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**Conclusion:** Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

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ORIGINAL ARTICLE

# Histopathology Study of Alginate Microspheres Containing Ovalbumin on Liver and Kidney Following Oral Administration and Evaluation of Uptake by Peyer's Plaque

## Oral Uygulamayı Takiben Ovalbümin İçeren Alginat Mikroküre lerinin Karaciğer ve Böbrekte Histopatoloji Çalışması ve Peyer Plakları Tarafından Alımının Değerlendirilmesi

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## ABSTRACT

**Objectives:** The development of oral vaccine formulations has been widely investigated to overcome oral route problems. This research investigated the *in vivo* immune response of ovalbumin-alginate microspheres by uptake compared with a commercial oral vaccine product.

**Materials and Methods:** Ovalbumin-loaded alginate microspheres were prepared using aerosolization. Ovalbumin antigen in vivo uptake was investigated in order to understand the distribution and uptake by Peyer's plaque (PP) after oral administration using fluorescence microscopy. The histopathology of ovalbumin-alginate microspheres in the liver and kidney was also investigated.

**Results:** The use of alginate microspheres to deliver vaccines could be a promising delivery system for the development of oral vaccines because uptake by PP is an essential step in oral vaccination.

**Conclusion:** Fluorescence visualization revealed the uptake of ovalbumin-loaded alginate microspheres with and without lyoprotectant maltodextrin by PP was equal to the oral vaccine product and no liver or kidney damage was found.

**Key words:** Vaccine delivery, microspheres, histopathology

## ÖZ

**Amaç:** Oral aşı formülasyonunun geliştirilmesi, oral kullanım problemlerinin üstesinden gelebilmek için geniş çapta araştırılmıştır. Bu araştırmada, ticari oral aşı ürününe kıyasla ovalbümin aljinat mikroküreleri alımındaki *in vivo* bağışıklık tepkisi araştırıldı.

**Gereç ve Yöntemler:** Ovalbümin yüklü aljinat mikroküreleri aerosolizasyon tekniği kullanılarak hazırlandı. Floresans mikroskobu kullanılarak oral uygulama sonrasında Peyer plakları (PP) ile alımın ve dağılımın anlaşılması için ovalbümin antijeninin *in vivo* alınımı araştırıldı. Ovalbümin aljinat mikrokürelerinin karaciğer ve böbrekteki histopatolojisi de araştırıldı.

**Bulgular:** Oral aşının geliştirilmesi için aşının salımında aljinat mikrokürelerin kullanılması umut vaad eden bir salım sistemi olabilir çünkü oral aşılamada PP tarafından alınım önemli bir adımdır.

**Sonuç:** Floresans görüntülemesi, lyoprotectant maltodextrin içeren veya içermeyen ovalbümin yüklü aljinat mikrokürelerin PP tarafından alımının oral aşıya eşit olduğunu ve karaciğer ve böbrekte hasar oluşturmadığını ortaya koymuştur.

**Anahtar kelimeler:** Aşı salınımı, mikroküreler, histopatoloji

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## INTRODUCTION

Oral delivery systems are one of the alternative routes of drug or vaccine administration, which are non-invasive and can avoid pain and discomfort and repeated administration is easy if required.[1] Peyer's plaques (PP) in the small intestine are the main target of oral delivery systems as a place for the transport of pathogens to lymphoid tissue.[2,3] This function is carried out by M-cells, which are located between epithelial cells, bringing antigens and microparticles measuring less than 10 μm.[4]

Microspheres contain biodegradable polymers and ideally have particle sizes of less than 200 μm.[5,6] Natrium alginate is a natural polymer that is non-toxic, biocompatible, and relatively inexpensive.[7] Alginates form a three-dimensional structure when reacted with a multivalent ion. Divalent cations such as calcium, barium and strontium bind between a collection G of alginate chains, and form bridges between the chains, which causes the gelling alginate solution. $Ca^{2+}$ is one of the best options as agents continually cross with alginate.[7] $Ca^{2+}$ is a two-dimensional planar binding poly guluronate acid group (G) of alginate that yields a so-called egg-box. In previous research, the production of ovalbumin-alginate microspheres using ionotropic gelation by aerosolization provided advantages of spherical-shaped, smooth, and small-sized particles (<30 m) that met the requirements of particles for oral delivery systems.[7,8] Maltodextrin was added to improve the stability of the microspheres during storage during freeze drying. The addition of maltodextrin lyoprotectant was found to form smooth surfaces and smaller microspheres (<6 μm) when compared with microspheres without a lyoprotectant.[8]

An alternative for oral antigen delivery systems is microspheres. The objective of this research was to determine the immune response after administration of ovalbumin-alginate microspheres as well as oral vaccine products. Furthermore, to determine the uptake and distribution of microspheres in the gastrointestinal tract as well as the target organ. Histology using fluorescence microscopy is a qualitative approach that may provide direct evidence of the existence and location of particles in the network.[9,10] This research evaluated ovalbumin-alginate microspheres with and without maltodextrin lyoprotectant and a commercial oral vaccine product. Unencapsulated ovalbumin was used as a negative control.

## MATERIALS AND METHODS

Ovalbumin, sodium alginate, protein quantification kit and BSA (Sigma Aldrich), $CaCl_2/2H_2O$ pharmaceutical grade (Solvay Chemicals Internationals), sodium citrate p.g, CMC Na p.g, and maltodextrin (Bratachem Chemicals), Rhodamin B (E Merck), vaccine product (i.m) from Sanovi Pasteur, Optimal Cutting Temperature (O.C.T) Compound (Sakura), phosphate-buffered saline pH 7.2, Na EDTA, aquadest, red gout blood cell, and mice mus musculus strain Balb C from Pusat Veterenaria Farma (PUSVETMA) Surabaya. Six mice of each group's formula was used in the in vivo study based on Federer calculation with the following animal criteria: healthy, no inflammation or irritation, 2-3 months old, and weight 20-30 grams. This research was approved by Animal Care Ethics Committee of Airlangga University in 2015.

