

## Delayed therapy of experimental global cerebral ischemia with acetyl-L-carnitine in dogs

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### Abstract

Acetyl-L-carnitine (ALCAR), when administered immediately following restoration of spontaneous circulation (ROSC) from experimental cardiac arrest (CA) has previously been demonstrated to promote normalization of brain energy metabolism and neurologic recovery following 10 min CA. In order to determine ultimate efficacy for this or other drugs, clinical trials must be performed in human subjects. In several human clinical trials, though, drug administration has been significantly delayed following resuscitation from CA. These experiments test the hypothesis that post-resuscitative delay in ALCAR administration will impair the ability of this drug to promote neurologic recovery. Neurological deficit scoring (23 h) as well as frontal cortex lactate levels (2 and 24 h) were compared following resuscitation from 10 min CA in dogs receiving either ALCAR or drug vehicle 30 min following ROSC. Dogs treated with ALCAR 30 min following ROSC from 10 min CA exhibited more normal cerebral cortex lactate levels than did vehicle control animals. There was no difference, however, in neurologic deficit scores between groups, with all animals demonstrating moderate to severe clinical neurologic impairment at 23 h following ROSC. A 30-min delay in ALCAR administration following ROSC from 10 min CA impairs the ability of this drug to promote neurologic recovery despite apparent normalization of brain lactate levels.

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Acetyl-L-carnitine (ALCAR) is a naturally occurring substance, which is widely distributed in human tissue, and shows great promise as a neuroprotective agent. When administered exogenously, ALCAR is readily transported across the blood–brain barrier, in part as a result of an organic cation/carnitine transporter, OCTN2 [18]. In primary neuronal cultures, incubation with ALCAR has been shown to prevent necrotic cell death induced through excitotoxicity [12], as well as apoptotic neuronal injury induced through serum deprivation [12,19]. In animals, ALCAR improves clinical neurologic outcome, and decreases weight loss following permanent focal cerebral ischemia in rats [22]. ALCAR appears to be similarly effective as a neuroprotectant

following global cerebral ischemia. Pre-treatment with ALCAR has been found to prevent neuronal death in the selectively vulnerable regions of cerebral cortex, hippocampus and striatum following forebrain ischemia in gerbils [29]. Additionally, when administered immediately following resuscitation from 10 min of experimental cardiac arrest (CA) in dogs, ALCAR has been shown to result in rapid normalization of brain energy metabolism in the absence of any adverse hemodynamic side effects [26]. In this same study, animals treated with ALCAR demonstrated significantly less neurologic impairment than vehicle-treated controls.

Previous human trials of post-resuscitative pharmacologic neuroprotection, based on promising pre-clinical results [4,24,31], have to date not demonstrated clinical efficacy, perhaps as a result of the prolonged time which elapsed following restoration of spontaneous circulation (ROSC) be-

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fore the experimental drug could be safely provided [7,8,23]. Despite the fact that ALCAR administration appears safe in both human shock [14] as well as immediately following CA/ROSC in dogs [26], delays in drug administration may be anticipated in the highly charged setting of the ambulance or resuscitation bay. It is thus important to better define the “window of opportunity” for ALCAR induced neuroprotection prior to embarking on human clinical trials. In light of the severe perturbations in brain energy metabolism which begin during cardiac arrest and persist long after resuscitation, and in light of negative human clinical trials where drug administration was significantly delayed, this study was designed to test the hypothesis that post-resuscitative delay in ALCAR administration will impair the ability of this drug to promote neurologic recovery following experimental CA/ROSC.

All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and approved by the Institutional Animal Care and Use Committee of the George Washington University Medical Center. The details of the animal model of cardiac arrest and resuscitation have previously been reported [26]. Anesthesia was induced initially with 17.6 mg/kg thiamylal sodium (Biotal) IV; prolonged anesthesia was then maintained with alpha chloralose (75 mg/kg). Following anesthesia, dogs were orotracheally intubated and ventilated on room air. Baseline ventilator settings were adjusted to maintain  $p\text{CO}_2$  between 25 and 35 Torr. Cut down catheters were placed into the right femoral artery and vein, with the femoral venous catheter advanced to the level of the right atrium under pressure tracing guidance. A left lateral thoracotomy was then performed through the fourth intercostal space. The pericardium was incised and reflected. Arterial blood pressure, pulse, EKG, and rectal temperature were monitored continuously. Heating blanket and lights were used to maintain rectal temperature  $>37^\circ\text{C}$ .

