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The Modulatory Effect of N-Acetyl Cysteine Supplementation on Hepatic Glutathione Concentration and Lipid Peroxidation Status in Old Rats Fed a Low-Protein Diet

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Abstract: There is growing evidence in the literature linking oxidant molecules and the degenerative and physiological changes that occur with advancing age. It is now well documented that oxidant molecules cause cell injury and have been shown to be a common factor in many of the age-associated diseases. On the other hand, the body has many antioxidant compounds that minimize oxidant molecules toxic effects. However, with advanced age, oxidant molecule production may overwhelm the antioxidant defenses, thus contributing to many degenerative diseases of aging. Aging is also a risk factor for protein-energy malnutrition; which was shown to be common among elderly populations. Insufficient intake of protein is frequently found in elderly populations. Glutathione (GSH) is one of the major antioxidant compounds in tissue. The presence of GSH in adequate amount may help in reducing the development of aging process and lead to healthy life. One of the most effective compounds used to serve as a cysteine delivery agent is N-acetyle-cysteine (NAC). The aim of the study is to investigate the effect of NAC and dietary protein on hepatic GSH and lipid peroxidation status in old rats. Rats fed a normal-protein (NP) diet, a low-protein (LP) diet, or a low-protein diet supplemented with the NAC (LP+NAC). GSH concentration in liver, serum albumin concentration and thiobarbituric acid reactive substances (TBARS), as an indication of oxidative tissue damage, were measured. Furthermore, a liver sample of each group was histologically examined using electron microscope. There was an increase in the weight gain of rats fed the LP+NAC diet compared to the rats fed the LP diet, despite the similarity of the daily food intake. Dietary supplementation of NAC to the LP diet restored GSH concentration in the liver to that level seen in rats fed the NP diet. The increase in hepatic GSH concentration in the LP+NAC group was parallel to the decrease in the plasma TBARS level. Furthermore, albumin level was increased in animals fed the LP+NAC diet. The study shows the effectiveness of NAC in restoring hepatic GSH concentration and in reducing plasma TBARS concentration in old rats fed low-protein diet.

Key words: Old rats, hepatic glutathione, lipid peroxidation, N-acetyl-cysteine, albumin

Introduction

The most popular theory trying to explain the deterioration in body functions that accompany advancing age is the free-radical damage. The theory postulates that aging is caused by free-radical reactions, which involved in production of the aging changes. Reactive-oxygen species (ROS), which is a general scientific term usually used to refer to free radical molecules and to non-radical molecules that are able to oxidized other biomolecules or are easily converted into radicals (Halliwell, 1996), causes cell injury by damaging cellular components, such as proteins, DNA and lipid membrane (Halliwell, 1987; Langseth, 1995; Wiseman and Halliwell, 1996). Advancing age cause alteration in cellular redox levels and dysregulated immune responses that could be responsible for inflammation and apoptosis (Hu *et al.*, 2000). ROS have been shown to be a common factor in many of the age associated diseases e.g. rheumatoid arthritis, heart disease, and Alzheimer's (Marry *et al.*, 1989; Halliwell, 1989; Balazs and Leon, 1994). The risk produced from the free-radical molecules can be minimised if there are enough antioxidant defenses to stop the oxidant chain reactions. There are a variety of antioxidant molecules and enzymes e.g. vitamins E and C, glutathione (GSH), GSH peroxidase, catalase, and superoxide dismutase, which can protect mammalian tissues from free radicals by converting them into non-harmful molecules. However, with advanced age, oxidant molecule production may overwhelm the antioxidant defenses (Hu *et al.* 2000), contributing to many degenerative diseases of aging.

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Table 1: Diet composition (g/kg)

Component	180g protein/kg (NP)	60g protein/kg (LP)	60g protein+N-acetyl cysteine/kg (LP+NAC)
Casein	204	68	68
L-methionine	3	0	0
N-acetyl-cysteine	0	0	8.16
Cellulose	100	100	100
Sucrose	319	388	385
Maize starch	319	389	384
Maize oil	30	30	30
Vitamins mix*	5	5	5
Minerals mix*	20	20	20

NP: Normal-protein diet; LP + M: Low protein diet; LP + NAC: Low protein + N-acetyl cysteine diet. *Vitamin and mineral mix (AIN-76, American Institute of Nutrition 1977).

