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Lipoic acid inhibits caspase-dependent and -independent cell death pathways and is neuroprotective against hippocampal damage after pilocarpine-induced seizures

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ABSTRACT

Alpha-lipoic acid has some neuroprotective properties, but this action has not been investigated in models of epilepsy. The aim of the present study was to investigate the protective efficacy of α-lipoic acid (lipoic acid) against pilocarpine-induced cell death through the caspase-dependent or -independent mitochondrial apoptotic pathways. Wistar rats were injected intraperitoneally with 0.9% saline (control group), pilocarpine (400 mg/kg, pilocarpine group) alone, or α-lipoic acid (20 mg/kg) in association with pilocarpine (400 mg/kg) 30 min before administration of α-lipoic acid. After the treatments all groups were observed for 24 h. Cell death was reduced in lipoic acid-treated rats. Cytosolic translocation of cytochrome *c* and subsequent activation of caspase-3 were reduced by lipoic acid treatment. AIF nuclear translocation and subsequent large-scale DNA fragmentation were also decreased in lipoic acid-treated rats. Our study suggests that lipoic acid inhibits both caspase-dependent and -independent apoptotic pathways and may be neuroprotective against hippocampal damage during pilocarpine-induced seizures.

1. Introduction

Alpha-lipoic acid is a disulphide derivative of octanoic acid and has been used safely for decades due to the presence of a crucial prosthetic group of various cellular enzymatic complexes (Packer et al., 1995; Tardif, 2008). In addition, α-lipoic acid has been characterized as an efficient antioxidant since it is a potential therapeutic agent in the treatment or prevention of different pathologies that may be related to an imbalance of the oxido-reductive cellular status (Han et al., 1997; Moini et al., 2002).

It has been reported that α-lipoic acid exerts protective effects in neurodegeneration, ischemia-reperfusion, polyneuropathy, diabetes, AIDS, seizures and hepatic disorder status (Maczurek et al., 2008; Packer et al., 1999, 1997). The precise mechanism of neuroprotective effects of α-lipoic acid remains unclear. It is known that α-lipoic acid can inhibit microglial activation and caspase-related apoptotic pathways (Bustamante et al., 1995). Recently, it was reported that inhibition of caspase-independent mitochondrial pathways, such as the nuclear translocation of apoptosis-inducing factor (AIF), is also involved in protective action of lipoic acid (Selvakumar et al., 2006).

Apart from stroke, epilepsy is the most common neurological disorder with 0.5% of prevalence (Browne and Holmes, 2001). The pilocarpine treatment in rats is an experimental model largely used to study the pathophysiology of seizures and status epilepticus (SE) (Freitas et al., 2003; Treiman, 1990). Although there are some contradictory reports, a potent neuroprotective effect of α-lipoic acid has been found in various animal models of neurological diseases (Ferreira et al., 2009; Holmquist et al., 2007; Militão et al., 2010). Thus, we investigated whether α-lipoic acid can reduce hippocampal cell death after pilocarpine injection in rats which has been accepted as a model of temporal lobe epilepsy (Freitas et al., 2003; Turski et al., 1983; Xavier et al., 2007). We further investigated whether caspase-dependent or -independent apoptotic pathway was involved in the neuroprotective effects of α-lipoic acid.

2. Material and methods

2.1. Animal procedures

Male Wistar rats (250-280 g; 2-month-old) were obtained from Central Animal House of the Federal University of Piaui, Piaui, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the official governmental guidelines in

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Table 1
Effect of pretreatment with lipoic acid on pilocarpine-induced seizures and lethality in adult rats.