### *Methods*

#### *Preparation of ovalbumin-loaded alginate microspheres*

Sodium alginate (2.5%) was dissolved in distilled water and ovalbumin (2.5%) was dissolved in it. This solution was then sprayed into a solution of 1.5 M $CaCl_2$ at a pressure of 40 psi. The mixture was stirred at 1000 rpm for 2 hours. Formed microspheres were collected and then separated using centrifugation at 2.500 rpm for 6 min and washed twice. The microspheres were resuspended in lyoprotectant solution (1 g/10 mL) with concentration according to the formula. The suspension was dried in a freeze dryer at a temperature of -80°C for 29 hours. For group preparation, formula was dispersed in CMC Na solution prior to administration.

Formulas in this study as follows:

F1.1: Formula of blank alginate microspheres 1st replicate,

F1.2: Formula of blank alginate microspheres 2nd replicate,

F3.1: Formula of ovalbumin-loaded alginate microspheres 1st replicate,

F3.2: Formula of ovalbumin-loaded alginate microspheres 2nd replicate,

K1: Control of ovalbumin 1st replicate,

K2: Control of ovalbumin 2nd replicate.

#### *Preparation of animal in vivo study*

The mice were adapted for a week in a room at 25°C±2°C in a separate cages. The mice were then orally given the formulas with administration volume adjusted to the body weight of mice. For histopathologic study, after administration, the mice were sacrificed through anesthesia with ketamine prior to cervical dislocation, and the liver and kidneys were then taken. The liver and kidneys were cut and sliced. The liver and kidney samples prepared for hematoxylin and eosin staining and visualized using a fluorescence microscope (FSX 100, Olympus).

#### *Histopathology study of ovalbumin-alginate microspheres in liver and kidney*

Histopathologic examination of the liver and kidneys aimed to show the degree of damage to the liver and kidneys from the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres. This evaluation used an optical microscope Nikon H600L complete with a DS Fi2 300 megapixel digital camera and Nikon Image System Software to analyze the data.

The scoring method for the degree of liver damage in this examination was performed according to the methods of Knodell et al.[9] and Klopfleisch[11], whereby the degree of damage of each sample was determined by adding the entire score of the four types of histopathologic lesions, as shown in Table 1.

The scoring method for the degree of kidney damage was performed according to the Klopfleisch[11] method, whereby the degree of damage was determined by adding the entire score of the four types of histopathologic lesions, as shown in Table 2.

**Table 1. Score based on histopathological lesions of liver**

| Histopathology of lesion | Score | Note |
|---|---|---|
| Degenerative | 0 | No degenerative occured |
| | 1 | Degenarative changes occured at less than 25% of all view areas |
| | 2 | Degenarative changes occured at 26-50% of all view areas |
| | 3 | Degenarative changes occured at 51-75% of all view areas |
| | 4 | Degenarative changes occured at above 76% of all view areas |
| Necrosis | 0 | No necrosis occured |
| | 4 | Necrosis occured at less than 25% of all view areas |
| | 6 | Necrosis occured at 26-50% of all view areas |
| | 8 | Necrosis occured at above 50% of all view areas |
| | 10 | Necrosis occured at 26-50% of all view areas along with bridging necrosis |
| | 12 | Necrosis occured at above 50% of all view areas along with bridging necrosis |
| | 14 | Diffuse necrosis >76% and distribute widely at almost all lobulus area (Multilobular necrosis) |
| Inflammation | 0 | No inflammation occured |
| | 1 | Inflammation area occured at less than 1/3 of total area Kiernan's triangle (portal area) |
| | 2 | Inflammation area occured at 1/3-2/3 of total area Kiernan's triangle |
| | 3 | Inflammation area occured at above 2/3 of total area Kiernan's triangle |
| Fibrosis | 0 | No fibrosis occured |
| | 2 | Intra sinusoidal fibrosis or periportal fibrosis occured at less than 25% of all areas |
| | 4 | Intra sinusoidal fibrosis or periportal fibrosis occured at 25-50% of all areas |
| | 6 | Intra sinusoidal fibrosis or periportal fibrosis occured at above 50% of all areas |
| | 8 | Intra sinusoidal fibrosis or periportal fibrosis occured at 50-75% of all areas |

Total degree of damage is the total amount of all the above lesion degree of damage is where the interval is betwen **0 - 28**

*Uptake of microspheres*

Formulas of ovalbumin-alginate microspheres with and without lyoprotectant were compared with ovalbumin and an oral vaccine product. Rhodamine B is a fluorochrome, which was used to label all groups. The mice were adapted for a week in a room at 25˚C ± 2˚C in separate cages. Mice were then fasted for 16 hours followed by oral administration. Volume oral administration was 500 µL/25-gram body weight. To determine the intestinal uptake in the mice 7 and 8 hours after oral administration, the mice were anesthetized using ketamine and sacrificed by cervical dislocation. After the mice were dead, the intestine samples were split and cut. The intestine samples were embedded in OCT. The intestine was cut transversely with a thickness of 5 µm using a cryotome (Tissue-Tek Cryo3, Sakura) at a temperature of -59˚C. Intestinal tissue histology was then observed using a fluorescence microscope with a red filter.

*Data analysis*

Data from the evaluation of ovalbumin-alginate microsphere characteristics are expressed as mean ± standard deviation from triplicate experiments (data not shown). The histology study was analyzed semi-quantitatively based on scores and presented in duplicate data. For the uptake study, triplicate experiments were conducted and selected micrograph figures were presented.[11]

## RESULTS AND DISCUSSION

The histopathologic examination of the livers of the mice showed the degree of damage caused by the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres.

The scores for the degree of damage to the liver can be seen in Table 3.

For the histopathology of kidney, the degree of damage to the kidneys caused by the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres is shown in Figure 1-7.

The scoring for the degree of damage to the kidneys can be seen in Table 4.

The histopathologic results in the liver and kidney showed damage/necrosis of the liver and the kidney was minimal or even absent.