GSH is one of the major antioxidant compounds in tissue, consisting of three amino acids, cysteine, glycine, and glutamate, in which cysteine is the most limiting amino acid for GSH synthesis (Meister *et al.*, 1986). GSH levels have been shown to be decline with age (Iantomasi *et al.*, 1993; Nuttall *et al.*, 1998). Thus, during aging the decrease of GSH production and the increase of oxidant molecules production may be responsible for the damages in structure and function of cell, which accompany advanced age in human being.

GSH, itself, is not an effective source to raise intracellular GSH levels, because it is mostly degraded in the extra cellular compartment by the enzymes γ-glutamyl transpeptidase and dipeptidase, and oxidative depletion can outpace synthesis (Meister *et al.*, 1986). Since cysteine is rapidly oxidized and is also toxic at high concentrations, it is not practical to use it as a supplement (Golden and Ramadath, 1987), several compounds have been used as a cysteine delivery agent. One of the most effective compounds used to serve as a cysteine delivery agent is N-acetyle-cystein (NAC) (Droge and Breitkreutz, 2000). NAC is a potent antioxidant with antimutagenic and anticarcinogenic properties. NAC increases intracellular GSH by reducing the cystine to cysteine and that cysteine is more efficiently trnasported than cystine into the cells (Deneke and Fanburg, 1989).

Aging is a risk factor for protein energy malnutrition (PEM). Poor nutritional status is one of the major factors associated with mortality in older persons (Morley, 2000). It is well documented that under nutrition impairs immunity, decreases resistance to infection and reduces the antioxidant-defense mechanisms of the body (Chandra, 1993). Tissue GSH concentration were decreased in PEM, which was correlated with increased susceptibility to oxidative stress (Deneke *et al.*, 1985; Taylor *et al.*, 1992). However, starting PEM rehabilitation with a protein rich diet may impose metabolic stress due to body's adaptation to the catabolic PEM state (Solomons, 1985; Badaloo *et al.*, 1999). As well insufficient intake of protein is frequently found in elderly populations (Moreiras *et al.*, 1996). Thus, it is desirable to restore tissue GSH without feeding a high protein diet, NAC might be effective for such a strategy.

The present study investigates the effect of NAC and dietary protein on hepatic GSH and lipid peroxidation status in old rats. Old rats were fed a normal-protein diet, a low-protein diet, or a low-protein diet supplemented with NAC. Hepatic GSH concentration, plasma thiobarbituric acid reactive substances (TBARS), as an indication of oxidative stress, and albumin were measured. Furthermore, liver samples were taken for electron microscopic examination.

Experimental design and diet composition: Animal procedures were conducted in accordance with legislation laid down by the College of Applied Medical Sciences-King Saud University. Thirty-six elderly Wistar rats (18 months old) were obtained from the Experimental Animal Care of King Saud University, Riyadh, Kingdom of Saudi Arabia. Rats were housed individually in cages and maintained at $22 \pm 1^{\circ}C$ with a 12-12 hour light-dark cycle (Lights on at 7:30 am).

All groups were fed casein as the sole dietary protein source, as a relative insufficiency of sulphur amino acid can be created by inclusion of protein in diets in amounts of less than 180g/kg diet (National Research Council, 1978). The amount of sulphur amino acids in casein was 2.76g methionine and 0.43g cystine/100g casein as determined by high pressure liquid chromatography.

All diets were prepared by mixing dry ingredients in a mixer; this was followed by addition of maize oil. To these diets, water was added to make the diets into small biscuits that were dried in oven at $80^{\circ}C$ for up to 48-72 hours.

Old rats were fed three different dietary groups for a paeriod of one month as follows (Table 1).

1) The first group, n=12, was fed a normal-protein diet (180g protein supplemented with 0.03g L-methionine/kg diet), an amount commonly used in rat studies. This group was called the normal-protein (NP) group.

2) The second group, n = 12, was fed a low-protein diet (6% protein diet). This group was called the low-protein (LP) group.