Groups	Latency to first seizures (min)	Percentage seizures	Percentage survival	Number of animals/group
Pilocarpine	35.00 ± 0.70	60	40	24
LA plus pilocarpine	89.00 ± 1.95[c]	10[a]	100[a]	24
LA	00	00[a,b]	100[a]	24

Animals were pretreated acutely, intraperitoneally, with lipoic acid and 30 min after receiving pilocarpine 400 mg/kg, i.p. Results for latency to first seizure are expressed as mean ± S.E.M. Result for percentage seizures and percentage survival are expressed as percentages of the number of animals from each experimental group.
[a] $p<0.0001$ compared with the pilocarpine group (χ^2-test).
[b] $p<0.0001$ compared with LA plus pilocarpine group (χ^2-test).
[c] $p<0.0001$ compared with the pilocarpine group (ANOVA and Student–Newman–Keuls test).

compliance with the Society Policy and was submitted by the Ethics Committee of the Federal University of Piauí, Brazil. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.).

In a set of experiments, the animals were divided into four groups and treated with α-lipoic acid (20 mg/kg, i.p., $n=24$) or 0.9% saline (i.p., $n=24$) and 30 min later, they received pilocarpine hydrochloride (400 mg/kg, i.p.). Within this 30-min interval, the rats were observed for the occurrence of any change in behavior. The treatments previously described represent the LA plus pilocarpine and pilocarpine groups, respectively. Other two groups received 0.9% saline (i.p., $n=24$, control group) or α-lipoic acid alone (20 mg/kg, i.p., $n=24$, LA group). After the treatments, the animals were placed in 30 cm × 30 cm chambers to record: latency to first seizure, any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonus, tonus, clonic–tonic seizures (Turski et al., 1983), and number of animals that died during 1 h after pilocarpine administration.

Previous work have shown that the numbers of convulsions and deaths occurring within 24 h post injection always follow the same pattern, so we decided to observe the animals for 24 h as pilocarpine-induced convulsions occur in 30–60 min and deaths within 24 h after pilocarpine injection. The survivors were killed by decapitation and their brains were dissected out on ice to remove hippocampus for caspase-dependent or -independent apoptotic determinations. The pilocarpine group was constituted by those rats that presented seizures.

The drug dosages were determined from both dose–response studies, including pilocarpine (data not shown), and observations of the doses currently used in animals studies in the literature (Freitas, 2009; Santos et al., 2008; Xavier et al., 2007). The doses used are not equivalent to those used by humans because rats have different metabolic rates.

2.2. Histopathological investigation in hippocampus

All groups were closely observed during 24 h for behavioral changes and convulsive state. Animals were sacrificed by decapitation 24 h after the treatment and brains were dissected out and fixed in formalin 10%. After an initial coronal section at the level of the optic nerve, 3–5 μm thick sections were prepared and stained with hematoxylin and eosin (HE) for light microscopy studies (100 ×). The degree of hippocampal damage severity was defined by a scale ranging from 0 (none) to 100 (total) by light microscopy and previously defined to be reliable for morphological analysis (Paxinos and Watson, 2007). Brain damage presence was confirmed if hippocampus showed at least 50% involvement. To detect DNA fragmentation, terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphatebiotin nick end-labeling (TUNEL) (Roche Diagnostics GmbH, Penzberg, Germany) was performed (Lee et al., 2005b). Using high-powered magnification (400×), the total number of cells and the number of TUNEL-positive cells within a grid were counted. The ratio of the number of TUNEL-positive neurons in CA1 and CA3 was calculated and expressed as percent of the TUNEL-positive cells in each group ($n=4$). After washing, 0.5 g/ml of propidium iodide (PI) (Sigma, St. Louis, MO, USA) was used for counterstaining. Thereafter, the sections were observed under LSM 510 confocal laser scanning microscopy (Carl Zeiss, Thornwood, NY, USA). Immunofluorescent staining was done as previously described (Lee et al., 2005a). Fixed sections were reacted to primary antibodies, rabbit anti-cytochrome *c* (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or goat anti-AIF (1:100; Santa Cruz Biotechnology). After washing, the sections were incubated with FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) or FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). After counterstaining with PI, the slides were observed using a confocal laser scanning microscope (Carl Zeiss).

