IN VIVO EVALUATION OF THE CHRONIC TOXIC EFFECTS OF REPEATED ADMINISTRATION OF THE C-4 COMPOSITION AT DIFFERENT DOSES IN RABBITS

Makhmudov Lazizbek[1], Yakubova Rana[1], Tagayalieva Nigora[1]
[1]Institute of Bioorganic Chemistry named after Academician O. S. Sodikov,
Academy of Sciences of the Republic of Uzbekistan, Tashkent, Uzbekistan

DOI: 10.67620/sai.57d0011

ABSTRACT

Background: Repeated-dose toxicity studies are required to identify cumulative adverse effects, target-organ injury, dose-response patterns, and the reversibility of treatment-related changes before a pharmacologically active composition advances in development. Objective: This study evaluated the safety of the C-4 composition after once-daily oral administration to rabbits at 4, 8, or 16 mg/kg for 90 consecutive days. Methods: Rabbits were assigned to a distilled-water control group or one of three dose groups. Clinical condition, behavior, body weight, peripheral blood indices, serum biochemical markers, relative organ weights, gross pathology, and hematoxylin-eosin histology of major organs were assessed. Quantitative figures in the source study report mean ± SEM for four animals per group. Results: No statistically significant treatment-related differences were reported for hemoglobin, erythrocytes, leukocytes, platelets, selected erythrocyte indices, serum total protein, glucose, urea, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, or body-weight gain ($p > 0.05$). Relative organ-weight coefficients were broadly comparable across groups. Most examined tissues retained normal architecture, with mild lymphoid activation and vascular congestion described as non-destructive findings. At 16 mg/kg, however, uneven myocardial congestion and microfoci of myolysis with little or no cellular reaction were reported. Conclusion: The available dataset shows no overt systemic, hematological, or serum-biochemical toxicity at 4-16 mg/kg over 90 days. The 4- and 8-mg/kg doses appear well tolerated. A definitive no-observed-adverse-effect level should not be assigned until the high-dose cardiac finding, sample-size discrepancy, and unreported recovery, coagulation, and renal-excretory outcomes are clarified.

ACCEPTED

02.08.26

Keywords: *C-4 composition; repeated-dose toxicity; rabbits; hematology; clinical chemistry; histopathology; preclinical safety.*

CC BY

INTRODUCTION

The development of a new pharmacologically active composition requires a staged evaluation of efficacy, exposure, and safety. Acute toxicity testing can identify immediate hazards and approximate dose-limiting effects, but it cannot adequately predict adverse reactions that arise through accumulation, repeated metabolic stress, adaptive responses, or delayed tissue injury. Repeated-dose studies are therefore designed to characterize the toxicological profile of a candidate substance, identify potential target organs,

ISSN: 2181-3337 | SCIENTISTS.UZ

evaluate exposure-response relationships, and determine whether observed effects persist or resolve after treatment is withdrawn [1-4]. A 90-day study in a non-rodent species is commonly classified as a subchronic repeated-dose investigation, even when the broader research programme refers to it as a chronic-toxicity assessment [1].

An integrated toxicological assessment normally combines daily clinical observations with body-weight and food-consumption data, hematology, clinical chemistry, urinalysis, gross necropsy, organ weights, and histopathology. Each domain contributes different information. Hematological variables may reveal effects on erythropoiesis, leukopoiesis, platelet production, inflammation, immune status, or oxygen-carrying capacity. Clinical chemistry can provide evidence of altered hepatocellular integrity, biliary function, protein synthesis, carbohydrate metabolism, or renal excretory function. Organ weights may identify treatment-related hypertrophy, atrophy, congestion, or proliferative change, while microscopic examination remains essential for detecting focal or subtle tissue lesions that are not reflected in circulating biomarkers [7-15].

The liver and kidneys are central to the disposition of xenobiotics and are consequently prominent targets in nonclinical safety assessment. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely used indicators of hepatocellular injury, although AST is less organ-specific and both enzymes must be interpreted together with histopathology and other clinical-pathology findings [10,11]. Alkaline phosphatase (ALP), total protein, glucose, and urea add information concerning hepatobiliary function, hepatic synthetic capacity, intermediary metabolism, and nitrogen handling. Traditional renal indices can be insensitive to early structural damage; accordingly, contemporary toxicology increasingly supplements urea and creatinine with urinalysis, urinary proteins, and qualified kidney-injury biomarkers when the study design and resources permit [12,13].

Rabbits are used in selected pharmacological, toxicological, medical-device, and translational investigations, but interpretation requires species-appropriate reference information. Rabbit hematological and biochemical values may vary with age, strain, sex, diet, handling, stress, circadian rhythm, and laboratory methods [8,9]. For this reason, comparison with a concurrent control group is more informative than reliance on a single published reference interval. Transparent reporting of animal allocation, numbers analyzed, randomization, blinding, ethics approval, exclusions, and adverse events is also essential for reproducibility and critical appraisal under the ARRIVE 2.0 framework [6].

The C-4 composition is a multicomponent pharmacologically promising formulation intended for further preclinical development. Before advancement, its safety under prolonged daily exposure must be characterized, with particular attention to cumulative and dose-related effects. The present study examined rabbits receiving 4, 8, or 16 mg/kg orally for 90 days. The available study record included clinical observations, body-weight measurements, peripheral-blood indices, serum biochemistry, relative organ weights, macroscopic examination, and histological images of major organs. The objective was to determine whether repeated administration produced systemic, hematological, biochemical, or morphological evidence of toxicity and to evaluate whether the findings supported a safe dose range.

2. Materials and Methods

2.1. Test article

The test article was identified in the source study as the C-4 composition. It was administered in distilled water by intragastric gavage. Detailed information on the qualitative and quantitative composition, active constituents, purity, batch number, stability, storage conditions, and preparation of the dosing formulation was not included in the available document. These details should be added from the original laboratory records before journal submission because they are necessary for reproducibility and interpretation.

ISSN: 2181-3337 | SCIENTISTS.UZ

2.2. Experimental animals and allocation

Rabbits of the same sex, with a reported initial body weight of 2.4 ± 0.5 kg, were maintained under standard vivarium conditions. The study record describes four experimental groups with 10 animals allocated to each group. This allocation corresponds to 40 rabbits, although one sentence in the source document reports a total of 30 animals. The group-based allocation is used in the present revision, but the final total must be verified against the original animal log. The source also does not specify the rabbit strain, exact sex, age, acclimatization period, or method used to generate the random allocation sequence.

Group	Treatment	Dose	Schedule
1	Distilled-water control	0 mg/kg	Once daily for 90 days
2	C-4 composition	4 mg/kg	Once daily for 90 days
3	C-4 composition	8 mg/kg	Once daily for 90 days
4	C-4 composition	16 mg/kg	Once daily for 90 days

Table 1. Experimental groups and dosing regimen.

