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Protection of the retina from ischemia-reperfusion injury by L-carnitine in guinea pigs

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PURPOSE. *To investigate the efficacy of L-carnitine in preventing retinal injury followed by ischemia-reperfusion.*
METHODS. *The eyes of 34 guinea pigs were used in this experiment. The guinea pigs were divided into two groups: the first group (n= 17) was given L-carnitine intraperitoneally (500mg/kg) and second group (n=17) received the same dose of saline solution. Under general anesthesia, peritomy was performed. Retro-orbital tissues were ligated for 90 minutes and ischemia was induced, followed by 4 hours of reperfusion. One of the enucleated eye was stained with hematoxylin and eosin (H&E) and retinal thicknesses were evaluated. Thiobarbituric acid reactive substances (TBARS) levels were determined in the retina of the other eye.*
RESULTS. *Mean TBARS levels in retinal tissue were found lower in L-carnitine group (2.77 ± 0.55 µM) than in the control group (6.57 ± 1.19 µM), (p<0.01). On the other hand, mean retinal thickness was found to be increased in the control group (47.47 ± 5.62 µm) when compared to the L-carnitine group (26.52 ± 4.65 µm), (p<0.01). In correlation analysis, significantly positive relationships were found between retinal TBARS level and retinal thickness both in the control and L-carnitine groups (r=0.981, p<0.01 and r= 0.967, p<0.01 respectively).*
CONCLUSIONS. *L-carnitine is effective in preventing retinal injury followed by ischemia-reperfusion. (Eur J Ophthalmol 2003; 13: 80-5)*

KEY WORDS. *Ischemia-reperfusion, L-carnitine, TBARS, Retinal edema*

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INTRODUCTION

Ischemia is defined as a local, temporary oxygen deprivation associated with inadequate removal of metabolites, caused by reduced tissue perfusion. In the eye, ischemia of the retina resulting in irreversible morphological and/or functional changes, was thought to be the consequence of depleted stores of adenosine triphosphate (ATP) resulting from inadequate supplies of glucose and oxygen (1-3). In the past, the tissue injury was attributed to the ischemic insult alone. As already shown in previous studies, both ischemia and reperfusion lead to much greater oxidative damage of the retina, even worse than ischemia alone for the same period (4).

During ischemia, there is a build-up of adenosine resulting from the catabolism of intracellular ATP; it is converted to hypoxanthine and at the same time, a Ca^{2+}-activated protease is thought to catalyse the conversion of xanthine dehydrogenase to xanthine ox-

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idase (5, 6). Reperfusion results in a burst of superoxide anion radicals generated from the oxidation of hypoxanthine to uric acid (4). Consequently, oxygen-derived free radicals formed are believed to lead to peroxidation of lipids in the cell membrane, modification of protein and nucleic acids, and change in cellular calcium homeostasis (7, 8). In addition to causing tissue damage directly, free radicals are also responsible for stimulating the release of excitatory amino acids (especially glutamate), which results in a rapid cellular efflux of potassium and influx of sodium, calcium, and chloride with obligated water (4). The combined effects of free radical release, osmotic swelling of cell, and activation of lytic enzymes lead to severe damage to cell and even cell death (2, 5, 9).

In lipid peroxidation, the fatty acid side chains of the membrane phospholipids are oxidised to unstable hydroperoxides and they breakdown to form many aldehydes such as malondialdehyde (8,10). Therefore, the TBARS, an index of lipid peroxidation level, reflects the severity of the lipid peroxidation (11,12).

Various pharmacologic agents might serve to speed up reenergisation after ischemic tissue injury. L-carnitine (3-hydroxy-4-N-trimethonium butyrate) is crucial for mitochondrial energy production via ß oxidation of fatty acids (13). It has been demonstrated in several different models that L-carnitine can increase the metabolic rate of mitochondria, thus improving mitochondrial oxygen utilization in these systems, which in turn may counteract some toxic effects to the ischemically injured tissue (14).

Up to date, we could not find any study investigating the protective effect of L-carnitine in retinal ischemia-reperfusion, in the literature. Therefore, in this study we aimed to investigate the efficacy of L-carnitine supplementation in preventing retinal injury followed by ischemia-reperfusion.

