial decrease in the examined genes in the microwave water except for the expression of amylase and HDAC3; Compared to magnetic and tap water, they displayed an uptick in activity. Microwave water induced changes in the gene expression of MUC7 and MUC19. It led to a decrease in gene expression and, consequently, a reduction in the protein. This decrease led to chronic dry mouth, difficulties chewing, swallowing, and tasting (Ohara, Hirano, Yoshida, & Suzuki, 2011), and a rise in yeast infection and caries [17]. In magnetic water, there was a significant down-regulation in MUC7 and MUC19 compared with tap water, but not as the outcome of microwaved water.

We showed for the first time that microwave-treated water decreases the gene expression of HTN1 and HTN3. These genes' downregulation is recorded in Sjögren's syndrome and the dysfunction of salivary glands [18]. On the other hand, magnetic water didn't differ from tap water in HTN1 and HTN3 genes.

Notably, we observed that the expressions of two cystatin family genes (CST2 and CST4) were significantly downregulated in microwave water. Also, magnetic water slightly reduces the gene expression of CST2 and CST4 genes compared to tap water. There is no reference to water's impact on the gene expression related to slavery glands.

When microwave-treated water was compared to tap water, the genes for amylase and HDAC3 were upregulated, indicating the microwave treatment's negative consequences. These impacts might result from modifications to the properties of water, including pH, conductivity, and water molecule movement [19]. As a result of reaching supercritical conditions, we have also observed salt precipitation after boiling water in a microwave [20]. It can be the primary factor behind the harmful consequences of microwave treatment. Other modifications to water's normalcy that other writers have noted caused essential changes in living cells. Salts can be dissolved in water because of their strong bipolarity. As a result, water molecules might cluster around the salt ions that have been separated. An increase in temperature causes a slow but noticeable shift in the solution's structure, including the hydrogen-bonding network and the clustering of water molecules around solutes. The cause of this is a decrease in molecular interaction and an increase in molecular kinetic energy.

In contrast to the water dipole moment, the applicable electrostatic force connected to ion charges decreases with the temperature significantly slower. Accordingly, the hydrogen-bonded network becomes less robust and can less shield the salt ions [20]. These changes in tap water's characteristics may impact the genes' epigenetic state. This is amply demonstrated by the significantly increased regulation of the HDAC3 gene in microwave water. Among the genes with a substantial impact on epigenetic changes is HDAC3. Histone deacetylase is a family of proteins that HDAC3 encodes. In comparison to tap and magnetic water, microwave water increased the expression of this gene. As a result,

we can say that the epigenetic effect was influenced by microwave water.

However, there were no discernible variations in the expression of the Amylase and HDAC3 genes between magnetic water and tap water. This refers to the high-power magnetic water having no side effect on salivary gland secretion in half of the studied genes. In the other genes, the result of magnetic water is not as bad as microwave water.

Our histological study for both H&E and Bcl2 found almost the same result. Compared to the microwave-water group, the submandibular salivary gland of the magnetic-water and tap-water groups displayed a substantial difference. On the other hand, the submandibular salivary gland of tap water was insignificant to the magnetic water. However, Bcl2 is considered a proto-oncogene concerning programming cell death by physiological apoptosis inhibitors [21]. So, Bcl2 expressions increase with the severity of dysplasia and vice versa [22].

To our knowledge, no paper was treated directly with the effect of magnetized or microwaved water on the salivary gland. So, we used other organs as a reference for our results.

Our findings were consistent with [23]; they demonstrated that the magnetized water had no pathologic impact on heart, lung, or spleen tissue compared with the control group. Another paper used magnetic water or Ginkgo biloba to treat nephropathy-induced type 2 diabetes in rats. The histological examination of the kidney found a decrease in the size of bowman's space and glomerular cellular composition compared to the group that had diabetes without any medication [24]. [25] found that low-frequency electromagnetic fields negatively affected the motility of rat sperm. However, the harmful impact of microwave ovens on water may be due to the destruction of the internal structures because of the increase in the heating rate. So, the higher rate of water loss generated many processes that were not easily controlled [26] and [27].

CONCLUSIONS

This is the first report to study the effect of different water types: tap water, magnetic, and microwave on the salivary gland activity involving the histopathological, immunohistochemistry, and intracellular molecular activity of the gland. This study concluded that microwaved water hurts the salivary gland activity on the molecular level through down-regulation in the mRNA expression of MUC7, MUC19, HTN1, HTN3, CST2, and CST4 genes accompanied by upregulation of the amylase and HDAC3 expression concerning the tap water and magnetic water as well as, the histopathological level.

Data Availability: All data generated or analysed during this study are included in this published article.

Declarations

Ethical Approval

The experiment was approved by delta university's Animal Ethical Committee in conformity with Egyptian regulations on the use of animals in scientific research. All procedures complied with applicable laws. The study strictly adhered to the ARRIVE guidelines. (FODMRC 2,021,000,100)

Informed Consent Statement: Not applicable.

Conflicts of Interest: No conflicts of interest.

Consent to Participate: All authors have contributed and agreed to participate in this paper.

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