LIPOFUSCIN ACCUMULATION IN NEURONS WITH RESTRAINT STRESS

A.Chaudhary*, S.P.Sharma and T.J.James

Department of Zoology, Kurukshetra University, Kurukshetra-132 119 Haryana (India).

Key Words

Cerebella, Lipofuscin, Restraint Stress, Wistar rats.

Abstract

Stress induced lipofuscinosis was studied in rat cerebellum. 3 month old wistar rats were subjected to restraint stress by keeping them immobile for 24, 48 and 72 hours duration. This was achieved in specially prepared cages which allowed no space for the rats to move; giving a stress to the animal. The cerebella from the stressed groups rats were removed after the experiment and were processed for fluorescent microscopical, histochemical and fluorimetric study of lipofuscin. The lipofuscin content in the Purkinje neurons was compared with that of the control rats which were of the same age, size and weight as of the experimental rats. The results showed that the lipofuscin content in the neurons of the experimental rats was more than that of the control ones. In gist, while 24 hrs. stress caused a 28.9% increase in lipofuscin content, 48 hrs. stress resulted in a 38.3% increase. This shows that restraint stress can be a good experimental model for lipofuscinogenesis and ageing studies.

Introduction

The concept of stress was first introduced in life sciences by Selye [1]. It is now widely used to refer either to a stimulus (external force acting on an organism) or to a response (change in physiology, morphology and anatomy), or to an interaction (mutual action between external force and resistance opposed to it). In other words, it is a combination of all the above factors [1]. The biological-oriented approach to stress is response-oriented. It views the interaction of the organism as an attempt to interact with the environment. In fact, each individual needs a moderate amount of stress to be alert and capable of functioning effectively in an organization [2]. This view has been supported by the studies of Mathew [3] and Pestonjee [4].

However, studies show that various stresses affect the body system unfavourably. Psychological and clinical studies of Khetri et al. [5] have proved that stress plays a vital role in inducing cancer. Moreover, experimental evidence has indicated that stress produces increased adrenocortical secretions, which results in initiation, formation and severity of gastric ulcers [6]. Similar studies of Tha et al. [7] revealed a relation between stress and asthma.

Many other problems have been known to be caused by stress like anxiety necrosis, peptic ulcer, hypertension or coronary heart problems. It has also been proved that endocrinal glands are very sensitive to environmental stress. Exposure to conditions of environmental stress, such as extreme cold is known to change the concentration of body fluids, haematology, stress hormones, cortisol, etc. [8] In the same way rats immersed in water for a long time were found to have developed gastric ulcers [9].

A series of new research has shown that malnutrition is the legitimate cause of nutritional stress, which produces several diseases like Kawashiorkor and marasmus [10]. Nutritional stress also influences the biochemical components like DNA, RNA, protein and lipid, which vary with the increased nutritional stress. Studies made three decades ago revealed that fasting stress followed by trauma increases plasma free fatty acids and glycerides in liver [11]. Similarly, fasting and surgical stress caused a rise in total lipids in the liver of rats [12]. Fasting and restraint stress has also been reported to cause fragmentation of ER in the retina. It was further observed to have caused autophagic vacuoles to grow in size [13].

Recent studies by Hasan [14] on rats which were kept under restraint stress, achieved by keeping the animals immobile for 24 hours, found an increase in MDA and lipofuscin in the brain tissue. Earlier, Kereneyi et al. [15] had reported such a finding after they studied the effect of psychological stress on rats. In the present study, the authors have endeavoured to investigate the effect of restraint stress continued for longer duration by histochemical, fluorescent microscopical and biochemical analysis. Lipofuscin is one of the cytological manifestations of ageing and, therefore, any factor that induce it in cells will have a direct bearing on ageing. Many experimental manipulations have been done to induce lipofuscinosis in cells [16], prominent being metal toxicity [14,17], nutritional deficiency [18-22] and leupeptin treatment [23]. Recently, behavioural and psychological stresses have also been reported to induce lipofuscin [14].

Material And Methods

Ten, three-month-old male wistar rats were subjected to restraint stress by keeping them immobile in specially designed wire-gauze cages. They were allowed to remain in the cage, which did not have any space for the animal's movement, for 24, 48 and 72 hours. Ten age-matched rats were kept in the same condition as of stressed animals except restraint stress. After the stipulated period of time, they were sacrificed and their cerebella removed carefully. The cerebella were then processed for histochemical, fluorescent microscopical and biochemical analyses.