METHODS

Induction of ischemia and reperfusion

All procedures involving guinea pigs adhered to the ARVO Resolution on the Use of Animals in Research.

The experiment was performed using 34 guinea pigs (250 -350 g body weight), which were randomly divided into two groups with 17 animals per groups. Forty-five minutes before ischemic insult, L-carnitine (Camitine amp, Sigma-tau, Rome) at a dose of 500 mg/kg body weight was given intraperitoneally to the first group and the same amount of saline solution (2.5 ml/kg) was given to the second group with the same route.

The pupils of the experimental animals were maximally dilated with tropicamide HCl (1%) and phenylephrine (2.5%), and guinea pigs were anesthetized with an intramuscular injection of ketamine HCl (Ketalar, Parke Davis, UK, 50 mg/kg) and anesthesia was maintained throughout the experiment.

The lids were retracted by suture. At the onset of each experiment, after the bulbar conjunctiva had been incised around the limbus and dissected posteriorly, a suture was placed behind the globe. The suture ends were inserted into a plastic tube, which was rounded on the side facing the globe; including the central retinal artery, ciliary arteries, and the retrobulbar connective tissue. Ischemia was induced by pulling the suture through the plastic tube. Reperfusion was performed by cutting off the top part of the tube and removing the suture and tube. The successful induction of ischemia and adequacy of reperfusion were confirmed visually with an ophthalmoscope.

Histopathological evaluation

In the present study, the eyes were subjected to 90 minutes of regional ischemia followed by 4 hours of reperfusion. At the end of reperfusion period, the animals were sacrificed with over doses of pentobarbital. After the sclera was marked superiorly and nasally with sutures, the eyes were enucleated and rapidly cut open and divided into two halves by coronal section through the ora serrata, and vitreous was removed. The posterior half of the eyes was fixed in 10 % neutral buffered formaldehyde solution, dehydrated in graded series of ethanol and embedded in paraffin. Sagittal sections of the whole eye were cut in 5 µm thickness and stained with hematoxylin and eosin. Beginning from the nasal part of the optic disc, 10 slides were selected from the slides passing from optic disc by systematic random sampling (one slide out of each 10 slides was taken). The images of the slides, examined under light-microscope at the high power magnification (x400), were monitorized and the retinal thickness was measured in micron level by a size

schedule with millimetric disc. The thicknesses of the monitorized images of the retina were measured blindly from three separate regions: far from of the optic disc at 500 and 2000 µm in the superior retina and at 2000 µm in the inferior retina. Three areas on each slide were measured containing the inner nuclear, inner plexiform and ganglion cells' layers in the sagittal sections (15) and then the average thickness value of the inner retina for each eye was determined by the mean of 30 measurements.

TBARS assay

The retinas of the other eyes were removed separately using operation microscope and put in a tamponade solution. Then it was homogenized in a vibrator for two minutes and frozen in liquid nitrogen. The specimen was stored at -80 °C until biochemical analysis. TBARS level was estimated according to the method of Satoh (16). Briefly, 2.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid are added to 0.5 ml of supernatant, and then the mixture is heated in boiling water for 30 minutes. The resulting chromogen is extracted with 4 ml of n-butyl alcohol and absorbance of the organic phase is determined at 530 nm. Malondialdehyde-bis-acetal was used as a standard and values were presented as µM. In this study we used the thiobarbituric acid method for determination of lipid peroxidation, and total thiobarbituric acid reactive materials were expressed as TBARS. Although this method is not specific for malondialdehyde, measurement of TBARS is an easy and reliable method, which is used as an indicator of lipid peroxidation and free radical activity in biological samples.

Statistical analysis

The results were given as mean ± standard deviation. Statistical analysis was carried out by Mann-Whitney U test for the comparison of the group. Correlation between variables was calculated by Spearman Rank's Correlation Analysis. A probability <0.05 was considered significant.

