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A novel therapeutic strategy for experimental stroke using docosahexaenoic acid complexed to human albumin

Ludmila Belayev^{1,*}, Larissa Khoutorova¹, Sung-Ha Hong¹ and Andre Obenaus²

¹ Neuroscience Center of Excellence, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

² Department of Pediatrics, School of Medicine, Loma Linda University, Loma Linda, CA 92354, USA

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Abstract – Despite tremendous efforts in ischemic stroke research and significant improvements in patient care within the last decade, therapy is still insufficient. There is a compelling, urgent need for safe and effective neuroprotective strategies to limit brain injury, facilitate brain repair, and improve functional outcome. Recently, we reported that docosahexaenoic acid (DHA; 22:6, n-3) complexed to human albumin (DHA-Alb) is highly neuroprotective after temporary middle cerebral artery occlusion (MCAo) in young rats. This review highlights the potency of DHA-Alb therapy in permanent MCAo and aged rats and whether protection persists with chronic survival. We discovered that a novel therapy with DHA-Alb improved behavioral outcomes accompanied by attenuation of lesion volumes even when animals were allowed to survive three weeks after experimental stroke. This treatment might provide the basis for future therapeutics for patients suffering from ischemic stroke.

Keywords: Omega-3 polyunsaturated fatty acids / neuroprotection / behavior / MRI / histopathology

Résumé – Une nouvelle stratégie thérapeutique pour les AVC expérimentaux utilisant l'acide docosahexaénoïque complexé à l'albumine humaine. En dépit des avancées de la recherche sur l'AVC ischémique et des améliorations significatives des soins apportés aux patients durant la dernière décennie, la thérapie de prise en charge demeure encore insuffisante. Ainsi, il est urgent de développer des stratégies neuroprotectrices sûres et efficaces afin de limiter le développement des lésions cérébrales et de restaurer les altérations cellulaires et fonctionnelles. Récemment, nous avons mis en évidence que l'acide docosahexaénoïque (DHA ; 22:6, n-3) complexé à l'albumine humaine (DHA-Alb) s'avère hautement neuroprotecteur chez le jeune rat après une occlusion cérébrale moyenne temporaire de l'artère (MCAo). Cette revue met en avant tout le potentiel d'une thérapie DHA-Alb chez des rats MCAo permanents et âgés, et soulève la question de la persistance de la neuroprotection en condition de survie prolongée. Cette nouvelle thérapie de traitement avec du DHA-Alb améliore par ailleurs les performances comportementales et s'accompagne d'une régression volume des lésions cérébrales même après 3 semaines suivant l'AVC expérimental. Ce type de traitement pourrait ainsi constituer une nouvelle base de prise en charge des patients atteints d'un AVC ischémique.

Mots clés : Oméga-3 / acides gras poly-insaturés / neuroprotection / comportement / IRM / histopathologie

1 Neuroprotective strategies for ischemic stroke

Stroke is the fifth leading cause of death and disability in the United States. Every year, more than 795 000 people in the United States have a stroke. On average, one American dies from stroke every 4 min (Mozaffarian *et al.*, 2015). Therapeutic options in the acute phase of a stroke are limited and the only approved treatment for ischemic stroke is intravenous recombinant tissue plasminogen activator (tPA), which opens channels for blood flow through the occluded thrombi and improves neurological outcome (Heuschmann *et al.*, 2004).

However only 3–5% of stroke patients are eligible for tPA therapy, which must be initiated within 4.5 h of stroke onset – otherwise effectiveness is limited and there is potential for hemorrhagic side effects (Fisher and Bastan, 2008).

While many neuroprotective drugs have been developed, none of these drugs have been translated to the clinical setting (O'Collins *et al.*, 2006; Tymianski, 2013). This failure could be explained by the lack of consideration for the risk factors for stroke, including aging, permanent occlusion and long-term follow up in pre-clinical studies (Turner *et al.*, 2013). Thus, animal studies which investigate the mechanisms and efficacy of novel treatments considering different risk factors for stroke remain ideal.