2.3. DNA fragmentation in hippocampus

To quantify DNA fragmentation, we used commercially prepared enzyme immunoassay kit to determine cytoplasmic histone-associated DNA fragments (Roche Diagnostics). After pilocarpine injection, hippocampus was quickly dissected and subfractionized in lysis buffer. A cytosolic volume containing 20 μg of protein was used for the ELISA, following the manufacturer's protocol ($n=5$). To quantify the caspase-3 activity, we used a commercially prepared enzyme immunoassay kit (Chemicon International, Temecula, CA, USA). The cytosolic samples were prepared as described for the Western blot method ($n=5$). Equal amount of protein (20 μg) was used to determine the cleaved bioluminescent substrate by active caspase-3 and ELISA.

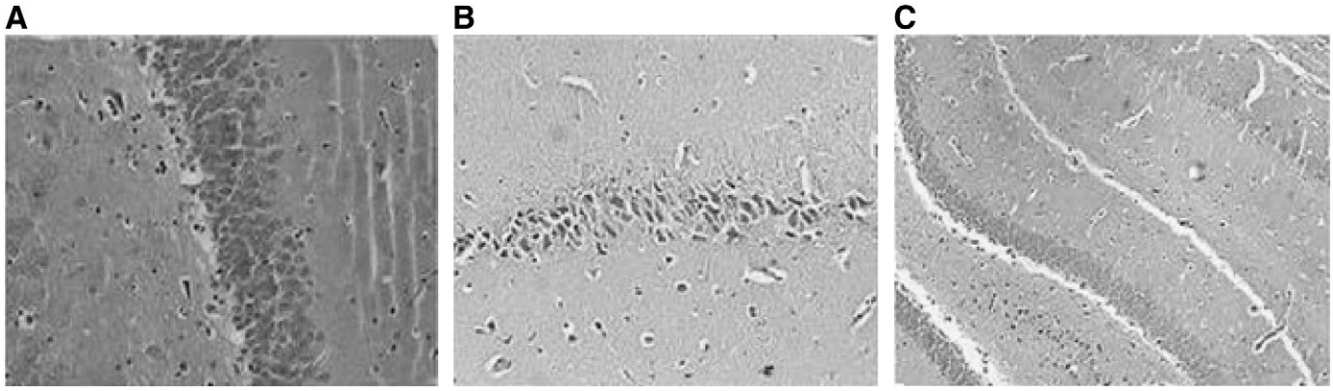
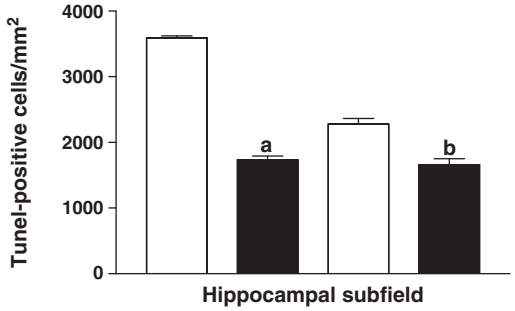
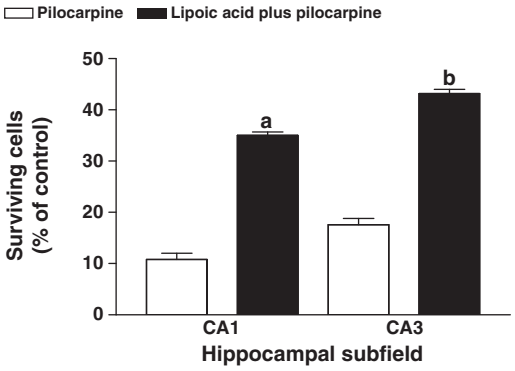


Fig. 1. Reduction of hippocampal cell death by lipoic acid. Comparison of severity of lesion was expressed as a mean ± S.E.M. of scores of damage based in a scale from zero (none) to 100 (total) percentage of hippocampus involvement. Brain damage was considered positive if there was at least 50% hippocampal involvement showed by hematoxylin and eosin staining (HE). Pictures (100 ×) shown are from one representative experiment of $n=5$. Legends: A: saline group; B: pilocarpine group; and C: lipoic acid plus pilocarpine group;



[a]p<0.001 as compared to pilocarpine group (t-Student-Neuman-Keuls test);
[b]p<0.001 as compared to pilocarpine group (t-Student-Neuman-Keuls test).