After preparation, dogs were separated into one of five experimental groups:

- 1) Non-ischemic controls ( $n = 10$ )  
or those undergoing 10 min of CA followed by ROSC for 2 h;
- 2) With ALCAR therapy ( $n = 5$ ); or
- 3) Without ALCAR ( $n = 11$ );  
or those undergoing 10 min of CA followed by ROSC for 24 h;
- 4) With ALCAR therapy ( $n = 7$ ); or
- 5) Without ALCAR ( $n = 7$ ).

ALCAR (Sigma Tau S.p.A., Rome) was administered at a dose of 100 mg/kg 30 min following ROSC from 10 min CA, then at a IV bolus dose of 50 mg/kg q6h in those dogs undergoing 24 h ROSC. Animals not receiving ALCAR were treated with identical volumes of drug vehicle (normal saline pH adjusted to 6.7) alone. Drug administration was randomly assigned in the 24 h treatment groups, with the animal treat-

ment team blinded during the entire 24 h period to the identity of the solution being infused.

Following surgical preparation, ventricular fibrillation was induced through application of a train of electrical current directly to the surface of the right ventricle. Ventricular fibrillation was allowed to persist for 10 min. At the end of this period, open chest CPR was begun at a rate of 50 compressions/min for 3 min. At the beginning of CPR, epinephrine (0.2 ml/kg of a 1/10,000 solution) and sodium bicarbonate (1 meq/kg) were infused rapidly through the central venous catheter. At the end of 3 min of CPR, ROSC was attained through internal defibrillation at 5 J. Following ROSC, arterial blood gas samples were measured frequently per protocol; ventilator settings were adjusted to maintain  $80 < p\text{O}_2 < 120$  mmHg and  $25 < p\text{CO}_2 < 35$  mmHg.

In animals receiving 24 h of intensive care, the chest was closed in four layers, a chest tube inserted, the chest evacuated, and the chest tube connected to a Heimlich chest valve. Post-resuscitation, analgesia/anesthesia was maintained with a constant infusion of morphine sulphate (0.1 mg/kg/h); additional boluses of morphine were provided as needed. Core temperature was maintained between 37 and 38.5 °C throughout the post-resuscitative period with the use of heating blankets, heating lights or external cooling as needed. Dogs were weaned from controlled ventilation between hours 20 and 23. At 23 h following ROSC, animals were awakened with an intravenous injection of naloxone (0.6 mg/kg). Animals were tested at that point for neurologic deficit (as previously described) by two observers blinded to treatment protocol and trained to criteria of the testing procedure [26]. The neurologic deficit scoring system was based on a well-accepted model previously described by Bircher and Safar [3]. In this test a result of 0% indicated normal neurologic function with a result of 100% indicating brain death. Recent results from our group demonstrate a strong correlation between NDS at 23 h and quantitative stereologic histopathology. There was close correlation between percent dying neurons in each animal with that animal's clinical NDS. Linear regression of this correlation was significant ( $R^2 = 0.71$ ,  $p = 0.005$ ) [25]. At the completion of the NDS examination, animals were once again deeply anesthetized with 17.6 mg/kg thiamylal sodium and 40 mg/kg alpha chloralose and subjected to controlled ventilation as previously described.

Animals were excluded from consideration for any of the following reasons:  $p\text{O}_2 < 60$  mmHg on room air ventilation before cardiac arrest, temperature  $<36^\circ\text{C}$  before cardiac arrest despite heating blanket, systolic arterial pressure  $<60$  mmHg for  $>1$  min at any time, systolic arterial pressure  $<80$  mmHg for 10 min at any time, or death from non-neurological cause before completion of the experiment. A total of three animals were excluded for one or more of these reasons.