**Table 2. Score based on histopathological lesions of kidney**

| Histopathology of lesion | Score | Note |
|---|---|---|
| Degenerative of tubular epithelial cell | 0 | No degenerative occured |
| | 1 | Degenarative changes occured at less than 25% of all view areas |
| | 2 | Degenarative changes occured at 26-50% of all view areas |
| | 3 | Degenarative changes occured at 51-75% of all view areas |
| | 4 | Degenarative changes occured at above 76% of all view areas |
| Necrosis of tubular epithelial cell | 0 | No necrosis occured |
| | 2 | Number of necrosis cell of less than 25% of all view areas |
| | 4 | Number of necrosis cell of 26-50% of all view areas |
| | 6 | Number of necrosis cell of above 50% of all view areas |
| | 8 | Number of necrosis cell of above 76% of all areas |
| Necrosis of glomerular | 0 | No necrosis glomerular occured |
| | 3 | Necrosis glomerular occured of less than 25% of all glomerulus |
| | 5 | Necrosis glomerular occured of 26-50% of all glomerulus |
| | 7 | Necrosis glomerular occured of above 50% of all glomerulus |
| | 9 | Necrosis glomerular occured of above 76 % of all glomerulus |
| Glomerular infiltration | 0 | No infiltration glomerular occured |
| | 1 | Infiltration glomerular occured of less than 25% of all glomerulus |
| | 2 | Infiltration glomerular occured of 26-50% of all glomerulus |
| | 3 | Infiltration glomerular occured of above 50% of all glomerulus |
| | 4 | Infiltration glomerular occured of above 76% of all glomerulus |
| Interstitial infiltration | 0 | No infiltration occured in interstitial |
| | 1 | Infiltration occured of less than 25% of all interstitial |
| | 2 | Infiltration occured of 26-50% of all interstitia |
| | 3 | Infiltration occured of above 50% of all interstitial |
| | 4 | Infiltration occured of above 76% of all interstitial |
| Mesangial proliferation and or hyalization (glomerular sclerosis) | 0 | No proliferation and or hyalization glomerular sclerosis occured |
| | 1 | Proliferation and or hyalization glomerular sclerosis occured at less than 25% of all glomerulus |
| | 2 | Proliferation and or hyalization glomerular sclerosis occured at 25-50% of all glomerulus |
| | 3 | Proliferation and or hyalization glomerular sclerosis occured at above 50% of all glomerulus |
| | 4 | Proliferation and or hyalization glomerular sclerosis occured at 50-75% of all glomerulus |
| Interstitial fibrosis | 0 | No fibrosis occured |
| | 3 | Fibrosis occured at less than 10% of all areas |
| | 5 | Fibrosis occured at 11-30% of all areas |
| | 10 | Fibrosis occured at above 30% of all areas |

Total degree of damage is the total amount of all the above lesion degree of damage is where the interval is betwen **0 - 28**

**Table 3. Scores of the degree of damage to the liver**

| Preparat code | Score | | | | Total score |
|---|---|---|---|---|---|
| | Degeneration | Necrosis | Inflammation | Fibrosis | |
| F1.1 | 1 | 4 | 1 | 0 | 6 |
| F1.2 | 0 | 4 | 2 | 0 | 6 |
| average | | | | | **6** |
| F3.1 | 2 | 4 | 3 | 0 | 9 |
| F3.2 | 0 | 4 | 3 | 0 | 7 |
| average | | | | | 8 |
| K1 | 2 | 4 | 1 | 0 | 7 |
| K2 | 2 | 4 | 3 | 0 | 9 |
| average | | | | | 8 |

**Table 4. Scores the degree of damage to the kidneys**

| Preparat code | Forms of lession | | | | | | | Total score |
|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | |
| F1.1 | 0 | 2 | 0 | 2 | 0 | 4 | 0 | 8 |
| F1.2 | 0 | 2 | 0 | 0 | 1 | 1 | 0 | 4 |
| average | | | | | | | | 6 |
| F3.1 | 0 | 4 | 5 | 2 | 2 | 2 | 0 | 15 |
| F3.2 | 0 | 4 | 2 | 2 | 2 | 2 | 0 | 12 |
| average | | | | | | | | 13.5 |
| K1 | 0 | 4 | 3 | 3 | 3 | 5 | 0 | 19 |
| K2 | 0 | 4 | 1 | 1 | 3 | 3 | 0 | 12 |
| average | | | | | | | | 15.5 |

The results of uptake of ovalbumin-alginate microspheres and oral vaccine product in the fluorescence microscopy examination 7 and 8 hours after application can be seen in Figure 8 and 9.

Observations of uptake, as one of the immune response indicators, were made using a fluorescent indicator, which produces a fluorescent color at a specific wavelength. Emission wavelength fluorescence results are captured and selected by the filter, which then presents them in an appropriate dye setting. The microscopy observations of the immune response of the ovalbumin control, ovalbumin-alginate microspheres, ovalbumin-alginate microspheres with 5% maltodextrin, and the oral vaccine product were expected to show an oral vaccine antigen ovalbumin protein in the target site, the PPs. A microscopy morphology overview demonstrated golden yellow fluorescence, which suggested the presence of ovalbumin in the intestine, especially in the PPs.

Observations of uptake in the ileum of the mice performed 7 and 8 hours after administration can be seen in Figure 8 and 9. The uptake of unencapsulated ovalbumin was not seen; this may suggest that unencapsulated ovalbumin was not taken in the ileum and did not induce an immune response in lymphoid tissue.[1]

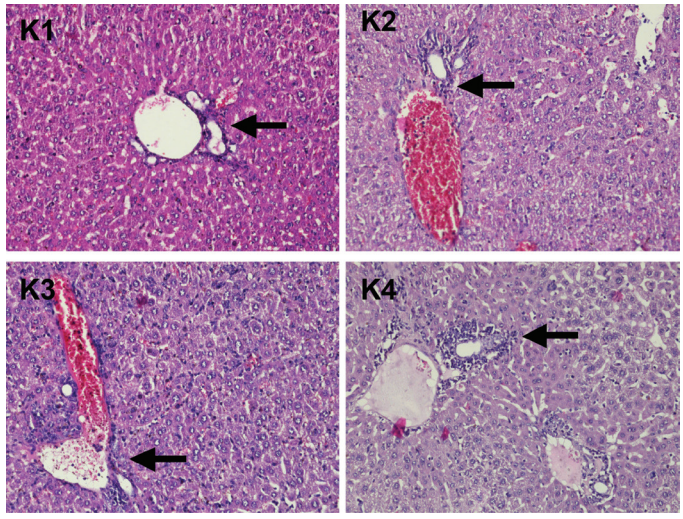


**Figure 1.** Differences of infiltration of inflammatory cells in the portal area (arrow) during treatment (H&E staining, Magnification 200x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

