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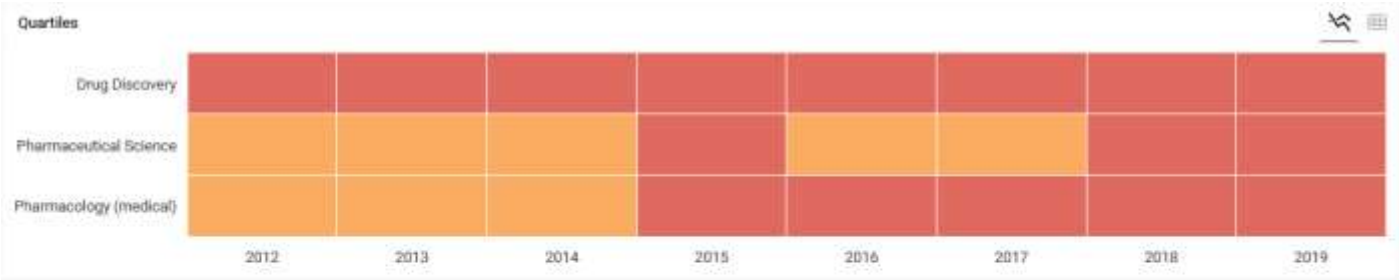
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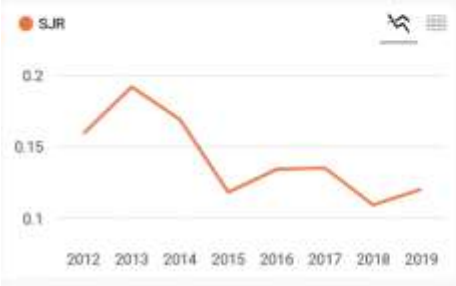
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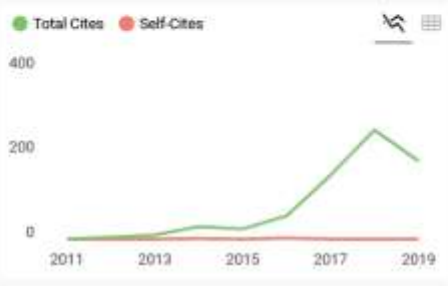
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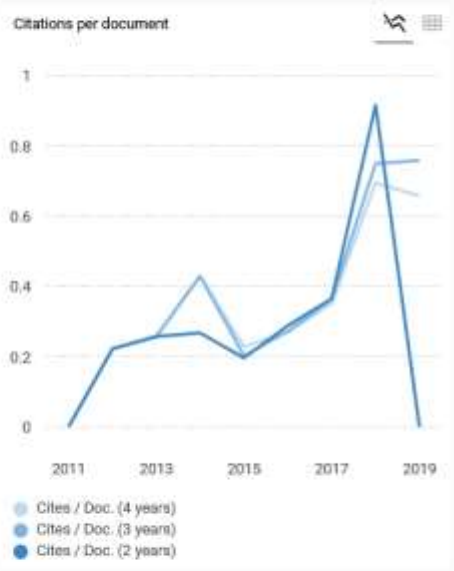
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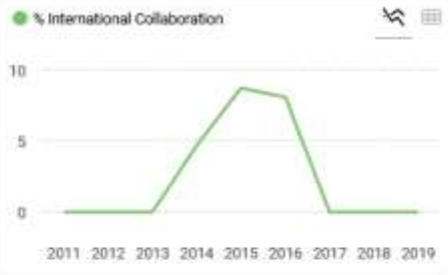
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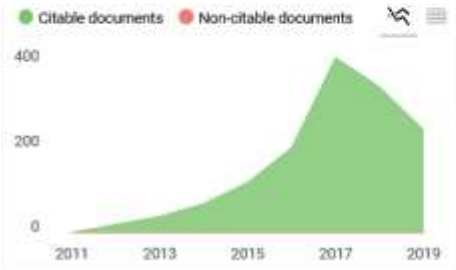
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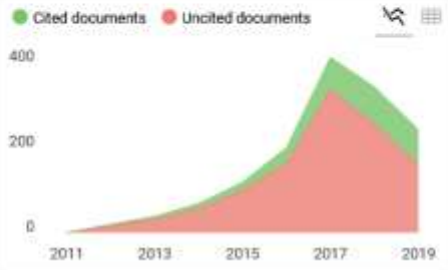
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Research Article