RESULTS

As seen from Table I, mean TBARS levels were 2.77 ± 0.55 µM (range: 1.47 -3.42 µM) in L-carnitine group and 6.57 ± 1.19 µM (range: 4.15 - 9.0 µM) in the control group. The difference between two groups was statistically significant ($p<0.01$). Mean retinal thickness was found to be significantly increased in the control group (47.47 ± 5.62 µm) when compared to the L-carnitine group (26.52 ± 4.65 µm), ($p<0.01$). In correlation analysis, there were positive correlations between TBARS levels and retinal thickness values in both of L-carnitine ($r=0.967$, $p<0.01$) and control groups ($r=0.981$, $p<0.01$). Figures 1 and 2 show the correlation between TBARS levels and retinal thickness of both groups. Edema in inner plexiform layers of L-carnitine and control groups were illustrated in Figures 3 and 4. Histopathological changes, such as edema of inner plexiform layers, were limited mostly to the inner part of retina.

DISCUSSION

The results of this study reveal that L-carnitine exerts a significant neuroprotective effect in the guinea pig retinas when administered before transient ischemia-reperfusion insult.

As already shown in previous studies, it was demonstrated that both ischemia and reperfusion lead to oxidative damage of the retina (2, 4). Tissues are normally protected from oxidative damage by two major mechanisms: antioxidant enzyme system (such as superoxide dismutase, catalase and the peroxidases),

TABLE I - TBARS LEVELS AND RETINAL THICKNESSES IN THE L-CARNITINE AND CONTROL GROUPS

	n	L-carnitine group	Control group	p
TBARS level (µM)	17	2.77 ± 0.55	6.57 ± 1.19	<0.001
Retinal thickness (µm)	17	26.52 ± 4.65	47.47 ± 5.62	<0.001

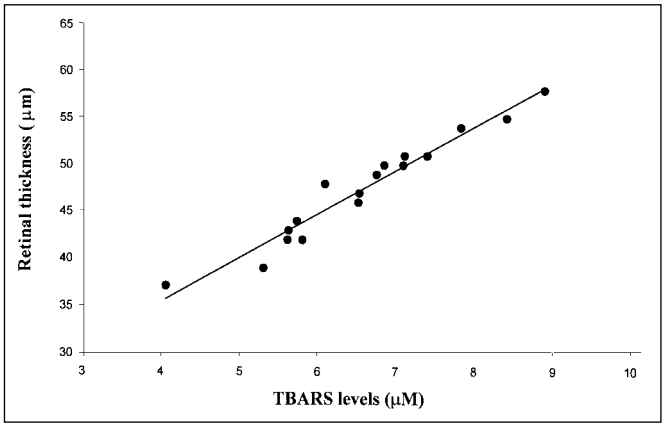

Fig. 1 - *TBARS levels and retinal thicknesses in the control group.*

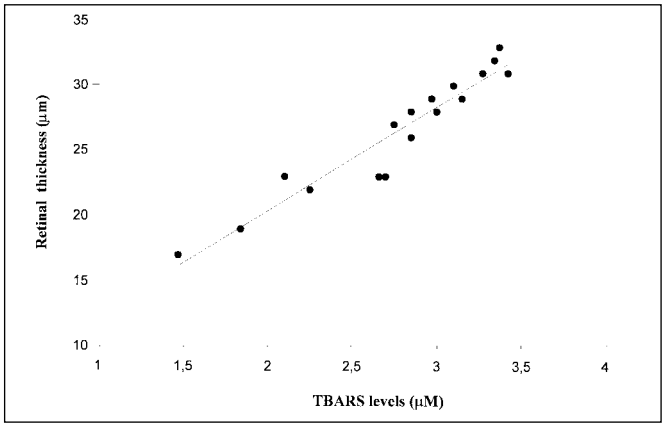

Fig. 2 - *TBARS levels and retinal thicknesses in the L-Carnitine group.*

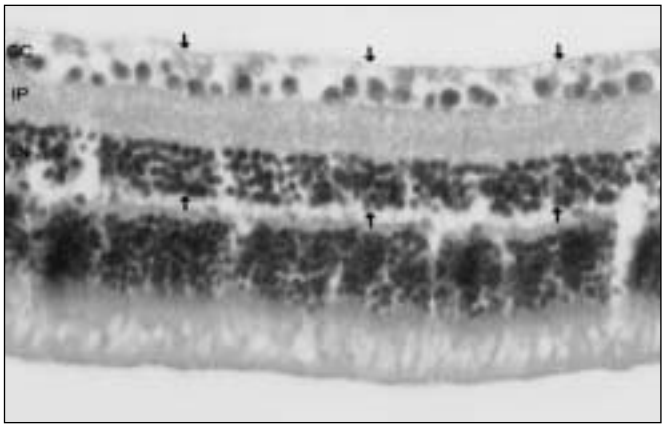

Fig. 3 - *Retinal thickness illustrated in the L-carnitine group (H&E x400). GC, Ganglion cell layer; IP, Inner plexiform layer; IN, Inner nuclear layer; Distance between the arrows, retinal thickness.*