* Correspondence: lbelay@lsuhsc.edu

2 Docosahexaenoic acid and stroke

Cerebral ischemia is characterized by a rapid accumulation (within minutes) of free fatty acids, including arachidonic acid (AA; 20:4, n-6) and docosahexaenoic acid (DHA; 22:6, n-3), due to increases in intracellular calcium and activation of phospholipases (Muralikrishna Adibhatla and Hatcher, 2006). This free pool of AA and DHA is then converted *via* enzymatic processes as well as free radical-mediated lipid peroxidation into a cascade of activated pro- and anti-inflammatory mediators, the makeup of which ultimately drives the cell toward survival or programmed cell death.

DHA is a natural element of our diet and is found in fish (salmon, tuna, mackerel, herring, sardines, halibut), other seafood (algae, krill). As a consequence, we depend on the dietary supply of DHA or of its precursor, linolenic acid (18:3, n-3). It is well established that DHA and other omega-3 fatty acids have an important role in heart health. Recent studies have established that omega-3 fatty acids reduce inflammation and may have beneficial effects in patients with coronary heart disease, asthma, rheumatoid arthritis, osteoporosis, sepsis, cancer, dry eye disease and age-related macular degeneration (Simopoulos, 2008).

DHA is a member of the essential omega-3 fatty acid family and is enriched in the membranes of the central nervous system (Bazan, 2006). It is also necessary for the development of the nervous system, including vision (Bazan, 2007). It has been shown to be involved in memory formation, synaptic membrane function, aging and neuroprotection (Bazan *et al.*, 2011). We have discovered that DHA is a critical component of endogenous mechanisms that protect the brain after injury (Bazan, 2005, 2006). An early increase in unesterified (free) DHA during brain ischemia initiates a pathway for docosanoid biosynthesis, specifically neuroprotectin D1 (NPD1) (Bazan, 2003; Marcheselli *et al.*, 2003). We have shown that NPD1 inhibits brain ischemia-reperfusion-mediated leukocyte infiltration and proinflammatory gene expression, and promotes cell survival both *in vitro* and *in vivo* (Bazan, 2007; Belayev *et al.*, 2011; Marcheselli *et al.*, 2003). Our recent studies demonstrated that DHA therapy in medium (5 mg/kg) dose improved neurological and histological outcome when treatment is initiated as late as 5 h after onset of stroke (Belayev *et al.*, 2009, 2001).

3 Docosahexaenoic acid complexed to albumin (DHA-Alb) as a possible superior neuroprotectant

High-dose human albumin (Alb) therapy is strongly neuroprotective in animal models of focal cerebral ischemia (Belayev *et al.*, 2001), as well as in global cerebral ischemia (Belayev *et al.*, 1999). The neuroprotective efficacy of albumin is attributed to its multifunctional properties, which include antioxidant action, hemodilution and oncotic effects, binding of copper ions, fatty-acid transport, preservation of endothelial integrity, platelet anti-aggregatory effects and decreased red blood cell sedimentation under low-flow conditions (Belayev *et al.*, 1997, 2002). In addition, Alb administration following MCAo induces a systemic mobilization of

free fatty acids (FFA) through the blood stream. Under these conditions, Alb may help to supply the n-3 polyunsaturated acids (PUFA) to the postischemic brain (Belayev *et al.*, 2005; Rodriguez de Turco *et al.*, 2002) and thereby contribute to replenishing PUFA lost from cellular membranes during ischemia (Bazan *et al.*, 1992) and/or allow these PUFA to serve as an alternative source of energy (Rapoport *et al.*, 2006). Pre-clinical studies in a rat focal cerebral ischemia stroke model (these were young rats) demonstrated that administration of Alb at high doses (2.5 g/kg) decreased infarct volume, reduced brain swelling (Belayev *et al.*, 1997, 1998) and improved local cerebral perfusion in affected tissue (Belayev *et al.*, 2002; Huh *et al.*, 1998). These extremely-promising results led to the development of a human clinical trial testing Alb as a neuroprotectant after acute ischemic stroke. Unfortunately, a phase III clinical trial in humans was stopped because the high dose of Alb (2 g/kg) used in this trial led to pulmonary edema in 13% of patients (Ginsberg *et al.*, 2011). This likely occurred because high-dose Alb expands intravascular volume and may exacerbate congestive heart failure in some elderly patients.

