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AMELIORATION OF ^{131}I INDUCED SALIVARY GLAND DAMAGE BY *OCIMUM SANCTUM* AND AMIFOSTINE PRE SUPPLEMENTATION IN RABBITS

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Keywords:

^{131}Iodine, *O. sanctum* extract, amifostine, salivary glands, radioprotection, xerostomia

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ABSTRACT: Oral administration of ^{131}I to the patients of differentiated thyroid cancer is routinely used to ablate remnant thyroid tissue or metastasis. In 30% of the patients, permanent salivary gland damage due to therapeutic ^{131}I exposure results in xerostomia, affecting their quality of life. Amifostine is the only standard FDA-approved radio protect ant available for damage control, albeit associated with side effects such as hypotension and allergic reactions. The present study was carried out to compare the radioprotective effect of *O. sanctum* extract with that of a standard radio protectant, amifostine in salivary glands of the rabbits exposed to high dose (1GBq) of ^{131}I internal radiation exposure. The study parameters included were salivary amylase, serum SGOT and SGPT, haematological parameters, 99m Technetium pertechnetate scintigraphy study, histopathology and ultrastructure of the salivary gland. The experimental rabbits were sacrificed after 6 months of ^{131}I exposure. The experimental observations, majorly histopathological and electron microscopic are suggestive of better preservation of cell morphology and ultrastructure after *O. santum* as well as amifostine pre supplementation. This indicates the beneficial effects of *O. sanctum* extract pre supplementation for the radioprotection of salivary gland against therapeutic ^{131}I exposure.

INTRODUCTION: Effective management of differentiated thyroid carcinoma involves ^{131}I oral administration for ablating the remnant thyroid tissue and treating recurrent disease [1].

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Though it is mostly concentrated in the thyroid gland, patients receive whole-body internal radiation exposure. In case of recurrence, whole-body exposure increases further due to repeated administration of ^{131}I depending on the disease severity.

Though the therapeutic dose administered is designed with all precautions ensuring the patient's safety, few side effects such as transient anaemia, transient salivary gland swelling, pulmonary fibrosis and hepatotoxicity are observed in this

patient population [2, 3]. In about 20-25% of thyroid cancer patients receiving therapeutic ^{131}I exposure, salivary gland damage of permanent nature is observed, leading to xerostomia and other dental problems affecting their quality of life [3, 4]. It has been observed that other than the thyroid, the salivary glands are another major receiving port for concentrating ^{131}I, as it also shows the presence of sodium iodide symporter, which helps in transporting ^{131}I from blood to inside of thyrocytes [5]. This particularly is a cause of concern for ^{131}I treated patients as they have to bear the discomforts of the dry mouth for the rest of their life. The strategies planned for ameliorating the salivary gland damage either improve the transit time of ^{131}I by using sour candies such as lemon candy, or using sialogogues such as pilocarpine, or controlling the cellular damage with the help of radio-protectants [6, 7].

So far, amifostine is the only FDA-approved radio protectant which has been used in clinical settings for salivary gland radioprotection in case of ^{131}I and external radiation exposure in the management of thyroid and head and neck cancer [8]. However, effective amifostine administration has led to hypotension in patients. Therefore, it needs to be administered under medical supervision [9]. This indicates the need of a still better choice of radio protectant for use in the patient population. Clinical reports of discontinuation of the drug in patients due to adverse effects are available in literature [9, 10]. Many natural dietary components and herbal products are under exploration for their usage as radio protectant [11, 12]. *O. sanctum (Ocimum sanctum) i.e.* Tulasi extract, has been extensively investigated for its radioprotective abilities against external gamma irradiation by Uma Devi *et al.* [13, 14].

It is also known for its antioxidant, anti-inflammatory, hepatoprotective, and many other beneficial properties [15, 16]. Previous work of our group has demonstrated the radioprotective ability of the Tulasi extract against internal therapeutic ^{131}I exposure of 3.7 MBq and 18.5 MBq of ^{131}I in mice and rats, respectively, indicating a beneficial effect on the salivary gland damage [17, 18]. In the present study, we have compared the effect of *O. sanctum* and amifostine pre supplementation on the salivary glands after high dose ^{131}I exposure in higher animals *i.e.,* rabbits.

