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A Biodistribution and Toxicity Study of Cobalt Dichloride–N-Acetyl Cysteine (C4) as an Implantable MRI Marker for Prostate Cancer Treatment

Steven J. Frank, MD^{*}, Mary J. Johansen, Pharm.D.[†], Karen Martirosyan, PhD[‡], Mihai Gagea, DVM[§], Carolyn S. Van Pelt, DVM, PhD[§], Agatha Borne, DVM, PhD[§], Yudith Carmazzi, MS[†], and Timothy Madden, PharmD[†]

^{*}Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center

[†]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center

[‡]Department of Physics and Astronomy, University of Texas Brownsville

[§]Department of Veterinary Medicine & Surgery, The University of Texas MD Anderson Cancer Center

Abstract

Purpose—C4, a cobalt dichloride–N-acetyl cysteine complex, is being developed as a positive-signal magnetic resonance imaging (MRI) marker to localize implanted radioactive seeds in prostate brachytherapy. We evaluated the toxicity and biodistribution of C4 in rats with the goal of simulating systemic effects of potential leakage from C4 MRI markers within the prostate.

Methods—9 μ l doses (equivalent to leakage from 120 markers in a human) of control (0.9% sodium chloride), 1% (proposed for clinical use) and 10% C4 solution were injected into the prostates of male Sprague-Dawley rats via laparotomy. Organ toxicity and cobalt disposition in plasma, tissues, feces and urine were evaluated.

Results—No C4-related morbidity or mortality was observed in the biodistribution arm (60 rats). Biodistribution was measurable following 10% C4 injection: cobalt was cleared rapidly from periprostatic tissue; mean concentrations in prostate were 163 μ g/g and 268 μ g/g at 5 and 30 minutes but were undetectable by 60 minutes. Expected dual renal-hepatic elimination was observed with % injected dose recovered in tissues of 39.0 $\pm$ 5.6% (liver) > 11.8 $\pm$ 6.5% (prostate) > 5.3 $\pm$ 0.9% (kidney) with low plasma concentrations detected up to 1 hr (1.40 μ g/ml at 5–60 minutes). Excretion in urine was 13.1 $\pm$ 4.6 % with 3.1 $\pm$ 0.54 % recovered in feces by 24 hours. In the toxicity arm, three animals died in the control group and 1 each in the 1% and 10% groups from surgical or anesthesia-related complications; all others survived to scheduled termination at 14 days. No C4-related adverse clinical signs or organ toxicity was observed.

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Corresponding Author: Steven J. Frank, MD, Department of Radiation Oncology, Unit 97, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel 713-563-8489; sjfrank@mdanderson.org.

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Conflicts of Interest Notification: S.J. Frank and K. Martirosyan are co-founders of C4 Imaging, LLC. All other authors declare no conflicts of interest.

Conclusion—C4-related toxicity was not observed at exposures at least 10-fold that proposed for human use. This data demonstrating lack of systemic toxicity with dual routes of elimination in the event of *in-situ* rupture suggests C4 warrants further investigation as an MRI marker for prostate brachytherapy.

Keywords

prostate brachytherapy; encapsulated contrast agent marker; C4; magnetic resonance imaging (MRI); C4 MRI Marker; post-implant dosimetry

INTRODUCTION

Brachytherapy is a standard-of-care approach for treating localized prostate cancer. Dosimetric precision after brachytherapy seed implantation is crucial to ensure the adequacy of the implant and the highest probability of cancer cure.¹ For example, if post-implant evaluation reveals that the radiation dose being delivered to the sites of biopsy-positive disease is inadequate, additional seeds could be implanted in the prostate to optimize that patients treatment. MRI is the optimal imaging modality for quality assurance in prostate brachytherapy. MRI sequences have been identified which reduce susceptibility artifacts for prostate delineation and provide the best visualization of the anatomy of the prostate and its surrounding normal tissue structures.² However, use of MRI for image-based dosimetry after seed implantation is complicated by the appearance of the titanium radioactive seeds as signal voids with susceptibility artifacts, making precise localization of each seed within the prostate and periprostatic tissue difficult.³ CT is still required for fusion with MRI to distinguish seeds from spacers and to assist with identification of extraprostatic seeds during post-implant dosimetric evaluation.² There are inherent problems in the accuracy of MR-CT fusion however, which subjects the patients to additional radiation exposure and the inconvenience of additional testing. One approach to ensuring the precise localization of each seed using MRI alone involves attaching a positive contrast marker to each seed that is easily visualized by MRI (Fig. 1). The use of an MRI marker could eliminate the need for CT as part of post-implant assessment and reduce the time for radiation oncologists and physicists to fuse MRI to CT to identify seeds by CT imaging.