Recently, we tested a novel therapy – Alb (0.63 g/kg) complexed to DHA – in a young rat stroke model (Eady *et al.*, 2012). The idea was to reduce the Alb dosage without reducing its protective effects. Excitingly, we found that this treatment with low/moderate doses of DHA-Alb led to improved neurological outcomes and significant reduction of infarct volume (especially in the salvageable penumbral region) even when treatment was initiated as late as 7 h after onset of MCAo (Eady *et al.*, 2012), compared to 4 h for Alb (1.25 g/kg) (Belayev *et al.*, 2001) and 5 h for DHA alone (Belayev *et al.*, 2001). Thus, when albumin is complexed with DHA, it is possible to achieve neuroprotection at *lower* albumin doses; this takes on great clinical importance as the administration of high-dose albumin, by expanding intravascular volume, may on occasion precipitate acute pulmonary edema and congestive heart failure, particularly in patients with compromised cardiovascular function.

4 DHA-Alb therapy reduces injury after permanent MCAo

While human thromboembolic stroke commonly involves some degree of early or late reperfusion, in many cases the arterial occlusion remains permanent. The rate of spontaneous recanalization of cerebral artery occlusion in humans is at 17% by 6–8 h from onset (Kassem-Moussa and Graffagnino, 2002). Models resulting in permanent ischemia mimic clinical stroke without reperfusion. Occlusion is usually induced by cauterization of the MCA via a craniotomy or advancing a suture into the ICA to occlude the MCA at its origin from the circle of Willis. However, because of greater mortality with permanent occlusion models, most preclinical studies use transient MCAo. During preclinical investigations, it is vital that the potential for new therapies to ameliorate the consequences of clinically significant permanent occlusions are assessed in an animal model. As recommended by Stroke Therapy Academic Industry Roundtable, permanent MCAo models should be studied first, followed by transient (reperfusion) models (Stroke Therapy Academic Industry Roundtable, 1999).