3) The third group, n = 12, was fed a 6% protein diet, similar to the LP group, but it was supplemented with NAC to bring the amount of total sulphur amino acids to that present in the NP group. This group was called the low-protein diet + N-acetyl cysteine (LP + NAC) group.

At the end of the experiment, blood was collected in EDTA tube by cardiac puncture under light ether anesthesia. The blood was then centrifuged at 2000 rpm/min for 15 minutes at 4°C. The plasma was collected, and stored at -20°C for further analysis of TBARS and albumin. The liver, from each rat was rapidly dissected out, cleaned, and weighed. Prior to snap freezing in liquid nitrogen, small samples were taken from liver for immediate analyses of GSH content. Small samples of liver tissues were also dissected from rats, and then immediately preserved in glutarldhyde for electron microscopy examination.

Materials and Methods

Glutathione assay: GSH in liver was measured by a colorimetric reaction, based on the oxidation of the reduced form of GSH by the aromatic disulphide compound, 5,5,-dithiobis-2-nitrobenzoic acid (DTNB), to form the oxidized form of GSH and the aromatic thiol, 5,thio-2-nitrobenzoic acid (Beutler, 1963). The yellow colour formed due to the reduction of DTNB was measured at 412 nm, and it is proportional to the amount of GSH present in the sample.

Albumin concentration: The bromocresol green dye method, using glycine-HCl as a buffer, was used to determine the concentration of albumin in the plasma (McPherson and Evard, 1972).

Thiobarbituric acid reactive substances: Plasma levels of lipid peroxides, as an indication of oxidative stress, was measured as reaction products of malondialdehyde, a compound generated as a result of lipid oxidation, with thiobarbituric acid. The formation of TBARS was measured at 532nm using a standard spectrophotometer (Armstrong and Browne, 1994). A TBARS Assay Kit was purchased from ZeptoMetrix Corporation (Buffalo, New York, USA).

Electron microscopy examination: Small samples of liver tissues were dissected from rats (one rat from each group), and then immediately preserved in glutarldhyde. Slides of these preserved sections were kindly prepared by the Department of Histology-King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia.

Statistical analysis: Results were expressed as mean values±standard error of the mean. The results were compared using one-way analysis of variance followed by Fisher's least significant differences procedure (Steel and Torrie, 1960) with a significant level of p <0.05.

Results

Body weight, food intake, and liver weight: No significant difference was found in the initial weights among various groups. The initial weights were 412± 7.3g, 413±7.0g, 413±6.9g for the NP, LP, and the LP + NAC group, respectively. Table 2 shows that the weight gain of the LP group is lower than the NP and the LP + NAC groups. However, there was no significant difference in the weight gain between the NP and LP + NAC groups. The results show no significant difference in the daily food intake among the three dietary groups (Table 2). This indicates that the reduction in the weight gain of rats fed the LP diet was not due to the decrease in the amount of food intake.

Table 3 shows a significant increase in the final body weight of the rats fed the NP and the LP+NAC compared to the rats fed the LP diet.

The liver weights in Table 4 were presented as total liver weights and as relative-liver weights (percentage of body weight) to allow differences due to body weight. The results show that the LP + NAC group had significantly higher total liver weight as well as relative-liver weight compared to the LP and the NP groups.

Glutathione, thiobarbituric acid reactive substances and albumin concentrations: Table 5 shows that rats fed the LP diet had lower hepatic GSH concentration compared to rats fed the NP and LP + NAC diets. Dietary supplementation of NAC to the LP diet restored GSH concentration in the liver to that level seen in rats fed the NP diet.

There was no significant difference in TBARS level between the NP and the LP + NAC groups (Table 6). The addition of NAC to the LP diet significantly decreased the plasma TBARS level compared to the corresponding animals fed the low-protein diet, but without NAC supplementation. The NP group also has lower plasma TBARS level compared to the LP group, but this was not statistically significant.

Albumin level was significantly decreased in the LP group compared to the NP and LP + NAC groups (Table 7). Addition of NAC to the LP diet significantly increased albumin level to that level seen in the NP group.

