Drug and Chemical Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/idct20

Single and repeat-dose toxicity and local tolerance assessment of newly developed oil emulsion adjuvant formulations for veterinary purposes

Karrar Ali Mohammed Hasan Alsakini, Ebru Sanci, Aylin Buhur, Altuğ Yavasoglu, N. Ülkü Karabay Yavasoglu & Ayşe Nalbantsoy

To cite this article: Karrar Ali Mohammed Hasan Alsakini, Ebru Sanci, Aylin Buhur, Altuğ Yavasoglu, N. Ülkü Karabay Yavasoglu & Ayşe Nalbantsoy (13 Dec 2023): Single and repeat-dose toxicity and local tolerance assessment of newly developed oil emulsion adjuvant formulations for veterinary purposes, Drug and Chemical Toxicology, DOI: 10.1080/01480545.2023.2291985

To link to this article: https://doi.org/10.1080/01480545.2023.2291985

Published online: 13 Dec 2023.

DRUG AND CHEMICAL TOXICOLOGY
https://doi.org/10.1080/01480545.2023.2291985

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Single and repeat-dose toxicity and local tolerance assessment of newly developed oil emulsion adjuvant formulations for veterinary purposes

Karrar Ali Mohammed Hasan Alsakini[a,*], Ebru Sanci[b], Aylin Buhur[c], Altuğ Yavasoglu[c], N. Ülkü Karabay Yavasoglu[d] and Ayşe Nalbantsoy[d]

[a]Graduate Faculty of Natural and Applied Science, Biotechnology Department, Ege University, Izmir, Turkey; [b]Center for Drug Development and Pharmacokinetic Applications, Ege University, Izmir, Turkey; [c]Department of Histology and Embryology, Ege University, Izmir, Turkey; [d]Department of Biology, Ege University, Izmir, Turkey

ABSTRACT

Adjuvants are components of vaccines that boost the intensity, duration, and breadth of the immune response. Insight into the mechanisms responsible for the immunotoxicity of both local and systemic adverse reactions following the use of adjuvants has been gained through research over the past twenty years. In the present study, single and repeated-dose toxicity and local tolerance of newly developed Water-in-Oil (W/O) and Water-in-Oil-in-Water (W/O/W) Emulsion adjuvants (Coralvac RZ 528, Coralvac RZ 506, Coralvac AT 318, Coralvac AT 318 SIS and Coralvac 252) by Coral Biotechnology Industry and Trade Incorporated Company were demonstrated after intramuscular injection in mice. In both toxicity studies, no adverse reactions such as death, general appearance, behavior, or weight loss were observed in the mice in the experimental groups. The results indicate that clinical chemistry parameters demonstrated normal function of the major organs and no irreversible damage to the mice in all adjuvant groups compared to the control group. In histopathologic investigation of single dose toxicity study, inflammation, edema, and large amounts of lipid droplets were observed on the 7th day in all experimental groups. On the 14th day, when the control group and the experimental groups were compared, it was seen that inflammation and edema had decreased considerably. Similarly, repeated dose toxicity study showed mild inflammation and edema in the control group, while quite widespread and severe inflammation, edema, and diffuse lipid droplets of varying sizes were observed in all adjuvant groups compared to the control group. These observations would be useful for the future development of oil-based adjuvants and their use in veterinary inactive vaccines.

ARTICLE HISTORY
Received 7 June 2023
Revised 13 November 2023
Accepted 21 November 2023

KEYWORDS
Adjuvants; veterinary vaccines; emulsions; single dose toxicity; repeated dose toxicity; local tolerance

1. Introduction

Vaccines are one of the most effective medical inventions of the previous century. Their values have currently changed and are now characterized from a broader perspective that includes prophylactic and therapeutic vaccines (Tamargo Santos et al., 2019). The current advancement of animal husbandry has been severely hampered by the emergence of animal serious diseases. However, vaccination serves a crucial role in preventing the spread of infectious diseases in livestock. Vaccines for animals are used for several reasons, including preventing the spread of disease, increasing animal productivity, and ensuring the safety of the food supply (Woodland, 2019). Improved antigen specificity and reduced toxicity have led to the creation of numerous new vaccines for animal diseases, however despite these advantages, their immunogenicity is low and the immune response they elicit in the body is insufficient. The health, social, environmental, and economic achievements obtained by these vaccinations in control programs or emergencies would not have been conceivable without the use of adjuvants in their formulation (Sander et al., 2020; Warimwe et al., 2021). Vaccine adjuvants are chemicals added to vaccinations to boost the immunogenicity of highly purified antigens that lack enough immunostimulatory qualities on their own (Di Pasquale et al., 2015). Therefore, a safe and effective adjuvant should be added to boost its immunogenicity (Bihua et al., 2017; Zeqian et al., 2012). The adjuvants allow, among other activities, to alter and control the interaction between antigens and the immune system of the body. However, it is known that novel adjuvants with predictable efficacy are clearly needed for future vaccines (Schijins and O'Hagan, 2006).

Vaccines for both humans and animals typically include a number of different immunologic adjuvants and other additives, all of which work in combination with the vaccine's antigen to accelerate, prolong, or otherwise improve the immune response to that antigen (Cerpa-Cruz et al. 2013, Heegaard et al. 2011, Sayers et al. 2012). These adjuvants can enhance immune responses in a number of ways. Immunostimulants [saponins, Toll-like receptor (TLR) agonists, cytokines] and delivery agents [emulsions, microparticles,

CONTACT N. Ülkü Karabay Yavasoglu ulku.karabay@ege.edu.tr; Ayşe Nalbantsoy nalbantsoya@egmail.com Faculty of Engineering, Department of Bioengineering, Ege University, Izmir, Turkey
*Additional Affiliation: Department of Microbiology, Baghdad University, Baghdad, Iraq.

mineral salts] can be roughly separated into two categories (Cox and Coulter, 1997; O'Hagan, 2015). Antigen-presenting cells (APCs) can be stimulated to secrete more cytokines with the help of immunoostimulants. However, delivery agents aid in keeping antigens in the correct conformation for presentation to APCs and in allowing for a gradual release to maintain immune activation over time. Emulsions and mineral salts can provide a depot effect at the injection site, resulting in a delayed release of the antigen and ongoing stimulation of immune cells, while TLR agonists and other immunostimulatory chemicals can boost immune cell recruitment and cytokine secretion (Burakova et al., 2018). In addition to this, they can slow the release of antigen, prevent it from being degraded by proteases, and direct it to mucosal associated lymphoid tissues (MALT) like Peyer's patches. They can also activate or modulate immune cells like dendritic cells and lymphocytes, improve MHC Class I presentation for optimal cytotoxic T lymphocyte (CTL) responses (Degen et al., 2003; McCluskie and Weeratna, 2001). In comparison to polymers like poly lactic-co-glycolic acid and chitosan, oil emulsions are utilized more frequently as animal vaccination carriers due to their lower cost and sustained release with antigen-protective action (Myc et al., 2003). When the antigen vaccine emulsion is manufactured, the oil adjuvant, which consists of oil and emulsifier, is usually covered in the microstructures formed by oil and water (Bielinska et al., 2007; Biruss et al., 2007). When administered intramuscularly, a vaccination can cause the formation of a reservoir, the delayed release of antigen from oil and water microstructures, and the induction of both cellular and humoral immune responses (Zhou et al., 2021).

The adjuvants whose safety tests were investigated within the scope of the study are the products formulated by Coral Biotechnology Company through R&D studies. Adjuvants include ultra-refined mineral oil and special emulsifier packages. The adjuvants used in the study form water-in-oil (w/o) and water-in-oil-in-water (w/o/w) emulsions. Water-in-oil type emulsions are the type of emulsion that is formed by keeping the antigen phase trapped in the oil phase as droplets. This type of emulsion keeps the antigen at the injection site and releases continuously, and this release triggers long-term humoral immunity in B cells (Jansen et al., 2006). Known as multiple or double emulsions, w/o/w type emulsions contain water droplets trapped in oil droplets dispersed in the outer water phase. The antigen can be found in the internal or external water phase of the structure (Fukanoki et al., 2000). They exhibit a rather weak character in terms of stability, but are more reliable in terms of effects compared to other types of emulsions (Degen et al., 2003).

The objectives of this study were to perform the safety tests of the Coral Biotechnology Industry and Trade Incorporated Company's newly developed Water/Oil (W/O) and Water/Oil/Water (W/O/W) emulsion adjuvants, which will be tested for the first time within the scope of this study in Turkey.

2. Materials and methods

2.1. Experimental design and animals

The study was carried out in accordance with the requirements of 'Part 3: Safety tests' of Annex 1, Title II to Directive 2001/82/EC and according to the requirements of Ph. Eur. 5.2.6 'Evaluation of safety of veterinary vaccines and immunosera' and WHO Guidelines on the evaluation of vaccine adjuvants and adjuvanted vaccine (2013). Toxicity studies were carried out with the approval of Ege University, Local Ethics Committee of Animal Experiments (EUHADYEK) with a permission number 2022-013.

