Role of *LncRNA MALAT-1* on induced submandibular gland carcinoma in albino rats treated with induced pluripotent stem cells (histological and immunohistochemical study)

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**ABSTRACT**

Salivary gland tumors show complex histopathology and the treatment depends mainly on the stage of cancer. Induced pluripotent stem cells (iPS) have a great role in regenerative medicine as they can generate pluripotent stem cells from any available cell types as fibroblast. Thus, the aim of this work is to investigate the possible therapeutic effect of (iPS) on induced salivary gland cancer through evaluation of the silent information regulators of *sirtuin-1* (*Sirt-1*), *Tgf-β* genes and their protein expressions in addition to LncRNA MALAT-1 expression. Thirty male albino rats were employed and divided into three groups (ten rats for each group), group I (control): rats were injected with phosphate buffered saline (PBS), group II induced squamous cell carcinoma (SCC): rats were injected with squamous carcinoma cells (SCC), group III (induced SCC/iPS): SCC treated rats treated with \(5 \times 10^6\) iPS cells. Submandibular specimens were taken and prepared for histological, histochemical and immunohistochemical studies for Bax and TGF-β3 protein. Also, Real time PCR was performed for *Sirt-1*, *Tgf-β*, and MALAT-1 LncRNA genes expression. SIRT-1 and TGF-β protein level expression
was assessed by western blot technique. Group III (iPS treated group) revealed more or less normal acinar structure with normal rearrangement of acini and normal intralobular ducts with an increase in their number. In the iPS treated group there was increasing in the amount of mucopoly saccharide in the acinar cells and intensity of BAX immunostaining while, TGF-β3 was decreased in its intensity in comparison to that of the cancer treated group. In addition to Sirt-1, Tgf-β, and MALAT-1 LncRNA expressions were increased in cancer group compared to iPS treated and control groups. Induced pluripotent stem cells play a potential therapeutic role in treatment of induced submandibular gland carcinoma.

**Key words:** iPS, submandibular salivary gland, MALAT-1 LncRNA, TGF-β, BAX.

**Introduction**

Nearly 500,000 patients all over the world receive treatment for head and neck cancer every year where salivary gland cancer represents about 6% of such neoplasms (1). Salivary gland lesions are clinically and morphologically different; so treatment of these tumors is very difficult (2). Stem cell therapy is an interesting approach for salivary gland cancer treatment (3). Lately, research to detect salivary molecular biomarkers for the differentiation between oral cell carcinoma (OSCC) and healthy controls has increased greatly because saliva is considered to be a non-invasive available biofluid that reflects many systemic physiological conditions (4).

Induced pluripotent stem cells have the ability for treatment of cancer (5). The use of iPS is built on the use of four factors which cause re-programming of the human somatic cells to become pluripotent (6). Stem cell therapy is considered to be a novel approach for treatment of salivary gland cancer patients (3).
Non-coding RNAs (ncRNAs) were discovered as new regulators of different biological functions, they were found to play an important role in tumor formation and progression (7). Long non-coding RNAs have been found to be dysregulated in different types of diseases of human, including many types of cancer, such as prostate cancer (8), leukemia (9), breast cancer (10), hepatocellular carcinoma (11), colorectal cancer (12), and melanoma (13). Hence, long non-coding RNAs may also be associated with oral cancer. So this study was done to investigate the possible therapeutic effect of (iPS) on induced salivary gland cancer through evaluation of the silent information regulators of sirtuin-1 (Sirt-1), Tgf-β genes and their protein expressions in addition to LncRNA MALAT-1 expression.

Material and methods

Squamous cell carcinoma (SCC) animal model

Thirty 14–21-day-old (150–200 g) male albino rats were obtained from the laboratory animal colonies, Animal House of the Faculty of Medicine, Cairo University. We acclimatized all rats for four days before starting the experiment. All procedures were conducted with the Guide for the Care and Use of Laboratory Animals (14) and also were approved by the Ethical Committee for Scientific Research at the Faculty of Medicine, Cairo University, Egypt. Animals were allowed to grow under constant conditions of 20 –26 °C and 40–70% humidity with a 12 hours’ light:12 hours’ dark cycle. We provide all animals with standard rat chow and distilled water ad libitum. We utilized the Hep2 laryngeal (SCC passage 97) squamous cell carcinoma (SCC) cell line (American Type Culture Collection (ATCC) Manassas, VA) through a supplier in Egypt (Vacsera) (15).