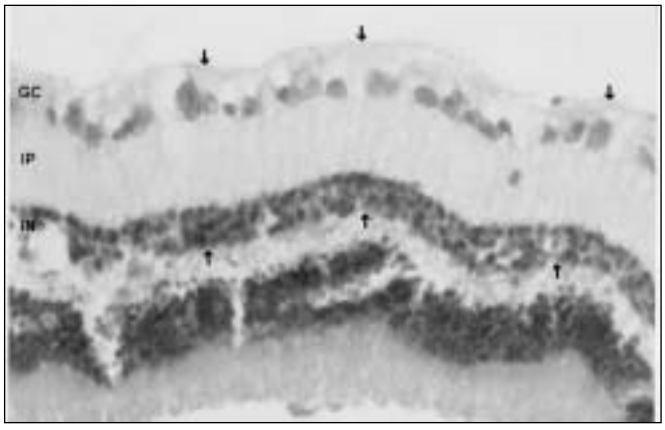

Fig. 4 - *Retinal thickness illustrated in the control group (H&E x400). GC, Ganglion cell layer; IP, Inner plexiform layer; IN, Inner nuclear layer; Distance between the arrows, retinal thickness.*

organic free-radical scavengers (such as ascorbate and vitamin E) (4, 9). However, after ischemia-reperfusion the load of free radicals can exceed the defensive capacity of endogenous scavenger mechanisms and result in tissue damage.

Many substances have been used in the prevention of oxidative damage due to production of free radicals exceeding the endogenous capacity of free radical scavenger mechanism during ischemia-reperfusion (4-6). In the present study, we investigated the possible preventive role of L-carnitine (which has been used previously to prevent ischemia-reperfusion injury in brain, heart and intestine tissues) in ischemia-reperfusion of retinal tissue (14,17-20).

Carnitine is required for the transport of long chain fatty acids into the mitochondrial matrix, allowing their catabolism by beta-oxidation (21). Thus carnitine is crucial for mitochondrial energy production. Many metabolic changes that occur with ischemia are apparently secondary to the intramitochondrial accumulation of acyl-CoA, which is known to be toxic to many different enzyme systems such as pyruvate dehydrogenase. L-carnitine is used to remove acyl groups from long-chain acyl-CoA, thereby restoring the normal intramitochondrial acyl-CoA/free CoA ratio (22).

For a tissue to recover from ischemic insult, the mitochondria must be rapidly re-energized during reperfusion so that ATP can once again be produced and ionic homeostasis restored. Unfortunately, this restoration is retarded even after a brief period of ischemia. Various pharmacological agents might serve

to speed up re-energisation after ischemic neuronal injury. It has been demonstrated in several different models that L-carnitine can increase the metabolic rate of mitochondria, thus improving mitochondrial oxygen utilization in these systems, making this a potentially useful drug to counteract some of the effects of ischemic brain injury (23). Rosenthal et al (14) investigated mechanisms of ischemia/reperfusion injury to brain tissue and the use of acetyl-L-carnitine in the canine cardiac arrest model. It is shown that normalization of cerebral cortex lactate levels and lactate/pyruvate ratios present at a very early stage of reperfusion in the carnitine group, suggested that carnitine may, at least partially, exert a therapeutic effect by potentiation of aerobic energy metabolism and inhibition of glycolytic lactic acidosis which is known to increase the ischemic injury (14, 24).