[a]p<0.001 as compared to pilocarpine group (t-Student-Neuman-Keuls test);
[b]p<0.001 as compared to pilocarpine group (t-Student-Neuman-Keuls test).

Fig. 2. Semi-quantitative analysis of TUNEL-positive cells in both CA1 and CA3 and surviving cells in hematoxylin and eosin (HE) staining in lipoic acid-treated rats after pilocarpine injection.

2.4. Western blot analysis for cytochrome c, AIF, and cleaved caspase-3 in hippocampus

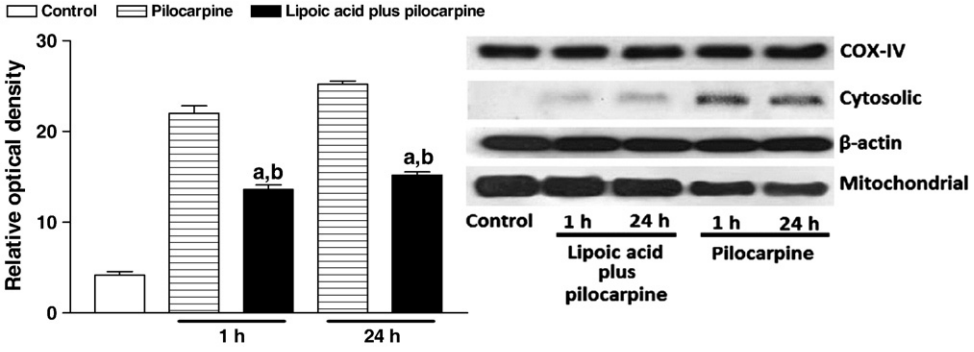
Hippocampal tissues were collected at 24 h after pilocarpine injection and subfractionized into the mitochondrial, cytosolic, and nuclear fractions ($n=5$) (Lee et al., 2005a). Transblots were incubated with primary antibodies such as rabbit anti-cytochrome *c* (1:200; Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 (1:1000; Cell Signaling Technologies, Beverly, MA, USA), or goat anti-AIF (1:200; Santa Cruz Biotechnology), respectively. To reveal large-scale DNA fragmentation, pulse-field gel electrophoresis (PFGE) was performed as previously described ($n=5$) (Zhang et al., 2002). Using a CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad, Hercules, CA, USA), DNA samples was prepared in agarose plugs.

2.5. Statistical analyses

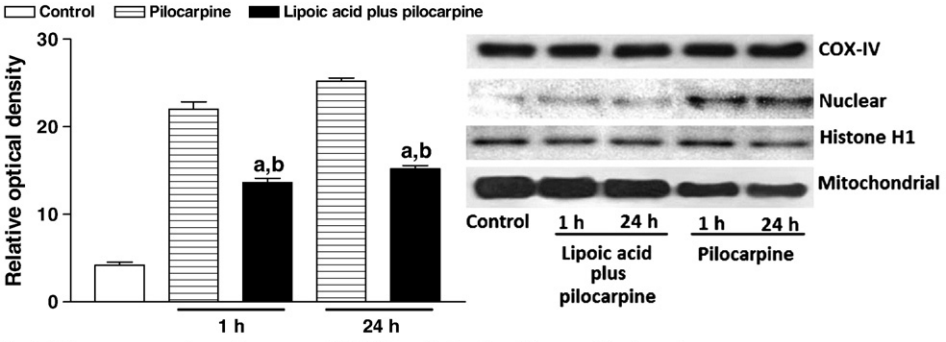
DNA fragmentation and Western blot analysis were compared using ANOVA and the Student–Newman–Keuls test as post hoc test, because these results show a parametric distribution. The histopathological analysis was calculated as percentages, and compared with the Mann–Whitney test. In all situations statistical significance was reached at p less-than-or-equals, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, Version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

