The Mammary Gland Structure after Submaxillectomy in Adult Female Albino Rats: A Histological Study
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Abstract
The surgical extraction of the submandibular salivary glands or depletion of its peptides led to some atrophic effects on the structure and function of various organs. Consequently, total submaxillectomy could be used as a preventive measure or approach of mammary gland neoplasia. To reveal the exact role(s) of the submandibular active peptides on the normal mammary glands structure; two groups of female rats were used, control group I: intact (Ia) and sham operated (Ib), and group II submitted to total submaxillectomy. Biochemical analysis of serum epidermal growth factor (EGF) was estimated. The mammary glands were prepared for whole mount Carmine alum, Hematoxylin and Eosin (H&E) and Masson’s trichrome (MT) stain procedures and analysis of tissue epidermal growth factor (EGF), insulin like growth factor 1 (IGF1) and transforming growth factor α (TGFα) tissue proteins. A significant decrease of all examined submandibular gland peptides in serum and in the mammary tissue was detected in the group II. The whole mount technique revealed an overall decreased size of the mammary glands ductal tree which appeared less arborescent. Abnormally directed tertiary ducts, stunted terminal end buds and defective lateral and alveolar buds were demonstrated. H&E and MT stained sections revealed overall decreased connective tissue elements in the form of thin capsule, fine interlobular septa and a decrease of fibroblastic stroma around the ducts and blood vessels. More abundant adipose tissue in the interstitium was noted. Less developed parenchyma in the form of fewer and thinner abnormally directed interlobular ducts and the vacuolated luminal epithelium were seen. A prominent number of inflammatory cells infiltrating the stroma and some ductules were recorded.

Keywords: Female rat, Mammary gland, Submaxillectomy, Whole mount.

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Introduction

It is well known that, several biologically active peptides and hormones are secreted from the submandibular gland e.g. epidermal growth factor (EGF), nerve growth factor (NGF), transforming growth factor-alpha and beta (TGFα, β) hepatocyte growth factor (HGF), insulin-like growth factors I and II (IGF-I, IGFII), and basic fibroblast growth factor (bFGF)(1).

These peptides are important in regulating growth, maturation, function, and maintenance of many tissues particularly, the gut and brain (2). The influence of the submandibular salivary glands on the morphology of many organs such as liver and thyroid was reported (3).

Like any tissues, the mammary gland has been known to be affected by these factors (4, 5). The submandibular EGF and TGFα are potent mitogenic for both normal and neoplastic mammary epithelium either in vitro (6) or in vivo (7). After their local administration in the mammary fat pad, both factors stimulate the lobulo-alveolar formation in mammary gland (8). Moreover, EGF is also involved in cellular motility, survival, and development of all organs (9). It acts by binding EGF receptor on the cell surface and stimulating the intrinsic protein-tyrosine kinase activity of the receptor, resulting in a variety of biochemical changes within the cell that ultimately lead to DNA synthesis and cell proliferation (10). EGF is accused in tumor development as it leads to a significantly increased intravasation and metastasis from the primary tumor (11). Over expression of EGF receptor in breast cancer was recorded (12). The highest level of IGF-I expression in the fat pad of mammary tissue was recorded during the pubertal growth phase and during late pregnancy (13).

Classically, submaxillectomy is a safe surgical technique (14) performed for the treatment of tumors or obstructive conditions of the submandibular gland, especially in the case of hilar-parenchymal obstructions (15).

Despite of a plethora of animal studies that had explored the effects of submaxillectomy on many organs; its precise role on the resting mammary gland structure is still not fully resolved. We hypothesized that histological examination of mammary glands in both groups via whole mount carmine alum technique; H&E and MT stains may clarify the effect of surgical extraction of the submaxillary salivary gland on mammary gland. So, this research was designed to have a glimpse of structural changes of the mammary gland that may occur after submaxillectomy and debate according to these changes, the possibility of using complete ablation of the
submandibular glands as a preventive procedure of breast tumorigenesis at high risk susceptible females.