Electron micrographs of hepatocytes: Electron micrographs showed a normal structural features of hepatocytes in the NP group (Fig. 1), increased deposition of fat globules in the LP group (Fig. 2), and a reversal of fat content with increased deposition of lipofuscin granules associated with smooth endoplasmic reticulum in the peribiliary ectoplasm in the LP + NAC group (Fig. 3).

Table 2: The effects of dietary supplementation of N-acetyl-cysteine on daily weight gain and food intake in old rats fed low-protein diet

Dietary groups (g protein/kg diet)	NP+M 180	LP 60	LP+NAC 60	one-way ANOVA p-value F ratio
Average daily weight gain(g)	0.797±0.38[a]	-0.812±0.25[b]	0.648±0.200[a]	P = 0.001 F = 9.44
Average daily food intake (g)	30.65±1.97[a]	27.74±1.42[a]	28.13±1.26[a]	P = 0.379 F = 1.00

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means within each row having different letter superscripts following the number differ significantly.

Table 3: Final body weight of rats fed normal-protein diet, low-protein diet or low-protein diet supplemented with N-acetyl-cysteine

Dietary groups (g protein/kg diet)	NP+M 180	LP 60	LP + NAC 60	one-way ANOVA p-value F ratio
Final body weight (g)	443.0 ± 14.2[a]	397.5 ± 12.7[b]	440.3 ± 11.8[a]	P = 0.030 F = 3.9

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means having different letter superscripts following the number differ significantly.

Table 4 : The effects of dietary supplementation of N-acetyl-cysteine on total and relative liver weight in old rats fed low- protein diet

Dietary groups (g protein/kg diet)	NP + M 180	LP 60	LP + NAC 60	one-way ANOVA p-value F ratio
Total liver weight (g)	14.4 ± 0.68[a]	13.0 ± 0.69[a]	18.8 ± 1.02[b]	P = 0.000 F =14.28
Relative liver weight (g/kg body weight)	32.35 ± 0.82[a]	32.61 ± 1.30[a]	42.68 ± 1.71[b]	P = 0.000 F = 19.68

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means within each row having different letter superscripts following the number differ significantly.

Table 5: The effects of dietary supplementation of N-acetyl-cysteine on glutathione (GSH) concentration in old rats fed low protein diet

Dietary groups (g protein/Kg diet)	NP+M 180	LP 60	LP+NAC 60	one-way ANOVA p-value F ratio
GSH (μmol/g liver)	8.364±0.361[a]	2.497±0.507[b]	8.375±0.407[a]	P = 0.000 F = 62.41

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means having different letter superscripts following the number differ significantly.

Table 6: The effects of dietary supplementation of N-acetyl-cysteine on Thiobarbituric acid reactive substances (TBARS) concentration in old rats fed low protein diet

Dietary groups (g protein/Kg diet)	NP+M 180	LP 60	LP+NAC 60	one-way ANOVA p-value F ratio
TBARS (nmol/ml)	11.92±0.36[ab]	12.64±0.42[a]	10.77±0.45[b]	P =0.010 F = 5.26

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means having different letter superscripts following the number differ significantly.

Table 7: The effects of dietary supplementation of N-acetyl-cysteine on albumin concentration in old rats fed low protein diet

Dietary groups (g protein/Kg diet)	NP+M 180	LP 60	LP+NAC 60	one-way ANOVA p-value F ratio
Albumin (g/l)	40.383 ± 0.778[a]	36.675 ± 0.666[b]	40.258 ± 0.462[a]	P = 0.000 F = 10.54