Pilocarpine induced the first seizure at 35.00 ± 0.70 min. All the animals studied showed generalized tonic–clonic convulsions with status epilepticus (SE) and 60% survived the seizures. All animals pretreated with the lipoic acid selected for this study were observed



[a]p<0.001 as compared to saline group (ANOVA and t-Student-Neuman-Keuls test);
[b]p<0.001 as compared to pilocarpine group (ANOVA and t-Student-Neuman-Keuls test).



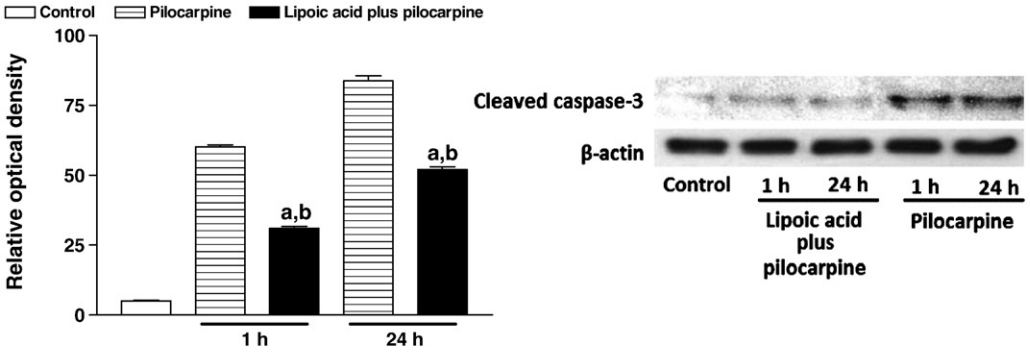
[a]p<0.001 as compared to saline group (ANOVA and t-Student-Neuman-Keuls test);
[b]p<0.001 as compared to pilocarpine group (ANOVA and t-Student-Neuman-Keuls test).

Fig. 3. Reduction of cytochrome *c* release and AIF nuclear translocation from mitochondria by lipoic acid. Cytosolic release of cytochrome *c* in Western blot analysis after pilocarpine treatment. AIF nuclear translocation after pilocarpine injection in Western blot analysis. COX, cytochrome oxidase. COX-IV, beta-actin and histone H1; internal controls of each mitochondrial, cytosolic and nuclear subfractions.

for 24 h before pilocarpine injection and they manifested alterations in behavior, such as peripheral cholinergic signs (100%), tremors (50%), staring spells, facial automatisms, wet dog shakes, rearing and motor seizures (10%), developed progressively within 1–2 h into a long-lasting SE (10%). Result shows that when administered at the dose (20 mg/kg) before pilocarpine, lipoic acid reduced by 50% the percentage of animals that seized, increased (154%) latency to the first seizure (89.00 ± 1.95 min) and increased the survival percentage (60%) when compared to the pilocarpine-treated group (Table 1). None of the animals that received injections of isotonic saline (control) or lipoic acid alone showed seizures or histopathological changes in hippocampus. However, neuronal loss, gliosis, and typical vacuolar degeneration are observed in the pilocarpine-treated hippocampus region. Importantly, no histopathological changes are observed in lipoic acid-treated after policarpine co- administered hippocampus (Fig. 1).