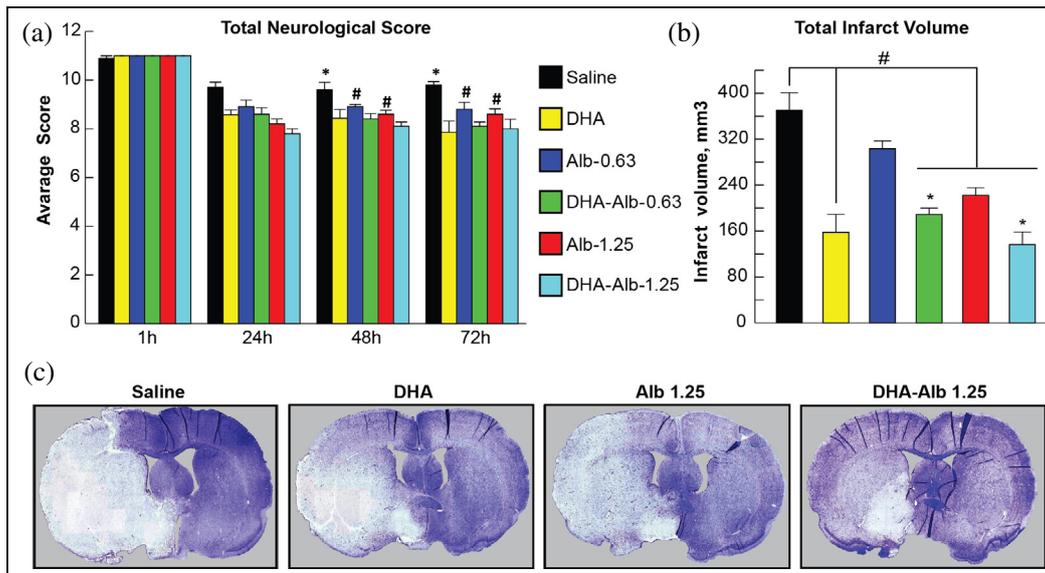


Fig. 1. Effect of DHA-Alb on permanent MCAo. (a) Total neurological score (normal score = 0, maximal score = 12) during MCAo (60 min) and on days 1, 2 and 3 after treatment; (b) total infarct volume and (c) computer-generated MosaiX-processed images of Nissl stained paraffin-embedded brain sections on day 3 after stroke. Treatment with DHA-Alb significantly improved neurological scores (48 and 72 h), showed less extensive damage, mostly in the subcortical area and reduced total lesion volumes compared to the corresponding Alb-treated group. Values shown are means \pm SEM. # $p < 0.05$ versus saline group; * $p < 0.05$ versus Alb (1.25 g/kg) group (two-way repeated-measures ANOVA).

An exception to this recommendation exists for drugs with mechanisms of action requiring successful reperfusion.

Our recent studies demonstrated that DHA-Alb is highly neuroprotective after temporary MCAo, but whether a similar effect occurs in permanent MCAo is unknown. The argument for applying DHA-Alb in human ischemic stroke would be strengthened by the demonstration of its efficacy in permanent arterial occlusion.

Male Sprague-Dawley rats underwent permanent MCAo and behavioral function was evaluated on days 1, 2 and 3 after MCAo. DHA (5 mg/kg), Alb (0.63 or 1.25 g/kg), DHA-Alb (5 mg/kg + 0.63 g/kg or 5 mg/kg + 1.25 g/kg) or saline were administered i.v. at 3 hours after onset of stroke. Histopathology was conducted on day 3. Our study demonstrated that rats treated with low and moderate doses of DHA-Alb showed improved neurological score compared to corresponding Alb groups on days 2 and 3 (Fig. 1a). In addition, the total corrected infarct volumes were reduced by both doses of DHA-Alb compared to the corresponding Alb groups (Fig. 1b). Histological evaluation of the brains exhibited a consistent pannecrotic lesion involving both cortical and subcortical (mainly striatal) regions of the right hemisphere in saline-treated rats. In contrast, infarct size was attenuated in rats treated with DHA-Alb (Fig. 1c). In conclusion, DHA-Alb therapy is neuroprotective in permanent MCAo in rats. This treatment can provide the basis for future therapeutics for patients suffering from ischemic stroke without reperfusion (Eady *et al.*, 2013).

5 DHA-Alb therapy provides neuroprotection after stroke in aged rats

Age is the most important independent risk factor for stroke. Both the incidence of stroke and level of negative outcomes associated with stroke (in terms of disability and mor-

tality rate) increase with age. With the growth of the global elderly population, the prevalence of stroke will rise also. Reports indicate that 75–89% of strokes occur in individuals aged > 65 years (Feigin *et al.*, 2003). Of these strokes, 50% occur in people who are aged ≥ 70 years and nearly 25% occur in individuals who are aged > 85 years. Risk factor profiles and mechanisms of ischemic injury vary between young and old patients with stroke. It is well known that elderly patients tend to have a worse outcome than younger patients because they have comorbidities that heavily affect outcome. In addition, elderly patients often receive less-effective treatment than younger individuals. Although age is one of the most significant prognostic markers for poor outcome, very few studies have been performed in aged animals, especially in animals over 15 months of age. Studies on aged animals (*e.g.* rodents) can closely mimic the clinical features of older patients with stroke. Aged animals show greater infarction volumes and a higher mortality rate than young animals following MCAo (Chen *et al.*, 2010). While animals of all ages have shown neurological deficits after MCAo, aged animals exhibited poorer performances than young animals in functional tests (Liu *et al.*, 2009). Aged mice of both sexes showed considerably reduced stroke-induced edema compared with young animals. This finding is consistent with the clinical observation that young patients with stroke are more likely to develop fatal brain edema than older patients (Wagner and Lutsep, 2005).

