Lehigh Valley Health Network

LVHN Scholarly Works

Toxicology Division

The effect of cyclosporine A on survival time in salicylate-poisoned rats.

Kenneth D. Katz MD
Lehigh Valley Health Network, kenneth_d.katz@lvhn.org

Steven C Curry

Daniel E Brooks

Richard D Gerkin

Follow this and additional works at: https://scholarlyworks.lvhn.org/toxicology

Part of the Medicine and Health Sciences Commons

Published In/Presented At

Katz KD, Curry SC, Brooks DE, Gerkin RD. The effect of cyclosporine A on survival time in salicylate-poisoned rats. J Emerg Med. 2004 Feb;26(2):151-5. doi: 10.1016/j.jemermed.2003.07.008.

This Article is brought to you for free and open access by LVHN Scholarly Works. It has been accepted for inclusion in LVHN Scholarly Works by an authorized administrator. For more information, please contact LibraryServices@lvhn.org.

The Journal of Emergency Medicine, Vol. 26, No. 2, pp. 151–155, 2004
Copyright © 2004 Elsevier Inc.
Printed in the USA. All rights reserved
0736-4679/04 $–see front matter

ELSEVIER

doi:10.1016/j.jemermed.2003.07.008

Original Contributions

THE EFFECT OF CYCLOSPORINE A ON SURVIVAL TIME IN SALICYLATE-POISONED RATS

Kenneth D. Katz, MD, Steven C. Curry, MD, Daniel E. Brooks, MD, and Richard D. Gerkin, MD

Department of Medical Toxicology, Good Samaritan Regional Medical Center, Phoenix, Arizona
Reprint Address: Kenneth Katz, MD, Department of Emergency Medicine, University of Pittsburgh, UPMC Presbyterian, 200 Lothrop St., Room D-L45, Pittsburgh, PA 15213

☐ **Abstract—Salicylate (SAL) produces mitochondrial membrane permeability transition (MPT) with resultant oxidative phosphorylation uncoupling. Cyclosporine A (CSA) inhibits SAL-induced MPT. This study determined if CSA pretreatment prolonged survival time in SAL-poisoned rats. Twenty-nine rats were randomized to receive pre-treatment with either 30 mg/kg CSA or equal volume of control diluent intraperitoneally (i.p.). Four hours later, all rats received 1700 mg/kg sodium salicylate i.p. Survival time, whole blood CSA ([CSA]), and serum sodium ([Na]), glucose and SAL ([SAL]) concentrations were determined. The results showed median survival time for controls was 18 min (95% CI 14–22 min) and for CSA animals was 14 min (95% CI 13–15 min). Univariate and multivariate analyses and Cox proportional hazard regression revealed CSA treatment was associated with higher [SAL], which was associated with shortened survival times. The CSA group also demonstrated shorter survival times for a given [SAL]. In conclusion, CSA pre-treatment shortened survival in SAL-poisoned rats. © 2004 Elsevier Inc.**

☐ **Keywords—salicylate; cyclosporine; mitochondrial permeability transition; oxidative phosphorylation**

INTRODUCTION

According to American Association of Poison Control Centers (AAPCC) data, salicylate (SAL) accounted for nearly 6000 poisonings in 2000, and analgesics were the primary substance in 30% of all reported fatalities, with acetaminophen, aspirin and other salicylates accounting for 72% of analgesic fatalities' (1). Much of salicylate's toxicity stems from its ability to halt mitochondrial synthesis of adenosine triphosphate (ATP) through uncoupling of oxidative phosphorylation (2,3). In vitro, one mechanism by which SAL uncouples oxidative phosphorylation is induction of the mitochondrial permeability transition (MPT). The MPT is a state in which a pore forms at contact sites between the mitochondrial outer and inner membranes from a complex of the adenine nucleotide translocator (ANT) and cyclophilin-D (CyP-D) (3). Evidence also exists for the incorporation of the voltage-dependent anion channel into this complex. When the MPT is present, the mitochondrial inner membrane becomes permeable to low-molecular weight solutes, such as protons, which disrupt the proton-motive force necessary for ATP formation (4). Thus, uncoupling of oxidative phosphorylation occurs, leading to cell apoptosis and death. Clinically, impaired ATP formation is manifested by metabolic acidosis, seizures, coma, shock and death (2).