2.3. Husbandry, dosing, and clinical observations

Animals were housed at 22 ± 2 °C and 55-65% relative humidity, received a standard laboratory diet, and had ad libitum access to drinking water. The C-4 composition or an equivalent volume of distilled water was administered orally by gavage once daily, seven days per week, for 90 consecutive days. General condition, posture, activity, grooming, behavior, food and water consumption, and visible clinical signs of toxicity were monitored during the study. Body weight was recorded on days 10, 30, and 90. The source record does not provide cage density, light-dark cycle, gavage volume, individual dose calculations, food-consumption data, or a formal clinical-observation scoring system.

2.4. Hematological, biochemical, and coagulation assessment

Peripheral blood was examined on days 10, 30, and 90. The reported hematological panel included hemoglobin concentration, erythrocyte count, reticulocyte percentage, platelet count, leukocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH). The source figures report mean ± SEM for n = 4 animals per group, but the relationship between this analytical subset and the 10 animals allocated to each group is not described.

Serum biochemical testing included urea, glucose, total protein, ALT, AST, and ALP. Analyses were performed with a semi-automated biochemical analyzer manufactured by Rayto Life and Analytical Sciences Co., Ltd., using commercial reagent kits from Cypress Diagnostics, Belgium. Prothrombin time, activated partial thromboplastin time, and fibrinogen concentration were reportedly measured using a HUMACLOT Junior single-channel coagulometer and Cypress Diagnostics reagents. However, numerical coagulation results were not included in the available manuscript and are therefore not interpreted in the Results section.

2.5. Necropsy, organ weights, and recovery period

At the end of the 90-day dosing period, animals selected for terminal evaluation were anesthetized with ether and euthanized by decapitation, as reported in the source record. The thoracic and abdominal cavities were opened, organs were examined macroscopically, and tissues were collected. Relative organ-weight coefficients were reported for the heart, lungs, spleen, kidneys, stomach, liver, and small intestine. The normalization formula and units used for these coefficients were not specified. A subset of animals was reportedly retained without further dosing for a recovery period to assess reversibility or persistence, but the duration, group sizes, and recovery findings were not present in the available dataset.

2.6. Histological processing and evaluation

Samples were collected from the liver, heart, lungs, stomach, small intestine, spleen, kidneys, thymus, lymph nodes, and adrenal glands. Tissues were fixed in 10% neutral buffered formalin for 48 hours, washed in running tap water for 2-4 hours, dehydrated through graded ethanol, cleared in

ISSN: 2181-3337 | SCIENTISTS.UZ

chloroform, and embedded in paraffin. Sections 5-8 μm thick were prepared. After deparaffinization, sections were stained with hematoxylin for approximately 3 minutes, washed under running water, counterstained with eosin for 0.2-3 minutes, dehydrated through 70-96% ethanol, cleared in carbol-xylene and xylene, and mounted in balsam. Hematoxylin stained nuclei blue to violet, whereas eosin stained cytoplasm and extracellular tissue components in shades of pink.

The available narrative describes the heart, lungs, kidneys, spleen, thymus, lymph nodes, adrenal glands, stomach, and small intestine. Although the liver was listed among collected tissues, a dedicated liver histology description or liver micrograph was not included. The record also does not specify whether the pathologist was blinded to treatment, whether lesions were graded for incidence and severity, or whether peer review was performed. These elements are recommended in toxicologic histopathology practice [14].

2.7. Ethical compliance

The source manuscript states that all experimental protocols were reviewed and approved by an Institutional Animal Care and Use Committee and that animal care complied with Directive 2010/63/EU on the protection of animals used for scientific purposes [5]. The committee name, approval number, approval date, and institution-specific protocol identifier were not provided and should be inserted from the original approval documentation. Reporting was revised with reference to ARRIVE 2.0 [6].

2.8. Statistical analysis

Experimental data were processed and visualized using OriginPro version 9.0 (OriginLab Corporation, USA). Results were expressed as mean ± standard error of the mean (SEM). At each observation time, groups were compared using one-way analysis of variance followed by Tukey's post hoc test. Statistical significance was defined as $p < 0.05$. The source document reports only the conclusion that group differences were not significant ($p > 0.05$); exact F statistics, degrees of freedom, adjusted p values, effect sizes, confidence intervals, assumptions checks, and individual-animal data were unavailable. No analysis of longitudinal within-animal correlation was reported.

3. Results

3.1. Clinical condition and body-weight dynamics

Throughout the 90-day administration period, rabbits in the control and C-4 groups were described as active and well groomed. Food and water intake appeared normal, coats remained smooth and glossy, and no visible treatment-related clinical signs were reported. Behavior in treated groups did not differ appreciably from that of control animals.

Body weight increased over time in all groups, consistent with expected growth during the study. Rabbits receiving C-4 showed a descriptive tendency toward greater weight gain than control animals, particularly at 4 mg/kg, but the differences were not statistically significant ($p > 0.05$). No dose-related loss of body mass or growth suppression was evident in the reported data. Because body weight is a broad indicator rather than an organ-specific endpoint, these findings support the absence of overt systemic intolerance but do not independently exclude focal tissue toxicity.