NP + M = Normal-protein diet + methionine; LP = Low-protein diet; LP + NAC = Low-protein diet + N-acetyl-cystiene. *Results are presented as mean ± standard error of the mean, n = 12 for all groups. Means having different letter superscripts following the number differ significantly.
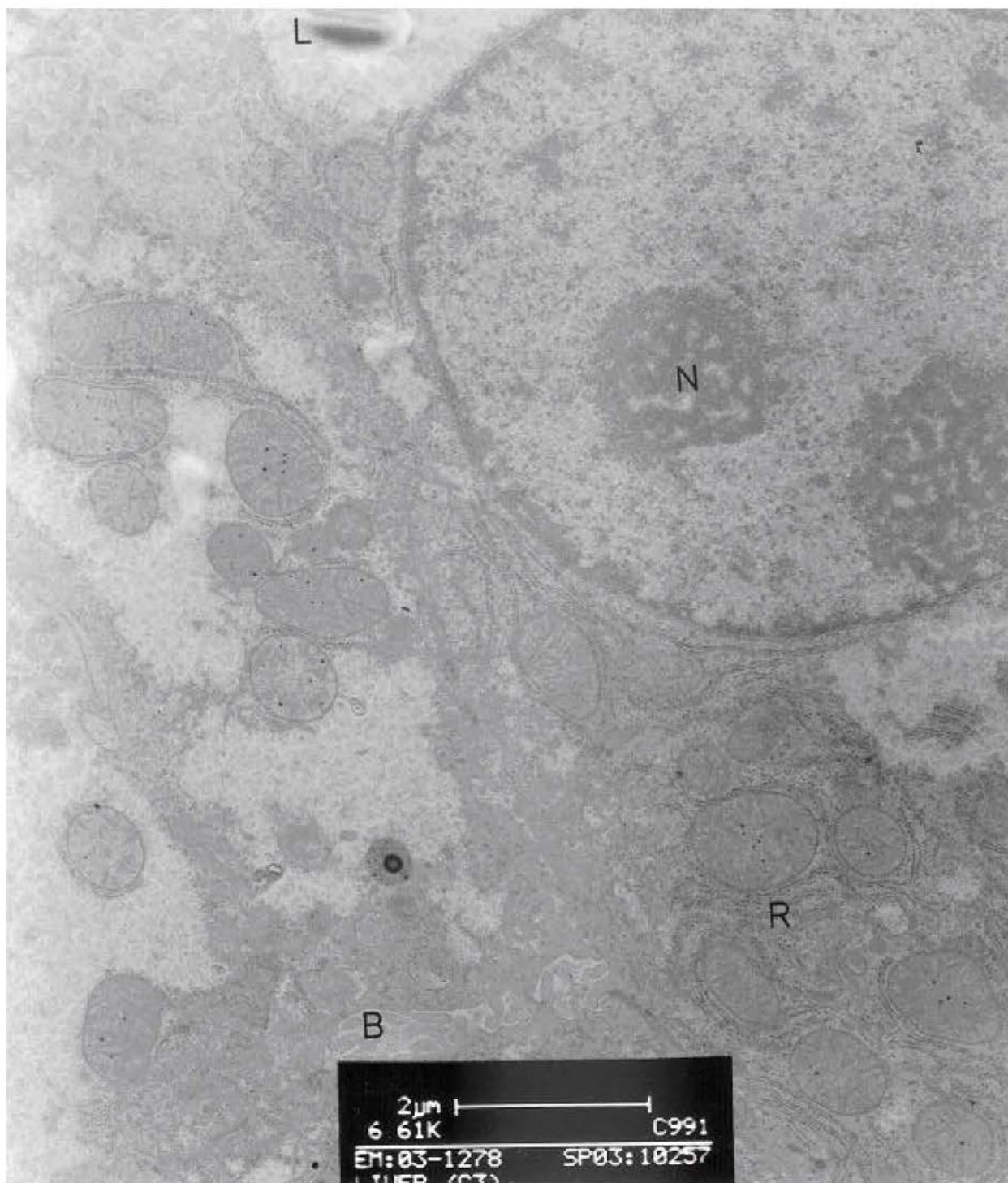
Fig. 1:   Electron micrograph from a section of a rat's liver fed normal-protein diet.
