

Serum cardiac troponin I as a biomarker in cardiac degeneration following experimental salinomycin toxicosis in sheep

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ABSTRACT

Salinomycin is an ionophore with antimicrobial properties. It is a dietary additive used as a growth promoter for ruminants and as a coccidiostat in chickens. However, over-dosage or misuse situations can lead to a series of toxic syndromes. Cardiac troponin I (cTnI) is the part of the troponin complex (I, C and T) within the sarcomere in myocardial cells that regulates contraction of the heart muscle. cTnI is released from injured myocytes into circulation, so it can be a specific biomarker in myocardial necrosis. The purpose of this study is to propose cTnI for diagnostic cardiac degeneration induced by experimental toxicosis with salinomycin in sheep. Twenty Iranian mixed breed adult female fat-tailed sheep (BW: 33.3 ± 3.4 kg) were used in this study. The sheep were randomly divided into five equal groups. Group I (control) received 20 mL normal Saline. Groups II, III, IV and V were orally administered 1 mg/kg (twice a day for two days), 2, 3 and 4 mg/kg (once a day for two days) salinomycin, respectively. Following drug administration, blood samples were collected at different time intervals (2, 5, 8, 14 and 21 days) in order to determine various biochemical parameters (cTnI, CK, LDH, ALT and AST). In all groups, the heart sounds of the animals were carefully heard and electrocardiogram (ECG) was taken to determine the type of probable arrhythmia. The results illustrated a significant increase in the activity of cTnI. Numerous arrhythmias were recorded, such as: sinus tachycardia, supraventricular tachycardia, sinus arrhythmia and supraventricular premature contraction. All animals with arrhythmias showed a significant increase in the activity of cTnI. Cardiac muscle necrosis observed macroscopically on post mortem examination revealed myocardial degeneration. Overall, the results of this study indicate that cTnI may be considered as a valuable biomarker in diagnosing cardiac degeneration due to salinomycin toxicosis.

Key words: salinomycin toxicosis, sheep, troponin I, biochemical parameters, arrhythmia

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Introduction

Salinomycin is a monocarboxylic polyether antibiotic produced by *Streptomyces albus*. It is used to prevent coccidiosis in chickens and is also added to feed to improve feed efficiency in beef cattle (BASTIANELLO et al., 1996; RIZVI, 1999). Due to the fact that the ionophorous anticoccidials currently used have a narrow margin of safety and some of them are highly toxic to several animal species, the considerable concern for their safe application is entirely justified (VACZI et al., 2006). Toxic doses of these drugs vary and depend on the ionophore compound and the species of animals (GALITZER et al., 1986). Moreover, because of its favorable hemodynamic profile, salinomycin has the potential as a drug to increase cardiac output, blood pressure and left ventricular contraction force, and for improving the myocardial blood perfusion and mechanical efficiency of the heart (OZAKI et al., 1982). Salinomycin has been shown to exert an inotropic effect (GUPTA et al., 2005; SATOH and TSUCHIDA, 1999). This effect has been mostly attributed to the release of endogenous catecholamines (GAIDE et al., 1984), although Rajaian et al have proposed that the inhibition of catecholamine metabolism may also be involved (RAJAIAN et al., 2009). In clinical medicine, numerous serum biomarkers are used, or proposed for use, in the diagnosis of myocardial injury, including acute myocardial infarction (AMI). All of these biomarkers are macromolecules found in cardiac myocytes and their appearance in the serum is indicative of myocyte injury. Only CK-MB, cTnI, cTnT, myoglobin, and LDH have achieved wide clinical acceptance, not only for AMI, but for other forms of myocardial injuries (APPLE, 1999). Cardiac troponin is the 'gold-standard' biomarker of myocardial injury in humans because of its high tissue specificity, diagnostic sensitivity, low basal blood concentration, rapid release, and persistence in the blood. Studies in humans (ADAMS et al., 1993) and rats (YORK et al., 2007) indicated good correlation between the severity of morphologically detectable myocardial cell damage and blood troponin I concentrations. Serum concentrations of cTn correlate well with the histopathological changes in the myocardium, cardiac pathophysiology, extent of cardiac injury, clinical signs and prognosis. Use of cTn in veterinary medicine and in pre-clinical toxicology is growing rapidly (O'BRIEN et al., 1997; WELLS and SLEEPER, 2008). cTnI can be a marker in trauma, including traumatic reticuloperitonitis in cattle (GUNES et al., 2008). GUNES et al. (2005) suggest that cardiac troponin tests designed for use in human beings could also be used in the diagnosis of myocardial degeneration due to foot-and-mouth disease in calves. Studies in lambs with white muscle disease demonstrated that cTn kits used for humans can effectively determine myocardial degeneration in these animals (GUNES et al., 2010).