Histochemical Tests : For histochemical analysis, the tissue was processed in various fixatives adopted from Bancroft [24] and Pearse [25]. Sections cut at 7 u thickness were stained in Sudan Black B (SBB), periodic acid-Schiff (PAS), Nile Blue sulphate (NBS), Schmorl and Ziehl Neelson (ZN) [25].

Fluorescent microscopical studies : Fluorescent microscopic study was prosecuted on 10% formaldehyde calcium (FCa)-fixed, deparaffinized sections of cerebellum using an Olympus Fluorescent microscope equipped with UV light source (Osram HBO-200) and a filter combination of UG_1 exciter filter and L-420 barrier filter. Sections were mounted in Olympus non-fluorescent immersion oil (nd 1.404 at 25°C) before observing under the microscope.

Biochemical estimation of lipofuscin : This was done according to Dillard and Tappel [26] and Malentiyeva and Antonova [27].

Lipofuscin was extracted from tissue homogenate in 2:1 chloroform-methanol before reading in a fluorimeter model 151 (Systronics, India). Lipofuscin content was calculated by using the formula :

$$X = (n-n_2)/(n_1-n_2).C$$

where, X = lipofuscin content
n = reading of fluorimeter for unknown solution
n_1 = reading of fluorimeter for standard solution
n_2 = reading fluorimeter for reference solution
C = concentration of standard solution
and expressed in per gm tissue wet weight.

Neurons prone to lipofuscinosis : This was determined by counting the number of Purkinje neurons bearing autofluorescent lipofuscin pigment in tissue sections. Five sections from each animal were scanned for pigmented neuron counting. The study was done on 10 animals, each from control and stressed group animals. The percentage of pigmented neurons was then calculated. The significance in the difference in the stressed animals from the age-matched controls was determined by Student's 't' test.

Observations

A 24-hour stress resulted in a rather quick formation of autofluorescent pigment bodies, which emitted a bluish-white to yellow autofluorescence. These bodies tended to aggregate at the bases of the principal dendrites, being loosely arranged in 24 hours stressed preparations and aggregated into clumps in the 48 and 72 hours stressed preparations. The pigment reacted positively to SBB, NBS, PAS, schmorl and ZN tests. The experiments showed the following two major findings:

1 Pigment formation was very prominent soon after 24 hours stress;
2 While the amount of pigment in the individual neurons did not change sharply, great variations were observed in chemical nature of the pigment and the number of neurons becoming prone to pigment deposition after different durations of exposure to stress.

Pigmented neurons : Cerebella of 3 month-old control animals showed hardly any pigment in most of the Purkinje neurons. However, a few cells had a sparse amount of pigment granules, which emitted a low autofluorescence. The total population of such cells was only 2% (Table 1).

The number of Purkinje neurons with detectable amount of pigment was raised from 2% to 6%, 6% to 17% and 17% to 30% after the animals were subjected to 24, 48 and 72 hours stress, respectively. There was also a significant increase in the pigment in comparison to the controls (Table 1).

Fluorimetric Analysis of Lipofuscin : Biochemical estimation of lipofuscin in the cerebella of young-control and young-stressed animals was also carried out to confirm the above results.

There was a linear increase in the quantity of the autofluorescent material in the cerebella of rats that underwent stress for various periods (Table 2). The percentage increase in lipofuscin content was to the tune of 28.9%, 38.3% and 39.9% in 24, 48 and 72 hours stressed

animals, respectively, with respect to the young controls. Increase in pigment was found to be statistically highly significant (P<0.001).

Table 1. Percentage pigmented cell population in the cerebella of young (YC) and young stressed (YS) animals

(mean ± SE; n = 5)

	Control (YC)	24 hrs st. (YS 24)	48 hrs st. (YS 48)	72 hrs st. (YC 72)
	2.0	6.0	17.7	30.0
SD	0.69	1.25	2.64	2.88
SE	0.4	0.6	1.2	1.3
't' values				
Control	0	5.55*	12.41	20.59
24 hrs st.		0	8.72	16.76
48 hrs st.			0	3.93**
72 hrs st.				0

* P<0.01; ** P<0.05; Others P < 0.001

Table 2 Lipofuscin content in the cerebella of young(YC) and stressed (YS) animals (expressed in ug/g tissue wet wt.) n=3

	Control (YC)	YS 24	YC 48	YS 72
	770.33	993.30	1065.67	1071.33
SD	2.5	4.16	3.06	3.03
SE	1.33	2.4	1.77	1.75
't' values				
Control	0	81.27	133.4	136.9
YS 24		0.0	24.3	26.3
YS 48			0.0	2.7*
YS 72				0

* P<0.05; Others P<0.001

Discussion

Immobilization (solitary confinement in a small space) or restraint stress is one of the many stresses that animals can be subjected to. Many types of stresses have been experimented on rats as well as on human beings, keeping in view the reality that man in his daily life faces many stresses, one way or the other till death.

