Systemic immune response development in Albino rats after retrograde instillation of COVID-19 vaccine to submandibular salivary gland: An experimental study

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ABSTRACT

Objective: This study aimed to investigate whether using the submandibular gland duct (SMD) as an alternative mucosal route for vaccine administration induced anti-COVID-19 specific immunity.
Material and methods: Forty rats were randomized equally into four groups; Group I: Rats did not receive any intervention. Group II: Rats were subjected to intramuscular (IM) injection of COVID-19 vaccine. Group III: Rats were subjected to ductal cannulation by retrograde instillation of sterile saline into right SMD. Group IV: Rats in this group who had 0.5 ml of COVID-19 vaccine retrogradely injected into the right SMD. Subsequently, rats were examined for anti-COVID-19 specific antibodies (IgG). Also, light microscopic observation of glandular changes and immunohistochemical staining for CD20 was performed.
Results: The obtained results demonstrated a significant increase in anti-COVID-19 IgG levels in all rats vaccinated via intraductal immunization (group IV) compared to group II. Histologically, ectopic follicles were found within the glandular lobules of the inoculated submandibular gland (SMG) in group IV. In addition, the nearby lymph node in group IV demonstrated reactive follicle characteristics in the form of activated secondary follicles with germinal centers (GCs). Immunohistochemically, CD20 was localized in group IV in GCs of the ectopic lymphoid tissue and the nearby lymph nodes while group I, group II, and III demonstrated negative immunoreactivity.
Conclusion: The immune response demonstrated by intraductal SG immunization is generally more significant than that elicited by IM inoculation of the same vaccine.

1. Introduction

COVID-19, which the Coronavirus causes, is spreading throughout the world and has resulted in a significant loss of life. Consequently, it represents an extraordinary challenge.[1] Therefore, individuals are vaccinated against Coronavirus to prevent severe pathological and life-threatening conditions. Globally, IM injection with the COVID-19 vaccine is a commonly used method. However, SGs could be used as an alternative mucosal route for vaccine administration. For instance, it has been proven that this route affords both mucosal and systemic immunity compared to IM vaccination.[2]

Interestingly, SGs are also reported to function as immune organs.[3] Since they have some immune characteristics, such as the presence of dendritic cells, together with an extensive lymphoid tissue distribution. These lymphoid tissues represent the site of B cell activation and the inducer for cytotoxic T-lymphocytes.[4] In addition to the immune function and the well-known exocrine function, many researchers have demonstrated that SGs have an endocrine function[5,6] such as secretion from rodent SMG into the blood has been demonstrated for glucagon,[7] EGF,[8] NGF,[9] renin[10], kallikrein[11], and sialorphin[12,13]. Additionally, parotid gland endocrine secretion has been confirmed for a protein complex originally extracted from bovine parotid glands[14] and amylase.[15] Consequently, SGs have dual exocrine and endocrine functions. Due to these distinct, diverse characteristics, some researchers used SGs as an optimal immunization site for vaccination via retrograde perfusion of the gland duct using a gene or vaccine.[3,16]

In an elegant study, Ponzio & Sanders[16] investigated SGs as an immunizing site using DNA or attenuated pathogen. They found that intraductal vaccination resulted in a robust salivary and enteric IgA, indicating a widespread mucosal immune response, as well as high

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levels of circulating IgG. Furthermore, vaginal and lung antibodies were also detected. They concluded that the strength of immune response induced by SG vaccination is generally more potent detected in response to the same vaccine at a comparison site.

Likewise, Liu et al.[2] reported that immunization of SMD with tissue culture-derived murine cytomegalovirus or recombinant adenoviruses differentially activates T helper (Th)-1, −2, and −17 cells in SGs. They also demonstrated that SMG differently expressed the transcription factor T-cell–specific T-box transcription factor, which regulates the expression of the hallmark Th1 cytokines release. SMG cells had significantly higher levels of this expression pattern than spleen and lymph node cells. Therefore, they concluded that SMG could be an alternative mucosal route for administering vaccines.