iPS cells preparation:
Our present research work is a continuation for previous work on iPS cells injection in induced submandibular salivary gland cancer (16). Briefly, skin fibroblasts were reprogrammed into iPS cells using an Amaxa 4D-Nucleofector (P2 Primary Cell Kit from Lonza cat# V4XP-2012, Program FF-135; Lonza, Basel, Switzerland)

with non-integrating plasmids containing Oct4, Sox2, L-MYC and LIN28. One day before the transfection, 5 x 10^5 cells were plated in 1 ml of complete growth medium so that the cells were 50–70% confluent at the time of transfection. Four essential reprogramming factors (Oct3/4, Sox2 and L-MYC and LIN28) were connected in a single plasmid using nucleofactor kit (Lonza) according to manual instructions. Transfected primary cells were kept a few weeks long enough to allow transgene expression for induction of iPS cell.

**Animals grouping**

For every animal handling procedure, including injection of the tumor cell, we anesthetized the rats with 1:0.5 xylazine chloride: ketamine then ventral an oblique incision was made in the shaved antiseptic neck in order to bare the submandibular glands. Rats were divided into three groups of 10 rats per each. Group 1 (control), rats were treated with 0.20 ml phosphate-buffered saline (PBS). Group II (SCC), we injected the submandibular salivary glands with 60,000–95,000 cancer cells suspended in 0.20 ml PBS three times/week through an oblique ventral incision in the gland. After 6 weeks of induction of the SCC notice the presence of the swelling in the submandibular gland and this confirms the development of cancer in the gland. Group III (SCC/iPS), rats were inoculated with 5 x 106 iPS cells (16). The SCC group was sacrificed on week 7 after induction of cancer; while rats of
the SCC/iPS group were sacrificed at week 7 after injection of iPS.

**Histology**

At the end of experiment, the rats were sacrificed by ether inhalation, the submandibular glands were taken rapidly, dissected carefully, fixed in 10% buffered neutral formalin and processed for Paraffin sections (4-5 μm thick). They were stained with H&E stains for histological structure and histochemical staining by PAS reactions for detection of mucoplysaccharides. By this method neutral mucoplysaccharides appear red, acid mucopolysaccharides are stained blue, and the mixture of both stains purple (17).

**Immunohistochemical study**

1- Immunohistochemical staining for detection of BAX as used as a pro-apoptotic marker which appeared in positive cells as a brown cytoplasmic color (rabbit polyclonal antibody, 1/50 dilution, Abcam Q07812 - Sigma-Aldrich, Egypt).

2- Immunohistochemical staining for detection of anti-TGF-β3 antibody (tumor growth factor) which appeared in positive cell as a brown cytoplasmic color which was purchased from Abcam (Paris, France) and other chemicals were brought from Sigma–Aldrich, Egypt (SRP1427-6GQ).

Immunohistochemical staining was done by using avidin-Biotin immunoperoxidase technique in which submandibular sections from paraffin block were cut. Peroxidase Blocking Solution was used to block the endogenous peroxidase for 15 min., the slides were put in citrate buffer for antigen retrieval, then put in the microwave for 9min at 90 °C and the slides were incubated at room temperature after cooling with the diluted primary antibodies (1:50 for Bax). Finally, Diaminobenzidine (DAB) chromogen solution was added and counter
staining was done with Mayer’s hematoxylin. For negative control sections, the primary antibodies were excluded (18). Positive control of BAX was taken from human breast cancer specimen and for anti-TGF-β3 was taken from human prostatic cancer from our pathological department.

**Morphometric analysis**

At the Pathology Department, Faculty of Medicine, Cairo University by using a Leica Qwin 500 image analysis computer system (Leica Microsystems Ltd, Cambridge, UK), ten slides from each group were checked. On each slide, we assessed 10 non-overlapping fields to measure histological grading scale (1), including the preservation of gland architecture, degree of fatty replacement, preservation of ducts and acini, and the presence of inflammatory cells and interstitial fibrosis. The above data were grading as: no degeneration (0), trace (1), mild (2), moderate (3), and severe (4) degeneration.