Cyclosporine A (CSA) is an immunosuppressant drug used to prevent organ rejection in transplant patients and to treat various other medical conditions. Addition of CSA to SAL-treated cell preparations results in prevention of MPT formation via binding to CyP-D (5). In vitro studies have demonstrated prevention of SAL-induced

Received: 6 December 2002; Final submission received: 12 June 2003;
Accepted: 8 July 2003

MPT formation at CSA concentrations of 1000 nmol/L (1201 μg/L). Transplant patients receiving therapeutic doses of CSA achieve peak whole blood levels up to 5824 nmol/L (7000 μg/L) and maintain whole blood concentrations above 832 nmol/L (1000 μg/L) for at least 4 h (6,7). Thus, doses of CSA used clinically achieve concentrations that might prevent SAL-induced MPT formation.

No studies to date have examined the ability of CSA to prevent SAL toxicity in vivo. The aim of this study is to elucidate whether pre-treatment with CSA will prolong survival in rats poisoned with lethal doses of SAL.

METHODS

Preliminary Study

Preliminary experimentation was performed to document lack of mortality for 8 h after injection of CSA and to confirm that adequate blood CSA concentrations were achieved 4 h after i.p. administration in our model. Ten Sprague Dawley male rats weighing between 364 and 438 g received 30 mg/kg (0.6 cc/kg) of CSA i.p. and were then observed in their cages with free access to water. Based on previously reported pharmacokinetic studies, this dose was expected to produce [CSA] in excess of 3328 nmol/L (4000 μg/L) 4 h after injection, which is at the end of the distribution phase for CSA after i.p. injection in rats (8). Five rats were euthanized with pentobarbital after 4 h, and heart blood was immediately obtained for measurement of whole blood CSA concentration ([CSA]). The remaining five animals continued to be observed and were euthanized after 8 h.

EXPERIMENT

Twenty-nine Sprague Dawley male rats weighing between 356 and 443 g were randomly assigned to one of two groups: a CSA group or a control group. A blinded investigator drew a number from an envelope corresponding to 29 pre-numbered syringes containing either CSA at a concentration of 50 mg/mL, or CSA diluent (650 mg Cremephor EL in each mL of 32.9% ethanol). CSA animals received 30 mg/kg (0.6 cc/kg) i.p. injections of CSA; control animals received 0.6 cc/kg of the diluent i.p.

Three hours and thirty minutes after injection of either CSA or control solution, all animals were given 50 cc/kg of 5% dextrose in water subcutaneously in their flank to ensure adequate hydration and to prevent excessive rise in serum sodium concentration from the subsequent administration of sodium salicylate. The dextrose solution was heated to 37°C before injection.

Exactly 4 h after i.p. injection of CSA or control solution, all animals received an i.p. injection of 1700 mg/kg sodium salicylate (17 cc/kg). The sodium salicylate solution (100 mg sodium salicylate/mL sterile water) had been adjusted to pH 7.0 with either sodium hydroxide or hydrochloric acid and was heated to 37°C before injection. Our previous experience with this dose of sodium salicylate indicated that death in all animals would be expected within 40 min of injection.

Death was determined by absence of spontaneous motion, respiratory effort and palpable cardiac activity. Time until death was rounded to the nearest minute.

Immediately after death, cardiac blood was obtained for measurement of concentrations of serum glucose ([glucose]), salicylate ([SAL]), sodium ([Na]), and whole blood cyclosporine ([CSA]). Whole blood specimens for CSA analysis were immediately frozen. Other blood samples were immediately centrifuged and separated serum was refrigerated at 3°C before analyses. All samples were analyzed within 48 h. Serum [SAL] was measured colorimetrically using the Roche Diagnostic Salicylate assay (Roche Diagnostics, Indianapolis, IN). Whole blood CSA concentrations were measured with the EMIT 2000 Cyclosporine Specific Immunoassay (Hoeschst Marion Roussel, Frankfurt, Germany), which specifically measures the CSA parent compound. We found the salicylate assay to be unaffected by the presence of CSA.

The Institutional Animal Care and Use Committee at Arizona State University approved the study protocol. The care and handling of animals were in accordance with NIH guidelines for ethical animal research.

Statistical Analyses

Mean values for weight, [CSA], [SAL], [Na], and [glucose] were compared between groups using independent *t*-tests.

A Kaplan-Meier analysis was used to examine survival data. Difference in group survival times was compared using the log rank test. Univariate Cox proportional hazard analyses of the effect of group assignment, [SAL], [glucose], and [Na] on survival time was performed. Then, forward step-wise regression (inclusion $p = 0.05$; exclusion $p = 0.1$) was performed using variables found significant in the univariate analyses. Because SAL concentrations were unbalanced between groups, we incorporated the [SAL]$_*$group interaction term in this regression. Backwards step-wise regression was then performed to determine whether the same results would be obtained.