There are two main categories of stress; and both can be caused in different ways. Restraint stress, as shown in the present study, causes both mental and physical types of pressure. Hasan [14] was the first to use this type of stress as an experimental model to enhance lipofuscinogenesis in 24 hours. Earlier psychological stress was found to cause lipofuscin accumulation [15]. The present study undertaken to

see the effect of further durations of stress like 48 and 72 hours, revealed that the rate of lipofuscin formation remained high during such phases.

The results of the present study shows that there is an abrupt hike in pigment content in 24 hours' stress; the study also revealed the harmful effect of stress. There was a further increase in lipofuscin content after 48 hours' stress. But the percentage increase in the age pigment content after 72 hours' stress was not as much as that seen in animal kept for 48 hours stress. However, the number of neurons prone to lipofuscinosis remained elevated in 72 hour stressed animals. Thus, it can be interpreted that after a period of time, animals get adjusted with the situation in such a way that the formed lipofuscin is either removed gradually from the neurons or its formation is restricted probably by improving the defence mechanism in cells. Nevertheless, the effect of the 72 hours' stress was such that the non-pigmented neurons, too got implicated thus revealing the fact that longer duration of stress may be harmful to the animals.

The study also shows that restraint stress can be a useful model in the study of ageing process. It may be stated that continuous or intermittent stress can decrease the life span of animals because it causes the damage to cells, especially neurons and may even result in their death.

The fact that any stress applied on animals directly affects the endocrine system of the concerned animal [28] draws attention to the importance of restraint stress, as shown in the present study. Environmental and emotional stresses trigger hypothalamo-adrenohypo-adrenocortical axis through complex neuronal pathways going to the hypothalamus which in turn, accelerate the secretion of ACTH, GH, TSH, FSH, prolactin and LH [28]. This reveals that the stress on the animals must have drastically effected their physiological and metabolic activities leading to lipofuscinosis. Stress for more than 24 hours not only increased the amount of pigment accumulation but also it made more neurons prone to lipofuscinosis. The present findings also reveal that stress for longer time can damage the cell functionally, leading to pathological conditions as well as to premature ageing.

Address for Correspondence

The authors thankfully acknowledge the financial assistance received from UGC, New Delhi, India and Kurukshetra University, Kurukshetra, Haryana, India.

References

1 Selye, H : The Stress of Life. New York : McGraw Hill 1956.

2 Kets de Vries : Quoted from Pestonjee DM 1992.

3 Mathew VJ : Job stress of a creative manager FPM written comprehensive examination Paper, O.B Area, Indian Institute of Management Ahmedabad 1985.

4 Pestonjee DM : 'Executive Stress' should it Always be Avoided ? Vikalpa 1987; 12(1):23-30.

5 KhatriS, Chansouria JPN and Udupa KN : Role of Stress in Malignant Diseases. Paper presented at the International Seminar on Stress in Health andDiseases, Banaras Hindu University, Varanasi 1977.

6. Ahmed RN and Rao MA : 'Stress and Steroid Induced Gastric Ulcers and their Prevention in Fore Stomachetomized Albino Rats', Paper presented at the International Seminar on Stress in Health and Diseases, Banaras Hindu University, Varanasi, February 1977.

7 Jha VK, Udupa KN and Kumar B : Studies of Neurohormones in Bronchial Asthama. Paper presented at the International seminar on Stress in Health and Diseases, Banaras Hindu University, Varanasi February 1977.

8. Ramasaram P, Sitaraman V, Shivaramakrishna S and George R : Multiple Effects of Norepinephrine on Hepatic Enzymes. Paper presented at the International Seminar on Stress in Health and Diseases, Banaras Hindu University, Varanasi, February 1977.

9 Kadota T and Tamakuma S : Experimental Study of Stress-Induced Ulcer and defensive Mechanisms. In J Natl Def Coll 1986; 11(3):135-139.

10 Rao KSJ : Nutritional Stress and Hormonal Adaptation. Paper presented at the International Seminar on Stress in Health and Diseases. Banaras Hindu University, Varanasi, Feb. 1977.

11 Carlson, L.A. and Liejhldahl SO : Acto-med-Scand 1963; 173:25-33.

12 Camargo ACM, Cornicell J and Cardoso SS : Alteration in lipid contents of the liver in rat, after partial hepatectomy in Proc Soc exp Biol Med 1966; 173:1151-1154.