In the present study, ECG parameters and the types of arrhythmias were examined in sheep intoxicated experimentally with salinomycin. In addition, changes in biochemical parameters were also studied in the intoxicated animals.

Materials and methods

Twenty Iranian mixed breed adult female fat-tailed sheep weighing 33.3 ± 3.4 kg were stable-fed *ad libitum* with a mixture of alfalfa hay, corn silage and barley. The sheep were randomly divided into five equal experimental groups. Sodium salinomycin was orally administered. Group one (control) received 20 mL normal saline. Groups II, III, IV and V were given 1 mg/kg (twice a day for two days), 2, 3 and 4 mg/kg (once a day for two days) salinomycin, respectively. Following drug administration, blood samples were collected from the jugular vein into vacutainers at different intervals (2, 5, 8, 14 and 21 days), in order to determine various biochemical parameters (cTnI, CK, LDH, ALT and AST). The sera were separated by centrifugation at $750 \times g$ and were kept at -20 °C until analysis. To ensure the expected concentrations of our parameters, the frozen samples were sent to the laboratory for analysis within 48 hours. After arrival at the laboratory, the samples were thawed at room temperature and analyzed immediately. Biochemical analysis was carried out to measure serum ALT and AST activities by the colorimetric method of Reitman and Frankel (REITMAN and FRANKEL, 1957), CK by the Sigma colorimetric (modified Hughes) method, and LDH by the Sigma colorimetric method. The biochemical parameters were measured using appropriate commercial laboratory kits with RA1000 spectrophotometer.

A commercially available ELISA kit, cTnI Accubind™ Elisa assay (Monobind Inc) was used to determine the concentration of cTnI according to the manufacturer's instructions. The optical densities of the samples were compared with a standard curve prepared for standards containing 0 to 3 ng/mL of cTnI derived from human hearts. All the enzyme activities were measured at 37 °C and the results are presented in U/L.

ECG recording. Heart sounds of the sheep in both groups were carefully monitored before and after the administration of either salinomycin or Saline solution. Electrocardiograms (ECGs) were recorded to evaluate the presence and types of arrhythmias. A base-apex lead was used to record the ECG by Kenz ECG recorder model 110 (Suzuken Co., Japan). This lead was attached by placing the positive electrode on the left thorax, located in the fifth intercostal space, at the level of the point of the elbow or where the apex beat was most readily palpated. The negative electrode was attached to the skin on the right jugular furrow, two-thirds of the way from the ramus of the mandible to the thoracic inlet. The ground electrode was attached to a location remote from the heart. A base-apex lead ECG was recorded from each animal, at a paper speed of 25 mm/s and calibration of 10 mm/mV (1 cm = 1 mV). The ECGs of control sheep were used to obtain normal values for heart rate, amplitude, duration, configuration, segments (PR and ST) and intervals (PP, RR, PR and QT).

This study was approved by the research committee of Shiraz University according to the animal welfare regulations.

Statistical analysis. All data are presented as mean \pm SEM and were analyzed by proc mixed procedure using SAS for Windows release 9.11 computer software (SAS Institute Inc., Cary, NC). Time and dose groups, as well as their interaction, were considered as the main effects in the statistical model. The Tukey test was used to determine the significant differences between group means. In all analyses, a P value less than 0.05 was considered significant.