In the study, albino Swiss mice obtained from Izmir Veterinary Control and Research Institute, sexually matured, 6–8 weeks old and weighting between 15–25 g were used. The animals were quarantined for 7 d at Ege University Drug Development & Pharmacokinetic Research-Application Center (ARGEFAR). Mice used in the experimental groups were randomly selected and were housed in steel cages with individual ventilation in a temperature-controlled environment (22 ± 2 °C). Relative humidity was set at 45–65% with a 12 h light/dark period. They fed with standard laboratory feed and water, *ad libitum*. Feed and water were changed daily.

2.2. Adjuvants

In this study, Water/Oil and Water/Oil/Water (W/O/W) Type Emulsion Adjuvants developed by Coral Biotechnology Industry and Trade Incorporated Company were used. The adjuvants are shown in Table 1.

2.3. Single dose toxicity study

In the study, 6–8 weeks old, albino Swiss mice were used. Experimental groups consisted of 5 adjuvant groups (5 mice in each group); and a control (0.9% NaCl) group Concentrations were administered intramuscularly in a single dose of 0.1 ml/mouse from each adjuvant and control group. The animals were given sufficient water along with standard laboratory pellet food. Mice were weighted on day 0, day 2, day 7 during the administration of the adjuvants, and after the completion of the administration (day 14). The animals were observed and examined daily for signs of abnormal local and systemic reactions. On the 2nd, 7th and 14th days of the

Table 1. Water/oil (W/O) and water-in-oil-in-water (W/O/W) type emulsion adjuvants to be used in the studies.

Trade name	Emulsion type	Application	Oil type	Immunostimulant	Antigen/ Adjuvant (%V/V)
Coralvac RZ 528	W/O	Chichen (IBV)	Non-metabolizable oils and emulsifiers	Not included	30/70
Coralvac RZ 506	W/O	Chichen (IBV)	Non-metabolizable oils and emulsifiers	Not included	40/60
Coralvac AT 318	W/O/W	Cattle (FMD)	Non-metabolizable oils and emulsifiers	Not included	50/50
Coralvac AT 318 SIS	W/O/W	Cattle (FMD)	Non-metabolizable oils and emulsifiers	Not included	50/50
Coralvac 252	W/O	Cattle (FMD)	Non-metabolizable oils and emulsifiers	Not included	50/50

study, postmortem macroscopic and microscopic examinations of the injection site were done. Other objective criteria such as death, changes of general appearance or behavior, water and food consumption or weight loss were recorded. When the study was completed, all organs are removed (brain, liver, lung, kidney, spleen), weighted and the organs' averages, standard deviations and relative organ weights were calculated. Relative organ weights (organ to total body weight ratio) were calculated by dividing organ weights by total body weights according to Lazic et al. (2020).

2.4. Repeated dose toxicity study

In the study, 6–8 weeks old, albino Swiss mice were used. Experimental groups consisted of 5 adjuvant groups (8 mice in each group; 4 males and 4 females) and a control (0.9% NaCl) group. In the adjuvant groups, 0.1 ml/mouse was administered intramuscularly to the mice at the same time per week for 28 d. Similarly, 0.1 ml of 0.9% NaCl was applied to the control group for 28 d. The animals were given sufficient water along with standard laboratory pellet food. Mice were weighted every week during the administration of the adjuvants and after the administration was completed. The animals were observed and examined daily for signs of abnormal local and systemic reactions. At the end of the study, postmortem macroscopic and microscopic examinations of the injection site were done. Other objective criteria such as death, changes of general appearance or behavior, water and food consumption or weight loss were recorded. When the study was completed, all organs are removed (brain, liver, lung, kidney, spleen), weighted and the organs' averages, standard deviations and relative organ weights were calculated. Relative organ weights (organ to total body weight ratio) were calculated by dividing organ weights by total body weights according to Lazic et al. (2020).

2.5. Biochemical analysis

In all toxicity study groups, blood samples were collected from the heart (about 0.7–1 ml each mouse) under ketamine and xylazine anesthesia. Blood samples were centrifuged at 2000 rpm for 10 min in order to separate serum. In the present study, biochemistry analysis was performed with blood serum on Fujifilm FUJI DRI-CHEM NX500V IC device with Comprehensive S Panel kit including 13 parameters (Total protein-TP, Albumin-ALB, Globulin-GLOB, Glucose-GLU, Alanine aminotransferase-ALT, Gamma-glutamyl transferase- GGT, Alkaline phosphatase-ALP, Total bilirubin- TBIL, Total cholesterol-TCHOL, Creatinine-CRE, Blood urea nitrogen-BUN, Calcium-Ca, Inorganic phosphate-IP).

2.6. Histopathological analysis

The preserved injection site sections (muscle) of mice from the control group (Group I) and the treated groups (from Group II to Group VI) in the single-dose toxicity and repeated-dose toxicity trials were subjected to histological examination. Tissues were collected in a falcon tube to washed 2 times with PBS. Then tissues were kept in 4% paraformaldehyde for 1 night. The tissues were then passed through a series of increasing degrees of alcohol and left to air dry. Dried samples were passed through Xylol 3 times for 30 min until they became transparent. Paraffin-infused samples were kept in an oven at 58 °C for 1 night, the paraffin was renewed and waited for 2 h. This process was repeated 2 times respectively. Then, the tissues were embedded in paraffin and routine hematoxylin-eosin staining was performed on 5-micron sections taken from paraffin blocks. The images were taken and examined under the microscopy (BX5, Olympus, Tokyo, Japan) (Yigitturk et al., 2017). Histopathological examinations of the injection site were performed at Ege University, Faculty of Medicine, Department of Histology and Embryology.

2.7. Statistical analysis

Statistical evaluations in the study were made using SSPS 25.0 (IBM Corp., Armonk, New York, USA). At the end of the experiment, means and standard deviations were calculated for measurement data in each group, which contained body weights, organ weights and organ weight/total body weight ratios (relative organ weights) of the mice. Data were expressed as the mean ± standard deviation (SD). The statistical significance was compared between control and experimental groups by one way analysis of variance (ANOVA) followed by LSD test. Values of $p \leq .05$ were regarded as statistically significant.

3. Results

3.1. Clinical signs and body weight changes in single-dose toxicity study

During the single-dose toxicity test, no adverse reactions such as death, general appearance, behavior or weight loss were observed in the mice in the experimental groups. Animal weights and % increase in weight gains according to the groups are presented in Table 2. There was no statistically significant difference in body weight in any of the adjuvant groups compared to the control group. After a single dose toxicity study, organ (brain, liver, lung, kidney and spleen) weights and relative organ weights are presented in Table 3 and 4. There was no statistically significant difference in organ weights except brain for Coralvac 252 group and lung for Coralvac RZ 528, RZ 506, and AT 318 groups compared to the control group. There was no statistically significant difference in relative organ weights except lung for Coralvac AT 318 and AT 318 SIS groups in any of the adjuvant groups compared to the control group. No differences were observed between experimental groups for water and food consumption (data not shown).

3.2. Clinical signs and body weight changes in repeated-dose toxicity study

During the repeated dose toxicity test, no adverse reactions such as death, general appearance, behavior or weight loss were observed in the mice in the experimental groups. Animal weights and % increase in weight gains according to the groups are presented in Table 5. There was no statistically significant difference in body weight in any of the adjuvant groups compared to the control group. After the

Table 2. Total body weight values of mice after a single dose toxicity study.

	Total body weight (grams) (mean ± SD)				
Treatment Groups	Day 0	Day 2	Day 7	Day14	% Body weight gain
Control	24.31 ± 2.95	25.36 ± 2.84	28.65 ± 2.03	31.40 ± 1.50	29.16 ± 3.63
Coralvac RZ 528	23.83 ± 2.11	24.20 ± 1.57	26.31 ± 1.19	27.35 ± 0.90	17.96 ± 4.62
Coralvac RZ 506	24.50 ± 1.76	25.43 ± 1.22	27.28 ± 1.40	28.79 ± 0.98	18.02 ± 4.52
Coralvac AT 318	28.69 ± 1.93	29.25 ± 2.10	30.03 ± 2.05	31.35 ± 2.21	10.05 ± 1.30
Coralvac AT 318 SIS	29.91 ± 2.83	30.35 ± 2.73	31.29 ± 2.84	32.01 ± 2.87	10.34 ± 0.62
Coralvac 252	27.37 ± 2.72	27.75 ± 2.78	28.85 ± 2.16	30.43 ± 1.96	11.33 ± 4.87
F	1.204	1.174	0.875	0.919	0.739
p	0.337	0.351	0.513	0.487	0.603

Table 3. Organ weights of mice after a single dose toxicity study.

	Organ weights (grams) (mean ± SD)				
Treatment Groups	Brain	Liver	Lung	Kidney	Spleen
Control	0.40 ± 0.03	2.03 ± 0.17	0.25 ± 0.05	0.49 ± 0.13	0.16 ± 0.02
Coralvac RZ 528	0.37 ± 0.02	1.59 ± 0.30	0.15 ± 0.05*	0.41 ± 0.14	0.14 ± 0.02
Coralvac RZ 506	0.38 ± 0.03	1.85 ± 0.21	0.16 ± 0.02*	0.43 ± 0.05	0.14 ± 0.01
Coralvac AT 318	0.40 ± 0.02	2.01 ± 0.50	0.18 ± 0.01*	0.46 ± 0.06	0.13 ± 0.01
Coralvac AT 318 SIS	0.40 ± 0.02	2.17 ± 0.56	0.21 ± 0.06	0.52 ± 0.18	0.18 ± 0.05
Coralvac 252	0.44 ± 0.02*	2.11 ± 0.36	0.19 ± 0.03	0.58 ± 0.09	0.12 ± 0.03
F	3.798	1.556	3.490	1.239	2.594
p	0.011	0.210	0.016	0.322	0.052

*Statistically significant difference compared to the control group ($p < 0.05$).

