EFFECT OF MAHARISHI AMRIT KALASH ON AGE DEPENDENT VARIATIONS IN MITOCHONDRIAL ANTIOXIDANT ENZYMES, LIPID PEROXIDATION AND MITOCHONDRIAL POPULATION IN DIFFERENT REGIONS OF THE CENTRAL NERVOUS SYSTEM OF GUINEA-PIGS

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SUMMARY

Age related changes in the mitochondria of different regions of the CNS of two age groups of guinea-pigs (10 months and 32 months) were studied. The activities of glutathione peroxidase (GPx) and glutathione reductase (GRd) decreased significantly ($p < 0.05$) with age in the mitochondrial fractions of cerebral cortex, hypothalamus, cerebellum and spinal cord. A significant ($p < 0.05$) age related decrease in mitochondrial numerical density was observed in all regions studied. Electron microscopic observations revealed various degenerative changes in the mitochondria with age. Treatment of the animals with the Ayurvedic herbal mixture "Maharishi Amrit Kalash" (MAK), 500

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mg/kg body wt. daily for 2 months, significantly induced the activity of antioxidant enzymes, and also reversed the pathological changes to a considerable extent. MAK increased the activity of GPx significantly only in the 32 month-old animals. This shows the specificity of the action of MAK.

KEY WORDS

mitochondrial numerical density, lipid peroxidation, Maharishi Amrit Kalash, guinea-pig

INTRODUCTION

Free radicals are thought to be involved in a number of degenerative processes and their role in the ageing process is established /1-4/. Since mitochondria are the site of production of free radicals, there is reason to believe that they are liable to oxidative damage and that this can lead to various pathological conditions. A number of studies has been carried out to correlate the activities of antioxidant enzymes with ageing and the maximum life-span of various organisms. So far there are few reports which differentiate cytoplasmic and mitochondrial forms of enzymes when measuring enzyme activities, especially in the nervous system /5-7/. In the present study an attempt was made to determine age dependent mitochondrial numerical density, ultrastructural changes and the activities of antioxidant enzymes in specified areas of the CNS.

Ayurveda, the 'science of life', is an ancient system of health care practiced in India. Among the various branches of Ayurvedic medicine, Rasayana Tantra (preventive medicine of promotion therapy) lists several hundred food preparations called the 'Rasayanas' (Charkasamhita). In general, these 'Rasayanas' are derived from a wide range of plants, minerals and dairy products. Traditionally, these food supplements are used to strengthen the immune system and retard ageing. In Ayurveda, it is general practice to administer drugs in balanced combinations (a process called 'Sam yoga'), which aims at having beneficial effects on multiple organ systems, reducing toxicity and increasing efficacy /8/. Maharishi Amrit Kalash (MAK) is a herbal mixture prepared according to the ancient ayurvedic formulation.

MAK is available in two forms, MAK-4 and MAK-5. MAK-4, in the form of tablets, is called ambrosia; MAK-5, in the form of paste, is called nectar. The different components of MAK-4 and MAK-5 have been described previously /10,11/. According to Charka Samhita (an ayurvedic medical text), there is no maximum dose of this formulation; it can be taken in any the amount that does not disturb normal food consumption. MAK has shown promise in protection against free radicals attack /9,10,11/, inhibition of the growth of mammary tumors and lung carcinoma /12,13/, enhancement of lymphoproliferative response /14/, and modulation of opiate receptors /15/. MAK has been found to increase the activity of cytosolic antioxidant enzymes in aged guinea-pigs /16/. Since MAK contains many antioxidants /12-14/, and it is described in the Charka Samhita as one preparation that has the power to retard ageing, an attempt was made to determine its effect on the activity of antioxidant enzymes and mitochondrial structural integrity in young and old guinea-pigs.

MATERIALS AND METHODS

Male guinea-pigs (Dunkin Harley) aged 8 months and 30 months were used in the present study. Each group was subdivided into two groups, each consisting of 40 animals. One subgroup served as control and the other as the experimental group and was given a mixture of MAK-4 and MAK-5 in the ratio of 1:20. MAK was given orally at a dose of 500 mg/kg body weight daily at 11.00 hours for two months. Both groups were fed pelleted food (Hindustan Lever Ltd., New Delhi) *ad libitum.*

Oxidized and reduced glutathione and glutathione reductase were purchased from Sigma Chemical Co., USA. Dithiobisnitrobenzoic acid was purchased from Fluka Chemical Co., Switzerland. Other chemicals were purchased from CSIR Center for Biochemicals, New Delhi, SRL or SD-Fine Chemical Co., Mumbai, India and were of analytical grade. The Ayurvedic preparation MAK was a generous gift from the Maharishi Ayurveda Corporation Ltd., Faridabad, India.