ISSN: 2181-3337 | SCIENTISTS.UZ

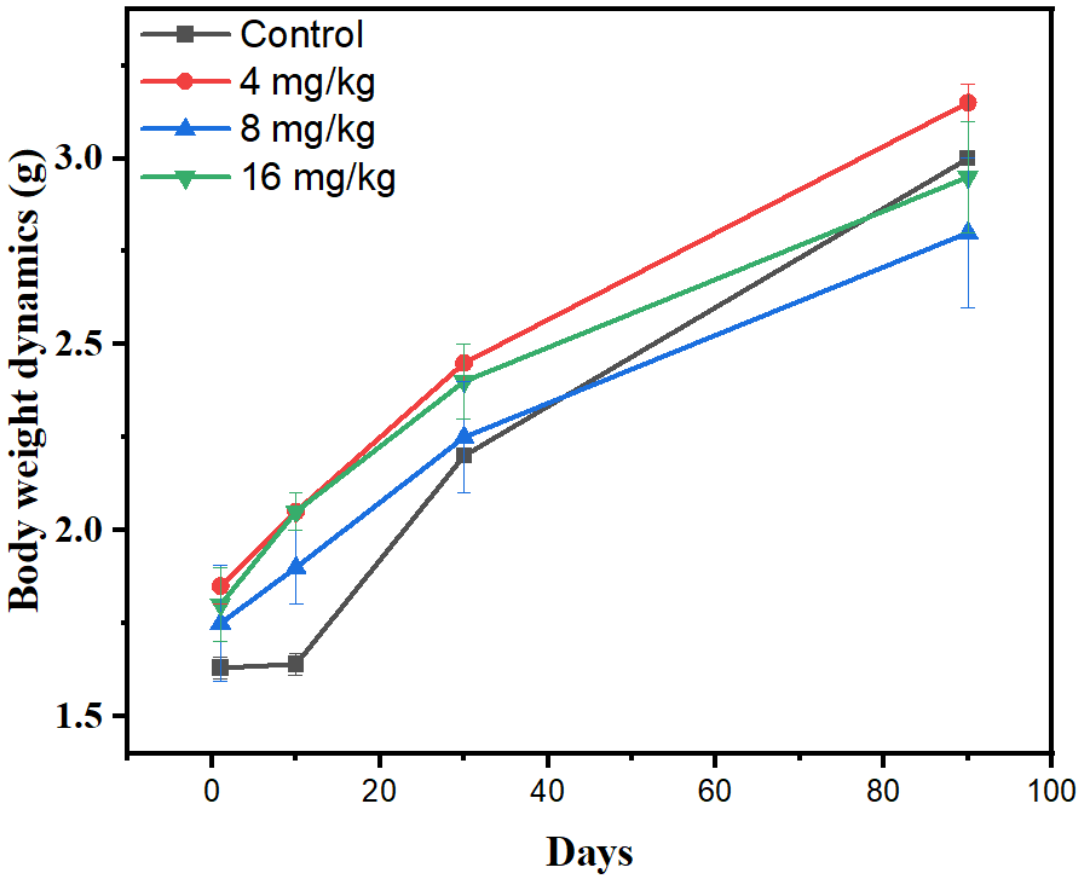


Figure 1. Body-weight dynamics in rabbits receiving distilled water or C-4 at 4, 8, or 16 mg/kg for 90 days. Data are presented as mean ± SEM, n = 4 per group as reported in the source figure.

3.2. Hematological findings

Repeated oral administration of C-4 did not produce statistically significant differences in the reported peripheral-blood variables at days 10, 30, or 90 compared with the control group ($p > 0.05$). Hemoglobin concentration, erythrocyte count, leukocyte count, platelet count, and erythrocyte indices were described as remaining within the physiological range for the study population. The absence of consistent dose-related changes suggests that the composition did not measurably suppress erythropoiesis, leukopoiesis, or thrombopoiesis under the conditions tested.

The reticulocyte-percentage panel in the source figure displays values of approximately 25-36%. These values are reproduced without reinterpretation because the underlying individual data and analyzer output were not available. The variable name, unit, and correspondence with the methods section should be checked against the laboratory worksheet before submission. This verification is important because species-specific hematological interpretation depends on both analytical methodology and correct parameter labeling [8,9].

ISSN: 2181-3337 | SCIENTISTS.UZ

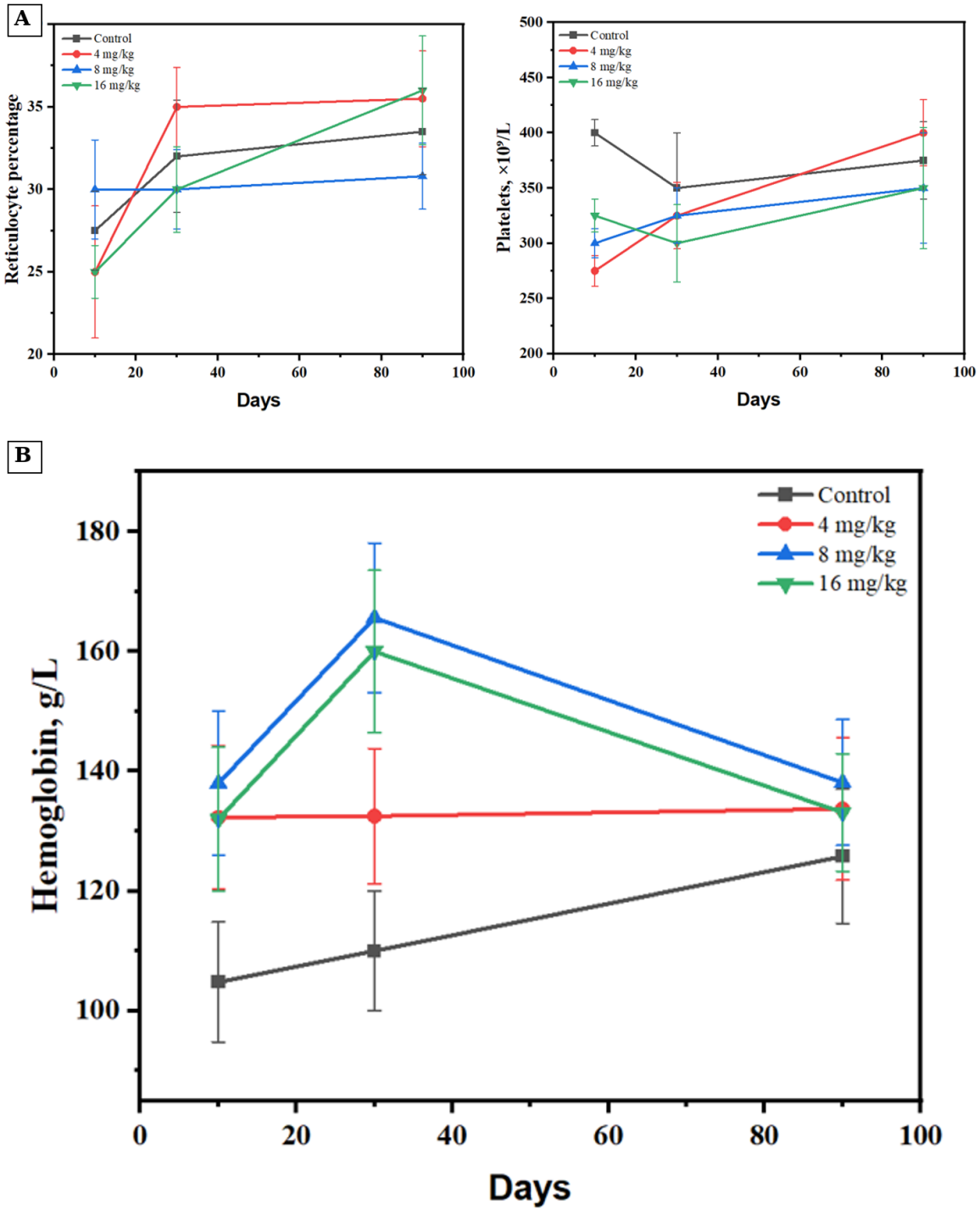


Figure 2. Selected hematological outcomes during repeated C-4 administration. (A) Reported reticulocyte percentage and platelet count; (B) hemoglobin concentration. Data are mean ± SEM, n = 4 per group as stated in the source figures. No statistically significant differences versus control were reported (p > 0.05).