Table 4. Relative organ weights (organ to total body weight ratio) of mice after a single dose toxicity study.

	Relative organ weights (grams) (mean ± SD)				
Treatment Groups	Brain	Liver	Lung	Kidney	Spleen
Control	0.012 ± 0.001	0.0566 ± 0.003	0.0055 ± 0.003	0.016 ± 0.001	0.0048 ± 0.001
Coralvac RZ 528	0.014 ± 0.002	0.0681 ± 0.010	0.0060 ± 0.010	0.015 ± 0.001	0.0051 ± 0.001
Coralvac RZ 506	0.014 ± 0.001	0.0696 ± 0.015	0.0063 ± 0.015	0.016 ± 0.001	0.0046 ± 0.001
Coralvac AT 318	0.013 ± 0.001	0.0688 ± 0.009	0.0068 ± 0.009*	0.016 ± 0.003	0.0059 ± 0.001
Coralvac AT 318 SIS	0.014 ± 0.003	0.0664 ± 0.004	0.0063 ± 0.004	0.018 ± 0.002	0.0039 ± 0.001
Coralvac 252	0.013 ± 0.001	0.0674 ± 0.006	0.0074 ± 0.006*	0.016 ± 0.002	0.0055 ± 0.001
F	0.886	0.843	2.710	0.713	2.450
p	0.507	0.534	0.047	0.620	0.066

*Statistically significant difference compared to the control group ($p < 0.05$).

repeated dose toxicity study, the organ (brain, liver, lung, kidney and spleen) weights of the groups are presented in Table 6, and relative organ weights are presented in Table 7. There was no statistically significant difference in relative organ weight in any of the adjuvant groups compared to the control group. No differences were observed between experimental groups for water and food consumption (data not shown).

3.3. Biochemical analysis

After the single dose and the repeated dose toxicity tests, the results of the biochemical analysis made from the blood taken from the mice in the experimental groups at the end of the experiment are presented in Tables 8 and 9. Rodents show variation in many clinical chemistry values. For this reason, although reference values cannot be specified for many parameters, in the evaluation made by considering the reference values of the specified breeds (Loeb and Quimby 1999); It was observed that Total protein (TP) and Albumin (ALB) values, which are indicators of general physical condition, were both within the reference values for animals in all experimental groups and the general health status of the animals was good. When ALT, GGT, ALP, and TBIL as hepatic system indicators, BUN, CRE, and IP as renal system indicators, TCHOL for vascular system and Ca, and IP as Bone system indicators were evaluated, it was determined that the application groups showed values compatible with the control groups.

3.4. Local tolerance of the injection sites

Injection site data on days 2, 7, and 14 after a single dose of adjuvant injection are presented in Figure 1. At 2 d and 7 d after the adjuvant injection, the local reactions at the injection site of the mice at all adjuvant treated groups were limited to edema only, and no local reactions were observed 14 d after the adjuvant injection. Injection site data on the 28th day after repeated dose adjuvant injection are presented in Figure 2. 28 d after the adjuvant injection, local reactions at the injection site of the mice at all adjuvant treated groups were observed as severe edema compared to the control group.

3.5. Histopathological analysis

After a single dose of adjuvant injection, the muscle tissues taken from the injection site of the mice on the 2nd, 7th and 14th days were evaluated in terms of general histological

Table 5. Total body weight values of mice after repeated dose toxicity study.

	Total body weight (grams) (mean ± SD)					
Treatment Groups	Day 0	Day 7	Day 14	Day 21	Day 28	% Body weight gain
Control	23.30 ± 1.05	26.13 ± 1.97	25.50 ± 1.87	26.96 ± 2.14	29.35 ± 2.81	27.70 ± 4.55
Coralvac RZ 528	24.68 ± 1.58	26.93 ± 0.98	27.85 ± 1.35	28.81 ± 1.64	30.14 ± 1.75	22.39 ± 3.87
Coralvac RZ 506	26.24 ± 2.40	27.10 ± 2.12	28.40 ± 2.04	28.98 ± 2.30	32.79 ± 2.83	25.83 ± 5.12
Coralvac AT 318	22.48 ± 2.24	24.39 ± 1.86	26.12 ± 1.58	27.47 ± 2.10	27.68 ± 1.99	22.98 ± 2.77
Coralvac AT 318 SIS	26.84 ± 3.43	29.92 ± 3.17	30.62 ± 2.94	30.90 ± 2.96	30.14 ± 1.75	18.83 ± 3.81
Coralvac 252	25.21 ± 2.60	26.25 ± 2.11	26.80 ± 2.14	27.11 ± 2.17	28.01 ± 2.27	12.30 ± 2 65
F	0.754	0.987	0.993	0.565	1.023	0.331
p	0.592	0.446	0.443	0.726	0.426	0.889

Table 6. Organ weights of mice after repeated dose toxicity study.

	Organ weights (grams) (Mean ± SD)				
Treatment Groups	Brain	Liver	Lung	Kidney	Spleen
Control	0.36 ± 0.02	1.91 ± 0.19	0.18 ± 0.01	0.49 ± 0.07	0.17 ± 0.02
Coralvac RZ 528	0.37 ± 0.04	2.33 ± 0.20	0.17 ± 0.03	0.46 ± 0.05	0.13 ± 0.03
Coralvac RZ 506	0.40 ± 0.02	2.19 ± 0.21	0.24 ± 0.04	0.50 ± 0.04	0.15 ± 0.02
Coralvac AT 318	0.39 ± 0.03	1.85 ± 0.36	0.16 ± 0.03	0.44 ± 0.05	0.12 ± 0.01
Coralvac AT 318 SIS	0.39 ± 0.05	2.30 ± 0.56	0.20 ± 0.05	0.44 ± 0.07	0.18 ± 0.05
Coralvac 252	0.36 ± 0.02	1.62 ± 0.30	0.25 ± 0.08	0.48 ± 0.05	0.12 ± 0.02
F	1.062	2.972	1.054	0.263	0.840
p	0.406	0.032	0.410	0.929	0.535

Table 7. Relative organ weights (organ to total body weight ratio) of mice after repeated dose toxicity study.

	Organ weights (grams) (Mean ± SD)				
Treatment Groups	Brain	Liver	Lung	Kidney	Spleen
Control	0.012 ± 0.002	0.067 ± 0.007	0.0065 ± 0.0006	0.015 ± 0.001	0.0061 ± 0.0008
Coralvac RZ 528	0.012 ± 0.001	0.077 ± 0.001	0.0056 ± 0.0004	0.015 ± 0.002	0.0045 ± 0.0004
Coralvac RZ 506	0.012 ± 0.001	0.067 ± 0.004	0.0074 ± 0.0005	0.016 ± 0.003	0.0047 ± 0.0007
Coralvac AT 318	0.014 ± 0.003	0.066 ± 0.002	0.0058 ± 0.0003	0.014 ± 0.002	0.0045 ± 0.0002
Coralvac AT 318 SIS	0.013 ± 0.001	0.077 ± 0.009	0.0068 ± 0.0009	0.017 ± 0.004	0.0060 ± 0.0017
Coralvac 252	0.014 ± 0.002	0.058 ± 0.002	0.0092 ± 0.0010	0.017 ± 0.002	0.0041 ± 0.0005
F	1.074	1.860	1.150	0.551	0.842
p	0.399	0.139	0.362	0.736	0.534

Table 8. Biochemical analysis values of mice after a single dose toxicity study.

	Treatment groups					
Biochemical parameters	Control	Coralvac RZ 528	Coralvac RZ 506	Coralvac AT 318	Coralvac AT 318 SIS	Coralvac 252
Total Protein (g/dL)	4.2	4.8	5.4	5.3	5.0	5.9
ALB (g/dL)	2.3	2.4	2.9	2.4	2.4	2.9
GLOB (g/dL)	1.9	2.4	2.5	2.9	2.6	3.0
GLU (mg/dL)	281	55	112	220	228	223
ALT (U/L)	100	78	41	40	32	47
GGT (U/L)	<10	<10	<10	<10	<10	<10
ALP (U/L)	<14	<14	<14	<14	<14	<14
TBİL (mg/dL)	0.7	0.6	0.8	0.2	0.7	0.4
TCHOL (mg/dL)	60	64	92	82	68	104
CRE (mg/dL)	0.48	1.03	0.60	0.64	0.31	0.31
BUN (mg/dL)	34.5	29.5	30.4	32.3	22.3	27.7
Ca (mg/dL)	<4.0	<4.0	4.0	4.0	4.0	<4.0
Inorganik P (mg/dL)	10.8	>15.0	>15.0	>15.0	8.2	8.9

parameters and histological images of the tissues are presented in Figure 3. Accordingly, mild inflammation and edema were observed in the muscle tissues of the Control group taken on the 2nd day, while edema and inflammation were not observed on the 7th and 14th days. The muscle tissues of the Control group taken on the 7th and 14th days were evaluated to have normal histological structure. When all adjuvant treated experimental groups were compared with the control group, it was observed that inflammation and edema were severe and widespread on the 2nd day, and inflammation and edema were more common and intensified on the 7th day. In addition to inflammation and edema, large amounts of lipid droplets were also observed in all adjuvant treated experimental groups. On the 14th day, when the control group and adjuvant treated experimental groups were compared, it was observed that inflammation and edema were considerably reduced.