Evaluation of the Antibody Response and Uptake of Ca-Alginate Microspheres Containing Model Antigen After Oral Immunization

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Abstract
Oral delivery system has numerous advantages; however some peptide and protein drugs may occurs degradation by gastrointestinal enzyme when given orally. Ca-alginate microspheres containing model antigen Ovalbumin were prepared to protect ovalbumin from degradation by forming microspheres to enhance immune response and uptake of microspheres by lymphoid tissue in mice's intestine. Ovalbumin-alginate microspheres were produced by aerosolization technique using Na-alginate polymer and $CaCl_2$ cross-linker. To increase stability during storage, microspheres were dried with 5% maltodextrin as lyoprotectant. To observe immunological evaluation, hemagglutination test by measuring antibody titre was conducted for all groups compared to vaccine product which administered via intra muscular route. In vivo uptake study of microsphere in mice's villi and Peyer's patches at different time series were performed by labelling microspheres with rhodamine B. IgG titre immune response of Ca-alginate microspheres containing ovalbumin increased when compared to blank microspheres and ovalbumin solution. BSA had similar titre as ovalbumin-alginate microspheres. In addition, lyophilized ovalbumin-loaded alginate microspheres with 5% maltodextrin produced the highest IgG titre. Interestingly, freeze-dried ovalbumin-loaded Ca-alginate microspheres showed equal immune response as intra muscular vaccine product. For uptake study in the intestine, it resulted both ovalbumin-alginate microspheres with and without 5% maltodextrin were able to be taken up by villi at 6 hours after given orally and taken up further by villi and Peyer's Patches at 7 to 10 hours. In conclusion, ovalbumin-loaded Ca alginate microspheres with 5% maltodextrin indicated that the Ca-alginate microspheres entrapping ovalbumin have potential to enhance immune response and facilitate the uptake.

Keywords: Ovalbumin, Alginate, Microspheres, Hemagglutination, Uptake

INTRODUCTION

Ovalbumin-loaded alginate microspheres used Peptide and protein drugs have generally given parenterally due to degradation by gastrointestinal enzymes[1]. However parenteral delivery system has disadvantages such as patient discomfort, the need of specialized personnel and high costs. Oral delivery systems is one of the alternative routes of drug or vaccine administration that is non-invasive, which can avoid the pain and discomfort when granting and easily if required repeated administration[2]. Peyer's patches (PP) is the main target of oral delivery systems in the small intestine as a place for the transport of pathogens to the lymphoid tissue. This function is carried out by M-cells which are located between epithelial cells, bringing antigens and microparticles measuring less than 10 μm[1].

Microspheres contain biodegradable polymer and ideally having a particle size of less than 200 μm[3]. Natrium alginate is a natural polymer that is non-toxic, biocompatible and and relatively inexpensive[4].

Alginates form a three-dimensional structure when reacted with a multivalent ion. Divalent cations such as calcium, barium and strontium binding between a collection G of alginate chains, forming bridges between the chains that cause gelling alginate solution. Ca^{2+} is one of the best options as agents continued cross with alginate[5]. Ca^{2+} is binding poly guluronate acid group (G) of the alginate in the form of two-dimensional planar, yields a so-called egg-box[6].

Previously, production of ovalbumin-alginate microspheres using gelation ionotropic technique by aerosolisation had the advantage of spherical shape, smooth with a small particle size (<30 m) that meets the requirements of particle size for oral delivery systems[7]. Maltodextrin was added to improve the stability of the microspheres during storage during freeze drying[8]. Addition maltodextrin lyoprotectant were found to form smooth surfaces and smaller microspheres (<6 μm) when compared to microspheres without lyoprotectant[9]. Ovalbumin was used as model antigen.