After the experimental period, the animals were sacrificed by decapitation, brains and spinal cords were removed immediately and rinsed in chilled normal saline. The different regions of the CNS, *viz.*, cerebral motor cortex, hypothalamus, cerebellum and cervical spinal cord, were separated. Samples were homogenized and 10% homo-

genate was prepared in 0.32 M sucrose solution using a glass homogenizer. The subsequent fractionation and washing were carried out at 0-4°C using a Remi high speed centrifuge. The purity of the mitochondrial fraction was determined by assay of the mitochondrial marker enzyme SDH and by electron microscopic observations.

The activity of glutathione peroxidase (GPx) was assayed by monitoring the oxidation of NADPH at 340 nm for 3 min using H_2O_2 as substrate /17/. The enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein. Glutathione reductase (GRd) activity was determined by measuring μmoles of reduced glutathione formed/min/mg protein /18/. Lipid peroxidation was quantified by the thiobarbituric acid color reaction /19/. Protein was measured by a standard method /20/.

For electron microscopy at the termination of experiment, transcardiac perfusion was carried out with a mixture of chilled 2% glutaraldehyde and 1% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains and spinal cords were immediately removed and placed in fresh chilled fixative. The specimens were stored in the same fixative for 2 h at 4°C. The motor cortex, cerebellum, hypothalamus and cervical spinal cord were then separated and diced into small blocks of tissue. These were rinsed in phosphate buffer (0.1 M, pH 7.4), post-fixed for 4 h in 1% osmium tetraoxide, dehydrated in graded ethanol series and embedded in epon 812. Ultra-thin sections were cut with an LKB ultratome using a glass knife, then stained with uranyl acetate and lead citrate.

Mitochondria were quantitatively analyzed by counting total recognizable mitochondria. The complete cross-sectional profiles of neuronal perikarya from 5-8 randomly selected micrographs of each group of animals were analyzed. Average numbers of mitochondria per cross-section were calculated. Neuronal cross-sectional size was not taken into account, in order to select micrographs as randomly as possible.

Statistical analysis

Results are expressed as means ± SEM and were analyzed by one way ANOVA followed by Duncan's multiple range test to detect inter-group differences.

RESULTS

GPx activity, shown in Table 1, was significantly decreased in the aged animals. The decrease was in the range of 22-50% in different tissues. MAK treatment induced increased GPx activity in all regions of the aged animals. It was effective in the younger animals. This shows the specificity of the action of MAK. GRd activity did not change with age except in the cerebellum of the aged animals (30% decline). MAK treatment increased GRd activity significantly in all regions (Table 2). The *in vitro* production of thiobarbituric acid reactive substances (TBARS) was lower in the older animals than in the younger ones. The maximum difference was observed in the cervical spinal cord (45.49%) and minimum in the hypothalamus (28.10%). MAK treatment, however, did reduce *in vitro* TBARS production in both age groups of guinea-pigs (Table 3).

Mitochondrial numerical density was found to be significantly decreased in the older animals. Mitochondrial numerical density increased after treatment with MAK (Table 4). The mitochondria in the neurons of older animals were swollen (Fig. 1); the structural integrity of the mitochondria was disturbed in the older neurons. Degeneration of cristae and deposition of membranous structures in the mitochondria were common in the older neurons (Fig. 2). In addition, a lot of lysosomal activity was observed near the degenerating mitochondria (Fig. 3).

DISCUSSION

The activity of GPx was highest in the spinal cord followed by the cerebellum, hypothalamus and cerebral cortex. However, GRd did not follow the same pattern. The highest GRd activity was observed in the hypothalamus, followed by the spinal cord, cerebellum and cerebral cortex. Variability in the activities of other antioxidant enzymes /16/ indicates marked variability in the degree of susceptibility to peroxidative reactions as a function of age in different regions of the CNS.