In the literature, there are different ischemia-reperfusion models for retinal tissue in terms of time duration. For example, Szabo et al (15) investigated rat eyes subjected to 30, 60, or 90 min ischemia followed by 0.5, 1, 2, 4 and 24 hr of reperfusion, respectively and observed that most serious retinal damage was detected in 90 min of ischemia followed by 4 hr of reperfusion; this retinal damage (edema) did not return to the preischemic values even after 24 hr of reperfusion. So, we also preferred a model with 90 min of ischemia followed by 4 hr of reperfusion. Szabo et al (15) observed marked cellular alterations especially in the inner part of retina at 90 min ischemia followed by reperfusion, using light microscope. However minimal changes were seen in the photoreceptor layer. Therefore, in our study we measured thickness of the inner part of retina in which ganglion cells, inner plexiform and inner nuclear layers were included.

As seen from Figures 3 and 4, retinal edema was more pronounced in the saline group than in the L-carnitine group; this finding implyed that retinal tissue was better preserved in the L-carnitine group.

Because free radicals and lipid peroxides occurring after oxidative stress are unstable and have a short life-span, TBARS with long life-span is used as a biochemical parameter evaluating the level of lipid peroxidation (11, 25). Therefore, we also used TBARS levels in the retinas of each group to determine lipid peroxidation. Positive correlations were observed between TBARS levels and retinal thickness values in L-carnitine and control groups. It was observed that retinal edema was increased as the TBARS levels increased. In L-carnitine group, both TBARS levels and retinal thicknesses were lower compared to the control group.

During our investigations, we could not find any previous reports about whether L-carnitine given intraperitoneally reaches the retina or not. In Literature, it has been indicated that L-carnitine administration by the intraperitoneal route reaches various tissues, such as brain and renal tissue, etc (26, 27). Recently, it has also been reported that L-carnitine transport systems exist in the brain capillary endothelial cells, retinal pigment epithelial cells and cerebral cortex neurons (28-30). These observations led us to speculate that L-carnitine given intraperitoneally may reach adequate concentrations in retinal tissues. In addition, our results show that low TBARS levels and retinal thickness values in the L-carnitine group compared with the controls could support that L-carnitine reaches retinal tissues and is effective on them.

It can be said that, as ATP level is adequate in the medium, the production of free oxygen radicals will be decreased, lipid peroxidation, cellular damage and retinal edema will therefore be minimized. In the control group, TBARS levels and retinal thicknesses were higher compared to the L-carnitine group. This may be due to the absence of mechanisms increasing ATP production mentioned in the L-carnitine group.

These data show that L-carnitine could be effective in preventing retinal injury followed by ischemia-reperfusion. Detailed studies are required to clarify this condition.