Author for Correspondence

Table 1: Groups of Alginate microspheres and controls for hemagglutination and uptake study

Group	G1	G2	G3	G4	G5	G6	G7	G8
Ovalbumin 2.5%		V			V	V		
Alginate 2.5%				V	V	V		V
$CaCl_2$ 1.5M				V	V	V		V
BSA 2.5%	V							
Maltodextrin 5%			V			V		
Vaccine product							V	
Rhodamine								V

G1: BSA solution as control
G2: Ovalbumin solution as control
G3: Maltodextrin solution as control
G4: Blank Ca-alginate microspheres
G5: Ovalbumin-Ca alginate microspheres
G6: Ovalbumin-Ca alginate microspheres with 5% maltodextrin
G7: Vaccine product (im)
G8: Blank Ca-alginate microspheres-rhodamine

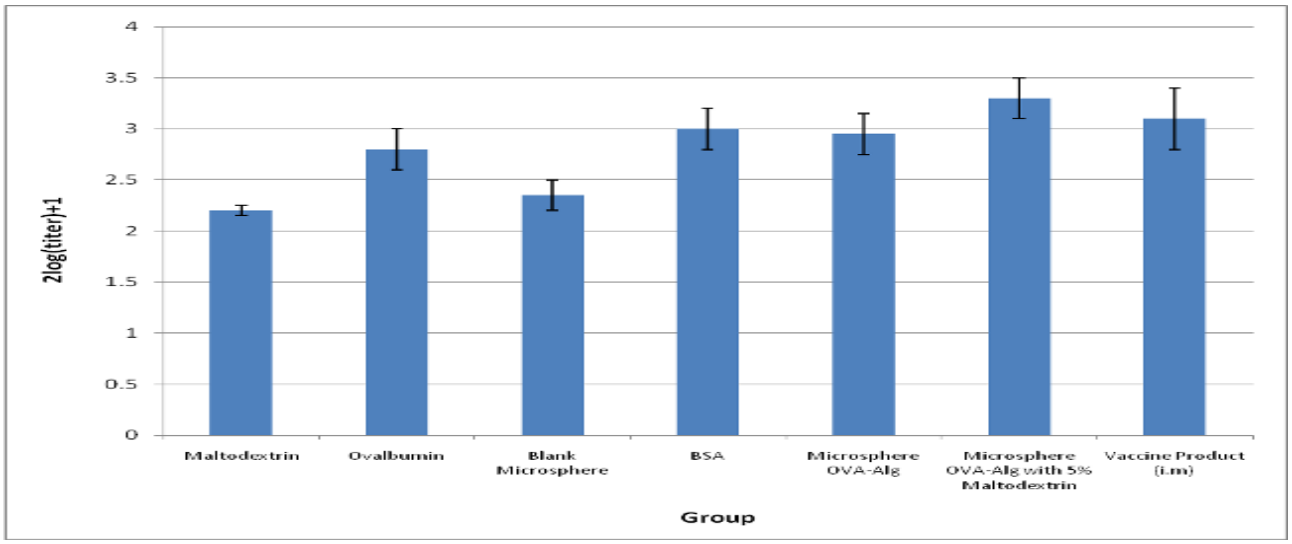

Figure 1: Hemagglutination IgG titre of groups

To evaluate immune response of ovalbumin entrapping in the alginate microspheres, hemagglutination test and uptake study were needed. The hemagglutination assay is essential to evaluate the formation of antibody and ability to stimulate immune response. Furthermore, to determine the uptake and distribution of microspheres in the gastrointestinal tract as well as the target organ, histology using fluorescent microscope is a qualitative approach that may provide direct evidence of the existence and location of the particles on the network[10].

This research evaluated Ca-alginate microspheres contain ovalbumin with and without maltodextrin lyoprotectant and vaccine product. Ovalbumin and blank microspheres were used as negative control.

MATERIALS AND METHODS

Materials

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), phospate buffer saline pH 7.2, Na EDTA, aquadest, red gout blood cell, and mus musculus strain Balb C from Pusat Veterenaria Farma (PUSVETMA) Surabaya.

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 solution of 1.5 M $CaCl_2$ solution at pressure of 40 psi. The mixture was stirred at 1000 rpm for 2 hours. Microspheres formed were collected and then separated by centrifugation at 2,500 rpm for 6 min and washed twice. Microspheres resuspended in lyoprotectant solution (1g/10mL) with concentration according to the formula. The suspension was dried by 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 of alginate microspheres, ovalbumin-loaded alginate microspheres and controls can be seen in Table 1.