Muscle tissues taken from the injection site on the 28th day after repeated dose adjuvant injection were evaluated in

Table 9. Biochemical analysis values of mice after repeated dose toxicity study.

	Treatment groups					
Biochemical parameters	Control	Coralvac RZ 528	Coralvac RZ 506	Coralvac AT 318	Coralvac AT 318 SIS	Coralvac 252
Total Protein (g/dL)	4.9	4.7	5.6	5.6	5.4	4.0
ALB (g/dL)	2.3	2.7	3.2	2.6	2.6	2.0
GLOB (g/dL)	2.6	2.0	2.4	3.0	2.8	2.0
GLU (mg/dL)	246	182	119	165	200	216
ALT (U/L)	48	100	90	48	53	118
GGT (U/L)	<10	<10	<10	<10	<10	<10
ALP (U/L)	<14	<14	<14	<14	<14	<14
TBİL (mg/dL)	0.5	1.1	2.4	0.8	0.6	<0.2
TCHOL (mg/dL)	<50	72	78	105	72	<50
CRE (mg/dL)	<0.20	0.36	0.59	0.48	0.69	<0.20
BUN (mg/dL)	27.7	30.2	28.2	24.8	33.0	22.8
Ca (mg/dL)	<4.0	<4.0	<4.0	<4.0	4.0	<4.0
Inorganik P (mg/dL)	9.6	12.1	>15.0	12.3	11.4	8.7

terms of general histological parameters and histological images of the tissues are presented in Figure 4. Accordingly, mild inflammation and edema were observed in the control group, while quite widespread, severe inflammation and edema were observed in all other adjuvant groups compared to the control group. In addition to these findings, diffuse lipid droplets of varying sizes are also observed.

4. Discussions

Both of Freund's complete and incomplete adjuvants, which are based on mineral (paraffin) oil, are the most widely used emulsion adjuvants for use on farm and laboratory animals. Despite their adjuvant properties in veterinary vaccinations, these W/O emulsions are too reactogenic for widespread application (Stills, 2005). It is thought that, O/W emulsion will likely be favored for the future generation of adjuvants because of the generally lower concentration of oils and superior safety and tolerability profiles. This study aimed to examine the systemic and local toxicity of W/O and W/O/W oil emulsion adjuvants in a mouse model. To our knowledge, our research is the first to directly compare five different adjuvants used in veterinary vaccines in Turkey. In the current study, no animals died from the doses used to determine single and repeated dose toxicity.

Changes in body weight are a sensitive indicator of toxicity after toxic chemical exposure (Vahalia et al., 2011). Symptoms of toxic consequences can be seen in animals' weight changes; losing more than 10% of the initial weight of animals is an important indicator of toxicity (Raza et al., 2002; Teo et al., 2002). Mice treated with single or repeated doses of oil-based emulsion adjuvants showed no statistically significant differences in body weight changes compared to the control group ($p > .05$). Mice in the adjuvant and control groups both gained weight during the course of the experiment, indicating that the oil emulsion adjuvants were safe to use (Blanco et al., 2009; Gebremickael et al., 2017). Changes in organ weight caused by medications or chemicals may be useful indications of test article-related changes in repeated-dose rodent investigations, regardless of comparable microscopic findings (Pritam et al., 2013). In our study, the weights of the brains and lungs in some adjuvant treatment groups differ statistically from the control group. However, in animal toxicity tests, organ weight changes are accepted as a sensitive indicator of chemically induced organ damage. It can be difficult to interpret because changes in organ weight might reflect chemically induced changes in overall body weight. Therefore, a common solution is to calculate the relative organ weight (organ to body weight ratio) (Lazic et al., 2020). For this reason, relative organ weights were calculated in our study and presented in Table 4. Although there was a difference in the lung relative weights of some groups in the single-dose toxicity study compared to the control group, this result was not considered significant since no difference was found in the repeated-dose study.

Serum biochemistry is an important indicator in the evaluation of a toxic substance (Eugine & Manavalan, 2013). Several serum chemistries can be used to evaluate liver function. Two of the most common serum transaminase assays are alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Increased levels of these enzymes in the blood are caused by damage to the liver, which allows them to leave and accumulate in the blood (Snell, 1958). Despite their high concentration in hepatocytes, AST and ALT are not exclusively liver enzymes; rather, AST is broadly prevalent in myocardial, skeletal muscle, brain, and kidney (Witthawaskul et al., 2003). In this study, the serum levels of ALT and AST did not differ significantly between the control and mice treated with any of the adjuvants group. This suggests that both single-dose and repeated-dose intramuscular injection of W/O and W/O/W oil emulsion adjuvants does not cause significant liver damage. The term 'kidney function test' is an inclusive term for a number of different diagnostic techniques used to assess renal function. The kidneys' ability to filter blood and regulate the body's fluid balance can be impaired by a wide variety of medical problems. When kidney function decreases or ceases altogether, metabolic waste products in the blood can accumulate and cause health problems. The origin and severity of renal dysfunction can be assessed by measuring blood concentrations of substances normally regulated by the kidneys. As a result, the concentrations of plasma creatinine, urea, and uric acid can be used as indicators of renal function (Hanisa et al., 2011). In the present study, serum creatinine and urea concentrations did not differ significantly in both single-dose and repeated-dose adjuvant groups compared to the control group after intramuscularly administration in mice. This shows that, contrary to certain toxicity studies in mice, adjuvants did not significantly affect renal function. No major distinctions were seen between the groups tested for any of the other biochemical markers.

During clinical development or post-approval, Particular surveillance is required by conducting appropriate animal investigations. In this way, by recognizing the mechanism of action of a particular adjuvant, the data on the adjuvant's toxicity obtained during pre-clinical and clinical investigations may provide some further information. Although few studies have focused on a single cause of adjuvant toxicity, much work has been put into understanding the processes by

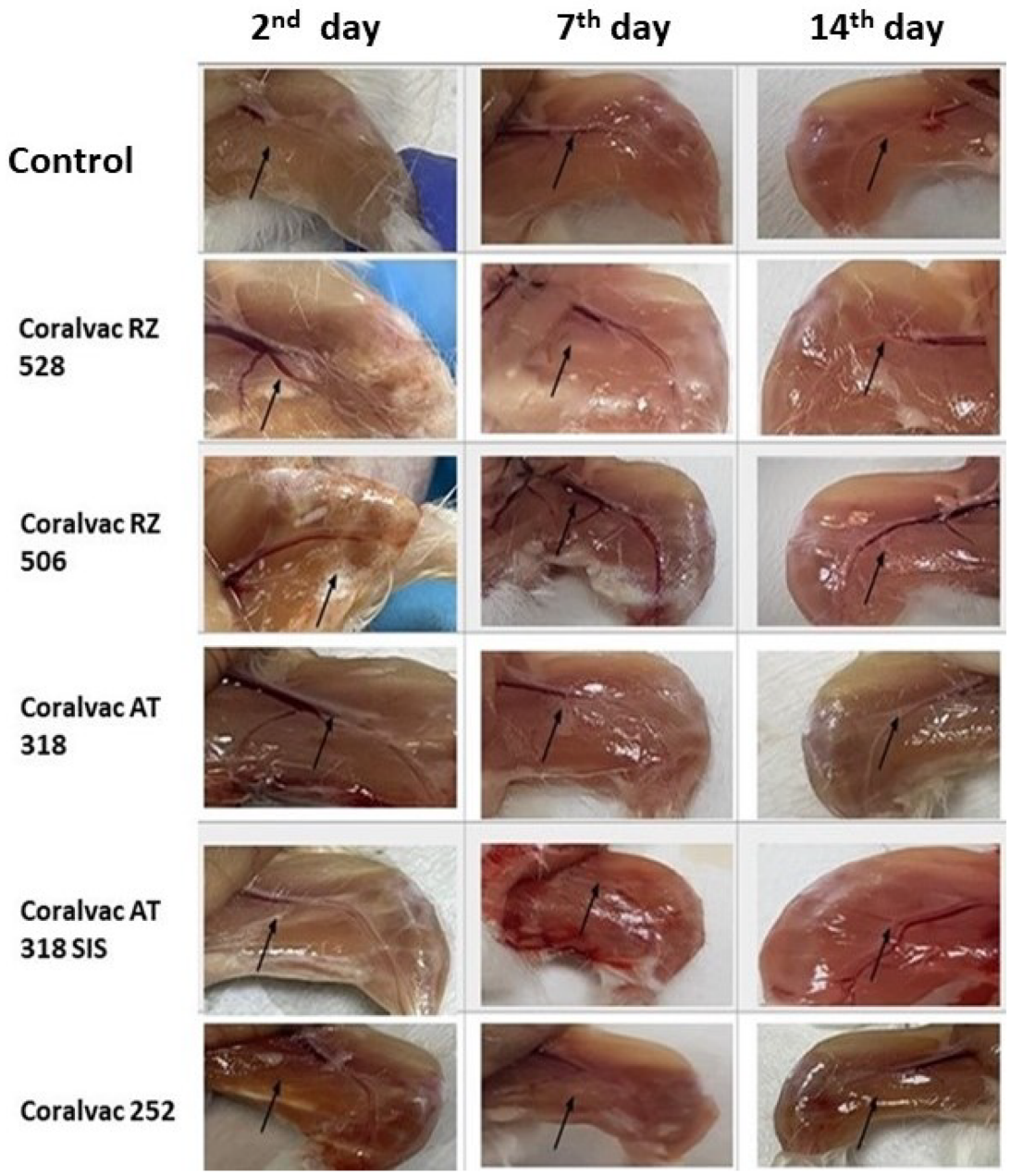

Figure 1. General view of the injection sites of mice after a single dose toxicity study (→: injection site). In 2nd and 7th day, only edema was determined at the injection site of mice at all adjuvant treated groups. In 14th day, there is no local reactions at the groups.