MATERIALS AND METHOD:

Animal Experiment: The project was approved by Institutional Animal (Project Ethics Committee No BAEC/21/10) [11]. Male New-Zealand white rabbits weighing around 1-1.5 kg were maintained in the Institutional animal house under controlled temperature, humidity, and light cycle with free access to water and a standard colony diet. Animal studies were performed in compliance with the Institutional animal ethics committee of BARC. Rabbits were divided into the following 4 groups,

Group I: Control (n=2)
Group II: Intravenous exposure to ^{131}I (1 GBq) (n=3)
Group III: Pre supplemented orally with *O. Sanctum* extract (40 mg/kg bwt) for 5 days and subsequent exposure to intravenous ^{131}I (1 GBq) (n=3)
Group IV: Pre supplemented with amifostine (200 mg/kg bwt) intravenous 30 min before ^{131}I (1 GBq) exposure (n=3)

^{131}I was obtained from the Board of Radiation & Isotope Technology (BRIT), Vashi, India. *O. sanctum* extract used in the study was obtained as a generous gift from Dr. Yogendra Nayak (Department of pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal India.) Amifostine was procured from NATCO Pharma Ltd.

At three months interval, blood samples of these rabbits were taken and tested for serum SGOT, SGPT and amylase. At the end of six months interval, salivary gland scintigraphy, haemato-logical profile and above parameters were repeated, and all rabbits were sacrificed. Their salivary glands were removed, part of it was stored in 10% neutral buffered formalin and subjected to pathological and electron microscopy analysis.

Saliva Collection: Rabbits were placed in a plastic restrainer cage and injected subcutaneously with pilocarpine (0.4 mg/kg). Saliva samples collected in a glass beaker and stored at -20 ºC were processed for estimation of amylase and protein using standard protocol [19, 20].

Salivary Gland Scintigraphy: Experimental rabbits were placed in a prone position directly on the low energy high resolution (LEHR) collimator of a large field of view gamma camera (SIEMENS signature E.CAM dual camera) After intravenous injection of 5 mCi of ^{99m}Tc pertechnetate, the image of experimental rabbits was acquired and stored in 256 × 256 matrix [21]. This was done to ensure the destruction of the thyroid gland.

Haematology & Serum Biochemistry Analysis: Blood collection was done by ear vein puncture method. 2 ml of blood was collected in heparinised vacutainer for haematology parameters [22]. 3 ml blood was collected in plain tube for serum SGOT and SGPT analysis (ERBA kits & IFCC method).

Histopathology: The excised parotid gland was rinsed in 0.1 M phosphate-buffered saline (0.1 M PBS) and preserved in 10% neutral buffered formalin (w/v) NBF for histology. Subsequently, the fixed tissue was rinsed, dehydrated in graded alcohol series (20%, 30%, 50%, 70%, 80%, 90%, 95% alcohol), and finally in absolute alcohol. The tissue was cleared from traces of water by immersion in xylene and embedded in molten paraffin. Blocks were trimmed of excess paraffin till the tissue was exposed [26]. Tissue sections about 3-5 µ were picked up on precoated poly-L-lysine slides. Slides stained with conventional H&E stains were evaluated for pathological changes with the light microscope (Olympus B × 60).

Electron Microscopy: The excised parotid glands were trimmed into smaller pieces and immersed in ice-cold modified Karnovsky fixative for 4 h at 40 °C. After 4 h, smaller segments 2-3 mm were trimmed. These pieces were further immersed in fresh fixative and maintained at 40 °C overnight. Surplus fixative was rinsed off from the fixed segments with two washes at 15 min intervals with 0.1M cacodylate buffer at 4 °C.

Thereafter, the rinsed specimens were post-fixed with 1% osmium tetroxide (1% OsO4, w/v, Ted Pella, USA) for 60-90 min at 4 ºC. Following post-fixation, the specimens were rinsed with two washes at 15 min intervals with 0.1M cacodylate buffer at 4 °C. Thereafter they were subjected to dehydration with two washes at 15 min intervals with graded series of dry acetone beginning with 30%, 50%, 70%, 80%, 90%, 95%, and dry acetone at 4 °C. The dehydrated specimens were gradually brought to room temperature. The specimens were cleared from traces of water using two washes of toluene for 30 min each. The specimens were exposed to increasing concentrations of the embedding medium prepared in toluene for 1 h each (1 part of embedding medium + 3 parts toluene). Further, the specimens were transferred to a cocktail of two parts of embedding medium and two parts of toluene for 1 h at room temperature. Finally, the specimens were subjected to a cocktail of 3 parts of embedding medium and one part of toluene under vacuum) for 1 h at room temperature.