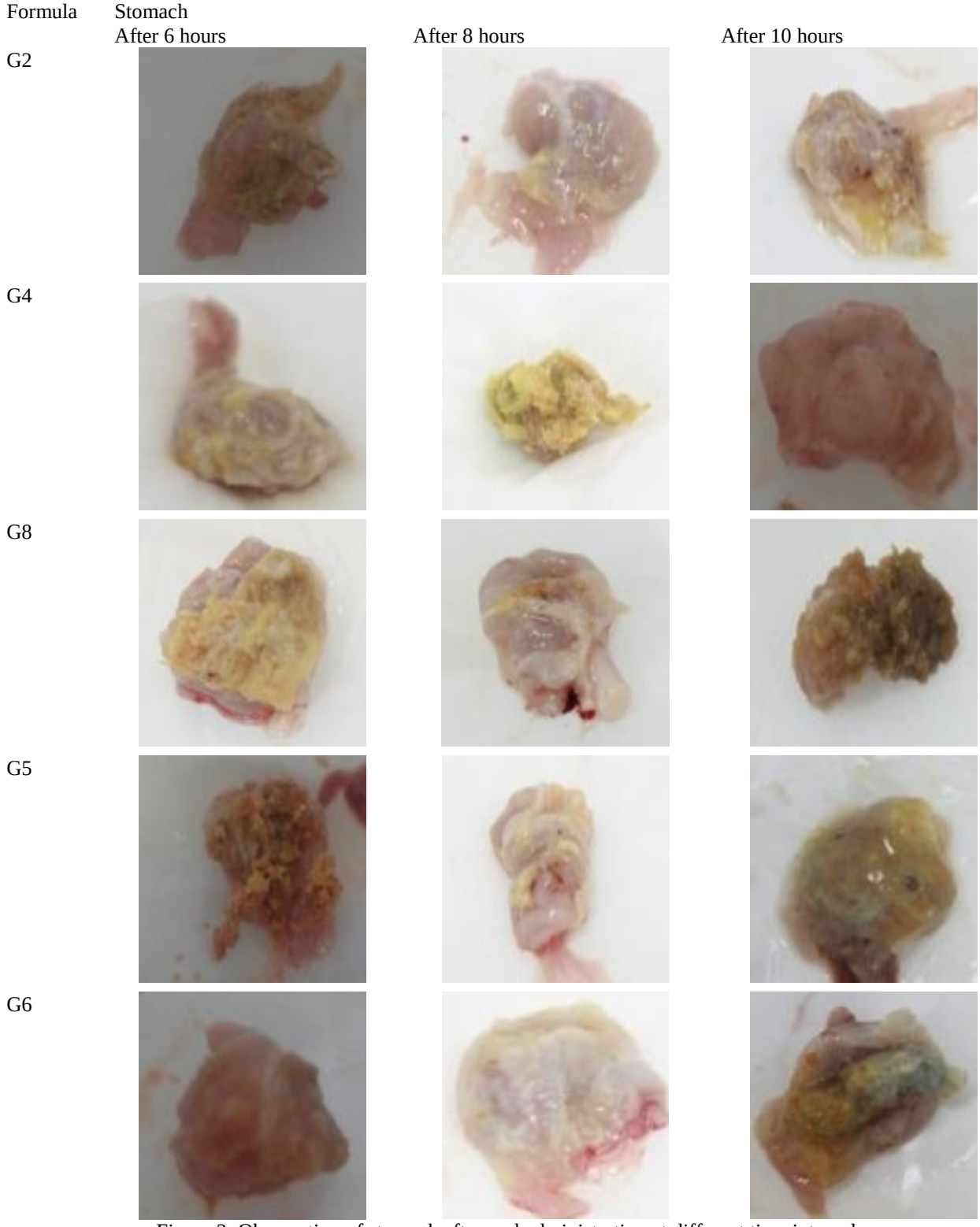

Figure 2: Observation of stomach after oral administration at different time interval

Hemagglutination test

Animals in vivo study has been approved by ethics committee and met National Ethic Standard by Faculty of Veterinary Universitas Airlangga. Mice were given orally ovalbumin-loaded alginate microspheres or control for five days for all groups of mice. At day 7, animals were injected intraperitonially using gout red blood cell suspension. At day 17, bloods were taken intracardially and were analysed for the serum or supernatant after centrifugation. Hemagglutination study was conducted to analyse immune response by measuring IgG titres. Vaccine product which was commonly used intra muscular was used to compare IgG titres.