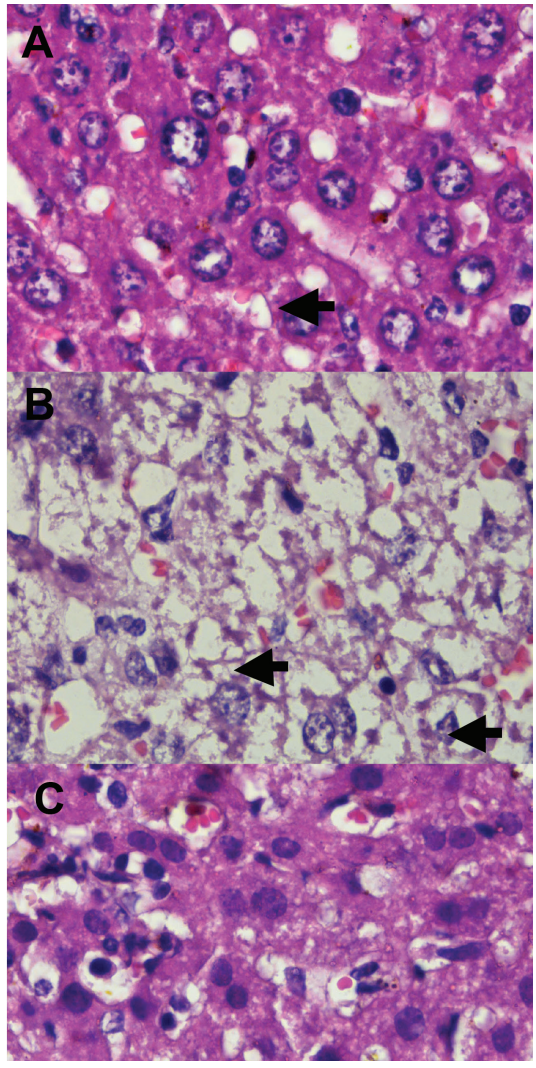


**Figure 2.** Normal hepatocyte cells (a), degenerative (b) and necrotic (c) (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

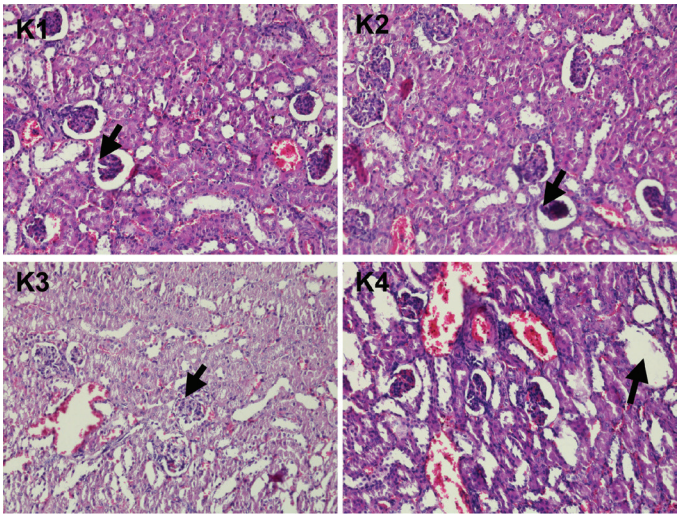


**Figure 3.** Different degrees of damage to the renal corpuscle in the renal cortex among treatments. Damage to renal corpuscle characterized by necrosis of glomerular cells. At a small magnification (100x-200x), visible wrinkle damaged glomeruli (black arrow) to Bowman's space is stretched, as compared to the normal renal corpuscle and even seen as an empty space (black arrow in K4) when composite of whole cell lysis by glomerular have cell activity phagosit. In this study, the glomerular injury in the group K3 relatively mild compared to other groups (H&E staining, Magnification 200x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

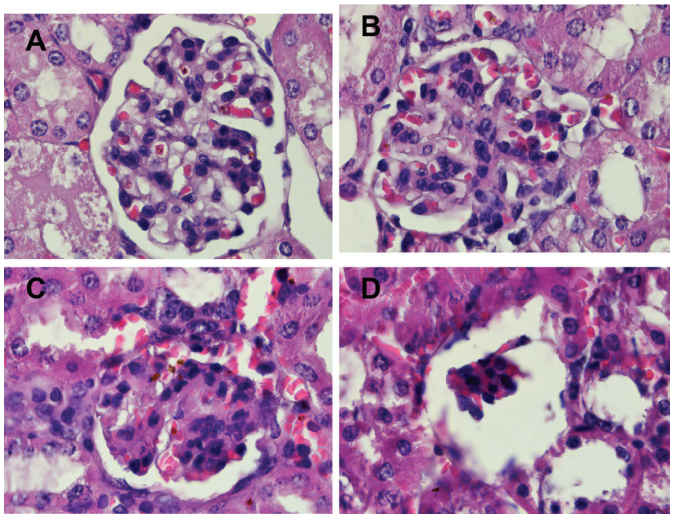


**Figure 4.** An overview of each glomerulus normal (a), hyperplasia (b), sclerosis (c) and necrosis (d) (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

For ovalbumin-loaded alginate microspheres, ovalbumin-alginate microspheres started entering through the villi at 7 hours, and entered deeper from seven to eight hours. Interestingly, uptake of ovalbumin-alginate microspheres with maltodextrin lyoprotectant showed deeper entry inside the villi at the 8$^{th}$ hour, the same as the oral vaccine product.

The uptake of ovalbumin-alginate microspheres in the villi toward the deeper part compared with unencapsulated microspheres indicated that the uptake of ovalbumin-loaded into the delivery system was more evident in the villi and PPs.