Although there has been a wide range of studies on the COVID-19 vaccine effect after IM injection, nonetheless, to our knowledge, no research has been conducted on the effect of the COVID-19 vaccine after SG intraductal administration. Accordingly, this study investigated the effect of SG intraductal perfusion of the COVID-19 vaccine as an alternative mucosal route for vaccine administration.

2. Materials and methods

2.1. Vaccine

COVID-19 vaccine BIBP (Sinopharm), or BBIBP-CorV (an inactivated vaccine produced by Beijing Bio-Institute of Biological Products (BBIBP) in china) was purchased from VACSERA vaccination center in Egypt. The final vaccine product in each 0.5 ml dose is composed of 6.5 U (4 μg) of inactivated SARS-CoV-2 antigens soluble in aluminium hydroxide adjuvant in phosphate-buffered saline (PBS).[17]

2.2. Animals and experimental design

Forty adult male albino rats aged between 8 and 10 weeks (200–250 g). The sample size was calculated using StatCalc under Epi-info software with a two-sided confidence level of 95% and a power of 80% with a ratio of control: immunized is 1:1. Therefore, we have used ten rats per group.

The rats were purchased and housed for two weeks and received a standard diet and water. They were kept in a 12-h light/dark cycle prior to the experimental procedures. These were performed at Oral Biology Laboratory, Oral Biology Department, Faculty of Dentistry, Tanta University. Animals were randomly divided into four groups (n = 10) as follows:

Group 1: (Control) rats did not undergo any intervention.

Group II (IM vaccine) rats were subjected to IM injection of 0.5 ml of a single dose of COVID-19 vaccine BIBP.

Group III (Intraductal saline) rats were subjected to retrograde instillation of 0.5 ml of sterile physiological saline into right SMG via ductal cannulation.

Group IV (Intraductal vaccine) rats in this group were subjected to retrograde instillation of 0.5 ml of COVID-19 vaccine BIBP into the right SMG via ductal cannulation.

All animal experiments were conducted in accordance with the ARRIVE guidelines and were conducted after getting permission from the Internal Research Ethics Committee (REC), Faculty of Dentistry, Tanta University, (Number #R-OB-10-21-1).

2.3. SMD cannulation & retrograde injection

The animals were anesthetized via intraperitoneal injection with Ketamine hydrochloride 10% (Sigma-Aldrich Pty Ltd) with xylazine hydrochloride 2% (Sigma-Aldrich Pty Ltd) at a dosage of 0.06 ml/g & 0.03 ml/g, respectively.[18] The duct cannulation was performed according to the following schedule[19]:

The rats were subcutaneously injected with 1 μl/g bodyweight of 0.5 mg/ml atropine sulfate monohydrate (Sigma-Aldrich Pty Ltd) to inhibit the salivary secretions. Afterward, the rats were placed in the ventral position, the maxillary incisors were bolted on a metal wire, and the mandibular incisors were hooked on an elastic string. Subsequently, ductal dilatation by lacrimal probes (Medline Industries, Inc) was performed. An insulin syringe (Sigma-Aldrich Pty Ltd) with a 29-gauge needle (Sigma-Aldrich Pty Ltd) was inserted into a 0.58 mm diameter polyethylene tube (Sigma-Aldrich Pty Ltd), with the tip of the needle 5 mm away from the tube tip. At this time, the polyethylene tube was inserted 3–5 mm inside SMD orifice while the needle has remained outside the duct. Either the saline or the vaccine was injected into SMD. The infusion was delivered gradually with positive pressure on the syringe piston.

2.4. Serum samples preparation

Serum samples were collected 28 days after vaccine injection to test for anti-COVID-19-specific IgG antibodies. Under ether anesthesia (Sigma-Aldrich Pty Ltd), blood samples were collected from the jugular vein of rats in all groups into centrifuge tubes. Blood was allowed to clot at room temperature for about 1 h before centrifuging at 3000 rpm for 10 min. Serum was separated and kept at −20 °C prior to analysis for antigen-specific ELISA.[20]

2.5. Rat euthanization & sample preparation

At the end of the experiment (at day 28), all the rats were treated with an overdose of ketamine anesthesia and sacrificed, and then SMG tissues were dissected and removed instantly for light microscopic (LM) investigation.