Results

Clinical signs. Clinical signs noticed after oral administration of salinomycin were: inappetence, non-coordination, dehydration, increased respiration, tachycardia, muscular weakness, salivation, continual panting, prostration and paralysis, groan, tremors, increased body temperature and heart rate, oral and nasal discharge and bruxism. Advanced clinical signs in sheep that later died included severe tachycardia and tachypnea, associated with hind limb ataxia. One sheep from group 2 (1 mg/kg), and one sheep from group 5 (4 mg/kg), died within 72 h after the administration of salinomycin. In the control group there were no abnormal clinical signs during the experiment.

Pathology. The two dead sheep were examined within 12 hours post mortem. Postmortem examination revealed congestion and edema of the skeletal muscles, swelling and hemorrhagic foci in peripheral lymph nodes, hydrothorax and hydropericardium, petechial hemorrhage in fat tissues of the heart base on the epicardium, severe pulmonary congestion and edema, and also the presence of moderate frothy fluid in the trachea and bronchi. In the heart muscle, myocardial degeneration and necrosis, without any inflammatory reaction or calcification, and epicardial hemorrhage within deposited fat cells were diagnosed.

Biochemical parameters. The various degrees of changes in several biochemical parameters in all these groups are demonstrated in Table 1.

AST. On day 2, there was a significant ($P < 0.01$) increase in the activity of AST in group V compared to all the other groups, except group II. The increase in AST activity on day 2 in group V was also significantly ($P < 0.01$) different from those in succeeding time intervals.

ALT. There were significant differences in the activity of ALT with different doses of salinomycin ($P < 0.01$). The serum ALT level in group V was significantly higher than all other groups, except group II. However, there were no significant ($P = 0.39$) differences with respect to different times ($P = 0.20$).

LDH. There were no significant ($P = 0.07$) differences in the activity of LDH with various doses of salinomycin and also with respect to different times ($P = 0.09$) in all groups.

Table 1. Serum biochemical parameters (mean \pm SEM; n = 4) after oral administration of different doses of salinomycin in sheep.

Groups	Days	AST (IU/L)	ALT (IU/L)	LDH (IU/L)	CK (IU/L)	cTnI (ng/mL)
Control	2	118 \pm 21 ^b	15 \pm 3	764 \pm 49	375 \pm 93 ^b	0.30 \pm 0.02
	5	86 \pm 9	14 \pm 1	404 \pm 91	236 \pm 31	0.39 \pm 0.06 ^b
	8	107 \pm 14	15 \pm 1	912 \pm 101	284 \pm 36	0.39 \pm 0.02
	14	218 \pm 22	30 \pm 9	986 \pm 30	275 \pm 19	0.39 \pm 0.02
	21	130 \pm 21	28 \pm 5	671 \pm 59	298 \pm 58	0.39 \pm 0.20
1 mg/kg	2	1232 \pm 690 ^{ab}	123 \pm 75	3690 \pm 2118	5492 \pm 3280 ^{aA}	0.51 \pm 0.05 ^B
	5	2818 \pm 1909	344 \pm 231	3506 \pm 1925	1169 \pm 657 ^B	1.93 \pm 0.81 ^A
	8	301 \pm 143	143 \pm 92	1863 \pm 801	261 \pm 60 ^B	0.76 \pm 0.05 ^B
	14	425 \pm 224	250 \pm 88	1907 \pm 740	328 \pm 64 ^B	0.52 \pm 0.06 ^B
	21	138 \pm 15	38 \pm 8	890 \pm 109	242 \pm 6 ^B	0.61 \pm 0.06 ^B
2 mg/kg	2	86 \pm 6 ^b	16 \pm 4	1313 \pm 329	320 \pm 28 ^b	0.64 \pm 0.08
	5	106 \pm 6	33 \pm 2	1017 \pm 152	293 \pm 40	0.86 \pm 0.03 ^b
	8	110 \pm 7	18 \pm 3	1053 \pm 54	229 \pm 32	0.71 \pm 0.06
	14	130 \pm 4	49 \pm 12	936 \pm 37	568 \pm 78	0.73 \pm 0.10
	21	123 \pm 8	16 \pm 2	880 \pm 67	366 \pm 13	0.60 \pm 0.08
3 mg/kg	2	73 \pm 10 ^b	11 \pm 0	980 \pm 84	306 \pm 20 ^b	0.71 \pm 0.02
	5	107 \pm 9	19 \pm 0	808 \pm 29	241 \pm 15	0.72 \pm 0.04 ^b
	8	118 \pm 14	16 \pm 1	682 \pm 154	270 \pm 16	0.78 \pm 0.05
	14	135 \pm 12	35 \pm 15	912 \pm 249	372 \pm 34	0.68 \pm 0.05
	21	146 \pm 22	13 \pm 2	813 \pm 50	263 \pm 34	0.63 \pm 0.05
4 mg/kg	2	4665 \pm 2566 ^{aA}	521 \pm 283	10656 \pm 7580	1560 \pm 1081 ^b	0.78 \pm 0.05
	5	685 \pm 583 ^B	749 \pm 554	1716 \pm 857	566 \pm 308	0.69 \pm 0.05 ^b
	8	320 \pm 207 ^B	316 \pm 226	1402 \pm 478	307 \pm 56	0.91 \pm 0.13
	14	150 \pm 30 ^B	20 ^a	1047 \pm 99	430 \pm 26	0.79 \pm 0.04
	21	150 \pm 32 ^B	27 \pm 7	1220 \pm 311	422 \pm 56	0.67 \pm 0.06