Ultrathin sections (60-70 nm) were cut using glass knives on an ultramicrotome (Leica Ultra cut R, Leica Microsystems, Wetzlar, Germany). Sections floated on water in the trough having silver & gold interference colours (60-75nm thickness) were picked up on 200 meshed copper grids. Sections were stained with uranyl acetate and lead citrate. Stained sections were observed under a transmission electron microscope (Tecnai G2-12, FEI, Hillsboro, United states) at an accelerating voltage of 80-100kV. Electron micrographs were captured using a Mega View III CCD (Charge Coupled Device) camera and analyzed using the analySIS™ 3.1 software provided with the TEM [24].

Statistical Analysis: Statistical significance was calculated using paired and unpaired Student's "T" test. Statistical significance was assigned to the observations at $p < 0.05$.

RESULTS:

Hematology and Biochemistry Parameters:

Total Erythrocyte Count (RBC): RBC count was found to be decreased significantly in rabbits of Gr II and Gr III as compared with that of rabbits in Gr I (p=0.009, p=0.005 respectively). Whereas a significant increase in RBCs (p =0.003) was observed in rabbits of Gr IV in comparison to Gr II and comparable with Gr I **Table 1.**

Hemoglobin (Hb): In comparison to Gr I, all other groups have shown a reduction in the Hb levels; however, Gr-II has exhibited pronounced reduction (p=0.043). Gr IV has exhibited a slightly significant rise in Hb levels when compared with Gr-II (p =0.055) **Table 1.**

Platelets: Blood platelet counts remained significantly low in Gr II rabbits, compared with Gr I (p=0.003), whereas comparatively milder reductions in platelet counts were observed in Gr III and Gr IV in comparison to Gr I **Table 1.**

TABLE 1: EFFECT OF O.SANCTUM AND AMIFOSTINE PRE SUPPLEMENTATION ON HAEMATOLOGICAL PARAMETERS IN ^{131}I EXPOSED GROUP (N=11)

Parameters	Gr I	Gr II	Gr III	Gr IV
RBC (x10^3 mm^3)	8.2 ± 0.2	5.6** ± 0.6	5.5** ± 0.5	9.2 ± 0.7 $^{\Delta}$
Hemoglobin (gm/dl)	14.5 ± 2.4	9.7* ± 0.8	11.1 ± 0.8	11.8 ± 1.1
Platelet (x10^3 mm^3)	600.5 ± 16.3	383.3* ± 36.7	391.3* ± 57.6	407.3 ± 10.3

Gr I: Control, Gr II: ^{131}I, GrIII: ^{131}I + O.sanctum ,GrIv: ^{131}I + Amifostine * p<0.05 , ** p<0.01 , vs Control Δ p<0.05, vs ^{131}I

Serum Glutamic Oxaloacetic Transaminase (SGOT) & Serum Glutamic Pyruvic Transaminase (SGPT): Statistically significant increase in serum SGOT levels of Gr III as compared with Gr I (p=0.003) was noted at 3 months time interval. Whereas significant reduction in serum SGOT levels was observed in GR IV as compared with Gr I & Gr II at 3 months of the time interval. (P=0.08). No alterations in SGPT levels were observed in the rest of the animal groups. At 6 months of an interval, no major changes in serum SGOT, SGPT levels were noted **Table 2**.

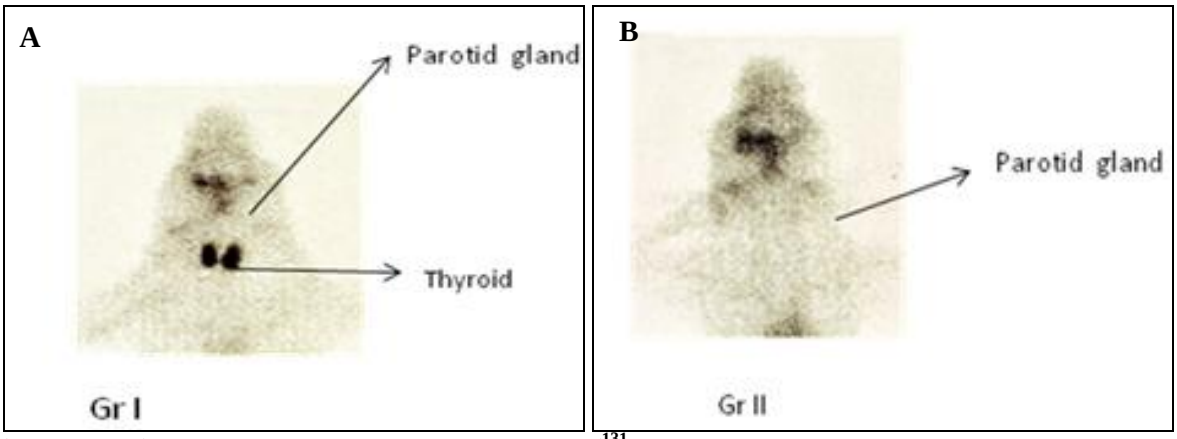
Salivary Amylase & Protein: No appreciable differences were noted in the salivary amylase levels in the rest of the experimental groups (Gr-II, Gr-III, Gr IV) as compared to control (Gr I) **Table 2**.