We also examined the influence of group assignment,

Table 1. Comparison of Parameters Between Groups

Group	[Na] mmol/L	[SAL] mmol/L (mg/dL)	[glucose] mmol/L (mg/dL)	[CSA] nmol/L (μg/L)	Weight kg
control (n = 14)	148 ± 4.9	19.6 ± 6.5	18 ± 5	0	.387 ± .15
		(273 ± 90)	(324 ± 90)	(0)	
	[139–156]	[162–424]	[195–573]		[0.356–0.405]
CSA (n = 15)	149 ± 5.2	25 ± 7.1*	19.7 ± 5.2	4658 ± 1736	.397 ± .19
		(347 ± 99)*	(355 ± 78)	(5599 ± 2087)	
	[143–163]	[257–635]	[219–510]	[980–9560]	[0.365–0.443]

Values represent mean ± SD. [Na] = serum sodium concentration; [SAL] = serum salicylate concentration; [glucose] = serum glucose concentration; [CSA] = whole blood cyclosporine A concentration. All aforementioned parameters were measured in heart blood immediately after death.
*$p = 0.03$. [] in third line of each variable indicates data ranges.

corrected for [SAL], on survival time using the Cox model.

With 14 animals in each group, and assuming a standard deviation in survival time reported previously for rats receiving the dose of i.p. salicylate used in this study, we calculated a power of > 0.8 to detect a doubling of survival time, a result we would consider statistically significant.

All statistical testing was performed using SPSS version 10.1 software (SPSS Inc., Chicago, IL). A two-tailed $p < 0.05$ was chosen to represent statistical significance. No corrections were made for multiple comparisons.

RESULTS

Preliminary Study

All 10 animals behaved normally after CSA injection, and all survived until time of euthanasia at either 4 or 8 h. [CSA] 4 h after injection ranged from 878.5 to 15, 208.9 nmol/L (1,056 to 18,280 μg/L).

Experiment

As described previously, death was immediately preceded by a brief generalized seizure, which usually lasted less than 30 s (9). Group means for weight, [SAL], [Na], [glucose], and [CSA] are shown in Table 1. Mean concentrations for SAL were significantly higher in animals receiving CSA as compared to controls ($p = 0.03$).

CSA animals survived a median time of 14 min (95% CI 13–15 min) with a range of 10 to 18 min. Control animals survived a median time of 18 min (95% CI 14–22 min) with a range of 13 to 83 min. The log rank test revealed a statistically significant shortened survival time for the CSA group ($p = 0.0008$; Figure 1).

Results of univariate analyses are shown in Table 2. Forward regression using the Cox proportional hazard analysis revealed a statistically significant association between survival time and both the [SAL]*group interaction (hazard ratio 1.005; $p < 0.001$) and [Na] (hazard ratio 1.18; $p = 0.004$). Whereas [SAL] alone was related to survival times (higher levels associated with shorter times, Table 2), this term dropped out of the regression when the [SAL]*group interaction term was added. Backwards stepwise regression produced identical results.

A Cox proportional hazards model revealed a statistically significant association between group assignments corrected for [SAL] (hazard ratio 3.19; $p < 0.001$).

Although [CSA]s demonstrated a wide range among experimental animals, they did not demonstrate a statistically significant association with outcome.

DISCUSSION

SAL impairs production of ATP by several mechanisms, including inhibition of dehydrogenases in the tricarbox-

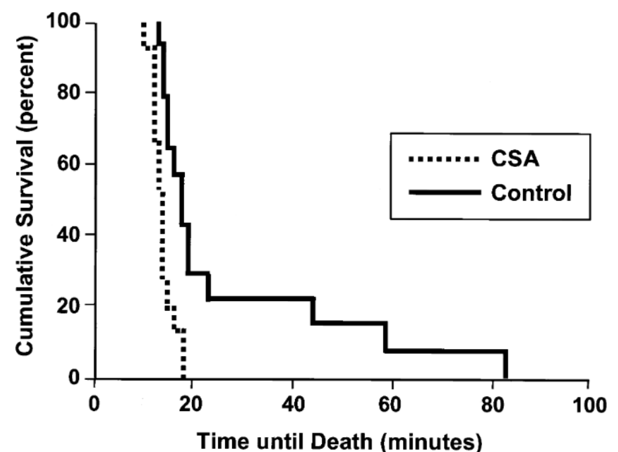

Figure 1. Comparison of cumulative survival percent between the CSA and control groups. CSA = cyclosporine A.