**Materials and Methods**

**Animal model**

Thirty two mature virgin female albino rats aged eight weeks (130-180gm) were obtained from animal house of Faculty of Medicine, Zagazig University, Zagazig, Egypt. They were housed (25 ± 2°C; 12:12 hours light/dark) with food and water *ad libitum* according to the guidelines of Faculty of Veterinary Medicine, Zagazig University, Egypt. Following acclimatization for a week, they were divided into two groups (n=16):

a- **Control group (I):** was further subdivided equally into two subgroups: intact subgroup (Ia) and sham operated subgroup (Ib), which underwent only cervical midline skin incision and full mobilization of the glands without ablation (16).

b- **Experimental submaxillectomized group (II):** animals were exposed to surgical ablation of their submandibular salivary glands. The surgical operation was performed under light ether anesthesia between 8.00 am and 13.00 pm to mitigate the effects of diurnal cycles. The submandibular glands were excised through a mid-line cervical incision and the main vessels were tied with 4-0 silk ligature. Care was taken to preserve sublingual and parotid glands, and not to interfere with their blood supply. The submandibular duct was ligated with 4-0 silk under the dissecting microscope. Skin wound was closed with 3-0 silk (16). After recovery from anesthesia the rats were left in their cages for about 90 days.

**Rats' weight:**

The rats' body weight was recorded at the beginning and at the end of experiment.

**Determination of stages of estrous cycle:**

Before the end of the experiment (90 days), all rats were examined daily to detect the estrous cycle stage. Their vagina was lavaged with physiologic saline (0.9% NaCl) to obtain vaginal smears. All smears were stained with papanicolaou stain. Proestrus stage was identified by presence of small, rounded nucleated, cornified epithelial cells with few to no leukocytes. Estrus stage was determined through the presence of numerous and clumped large cornified cells with degenerated nuclei. Metestrus stage was determined by the presence of many leukocytes and few cornified cells, and the diestrus stage smear was composed of many leukocytes (17). At estrus stage, the rats were sacrificed after ether inhalation.

**Biochemical analysis:**
For analysis of serum EGF: venous blood samples were withdrawn from the retro-orbital sinus of all rats and collected in clean glass centrifuge tubes. Blood samples were allowed to clot at room temperature for 30 minutes and clarified by centrifugation, then; the sera were stored at -20°C.

**Histochemical analysis:**
For analysis of mammary EGF, IGF I and TGFα tissue protein; small pieces of right mammary glands were weighed, homogenized in 20 volumes (wt/vol) of phosphate buffer saline (PBS) at 37°C, and centrifuged at 15,000 rpm for 20 min at 4°C; then the supernatants were stored at −20°C.

**N. B.:** Both the sera and the supernatants were processed according to the method described by (18).

**Light microscope study:**
The left inguinal mammary glands were prepared for whole mount Carmine alum staining technique (19). The remaining parts of right glands were fixed overnight in 10% neutral buffered formalin, processed for paraffin technique, sectioned (5µm) and stained with H&E and MT (20).

**Whole mount Carmine alum staining technique:**
The left dissected mammary glands were immediately spread into glass slides, carefully corresponding to their original size and shape in situ. The glands were fixed in Carnoy's fixative (25% glacial acetic acid, 75% absolute ethanol alcohol (EtOH)), at room temperature in the fume hood for 2 days or longer, washed in 70% EtOH for 1 hour, rinsed in distilled water for 30 min, stained in carmine alum stain for 2 days or longer, dehydrated in ascending grades of alcohol and cleared in xylene for at least for 2 days.

**N. B.** The fattier the gland, the longer clearing time is required.

**Morphometrical analysis:**
Using whole mount carmine alum stained slides, total mammary gland, epithelial area and interductal fat pad area, were measured by Leica Qwin 500 image analyzer computer system (Leica Imaging system, Ltd, Cambridge, England). The lateral and alveolar buds were counted by stereomicroscope. All parameters were measured in the image analyzing unit of the Pathology Department, Faculty of Dentist, Cairo University, Cairo, Egypt.

**Statistical analysis:**
Statistical analysis was performed on all previous parameters and the obtained data were expressed as mean values ± standard deviation and analyzed using unpaired student’s T-test. Differences were considered to be significant at P<0.05 against the
control group.