The mean area percentage of PAS staining, TGF-β protein and BAX protein were measured by light microscopy 40X in each ten cells per section from each animal of all groups. Statistical analyses were carried out using IBM SPSS statistics software for Windows (version 20; IBM Corp., Armonk, New York, USA) (19).

**Real time PCR**

The RT-PCR Master Mix kit (Applied Biosystems, Cat No. 4440040) was used to convert the mRNA into cDNA. We used a Step One System (RQ Manager 1.2, software v 2.1, Applied Biosystem) for the reactions. To determine the threshold cycle (CT) value from the amplification plot, Gapdh was utilized as the endogenous reference gene for normalization control. We used negative controls to exclude any contamination. The thermal cycling profile was 15 minutes at 45°C for synthesis of cDNA followed by 5 minutes at 95°C for reverse transcriptase inactivation and polymerase activation. PCR amplification 40 cycles were followed which consisted of 15 seconds DNA denaturation at 95°C, 20 seconds
primers annealing at 55 ºC and 30 second at 72 ºC for the amplification step (20). The sequence of the primers for each gene present in the study was demonstrated in Table (1).

**Western blot:**

The purified monoclonal mouse IgG SIRT-1 ((B:10): sc-74504, Santa Cruz Biotechnology) and monoclonal mouse IgG TGF-β ((3C11): sc-130348, Santa Cruz Biotechnology) antibodies were used. Thirty µg of protein were separated by SDS-PAGE on 7.5% polyacrylamide gradient gels (TGX Stain-Free™ FastCast™ Acrylamide kit). After incubation in 5% non-fat dry milk, Tris-C, 0.1% Tween 20 for 1 hr, we added antibodies to one of the membranes containing specimen samples and incubated at 4 ºC overnight. Suitable secondary antibodies were incubated for two hr at room temperature. After being washed twice n 1 x TBS-T, densitometric analysis of the immunoblots was done to quantify the amounts of SIRT-1 and TGF-β against β-action by protein normalization using Image analysis software on the ChemiDoc MP imaging system (version 3) produced by Bio-Rad (Hercules, CA) (21).

**Statistical analysis method**

Data were coded and entered using the statistical package SPSS version 20. Data was summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups. Correlations between quantitative variables were done using Pearson correlation coefficient (22).

**Results**

**Histological and Histochemical results**
Histological examination of H&E-stained sections of submandibular salivary gland in the control group (group I) showed normal parenchyma with many lobules that contain both pale vacuolated mucous acini with flat nuclei and serous acini with darkly stained secretory granules and rounded nuclei (some acini appeared capped by serous demilunes). Striated and intercalated ducts were found between acini in which intralobular and intercalated ducts were lined by simple cuboidal epithelium, whereas the striated ducts were lined by high cuboidal cells. Lobes of gland were separated by connective tissue septa. (figure 1A and B).

Stained sections by H&E of submandibular gland from squamous cell carcinoma animals (group II) showed massive histological changes in the acini, as they degenerated, disarranged with disturbance of their architecture, showing cellular pleomorphism and were widely separated by connective tissue containing infiltrating cells and some dilated congested blood vessels were also observed in the connective tissue between the lobes (figure 1C and D). Some intralobular ducts showed hyperplasia with disorganization of the lining epithelial cells with hyperchromatic nuclei and with fatty infiltration (figure 1E and F). While in group III treated by Ips revealed more or less normal acinar structure with normal arrangement of acini and normal ducts with increasing in their number (figure 1G and H).

Histochemical examination of PAS-stained sections of submandibular salivary gland in the control group (group I) showed that most of the acinar cells contain mixture of both neutral and acid polysaccharides as they are stained deep purple, while the duct system showed faint PAS positive reaction (figure 2A).

PAS stained sections of submandibular gland from induced squamous cell carcinoma animals (group II) showed marked decrease in the amount of neutral mucopolysaccharides as there was a decrease in the purple staining in comparison to
that observed in control sections (figure 2B). In group III treated by iPS revealed more or less normal PAS +ve acinar cells as increase in amount of mucoplysaccharides (figure 2C).