Table 2. Univariate Associations Using Cox Proportional Hazard Analysis

	p	Hazard Ratio
Group	0.003	.272
[Na]	<0.001	1.207
[SAL]	<0.001	1.013
[glucose]	.345	.998
[SAL]×group	<0.001	1.005

[Na] = serum sodium concentration; [SAL] = serum salicylate concentration; [glucose] = serum glucose concentration. [Na], [SAL], and [glucose] parameters were measured on heart blood drawn immediately after death. death.

ylic acid cycle, impairment of gluconeogenesis, and, importantly, uncoupling of oxidative phosphorylation (2). Like most uncoupling agents, SAL diminishes the proton gradient across the mitochondrial inner membrane, which increases oxygen consumption while impairing ATP formation. In vitro studies reveal that one mechanism by which SAL uncouples is induction of the MPT, a state in which the inner membrane becomes permeable to protons and other molecules less than about 1500 Da. This increased permeability may be due to a functional change in ANT and CyP-D, a matrix protein thought normally to bind to ANT.

In vitro studies have also shown that CSA binds to CyP-D to prevent SAL-induced MPT. Hepatocytes incubated in CSA at concentrations of 1000 nmol/L (1201 ug/L) are protected from MPT formation by SAL. These studies suggest that SAL toxicity in vivo might be attenuated by CSA, but no in vivo animal studies have been performed to date. Therefore, we performed this study to examine the ability of CSA to prolong survival from SAL in rats. If animal studies indicated that CSA pretreatment could delay or prevent death from SAL, this would not only support studies using CSA to treat SAL-poisoned animals, but human clinical trials as well, in which CSA therapy might be used to prevent death in critically ill patients until emergency hemodialysis could be commenced.

Rats have been frequently used as an animal model for studying SAL toxicity (9–11). Toxic concentrations of SAL in rats are similar to those found in human beings. As in human beings, rats develop severe central nervous system toxicity characterized by seizures and coma shortly before death (9). Treatment strategies (e.g., sodium bicarbonate, glucose) that prevent death in human beings have been shown to prevent death in rats (10).

Cyclosporine A is well absorbed after i.p. injection in rats, with peak blood levels achieved within 2 h, complete distribution within 4 h, and an elimination half-life over 24 h (12). Peak CSA concentrations in human beings receiving immunosupression for transplantation may reach 5824 nmol/L (7000 μg/L). Furthermore, limited data indicate that human brain CSA concentrations seem to be about one-half of those in serum, suggesting that interstitial or intracellular CSA brain concentrations could exceed those that protect against in vitro MPT formation with therapeutic doses (13). We chose a CSA dose that would produce post-distribution [CSA] in blood of approximately 3328 nmol/L (4000 μg/L) in rats. These doses are well below the i.p. LD_{50} in rats of 147 mg/kg and, as expected, our preliminary experiment revealed no mortality from injection of 30 mg/kg CSA alone (12).

Sodium salicylate is also well absorbed after i.p. injection (10). Hill reported that 1380 to 1500 mg/kg sodium salicylate i.p. in rats resulted in death within 34 min (9). These data are in keeping with slightly shorter survival times in the majority of our rats when 1700 mg/kg sodium salicylate was injected. The fact that a control animal survived for 83 min is difficult to explain, but may represent a chance occurrence.

We achieved peak [CSA]s in a rat model that are similar to those measured in human beings receiving CSA for immunosupression. However, we were unable to demonstrate CSA's efficacy in prolonging survival time in rats poisoned with SAL.

CSA animals died sooner and exhibited higher [SAL] at the time of death. Cox proportional hazard regression indicated that the [SAL]*group interaction term was related to survival time. This indicates that there were two effects of CSA. The first effect was that CSA treatment was associated with higher concentrations of SAL, which, in turn, were associated with shortened survival times. The second effect was that CSA animals exhibited shorter survival times for a given [SAL] (hazard ratio = 3.19), suggesting a direct adverse effect of CSA on survival.

With regard to higher [SAL] in CSA animals, perhaps CSA caused more rapid absorption of SAL across the peritoneum or limited the distribution of SAL to non-critical organs to change SAL's apparent volume of distribution. With regard to shortened survival corrected for [SAL] in CSA animals, one could hypothesize that CSA enhanced SAL distribution into the brain or heart, or produced a toxic effect on these organs apart from SAL.