**Results**

**General observations**
Throughout this study no deaths of rats occurred. During dissection, the control (intact and sham operated) mammary glands appeared slightly tough and rigid while, the submaxillecotomized mammary glands appeared very soft and friable. No biochemical, morphometrical, statistical or histological differences were detected between both intact and sham operated groups.

**Biochemical and Histochemical results:**
The submaxillecotomized group showed a statistically significant decrease of serum EGF as P <0.05 (Table 1 and Histogram 1). A highly statistical significant decrease of tissue EGF as P <0.001 and a significant decrease of tissue IGF I and TGF α as P < 0.05 were detected in submaxillecotomized group in comparison with control group (Table 2 and Histogram 2).

**Table 1:** Statistical analysis of mean values of serum EGF (ng/ ml) in control and submaxillecotomized groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ±SD</th>
<th>Range</th>
<th>T test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.5±9</td>
<td>46.5-82.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submaxillecotomized</td>
<td>10.32±3</td>
<td>4.32-16.38</td>
<td>18.06</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

This table shows a statistically significant decrease of serum EGF in submaxillecotomized group as P<0.05 in comparison with control group.
Histogram 1: Mean values of serum EGF in control and submaxillectomized groups.

Table 2: Statistical analysis of mean values of tissue EGF, IGF I and TGFα (ng/mg):

<table>
<thead>
<tr>
<th>factors</th>
<th>Control</th>
<th>Submaxillectomized</th>
<th>T test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Range</td>
<td>Mean ±SD</td>
<td>Range</td>
</tr>
<tr>
<td>EGF</td>
<td>0.47±0.05</td>
<td>0.37-0.57</td>
<td>0.12±0.02</td>
<td>0.08-0.16</td>
</tr>
<tr>
<td>IGF I</td>
<td>0.62±0.1</td>
<td>0.42-0.82</td>
<td>0.08±0.02</td>
<td>0.04-1.02</td>
</tr>
<tr>
<td>TGFα</td>
<td>0.24±0.1</td>
<td>0.04-0.44</td>
<td>0.04±0.01</td>
<td>0.02-0.06</td>
</tr>
</tbody>
</table>

This table shows that there is a highly statistical significant decrease of tissue EGF as P <0.001 and a significant decrease of tissue IGF I and TGFα as P<0.05 in submaxillectomized group in comparison with control group.
Histogram 2: Showing mean values of tissue EGF, IGF I and TGFα in control and submaxillectomized groups.

Histological results
Control group (I)
Examination of carmine alum staining whole mount slides revealed that, the fat pad of the gland was completely laced with the ductal tree nearly and reached the edge of the fat pad (Figure 1A). The ductal tree appeared highly arborescence (Figure 2A). Large lactiferous ducts were branched and expressed extensive lateral branching along their length (Figure 3A). An overall prominent number of lateral and terminal ductal branches were observed (Figure 3C). Many florid terminal ductules with a prominent number of alveolar buds were demonstrated on expense of little amount of fat pad (Figure 3E).

Examination of H&E stained sections revealed that the rat's mammary gland was formed of two different compartments: connective tissue stroma and epithelial tissue parenchyma. The stroma was consisted of thick capsule, many septa dividing the mammary gland into lobules and dense fibrous connective tissue surrounding the ducts and the blood vessels (Figures 4A,B&5A,C,E). The adipose connective tissue ensheathed the ductal dense fibrous connective tissue was formed of unilocular adipocytes(Figures 4,5&6). The parenchyma of the mammary gland was mostly composed of scattered tubular ducts enclosed with relatively thick, intensely eosinophilic connective tissue. While, the alveolar buds were enclosed with
obviously thin layer of connective tissue (Figures 4A, B&5A, C). Their lining epithelium was formed of two layers. The luminal layer was consisted of deeply basophilic teethed shaped high cuboidal cells and sparse cytoplasm, while the outer one was rounded to flat shaped cells. At the poles of terminal ductules (terminal end bud), a mass of cells was detected. Numerous flat nuclei of fibroblasts were embedded within the fibrous stroma (Figure 5C).

Masson's trichrome stained sections revealed that the connective tissue around the ducts was collagenous (Figure 4C).