Uptake of microspheres

Formulas of Ca-alginate microspheres with and without lyoprotectant compared to ovalbumin, maltodextrin and

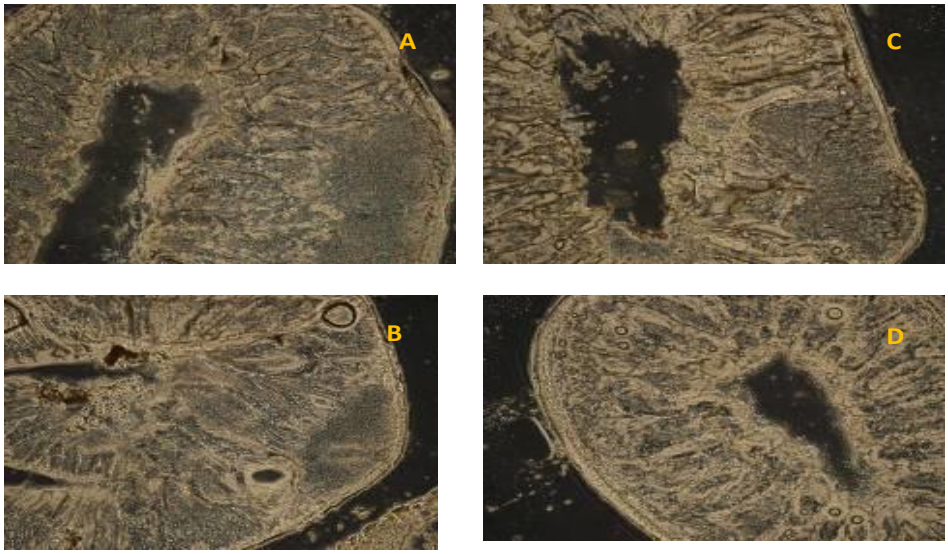

Figure 3: Uptake of maltodextrin (G3) at 6 hour (A) and 8 hour (B); blank microspheres (G4) at 6 hour (C) and 8 hour (D) after oral administration