TABLE 2: EFFECT OF RADIATION EXPOSURE ON THE LIVER ENZYMES AND SALIVARY ENZYMES IN THE EXPERIMENTAL ANIMALS (N=11)

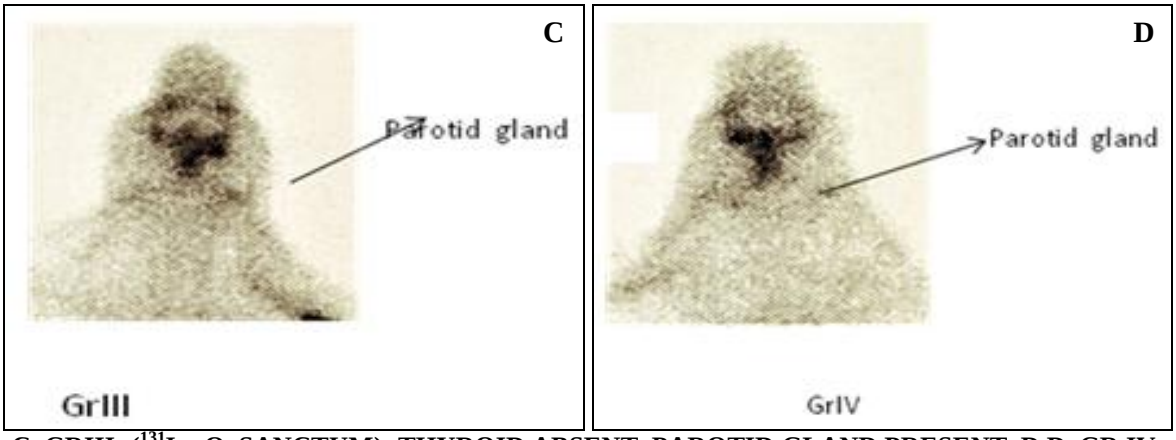
Group	Gr I		Gr II		Gr III		Gr IV	
Period	3M	6M	3M	6M	3M	6M	3M	6M
SGOT	15.0	14.8	14.2	17.2	26.7*	15.3	9.3**	15.2
(IU/ml)	± 0.1	± 4.1	±2.5	±5.9	±3.5	±4.9	±0.7	±5.5
SGPT	23.1	16.0	34.0	26.6	32.8	23.1	22.2	22.2
(IU/ml)	±2.8	±1.4	±8.2	± 9.1	±8.5	±10.1	±3.3	±6.9
Salivary Amylase	952 .0	784.0	1133.0	978.0	855	681.0	948.0	863.0
(U/ml)	±316	± 218	±180	± 65	±311	±43.6	±199	±183.3
Salivary Protein	135.0	160.7	96.9	135.2	127.8	125.6	110.7	169.1
(mg%)	±71	±26.6	±57.8	±25.8	± 58.6	±60.8	±13.3	±68.7

Gr I: Control, GrII: ^{131}I, Gr III: ^{131}I + O.sanctum , Gr IV: ^{131}I + amifostine p<0.05 *p<0.05 vs Control, ** p<0.01 ***p<0.001 Δ p<0.05 vs ^{131}I

99mTc Pertechnetate Scintigraphy Imaging: The absence of thyroid gland was confirmed by 99mTc scintigraphy in rabbits receiving internal ^{131}I exposure (Gr-II, Gr-III, Gr-IV). Control (Gr-I) rabbits exhibited the presence of the thyroid gland in scintigraphy study.