Submaxillecetomized group (II)

Examination of carmine alum stained whole mount slides revealed an overall decrease in the length of ductal tree. It occupied nearly half of the fat pad. The fat pad enclosing the mammary tree appeared unaffected (Figure 1B). The ductal tree appeared thin and less arborescence (Figure 2B). The large ducts with nearly absent arborization were seen (Figure 3B). Less and thinner tertiary ducts with abnormally directed lateral branching ducts were predominant. The terminal end buds were stunted and their ends were blunted (Figures 3D &F).

The examination of H&E stained sections revealed thin capsule, more abundant adipose tissue in the interstitium (Figure 4D) and thin interlobular septa (Figure 4E). The periductal fibrous connective tissue was moderately thick. While, the amount of stroma surrounded the alveolar buds appeared unaffected (Figures 4E, F). A little amount of pale eosinophilic fibrous connective tissue surrounded abnormally directed terminal ducts was noticed (Figure 5B). The subtending ducts were curved. The blood vessels were congested and dilated. Some mast cells and fat cells invaded the periductal stroma around the deformed subtending ducts (Figure 5D). The fibrous connective tissue around the blood vessels was thin (Figure 5F). Disintegrated foamy luminal cells lining subtending ducts and some foamy macrophages were detected (Figures 6A&B). Heavy cellular infiltration of the stroma like, eosinophilis and monocytes was observed (Figures 6C &7D). Blood capillaries and mast cells invaded the stroma around collapsed alveolar buds (Figure 7A). The mast cells in the stroma were shown either singly (Figure 7B) or in clusters (Figure 7C).

Masson's trichrome stained sections revealed a thin layer of collagen fibers surrounding deformed dilated ducts (Figure 4G).
Figure 1: Photographs of whole mount Carmine alum of mammary glands of control group (A) and submaxillectomized group (B) according to whole-mount Carmine alum stain. (A): Indicating the ductal tree nearly reaching the edge of the fat pad. (Carmine Alum stain). (B): Indicating the ductal tree nearly filling half of the fat pad. The mass of fat pad appeared nearly unaffected in both groups. Notice the chain of lymph nodes, the landmark of mammary gland in the center is prominent in the control group. (Carmine Alum stain). (using a Hitachi KP-D50 color digital camera).

Figure 2: Photomicrographs of mammary glands of control group (A) and submaxillectomized group (B) according to whole mount Carmine alum stain.

(A): Indicating a highly arborization of ductal tree. (Carmine Alum stain, X 20).

(B): Indicating a little arborization of ductal tree. (Carmine Alum stain, X 20).
Figure 3: Photomicrographs of mammary glands of control group (A,C,E) and submaxillectomized group (B, D, F) according to whole mount Carmine Alum stain. (A): Indicating branching large ducts (arrows) with extensive lateral buds (arrow heads) along the ducts. (Carmine Alum stain, X 40). (B): Indicating large ducts(arrows) with unapparent lateral budding (arrow heads). (Carmine Alum stain, X 50). (C): Indicating arborization of tertiary ducts(arrows) alveolar buds (arrowheads) interlacing with little amount of fat pad (FP). (Carmine Alum stain, X 30). (D): Indicating fewer thin arborization of tertiary duct(arrows)and lateral branching ducts(arrowheads) (Carmine alum stain, X40). (E): Indicating large bulbous florid terminal alveolar buds(arrows) and lateral bulbous end buds (arrowheads),(Carmine Alum stain, X50). (F): Indicating irregularly directed blunt ended lateral branching ducts and nearly absence of terminal end buds (arrow heads) were seen interlacing with apparent amount of fat pad(FP). (Carmine Alum stain, X40).
Figure 4: Photomicrographs of mammary glands’ sections of control group (A-C) and submaxillectomized group(D–G). (A): Indicating a thick capsule, many septa (arrows) and adipose stroma(H&E, X100). (B): Indicating scattered tubular duct system (arrows) surrounded by dense eosinophilic connective tissue and enclosed by adipose stroma (H&E, X 100). (C): Indicating a thick layer of collagen fibers (arrows) around the ducts and a thin layer of the collagen fibers around alveolar buds (arrow heads) (MT, X100). (D): Indicating a thin capsule facing the abdominal muscles (H&E, X 100). (E): Indicating thin septa (H&E, X 100). (F): Indicating a decrease of the amount of fibrous stroma around the deformed dilated ducts and the alveolar buds (H&E, X100). (G): Indicating thinning of collagen fibers (arrows) surrounding the ducts (MT, X 100).
Figure 5: Photomicrographs of mammary glands' sections of control group (A,C,E) and submaxillectomized group(B, D, F). (A): Indicating a straight large duct with distinct lumen and many alveolar buds (arrows) (H&E,X400). (B): Indicating an abnormal directed duct (arrows) (H&E,X400). (C): Indicating a subtending duct surrounded by homogenous dense eosinophilic fibroblastic stroma containing numerous flat nuclei of fibroblasts (arrows heads). A mass of deeply basophilic cells surrounded by cap cells located at its periphery (terminal end bud) (open arrow) is seen (H&E,X1000). (D): Indicating an abnormal shaped subtending duct (open arrow) invaded by fat cells, blood capillary (bv), abnormal alveolar buds (ab) and mast cell (arrow head). Note abnormal shaped lateral end bud (arrows) and pale eosinophilic fibrous stroma around the duct (H&E,X400). (E): Indicating a thick dense stroma around the artery (arrow) and the vein (arrow head) (H&E,X400). (F): Indicating a thin stroma around the artery (arrow) and the vein (arrowhead) (H&E,X400).
Figure 6: Photomicrographs of mammary glands' sections of submaxillectomized rats.