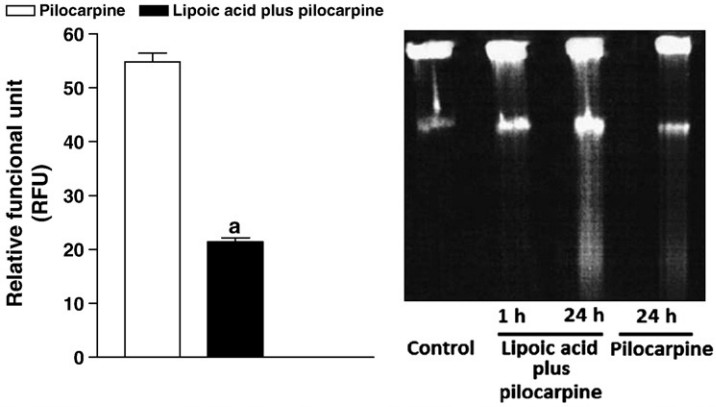
In histopathological analysis, surviving cells in hippocampus were significantly increased in lipoic acid-treated rats at 24 h after seizures compared to pilocarpine-treated rats ($p<0.001$) (Fig. 1). TUNEL-positive cells in both CA1 ($p<0.001$) and CA3 ($p<0.0001$) were significantly reduced in lipoic acid-treated rats after seizures compared to the pilocarpine-treated group (Fig. 2). Semi-quantitative analysis of surviving CA1 ($p<0.001$) and CA3 ($p<0.001$) cells in hematoxylin and eosin (HE) staining was significantly increased in lipoic acid-treated rats after seizures (Fig. 2).

In Western blot analysis, cytochrome *c* immunoreactivity was evident in the cytosolic fraction of seized rats after pilocarpine injection, whereas immunoreactivity of cytochrome *c* in cytosolic fraction of normal control was barely detected (Fig. 3). The amount of cytosolic cytochrome *c* was significantly reduced in lipoic acid-treated rats at 1 h and 24 h after seizures compared to the pilocarpine group ($p<0.001$) (Fig. 3).

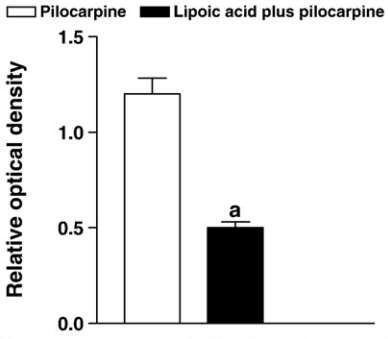


[a]p<0.001 as compared to saline group (ANOVA and t-Student-Neuman-Keuls test);

[b]p<0.001 as compared to pilocarpine group (ANOVA and t-Student-Neuman-Keuls test).



[a]p<0.001 as compared with pilocarpine group (ANOVA and Student–Newman–Keuls test).



[a]p<0.001 as compared with pilocarpine group (ANOVA and Student–Newman–Keuls test).

Fig. 4. Inhibition of caspase-dependent and -independent pathways by lipoic acid after pilocarpine treatment in hippocampus. Expression of cleaved caspase-3 was reduced by lipoic acid in Western blot analysis. Pulse-field gel electrophoresis; large-scale DNA fragmentation (mainly ~50 kbp) was shown as early as 1 h and further 24 h after pilocarpine treatment, and it was decreased by lipoic acid at 24 h after pilocarpine treatment. Caspase-3 activity assay after pilocarpine and lipoic acid treatment. Comparison of DNA fragmentation among pilocarpine and lipoic acid-treated rats, using cell death assay.

The injection of lipoic acid alone did not change the amount of cytochrome *c* in mitochondria in the control rats (without pilocarpine). In contrast, lipoic acid-treated rats showed reduction of cytochrome *c* immunoreactivity in cytosol, compared to pilocarpine-treated rats (Fig. 3).

In Western blot analysis, mitochondrial AIF in pilocarpine-treated rats increased time-dependently after seizures, whereas nuclear AIF in lipoic acid-treated rats correspondingly decreased after pilocarpine-induced seizures (Fig. 3). The amount of nuclear AIF in lipoic acid-treated rats was significantly decreased compared to pilocarpine-treated rats ($p<0.001$) (Fig. 3). In Western blot analysis, the immunoreactivity of cleaved caspase-3, the active form of caspase-3, was increased in seized rats after pilocarpine injection.