Unfortunately, most experimental studies are conducted on healthy young animals under rigorously controlled laboratory conditions. However, the typical stroke patient is elderly with numerous risk factors and complicating diseases (*e.g.* diabetes, hypertension and heart diseases). Stroke researchers frequently avoid using aged animals for MCAo due to the more complex surgical procedure and high cost of purchasing and raising animals. Very few experimental studies exist in the literature that

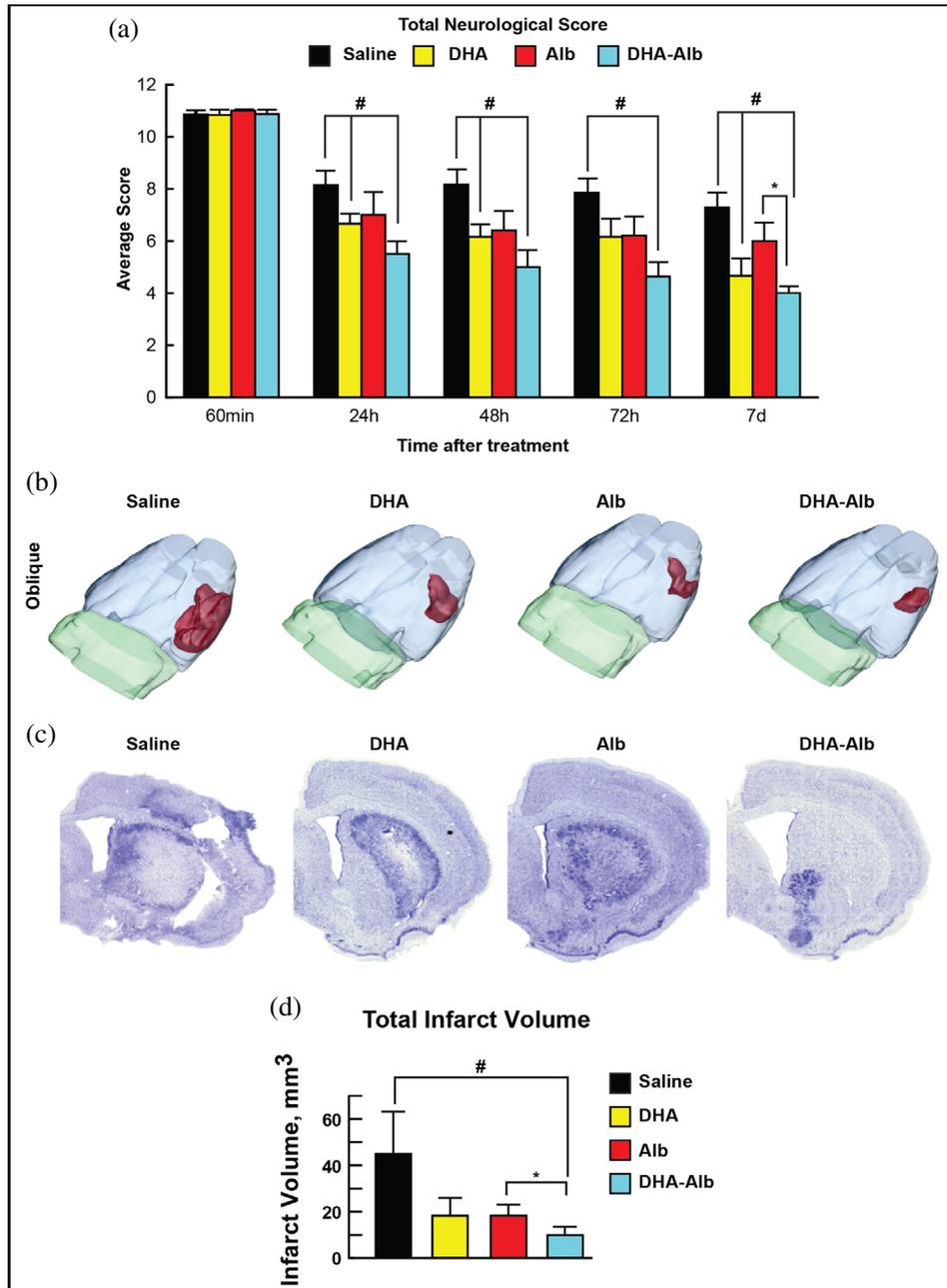


Fig. 2. Effect of DHA-Alb after MCAo in aged rats. (a) Total behavioral score (normal score = 0, maximal score = 12) during MCAo (60 min) and on days 1, 2, 3 and 7 after treatment; (b) 3D reconstructions of MRI-derived lesion volumes from high resolution T2 weighted images; (c) computer-generated MosaiX processed images of Nissl stained paraffin-embedded brain sections; (d) total infarct volume. DHA-Alb treatment significantly improved neurological score, reduced MRI lesion volume and attenuated infarct size compared to the saline group during the 7-day survival period. Values shown are means \pm SEM. # $p < 0.05$ versus saline group; * $p < 0.05$ DHA-Alb versus Alb group (repeated measures ANOVA followed by Bonferroni tests).

have examined middle aged and aging animals, and these have led to somewhat inconsistent results (Liu and McCullough, 2011). This might partly explain the translational failure of neuroprotective drugs in humans.

Recently we have shown that DHA-Alb is neuroprotective after experimental stroke in young rats. To determine whether treatment with DHA-Alb would be protective in aged rodents, SD aged rats (18-months old) received 2 h of MCAo. The behavior was conducted on days 1, 2, 3 and 7 after MCAo. DHA

(5 mg/kg), Alb (0.63 g/kg), DHA-Alb (5 mg/kg + 0.63 g/kg) or saline was administered i.v. 3 h after onset of stroke. *Ex vivo* T2-weighted imaging (T2WI) and histopathology were conducted on day 7. DHA-Alb treatment improved behavioral scores on day 1, 2, 3 and day 7 (Fig. 2a). A reduction in lesion volume was observed in DHA-Alb-treated rats by *ex vivo* MRI (Fig. 2b) and histopathology (Figs. 2c and 2d) compared to saline-treated rats. In conclusion, DHA-Alb therapy is highly neuroprotective in aged rats following focal cerebral ischemia