(A): Indicating disintegrated luminal epithelium (arrows). The luminal cells are pale, basophilic and vacuolated (v) (H&E, X 1000). (B): Indicating foamy macrophages invading the luminal epithelium. A dilated small blood vessel (bv) is seen (H&E, X 1000). (C): Indicating a stroma populated by many immune cells particularly many eosinophils (arrows) and monocytes (arrow heads) (H&E, X 400).

Figure 7: Photomicrographs of mammary glands' sections of submaxillectomized rats (A): Indicating irregular shaped, collapsed alveolar buds (arrows). Note a mast cell (arrow head) near a blood capillary (bv) (H&E, X 1000). (B): Indicating a prominent number of scattered mast cells (arrows) (H&E, X 1000). (C): Indicating a cluster of mast cells (arrow heads) (H&E, X 1000). (D): Indicating eosinophils (arrows) and monocytes (arrow heads) (H&E, X 1000).
Morphometrical results:
The submaxillectomized group showed a significant decrease of epithelial area, a significant increase of interductal fat pad area (Table 3 and Histogram 3) and a significant decrease of number of lateral and alveolar buds (Table 4 and Histogram 4).

**Table 3:** Statistical analysis of mammary glands areas (cm²) between control and submaxillectomized groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total mammary gland area</th>
<th>Epithelial area</th>
<th>Interductal fat pad area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.45 ± 0.67</td>
<td>8.35 ± 0.89</td>
<td>7.10 ± 0.30</td>
</tr>
<tr>
<td>Submaxillectomized</td>
<td>14.52 ± 0.57</td>
<td>4.30 ± 0.61</td>
<td>10.22 ± 0.12</td>
</tr>
<tr>
<td>P value</td>
<td>0.003*</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>

This table shows a highly significant decrease of epithelial area and a highly significant increase of interductal fat pad area of the submaxillectomized mammary glands.

**Histogram 3:** Shows Mammary glands areas analysis.
Table 4: Statistical analysis of body weights, lateral and alveolar buds of mammary glands in control and submaxillectomized groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weights(gm)</th>
<th>Lateral buds</th>
<th>Alveolar buds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150±30</td>
<td>190±40</td>
<td>195±40</td>
</tr>
<tr>
<td>Submaxillectomized</td>
<td>170±40</td>
<td>47.5±10</td>
<td>32.5±11</td>
</tr>
<tr>
<td>P value</td>
<td>0.182</td>
<td>0.008*</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

This table shows a significant decrease of number of lateral and alveolar buds of the submaxillectomized mammary glands.

Histogram 4: Shows rat weights, lateral and alveolar buds of mammary glands in control and submaxillectomized groups.

Discussion

The mammary gland is a tool for examining many aspects of developmental, endocrine, and tumor biology. It is a complex tissue, its complexity is attributed to the continual undergoing developmental changes in structure and function(21).