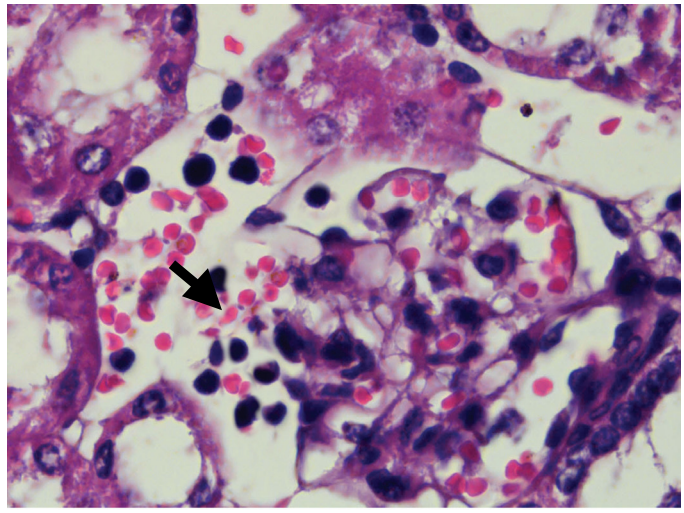

**Figure 5.** Infiltration of inflammatory cells (arrows) in the glomerulus (H&E staining. Magnification 1000x; Nikon microscope H600L; Fi2 300 megapixel camera DS).

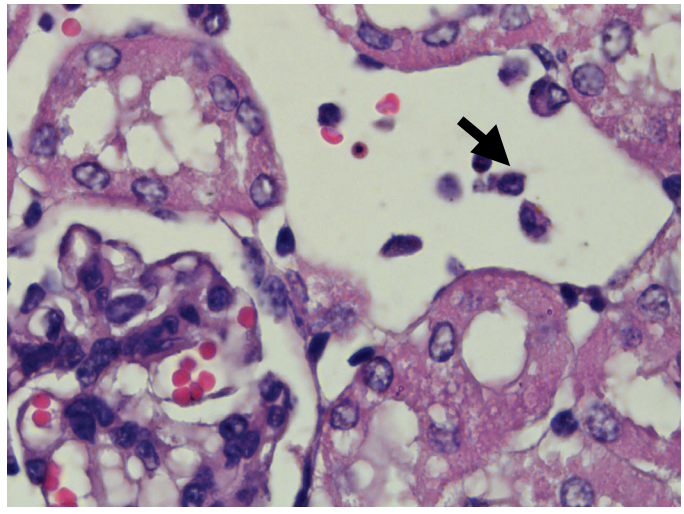

**Figure 6.** Infiltration of inflammatory cells (arrows) in the interstitial space (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

Uptake of microparticles in the intestine was influenced by particle size and hydrophobicity.[4] Microspheres smaller than 5 µm were transported to the lymph, where the antigen contained would be released and produce an immune response, whereas particles sized larger than 5 µm would stay in PPs and release antigen.

From the observations, a fluorescent golden yellow glow indicated the presence of ovalbumin in the network of PPs. However, the ovalbumin control group showed a lower intensity compared with formula ovalbumin-alginate microspheres both with and without lyoprotectant maltodextrin or oral vaccine product. Ovalbumin uptake in PPs was clearly shown for the ovalbumin-alginate microspheres with lyoprotectant and the oral vaccine product. This illustrated that ovalbumin had reached the target site and been taken up by M cells in PPs. In terms of particle size in ovalbumin-loaded alginate microspheres, small-

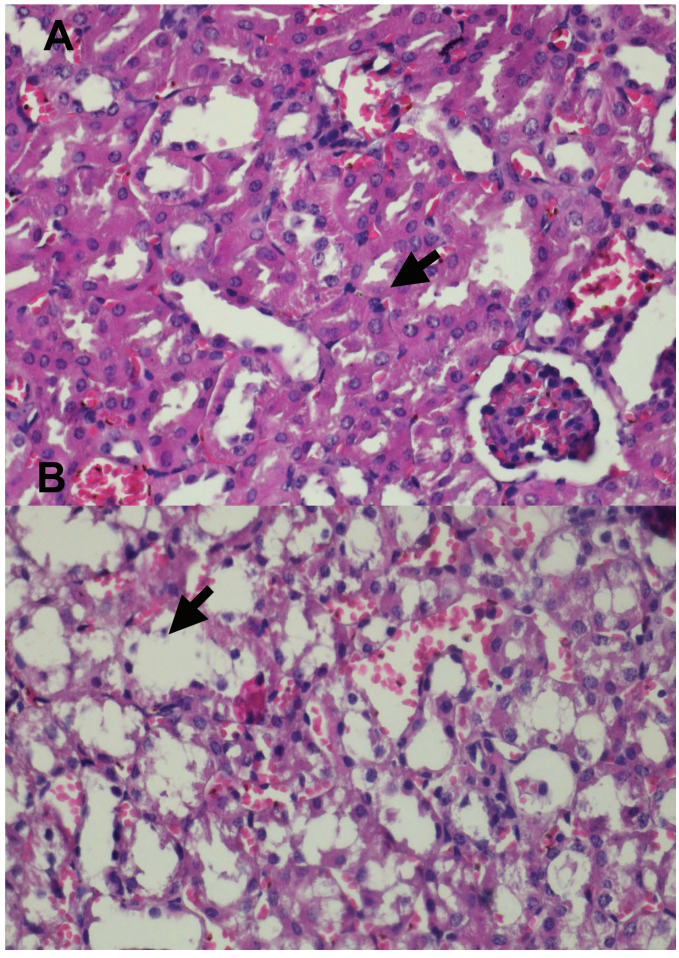


**Figure 7.** Cells of (a) tubular epithelial normal (black arrow) and (b) epithelial cells of tubular necrotic (black arrows) (staining H&E. Magnification 400x; microscope Nikon H600L; camera DS Fi2 300 megapixels).

sized particles passed directly into glands in addition to PPs, and were suitable to induce response.[12,13] Antigen to the target site and microspheres can bypass all barriers in the gastrointestinal tract and enter the epithelial tissue in PPs.

From the description above, it is summarized that the in vivo immune response test conducted on mice showed that microspheres as delivery systems of oral vaccines can provide an immune response equal to that of oral vaccine products.