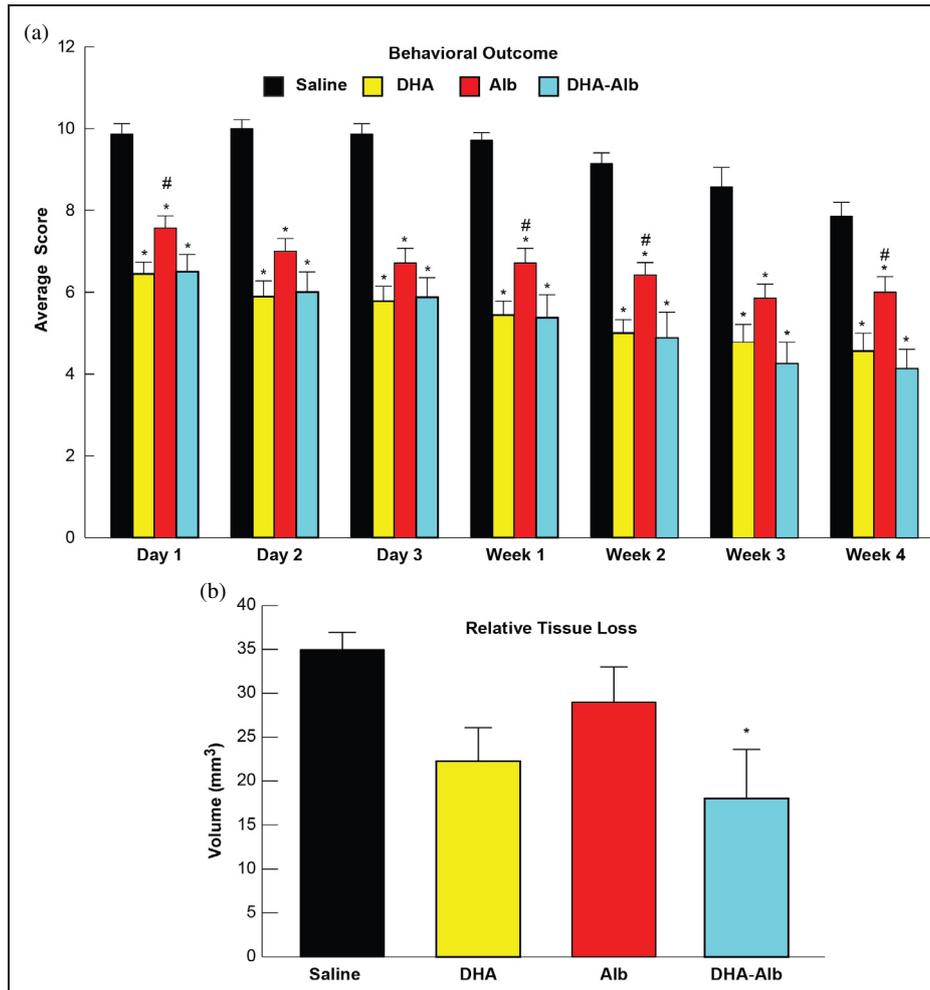


Fig. 3. Effect of DHA-Alb on long term survival after MCAo. (a) Behavioral outcome after stroke (normal score = 0, maximal deficit = 12) during 60 min of MCAo and various time after MCAo. (b) Histopathology at three weeks after stroke. DHA-Alb treatment improved total neurological score and reduced tissue loss on week four after stroke. Values shown are means \pm SEM. # $p < 0.05$ versus saline group; * $p < 0.05$ DHA-Alb *versus* Alb group (repeated measures ANOVA followed by Bonferroni tests).

and has potential for the effective treatment of ischemic stroke in aged individuals.

6 DHA-Alb treatment confers enduring neuroprotection

Postischemic histologic and neurologic responses to ischemia persist for weeks after perfusion has been restored (Sheng *et al.*, 2009). This is relevant to translation of preclinical studies to clinical trials, which typically assess outcome at intervals of several months after insult. Thus, observations made in the first few days after experimental stroke may not predict efficacy in long-term outcome clinical trials. Chronic or late-stage behavioral testing is especially critical when assessing neurorestorative agents (Herson and Traystman, 2014). Not only may benefits of some agents appear only after a period of time, but extended testing can determine whether the beneficial effects of a compound are sustained or simply delay the appearance of a deficit. There are examples of compounds shown to be initially effective after a short survival time but

those initial beneficial effects were lost over time (Valtysson *et al.*, 1994). It should be emphasized that some behaviors initially impaired by stroke recover naturally in rodent models. Thus, it is especially important to be able to distinguish between a test compound's ability to speed normal recovery versus its ability to produce recovery of functions otherwise lost. Several studies have now demonstrated that it is necessary to follow animals for much longer time periods.