            Electron micrograph from control group (the normal-protein diet) showing normal structural features of
            hepatocytes, observe mitochondrial that is surrounded by regular arrays of rough endoplasmic reticulum
            (R). Hepatocytic nucleus is characterized by two apparent nucleoli (N). (L); lipid droplet, (B); bile
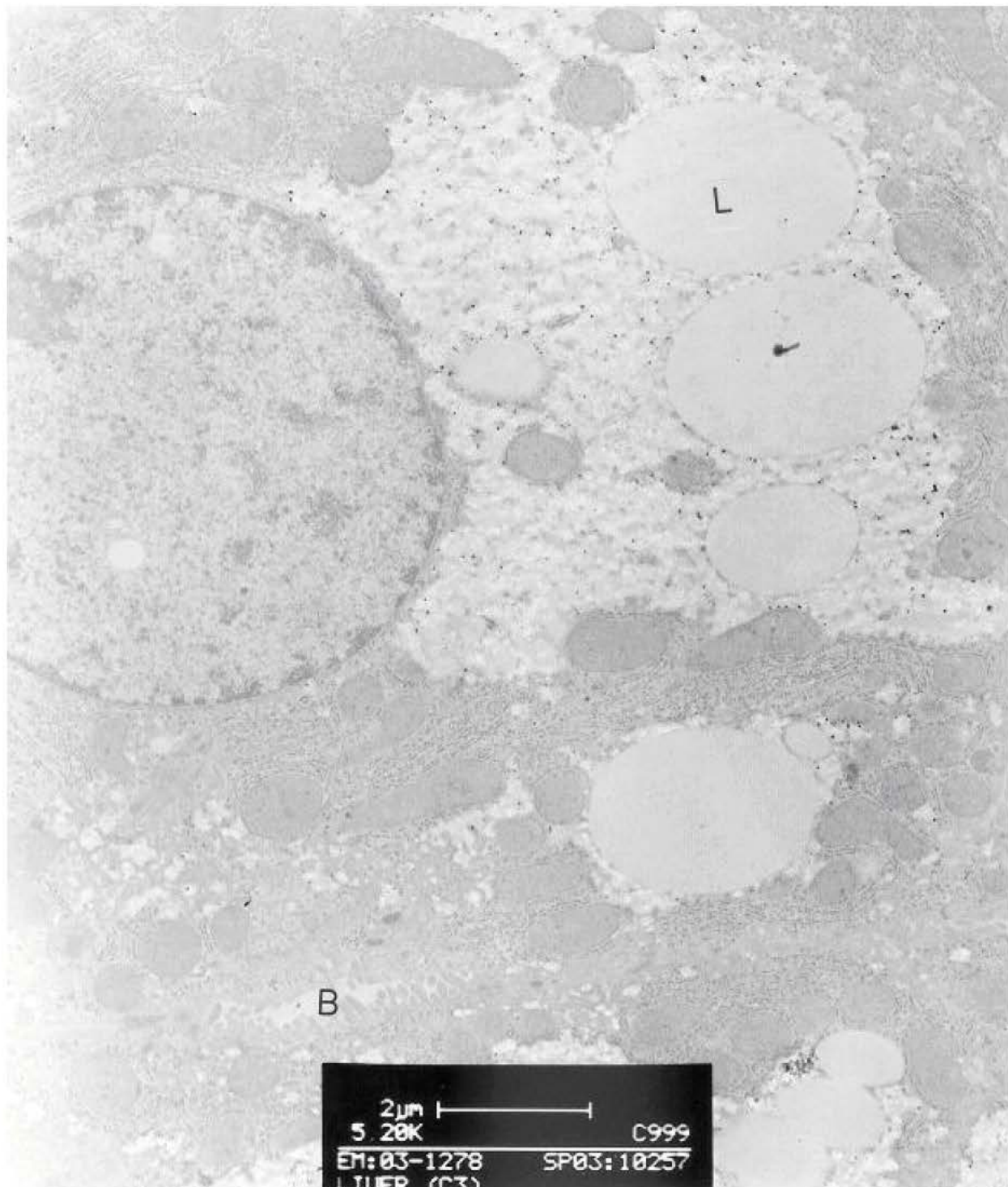   canaliculi.