Mitochondria are considered as the major source of free radicals, even in the absence of pathological conditions, in the form of O_2^- and H_2O_2. In addition, a large body of evidence exists which states that mitochondrial genomic abnormalities occur with ageing and age-

TABLE 1

Effect of MAK on the activity of mitochondrial glutathione peroxidase

Tissue	Young animals (10 months)		Old animals (32 months)	
	Control	Treated	Control	Treated
Cerebral cortex	25.90±1.67	27.97±2.31	20.08±1.15**	26.84±2.87*
Hypothalamus	30.00±2.26	31.47±3.40	20.76±1.66**	29.29±1.38*
Cerebellum	36.90±2.27	38.82±2.33	24.21±2.19**	35.23±1.56*
Spinal cord	40.97±2.51	42.38±1.82	30.13±1.53**	37.52±1.32*

Data are means±SEM of 5 animals.
The activity of enzyme is expressed as μmoles NADPH oxidized/min/mg protein.
* $p < 0.05$ vs controls within the same age group.
** $p < 0.05$ vs young controls.

TABLE 2

Effect of MAK on the activity of mitochondrial glutathione reductase

Tissue	Young Animals (10 months)		Old Animals (32 months)	
	Control	Treated	Control	Treated
Cerebral cortex	0.19±0.02	0.27±0.04*	0.17±0.01	0.26±0.01*
Hypothalamus	0.22±0.02	0.28±0.01*	0.21±0.01	0.28±0.02*
Cerebellum	0.20±0.02	0.32±0.02*	0.14±0.03**	0.26±0.01*
Spinal cord	0.21±0.05	0.22±0.01	0.20±0.04	0.32±0.04*

Data are means±SEM of 5 animals.
The activity of enzyme is expressed as μmoles GSH formed/min/mg protein.
* $p < 0.05$ vs controls within the same age group.
** $p < 0.05$ vs young controls.

TABLE 3

Effect of MAK on thiobarbituric acid reactive substances (TBARS) produced after incubation with mitochondrial protein in air for one hour

Tissue	Young animals (10 months)		Old animals (32 months)	
	Control	Treated	Control	Treated
Cerebral cortex	13.43±0.19	10.11±0.33*	7.83±0.55**	5.87±0.34*
Hypothalamus	9.43±0.17	7.23±0.22*	6.78±0.13**	4.80±0.27*
Cerebellum	14.78±0.19	10.43±0.31*	8.98±0.23**	6.10±0.21*
Spinal cord	11.98±0.67	8.64±0.45*	6.53±0.46**	4.61±0.68*

Data are means±SEM of 5 animals.
The figures in the table are μmoles of TBARS/mg protein.
* $p < 0.05$ vs controls within the same age group.
** $p < 0.05$ vs young controls.

TABLE 4

Effect of MAK on numerical density of mitochondria

Tissue	Young animals (10 months)		Old animals (32 months)	
	Control	Treated	Control	Treated
Cerebral cortex	23.36±1.41	24.37±1.53	17.14±1.71**	21.25±1.43*
Hypothalamus	29.43±2.14	30.46±1.84	21.23±2.13**	26.38±1.54*
Cerebellum	35.52±1.85	37.53±1.76	27.20±1.98**	34.30±1.75*
Spinal cord	33.50±1.52	35.50±1.87	27. 30±1.76**	32. 38±1.24*

Data are means±SEM of 6 animals.
The values in the table are the number of mitochondria per neuronal perikaryon from 6 micrographs.
* $p < 0.05$ vs controls within the same age group.
** $p < 0.05$ vs young controls.

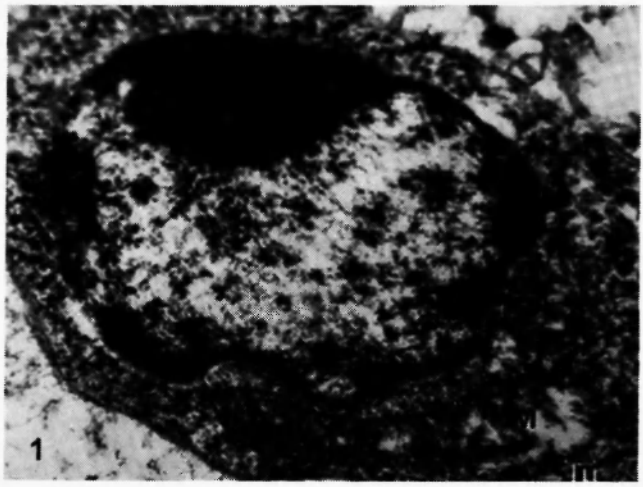

Fig. 1: An exceptionally large swollen mitochondrion (M) covering a considerable cytoplasmic area in a Purkinje neuron of a 32 month-old guinea-pig's cerebellum.