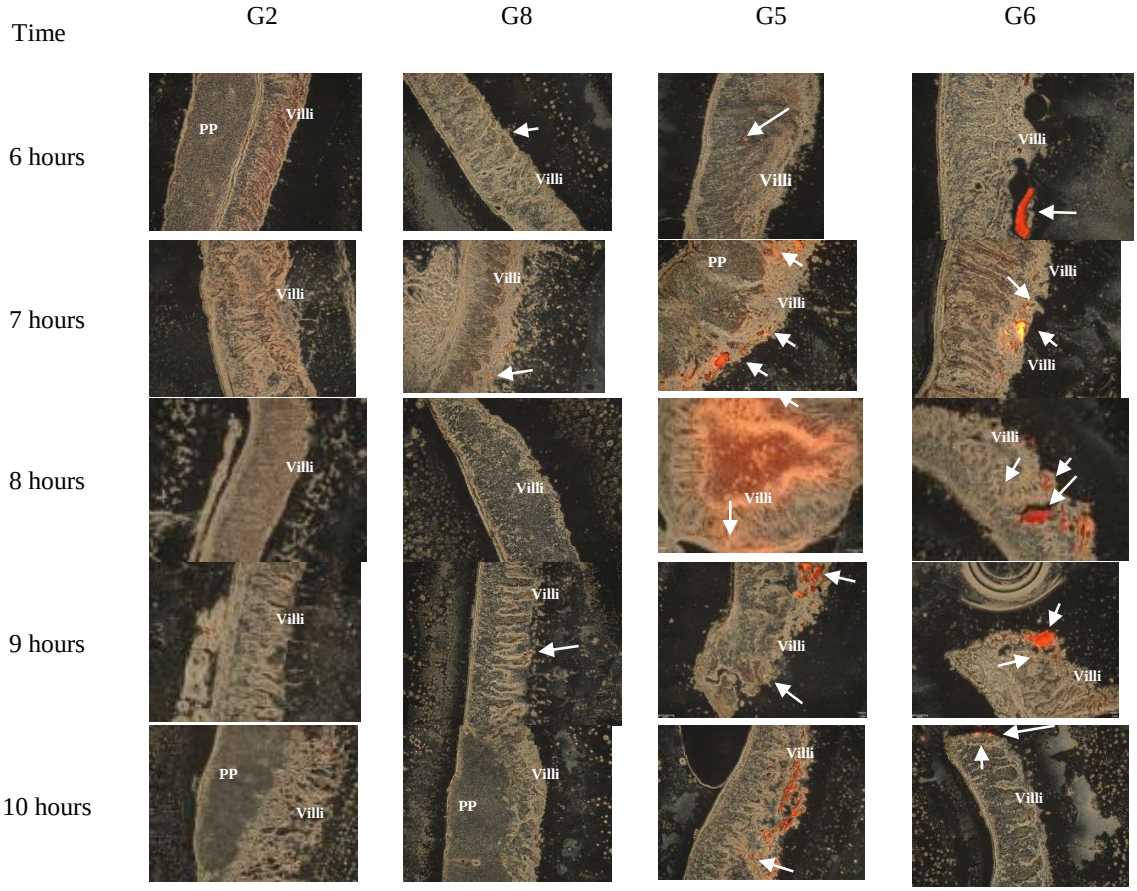

Figure 4: Uptake of labelled-ovalbumin (G2), labelled-blank microspheres (G8), labelled-ovalbumin-alginate microspheres (G5) and labelled ovalbumin-alginate microspheres with 5% maltodextrin (G6)

blank microspheres control were used. Rhodamine B was a fluorochrome and was used to label all groups. All groups were dispersed into CMC Na in aqueous solution as control. Prior sacrificed, Mice were adapted for a week in a room with a temperature of 25 ºC ± 2 ºC in a separated cage. Mice were then fasted for 16 hours followed by orally administered. Volume oral administration was 500 µL/ 25 gram body weight. To determine the uptake in intestinal mice, following after 6,7,8,9 and 10 hours after oral administration, mice were sacrificed. Mixture intestinal tissue histology was then observed with a fluorescent microscope using a red filter.