Fig. 2: Electron micrograph from a section of a rat's liver fed the low-protein diet.
Electron micrograph from low-protein group demonstrated increased deposition of fat globules (L). Observed proliferated bile canaliculi (B).
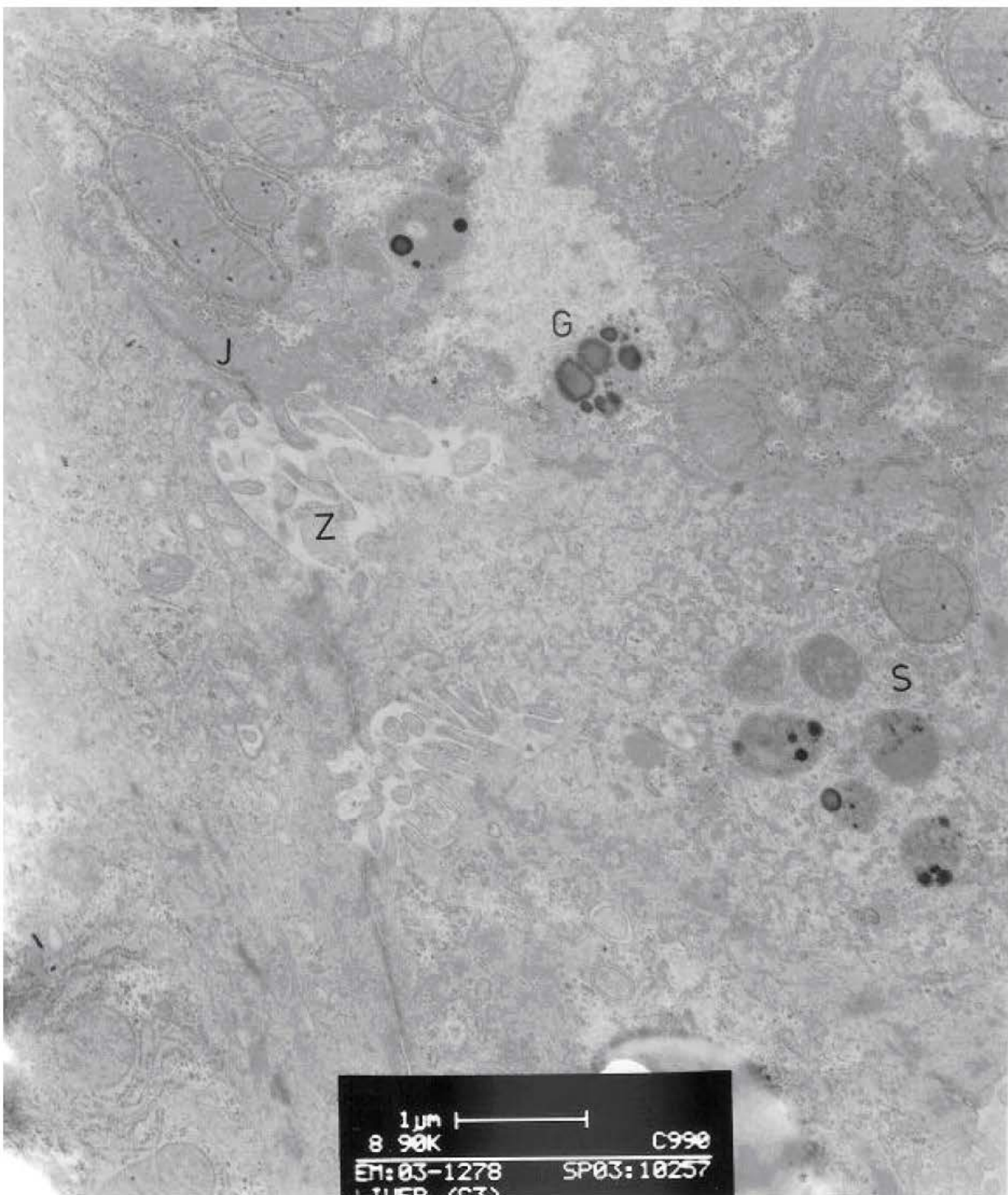
Fig. 03: Electron micrograph from a section of a rat's liver fed the low-protein diet supplemented with N-acetyl cysteine group.