For each parameter, small superscripts show significant difference between various groups in each day of experiment, and large superscripts show significant difference between various days of experiment in each group ($P < 0.05$).

CK. The significant ($P = 0.03$) interaction between various groups and times for CK is demonstrated in Table 1. There were significant differences in the activity of CK in group II compared with other groups at day 2 of the experiment ($P < 0.05$). CK increase at day 2 in group II was also significantly different when compared with succeeding time intervals in the same group ($P \leq 0.01$).

cTnI. The significant ($P = 0.004$) interaction between various groups and times for cTnI is demonstrated in Table 1. On day 5, there was a significant ($P < 0.01$) increase in the

cTnI in group II compared with other groups. cTnI serum levels also showed a significant ($P \leq 0.001$) increase on day 5 compared with the succeeding time intervals in group II.

ECG records (arrhythmias). ECG records indicate that the heart rate in the control group (122 ± 18 beats/min) was significantly ($P < 0.05$) lower than in the experimental sheep (210 ± 36 beats/min). In the control animals, a normal heart rhythm was noticed in all cases except for sinus tachycardia (3 cases) and sinus arrhythmia (2 cases). Numerous arrhythmias were recorded in the experimental sheep, such as: sinus tachycardia (15 cases), supraventricular tachycardia (7 cases), sinus arrhythmia (3 cases) and supraventricular premature contraction (2 cases). Significant increase in cTnI activity was demonstrable in all animals showing various types of cardiac arrhythmias.

Discussion

Degenerative myopathy and myocardiopathy are the main injuries reported in the intoxicated animals with salinomycin (KHODAKARAM TAFTI et al., 2008). Ionophores form complexes with cations and mediate their transport across the cell membrane in response to diffusion gradient, and so mitochondrial failure and depletion of cellular adenosine triphosphate (ATP) may occur as ATP-dependent pumps attempt to compensate for the influx of ions. In addition, failure of calcium ion retrieval from the cytosol and ultimate myofibril hypercontraction, degeneration and necrosis may occur. Therefore, highly energetic tissues in the body, such as myocardium and skeletal muscles, are primarily affected. It should be mentioned that changes in transmembrane ion gradients and electrical potential often produce profound effects on cellular functions and metabolism (BLANCHARD et al., 1993). Toxicity is seen when ionophores are administered at the wrong dose and to the wrong species (McKELLAR and LAWRENCE, 1996). This is reflected in the emergence of numerous arrhythmias in the sheep intoxicated with salinomycin in the present study. This finding is consistent with the observations by AGAOGLU et al. (2002).