## CONCLUSION

It can be concluded that formula ovalbumin-alginate microspheres with lyoprotectant maltodextrin showed delivery of antigen to the target site, PP, at the same intensity as an oral vaccine. Furthermore, histopathology tests showed no necrotic damage of the liver and kidneys.

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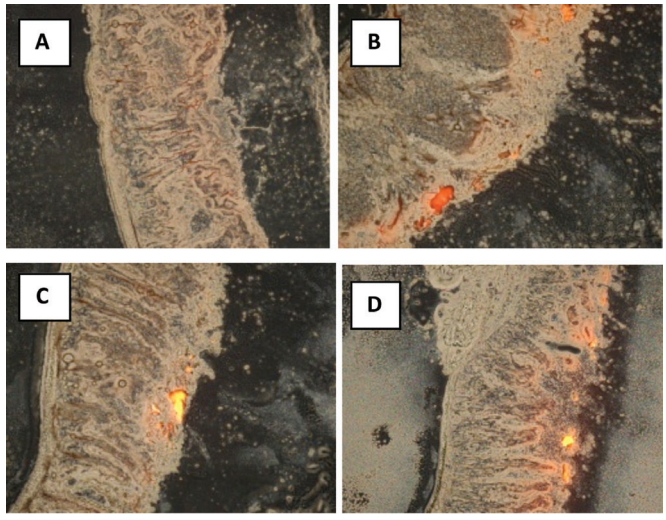


**Figure 8.** Uptake after 7 hours application (a) Ovalbumin, (b) ovalbumin-alginate microspheres, (c) ovalbumin-alginate microspheres with 5% maltodextrin, and (d) an oral vaccine product.

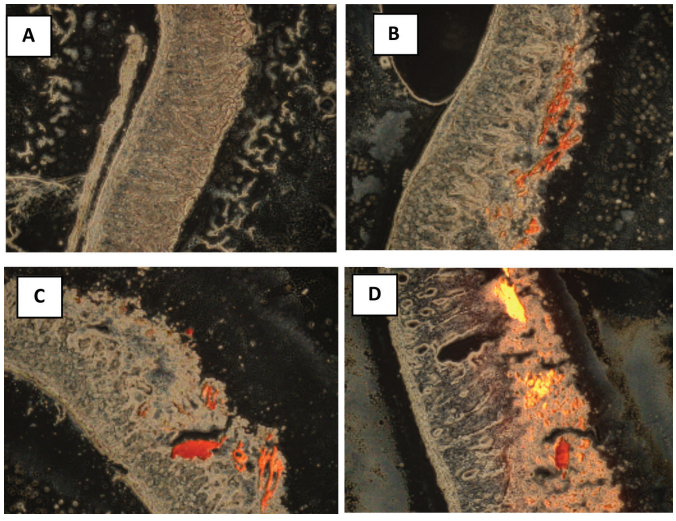


**Figure 9.** Uptake after 8 hours application (a) ovalbumin, (b) ovalbumin-alginate microspheres, (c) ovalbumin-alginate microspheres with 5% maltodextrin, and (d) an oral vaccine product.

*Conflict of Interest: No conflict of interest was declared by the authors.*

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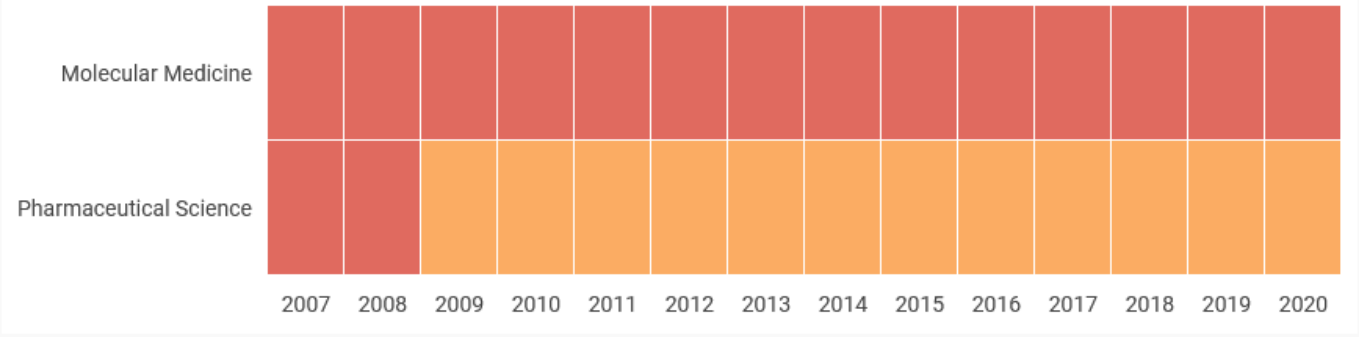