The submandibular gland is a “duacrine” gland. Its biologically active peptides and hormones are shown to modify a variety of functions including growth, differentiation, enzymatic control, homeostatic regulation, and adaptation to stress (22,23). Normally, the salivary glands synthesized subtle amounts of these
proteins and peptides, but a significant impact occur on systemic response to a variety of stresses and pathologies (1,3). So, these peptides are important factors in the etiology of glandular inflammation and malignancies (23).

In the present study, the sacrifice time was at estrus stage (the end of the luteal phase of estrous cycle), to control the fluctuations in hormone levels. It represents the most differentiated mammary gland state (maximum alveolar proliferation)(16, 24).

In the current study, all animals showed a trivial change in their body weight. Yah and Jang(25) suggested that submaxillectomy is an endocrinal and not a nutritional problem.

As serum EGF was declined after the fourth week of submaxillectomy(26), we chose the sacrifice time after 90 days of operation. In the present work, a significant decrease of tissue EGF, IGF and TGFα and a significant reduction in serum EGF concentration in submaxillectomized group were detected(27). Similar serum and urine levels of EGF in submaxillectomized mice after more than three weeks of surgery in comparison with the control and sham-operated animals were reported(3). Surprisingly, Byyny and coauthors (28) reported unchanged serum level of EGF after submandibular extraction. In submaxillectomized mice, the parotid glands EGF content was lower while EGF content in thyroid glands and kidneys was higher (3). The submandibular gland is considered a major source of circulating EGF (26). Recently, local synthesis of EGF in mammary tissue and in milk was reported (29).

The best technique used to visualize the mammary gland architecture is known as whole-mounting, in which the entire tissue is spread on a glass slide and stained while preserving the 3D structures and allowing accurate quantitative measurements (18). The present hypothesis tested by (whole mount technique) revealed an overall reduction in ductal tree nearly to half in comparison to rats whose submandibular glands had not been removed (intact and sham operated animals). These data were in accordance with (31). They hypothesized that the biologically active factors secreted by the submaxillary gland could prevent either cell degeneration or cell differentiation and death.

Poorly branched mammary glands with rudimentary thin ducts and non visible TEBs are documented after a significant decrease of fibroblast growth factor FGFb2. Ductal branching, formation and maintenance of terminal end buds in the mammary gland are dependent upon FGFR2b signaling, especially in the immature and after weaning mammary glands(32). In contrast, ablation of the submaxillary glands increased tumor infiltrating natural killer cells (33). Moreover, there was a reduction of about 30% in Ehrlich tumor size when the tumor was previously incubated with
submaxillary gland extract (34).

An apparent discrepancy between the present results which showed no effect on the size of the fat pad in all groups and the observations of (35) who revealed proportional relation between the size of fatty stroma and ductal length. Normally, the TEBs when reach the connective tissue surrounding another epithelial structure or the capsule at the edge of the fat pad, they regress to form terminal ductules. The regressed TEB becomes encased in their basement membrane and stroma (36). Retarded ductal elongation, abnormal appearance of both terminal and lateral end buds which were irregularly directed with stunted blunted ends without formation of terminal ductules and defective, non bulbous alveolar buds in the present observations occurred unrelated to the edge of fat pad or the connective tissue surrounded other ducts. Similar results were observed after inactivation of EGF and amphiregulin genes (4).

The cellular basis of ductal elongation and bifurcation of mammary gland remained obscure. Initiation and elongation of new mammary ducts need fibroblast growth factor II (FGFII) (33,38). Some studies had attributed these changes in the architecture of the mammary glands to the decrease of EGF and FGF after submaxillectomy (21). EGF may stimulate epithelial synthesis of type IV collagen (39), an important component of the basal lamina required for epithelial attachment and proliferation (40). However, EGF is responsible for tubuloalveolar development and differentiation in lactogenic mammary gland only (4). They added that, amphiregulin (AR) (an EGF family growth factor which binds and activates EGF receptor) is the principle factor in mammary ductal branching. However, additional loss of EGF and TGFα may further exacerbate delay in ductal outgrowth in absence of AR. They hypothesized that, these changes were due to the imbalance between DNA synthesis and apoptosis caused by absence of these active peptides. Lower pregnancy rate and increased occurrence of abortion in addition to delayed total duration of parturition as reflected in longer duration of the expulsion of their pups and placenta and longer time interval between expulsion were reported submaxillectomized females (41). The submaxillectomized rats could not nurse their pups (42).