At the end of the appropriate experimental period, the animals were placed in the prone position, and a 5 cm  $\times$  8 cm wedge of skull surgically removed following reflection of scalp musculature. A 2 cm  $\times$  1 cm by 1 cm thick wedge

of right frontal cortex was then removed and immediately immersed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Samples (0.1 gm) of frozen tissue were accurately weighed and homogenized in 3% perchloric acid. Homogenates were briefly centrifuged and the supernatant used for lactate analysis with a YSI Model 2300 Stat glucose and lactate analyzer.

All data are reported as mean  $\pm$  S.E.M. Physiological data as well as lactate levels were compared among animal groups using ANOVA followed by Neuman–Keuls post hoc testing for planned comparisons where appropriate. The neurological deficit scores of drug treated dogs were compared with values of vehicle-treated dogs through the use of the Mann–Whitney  $U$  two-tailed  $t$ -test. Values of  $p < 0.05$  were considered significant for all tests.

Physiological variables for the various dog groups were compared immediately before the onset of cardiac arrest. The results are displayed in Table 1. Rectal temperature was minimally lower in the non-ischemic control animals when compared to animals undergoing 2 h of ROSC with ALCAR as well as when compared to both groups of animals undergoing 24 h ROSC ( $p < 0.05$ ). Temperatures in all animals, however, were well within the physiologic ranges previously determined as acceptable for inclusion. It is important to note that no differences among groups were found in any other physiologic parameter, nor were any differences in temperature noted between different treatment groups at the same time period.

The results of lactate measurements performed on acid extracts of fast frozen samples of frontal cortex are described in Fig. 1. It has previously been demonstrated that cardiac arrest results in severe cerebral lactic acidosis with a six-fold increase in cortical lactate concentrations seen following 10 min of cardiac arrest in dogs [26]. Results from this study further demonstrate that in untreated dogs brain lactate levels remain elevated at both 2 h ( $p < 0.001$ ) and 24 h ( $p < 0.001$ ) following resuscitation from experimental cardiac arrest. In contrast, those animals receiving ALCAR 30 min following ROSC demonstrated cortical lactate levels statistically similar to control values at both 2 and 24 h following ROSC. At each of these time points, cortical lactate levels in ALCAR treated dogs were significantly lower than vehicle-treated controls ( $p < 0.001$ ).

The neurologic deficit score exam (NDS) was performed in all dogs undergoing 10 min of CA followed by 24 h of in-

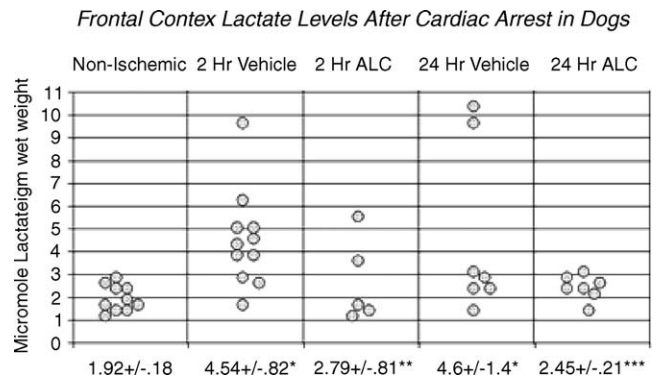


Fig. 1. ALC (acetyl-L-carnitine) or drug vehicle were administered 30 min following resuscitation from 10 min cardiac arrest. Headings denote time of lactate measurement following resuscitation. Non-ischemic group received drug vehicle 90 min before lactate measurement, but did not undergo cardiac arrest. \*  $p < .001$  compared to non-ischemic control, \*\*  $p < .001$  compared to 2 h vehicle ROSC, and \*\*\*  $p < .001$  compared to 24 h vehicle ROSC.