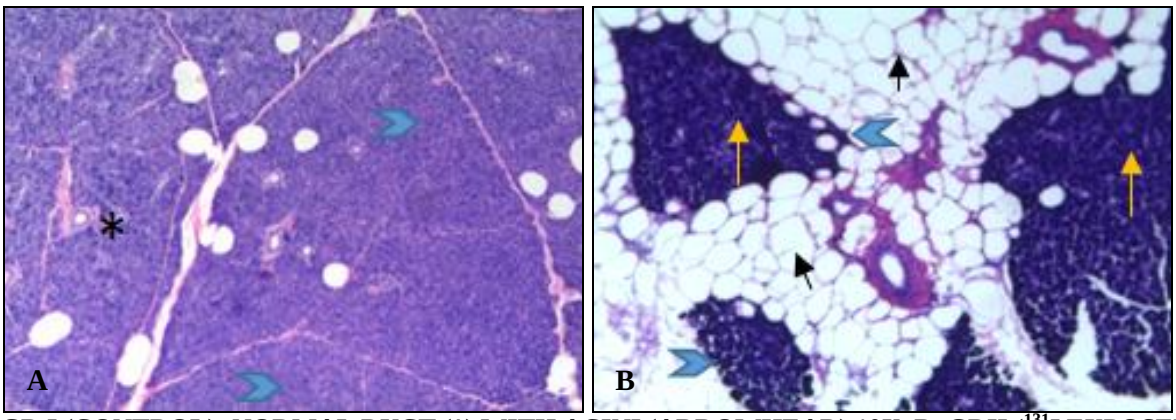
A. GRI (CONTROL): PRESENCE OF THYROID B. GRII (^{131}I EXPOSED: THYROID ABSENT, PAROTID GLAND PRESENT



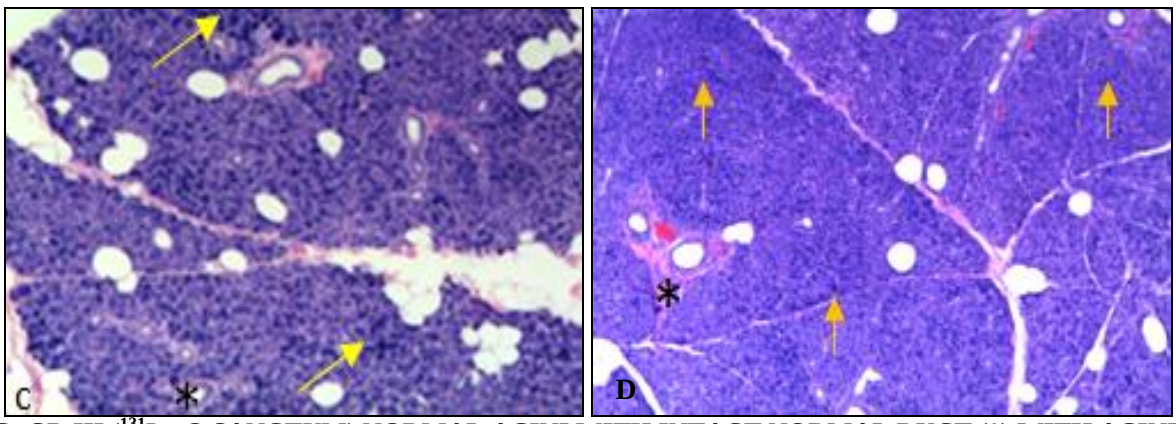
C. GRIII: (^{131}I + O. SANCTUM): THYROID ABSENT, PAROTID GLAND PRESENT, D D. GR IV: (^{131}I+AMIFOSTINE): THYROID ABSENT, PAROTID GLAND PRESENT

FIG. 1: 99M TC PERTECHNETATE UPTAKE IN EXPERIMENTAL RABBITS AT 6 MONTHS OF TIME INTERVAL

Histopathology: The hematoxylin stained tissue sections revealed appreciable cell architecture and morphology changes in rabbit parotid glands. Multiple focal areas of lipomatosis and atrophy were observed in Gr II rabbit parotid glands **Fig. 2B.** Gr III and Gr IV rabbits' parotid glands exhibited minimal lipomatosis and near-normal cell architecture **Fig. 2C, D Table 3.**