which adjuvants produce their immunostimulatory and, potentially, harmful effects. The wide variety of adjuvants makes it challenging to conduct a reductionist analysis of their efficacy and safety. Therefore, each analysis needs to be performed after a distinct adjuvant has been applied. Identification of appropriate biomarkers and bio-models with the ability to analyze potency, immunogenicity, toxicity, and subject-specific patterns is a key challenge for the development of future adjuvants and adjuvanted vaccines (Sykes et al., 2012). In addition to regulating the development of new vaccines, this data can be utilized to continually ascertain the benefit-risk outline of the vaccine adjuvant throughout its biological schedule. The rational design of adjuvants is a rapidly developing field with the goal of enhancing the efficacy, safety, and immunogenicity of future vaccines through a variety of methods (Habib et al., 2023).

In some vaccines containing adjuvant, side effects such as pain, swelling, redness, and burning occur at the injection site (Garçon, Leroux-Roels, et al., 2011; Garçon, Segal, et al., 2011). In comparative studies with vaccines with and without adjuvant, it was observed that vaccines containing adjuvant increased reactogenicity at the injection site (Kosalaraksa et al., 2014; Levie et al., 2002). The reactogenicity seen in adjuvanted vaccines is a local inflammatory response, an innate immune response, induced by the adjuvant at the vaccine site (Tavares Da Silva et al., 2013; Gebremickael et al., 2017). However, studies with all licensed adjuvanted vaccines have yielded a positive benefit-risk ratio.

Previous investigations using assays with various cancer vaccines and the adjuvant Montanide ISA 51 have demonstrated local harm at the injection site in animals treated with the adjuvant plus the vaccination (Bada et al., 2002; Mancebo

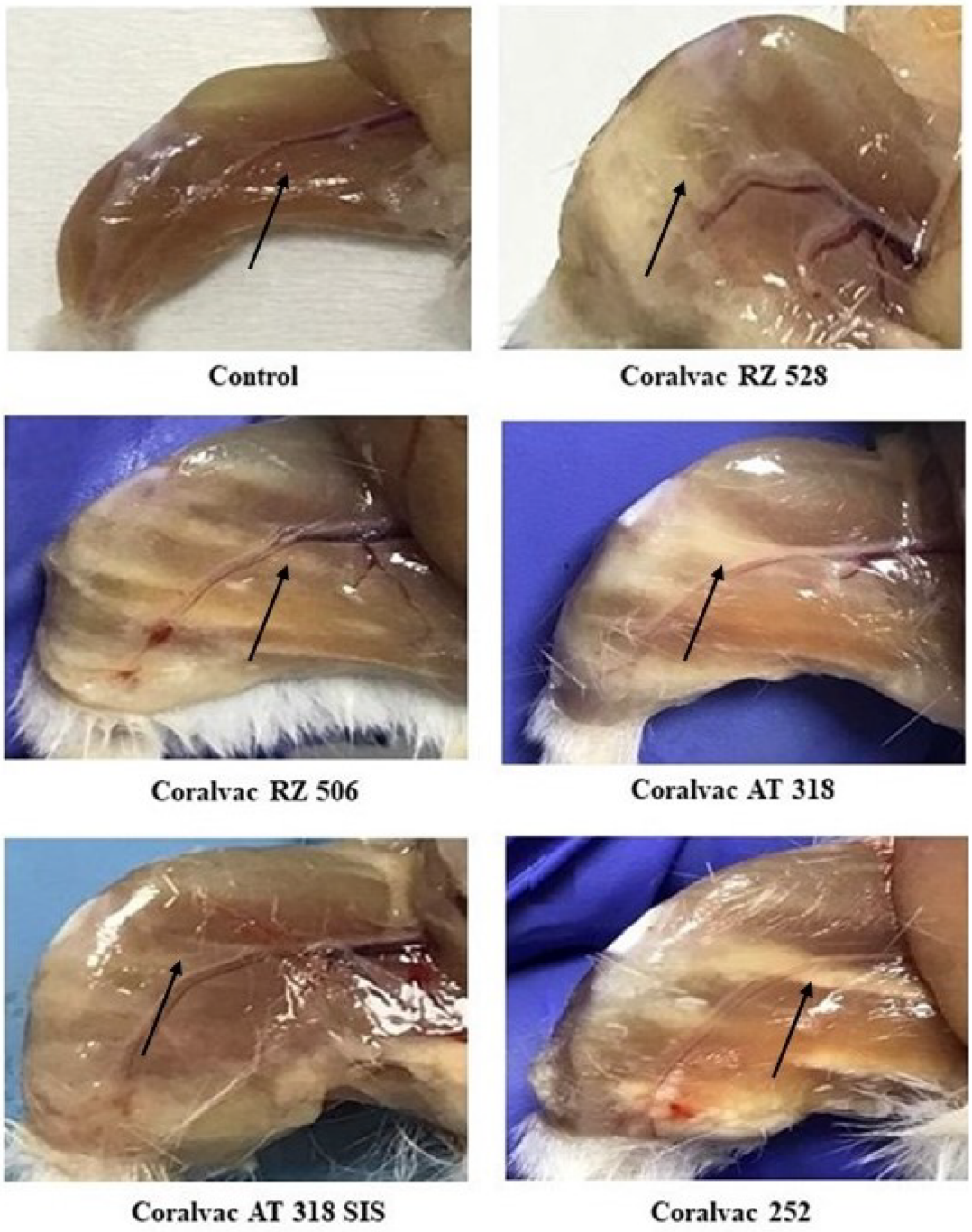

Figure 2. General view of the injection sites of mice on the 28th day of the repeat dose toxicity study (→: injection site). On the 28th day, severe edema was observed at the injection sites of mice at all adjuvant treated groups compared to the control group.

et al., 2012). Oil-based adjuvants like Montanide ISA 51 have been linked to adverse local reactions such abscesses and granulomas (Jisaka et al., 1992; Leenaars et al., 1998). Oil adjuvant-based vaccines work by forming a depot at the injection site, where the antigen is released slowly and antibody-producing plasma cells are stimulated (Aucouturier et al., 2001). These adjuvants are added to vaccines so that the antigen stays at the injection site for a long time after vaccination, a phenomenon known as the depot effect (Graham et al., 2010; Miles and Saul, 2005). The specific composition of Montanide ISA 51 is mineral oil and a surfactant called mannide monoleate, which is produced by reacting oleic acid with the sugar mannitol. Although rapidly metabolized and cleared from the body, the emulsifier mannide monooleate has the potential to generate harmful fatty acids via the enzymatic breakdown of native lipid chains, resulting in local inflammatory reactions (Jerome Aucouturier et al., 2006). When investigated data from the Novartis vaccines (Schultze et al., 2008) and Diagnostics data file (Report, 2008), it was showed that intramuscular administration the squalene-based adjuvant MF59 containing 4.3% squalene in rabbits showed minor inflammatory and degenerative changes at the injection site. It