Electron micrograph from the LP + NAC group showing reversal of fat content with increased deposition of lipofuscin granules (G) associated with smooth endoplasmic reticulum (S) in the peribiliary ectoplasm. Observe junction complex that hold the bile canaliculi and bizarre-shaped biliary microvilli (Z).

Discussion

The study presupposes that elderly might require special demands for sulphur amino acids. A rat model was used to examine this hypothesis. Although cysteine can be synthesized from methionine, there are indications that there is a requirement of cysteine in the diet of rats. When methionine was provided at the minimum requirement needed to support growth (0.17%), the addition of cysteine to such a diet improves the growth performance of growing rats (Sowers et al., 1972 and Stockland et al., 1973). Cho, et al. (1984) have observed that the mean daily weight gain of rats fed diets providing adequate or excess amounts of sulphur amino acids, in the form of methionine or cystine, was significantly higher than rats fed diet providing only the absolute minimum methionine requirement of growing rats (0.17%) recommended by Sowers, et al. (1972) and Stockland et al. (1973). Alhamdan and Grimble (2003) have shown that the average daily weight gain of growing rats fed a low-protein diet (80g protein/kg diet), supplemented with the sulphur amino acids (cysteine or methionine) was higher than animals fed a low-protein diet, but without sulphur amino acid supplementation. Similarly in old rats, the study showed that there was an increase in the weight gain of the rats fed the LP diet supplemented with NAC compared to the rats fed the LP diet, despite the similarity in the amount of the daily food intake between the two groups. This indicates that the reduction in the weight gain of rats fed the LP diet was not due to the decrease in the amount of food intake.

GSH serves as a reservoir of cysteine to be used mainly in the synthesis of serum proteins such as albumin (Tateishi et al., 1977; Tateishi et al., 1980; Higashi et al., 1977). This might give an explanation to the significant increase in albumin level in the LP + NAC group.

NAC supplementation to the low-protein diet restored GSH concentration in the liver to the level seen in the normal-protein diet. This was in agreement with the study of Li et al. (2002) that has shown that the short term (1 week) supplementation of cysteine prodrugs (methionine, NAC and L-2-oxo-4-thiazolidine-carboxylate) as a dietary component to malnourished mice, restored GSH concentrations in liver, lung, heart, and spleen. In a human study, Gavish and Breslow have shown the efficiency of NAC supplementation (2-4 gm daily for 8 weeks) in increasing tissue GSH (Gavish and Breslow, 1991).

GSH in liver was found to be decreased with age (Liu and Choi, 2000; Arivazhagan et al., 2001) or unchanged (Klausner et al., 1996; Stio et al., 1994; Mosoni et al., 2004). For example, Mosoni's study (2004) shows no significant difference in GSH concentration between rats aged 6 months and 9-25 months, while Arivazhagan et al. (2001) shows a decrease in GSH concentration with advancing age, in which they relate this decrease to the enhancement of oxidative damage caused by free
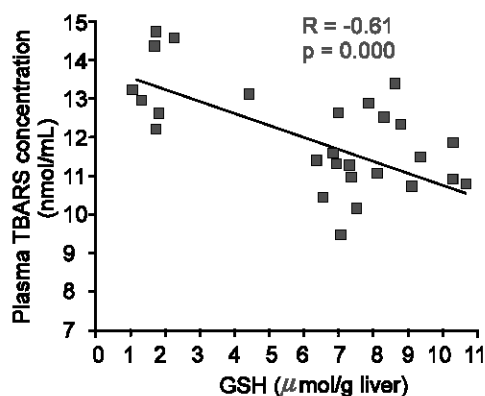

Fig. 4: Correlation between hepatic glutathione concentration and plasma thiobarbituric reactive substances (TBARS)

radicals during aging. In yargicoglu et al. (2001) study, a significant increase in the level of TBARS was observed with advanced age and they suggest that it was due to the decrease in the antioxidant protective system, as free radical might be an important cause of aging.

In the present study, TBARS concentration as an indication of lipid peroxidation level was found to be higher in animals fed the low-protein diet compared to animals fed the low-protein diet supplemented with NAC. The decrease of plasma TBARS level that was found in animals fed the LP+NAC was parallel to the increase in the hepatic GSH concentration. This was supported with the significant negative correlation that was found between hepatic glutathione concentration and plasma TBARS (Fig. 4).

To conclude, the study shows the ability of NAC to enhance hepatic GSH concentration and reduce plasma TBARS concentration in old rats fed a low-protein diet.

Acknowledgment

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