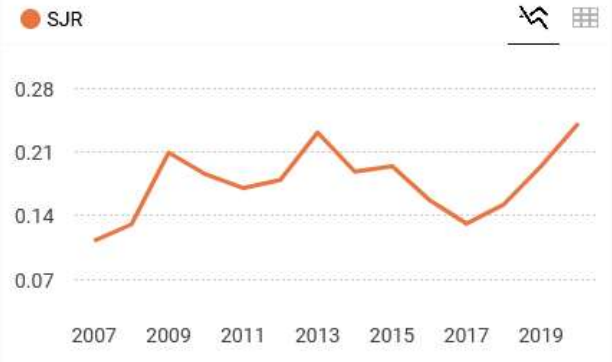


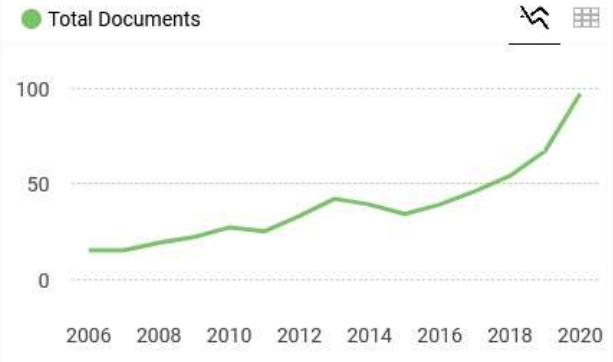


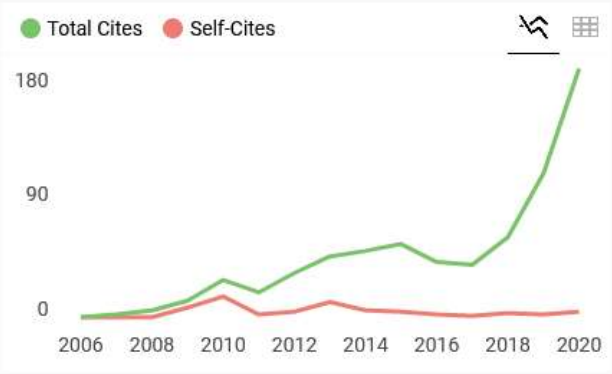


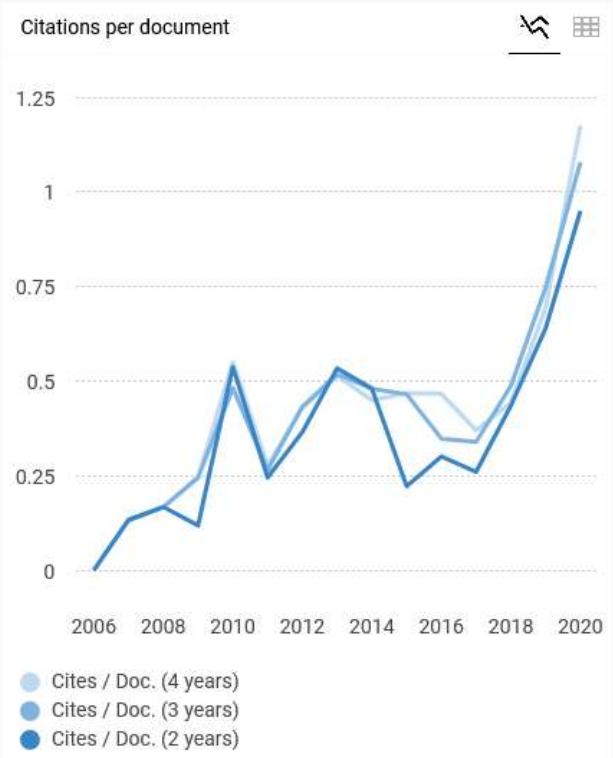


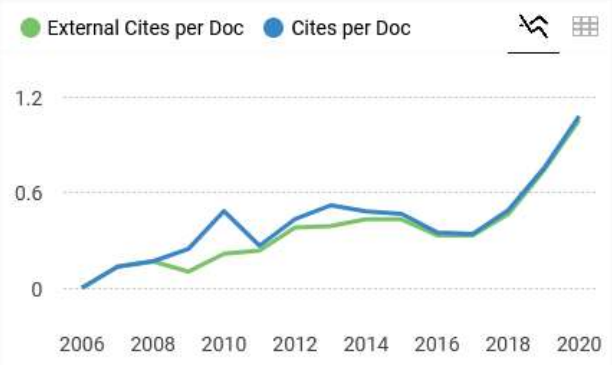


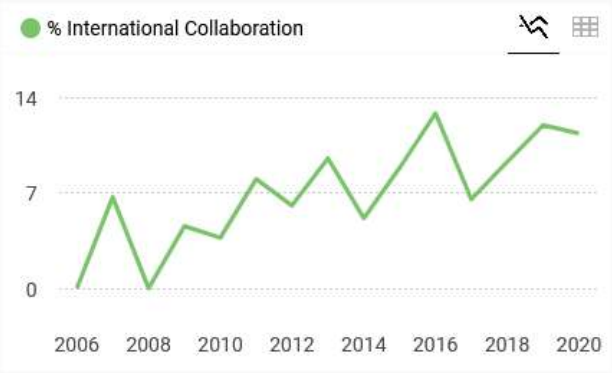


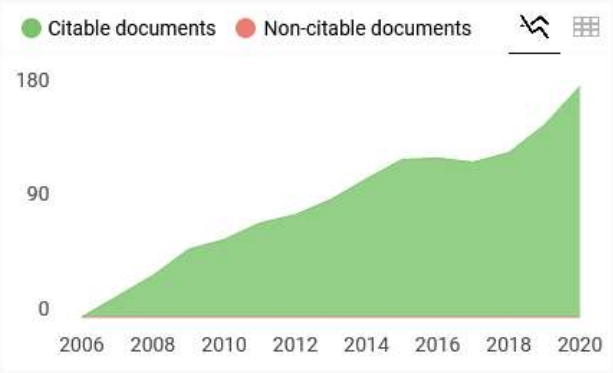


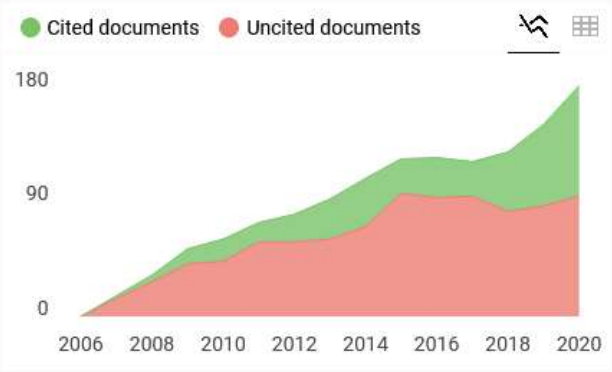



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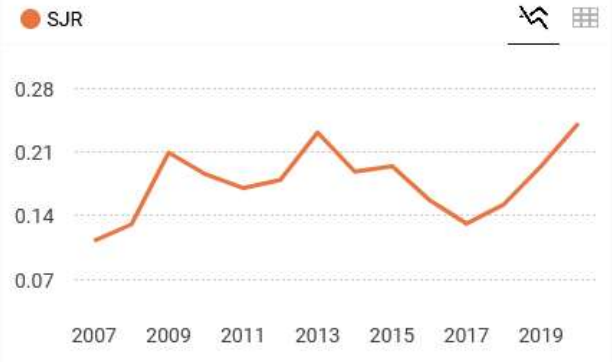