tensive care (Fig. 2). The mean NDS for seven vehicle-treated animals was  $60.3 \pm 6.2$ . All vehicle-treated dogs demonstrated moderate to severe neurologic impairment. All dogs breathed spontaneously, but had marked impairments in their levels of consciousness; most appeared totally unaware of their surroundings. No dog could stand or walk unassisted. Several dogs demonstrated severe purposeless running motions on examination. No overt seizure activity was noted. Similarly, the average NDS for seven dogs receiving delayed administration of ALCAR was  $57 \pm 5.2$ . The range of NDS seen in this treatment group was virtually identical to the range seen in vehicle-treated animals. All dogs in this group demonstrated moderate to severe neurologic impairment, with a spectrum of behavior identical to that described above for vehicle-treated control animals.

Acetyl-L-carnitine, when administered immediately following resuscitation from experimental cardiac arrest, has previously been shown in our laboratory to afford significant neuroprotection [26]. One factor, which contributes to neurologic impairment following CA is the rapid failure of oxidative metabolism of glucose. With the cessation of blood flow to the brain, cerebral glucose is rapidly depleted through anaerobic metabolism; severe cerebral lactic acidosis rapidly develops [11]. Once blood flow to the brain is restored following CPR, however, cerebral aerobic metabolism may remain deranged for prolonged periods of time despite reinstatement

Table 1  
Physiologic variables measured prior to cardiac arrest in dogs

Experimental group	SAP	DAP	Pulse	T	pH	$p\text{CO}_2$	$p\text{O}_2$
Control	$155 \pm 6$	$117 \pm 5$	$140 \pm 6$	$37.1 \pm .2$	$7.37 \pm .04$	$30 \pm 2$	$91 \pm 4$
2 h ROSC	$145 \pm 5$	$112 \pm 4$	$124 \pm 11$	$37.1 \pm .2$	$7.42 \pm .02$	$30 \pm 2$	$77 \pm 5$
2 h ROSC/ALCAR delay	$160 \pm 6$	$115 \pm 5$	$151 \pm 12$	$37.8 \pm .1^*$	$7.46 \pm .02$	$28 \pm 2$	$86 \pm 4$
24 h ROSC	$167 \pm 6$	$122 \pm 5$	$113 \pm 8$	$37.8 \pm .2^*$	$7.39 \pm .07$	$30 \pm 2$	$87 \pm 3$
24 h ROSC/ALCAR delay	$159 \pm 5$	$120 \pm 4$	$143 \pm 9$	$37.7 \pm .1^*$	$7.45 \pm .02$	$29 \pm 1$	$83 \pm 3$

Values are mean  $\pm$  S.E. SAP, systolic arterial pressure (mmHg); DAP, diastolic arterial pressure (mmHg); ROSC, restoration of spontaneous circulation; ALCAR delay, acetyl-L-carnitine administered 30 min following ROSC.

\*  $p < 0.05$  compared with control group.