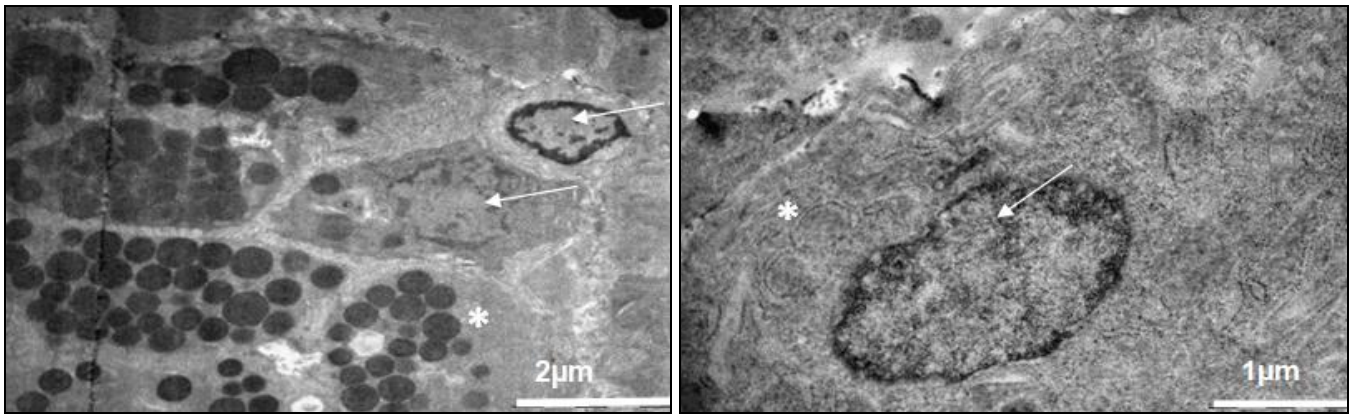
A: GR I (CONTROL): NORMAL DUCT (*) WITH ACINI (ARROWHEAD) 10X, B: GRII (^{131}I EXPOSED): MULTIFOCAL DIFFUSE AREA OF LIPOMATOSIS (BLACK ARROW) WITH ATROPHIC ACINI (ARROWHEAD) AND INCREASED BASOPHILIC DEGENERATIVE CHANGES (YELLOW ARROW)



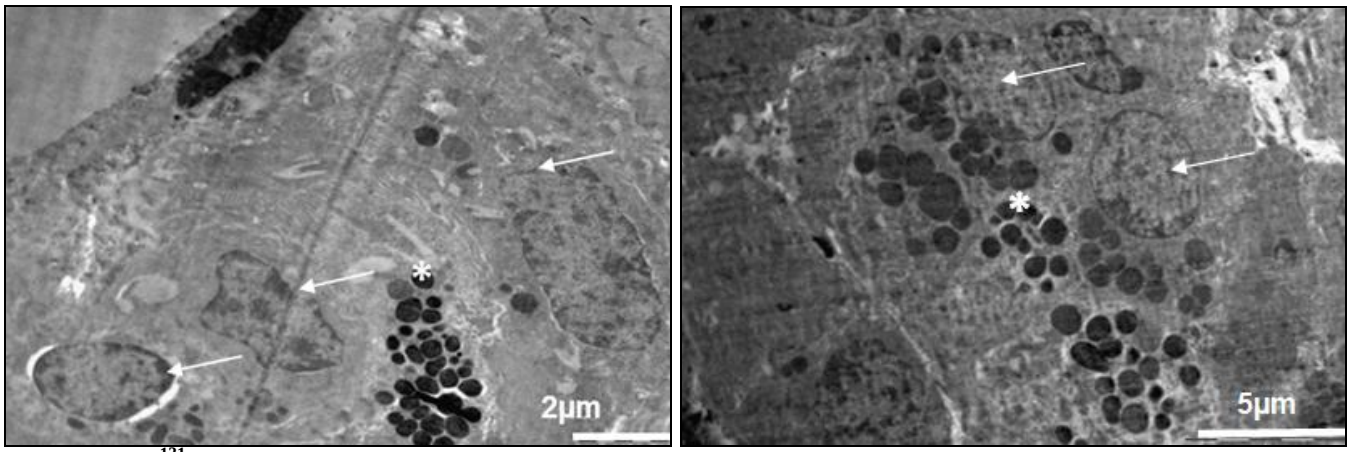
C: GR III (^{131}I + O.SANCTUM) NORMAL ACINI WITH INTACT NORMAL DUCT (*) WITH ACINI. MULTIFOCAL AREAS OF MILD BASOPHILIC CHANGES (YELLOW ARROW) ARE OBSERVED. 10X D: GR IV (^{131}I+AMIFOSTINE): NORMAL ACINI WITH INTACT NORMAL DUCT (*) WITH ACINI. MULTIFOCAL AREAS OF MILD BASOPHILIC CHANGES (YELLOW ARROW) ARE OBSERVED. 10X