Sample preparation was performed according to Bancroft et al.[21] For three days, the dissected tissues were kept and fixed in 10% neutral buffered formalin at 4 °C. After fixation, they were embedded in paraffin, and serial tissue sections of 4 μm thickness were obtained from all groups. Each group received two sets of tissue sections. One was stained with hematoxylin and eosin (H&E) for histopathological examination. The second was loaded on positively charged glass slides for immunohistochemical analysis to detect CD20 markers (cluster of differentiation 20). It is a transmembrane glycoprotein predominantly expressed on the surface of B cells.

2.6. Statistical analysis

Serum analysis data were expressed as mean ± SD using SPSS (Statistical Package for Social Sciences) 22.0 software. One-way analysis of variances (ANOVA) was used to compare all experimental groups. Then, Tukey's honestly significant difference (HSD) test was used to determine if there was a significant difference between the various pairs of means. The level of significance was determined at a P-value < 0.

3. Results

3.1. Body weight changes in all studied groups

The bodyweight of all experimental rats was recorded throughout the experiment. It was observed that group II, group III, and group IV demonstrated no changes in body weight when compared to group I (control group).

3.2. Serum levels of anti-COVID-19-specific IgG antibodies

The obtained results demonstrated a significant increase in anti-COVID-19 IgG levels in group IV (intraductal vaccine), which had the highest antibody titer compared to group I (control group), group II (IM vaccine), and group III (intraductal saline). Also, group II exhibited significant anti-COVID-19 IgG levels than group I and group II.

Nevertheless, the anti-COVID-19 IgG levels were insignificant in groups I and III (Table 1).

3.3. Histopathological results

3.3.1. Hematoxylin and Eosin stain

- **Groups (I, II, and III):** The histological features of SMG in group I and group III disclosed similar connective tissue (CT) and parenchymal features with no detectable difference and without any abnormal structures. In these groups, the normal acini (A) were closely packed with intralobular ducts (D) in between (Figs. 1 and 2). The acinar cells were mainly seromucous with few numbers of mucous acini. Serous acini were almost spherical and comprised pyramidal cells surrounding a narrow lumen with granular basophilic cytoplasm and rounded basal nuclei, whereas the intralobular ducts had rounded nuclei and light acidophilic cytoplasm. Also, the nearby lymphoid tissue appeared normal in the form of primary lymphoid aggregates (Figs. 1 and 2). Similarly, in group II, SMG parenchymal elements and the closely associated lymph node demonstrated normal structures (Fig. 2).
- **Group IV:** The SMG of group IV (intraductal vaccine) SMG displayed ectopic lymphoid tissue with GCs. Also, the nearby lymph node displayed reactive follicle characteristics in the form of activated secondary follicles with GCs (Fig. 3).

3.3.2. Immunohistochemical results

In both groups (I, II, and III), immunohistochemical localization of CD20 as a marker for B cells in the nearby SMG lymph nodes revealed negative immunoreactivity in both groups (I, II, and III) compared to group IV (Fig. 4 a-b). Group IV displayed strong immunoreactivity to CD20 in the active lymphoid GCs in the nearby lymph nodes and the ectopic lymph nodes located within the SMG lobules (Fig. 4 c-d and Fig. 5).

4. Discussion

Mucosally administered vaccines have gained significant attention due to their ease of administration and their ability to protect multiple mucosal sites as well as systemic sites. Furthermore, when dealing with pathogens, particularly viruses, effective immunization approaches are required to understand the defense mechanisms in the tissues, which is a crucial issue in vaccine development.[4]

Traditionally, most vaccines are administrated parentally via injection, such as IM and subcutaneous injections. Interestingly, these immunization sites can induce humoral immune responses in systemic (e.g., spleen, blood, and draining lymph nodes) but not in mucosal compartments, the immune response at mucous membranes (e.g., SGs, genitourinary tracts (GIT), and alimentary canals). Consequently, some trials to induce mucosal immune responses have been performed.[3]

Therefore, this study emphasized evaluating another vaccination address that could induce both mucosal and systemic immunity.