Over the past 5 years, our group has developed an MRI marker (C4), an encapsulated contrast-agent incorporating 1% cobalt-dichloride-N-acetyl cysteine ($\text{CoCl}_2\text{-NAC}$), that when placed adjacent to radioactive seeds permits precise identification of implanted radioactive seeds using MRI. When hydrated, cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) has paramagnetic properties that make it an effective positive contrast agent marker for MRI. The efficacy of this marker for this purpose was recently demonstrated in an *ex vivo*⁴ and *in-vivo* canine model (Fig. 2). We further showed that the C4 MRI marker does not affect the anisotropy of the radioactive seeds and therefore can be placed next to the titanium seeds for post-implant identification on MRI.⁵ In the current study, we evaluated the local and systemic toxicity of C4, as well as the biodistribution of cobalt, in a model mimicking leakage of C4 solution from MRI markers into the prostate or periprostatic tissue. Using this model, we were able to investigate the potential for toxicity from possible *in situ* rupture of the MRI markers and provide initial insight into the safety of this approach and the distribution and elimination of cobalt following systemic exposure to C4.

METHODS AND MATERIALS

This study was conducted with strict adherence to the National Research Council's Guide for the Care and Use of Laboratory Animals. All methods were reviewed and approved by the Institutional Animal Care and Use Committee. The study involved tissue distribution and

toxicity analyses in 8- to 12-week-old male Sprague Dawley rats (supplied by Harlan Laboratories) after intraprostatic injection of control solution or one of two dosages of C4. Rats used for toxicity analyses were sexually mature males (i.e., at least 10 weeks old) at the time of dosing.

Dosing

C4 solutions were freshly prepared on the day of administration. Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and N-Acetyl-Cysteine (NAC, $\text{C}_5\text{H}_9\text{NO}_3\text{S}$) were purchased from Sigma-Aldrich. 1% C4 solution (1% CoCl_2 -2% NAC) was prepared by dissolving 0.42 mmol $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 1.18 mmol of NAC in 10 mL distilled water. 10% C4 solution (10% CoCl_2 -2% NAC) was prepared by dissolving 4.2 mmol $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 1.18 mmol of NAC in 10 mL distilled water. Solutions were sonicated at room temperature for 15 minutes to completely dissolve. Adequate amounts of each solution were retained for quantification of total cobalt content by inductively coupled plasma analysis. All solutions were within 10 % of nominal concentrations.

On study day 0, the rats were anesthetized with isoflurane and either control or C4 solution was injected into the prostates of each rat through a 1.0-cm incision in the abdominal wall (laparotomy) in the inguinal region adjacent to the ventral prostate. The injection volume (9 μL) and administration site (the ventral and lateral lobes of the prostate) in rats were selected to mimic the conditions experienced if all marker seeds implanted in the center and outer capsule of prostate in a human subject were to rupture simultaneously. The outer dimensions of the MRI marker are 5.5 mm $\times$ 0.8 mm, therefore, assuming a maximum active length of 5.5 mm and 0.4 mm radius, the total volume of C4 solution in each MRI marker would be 2.5 μL . For a typical implant of 80–120 titanium seeds, a maximum of 300 μL of 1% C4 could seep into the interstitial space surrounding the prostate, which typically weighs about 60 g in human males. For this rat model we extrapolated the dose to a 0.16-g rat prostate if 120 markers were used and filled to their capacity volume of 2.5 μL [300 μL 1% C4 in 60-g human prostate = 0.8 μL 1% C4 in 0.16- g rat prostate] and tested doses of 9 μL of 1% (21 μg) or 9 μL of 10% C4 complex given as a periprostatic injection, thus exceeding the maximum amount that could be released in the event of marker rupture in a human by factors of 10 and 100, respectively.