FIG. 2: HISTOPATHOLOGICAL CHANGES IN PAROTID GLAND OF THE EXPERIMENTAL RABBITS

Electron Microscopy: The electron microscopic study revealed degenerative changes in cell organelles such as the nucleus, zymogen granules, nuclear membrane, and endoplasmic reticulum in parotid glands of Gr-II rabbits **Fig. 3B.** as compared to Gr I **Fig. 3A**. However, the degenerative changes were less pronounced in Gr III and Gr IV rabbit parotid glands **Fig. 3C, D.** as compared to Gr-II. Amongst all, nuclear pyknosis, destruction of zymogen granules, and loss of cellular junctions are the major changes observed in Gr II rabbits as compared to Gr I; the electron microscopic study revealed degenerative changes in cell organelles such as the nucleus, zymogen granules, nuclear membrane and endoplasmic reticulum in parotid glands of Gr-II rabbits **Fig. 3B.** as compared to Gr I **Fig. 3A**. However, the degenerative changes were less pronounced in Gr III and Gr IV rabbit parotid glands **Fig. 3C, D** as compared to Gr-II. Amongst all, nuclear pyknosis, destruction of zymogen granules, and loss of cellular junctions are the major changes observed in Gr II rabbits as compared to Gr I **Fig. 3.**



A: GR I (CONTROL): INTACT ULTRASTRUCTURE, HEALTHY NUCLEUS (ARROW) WITH INTACT NUCLEAR MEMBRANE AND CHROMATIN. UNIFORMLY SCATTED DARK SECRETORY GRANULES (*). INTACT CELL JUNCTION BETWEEN TWO CELLS MAGNIFICATION=9900X, B: GR-II (^{131}I EXPOSED): PERTURBED ULTRASTRUCTURE, DEGENERATING NUCLEUS WITH SIGN OF PYCNOSIS (ARROW), IRREGULAR NUCLEAR BOUNDARY WITH FRAGMENTED CHROMATIN, AND LOSS OF SECRETORY GRANULES AND OTHER INTERNAL CELL ORGANELLES (*). MAGNIFICATION=11500X



C: GR III (^{131}I EXPOSED+ O. SANCTUM): INTACT NUCLEUS (ARROW) WITH BASEMENT MEMBRANE SCATTERED VARIABLE SECRETARY GRANULES (*). INTACT BASEMENT MEMBRANE MAGNIFICATION = 8200X D: GR IV (^{131}I EXPOSED+ AMIFOSTINE): INTACT NUCLEUS (ARROW) WITH SCATTED UNIFORM SECRETARY GRANULES (*).MAGNIFICATION=4200X

FIG. 3: ELECTRON MICROSCOPIC SECTIONS OF PAROTID GLAND IN EXPERIMENTAL RABBITS

DISCUSSION: Radioprotective abilities of *O. sanctum* extract are well documented by Umadevi and Ganausoundari *et al.* in mice receiving external high dose gamma radiation exposure [13,14]. We have observed radioprotection against internal ^{131}I exposure in our earlier work using rat and mice model [17, 18]. In the present study we have tried to compare the radio protective effect of *O. sanctum*

extract with standard radioprotectant amifostine against high dose internal exposure of ^{131}I by studying hematological profile, biochemical parameters and histopathology of the salivary gland in rabbit model. In the current experiment, a significant reduction in total erythrocyte count, platelets, and hemoglobin were seen in the rabbits exposed to a high dose (1 GBq) of ^{131}I (Gr-II) as compared to the control group (Gr I). Transient depression in bone marrow activity leading to reduction in total blood count is observed in some of the differentiated thyroid carcinoma patients treated with ^{131}I [1, 2]. Significant increase in hemoglobin, platelets, and erythrocytes in Gr-IV and marginal raise in the same in case of Gr-III indicates encouraging effect of *O. sanctum* and amifostine pre supplementation on hematological profile. Radioprotective properties of amifostine and *O. sanctum* against external gamma radiation exposure have been reported by Ganousundari *et al*. on mice bone marrow cells exposed to radiation [13, 14].