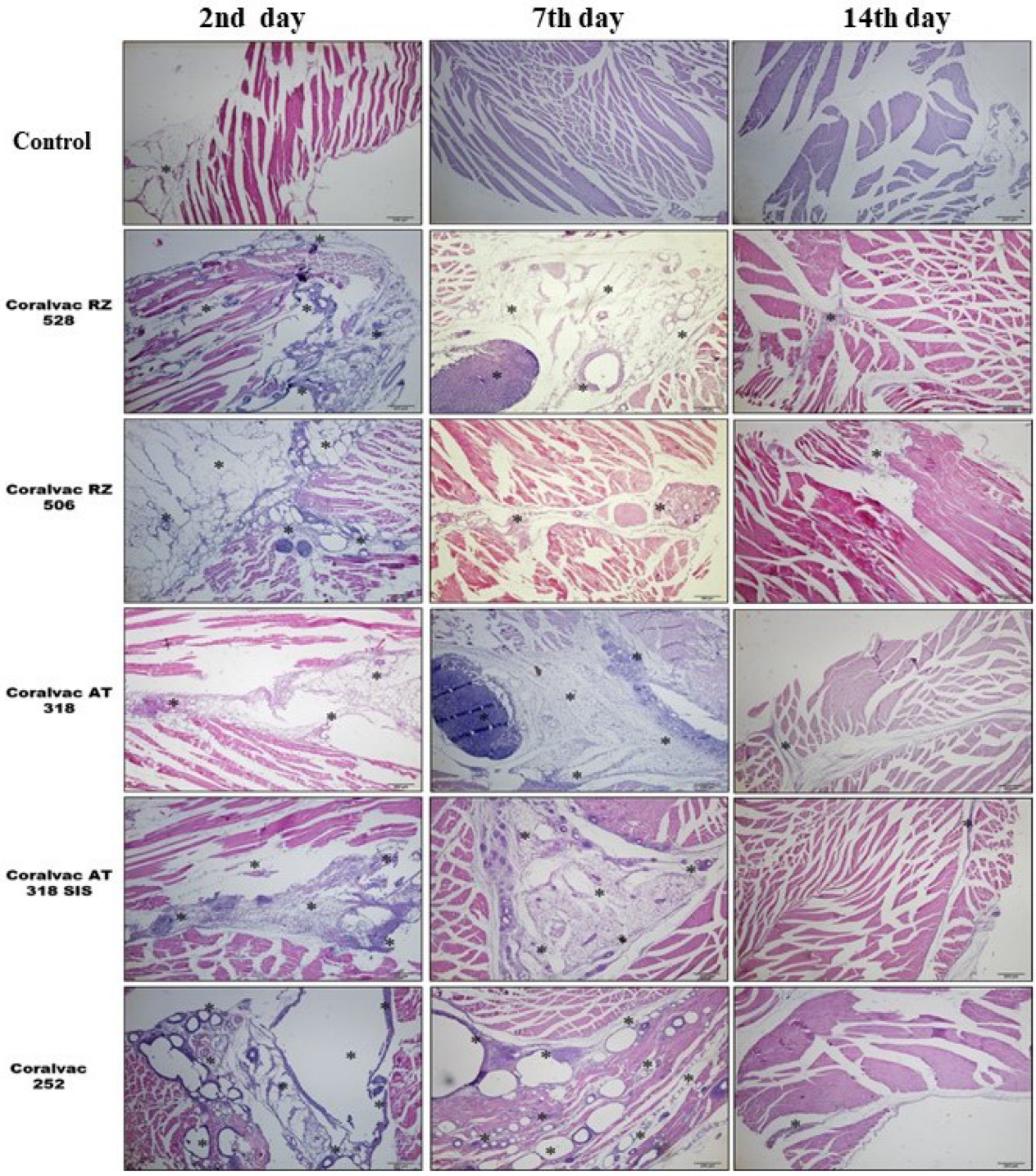

Figure 3. Histological view of the injection sites of mice after a single dose toxicity study (*: area of inflammation; magnification: x4; stain: Hematoxylin-Eosin). Mild inflammation and edema in muscle tissues of the control group on the 2nd day, while the tissues taken on the 7th and 14th days have normal histological structure. On all adjuvanted experimental groups, widespread severe inflammation and edema on the 2nd day, inflammation and edema were more common and intensified on the 7th day. Large amounts of lipid droplets in all adjuvanted experimental groups. In these groups, inflammation and edema were considerably reduced on the 14th day.

has been concluded that squalene emulsion is a safer adjuvant because it dissolves partially or completely over 7–14 d. In our study, newly developed oil emulsion adjuvants were found to be safe after testing; a single dose elicited mild to moderate inflammation within 7 d, and this had decreased significantly by 14 d. Muscle tissue from mice given a single dosage of adjuvant revealed significant edema and an increase in inflammatory cells when examined histologically.

Mineral oil W/O emulsions are more effective adjuvants, but O/W are safer. They are less irritating and hazardous than water in oil emulsions. Both types of emulsion damage cells and are damage-associated molecular patterns (DAMPs) type adjuvants. A water-in-oil emulsion with a surfactant like Tween, Span, or lecithin can be used to generate a slow-release antigen depot. Light mineral oil causes a local, chronic inflammatory reaction and a granuloma or abscess around the

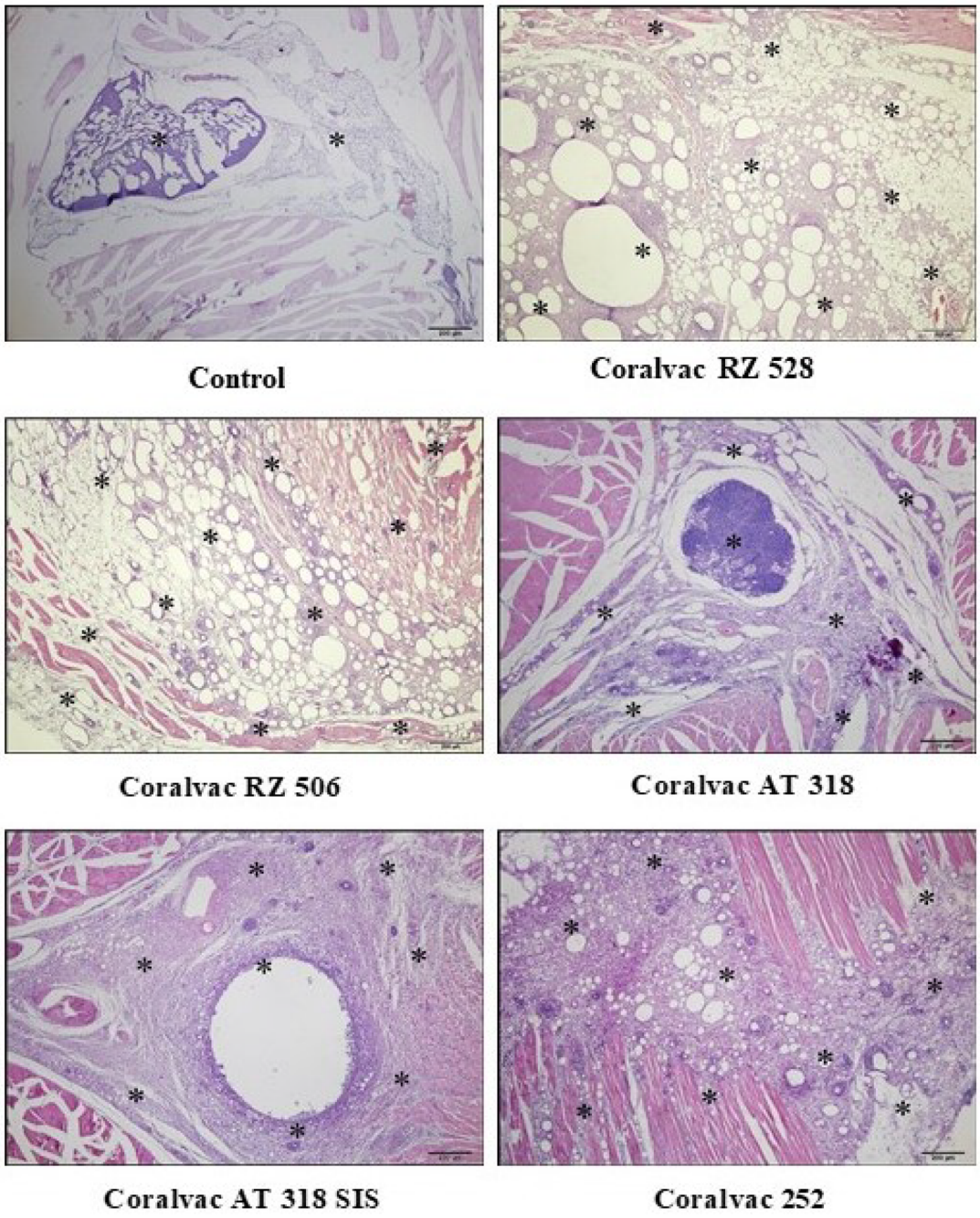

Figure 4. Histological view of the injection sites of mice after a repeat dose toxicity study (*: areas of inflammation; magnification: x4; stain: Hematoxylin-Eosin). Mild inflammation and edema in the control group, while quite widespread severe inflammation and edema in all adjuvanted groups compared to the control group. Diffuse lipid droplets of varying sizes in all adjuvanted groups.

injection site. The emulsion's aqueous phase slowly releases antigens. Emulsion adjuvants may cause severe tissue damage. Nonmineral oils are less irritating but less effective adjuvants. DAMPs from tissue injury activate dendritic cells and macrophages (Tizard, 2021). In present study, local damage at the administration site of adjuvants is thought to be a result of the weekly intramuscular injections of the oil emulsion adjuvants, enhanced by the immunogenic mechanism of action of these adjuvants, due to the unique characteristic of the lesions at the administration site of repeated dose in all adjuvant groups, with severe inflammation, edema, and diffuse lipid droplets compared to the control group.