Data Analysis

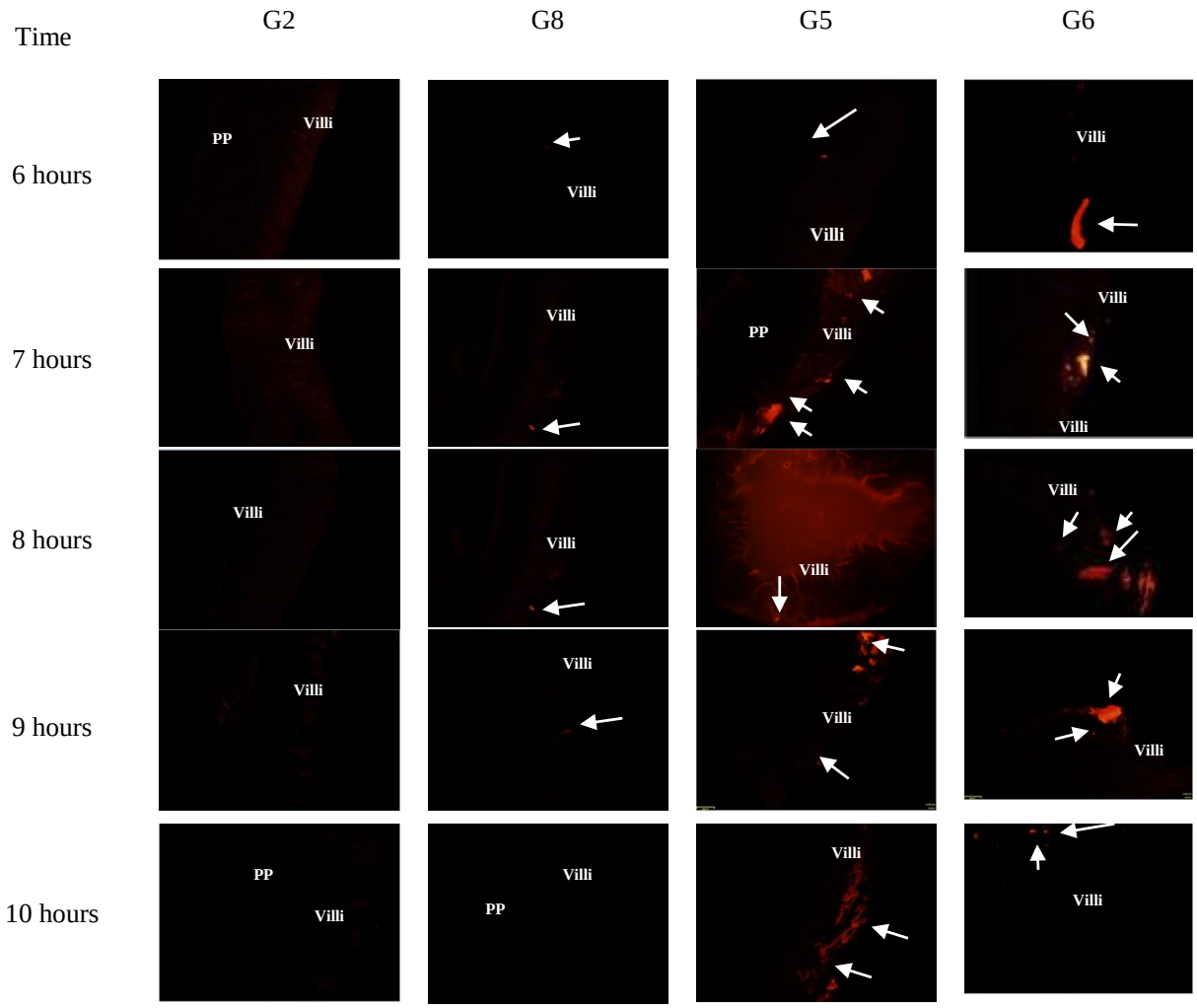

Figure 5: Uptake by red filter of labelled-ovalbumin (G2), labelled-blank microspheres (G8), labelled-ovalbumin-alginate microspheres (G5) and labelled ovalbumin-alginate microspheres with 5% maltodextrin (G6)

The data are expressed as the mean ± SD from triplicates experiments. The uptake study was analysed qualitatively using three replicates experiments.

RESULTS AND DISCUSSION

Hemagglutination study

From hemagglutination study as shown in Figure 1, lyophilized ovalbumin-Ca alginate microspheres with 5% maltodextrin exhibited higher antibody titre compared to ovalbumin-Ca-alginate microspheres without lyoprotectant, whereas ovalbumin-alginate microspheres produced higher IgG than blank microspheres, ovalbumin control and BSA. Importantly, freeze dried ovalbumin-Ca alginate microspheres using 5% maltodextrin produced the highest titre compared to other groups and an equal IgG titre as vaccine product. This indicated that ovalbumin antigen has arrived at the target site and ovalbumin microspheres was able to across the GI tract barrier in peyer's patches produced immune response and potential for oral vaccine delivery system. Maltodextrin lyoprotectant seemed stabilized microspheres due to hydrogen bonding between sugar or alcohol sugar and protein during freeze drying and avoid aggregation [8]. Vaccine product which was given via intra muscular route produced similar level of IgG titre as microspheres. However, use of oral vaccine product was also needed for further study.

Uptake of microspheres using fluorescence microscope

From observation of the stomach after two and three hours oral administration, both of microspheres with and without lyoprotectant, the colour of stomach were still pink, but after six hours less colour were found (Fig. 2). This indicated that Ca-alginate microspheres have entered the intestine. The investigation was continued to 10 hours.

Several other studies showed that microspheres started to be observed its existence in the ileum after three hours[1], four[11] and six hours[12] after oral administration. Factors which affected gastric emptying time included dosage volume, viscosity, osmotic pressure, chemical composition and pH[13].

In observation using fluorescent microscopy, the selection of the proper exposure time is important to overcome the auto fluorescent. The observation of maltodextrin (G3) at six and eight hours after oral administration showed no red luminescence in the ileum, so that the presence of maltodextrin did not affect the existing red luminescence in ovalbumin-alginate

microspheres with 5% maltodextrin lyoprotectant (G6) (Fig. 3). In case of blank microspheres (G4), there was also no red luminescence. This means no blank microspheres undergo auto-fluorescent when observed with a red filter.