The immunomodulatory property of *O. sanctum* is reported in the literature, indicating a beneficial effect on CBC parameters and bone marrow cells [14, 25, 26]. Our observations are in agreement with these reports. Marginal incidence of hepatotoxicity due to ^{131}I therapy is observed in the case of patients with thyroid disorders [27, 28]. The absence of long-term major alterations in liver enzyme SGOT and SGPT in the current experimental group affirms the same. Salivary amylase is an important digestive enzyme secreted by salivary glands. It is often used as one marker for assessing salivary gland damage post-radiation exposure [32, 33].

In our study, salivary amylase activity remained unaltered in ^{131}I exposed groups irrespective of their state of radio protect ant supplementation compared with the unexposed control group at 3 and 6 months duration. In literature, controversial findings are reported regarding amylase activity post ^{131}I exposure. Chitra *et al.* have reported a decrease in salivary amylase and protein in patients of oral cavity carcinoma (OCC) receiving radiation therapy [29]. On the contrary, Blakely *et al.* have reported an increase in salivary amylase activity post-radiation exposure in experimental rhesus monkey model [30]. In few other studies, peaks in salivary amylase are observed, which got normalized over a period of time [31, 32]. Our findings are in agreement with those observations. Salivary secretion is a result of stimulation of parotid, submandibular, lingual, and salivary glands [32]. Amongst them, parotid glands are majorly functional and radiosensitive in nature. However, other glands also contribute towards salivary secretion [32]. Hence, post ^{131}I exposure through parotid glands is likely to be adversely affected; other salivary glands may contribute to the salivary output. In the present study, saliva collection was not exclusively done from the parotid gland by cannulation but was a result of stimulation of all salivary glands under pilocarpine stimulation. All these factors may be responsible for observed unaffected salivary amylase levels.

histopathological examination of the salivary gland has revealed major atrophy and lipomatosis in acinar cells of the parotid glands of the rabbits exposed to only ^{131}I as compared to control rabbits. *O. sanctum* and Amifostine pre supplemented rabbits have exhibited low lipomatosis and near-normal acinar cell morphology in their parotid glands compared with ^{131}I exposed rabbits, which agrees with the findings of Bohuslavzki *et al.* [21]. Electron microscopy study of the parotid glands of the rabbits exposed to only ^{131}I has exhibited nuclear pyknosis and depletion of secretory granules, whereas *O. sanctum* and amifostine pre supplemented rabbits have shown intact nucleus and less degenerative changes in secretory granules, cell junctions and other cell organelles **Fig. 2.** Kutta *et al.* have reported similar observations regarding the parotid gland of rabbits pretreated with 1 GBq [33]. This indicates the protective effect of amifostine and *O. sanctum* extract pre supplementation at the cellular level in experimental rabbits.

Various strategies are planned to increase the transit time of ^{131}I to circumvent or attenuate the salivary gland damage, which includes glandular massage after oral ^{131}I administration, sucking lemon candy or pilocarpine stimulation to increase the salivation [6, 7]. However, these are of limited utility and are also controversial in some instances. Amifostine is the only FDA-approved drug available for radioprotection of salivary glands, which has well-known side effects needing its discontinuation in adversely affected patients.

Kim *et al*. have reported controversial findings of cytoprotection and parenchymal radioprotection by amifostine pre-treatment in salivary glands of patients receiving ^{131}I therapy questioning its effectiveness in clinical settings [35]. As entry of ^{131}I in salivary glands cannot be prevented due to the presence of sodium iodide symporters, the other alternative approach for radioprotection is to increase the scavenging of the reactive species generated by suitable means. In such circumstances, antioxidant pre supplementation presents an appropriate means for controlling the cell damage within the gland. *O. sanctum* is a potent antioxidant that is radioprotective for hematopoietic system as well as an anti-inflammatory in nature. It's found to be nontoxic even at higher doses (2 gm/kg) and is known to have no side effects *in-vivo*. Our study has demonstrated a comparable radioprotective effect of *O. sanctum* extract pre supplementation with FDA-approved radio protect ant amifostine in rabbits. In conclusion, *O. sanctum* extract needs to be further tested in higher concentrations for its effect on the functional ability of the salivary gland in higher animals towards exploring its usage in clinical settings.

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CONFLICTS OF INTEREST: Authors declare that there is no conflict of interest

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