In the current study, salivary glands were chosen as an alternative site for vaccination because they are important mucosal tissues in the oral cavity and upper GIT which physiologically produce and secrete a variety of beneficial proteins in both saliva and blood that play a significant role in maintaining tissue homeostasis and integrity.[22] Moreover, SGs can potentially be a unique target site for the induction of both mucosal and systemic immunity because of their several physical and biological characteristics.[23] Depending on these unique characters, we used SMG as depot organ for vaccination. Dissimilar to the skin or muscle, the anatomy and physiology of the SG makes them perfect model for gene delivery and enhanced protein expression.[24]

Retrograde injection technique through SMD was performed, as retrograde perfusion with modified vectors in animal models successfully led to the expression of human keratinocyte growth factor,[25] alpha-1-antitrypsin[26], and human erythropoietin[27]. Furthermore, Liu et al.[28] concluded that SMGs could act as an inductive site for the mucosal immune system. It was found that SMD retrograde injection with tissue culture-derived murine cytomegalovirus (tc-MCMV) resulted in the development of MCMV-specific B and T-cell immunity, including MCMV-specific IgA in saliva and vagina together with systemic neutralizing MCMV-specific IgG in the serum.

Interestingly, the main histological and immunohistochemical findings in this study included the following:

First, GCs, a specialized microstructure that forms in secondary lymphoid tissues such as lymph nodes, produce long-lived antibody-secreting plasma cells and B cells, protecting against reinfection. Also, GCs allow interactions between T and B cells that are critical for developing most humoral immune responses that can be protective in response to vaccination or infection.[29]

Second, ectopic GCs are located within the SMG lobules. This finding is consistent with previous studies, which found that ectopic GCs could be formed in nonlymphoid tissue such as SGs.[30–32] In addition, Pitzalis et al.[33] stated that in multiple inflammatory states, including autoimmune disease, cancer, and during infection, these ectopic lymphoid aggregates range from tight clusters of B cells and T cells to highly organized structures that are considered functional GCs. Ectopic GCs have been vital as they support local GCs reactions, including clonal expansions, immunoglobulin class switching, antibody production, and the generation of cytotoxic T-cell responses to specific target antigens.[34]

Thirdly, positive CD20 immunostaining within GCs. Noteworthy, CD20 is a protein expressed on the surface of B cells, starting at the pre-B cell stage and on mature B cells in the bone marrow and the periphery.[35] CD20 is usually expressed on B cells during B-cell differentiation, B-cell development, B-cell receptor signaling, and cell-cycle initiation events,[36] confirming the presence of B cells within GCs and ectopic GCs.

On the contrary, B cells are involved in the GCs response after vaccination through the following mechanisms: they first recognize their cognate antigen via their B cell receptors. Then, they directly bind

Table 1

Serum levels of anti-COVID-19 specific IgG antibodies. Table abbreviations.[1].

	Group I (Control) (Anti-COVID IgG)	Group II (IM Vaccine) (Anti-COVID IgG)	Group III (Intraductal Saline) (Anti-COVID IgG)	Group IV (Intraductal Vaccine) (Anti-COVID IgG)
Range	0.1–0.31	0.85–1.57	0.2–0.41	1.2–2.2
Mean ± SD	0.01 ± 0.04	1.2 ± 0.29	0.3 ± 0.08	1.7 ± 0.41
f. test	29.011			
P. value	0.001*			

Control & Intraductal saline	Control & Intraductal Vaccine	Control & IM Vaccine	IM Vaccine & Intraductal Saline	Intraductal Vaccine & Intraductal Saline	Intraductal Vaccine & IM Vaccine
0.068[NS]	0.001*	0.001*	0.001*	0.001*	0.020*