The total amount of cleaved caspase-3 was significantly reduced in lipoic acid-treated rats compared to seized rats ($p<0.001$) (Fig. 4). In PFGE, large-scale DNA fragmentation (~50 kbp) was increased at 1 h and further increased at 24 h after pilocarpine treatment. However, large-scale DNA fragmentation reduced in lipoic acid-treated rats at 24 h after pilocarpine treatment (Fig. 4). Caspase-3 activity was significantly decreased in lipoic acid-treated rats compared to seized rats at 24 h after pilocarpine treatment ($p<0.001$). Nevertheless, there was no significant difference in caspase-3 activity between saline and lipoic acid treatments (Fig. 4). The amount of DNA fragmentation in cell death assay was significantly decreased by lipoic acid-treatment compared with pilocarpine-treated rats ($p<0.001$). In addition, the apoptotic DNA fragmentation in lipoic acid-treated rats was significantly less than that of the pilocarpine-treated rats ($p<0.001$) (Fig. 4).

4. Discussion

All the animals treated with pilocarpine showed generalized tonic–clonic convulsions with status epilepticus (SE), and 60% survived the seizures. All animals pretreated with the lipoic acid selected for this study were observed for 1 h and 24 h before pilocarpine injection and its manifested alterations in behavior, such as peripheral cholinergic signs, tremors, staring spells, facial automatisms, wet dog shakes, rearing and motor seizures, which develop progressively within 1–2 h into a long-lasting SE. Result shows that when administered at a dose of 20 mg/kg before pilocarpine, lipoic acid reduced the percentage of animals that seized, increased latency to the first seizure and increased the survival percentage as compared with the pilocarpine-treated group. Pretreatment with lipoic acid, had similar effects in two observation periods. Lipoic acid presents an anticonvulsant effect, improving some parameters related to pilocarpine-induced convulsions.

In this study, we demonstrated the first evidence that α-lipoic acid may provide a neuroprotective effect against rat hippocampal cell death after pilocarpine treatment through the inhibition of caspase-dependent and -independent apoptotic pathways. First, α-lipoic acid reduced DNA fragmentation after pilocarpine treatment. Second, α-lipoic acid inhibited both the caspase-dependent (cytochrome *c* release and the subsequent cleavage of caspase-) and independent pathways (nuclear translocation of AIF) after pilocarpine-induced seizures. Furthermore, caspase-3 activity was similarly inhibited between pan-caspase inhibitor and lipoic acid-treated rats, but the amount of apoptosis associated DNA fragmentation in lipoic acid-treated rats was lower than that of the pan-caspase inhibitor-treated rats. It suggests that the reduction of apoptosis by α-lipoic acid treatment is related with additional inhibitory function against the caspase-independent apoptosis along with blocking caspase-dependent pathways. Although present results show that apoptosis might be critical in pilocarpine-induced hippocampal damage, other cell death types such as necrosis need to be evaluated.

The recent reports showed that nuclear translocation of AIF (Cheung et al., 2005), a caspase-independent pathway, as well as the caspase-dependent cell death pathway (Henshall et al., 2000), is involved in seizure induced apoptotic cell death, and both caspase-dependent and -independent pathways are inhibited by lipoic acid treatment (Lovell et al., 2003; Poon et al., 2005). Thus, these reports support our speculation that lipoic acid may have a neuroprotective role by reducing apoptosis in both caspase-dependent and -independent manner after pilocarpine treatment. However, other mechanisms such as inflammation and microglial activation still remain to be evaluated in this model.

In conclusion, our results suggest that lipoic acid inhibits both caspase-dependent and -independent apoptotic pathways, and the inhibition of these pathways may contribute to the hippocampal damage reduction after pilocarpine treatment.

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