Biodistribution

Sixty male rats were divided into 3 groups: 20 underwent intraprostatic injection of control solution, 20 of 1% C4 solution, and 20 of 10% C4 solution. Rats were weighed upon arrival and before dosing, and all rats were observed for any signs of toxicity during the entire course of the in life phase. Five rats from each dose group were euthanized at 5 minutes, 30 minutes, 60 minutes, or 6 hours after dosing and blood samples collected by cardiac puncture were placed into heparinized tubes, after which the plasma was separated and frozen at -80°C until analysis. Spleen, heart, brain, prostate, lung, kidney, liver, and gut from all animals were harvested, blotted, and weighed. Tissue samples from each organ were placed in cryotubes and frozen at -80°C until analysis; prostate, kidney, liver, and plasma samples were evaluated immediately, and other tissues were archived. In a separate cohort, an additional 3 rats were given the highest dosage (10% C4) and maintained individually in metabolic cages for collection of urine and feces. Urine and feces were collected during the 24 hours before dosing and at 60 minutes, 6 hours, and 24 hours after dosing. Urine volume and fecal weight of each collection were recorded and the samples frozen at -80°C for analysis.

Toxicity

Thirty male rats were used for toxicity evaluation, 10 received intraprostatic injection of 9 μ L control solution, 10 of 1% C4, and 10 of 10% C4. Clinical observations were recorded at baseline and twice daily thereafter; rats were weighed at baseline, twice weekly (at least 2 days apart) thereafter, and terminally before blood collection and necropsy. Five rats were sacrificed per group at 24 hours (acute phase) and 14 days (recovery phase) after dosing.

Rats were anesthetized and weighed before and after terminal blood collection via cardiac puncture for clinical pathology, and a complete necropsy was performed on all animals. Animals that appeared moribund before their scheduled time for termination were anesthetized and subjected to the same procedures. Organs were fixed in 10% neutral buffered formalin for microscopic analysis. Male accessory sex glands (seminal vesicles, coagulating glands, ventral, lateral and dorsal prostate, ampullary glands and bulbourethral glands) were collected together, and urinary bladder, urethra and penis were collected together; these sets of organs were each attached to a small piece of dry cardboard before immersing into formalin in order to preserve the normal anatomical orientation of the organs. The testes and epididymides were collected separately. These tissues were fixed in 10% buffered formalin along with the other organs.

Histopathology

All major organs (40 tissues) of the digestive, urinary, respiratory, cardiovascular, hematopoietic (including bone marrow assessed via the sternum and femur/knee joint), endocrine, nervous, musculoskeletal, skin, and reproductive systems, and the site of abdominal laparotomy were examined grossly and collected in 10% neutral buffered formalin at necropsy. Formalin fixed tissues were processed, embedded in paraffin blocks, sectioned, and mounted on glass slides which were stained with hematoxylin and eosin (H&E), and examined microscopically. Any tissues not processed to slides were archived for later processing if necessary. The formalin-fixed seminal vesicles, coagulating glands, and urinary bladder were separated from the reproductive tract and processed together in one paraffin block. The prostate (ventral lateral and dorsal lobes) and attached genital organs were sectioned in a transverse (dorsoventral) plane and were processed in a separate paraffin block.

RESULTS

Biodistribution

All rats in the biodistribution group survived to their scheduled terminations, with no morbidity or mortality noted. Low endogenous cobalt concentrations (0.03–0.06 μ g/g tissue) were detectable only in liver tissue in control animals receiving saline. In rats given 1% C4, cobalt could be measured in the prostate tissue in 2 of 5 animals and only at 5 minutes, and was not detected in the plasma or kidney (Table 1). Due to largely undetectable concentrations observed in other tissues at 1% C4, only control and high dose (10% C4) liver samples were analyzed. Cobalt distribution was evaluable in rats receiving 10% C4, with a total percent of injected dose recovered of 80.8 % (Table 2). Mean cobalt concentrations measured in prostate tissue following 10% C4 injection were 163.6 μ g/g and 268.6 μ g/g at 5 and 30 minutes, but were undetectable in most animals by 60 min post injection. Plasma cobalt concentrations at 5 to 60 minutes after injection of 10% C4 were a mean of 1.40 μ g/mL and undetectable by 60 minutes. The greatest amount of cobalt was detected in the liver (39.0 $\pm$ 5.6 % of injected dose), with 5.3 $\pm$ 0.9 % recovered in the kidney. Concentrations in liver and kidney were half their peak concentration at 30 minutes (3.4 μ g/g) by 6 hours (1.4–1.5 μ g/g).