Sodium concentration was also independently related to survival time. This may not be surprising because we administered sodium salicylate, and one would expect more rapid absorption of SAL to be accompanied by more rapid absorption of sodium to maintain electrical neutrality. Variations in mean values for [Na] between groups were much less than for [SAL]. The relation of

[Na] to survival times, given the narrow range of [Na] values, may simply be serendipitous.

Several limitations of this study are apparent. Data from animal studies cannot always be extrapolated to human beings. We do not know that central nervous system CSA concentrations reached a value high enough to prevent MPT in the brain. In fact, rat studies have reported that brain [CSA]s are about 16% of those in blood (14). There was large variability in [CSA]s seen after i.p. CSA injections, both in preliminary study and in the controlled experiment. Except for the possibility of large variations in CSA's rate of absorption and volume of distribution, we cannot offer an explanation for this finding. Finally, the SAL dose we used may have been so high that no amount of CSA could have prevented death, leaving open the possibility that CSA might be effective in prolonging survival at lower, lethal doses of SAL. Importantly, in vitro studies demonstrate that MPT induction accounts for only part of uncoupling produced by SAL (15). SAL is a weak, lipophilic acid that can disrupt the proton gradient across the inner mitochondrial membrane through diffusion into the matrix, possibly explaining death from salicylate, even if MPT induction was prevented by CSA.

CONCLUSION

Pre-treatment with CSA in SAL-poisoned rats did not prolong survival, and was, in fact, associated with shortened survival and higher [SAL]. Future studies examining CSA's influence on SAL mortality should use i.v. rather than i.p. SAL to limit possible differences in SAL absorption and should use various [SAL]s to examine CSA's effect at lower SAL doses. In addition, administration of lower doses of SAL by gavage could be considered to more closely simulate human SAL exposure.

Acknowledgments—This study was funded by a grant from the Palms Clinic and Hospital Corporation Foundation, Phoenix, AZ.

REFERENCES

1. Litovitz TL, Klein-Schwartz W, White S, et al. 2000 Annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2001;19:337–95.
2. Curry SC. Salicylates. In: Reisdorff EJ, Roberts MR, Wiegenstein JG, eds. Pediatric emergency medicine. Philadelphia: W.B. Saunders; 1993:667–73.
3. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J 1999;341:233–49.
4. Gutknecht J. Salicylates and proton transport through lipid bilayer membranes: a model for salicylate-induced uncoupling and swelling in mitochondria. J Membr Biol 1990;115:253–60.
5. Testa G, Klintmalm GB. Cyclosporine and tacrolimus. Clin Liver Dis 1997;1:417–37.
6. Trost LC, Lemasters JJ. Reye's syndrome and related chemical toxicity. In: Lemasters JJ, Nieminen AL, eds. Mitochondria in pathogenesis. New York: Academic/Plenum Publishers; 2001:425–50.
7. Fahr A. Cyclosporine clinical pharmacokinetics. Clin Pharmacokinet 1993;24:472–95.
8. Wassef R, Cohen Z, Langer B. Pharmacokinetic profiles of cyclosporine in rats. Transplantation 1985;40:489–93.
9. Hill JB. Salicylate intoxication. N Engl J Med 1973;288:1110–3.
10. Hill JB. Experimental salicylate poisoning: observations on the effects of altering blood pH on tissue and plasma salicylate concentrations. Pediatrics 1971;47:658–65.
11. Drake JJ, Guant IF, Butterworth KR, et al. Short-term toxicity of isoamyl salicylate in rats. Food Cosmet Toxicol 1975;13:185–93.
12. National Toxicology Program Chemical Health & Safety Data. Cyclosporin A. Available at: http://ntp-server.niehs.nih.gov/Main_Pages/Chem-HS.html. Accessed May 25, 2002.
13. Ried M, Gibbons S, Kwok D, Van Buren CT, Flechner S, Kahan BD. Cyclosporine levels in human tissues of patients treated for one week to one year. Transplant Proc 1983;15:2434–7.
14. Bernareggi A, Rowland M. Physiologic modeling of cyclosporine kinetics in rat and man. J Pharmacokinet Biopharm 1991;19:21–50.
15. Trost LC, Lemasters JJ. Role of the mitochondrial permeability transition in salicylate toxicity to cultured rat hepatocytes: implications for the pathogenesis of Reye's syndrome. Toxicol Appl Pharmacol 1997;147:431–41.