Recently we demonstrated that DHA-Alb is highly neuroprotective when animals were allowed to survive during one week. To determine whether treatment with DHA-Alb would persist with chronic survival, SD rats (4-months old) received 2 h of MCAo. The behavior was conducted on days 1, 2, 3 and weeks 1, 2, 3 and 4 after treatment. DHA (5 mg/kg), Alb (0.63 g/kg), DHA-Alb (5 mg/kg + 0.63 g/kg) or saline was administered *i.v.* 3 h after onset of stroke. All treatments improved neurologic score beginning on day 1, which persisted throughout four weeks survival period compared to the saline-treated group (Fig. 3a). DHA-Alb complex enhanced recovery of behavioral function on day 1 and weeks 1, 2 and 4 and reduced tissue loss on week 4 compared to corresponding

Alb-treated group (Fig. 3b). In conclusion, DHA-Alb therapy confers marked and enduring neuroprotection, as assessed by both neurobehavioral and histological methods, in animals followed for 30 days after stroke.

7 Conclusions

Stroke research in animal models has made useful contributions to our understanding of the disease, but animal research and clinical practice still seem to be distant from each other. The clinical failure is mostly caused by the choice of drug and its pre-clinical evaluation, or clinical trial design and analysis. Crucial issues remain unresolved regarding the translation of preclinical developments to the bedside.

We reported here that a novel DHA-Alb therapy is highly neuroprotective in permanent and temporary MCAo in middle-aged rats and persist with chronic survival. This treatment might provide the basis for future therapeutics for patients suffering from ischemic stroke.

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