The percent excreted in the urine by 24 hours post injection was $13.1 \pm 4.6\%$, with $3.1 \pm 0.5\%$ recovered in the feces (Table 2). After injection of 10% C4, cobalt was detected in the urine within 60 minutes, with mean peak concentrations in urine of $11.6 \mu\text{g/ml}$ at 6 hours. Cobalt concentrations were negligible in the urine and feces samples collected from 3 rats the day before injection; only one rat had detectable fecal levels ($0.46 \mu\text{g/g}$) at 6–24 hours before injection. Feces were not available for collection at 60 minutes and 6 hours after injection (likely secondary to surgical manipulation or anesthesia), but mean cobalt concentration in feces collected 6–24 hours after injection was $3.28 \mu\text{g/g}$, indicating that fecal elimination was 8 times higher after dosing than in normal turnover. These findings are consistent with the reported main storage sites for cobalt and dual routes of hepatic and renal elimination.²⁰

Toxicity

No test article-related morbidity or mortality was noted in the 30 rats tested for toxicity, although three rats in the control group, one in the 1% group, and one in the 10% group died of surgery-and/or anesthesia-related causes. All other animals survived to their scheduled terminations. The single intraprostatic injection of control solution, 1% C4, or 10% C4 did not result in any toxicologically-important changes in the hematologic (Table 3) or clinical chemistry (Table 4) parameters, either at day 1 or at day 14 after intraprostatic administration. All group mean values were within the normal reference range for these animals. Serum transaminase values were falsely elevated in one rat in the control group and two rats in the 10% group at study day 14 due to hemolysis of the blood samples. These three rats had no significant histologic changes observed in the liver that would support hepatocellular injury or compound toxicity. Similarly, no gross or microscopic lesions attributable to C4 were detected in any of the examined tissues at either 1 day or 14 days post administration. Non C4-related inflammatory lesions consistent with reactions related to surgical manipulation and tissue injection were observed in the connective tissue surrounding the prostate glands, bladder, epididymides, testicles, and surgical margins of the skin, abdominal wall, and peritoneum in both the control and treated animals with similar incidence. Splenic lymphocytic hyperplasia and extramedullary hematopoiesis were also observed in the control and treated animals that were consistent with immunohematologic reactions due to chronic inflammation of the surgical site and not related to the test article. No differences in mean terminal body weights or organ weights (relative to body weight) were detected among groups at either 1 day or 14 days after the intraprostatic injection.

DISCUSSION

To our knowledge, this is the first study to analyze the biodistribution and toxicity of C4. Even with direct injection of the material into the prostate and surrounding tissues at doses 10 to 100- fold that proposed to be used in humans, no C4-related morbidity or mortality was observed. The greatest amount of cobalt was detected in the liver ($39.0 \pm 5.6\%$ of injected dose), with $5.3 \pm 0.9\%$ recovered in the kidney tissue and 13% excreted in the urine; confirming that this conjugate was eliminated via the kidneys and liver. No C4-related alterations were noted in the hematologic or clinical chemistry parameters, and no histopathologic lesions related to C4 administration were detected in rats at either of the tested dose levels.

The lack of evidence of toxicity from cobalt at the concentrations and volume used in this study is not surprising. Cobalt is an essential element and an important component of vitamin B12 (cyanocobalamin), which is required to maintain good health in animals and humans.⁷ Vitamin B12 is also a component of enzymes involved in hematopoiesis, and cobalt deficiencies can lead to pernicious anemia.⁸ Cobalt chloride is added to animal feed as a nutritional dietary supplement and is generally recognized as safe at levels consistent

with good feeding practices.⁷ The average person consumes about 11 µg of cobalt per day from dietary sources, and the U.S. recommended dietary allowance for vitamin B12 for adults is 2.4 µg/day, which contains 0.1 µg of cobalt.^{7, 8} According to reports from the U.S. Department of Health and Human Services and the United Kingdom National Poisons Information Service on the toxicological profile of cobalt, neither genotoxicity nor carcinogenicity has been reported in humans from cobalt chloride.^{7, 9}