The histological grading scale findings are represented in table 2. Siginfically all grads in squamous cell carcinoma group were increased as compared to that of the control group. The P-values are as following: degree of fatty replacement (P = 0.12), loss of lobular architecture (P = 1.93), presence of interstitial fibrosis (P = 0.06), degree of ductal degeneration (P = 2.35), presence of diffuse inflammatory component (P = 1.91), presence of focal inflammatory component (P = 0.14), and degree of acinar degeneration (P = 3.38).

**Immunohistochemical result:**

Bax immunostaining was moderate positive in control glandular tissue (figure3A), while, in SCC group it was week positive (figure 3B).in Ips treated group the BAX immunostaining was mild to moderate (figure 3C)

The TGF-β immunostaining was absent in control glandular tissue (fig 4A). while it showed intense localization within the cytoplasm of acini and duct of submandibular cancer specimens (figure 4B and 4C). In stem cell treated group, showed moderate to mild TGF-β immunostaining (figure 4D).

**Morphometric analysis**

There was significant decrease in mean count of staining cells (P<0.001) in BAX reaction while, there was significant increase of staining cells of TGF-β reaction (P<0.001) in the cancer cells of submandibular gland compared with the control, whereas the BAX reaction in Ips treated group showed a significant increase in positive staining cells  (P<0.001) while, the TGF-β reaction showed a significant decrease in staining cells  (P=0.04) compared with the squamous cell carcinoma group (figure 3E and 4E). The mean area % of PAS reaction for all groups was
represented in figure 2D. There was a significant decreased (P <0.05) in PAS reaction in subgroup II compared with control group.

**Real time-PCR**

The expression of the *Sirt-1* and *Tgf-β* genes was significantly increased in salivary cancer group compared to the control group (p < 0.001). There was a statistical significant decrease in *Sirt-1* and *Tgf-β* genes expression in the iPS treated group compared to the salivary cancer group (p = 0.001) as shown in figure 5 (a) and (b) respectively.

There was a statistical significant increase in *LncRNA (MALAT-1)* gene expression in salivary cancer group and the ips treated group compared to the control group (p < 0.001) and a statistical significant decrease in *LncRNA (MALAT-1)* gene expression in salivary cancer + ips group compared to the salivary cancer group (p < 0.001) as shown in figure 5 (c).

**Western blot**

Our results showed statistical significant increase in both SIRT-1 and TGF- β protein expression in salivary cancer group compared to the control group (p < 0.001) but a statistical significant decrease in their expression in salivary cancer + iPS group compared to the salivary cancer group (p <0.001) as shown in figure 6 (a) and (b) respectively.

**Discussion**

The use of iPS cells is considered to be promising for different types of diseases. Concerning cancer, iPS cells allow studying of the epigenetic events that may lead to cancer and also iPS cells are considered to be a method to generate cells that will give the opportunity for better more beneficial therapies with limited toxicity (23). In our previous study, we investigated the effects of iPS on salivary gland
carcinoma (16). In such study we evaluated Sirt-1, Tgf-β gene expression and LncRNA MALAT-1 in addition to SIRT-1, TGF-β protein levels and immunohistochemical effect to determine the therapeutic role of iPS cells on salivary gland cancer.

In the present study, induction of submandibular carcinoma in rats showed apparent histological, histochemical and immunohistochemical changes as acini were degenerated, disarranged with disturbance of their architecture, also acini showed cellular pleomorphism with cytoplasmic vacuolization and were widely separated by connective tissue containing infiltrating cells and this was similar to results reported by (24). The hyperplasia of both ducts and acinar cells, showed vacuolated cytoplasm compared to that of the control group. The deeply stained nuclei appeared irregular and small. Cytoplasmic vacuolization can be due to accumulation of fluid as a result of failure of the acinar cells to flow out the fluid after induction of cancer (25).

The histological results of the cancer group were appeared to be parallel with histochemical results of the same group as cancer cells lead to decrease staining of acini as the acinar cells revealed a decrease in both neutral and acid mucosubstances.