No experimental animals died or showed significant changes in outward indicators and physiological measures during this study. In mice, intramuscular administration of newly formulated oil adjuvants caused light reversible local inflammation in single doses and local inflammation and lipid droplets in repeated doses. In cases where repeated dosing of vaccines containing these adjuvants is required after completion of efficacy studies in target strains, administration should be preferred at least 2 weeks apart to minimize local reactions. However, oil emulsion adjuvant mechanism, mode of action, hypersensitivity, and inflammatory response induction investigations are needed (Pellegrino et al., 2015).

5. Conclusions

To sum up, a new oil emulsion adjuvants formulation (Coralvac RZ 528, Coralvac RZ 506) for poultry and (Coralvac AT 318, Coralvac AT 318 SIS and Coralvac 252) that developed by Coral Biotechnology company in Turkey including for FMDV did not cause local or systematic toxicity on any of the body organs, according to the first time evaluations with this study. They showed safety, which is an advantage with respect to adjuvant formulations that used W/O and W/O/W oil emulsion in veterinary vaccines.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported by Ege University Research Foundation (FDK-2023-30779).

ORCID

N. Ülkü Karabay Yavasoglu http://orcid.org/0000-0002-7483-0184
Ayşe Nalbantsoy http://orcid.org/0000-0002-9108-4451

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

References

Aucouturier, J., Dupuis, L., & Ganne, V. (2001). Adjuvants designed for veterinary and human vaccines. *Vaccine*, *19*(17–19), 2666–2672. https://doi.org/10.1016/S0264-410X(00)00498-9

Aucouturier, J., Ascarateil, S., & Dupuis, L. (2006). The use of oil adjuvants in therapeutic vaccines. *Vaccine*, *24*(Suppl. 2), S2. https://doi.org/10.1016/j.vaccine.2005.01.116

Bada, A., Parada, A. C., Arteaga, M., Martínez, J., León, A., Santana, E., Hernández, O., Orphee, R., González, A., Mesa, C., González, C., Montero, E., & Fernández, L. E. (2002). Toxicity of a GM3 cancer vaccine in *Macaca fascicularis* monkey: A 12-month study. *Human & Experimental Toxicology*, *21*(5), 263–267. https://doi.org/10.1191/0960327102ht248oa

Bielinska, A. U., Janczak, K. W., Landers, J. J., Makidon, P., Sower, L. E., Peterson, J. W., & Baker, J. R. (2007). Mucosal immunization with a novel nanoemulsion-based recombinant anthrax protective antigen vaccine protects against Bacillus anthracis spore challenge. *Infection and Immunity*, *75*(8), 4020–4029. https://doi.org/10.1128/IAI.00070-07

Bihua, D., Jun, Z., Heyu, M., Hai, X., & Yu, L. (2017). Research progress on adjuvant of veterinary vaccine. *Experimental Research*, *12*(003), 5–6.

Biruss, B., Kählig, H., & Valenta, C. (2007). Evaluation of an eucalyptus oil containing topical drug delivery system for selected steroid hormones. *International Journal of Pharmaceutics*, *328*(2), 142–151. https://doi.org/10.1016/j.ijpharm.2006.08.003

Blanco, M. M., Costa, C. A. R. A., Freire, A. O., Santos, J. G., & Costa, M. (2009). Neurobehavioral effect of essential oil of *Cymbopogon citratus* in mice. *Phytomedicine*, *16*(2–3), 265–270. https://doi.org/10.1016/j.phymed.2007.04.007

Burakova, Y., Madera, R., McVey, S., Schlup, J. R., & Shi, J. (2018). Adjuvants for Animal Vaccines. *Viral Immunology*, *31*(1), 11–22. https://doi.org/10.1089/vim.2017.0049

Cerpa-Cruz, S., Paredes-Casillas, P., Landeros Navarro, E., Bernard-Medina, A. G., Martínez-Bonilla, G., & Gutiérrez-Ureña, S. (2013). Adverse events following immunization with vaccines containing adjuvants. *Immunologic Research*, *56*(2–3), 299–303. https://doi.org/10.1007/s12026-013-8400-4

Cox, J. C., & Coulter, A. R. (1997). Adjuvants – a classification and review of their modes of action. *Vaccine*, *15*(3), 248–256. https://doi.org/10.1016/S0264-410X(96)00183-1

Degen, W. G. J., Jansen, T., & Schijns, V. E. J. C. (2003). Vaccine adjuvant technology: From mechanistic concepts to practical applications. *Expert Review of Vaccines*, *2*(2), 327–335. https://doi.org/10.1586/14760584.2.2.327

Di Pasquale, A., Preiss, S., Da Silva, F. T., & Garçon, N. (2015). Vaccine adjuvants: From 1920 to 2015 and beyond. *Vaccines*, *3*(2), 320–343. https://doi.org/10.3390/vaccines3020320

Fukanoki, S., Matsumoto, K., Mori, H., & Takeda, R. (2000). Relationship between antigen release and antibody response of infectious Coryza water-in-oil-in-water emulsion vaccines. *Avian Diseases*, *44*(4), 869–873.

Garçon, N., Leroux-Roels, G., & Cheng, W. (2011). Vaccine adjuvants. In N. Garçon, P. L. Stern, & A. L. Cunningham (Eds.), *Understanding modern vaccines: Perspectives in vaccinology* (pp. 89–113). Elsevier. https://doi.org/10.1016/j.pervac.2011.05.004

Garçon, N., Segal, L., Tavares, F., & van Mechelen, M. (2011). The safety evaluation of adjuvants during vaccine development: The AS04 experience. *Vaccine*, *29*(27), 4453–4459. https://doi.org/10.1016/j.vaccine.2011.04.046

Gebremickael, A., Gebru, G., Debella, A., & Addis, G. (2017). Acute and sub-chronic oral toxicity evaluation of eucalyptus globulus essential oil-water emulsion in mice. *Journal of Cytology & Histology*, *08*(02), 1000459. https://doi.org/10.4172/2157-7099.1000459

Graham, B. S., Mcelrath, M. J., Keefer, M. C., Rybczyk, K., Berger, D., Weinhold, K. J., Ottinger, J., Ferarri, G., Montefiori, D. C., Stablein, D., Smith, C., Ginsberg, R., Eldridge, J., Duerr, A., Fast, P., & Haynes, B. F. (2010). Immunization with cocktail of HIV-derived peptides in montanide ISA-51 is immunogenic, but causes sterile abscesses and unacceptable reactogenicity. *PLOS One*, *5*(8), e11995. https://doi.org/10.1371/journal.pone.0011995

Habib, A., Anjum, K. M., Iqbal, R., Jaffar, G., Ashraf, Z., Khalid, M. S., Taj, M. U., Zainab, S. W., Umair, M., Zohaib, M., & Khalid, T. (2023). Vaccine adjuvants: Selection criteria, mechanism of action associated with immune responses and future directions. *Iranian Journal of Immunology, 20*(1), 1–15. https://doi.org/10.22034/iji.2023.94097.2284

Hanisa, H., Hadijah, H., Rasedee, A., & Tarmizi, A. S. (2011). Sub-acute oral administration of Cymbopogon citratus stem infusion and its effects on blood biochemical parameters, body and organ weights in rats. *Journal of Tropical Agriculture and Food Science*, *39*(1), 1–7. http://jtafs.mardi.gov.my/jtafs/39-1/Cymbopogon citratus.pdf

Heegaard, P. M. H., Dedieu, L., Johnson, N., Le Potier, M. F., Mockey, M., Mutinelli, F., Vahlenkamp, T., Vascellari, M., & Sørensen, N. S. (2011). Adjuvants and delivery systems in veterinary vaccinology: Current state and future developments. *Archives of Virology*, *156*(2), 183–202. https://doi.org/10.1007/s00705-010-0863-1

Jansen, T., Hofmans, M. P. M., Theelen, M. J. G., Manders, F., & Schijns, V. E. J. C. (2006). Structure- and oil type-based efficacy of emulsion adjuvants. *Vaccine*, *24*(26), 5400–5405. https://doi.org/10.1016/j.vaccine.2006.03.074

Jisaka, M., Kawanaka, M., Sugiyama, H., Takegawa, K., Huffman, M. A., Ohigashi, H., & Koshimizu, K. (1992). NII-electronic library service. *Bioscience, Biotechnology, and Biochemistry*, *56*(5), 845–846. https://doi.org/10.1271/bbb.56.845

Kosalaraksa, P., Jeanfreau, R., Frenette, L., Drame, M., Madariaga, M., Innis, B. L., Godeaux, O., Izurieta, P., & Vaughn, D. W. (2014). AS03B-adjuvanted H5N1 influenza vaccine in children 6 months through 17 years of age: A Phase II/III randomized, placebo-controlled, observer-blind trial. *The Journal of Infectious Diseases*, *211*(5), 801–810. https://doi.org/10.1093/infdis/jiu548

Lazic, S. E., Semenova, E., & Williams, D. P. (2020). Determining organ weight toxicity with Bayesian causal models: Improving on the analysis of relative organ weights. *Scientific Reports*, *10*(1), 6625. https://doi.org/10.1038/s41598-020-63465-y

Leenaars, P. P. A. M., Koedam, M. A., Wester, P. W., Baumans, V., Claassen, E., & Hendriksen, C. F. M. (1998). Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. *Laboratory Animals*, *32*(4), 387–406. https://doi.org/10.1258/002367798780599884

Levie, K., Gjorup, I., Skinhøj, P., & Stoffel, M. (2002). A 2-dose regimen of a recombinant hepatitis B vaccine with the immune stimulant AS04 compared with the standard 3-dose regimen of Engerix-B in healthy young adults. *Scandinavian Journal of Infectious Diseases*, *34*(8), 610–614. https://doi.org/10.1080/00365540110080881

Loeb, W. F., & Quimby, F. W. (1999). *The clinical chemistry of laboratory animals*. (2nd ed.). Taylor & Francis USA.