Blood tests for the diagnosis of cardiac injury in animals are inadequate. Although troponin complex proteins have been determined in animals, there is a limited number of studies concerning their clinical or paraclinical use. Studies in animals have mainly included laboratory animals and have been designed on the basis of myocardial injury detection in human beings (CHARLES et al., 2000; FREDERICKS et al., 2001). Clinical trials have indicated that serum cardiac troponins are the first biochemical markers to appear during the course of acute coronary disease in human beings (BOCCARA et al., 2000). Detection of cTnI and cTnT is correlated with loss of cardiac function in toxicity studies in rodents and rabbit (SERRA et al., 2010). Use of cTnI as a diagnostic tool in cattle has been mentioned in a few studies. Early and accurate identification of myocardial injury may be important for the diagnosis, risk stratification, and treatment of cattle with primary or secondary myocardial diseases (VARGA et al., 2009). Utilization of tests for the evaluation

of cardiac troponins may enable veterinarians to diagnose myocardial degeneration due to FMD in cattle herds (GUNES et al., 2005). Myocarditis in domestic animals may result in sudden death. The diagnosis of myocarditis in farm animals is important, because in such cases the animals may be slaughtered before sudden death. Therefore, cardiac markers, such as troponins, could be used to diagnose myocarditis in animals (GUNES et al., 2005). All cTnI concentrations in clinically healthy dairy cows are found to be ≤ 0.03 ng/mL, resembling those concentrations reported by others in cattle (JESTY et al., 2005; MELLANBY et al., 2007; PEEK et al., 2008). The half-life of troponin and its complex in the circulation is about 2 hours (KATUS et al., 1989). In humans with acute myocardial infarction (AMI), the cTn level begins to rise 4-12 hours after the infarction, and it reaches its peak value 12 to 48 hours later (BABUIN and JAFFE, 2005). The initiation of necrotic process in the myocardium due to salinomycin intoxication was not revealed in this study. Levels of cTnI in the blood increased dependent on salinomycin intoxication, and maximum levels were achieved 48 to 72 hours following the administration of the ionophore in all groups. In human cardiomyocytes, approximately 6-8% of the total cellular cardiac troponin is cytosolically dissolved and thus unbound in the cytoplasm (ADAMS et al., 1994). Soon after myocardial cell injury, by affecting cell membrane permeability, some of this free pool is liberated into the blood stream. However, the majority of cTnI is retained intracellularly because of its structural linkage to the contractile apparatus. Thus, the release of cTnI may occur either monophasically, with only a minor increase after reversible myocyte injury, or polyphasicly with more severe injury of the myocardium, affecting the structurally bound portion of cTnI (VARGA et al., 2009). A persistent increase in cTnI concentrations in the blood suggests irreversible and active cardiomyocyte damage (O'BRIEN et al., 2006; STANTON et al., 2005; WELLS and SLEEPER, 2008). In this study, cTnI concentrations in all intoxicated groups were higher than in the control group. Myocardial damage caused by salinomycin poisoning is irreversible. The peak release of cTnI into the blood was achieved on the fifth day after intoxication in group II (1.93 ± 0.81 ng/mL) and on day 8 in group V (0.91 ± 0.13 ng/mL). In addition, the major cardiac injury was seen in these two groups. Cardiac muscle necrosis found in post mortem examination in two cases of these groups revealed myocardial degeneration and, therefore, justifies our conclusion.

Remarkable differences in the serum activity of CK were found on day 2 between group II (1 mg/kg twice a day for two days), with other groups receiving salinomycin. CK increase on day 2 in group II was significant, when compared with the following days in the same group. Increased serum activity of CK is probably due to a characteristic myopathy and probable necrosis, or reversible myocyte damage (STOCKHAM and SCOTT, 2002). The higher sensitivity of cTnI for the detection of myocardial injury was noticed compared with that for CK as described by other investigators (OOI et al., 2000). Serum cTnI and CK appeared to increase soon after salinomycin intoxication (day 2) and an

association between cTnI concentration, cardiac arrhythmias, and myocardial necrosis was found in this study, which is in accordance with other studies (AGAOGU et al., 2002; KHODAKARAM TAFTI et al., 2008). There were remarkable differences between the activity of ALT with various doses of salinomycin, and in the dose group V (4 mg/kg) it was significantly higher than all other groups, except group II (1 mg/kg twice a day for two days). The highest levels of cTnI, CK, AST and LDH in the serum were observed on the second day of sampling in group II (1 mg/kg twice a day for two days).