[1] * means there is a significant difference when P. value < 0.05, and [NS] means no significant difference when P. value > 0.05.
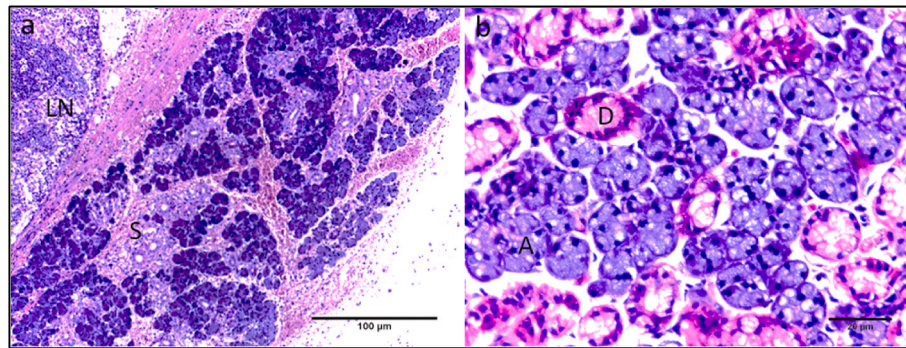
Fig. 1. Light micrograph illustrates SMG parenchymal elements and the closely associated lymph nodes in group I (control group), **a.** It displays normal glandular elements (S) together with primary lymphoid follicles (LN). **b.** The SMG shows closely backed acinar cells (A) with ductal elements in-between (D), (H&E, a x100; b x400).
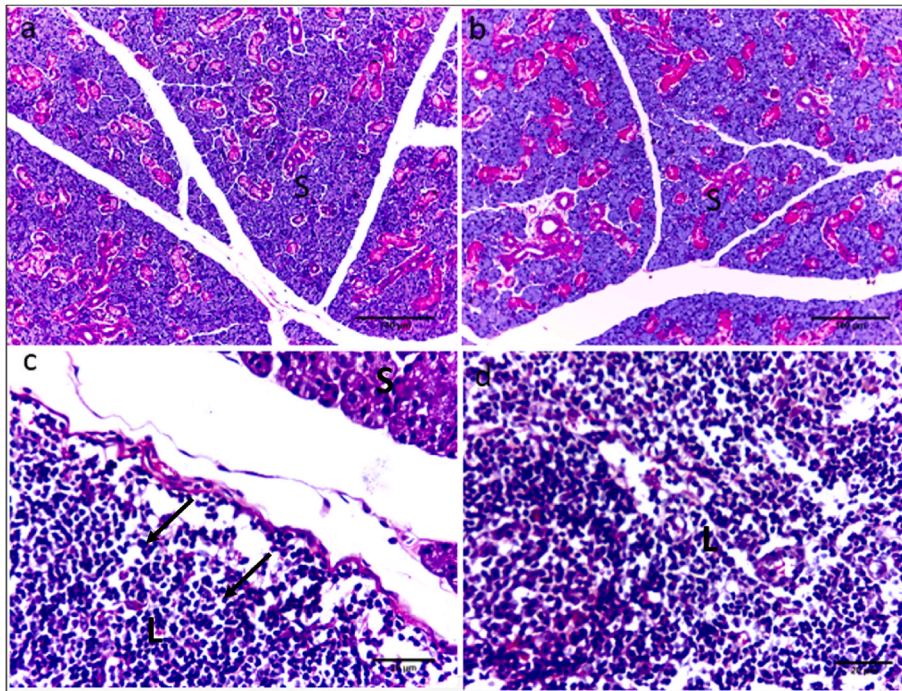

Fig. 2. Light micrograph illustrates normal SMG parenchymal elements (S) and the closely associated primary lymphoid follicles (L) at (c and d) **a.** The seromucous acini of group II (IM vaccine). **b.** The SMG of group III (intraductal saline) shows closely backed acinar cells with ductal elements in-between. **c.** Normal primary follicles of the closely associated lymph node in group II that are composed of small, round, inactive lymphocytes (arrows). **D.** Similarly, normal primary lymphoid tissue in group III, (H&E, a & b x 100; c & dx 400).

to soluble antigens in the vaccine and become activated.[37] Afterward, the activated B cells upregulate the chemokine receptor 7, which facilitates the migration of B cells in the T cell zone. B cells either initiate the GC response at the border of B and T cell zones or differentiate into short-lived extrafollicular plasma cells or memory B cells.[38] The GC response includes B cells migrating to the center of GC, where they begin to rapidly divide and undergo clonal expansion to form two distinct compartments. The dark zone contains rapidly diving B cells (centroblasts) and the light zone. The centroblasts migrate and become centrocytes, which capture the internalized antigen in GC and present it to T follicular helper cells are also present in the light zone to undergo selection.