Mancebo, A., Casacó, A., González, B., Ledón, N., Sorlozabal, J., León, A., Gómez, D., González, Y., Bada, A. M., González, C., Arteaga, M. E., Ramírez, H., & Fuentes, D. (2012). Repeated dose intramuscular injection of the CIMAvax-EGF vaccine in Sprague Dawley rats induces local and systemic toxicity. *Vaccine*, *30*(22), 3329–3338. https://doi.org/10.1016/j.vaccine.2012.01.092

McCluskie, M. J., & Weeratna, R. D. (2001). Novel adjuvant systems. *Current Drug Targets*, *1*(3), 263–271. https://doi.org/10.2174/1568005014605991

Miles, A. P., & Saul, A. (2005). Extraction and characterization of vaccine antigens from water-in-oil adjuvant formulations. *Methods in Molecular Biology*, *308*, 293–300. https://doi.org/10.1385/1-59259-922-2:293

Myc, A., Kukowska-Latallo, J. F., Bielinska, A. U., Cao, P., Myc, P. P., Janczak, K., Sturm, T. R., Grabinski, M. S., Landers, J. J., Young, K. S., Chang, J., Hamouda, T., Olszewski, M. A., & Baker, J. R. (2003). Development of immune response that protects mice from viral pneumonitis after a single intranasal immunization with influenza A virus and nanoemulsion. *Vaccine*, *21*(25-26), 3801–3814. https://doi.org/10.1016/S0264-410X(03)00381-5

O'Hagan, D. T. (2015). New-generation vaccine adjuvants. *ELS*, 1–7. https://doi.org/10.1002/9780470015902.a0020177.pub2

Pellegrino, P., Clementi, E., & Radice, S. (2015). On vaccine's adjuvants and autoimmunity: Current evidence and future perspectives. *Autoimmunity Reviews*, *14*(10), 880–888. https://doi.org/10.1016/j.autrev.2015.05.014

Pritam, S., James, A., Jerry, F., & Chirukandath, G. (2013). Toxicologic pathology (nonclinical safety assessment). In E. Judi, R. Page, J. Christopher, & N. Donald (Eds.), *Introduction to Toxicologic Pathology* (pp. 77–96). CRC Press.

Raza, M., Al-Shabanah, O. A., El-Hadiyah, T. M., & Al-Majed, A. A. (2002). Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. *Scientia Pharmaceutica*, *70*(2), 135–145. https://doi.org/10.3797/scipharm.aut-02-16

Report, A. (2008). Annual report 2007. *EPPO Bulletin*, *38*(3), 534–543. https://doi.org/10.1111/j.1365-2338.2008.01243.x

Eugine, L. P. S., & Manavalan, R. (2013). Acute toxicity studies of Andrographolide Research Journal of Pharmaceutical. *Biological and Chemical Sciences Acute Toxicity Studies of Andrographolide*, *2*, 547–552.

Sander, V. A., Sánchez López, E. F., Mendoza Morales, L., Ramos Duarte, V. A., Corigliano, M. G., & Clemente, M. (2020). Use of veterinary vaccines for livestock as a strategy to control foodborne parasitic diseases. *Frontiers in Cellular and Infection Microbiology*, *10*, 288. https://doi.org/10.3389/fcimb.2020.00288

Sayers, S., Ulysse, G., Xiang, Z., & He, Y. (2012). Vaxjo: A web-based vaccine adjuvant database and its application for analysis of vaccine adjuvants and their uses in vaccine development. *Journal of Biomedicine & Biotechnology*, *2012*, 831486–13. https://doi.org/10.1155/2012/831486

Schultze, V., D'Agosto, V., Wack, A., Novicki, D., Zorn, J., & Hennig, R. (2008). Safety of MF59™ adjuvant. *Vaccine*, *26*(26), 3209–3222. https://doi.org/10.1016/j.vaccine.2008.03.093

Schijins, V., & O'Hagan, D. (2006). *Immune potentiators in modern vaccine* (1st ed.). Elsevier Academic Press.

Snell, A. M. (1958). Liver function tests and their interpretation. *Gastroenterology*, *34*(4), 675–682. https://doi.org/10.1016/S0016-5085(58)80052-9

Stills, H. F. (2005). Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. *ILAR Journal*, *46*(3), 280–293. https://doi.org/10.1093/ilar.46.3.280

Sykes, L., MacIntyre, D. A., Yap, X. J., Ponnampalam, S., Teoh, T. G., & Bennett, P. R. (2012). Changes in the Th1:Th2 cytokine bias in pregnancy and the effects of the anti-inflammatory cyclopentenone prostaglandin 15-deoxy-Δ 12,14- prostaglandin J 2. *Mediators of Inflammation*, *2012*, 416739–416812. https://doi.org/10.1155/2012/416739

Tamargo Santos, B., Bungau, S., Fleitas Perez, C., Napoles, Y. M., Infante Bourzac, J. F., Hernandez, R. O., Gonzalez, W. R., Cioca, G., Aleya, L., & Gustavo Sierra Gonzalez, V. (2019). Immuno-toxicological evaluation of the adjuvant formulations for experimental anti-meningococcal vaccines without aluminium hydroxide. *Revista de Chimie*, *70*(4), 1251–1257. https://doi.org/10.37358/RC.19.4.7103

Tavares Da Silva, F., de Keyser, F., Lambert, P.-H., Robinson, W. H., Westhovens, R., & Sindic, C. (2013). Optimal approaches to data collection and analysis of potential immune mediated disorders in clinical trials of new vaccines. *Vaccine*, *31*(14), 1870–1876. https://doi.org/10.1016/j.vaccine.2013.01.042

Teo, S., Stirling, D., Thomas, S., Hoberman, A., Kiorpes, A., & Khetani, V. (2002). A 90-day oral gavage toxicity study of D-methylphenidate and D,L-methylphenidate in Sprague-Dawley rats. *Toxicology*, *179*(3), 183–196. https://doi.org/10.1016/S0300-483X(02)00338-4

Tizard, I. R. (2021). Adjuvants and adjuvanticity. *Vaccines Vet*, 75–86.e1. https://doi.org/10.1016/B978-0-323-68299-2.00016-2

Vahalia, M. K., Thakur, K. S., Nadkarni, S., & Sangle, V. D. (2011). Chronic toxicity study for Tamra Bhasma (A generic ayurvedic mineral formulation) in laboratory animals. *Recent Research in Science and Technology*, *3*(11), 2076–5061. https://updatepublishing.com/journal/index.php/rrst/article/view/834

Warimwe, G. M., Francis, M. J., Bowden, T. A., Thumbi, S. M., & Charleston, B. (2021). Using cross-species vaccination approaches to counter emerging infectious diseases. *Nature Reviews. Immunology*, *21*(12), 815–822. https://doi.org/10.1038/s41577-021-00567-2

Witthawaskul, P., Panthong, A., Kanjanapothi, D., Taesothikul, T., & Lertprasertsuke, N. (2003). Acute and subacute toxicities of the saponin mixture isolated from *Schefflera leucantha* Viguier. *Journal of Ethnopharmacology*, *89*(1), 115–121. https://doi.org/10.1016/S0378-8741(03)00273-3

Woodland, D. L. (2019). Veterinary vaccines. *Viral Immunology*, *32*(9), 361–361. https://doi.org/10.1089/vim.2019.29043.dlw

World Health Organization (WHO). (2013). Guidelines on the evaluation of vaccine adjuvants and adjuvanted vaccine, Technical Report Series No 987, Annex 2. Geneva. https://www.who.int/publications/m/item/nonclinical-evaluation-of-vaccine-adjuvants-and-adjuvanted-vaccines-annex-2-trs-no-987

Yigitturk, G., Acara, A. C., Erbas, O., Oltulu, F., Yavasoglu, N. U. K., Uysal, A., & Yavasoglu, A. (2017). The antioxidant role of agomelatine and gallic acid on oxidative stress in STZ induced type I diabetic rat testes. *Biomedicine & Pharmacotherapy*, *87*, 240–246. https://doi.org/10.1016/j.biopha.2016.12.102

Zeqian, G., Jiannan, S., Yi, W., & Chenghui, L. (2012). Progress in veterinary vaccine adjuvants. *Journal of Tangshan Teachers College*, *34*(2), 48–51.

Zhou, M., Li, Y., Chen, X., Zhou, H., Yang, S., & Qu, X. (2021). Preparation and characterization of polyoxyethylene dehydrated mannitol mono oleate as hydrophilic emulsifier potentially used in w/o/w type adjuvants. *Journal of Dispersion Science and Technology*, *42*(4), 614–621. https://doi.org/10.1080/01932691.2019.1701488