3.3. Serum biochemical findings

Serum total protein, glucose, and urea concentrations and the activities of ALT, AST, and ALP did not differ significantly among the control and treated groups at the reported observation points ($p > 0.05$). Total protein remained comparatively stable, providing no evidence of a marked reduction in hepatic protein-synthetic function. The absence of a consistent elevation in ALT or AST provided no serum-biochemical signal of hepatocellular injury, while stable ALP activity provided no evidence of a major cholestatic or hepatobiliary disturbance. Glucose and urea also remained broadly comparable with control values.

ISSN: 2181-3337 | SCIENTISTS.UZ

These findings should be interpreted as an absence of detectable changes in the measured conventional markers, rather than proof that no microscopic or mechanistic injury occurred. ALT and AST are useful but imperfect indicators, and urea is relatively insensitive to early renal tubular injury [10-13]. The source introduction refers to renal excretory evaluation, but quantitative diuresis, urinalysis, creatinine, electrolyte, or urinary-biomarker results were not included in the document.

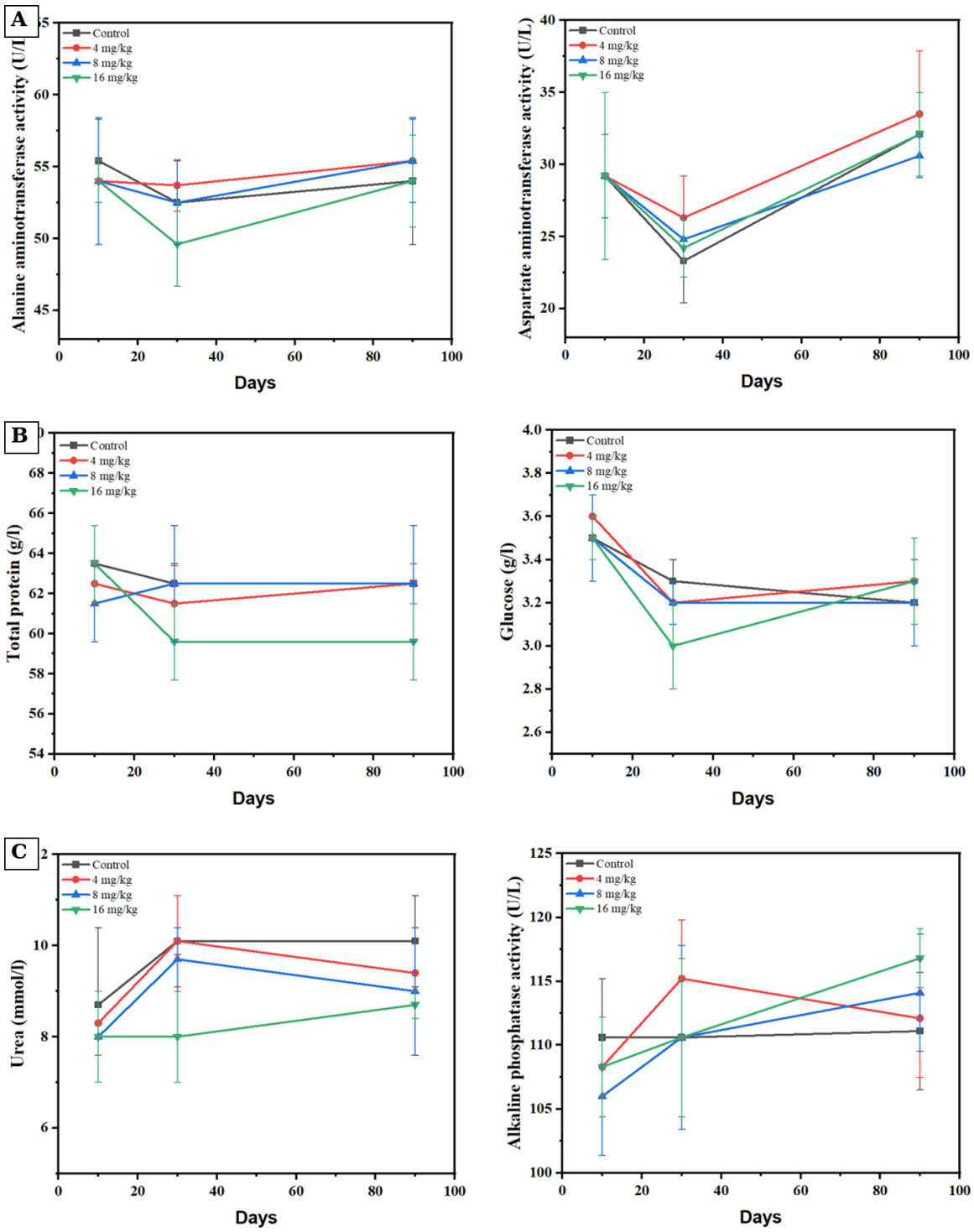


Figure 3. Serum biochemical parameters after 10, 30, and 90 days of repeated C-4 administration. Panels show ALT and AST activities, total protein and glucose concentrations, and urea and ALP values. Data are mean ± SEM, n = 4 per group as reported. No statistically significant differences from control were reported (p > 0.05).

3.4. Relative organ-weight coefficients

Relative organ-weight coefficients after 90 days were generally similar across the four groups (Table 2). No monotonic dose-related increase or decrease was apparent for the heart, kidneys, stomach,

ISSN: 2181-3337 | SCIENTISTS.UZ

liver, or small intestine. Lung and spleen coefficients were numerically lower in treated animals than in controls, but the source manuscript did not report statistical significance, confidence intervals, or the normalization formula. Organ-weight interpretation should integrate absolute weights, terminal body weights, macroscopic observations, and histopathology rather than rely on isolated relative coefficients [15].

Organ	Control	4 mg/kg	8 mg/kg	16 mg/kg
Heart	9.5 ± 1.0	9.0 ± 0.5	8.75 ± 0.75	8.85 ± 0.95
Lungs	13.29 ± 0.36	11.9 ± 0.6	11.95 ± 0.45	11.08 ± 0.725
Spleen	1.23 ± 0.025	1.15 ± 0.05	1.13 ± 0.025	1.08 ± 0.025
Kidneys	20.75 ± 1.25	19.50 ± 2.0	20.25 ± 2.25	19.35 ± 2.65
Stomach	36.0 ± 4.0	35.15 ± 3.1	35.82 ± 3.065	37.15 ± 1.55
Liver	99.1 ± 4.0	95.43 ± 4.5	98.10 ± 2.6	99.54 ± 7.24
Small intestine	3.01 ± 0.14	3.05 ± 0.75	2.9 ± 0.6	2.8 ± 0.5

Table 2. Relative organ-weight coefficients after 90 days of C-4 administration. Values are reproduced from the source document as mean ± SEM; the coefficient formula and units were not specified.