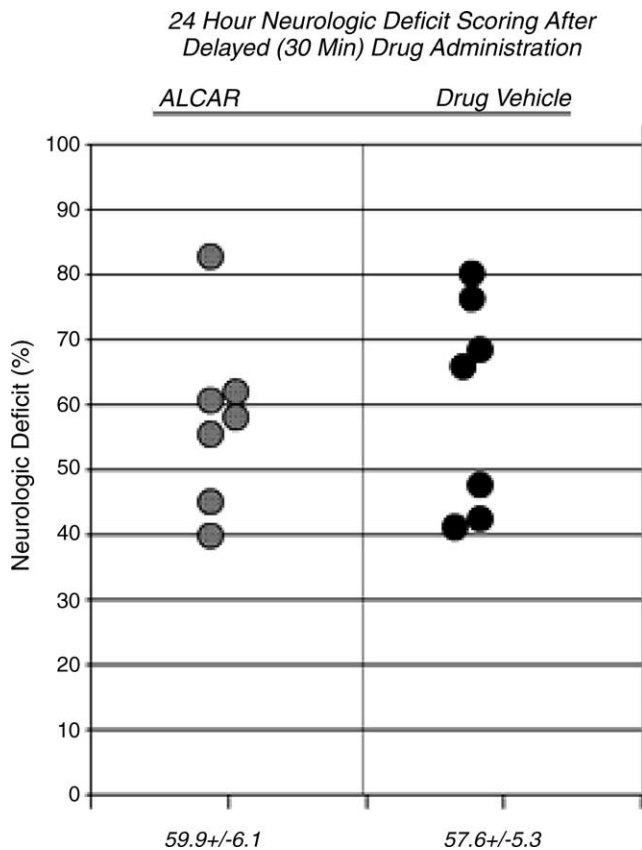


Fig. 2. Plot showing comparison of individual neurological deficit scores (NDS) 23 h following resuscitation from 10 min cardiac arrest in dogs. Values shown at bottom are mean  $\pm$  S.E. ALCAR or vehicle administered 30 min following resuscitation.

of normal cardiac function, thus aggravating post-ischemic neuronal injury through various mechanisms related to loss of ionic integrity [5,6]. This prolonged derangement in energy metabolism may be due in great part to the fact that reperfusion causes a significant decrease in the maximal activity [5,13,33–35], as well as immunoreactivity [6] of brain pyruvate dehydrogenase complex (PDHC) which catalyzes the critical oxidation/reduction reaction that links glycolysis to the tricarboxylic acid cycle. In vitro studies have suggested that inhibition of PDHC could be due to either oxidation of critical sulfhydryl groups, [30] or metal-catalyzed site-specific oxidation of any one of a number of susceptible amino acids [5].

The mechanisms by which ALCAR promotes post-ischemic neuroprotection remain speculative. Available evidence suggests that ALCAR acts to rapidly correct perturbations in energy metabolism seen following cerebral ischemia/reperfusion. Following CA/ROSC in dogs, ALCAR administration significantly lowered cortical lactic acidosis when compared to vehicle-treated controls [26]. With  $^{31}\text{P}$  and  $^1\text{H}$  NMR, Aureli demonstrated faster recovery and improvement of brain energy production following four vessel occlusion in rats [1]. Additionally, PET studies of  $2\text{-}^{11}\text{C}$  ALCAR administration suggested that endogenous serum AL-

CAR has some role in conveying an acetyl moiety into the brain, especially under an energy crisis [20].

In order to more rapidly normalize brain energy metabolism, and thus provide optimum neuroprotection, it would seem that ALCAR should be administered as soon as possible following ROSC, without otherwise compromising clinical care. In clinical practice, however, it may be difficult to administer some neuroprotective drugs safely in the immediate post-resuscitative period. Several clinical trials of post-resuscitative drug administration have been completed to date. In two of these studies, drug administration was significantly delayed in a large number of patients, because the drugs to be studied could not be administered until functional hemodynamic stability was restored [7,8]. In another study, although nimodipine was administered immediately following ROSC, patients treated with nimodipine demonstrated significantly lower blood pressures at several time points following ROSC, thus potentially compromising the ability of the study drug to exert positive effects on neurologic outcome [23]. In contrast, pre-clinical studies demonstrate that acetyl-L-carnitine can be administered safely immediately following resuscitation, making this an attractive post-resuscitative neuroprotective agent [26].

It must be realized, however, that post-resuscitative drug delivery might easily be delayed unless careful attention is paid to the window of opportunity available for drug effectiveness. As such, the time period available for effective neuroprotection must be determined for each treatment regimen before human clinical trials can be considered. Experimentally, the window of opportunity for effective neuroprotection varies significantly among experimental agents as well as between different types of cerebral ischemia. Following focal cerebral ischemia, neurons in the penumbral area remain at risk for many hours following initial vessel occlusion. As such, neuroprotective agents might be expected to exert a positive effect for long periods following the onset of stroke. In one experimental model, for example, nimesulide, a COX-2 inhibitor, demonstrated significant neuroprotection when administered up to 24 h following focal cerebral ischemia in rats [9]. Following human stroke, thrombolysis has proven effective in returning neurologic function to selected individuals. The effective time period for thrombolysis is much more limited, however, with efficacy seen only if lytic treatment is begun in less than 6 h (and perhaps as short as 3 h) following onset of symptoms [28]. Cardiac arrest differs from stroke fundamentally in that the entire brain is exposed to a short period of global ischemia. Many of the degradative processes following cardiac arrest begin during the period of ischemia and are intensified during reperfusion. As such, one would expect that neuroprotection might only prove effective if begun immediately following resuscitation. Human trials, however, demonstrate that induced hypothermia (the only effective neuroprotective strategy in humans) is effective in promoting significant neuroprotection even when begun several hours following resuscitation [2,17].