Observations uptake of G2, G5, G6, and G8 on the ileum of mice was performed on 6 to 10 hours after administration can be seen in Figure 4 and 5. The uptake of ovalbumin (G2) in Figure 4 showed that in the 6th and 7th hours after oral administration, red luminescence was seen in the intestinal villi, but the luminescence was disappeared from the villi in the 8,9 and 10 hours. Unencapsulated ovalbumin may not be seen to be up taken on the deeper ileum due to inefficient and was not strong enough to induce an immune response in lymphoid tissue[1]. In the study conducted by Borges et al[14], the amount of ovalbumin in uptake by a network was very little. Moreover, in previous research, ovalbumin administered orally produce Ig G titer was low compared to ovalbumin were trapped in the microsphere delivery system because ovalbumin was degraded by stomach acid[15].

For blank microspheres-rhodamine (G8) on the 6 to 10 hours, weak luminescence were derived from the microspheres and only limited to the villi surface (Fig. 4 and 5). In terms of ovalbumin-loaded alginate microspheres (G5) in both figures showed that at 6 hours, ovalbumin-alginate microspheres started entering through the villi. From seven to ten hours, they entered deeper. Interestingly, uptake of ovalbumin-Ca alginate loaded microspheres with maltodextrin lyoprotectant (G6) in both figures showed that at the 6th hour until the 9th hour, ovalbumin-alginate microspheres contains maltodextrin started to enter into the villi and go went through deeper inside the villi.

The uptake of G5 and G6 in the villi toward the deeper part compared to blank microspheres (G8) indicated that there was a correlation between ovalbumin with rhodamine, where the red luminescence of rhodamine described the presence of ovalbumin. The uptake of G5 and G6 was proved to be more in the villi and Peyer's Patches (Fig. 4 and 5). This is in line with the results of research conducted previously obtained measurements of Ig G titer immune response of blank microspheres was very low, this was due to the polymer only which was used in the microspheres production was not antigenic[15].

Uptake of microparticles in the intestine were influenced by particle size[12] and hydrophobicity[16]. From these results, G5 had a particle size of 7.43 μm and G6 had the size of 5.46 μm. In the study conducted by Tabata et al[12], after the uptake in Peyer's Patches, the particle size of the particles was less than 5μm was transported to the lymph, which is a lymphoid tissue systemic, where the antigen contained would be released and produce an immune response, whereas particles with size larger than 5μm would stay in Peyer's Patches and released antigen. Because of the particle size of G5 and G6 were more than 5μm, both formulas may stay longer in Peyer's Patches and delivered ovalbumin that can cause an immune response. Moreover, addition of lyoprotectant in G6 was used to protect the microspheres during lyophilisation and produced microspheres with a smaller size, spherical shape, and smooth morphology[9]. Results of uptake of ovalbumin-Ca alginate microspheres with 5% maltodextrin which was seemed to be slower release of ovalbumin and longer stay in villi was correlated with its in vitro release testing, which was at pH 7.4 as simulated intestinal condition at the 11th hour, the release amount of ovalbumin were only about 29,92% (data was not shown). From in vivo and in vitro release study, this can be attributed the possibility that the microparticles released ovalbumin slowly and may stay longer in Peyer's Patches.

CONCLUSION

Ovalbumin-loaded Ca alginate microspheres containing maltodextrin were found to enhance higher in vivo IgG titre compared to non-encapsulated ovalbumin or BSA and Ca-alginate microspheres with no lyoprotectant. However, vaccine product enhanced as same level IgG titre as Ovalbumin-Ca alginate microspheres containing maltodextrin. For uptake study, it was visualized that alginate microspheres with and without lyoprotectant delivered ovalbumin into intestinal villi and peyer's patches successfully. Moreover, vaccine product also resulted uptake into intestinal villi as same as ovalbumin-alginate microspheres with and without maltodextrin. These results indicated that the freeze-dried ovalbumin-alginate microspheres with maltodextrin are potential for oral vaccine delivery system.

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AUTHOR(S)' STATEMENT(S)

The authors declare no conflict of interest.

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