3.5. Macroscopic examination

At terminal necropsy, the major organs were reported to occupy normal anatomical positions. No free fluid was observed in the pleural or abdominal cavities. The mucosal surfaces of the oral, thoracic, and abdominal cavities were described as clean, moist, and normally colored, without visible hemorrhage. The lungs, stomach, and intestines showed no gross erosion, ulceration, or hemorrhage. No visible pathological changes were reported in the pancreas, kidneys, or adrenal glands.

3.6. Histopathological findings

3.6.1. Heart

At 4 and 8 mg/kg, the endocardial, myocardial, and epicardial layers were clearly distinguishable. Cardiomyocytes formed regularly oriented fibers with preserved transverse striations, centrally located oval or elongated nuclei, and visible intercalated discs. No morphological signs of myocardial ischemia or hypoxia were described at these doses. Mild reactive edema and occasional endothelial swelling were noted in some microvessels without marked alteration of the vascular wall.

At 16 mg/kg, the source histology caption reported uneven myocardial congestion and microfoci of myolysis accompanied by little or no cellular reaction. This is the most important potentially adverse microscopic observation in the dataset. The record does not provide lesion incidence, severity grade, distribution among animals, comparison with controls, or an independent pathology review. Consequently, the finding cannot be dismissed as non-treatment-related, but its biological and toxicological significance cannot be established from the available information.

3.6.2. Lungs

Pulmonary architecture was largely preserved. The walls of large, medium, and small intrapulmonary bronchi retained their expected tissue components, and no destructive changes were described in respiratory bronchioles, alveolar ducts, type I or type II pneumocytes, or interalveolar connective tissue. Moderate lymphoid-cell infiltration was present in the mucosa of some bronchi, together with isolated macrophages, mast cells, and plasma cells. Isolated macrophages containing dense cytoplasmic inclusions were observed in interalveolar tissue and air spaces. These observations were not accompanied by a clear dose-related inflammatory or destructive pattern.

3.6.3. Kidneys

The renal capsule was thin and intact, and the cortical region contained numerous renal corpuscles. Glomerular capillary loops remained patent, Bowman's spaces were of normal width, and no blood cells or pathological deposits were reported. Epithelial cells of the proximal, thin, and distal nephron segments retained their expected structure. Collecting ducts contained principal and intercalated cells in normal

ISSN: 2181-3337 | SCIENTISTS.UZ

proportions. Tubular lumina lacked cellular debris or abnormal deposits, and the cortical and medullary interstitium showed no edema or inflammatory infiltration. These findings provide no morphological evidence of overt nephrotoxicity in the examined sections.

3.6.4. Spleen, thymus, and lymph nodes

Splenic red and white pulp remained distinguishable and broadly maintained the normal adult proportion. Some follicles contained germinal or reactive centers with mitotic activity, and macrophages with hemosiderin pigment were present in the red pulp. At 8 mg/kg, foci of hyperplasia were described in subcapsular white pulp. The thymus preserved a clear cortico-medullary boundary, densely arranged cortical lymphocytes, medullary reticuloepithelial cells, and Hassall's corpuscles. Mild lymphocyte blast transformation was noted at 16 mg/kg.

Lymph nodes showed mild secondary activation of lymphoid, reticular, and macrophage populations, with activated lymphocytes and plasma cells in cortical and paracortical regions. These findings were not described as destructive and may represent reactive immune activity. Nevertheless, without incidence data, baseline infectious screening, or blinded lesion scoring, a treatment relationship cannot be established or excluded.

3.6.5. Adrenal glands

The adrenal capsule remained intact. The zona glomerulosa, zona fasciculata, and zona reticularis maintained their characteristic proportions, and chromaffin cells in the medulla retained their expected structure and dimensions. Venous sinusoids were unchanged or mildly dilated. No dystrophic or destructive alteration was reported.

3.6.6. Stomach and small intestine

The gastric surface epithelium and glands were largely preserved. The mucosal surface was covered by mucus and contained occasional exfoliated cells. The lamina propria contained isolated lymphocytes, plasma cells, and lymphoid follicles. Gastric glands retained their tubular structure. Mild stromal edema, vascular congestion, and focal neutrophil accumulation were described in the submucosa, but no ulceration, erosion, glandular destruction, or major disorganization was reported.

Small-intestinal villi were covered by a single layer of columnar epithelium with numerous goblet cells. The lamina propria contained lymphocytes, plasma cells, and lymphoid follicles, with moderate vascular congestion in some sections. The mucosal and submucosal architecture remained preserved, and no dystrophic change was described.