Creatine kinase and or LDH may exhibit several of the characteristics of an ideal biomarker with respect to the identification of myocardial injury. In addition, methodologies for their measurement are reasonably standardized and readily available. However, these lack specificity for cardiac damage, and these enzymes may be elevated in various disease states associated with skeletal muscle, kidney, and/or central nervous system injury (CHRISTENSON and AZZAZY, 1998; FREDERICKS et al., 2001). The kinetics of these proteins following myocardial injury also limits their use as cardiac biomarkers. Furthermore, measurement of the serum levels of these proteins cannot be substantially correlated with the extent of myocardial injury or disease progression (BERTINCHANT et al., 2000). It is clear that CK, ALT, AST, and LDH have limitations as biomarkers of myocardial injury and, therefore, more specific and more sensitive cardiac biomarkers could improve safety assessment and monitoring in preclinical and clinical research (WALLACE et al., 2004).

In conclusion, the results of this study may indicate that cTnI is a valuable biomarker in the diagnosis of cardiac degeneration due to salinomycin toxicosis in sheep.

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HAJIMOHAMMADI, A., H. RAJAIAN, E. KHALIJI, S. NAZIFI, M. ANSARI-LARI: Serumski srčani troponin I kao biljeg srčane degeneracije uzrokovane pokusnim trovanjem ovaca salinomycinom. *Vet. arhiv* 84, 41-51, 2014.

SAŽETAK

Salinomycin je ionofor s antimikrobnim svojstvima. Rabi se kao dodatak hrani koji u preživača ima ulogu promotora rasta, a u pilića ulogu kokcidiostatika. Prevelike količine i pogrešna uporaba salinomicina mogu dovesti do teških sindroma trovanja. Srčani troponin I (cTnI) je dio troponinskog kompleksa (I, C i T), unutar sarkomere srčanih mišićnih stanica, koji regulira kontrakcije srčanog mišića. Budući da se oslobađa iz oštećenih miokardocita u krvotok, cTnI može biti specifičan biomarker kod nekroze srčanog mišića. Svrha istraživanja bila je predložiti da se cTnI primjeni u dijagnostici srčane degeneracije uzrokovane pokusnim trovanjem ovaca salinomycinom. U istraživanje je bilo uključeno 20 odraslih, masnorepih ovaca, križanki iranskih pasmina (TM: $33,3 \pm 3,4$ kg). Ovce su metodom slučajnog izbora bile podijeljene u pet skupina iste veličine. Skupina I (kontrola) dobila je 20 mL otopine soli. Skupine II, III, IV i V dobile su oralnim putem salinomycin i to u količini od 1 mg/kg (dva puta dnevno kroz dva dana), odnosno 2, 3 i 4 mg/kg (jednom dnevno kroz dva dana). Odmah nakon toga uzimani su uzorci krvi u različitim vremenskim razmacima (2, 5, 8, 14 i 21 dana) s ciljem određivanja različitih biokemijskih pokazatelja (cTnI, CK, LDH, ALT i AST). U svim skupinama pažljivo su osluškivani srčani tonovi i određivan elektrokardiogram s ciljem otkrivanja tipa moguće srčane aritmije. Rezultati pokazuju značajno povećanje aktivnosti cTnI. Zabilježene su i mnoge aritmije kao što su sinusna tahikardija, supraventrikularna tahikardija, sinusna aritmija i supraventrikularna preuranjena kontrakcija. Sve su životinje uz aritmiju očitovale i značajno povećanje aktivnosti cTnI. Razudbom nakon uginuća makroskopski je opažena nekroza i degeneracija srčanog mišića. Zaključno, rezultati istraživanja pokazuju da cTnI može biti vrijedan biomarker u dijagnostici srčane degeneracije prouzročene trovanjem salinomycinom.

Ključne riječi: trovanje salinomycinom, ovca, troponin I, biokemijski pokazatelji, aritmija