Excessive cobalt concentrations, however, could be harmful.¹⁰ In the 1960s, some breweries added cobalt salts to beer to stabilize the foam, resulting in exposures of 0.04–0.14 mg cobalt/kg per serving.¹¹ To date, this is the only information available for intermediate-duration exposure via oral ingestion in humans.⁷ The most sensitive endpoint after oral exposure to cobalt in humans seems to be an increase in erythrocyte numbers (polycythemia).¹⁰ Davis and Fields reported 16%–20% increases in erythrocyte levels in all six healthy men given cobalt chloride orally at about 1 mg Co/kg-day.¹⁰ However, giving daily doses of cobalt chloride to pregnant women for 90 days did not prevent the expected reductions in hematocrit and hemoglobin levels associated with pregnancy.¹² In this study, no evidence of hematologic toxicity was observed. All hematologic parameters remained within normal range for this species and no abnormalities were observed in the bone marrow.

In a further effort to reduce the potential body burden of cobalt should the MRI markers be compromised inside a patient, we chelated the salt solution to NAC.¹³ CoCl₂ is a hypoxia-mimetic and NAC is a reactive oxygen species scavenger.¹⁴ NAC is used as a chelating agent to reverse heavy metal poisoning because of its ability to safely chelate metals such as cobalt and assist in their elimination.^{14, 15, 16, 17} Chelation of NAC with CoCl₂ results in the formation of an inert compound with no known biological activity. Several studies have investigated the effectiveness of various chelating agents for mitigating the toxicity of cobalt,^{15, 16, 18} and NAC was found to be the most effective chelator because it increased both urinary and fecal excretion of cobalt as well as decreased the levels of cobalt in the liver and spleen.¹⁵

For intermediate-duration oral exposure (<365 days) to cobalt, a minimal risk level has been derived of 0.01 mg Co/kg-day by dividing the low-observed-adverse-effect-level (1 mg Co/kg-day) by an uncertainty factor of 100 (10 for use of a low-observed-adverse-effect-level and 10 for human variability). Therefore, for a standard 75-kg male, the daily allowable cobalt consumption would be 0.75 mg/day.⁷ If an individual MRI marker were to be compromised after implantation, the amount of cobalt released would be 375 times *lower* than the daily minimal risk level. Comparing oral exposure levels with bolus injection into the prostate may be less than optimal, however, at this time, adequate studies are not available on the chronic toxicity of systemically ingested cobalt or cobalt compounds in humans and animals. Additional studies including evaluation of stability, the potential for promotion of reactive oxygen species formation, and the biocompatibility of C4 are ongoing to further evaluate its safety and to support its use for brachytherapy seed localization.

CONCLUSIONS

This study represents the first to evaluate the biodistribution and local and systemic toxicity of C4, a novel MRI visible marker under development for use in prostate brachytherapy. The lack of demonstrated toxicity coupled with rapid elimination as observed in this model mimicking systemic exposure to C4 provides evidence that it may be a viable candidate as an imaging marker to improve the accuracy of MRI-based dosimetry.

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Figure 1.

The C4 solution is placed within a polymer (Polyether ether ketone or PEEK) that is the size of a standard spacer used in strands for prostate brachytherapy. The C4 MRI markers are visualized within strands adjacent to the titanium non-radioactive seeds in a prostate phantom under MRI (oblique view)

Figure 2.

A strand of C4 MRI markers with titanium non-radioactive seeds are placed within an in-vivo canine prostate and visualized under MRI (sagittal view).

Table 1 Plasma and Tissue Cobalt Concentrations (Mean $\pm$ SD)

Cobalt [$\mu\text{g/mL}$]				
	Plasma ($\mu\text{g/mL}$)	Prostate ($\mu\text{g/g}$)	Liver ($\mu\text{g/g}$)	Kidney ($\mu\text{g/g}$)
<u>Control</u>				
5 min	nd	nd	0.03 $\pm$ 0.004	nd
30 min	nd	nd	0.04 $\pm$ 0.01	nd
60 min	nd	nd	0.06 $\pm$ 0.03	nd
6 hr	nd	nd	0.06 $\pm$ 0.04	nd
<u>1% C4</u>				
5 min	nd	17.3 $\pm$ 7.0	na	nd
30 min	nd	nd	na	nd
60 min	nd	nd	na	nd
6 hr	nd	nd	na	nd
<u>10% C4</u>				
5 min	1.3 $\pm$ 0.2	163.6 $\pm$ 141.6	2.1 $\pm$ 0.7	2.1 $\pm$ 0.6
30 min	1.4 $\pm$ 0.4	268.6 $\pm$ 500.1	3.4 $\pm$ 1.1	3.4 $\pm$ 1.0
60 min	1.5 $\pm$ --	30.9 $\pm$ --	1.9 $\pm$ 1.0	2.0 $\pm$ 1.5
6 hr	--	--	1.5 $\pm$ 0.7	1.3 $\pm$ 0.3

nd= not detected or below the limits of quantification; minimum detection limit 0.004 $\mu\text{g/mL}$ na = not analyzed