ISSN: 2181-3337 | SCIENTISTS.UZ

3.6.7. Histology plates

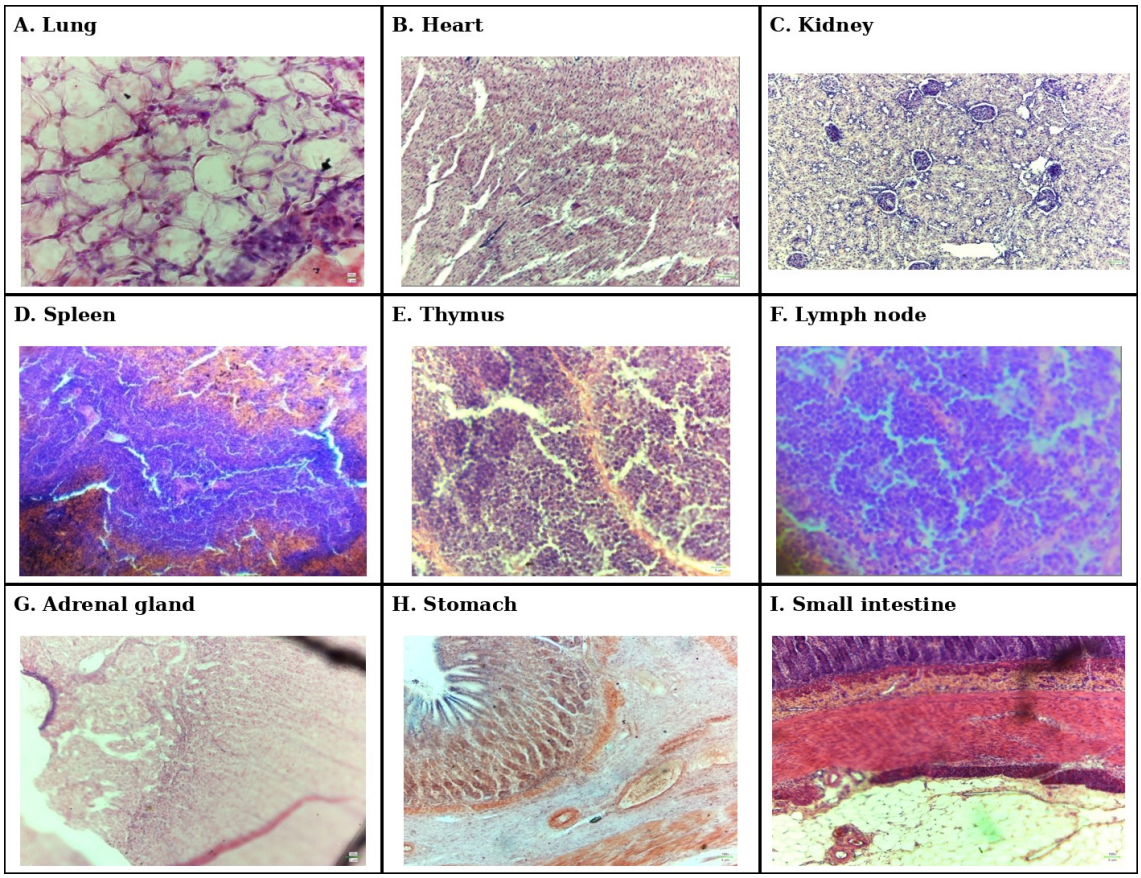


Figure 4. Representative hematoxylin-eosin micrographs after 90 days of C-4 at 4 mg/kg: (A) lung, (B) heart, (C) kidney, (D) spleen, (E) thymus, (F) lymph node, (G) adrenal gland, (H) stomach, and (I) small intestine. Original magnifications were reported as 10×40.

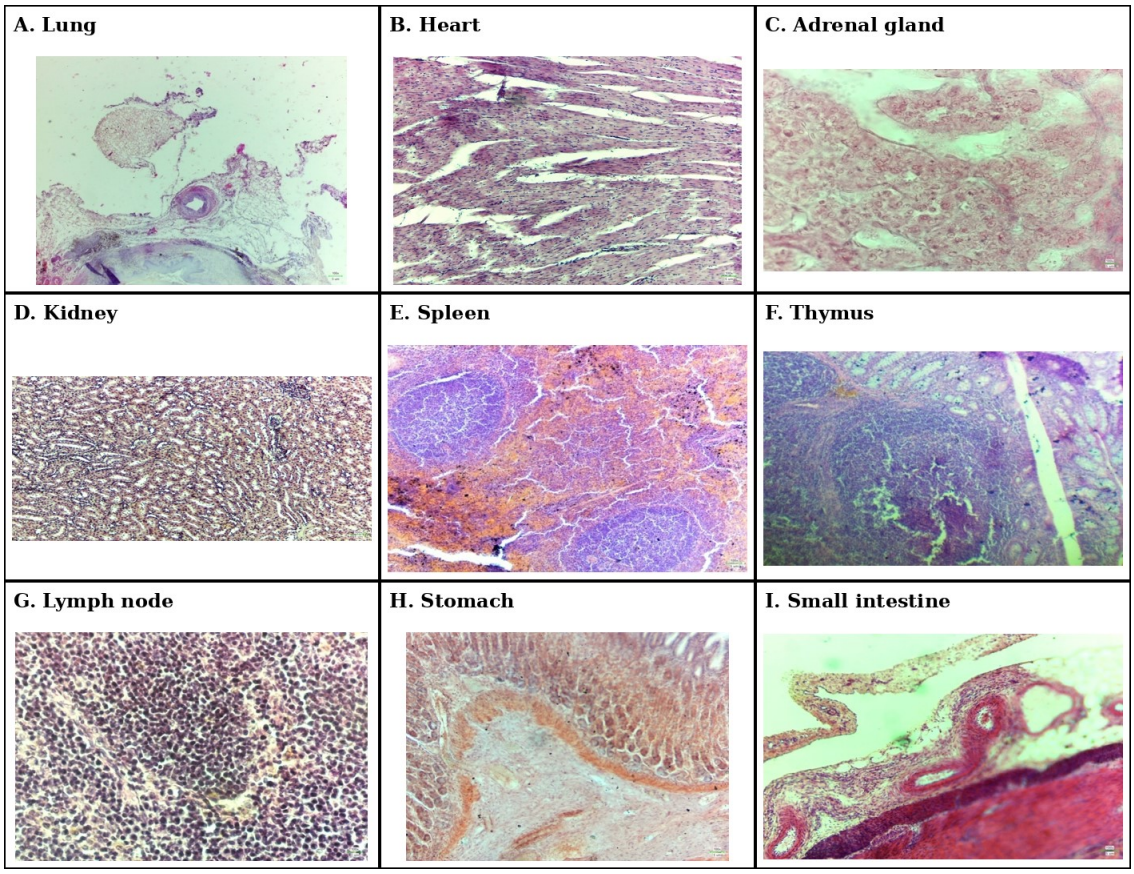


Figure 5. Representative hematoxylin-eosin micrographs after 90 days of C-4 at 8 mg/kg: (A) lung, (B) heart, (C) adrenal gland, (D) kidney, (E) spleen, (F) thymus, (G) lymph node, (H) stomach, and (I) small intestine. Reported magnifications varied from 10×4 to 10×40.