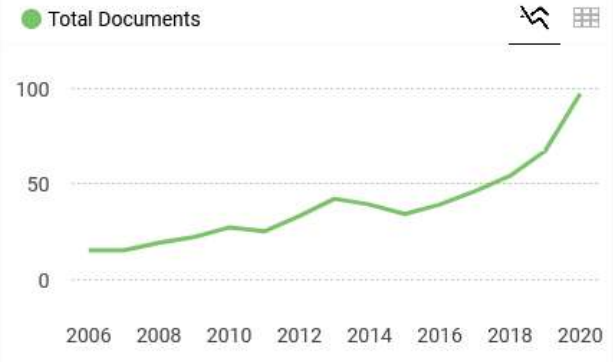


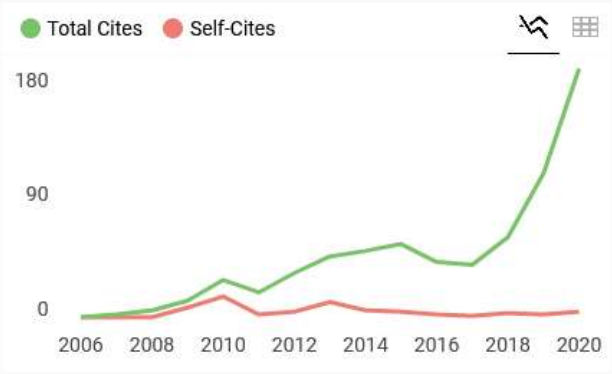


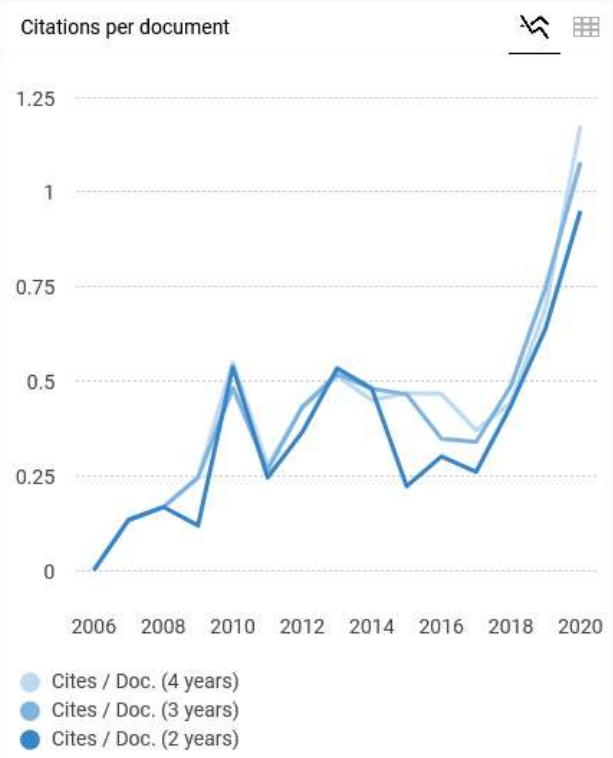


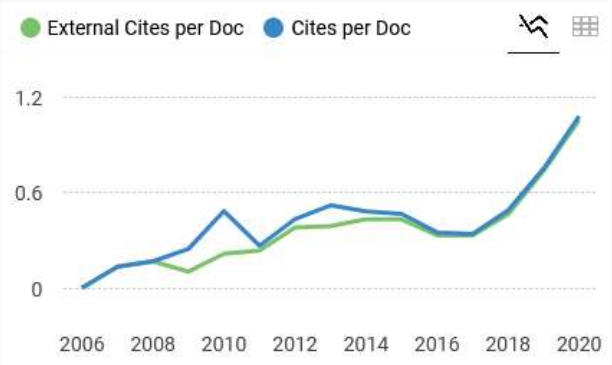


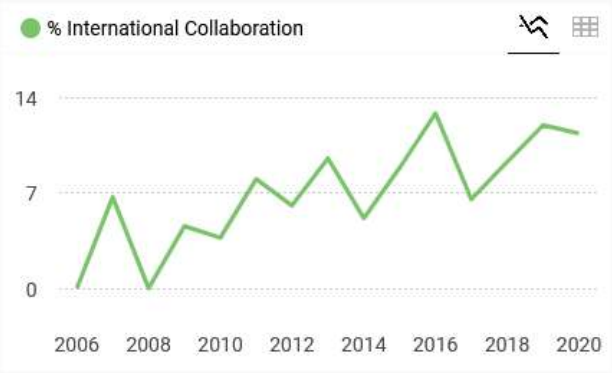


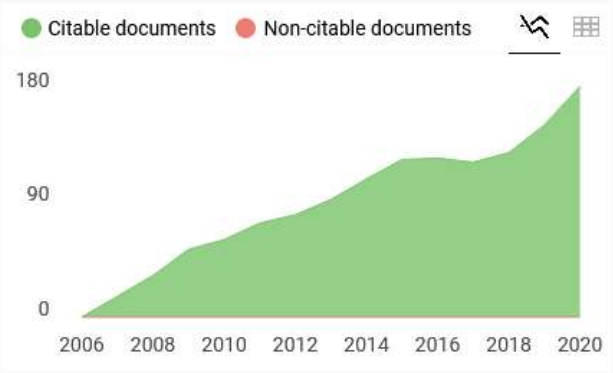


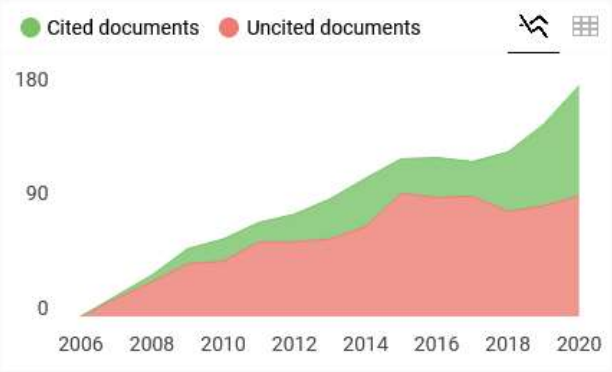



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