The submaxillectomized mammary glands showed thin capsules, fine interlobular connective tissue septa, more abundant adipose tissue in the interstitium and less and thinner tertiary interlobular ducts. An apparent discrepancy between these data and those reported in a previous study on submaxillectomized lactating mammary glands (42). They demonstrated thickening of capsule, abundant adipose tissue, a less
extensive parenchyma with very few secretory alveoli devoid of milk secretion, corpora amylacea and constricted lumens of the secretory tubules and interlobular ducts. The epithelial synthesis of type IV collagen of the basal lamina required EGF(40). The extracellular matrix (ECM) has long been recognized as providing signals during mammary gland branching (43). A study involving development of the mammary gland in vivo showed that inhibition or augmentation of either collagen fibers or glycosaminoglycan deposition attenuates mammary gland branching (44).

Histological study of the submaxillectomized rats revealed disintegrated luminal epithelium lining subtending ducts. Parsa and coauthors (32) showed that, survival and proliferation of the luminal epithelial progenitor cells are controlled by FGFb2. Another investigation referred disintegrated epithelium to activation of other ECM proteases by mast cells (45). The experimental group in the current work showed decreased numbers of fibroblasts and thickness of fibroblastic stroma surrounding the ducts. The growth factors secreted from activated platelets(46) and FGFb2, but not EGF, are sufficient to drive mitosis of fibroblasts(47).

The unique position of eosinophils(at TEBs) in the normal mammary gland and the preferential effect of their depletion on ductal branching strongly suggest that their role in regulating branching complexity, perhaps occurred by providing inhibitory signals at branching points(35). In the current work, both eosinophils and monocytes were observed in the interstitium. The eosinophils situated at the TEBs express the monocyte chemoattractant which attracts macrophages. The macrophages in ductal epithelium are responsible for engulfment of apoptotic cells and in their absence, this process may be inefficient (48, 35). In addition, macrophages synthesize many proteases including matrix metalloprotease (an enzyme that remodels collagen) that might be necessary for processing of ECM and in particular, the collagen fibers (49).

Normally, in the mammary gland, the mast cells are scattered throughout the stromal tissue, but in relatively small numbers, and did not appear to localize at any particular structure (50). Their proteases have been implicated in angiogenesis (51) and ECM protein degradation either directly (52) or by activation of other ECM proteases (45). The present data showed an apparent number of mast cells near blood capillaries, in the disintegrated stroma, around abnormally directed subtending ducts and alveolar buds. Appearance of mast cells in unusual sites in the mammary gland was found to compensate the decrease of submandibular growth factors as mast cells are a rich source of growth factors and also produce a host of serine proteases, pro-inflammatory cytokines, proangiogenic factors such as vascular endothelial
growth factor (VEGF) and chemokines. Their deficiency results in fewer duct ends and TEBs (49).

Although a reduction in mammary epithelial mass after ovariectomy (due to loss of estrogen) was reported (30). However, further evidence suggested that the estrogen-induced breast carcinoma in human is mediated by EGF (6). Submaxillectomy was shown to affect the ovarian hormone (26) and decrease size, local invasion, and lymph node metastasis of human gastric tumor (53).

The control of proliferation, differentiation and maintenance of normal mammary epithelial cells is probably occurring via very complex interactions between multiple growth factors, hormones and cells. Tries to understand normal breast development and how external risk factors alter breast development are important antecedents to control breast cancer incidence. However, it is difficult to assign the most influential factor on the mammary gland.

**Conclusion:**
The present study confirmed that the normal structure of mammary glands is dependent on submandibular gland’s peptides. Generalized atrophy of both stroma and parenchyma of mammary gland recorded after submaxillectomy may give a hope for a novel preventive measure or approach to neoplastic mammary gland especially, at high risk females. Further investigations about effects of submaxillectomy on the neoplastic mammary gland are recommended.

**Competing interests**
The authors declare that they have no competing interests.

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