ISSN: 2181-3337 | SCIENTISTS.UZ

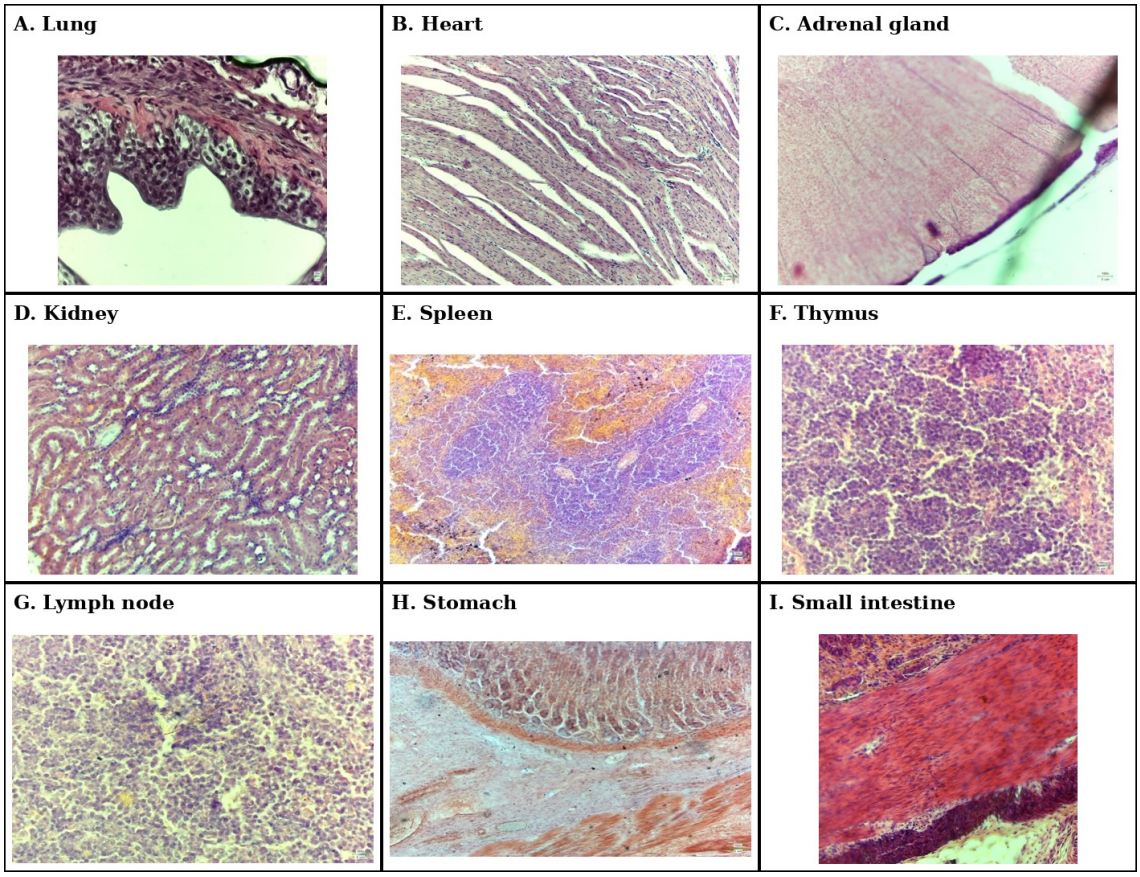


Figure 6. Representative hematoxylin-eosin micrographs after 90 days of C-4 at 16 mg/kg: (A) lung, (B) heart, (C) adrenal gland, (D) kidney, (E) spleen, (F) thymus, (G) lymph node, (H) stomach, and (I) small intestine. The heart panel was described as showing uneven congestion and microfoci of myolysis. Reported magnifications varied from 10×4 to 10×40.

4. Discussion

This 90-day repeated-dose study was intended to determine whether daily oral administration of the C-4 composition produced cumulative systemic toxicity in rabbits. Across the clinical, body-weight, hematological, and serum-biochemical endpoints reported, no statistically significant treatment-related differences were detected at 4, 8, or 16 mg/kg. The absence of reduced activity, impaired grooming, abnormal food or water consumption, weight loss, anemia, leukocyte disturbance, thrombocytopenia, or consistent liver-enzyme elevation suggests that the composition was generally tolerated during the dosing period. This broad pattern is relevant because repeated-dose guidelines emphasize integrated interpretation across in-life observations, clinical pathology, necropsy, organ weights, and histopathology rather than reliance on any single endpoint [1-4,7].

The hematological findings provide no clear evidence of bone-marrow suppression or peripheral blood-cell destruction. Stable hemoglobin and erythrocyte measures argue against clinically meaningful anemia, while the absence of consistent leukocyte and platelet changes argues against major leukopoietic or thrombopoietic toxicity. However, interpretation is limited by the lack of individual-animal data, differential leukocyte counts, blood-smear morphology, sex-specific results, and exact reference intervals for the colony. Rabbit blood parameters are particularly sensitive to handling and stress, and published reference values should be used cautiously because laboratory conditions and analytical platforms differ [8,9]. The apparent reticulocyte percentages in the figure also require confirmation of labeling and units before the result can be considered reliable.

The serum-biochemical results similarly show no conventional signal of hepatocellular or hepatobiliary injury. ALT is generally the most frequently used circulating indicator of hepatocyte membrane damage, whereas AST is less specific and can also increase after muscle injury. ALP may increase with cholestasis or other tissue sources, while total protein reflects a combination of hepatic synthesis, hydration, and immunoglobulin status [10,11]. In the present study, no dose-related rise in ALT,

ISSN: 2181-3337 | SCIENTISTS.UZ

AST, or ALP was reported, and total protein remained stable. These findings are reassuring, but they do not replace liver histopathology. The source record listed liver sampling and reported liver organ-weight coefficients, yet provided no dedicated liver microscopic description or micrograph. Accordingly, the conclusion should be restricted to the measured serum markers and organ weights rather than extended to a definitive absence of hepatic microscopic injury.

Renal assessment was more limited. Urea remained comparable across groups, and kidney histology did not show glomerular, tubular, or interstitial destruction in the representative sections. Nevertheless, urea and serum creatinine can remain unchanged until substantial functional reserve is lost, and modern preclinical safety programmes may incorporate urinalysis and qualified urinary biomarkers when renal liability is suspected [12,13]. The source manuscript refers to renal excretory function, but diuresis, urine chemistry, creatinine, electrolytes, or urinary biomarkers were not reported. The available evidence therefore supports the absence of overt histological nephrotoxicity, not a comprehensive demonstration of preserved renal function.

Organ-weight data did not reveal an obvious monotonic dose response. Liver and heart coefficients were similar to controls, and the small changes in lungs and spleen were not accompanied by a clear gross pathological correlate. Society of Toxicologic Pathology recommendations stress that organ weights should be interpreted with terminal body weight, absolute organ weight, relative weight, macroscopic findings, and microscopy [15]. Because the normalization formula, absolute weights, animal numbers, and statistical analysis were not provided, the coefficients in this manuscript are best regarded as descriptive supporting data.

Most histological descriptions were compatible with preserved tissue architecture. Mild bronchial lymphoid infiltration, reactive splenic follicles, lymph-node activation, mild thymic blast transformation, gastric vascular congestion, and scattered inflammatory cells can occur as background or reactive findings. Toxicological attribution usually requires a consistent increase in incidence or severity, a plausible dose response, concordant findings in related endpoints, and comparison with concurrent controls. The source record did not provide lesion tables or severity grades, so these mild observations cannot be classified definitively as spontaneous, adaptive, or treatment related. Best-practice toxicologic pathology also recommends clear diagnostic terminology, lesion incidence and severity, documentation of the pathologist's approach, and appropriate peer review when findings affect the study conclusion [14].

The high-dose cardiac observation deserves particular attention. Uneven myocardial congestion may be influenced by terminal procedures, anesthesia, agonal changes, tissue handling, or true cardiovascular effects. Microfoci of myolysis, however, describe focal loss or dissolution of myofibrillar structure and may represent a meaningful lesion depending on severity, distribution, and incidence. The absence of serum AST elevation does not eliminate the possibility of focal cardiac injury, especially because cardiac-specific biomarkers and electrocardiographic or functional data were not collected. Before the 16-mg/kg dose is declared non-adverse, the original slides should be reviewed blindly by an experienced toxicologic pathologist, with lesion grading and animal-level tabulation. If the finding is confirmed and exceeds the control background, the high dose may represent a lowest-observed-adverse-effect level rather than a no-observed-adverse-effect level.