The results from this series of experiments, though, demonstrate that as little as 30 min of delay in drug administration following reperfusion severely compromises the ability of acetyl-L-carnitine to exert its clinical neuroprotective effect. These results are qualitatively similar to those of Shuaib who found no neuroprotection in a gerbill model of cerebral ischemia when ALCAR was administered 30 min following reperfusion, although ALCAR provided significant neuroprotection when administered prior to the insult [29]. It is interesting to note that although delayed ALCAR administration failed to improve clinical neurologic outcome, drug treated animals did demonstrate reversal of cerebral lactic acidosis at both 2 and 24 h following cardiac arrest. It should be noted, however, that statistical significance was achieved at 24 h between groups only because of two “outlier” values of lactate, which did not correlate with clinical neurological outcome. The absolute values for lactic acid in each group, however, were virtually identical to values previously published for dogs receiving ALCAR immediately following ROSC, where a marked difference in clinical neurological outcome was noted at 24 h [26]. The fact that this drug appears to continue to be effective at the biochemical level, while affording little neurologic recovery at this time point, suggests that ALCAR exerts its effects through interruption of otherwise irreversible events which may be occurring during the early phases of reperfusion, but may be potentially modifiable through appropriate drug therapy. One possibility is that the effects of ALCAR on brain lactate and energy metabolism during the initial phase of reperfusion, i.e., the first 30–60 min is critically important for neuroprotection. Alternatively, we know, for example, that following 10 min CA in dogs, the concentration of cortical L-type voltage dependent  $Ca^{2+}$  channels in crude synaptosomal membranes is increased by 250%, with concentrations remaining elevated for up to 2 h following ROSC [16]. This increase in calcium channels may result in prolongation of the increased intracellular  $Ca^{2+}$  concentration which begins during ischemia and which has been linked to activation of degradative enzymes, and eventual cellular death [10,32]. Administration of acetyl-L-carnitine immediately following ROSC, has been demonstrated to result in normalization of cortical  $Ca^{2+}$  channel concentration by 30 min of reperfusion [15]. A 30-min delay in ALCAR administration may, therefore, result in a more prolonged period of elevated intracellular  $Ca^{2+}$  relative to that which may occur following immediate post-resuscitative ALCAR administration. Early treatment with ALCAR has also been shown to protect against free radical damage to soluble brain proteins that is evident following 2–24 h ROSC [21]. Since initiation of abnormal free radical generation can begin as soon as the brain is reperfused, [27,36] it is also possible that delayed ALCAR therapy is ineffective at alleviating this critical form of molecular injury. Further support for this hypothesis may be provided in the future using imaging techniques capable of providing continuous monitoring of brain energy metabolism in the post-resuscitative period, and allowing comparison of

multiple time points with eventual clinical neurological outcome.

Previous studies demonstrate that acetyl-L-carnitine can be administered safely and effectively immediately following resuscitation. When administered in this fashion ALCAR affords significant neuroprotection following experimental cardiac arrest. The results of these experiments, however, demonstrate that ALCAR does not promote clinically measurable neuroprotection if administration is significantly delayed following restoration of spontaneous circulation. In order to maximize the chances of effective neuroprotection, clinical trials should be designed with reference to the findings of this study. We postulate that for optimal neuroprotective benefit, acetyl-L-carnitine should be administered as shortly as possible following resuscitation, most definitely within 30 min of reperfusion.

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