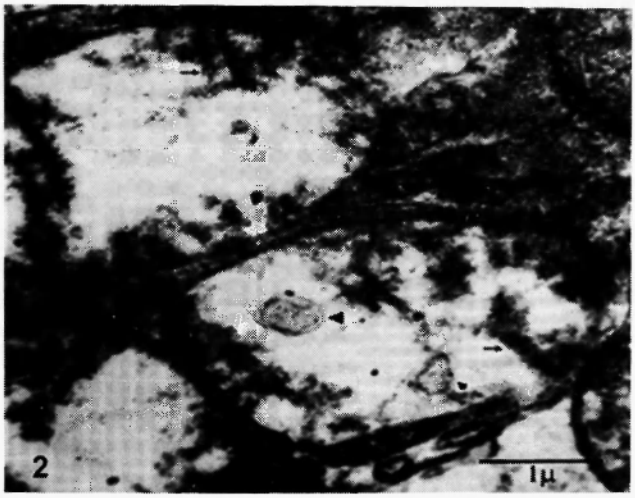

Fig. 2: Mitochondria whose cristae have been lost, harboring small membranous inclusions in the matrix (arrow).

Fig. 3: Mitochondrion with degenerated cristae. Accumulation of lysosomes (L) around the degenerating mitochondrion (M). The lysosomal material appears to be leaching out into the mitochondrial matrix.

associated neurological disorders /21,22/. Some mitochondrial deletions (such as the 5 kb deletion) have been found to be specifically localized in abnormally swollen and degenerating (lacking cristae) mitochondria, fused with lipofuscin /23/. This strongly suggests the involvement of free radicals in mitochondrial degeneration. The present study suggests that the age-related decline in mitochondrial antioxidant enzymes is associated with an increased number of swollen mitochondria in the studied regions of the CNS. Thus the abnormal mitochondria may produce more H_2O_2 which can diffuse into the cytoplasm and lead to the formation of peroxides in the cytoplasm in aged animals /16/.

The incubation of mitochondrial proteins in the presence of air and reaction with thiobarbituric acid provides an assessment of potential substrate available for peroxidation /16/. Lower amounts of TBARS production *in vitro* in the older animals than in the younger ones indicate decreased susceptibility to lipid peroxidation in older animals. The decrease in the mitochondrial numerical density with aging is in accordance with studies on the liver and heart in the mouse /24,25/, but no such study has yet been carried out on the CNS. The reduction

of mitochondrial numerical density and loss of mitochondrial cristae may be connected to the fact that the activity of various antioxidant enzymes is decreased with the advance of age, thus making the mitochondria more vulnerable to free radical damage. The accumulation of lipofuscin in the mitochondria of older animals also supports this assumption. The appearance of the membranous complex in the mitochondria of older animals can be compared with the increased proportion of membranous complex in the cytoplasm of aged neurons /26-28/. The origin of such membranous complexes is still unclear, though they appear to be one of the stages of disintegration due to enhanced free radical attack. MAK treatment was helpful in increasing mitochondrial numerical density, which might be due to the induction of mitochondrial division of the large swollen mitochondria, which were also observed in the liver of rats /25/. It has been observed that increased free radical accumulation due to decrease in the antioxidant enzymes can arrest cell division, ultimately leading to degeneration through membrane damage /4/. This situation can be reversed by increasing the level of antioxidant enzymes and detoxifying the free radicals. MAK successfully induced the activity of antioxidant enzymes. It also contains potent antioxidant components and hence it could increase the mitochondrial numerical density. Thus it is concluded that MAK can be helpful in protecting against free radical mediated damage to cell organelles and thereby it can thwart various age-associated pathological conditions.

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