Furthermore, centrocytes re-enter the dark zone for additional rounds of proliferation before exiting the GC as memory B cells or high-affinity antibody-secreting plasma cells.[29] The extrafollicular plasma cells produce the first wave of antibodies before undergoing apoptosis within a few days, providing an initial burst of antibodies essential for early infection control.[39] Memory B cells are one component of memory in the antibody system**.** B cell memory is generated along two fundamentally distinct cellular differentiation pathways. The first is the

generation of high-affinity somatic antibody mutants, while the second preserves germline antibody specificities and may prepare the body for rapid responses to antigenic variants of the invading pathogen.[40] Grewal et al.[3] illustrated that SGs inoculation acts as mucosal inductive sites against MCMV infection via the formation of ectopic GCs that function as ectopic mucosal inductive sites. When combined with transforming SGs into a mucosal inductive site that partially depends on the lymphoid neogenesis within them, these follicles were made up of B and T cells interacting with follicular dendritic cells proliferating cells, class switching, and plasma cell differentiation.

Significantly, multiple levels of circulating anti-COVID-19 IgG were detected in the serum after retrograde instillation of the COVID-19 vaccine, which is composed mainly of inactivated SARS-CoV-2 antigens SMD as compared to controls and IM groups. This result was similar to another result obtained from targeted SG injection with plasmid DNA encoding Porphyromonas gingivalis fimbriae that effectively stimulated systemic IgG antibodies.[41] Likewise, Sankar et al.[24] concluded that retrograde instillation of plasmid DNAs (pDNAs) complexed with Vaxfectin that acts as an adjuvant with vaccine plasmids to facilitate their action in eliciting immune response into SMG stimulated strongest
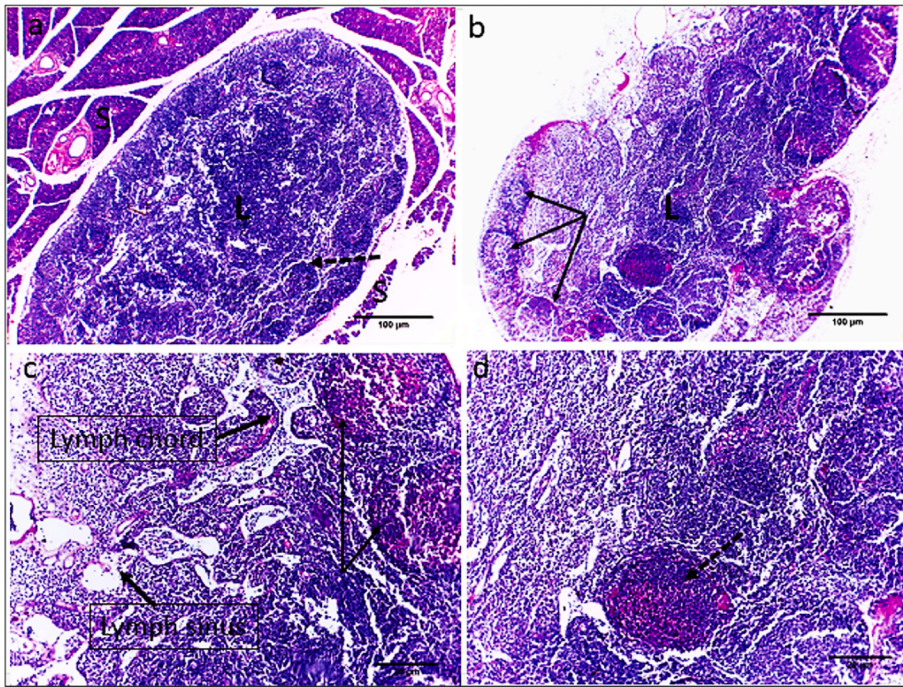
Fig. 3. (a–c) Light micrograph of SMG in group IV (Intraductal vaccine). **a.** Shows activated lymphoid tissue aggregates (secondary follicles) (L) with GCs (dashed arrow) that are closely approximated to the glandular tissue (S). **b.** Shows active lymphoid tissue (L) containing lymph follicles (branched arrow). **c.** Displays a band of activated lymphoid tissue, containing secondary lymphoid follicles (branched arrow) with GCs together with lymphoid medullary lymph sinuses separated by medullary lymph chords. **d.** Reveals activated lymph node with GCs (dashed arrow) (H&E, a, b x 100; c, d x400).
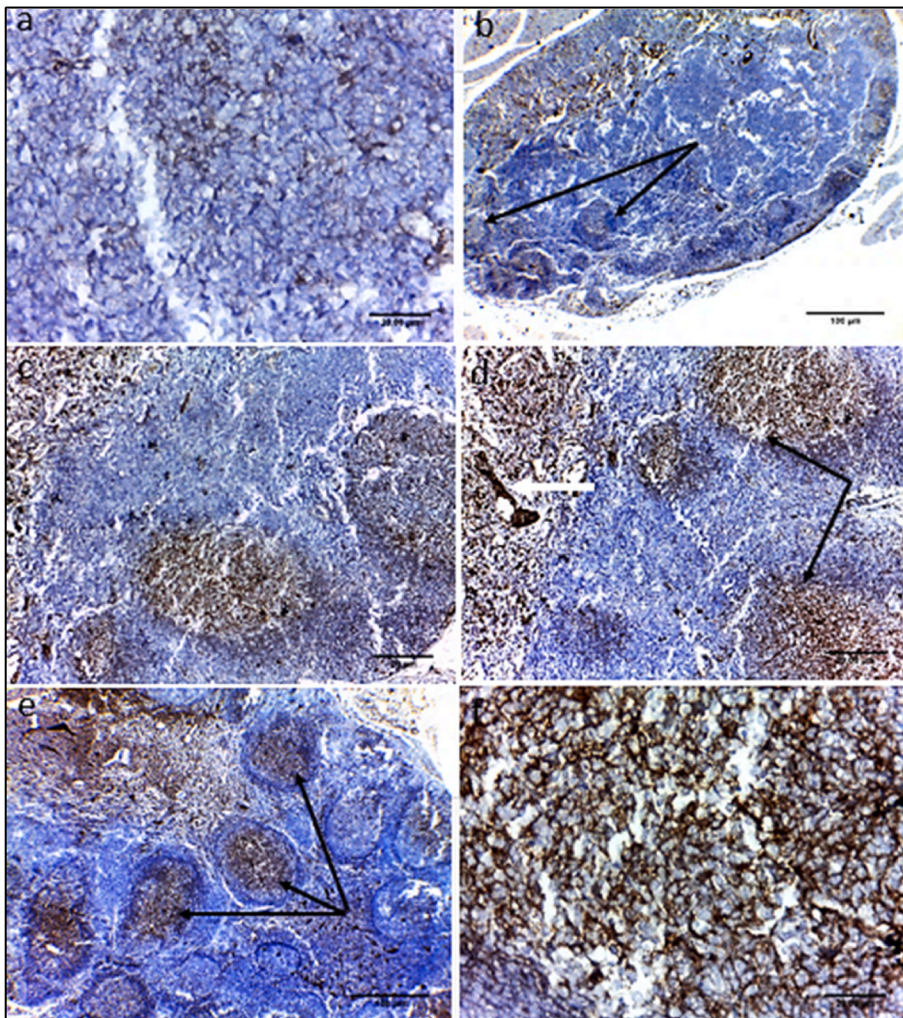