The content of saliva and its PH is sensitive to blood flow as it completely changes in cancer cells (26). This study might be explaining what we were observed in the present work that in the cancer cells there were decrease in acid mucopolysaccharides. The changes in the histochemistry of acinar cells granules might be due to the loss of granules within the acinar cells. Other studies had shown that the accumulation of secretion of the submandibular gland is decreased with corn fed rats for 2 months (27&28).
carcinoma causes a significant increase in number of cells and decrease in secretion amount, concentration of sialic acid and phosphate in the saliva, also the decrease in the relation of protein to sialic acid shows a disruption of glycoproteins biosynthesis (29).

Sirt genes have been involved in the genomic stability preservation, prolongation of life span, and linkage of DNA repair and metabolism, though many of their interactions are not well known (30). Sirt genes apparently show great controversy in different types of neoplasms according to latest findings, it was found that their expressions and activities appear to be downregulated in some types of cancer and upregulated in others (30).

We reported that Sirt-1 gene and its protein expression was significantly increased in salivary cancer group compared to the control group and was significantly decreased in the iPS treated group compared to the salivary cancer group confirming the role of iPS in treating salivary cancer. Similar to our results it was found that Sirt-1 gene were upregulated in different cancer types, such as prostate cancers, acute myeloid leukemia, non-melanoma skin cancers and primary colon cancer (31).

Noh et al. Positive SIRT-1 expression was observed in 67% of patients with invasive non-small cell lung cancers (32). SIRT-1 was found to be highly expressed in 24.5% of the specimens analyzed. High Sirt-1 expression showed a strong correlation with vascular invasion (33).

Xai and Zhou found that Sirt-1 gene and its protein expression were higher in paclitaxel-sensitive cervical cancer tissues than in normal tissues, and significantly higher in Paclitaxel-resistant cervical cancer tissues than in Paclitaxel-sensitive cervical cancer tissues (34).

On the contrary Lai et al. reported that Sirt-1 gene expression was downregulated in head and neck SCC (30).
TGF-β plays an important regulatory role by functioning as tumor suppressor factor in epithelial cells, where it acts as an early inhibitor of growth which induces apoptosis. However, in many cells, TGF-β induces proliferative and anti-apoptotic signals. It was found that the PI3K/Akt pathway is activated by TGF-β in different models and antagonizes the effects mediated by Smad. For example, activation of Akt or PI3K protects cells from apoptosis and proliferation inhibition induced by TGF-β (35).

In this study, the cancer exposed group showed mild positive staining for BAX in comparison to that of the control group. Rats exposed to cancer cells developed hyperplasia of both duct and acinar cells (36). It is assumed that exposure to cancer cells inhibit the apoptotic processes. Whereas, in the Ips treated group showed increase in the BAX immunostaining in comparison to that of the cancer group. In accordance with our study, other studies observed that the BAX immunostaining was decreased in submandibular glandes in rats fed a liquid diet (37).

So we examined Tgf-β gene and its protein expression which was assessed by western blot and immunohistochemistry, its expression was found to be significantly increased in salivary cancer group compared to the control group. Our finding is supported by different studies which declared that TGF-β1 is overexpressed in different types of human neoplasms, which correlates with tumor progression, angiogenesis, metastasis and poor prognostic outcome (38). Reis et al. found that Tgf-β1 was underexpressed in malignant prostatic tissue. Thus, assuming that TGF-β plays an important role in the development of prostate cancer by acting as a cell proliferation inhibitor and an inducer of apoptosis. Furthermore, they identified higher expression levels of Tgf-β in tumors with higher Gleason scores suggesting that Tgf-β may play a very important role in prostate cancer progression and prognosis (39).
We reported that Tgf-β gene expression was significantly decreased in the iPS treated group compared to the salivary cancer group confirming the role of iPS in treating salivary cancer.

It has been documented that several LncRNAs have the ability to control transcriptional alteration, concerning that the difference of the profiling of LncRNA between normal and cancer cells is not considered to be the secondary effect of cancer transformation and LncRNAs are strongly linked with the progression of cancer (32). Thus, profiling of the differential expression of LncRNAs may help in cancer diagnosis, prognosis and selection of effective lines of treatment (7).

We evaluated the expression of LncRNA MALAT-1 and found that LncRNA (MALAT-1) expression was significantly increased in salivary cancer group and iPS treated group compared to the control group but there was a statistical significant decrease in LncRNA (MALAT-1) gene expression in the iPS treated group compared to the salivary cancer group, confirming the ability of stem cells to improve salivary gland carcinoma.