Table 2 Distribution of 10% C4- % Injected Dose Recovered (Mean $\pm$ SD)

	% Recovery				
	Total	5 min	30 min	60 min	6 hr
Liver	39.0 $\pm$ 5.6	9.6 $\pm$ 2.5	15.1 $\pm$ 3.1	7.9 $\pm$ 4.3	6.3 $\pm$ 2.8
Prostate	11.8 $\pm$ 6.5	10.9 $\pm$ 8.1	3.4 $\pm$ 4.3	2.0 $\pm$ --	nd
Plasma	8.4 $\pm$ 1.3	4.0 $\pm$ 0.5	4.3 $\pm$ 1.1	4.5 $\pm$ --	nd
Kidney	5.3 $\pm$ 0.9	1.1 $\pm$ 0.3	2.1 $\pm$ 0.8	1.4 $\pm$ 1.1	0.8 $\pm$ 0.5
Urine	13.1 $\pm$ 4.6	4.0 $\pm$ 4.7	4.6 $\pm$ 1.1	4.5 $\pm$ 1.4	
Feces	3.1 $\pm$ 0.5	nd	nd	3.1 $\pm$ 0.5	

nd = not detected or below the limits of quantification; minimum detection limit 0.004 μ g/ml

Table 3

Hematology (Mean ±SD) at scheduled termination days 1 and 14

	WBC (10 ³ /μl)	RBC (10 ⁶ /μl)	HGB (g/dl)	HCT (%)	PLT (10 ³ /μl)
DAY 1					
Control	8.8±4.1	6.7±0.7	13.3±1.4	40.2±4.2	889±252
1% C4	9.4±4.5	7.2±0.1	13.9±0.2	42.4±1.1	1023±165
10% C4	9.9±1.2	7.2±0.2	14.2±0.5	43.0±0.9	921±475
DAY 14					
Control	11.9±0.8	7.8±0.4	14.8±0.5	46.2±2.0	1001±91
1% C4	10.2±1.8	7.8±0.3	14.3±0.3	44.1±0.9	1177±202
10% C4	10.5±0.2	7.6±0.2	13.9±0.9	42.9±2.1	1005±826

[WBC-White Blood Cells; RBC-Red Blood Cells; HGB-Hemoglobin; HCT-Hematocrit; PLT-Platelets]

Table 4

Clinical chemistry (Mean ±SD) at scheduled termination days 1 and 14

	T. BILI (mg/dl)	Cr (mg/dl)	BUN (mg/dl)	AST (mg/dl)	ALT (IU/L)	ALK PHOS (IU/L)	T. P. (IU/L)	ALB (gm/dl)	GLOB (gm/dl)
DAY 1									
Control	0.1	0.34±0.1	13.7±2.0	140±31	67±9	237±44	5.5±0.3	3.5±0.2	1.9±0.1
1% C4	0.1	0.39±0.1	15.1±1.8	137±13	70±7	250±33	5.8±0.0	3.7±0.0	2.1±0.0
10% C4	0.1	0.34±0.0	16.6±0.9	136±28	69±8	257±23	5.9±0.3	3.8±0.1	2.1±0.1
DAY 14									
Control	0.1	0.38±0.0	19.7±1.6	191±151	174±138	226±41	6.3±0.1	4.1±0.2	2.2±0.2
1% C4	0.1	0.36±0.0	19.6±1.5	90±10	68±7	223±50	6.3±0.2	4.1±0.1	2.2±0.1
10% C4	0.1	0.33±0.0	17.9±2.4	334±345	214±233	271±16	6.4±0.2	4.0±0.2	2.3±0.1

[T.BILI – Total Bilirubin; Cr – Creatinine; BUN- Blood Urea Nitrogen; AST-Aspartate Aminotransferase; ALK PHOS- Alkaline Phosphatase; ALB-Albumin; GLOB-Globulin]