Fig. 4. Light micrographs display immunohistochemical staining of CD20 expression in lymph nodes associated with SMG. **a.** Group I (control) shows negative CD20 immunostaining. **b.** Group II (intramuscular vaccine) shows negative CD20 immunostaining in the lymphoid follicles (branched arrow). **c.** Group III (intraductal saline) displays negative CD20 immunostaining. **d.** Group IV (Intraductal vaccine) reveals positive immunostaining within GCs (branched arrow) together with positive cellular staining within the lymphoid sinus (white arrow). **e.** Group IV reveals positive immunostaining within GCs (branched arrow). **f.** Higher magnification of lymphoid GCs in group IV display the positive B cells membranous immunostaining (CD20 immunostaining counterstain with Mayer's hematoxylin. a, c, f x400, b, d, e x100).
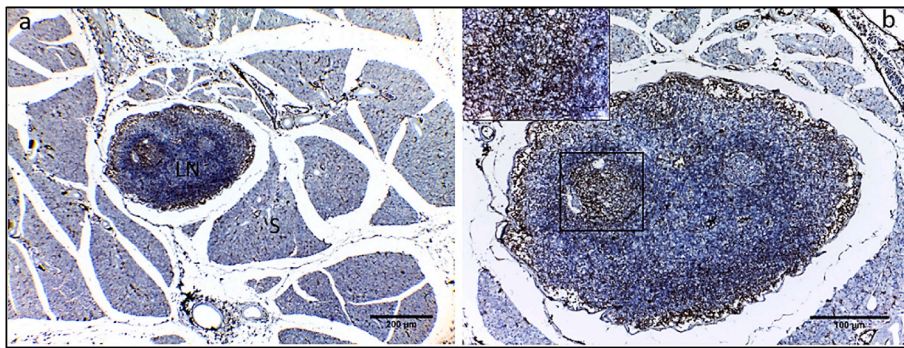
Fig. 5. Light micrographs display immunohistochemical staining of CD20 expression in the newly formed ectopic lymphoid tissue in group IV (Intraductal vaccine). **a.** Reveals ectopic lymphoid follicles (LN) within the SMG lobules (S). Notice, the LN displays strong immunostaining of CD20 within the GCs. **b.** Displays ectopic secondary lymphoid tissue with GCs (boxed area) with inset reveals higher magnification of the boxed area viewing positive B cell membranous immunostaining, (CD20 immunostaining counterstain with Mayer's hematoxylin. a x100, bx100, inset x400).

cytotoxic and humoral responses to influenza that detected by highest antibody levels (both IgG2a and IgG2b). Interestingly, Tucker et al.[42] reported that animals vaccinated with DNA by SG retrograde perfusion were significantly more resistant to the effects of lethal anthrax infection than IM DNA-vaccinated animals. Also, Liu et al.[28] found that after SMG retrograde injection with tc-MCMV, they demonstrated higher levels of specific MCMV-IgA at saliva, feces, and vagina, and systemic neutralizing MCMV-specific IgG in the serum. This could be attributed to memory B cells in GCs that can elicit more rapid and robust antibody responses upon antigenic stimulation than antigen-inexperienced naive B cells.[43,44] Additionally, Tucker et al.[45] found a strong immune response after DNA vaccination after both 6, 8, 9, and 14 weeks from parotid salivary glands intraductal vaccination. While, El Helou et al.[46] found that tetravalent dengue plasmid DNA vaccine is not immunogenic after 4 week from retrograde infusion into parotid gland in a non-human primate model. They attributed these results to the presence of zinc or polyethylenimine vectors into the plasmid DNA.

There are several limitations to this study. The main limitation of this study is the lack of previous research studies on the same topic. Additionally, the small mouth opening of some rats caused difficulty in detecting SMD opening. Over and above, some limitations for the future suggestions based on the results of this study include patient agreement for SMD injection instead of IM, SG diseases that may avert this route of administration, and SG delivery technology that would necessitate more workers time of a trained medical professional than IM vaccination by the needle.

5. Conclusion

The strength of immune response bestowed by intraductal SG vaccination is generally more significant than that d elicited by IM vaccination with the same vaccine. Therefore, further studies should be conducted in the future, focusing on this novel approach and detecting the mucosal immune response associated with this approach.

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Declaration of competing interest

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