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REFERENCES

1. Ducker JS. Vascular disorders, retinal arterial obstruction. In: Yanoff M, Ducker JS, eds. Ophthalmology. London: Mosby 1999; 17:1-8.
2. Ophir A, Berenshtein E, Kitrossky N, et al. Hydroxyl radical generation in the cat retina during reperfusion following ischemia. Exp Eye Res 1993; 57: 351-7.
3. Kohner EM, Chibber R. Retinal ischemia. In: Ryan SJ, Schachat AP, eds. Retina, vol 2. St. Louis: Mosby, 2001; 982-94.
4. Nayak MS, Kita M, Marmor MF. Protection of rabbit retina from ischemic injury by superoxide dismutase and catalase. Invest Ophthalmol Vis Sci 1993; 34: 2018-22.
5. Ophir A, Berenshtein E, Kitrossky N, Averbukh E. Protection of the transiently ischemic cat retina by zinc-desferrioxamine. Invest Ophthalmol Vis Sci 1994; 35: 1212-6.
6. Agustin AJ, Spitnas M, Koch F, Grus F, Lutz J. Effects of perfluorooctylbromide and vitamine E on ischemia induced retinal oxidative tissue damage. Exp Eye Res 1998; 66: 19-24.
7. Farber JL. Biology of the disease, membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. Lab Invest 1982; 47: 114-23.
8. Anderson RE. Biochemistry and metabolism, free radicals and antioxidants. In: Hecht KA, Straus H, Denny M, Taylor F, Gamet M. eds. Fundamentals and Principles of Ophthalmology. San Francisco: Am Acad Ophthalmology 1999; 372-80.
9. Kim SY, Kwak JS, Shin JP, Lee SH. The protection of the retina from ischemic injury by the free radical scavenger Egb 761 and zinc in the cat retina. Ophthalmologica 1998; 212: 268-74.
10. Halliwel B, Gutteridge JMC. Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet 1984; 8391: 1396-2007.
11. Siu AW, Reiter RJ, To CH. Pineal indoleamines and vitamin E reduce nitric oxide-induced lipid peroxidation in rat retinal homogenates. J Pineal Res 1999; 27: 122-8.
12. Uchiyama M, Mihara M. Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Ann Biochem 1978; 86: 271-8.
13. Swamy-Mruthinti 5, Lee Carter A. Acetyl-L-Carnitine decreases glycation of lens proteins: *in vitro* studies. Exp Eye Res 1999: 69: 109-15.
14. Rosenthal RE, Williams R, Bogaert YE, Getson PR, Fiskum G. Prevention of post ischemic canine neurologicai injury through potentiation of brain energy metabolism by acetyl-L-carnitine. Stroke 1992; 23: 1312-8.
15. Szabo EM, Droy-Lefaix MT, Doly M, Carre C, Braquer P. Ischemia and reperfusion-induced histologic changes in the rat retina. Invest Ophthalmol Vis Sci 1991; 32: 1471-1478.
16. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta 1978; 90: 37-43.
17. Slivka A, Silbersweig D, Pulsinelli W. Carnitine treatment for stroke in rats. Stroke 1990; 21: 808-11.
18. Loster H, Keller T, Grommisch J, Grunder W. Effects of L-carnitine and its acetyl and propionyl esters on ATP and PCr levels of isolated rat hearts perfused without fatty acids and investigated by means of 31P-NMR spectroscopy. Mol Cell Biochem 1999; 200: 93-102.
19. Ferrari R, Cucchini P, Visioli O. The metabolic effects of L-camitine in angina pectoris. Int J Cardiol 1984; 5: 213-6.
20. Stroh R, Chrostoper TA, Lopez BL, Guo YP, Amico-Roxas M, Ma XL. L-propionyl carnitine, an endogenous ester in fatty acid metabolism, exerts anti-shock and endothelial protective effects in rat splanchnic ischemia-reperffision injury. Shock 1998; 9: 216-22.
21. Cotter MA, Cameron NE, Keagan A, Dines KC. Effects of acetyl- and proprionyl-L-Carnitine on peripheral nerve function and vascular supply in experimental diabetes. Metabolism 1995; 44: 1209-14.
22. Bohan TP. Editorial comment. Stroke 1992; 23: 1317-8.
23. Bellei M, Battelli D, Guarriero DM, et al. Changes in mitochondrial activity caused by ammonium salts and the protective effect of carnitine. Biochem Biophys Res Commun 1989; 158: 181-8.
24. Hansford RG. Control of mitochondrial substrate oxidation. Curr Topics Bioenerg 1980; 10: 217-78.
25. Chida M, Suziki K, Nakanishi-Ueda T, et al. *In vitro* testing of antioxidants and biochemical end-points in bovine retinal tissue. Ophthalmic Res 1999; 31: 407-15.
26. Koudelova J, Mourek J, Drahota Z, Rauchova H. Protective effect of carnitine on lipoperoxide formation in rat brain. Physiol Res 1994; 43: 387-9.
27. Palomba D, Pes GM, Demontis MP, Varoni MV, Deiana L, Anania V. Metabolic and renal effects of Laevo-carnitine and propionyl-carnitine in rats with subtotal nephrectomy. Pharmacol Res 1996; 34:161-5.
28. Kido Y, Tamai I, Ohnari A, et al. Functional relevance of carnitine transporter OCTN2 to brain distribution of L-carnitine and acetyl-L-carnitine across the blood-brain barrier. J Neurochem 2001; 79: 959-69.
29. Nakanishi T, Hatanaka T, Huang W, et al. Na+- and C1--coupled active transport of carnitine by the amino acid transporter ATB (0, ±) from mouse colon expressed in HRPE cells and Xenopus oocytes. J Physiol 2001; 532: 297-304.
30. Wawrzenczyk A, Sacher A, Mac M, Nalecz MJ, Nalecz KA. Transport of L-carnitine in isolated cerebral cortex neurons. Eur J Biochem 2001; 268: 2091-8.