13 Salas M and Tuchweber B and Kourounkis P : Proc Can Fedn Biol Soc 1975; 19:15.

14. Hassan M : Age related changes in various regions of brain : correlation with neurotoxicological alterations. Ann Nat Acad Med Sci (India) 1985; 22:69-91.

15. Kerenyi M, Haranghy L and Huttner I : Investigations on experimentally produced age-pigment in the nervous system. Exp Geront 1968; 3:155.

16. Zs Nagy I : An attempt to answer the questions of theoretical gerontology on the basis of membrane hypothesis of aging. In: Advances in the Biosciences Pergamon, London 1987, vol.64, 393-413.

17. Zs Nagi I, Steiber J and Jeney F : Induction of Age Pigment accumulation in the brain cells of young male rats through iron-injection into the cerebrospinal fluid : International Gerontology Week - Tokyo 1995 : Fifth International Symposium for Ceroid and Lipofuscin Pigments...Biomarkers for Aging August 23-26, 1995; 15.

18 Katz ML and Robison WG : Nutritional influences on autoxidation, lipofusscin accumulation, and ageing. Free Radicals, Ageing and Degenerative diseases (eds.) Johnson JE, Walford R, Harman D and Miquel J. 1986; 221-59.

19 Brizzee KR, Ordy JM : Cellular features, regional accumulation and prospects of modification of age pigment in mammls. In Sohal RS (ed.) Age Pigments. New York Elsevier/North Holland Biomedical Press 1981; 101-154.

20 Patro IK, Sharma SP, Patro N : Neuronal lipofuscin : Its formation and reversibility. Ind Rev Life Sci 1988; 8:95-120.

21 Sharma SP, Gupta SK, James TJ, Patro IK : Influence of centrophenoxine on protein malnourishment induced lipid peroxidation nd lipofuscine in rat spinal cord. In Singh R, Singhal GS (eds.) Perspectives in ageing research, New Delhi, Today and Tommorrow 1990; 117-121.

22 James TJ, Sharma SP, Gupta SK, Patro IK : 'Dark' cell formation under protein malnutrition : process of conversion and concept of 'semi dark' type Purkinje cells. Ind Jr Exptl Biol 1992; 30:470-473.

23 Ivy GO, Schottler F, Baudry M and Lynch G : Inhibitors of lysosomal enzymes. Accumulation of lipofuscin like dense bodies in the brain. Science, 1984; 226:985-987.

24 Bancroft JD : An Introduction to histochemical technique. Butterworths, London 1967; pp.83-84.

25 Pearse, AGE : Histochemistry, Theoretical and Applied. Churchill London 1972; vol.II, pp432-434.

26. Dillard, CJ and Tappel AL : Fluorescent products of lipid peroxidation of mitrochondria and microsomes. Lipids 1971; 6:715-721.

27 Malentyeva G and Antonova L : Pharmaceutical chemistry, Mir Publishers, Moscow 1988; pp.46-47.

28. Pestonjee DM : Stress and Coping "The Indian Experience" SAGE Publications, Pondichery, India 1992:1-200.

Discussion after the talk of Dr. Chaudhary

Zs.-Nagy: How can you tell that the animals were "stressed" by the cage used, while they were also starving and did not have access to water for 3 days?

Chaudhary: It is true that during the experiment the rats did not get food or water. Considering the fact that it is rather difficult to provide the rats food and water during the stress, or even if it was provided, the animals were not able to take food and water. We thus kept a control group of animals of the same age, weight and sex without food and water.

Zs.-Nagy: Was the stress-induced lipofuscin followed for a longer time after the stress was suspended?

Chaudhary: Sorry, I have not worked on this aspect. In fact, our main aim was to find a new model for lipofuscinogenesis. However, as you suggested the work can be continued to find out the nature of the induced pigment after the stress period.

Alho: I do not agree with you that restraint stress is a good experimental model for lipofuscinogenesis; this stress is a multifactorial stress and can affect rats in many different ways.

Chaudhary: It is true that other factors may influence lipofuscinogenesis in this case. However, a control group of animals kept under the same conditions as those of the stressed animals, except for restraint stress, revealed the difference in the degree of lipofuscinogenesis.

Ivy: Your results are very interesting. As you know, protein deprivation (which is included in your paradigm due to food deprivation) can itself cause increased LF. I believe that your results would be more valid if you did not have this confounding factor. Although your control rats were also not fed, there is still a possibility of stress interacting with food deprivation (an additional stress) in your experimental animals.

Chaudhary: Thanks for your comment, and I fully agree with you, protein deprivation does cause increased LF. To nullify this factor, a control group of animals kept with conditions identical to those of the stressed animals was also used in our experiment.