Similar to our results, there was an increase in the expression of the LncRNA MALAT-1 which was firstly detected in metastatic non-small cell lung cancer (40), followed by uterine endometrial stromal sarcoma (41&42) and in 6 other different cancer types, including breast, pancreas, hepatocellular carcinoma, prostate, lung, and colon cancer (43).

Han et al. found that there is an over-expression in MALAT-1 in laryngeal squamous cell carcinoma, and MALAT-1 gene knocking down significantly suppress the growth, invasion and migration of the malignant cells and promotes their apoptosis (44).

Han et al. observed that there is an upregulation in MALAT-1 in bladder urothelial carcinoma as compared with the matched normal urothelium (45).
On the other hand, Tang et al. reported that there was no significant difference in *LncRNA MALAT-1* expression between metastatic and nonmetastatic tissues (7). So SIRT-1, TGF-β and *LncRNA MALAT-1* are considered to be important biomarkers detecting the role of iPS which may be useful in treating salivary gland carcinomas however more research is required to detect the side effects of utilizing iPS in treatment of oral carcinoma including long term follow up of transplanted iPS into SCC induced rats.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**


**Figure legends:**
Figure 1: A) A photomicrograph of rat submandibular gland from the control group showing many lobules (circle) containing mainly serous acini (s), mucous acini (m) and intralobular ducts (d). Notice connective tissue septa (c) between lobules and contain blood vessels (v). H&E, microscopic magnification x 200. B) Higher magnification photomicrograph of rat submandibular gland from the control group showing dark serous acinar cells (s) and pale mucous cells (m). Notice that some mucous acini are capped by serous demilunes (D). The intercalated ducts (ID) are lined by cuboidal epithelium, the myoepithelial cells are seen related to the serous acini (my). H&E, microscopic magnification x 400. C) A photomicrograph of rat submandibular gland from group II showing loss of acinar architecture with hyperchromatic nuclei (arrow). Notice inflammatory cells infiltration (i), dilated congested blood vessels (v) in between the acini. H&E, microscopic magnification x200. D) Higher magnification of group II showing massive inflammatory cell infiltration between acini (i) and presence of dilated congested blood vessels (v). Notice duct hyperplasia (arrow). H&E, microscopic magnification x400. E) A photomicrograph of rat submandibular gland from group II showing duct hyperplasia (arrows). Notice that the inflammatory cells infiltration (i), dilated congested blood vessels (V) and wide separation between the lobules (W). H&E, microscopic magnification x 200. F) higher magnification of group II showing congested blood vessels (v) and ductal hyperplasia (d). Notice that the nuclear pleomorphism (arrow) and fatty infiltration (black star). H&E, microscopic magnification x 400. G) A photomicrograph of rat submandibular gland from group III (stem cell treated group showing normal or less glandular cells (arrow). Notice that congested blood vessels (v) and some duct showing hyperplasia with increase in their number (d) H&E, microscopic magnification x 200. H) higher magnification of group III showing both normal mucous and serous acini (arrow). H&E, microscopic magnification x 400.
Figure 2: A) A photomicrograph of rat submandibular gland from the control group showing intense PAS stain in acinar cells (arrow) and duct (d). B) A photomicrograph of rat submandibular gland from group II showing marked decrease in the staining intensity of the secretory granules (arrow). Notice that increase numbers of intercalated ducts with increase intensity of PAS stain (d). C) A photomicrograph of rat submandibular gland from group III (stem cell treated group showing moderate decrease of PAS staining acini (arrow). PAS, microscopic magnification x 400. D) Mean area percentage of PAS staining.
Figure 3: A) A photomicrograph of rat submandibular gland from the control group showing moderate BAX reaction in both acini and ducts (arrows). B) Photomicrographs of rat submandibular gland from group II showing week positive BAX reaction in acini and ducts (arrow). C) A photomicrograph of rat submandibular gland from group III (iPS treated group showing mild to moderate Bax reaction in acini and duct (arrow). Immunostaining for BAX microscopic magnification x 400. D) Mean number of BAX immunopositive cells.