The 4- and 8-mg/kg groups showed no comparable cardiac lesion in the available descriptions and no consistent adverse findings in the other measured systems. These doses therefore appear well tolerated under the conditions of the study. Formal assignment of a no-observed-adverse-effect level nevertheless requires a complete, internally consistent dataset, verified exposure, adequate group numbers, and clear distinction between incidental and treatment-related pathology. Toxicokinetic measurements were not reported, so systemic exposure and dose proportionality cannot be evaluated. This is important because administered dose alone does not establish internal exposure, and multicomponent formulations can show nonlinear absorption, metabolism, or interaction among constituents.

ISSN: 2181-3337 | SCIENTISTS.UZ

The manuscript was revised to avoid the original overstatement that no pathological changes occurred at any dose. A more defensible interpretation is that no overt systemic toxicity or conventional serum-biochemical toxicity was demonstrated, most tissues appeared morphologically preserved, and the low and medium doses were tolerated. The 16-mg/kg group requires additional pathological clarification. This cautious interpretation is consistent with regulatory principles that repeated-dose studies should identify target organs, characterize dose-response relationships, and assess reversibility rather than simply classify a product as safe or unsafe [1-4].

5. Study Limitations

Several reporting limitations prevent a definitive regulatory conclusion. First, the test article is identified only as C-4; composition, purity, batch, stability, and formulation details are missing. Second, the stated total number of animals is inconsistent with the described group allocation. Third, the sex, strain, age, acclimatization, randomization method, blinding procedures, and sample-size justification are not reported. Fourth, the quantitative figures state n = 4 per group, although 10 animals per group were reportedly allocated, and no explanation is provided for the analytical subset.

Fifth, exact numerical hematological and biochemical tables, individual-animal data, effect sizes, confidence intervals, and complete statistical outputs are unavailable. Sixth, the reticulocyte panel requires verification of the parameter name and units. Seventh, coagulation outcomes, renal excretory measurements, urinalysis, and recovery-period results are mentioned in the methods or aims but are not reported. Eighth, the organ-weight coefficient formula and units are missing. Ninth, liver tissue was reportedly collected but no liver histology narrative or image was supplied. Tenth, the high-dose myocardial lesion lacks incidence, severity, animal identifiers, and pathology peer review.

These limitations do not invalidate the reported absence of major clinical or biochemical changes, but they substantially restrict reproducibility and the strength of the safety claim. The missing information should be recovered from laboratory notebooks, analyzer printouts, animal logs, raw-data files, and histopathology records. A revised statistical analysis using animal-level data and an appropriate repeated-measures or mixed-effects framework would strengthen the longitudinal interpretation. The final report should also include the ethical approval number, adverse-event record, inclusion and exclusion criteria, and a clear account of all animals analyzed in accordance with ARRIVE 2.0 [6].

6. Conclusions

Daily oral administration of the C-4 composition at 4, 8, and 16 mg/kg for 90 days produced no reported mortality, overt clinical toxicity, growth suppression, significant hematological disturbance, or significant change in the measured serum biochemical markers. Relative organ-weight coefficients were broadly comparable with control values, and most examined tissues retained their normal architecture.

The available evidence supports a favorable preliminary tolerability profile at 4 and 8 mg/kg. At 16 mg/kg, the reported myocardial congestion and microfoci of myolysis require confirmation and grading before the dose can be classified as non-adverse. Therefore, a definitive no-observed-adverse-effect level cannot be assigned from the current manuscript alone. Completion of the missing raw-data, pathology, recovery, coagulation, renal, and test-article documentation is necessary before the study is used to support subsequent clinical development.

REFERENCES

1. Organisation for Economic Co-operation and Development (OECD). Test No. 409: Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents. OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publishing; 1998. https://doi.org/10.1787/9789264070721-en
2. European Medicines Agency, Committee for Medicinal Products for Human Use. Guideline on repeated dose toxicity. CPMP/SWP/1042/99 Rev 1 Corr*. London: European Medicines Agency;

ISSN: 2181-3337 | SCIENTISTS.UZ

2010. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-repeated-dose-toxicity-revision-1_en.pdf

3. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. M3(R2): Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. Geneva: ICH; 2009. https://database.ich.org/sites/default/files/M3_R2__Guideline.pdf
4. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. S4: Duration of Chronic Toxicity Testing in Animals (Rodent and Non-Rodent Toxicity Testing). Geneva: ICH; 1998. https://database.ich.org/sites/default/files/S4_Guideline.pdf
5. European Parliament and Council of the European Union. Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union. 2010;L276:33-79. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063
6. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biology. 2020;18(7):e3000410. https://doi.org/10.1371/journal.pbio.3000410
7. Organisation for Economic Co-operation and Development (OECD). Guidance Document 116 on the Conduct and Design of Chronic Toxicity and Carcinogenicity Studies, Supporting Test Guidelines 451, 452 and 453. 2nd ed. OECD Series on Testing and Assessment No. 116. Paris: OECD Publishing; 2014. https://doi.org/10.1787/9789264221475-en
8. Wolford ST, Schroer RA, Gohs FX, et al. Reference range data base for serum chemistry and hematology values in laboratory animals. Journal of Toxicology and Environmental Health. 1986;18(2):161-188. https://doi.org/10.1080/15287398609530859
9. Melillo A. Rabbit clinical pathology. Journal of Exotic Pet Medicine. 2007;16(3):135-145. https://doi.org/10.1053/j.jepm.2007.06.002
10. Boone L, Meyer D, Cusick P, et al. Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies. Veterinary Clinical Pathology. 2005;34(3):182-188. https://doi.org/10.1111/j.1939-165X.2005.tb00041.x
11. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. Toxicology. 2008;245(3):194-205. https://doi.org/10.1016/j.tox.2007.11.021
12. Dieterle F, Sistare F, Goodsaid F, et al. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. Nature Biotechnology. 2010;28(5):455-462. https://doi.org/10.1038/nbt.1625
13. Vaidya VS, Ozer JS, Dieterle F, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nature Biotechnology. 2010;28(5):478-485. https://doi.org/10.1038/nbt.1623
14. Crissman JW, Goodman DG, Hildebrandt PK, et al. Best practices guideline: Toxicologic histopathology. Toxicologic Pathology. 2004;32(1):126-131. https://doi.org/10.1080/01926230490268756
15. Sellers RS, Morton D, Michael B, et al. Society of Toxicologic Pathology position paper: Organ weight recommendations for toxicology studies. Toxicologic Pathology. 2007;35(5):751-755. https://doi.org/10.1080/01926230701595300