Mean BAX immunopositive cells in all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean BAX immunopositive cells</th>
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<tbody>
<tr>
<td>Group I</td>
<td>2.5</td>
</tr>
<tr>
<td>Group II</td>
<td>0.5</td>
</tr>
<tr>
<td>Group III</td>
<td>1.5</td>
</tr>
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* denotes a significant difference from Group I. **# denotes a significant difference from Group II.
Figure 4: A) A photomicrograph of rat submandibular gland from the control group showing negative TGF-β reaction in both acini and ducts. B) and C) photomicrographs of rat submandibular gland from group II showing intense positive TGF-β reaction in acini and ducts (arrow). D) A photomicrograph of rat submandibular gland from group III (stem cell treated group showing mild to moderate TGF-β reaction in acini and duct (arrow). Immunostaining for TGF-β microscopic magnification x 400. E) Mean number of TGF-β immunopositive cells.
Figure 5: Real time PCR results for genes expression of a) Sirt-1, b) Tgf-β and c) MALAT-1 LncRNA in all studied groups. Data were expressed as Mean ± SD, p value <0.05 was significant. (*) Denotes significant difference versus control group. (#) Denotes significant difference versus salivary cancer group.
Figure 6: Western blot results for protein expression of a) SIRT-1 and b) TGF-β in all studied groups. Data were expressed as Mean ± SD, p value <0.05 was significant. (*) Denote significant difference versus control group. (#) Denotes significant difference versus salivary cancer group

Table (1): Primers sequence of all studied genes:

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence from 5′- 3′</th>
<th>Gene bank accession number</th>
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</thead>
<tbody>
<tr>
<td><strong>Sirt-1</strong></td>
<td>F: GGGATCTCTAGGCCCCAGTTTC</td>
<td>NM_001107073.1</td>
</tr>
<tr>
<td></td>
<td>R: CTTTGGGGAGAGGGGGAC</td>
<td></td>
</tr>
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<td><strong>Tgf-β</strong></td>
<td>F: TGCGCCTGCAGAGATTCAAG</td>
<td>NM_021578.2</td>
</tr>
<tr>
<td></td>
<td>R: AGGTAACGCCAGGAATTGGCTA</td>
<td></td>
</tr>
<tr>
<td><strong>MALAT-1</strong></td>
<td>F: ACAGGAACTCCATGGCAAACG</td>
<td>FO181540.13</td>
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<tr>
<td><strong>LncRNA</strong></td>
<td>R: AACCGAATTGCTATGGG</td>
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<td><strong>Gapdh</strong></td>
<td>F: AATGGTGAAGGTCG GTGTAAC</td>
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<td>R: AGGTAATGAAAGGGGTGCTTG</td>
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Table (2): The histological grading scale

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<th>Parameter/groups</th>
<th>Control group</th>
<th>Squamous cell carcinoma group</th>
<th>Stem cell treated group</th>
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<tbody>
<tr>
<td>Fatty replacement</td>
<td>None</td>
<td>moderate</td>
<td>None</td>
</tr>
<tr>
<td>Lobular architecture</td>
<td>None</td>
<td>Severe</td>
<td>Mild to moderate</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ductal degeneration</td>
<td>None</td>
<td>severe</td>
<td>Mild to moderate</td>
</tr>
<tr>
<td>Inflammatory component</td>
<td>None</td>
<td>moderate</td>
<td>mild</td>
</tr>
<tr>
<td>Acinar degeneration</td>
<td>None</td>
<td>severe</td>
<td>Moderate</td>
</tr>
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</table>

showed various histological changes in the experimental group compared to the control group. The results indicated significant differences in the grading scale parameters, particularly in terms of inflammation, acinar degeneration, and cellular proliferation. The histological analysis revealed a more severe cellular response in the experimental group, suggestive of an aggressive process.

The histological grading scale is a tool used to quantitatively assess the extent of various histopathological changes in tissue samples. This scale often includes parameters such as inflammation, fibrosis, and the degree of cell proliferation. The results from the histological grading scale can be critical in determining the effectiveness of therapeutic interventions, guiding clinical decisions, and providing insights into the underlying biological mechanisms.

In conclusion, the histological analysis provided valuable insights into the histopathological changes induced by the treatment, highlighting the need for further research into the mechanisms underlying these changes and their implications for patient management.