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Original

Behavioral, morphological and physiological shift in the rats administered with tryptophan deficient regimen

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Abstract

Protein-restriction or deficiency is associated with many pathological disorders. We have made an attempt to study the effect of marginal tryptophan deficiency and supplementation of adequate tryptophan on the activity of antioxidant enzymes in the liver and neuronal tissue of rats. Marginal tryptophan deficiency was created in the animals (group-C) by feeding them with diet consisting of casein (6%) and gelatin (12%). Control animals (group-A) received 20% casein in their diet. Another set of animals (group-B) received the marginal tryptophan deficient diet with 0.05% tryptophan. We have observed a decrease in body weight and organ development in the deficient animals. However, a protective mechanism has been observed in the tryptophan deficient animals that received 0.05% tryptophan. Biochemical studies have shown a decrease in protein content, reduced glutathione (GSH) levels, activities of catalase, glutathione-s-tranferase (GSTs) and tryptophan-fluorescence in tryptophan deficient rats. There is an increase in lipid peroxidation and AGE-fluorescence suggesting the oxidative stress due to tryptophan deficiency. However, in deficient rats that received 0.05% tryptophan in diet there was an increase in protein content, glutathione levels, catalase, glutathione-s-tranferase (GSTs) levels, tryptophan-fluorescence and inhibition in AGE-fluorescence and lipid peroxidation. Our findings suggest that adequate tryptophan administration to tryptophan deficient animals has a protective influence as revealed in the activity levels of antioxidant enzymes in relation to deficient animals.

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Key words: *Tryptophan. Reduced glutathione. Antioxidant enzymes. AGE-fluorescence. Tryptophan fluorescence. Protein carbonyls.*

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CAMBIOS CONDUCTUALES, MORFOLÓGICOS Y FISIOLÓGICOS EN LAS RATAS CON UN RÉGIMEN DEFICITARIO EN TRIPTÓFANO

Resumen

La restricción o deficiencia de proteínas se asocian con muchos trastornos patológicos. Hemos intentado estudiar el efecto de una deficiencia marginal de triptófano y la complementación adecuada con triptófano sobre la actividad de enzimas antioxidantes del hígado y tejidos neurales de la rata. Se consiguió una deficiencia marginal de triptófano en los animales (grupo C) alimentándoles con dieta consistente en caseína (6%) y gelatina (12%). Los animales control (grupo A) recibieron caseína al 20% en la dieta. Otro conjunto de animales (grupo B) recibió dieta con deficiencia marginal de triptófano al 0,05%. Hemos observado una disminución del peso corporal y del desarrollo en los animales con deficiencia. Sin embargo, se ha observado un mecanismo protector en los animales con deficiencia de triptófano que recibieron triptófano al 0,05%. Los estudios bioquímicos han mostrado una disminución del contenido en proteínas, una reducción de la concentración de glutatión (GSH), las actividades catalasa, glutatión-S-transferasa (GST), y fluorescencia de triptófano en las ratas con deficiencia de triptófano. Hubo un aumento de la peroxidación lipídica y la fluorescencia AGE, lo que sugiere un estrés oxidativo por una deficiencia de triptófano. Sin embargo, en las ratas deficientes que recibieron 0,05% de triptófano en la dieta, hubo una aumento del contenido proteico, las concentraciones de glutatión, la catalasa, las concentraciones de glutatión-s-transferasa, la fluorescencia de triptófano, y una inhibición de la fluorescencia AGE y de la peroxidación lipídica. Nuestros hallazgos sugieren que una administración adecuada de triptófano tiene una influencia protectora como viene mostrado por los niveles de actividad de enzimas antioxidantes en relación con los animales deficitarios.

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Palabras clave: *Triptófano. Glutatión reducido. Enzimas antioxidantes. Fluorescencia AGE. Fluorescencia de Triptófano. Carbonilos proteicos.*

Introduction

It is known since long-time that carbohydrates, proteins and lipids are biologically active macromolecules involved in various metabolic and energy yielding processes of cellular systems. Metabolic disturbance of any of these molecules is known to contribute to several chronic pathological states. Amino acids, free as well as in the form of constituents of proteins, play an important role in maintaining the structural integrity and folding nature of proteins, and any imbalance or inadequacy of these components influence protein synthesis, there by affecting the protein turnover. It has been reported that selective elimination of any one of the essential amino acids (i.e. L-tryptophan, L-phenylalanine and L-histidine etc.) is known to cause various pathologies. Tryptophan is an indispensable amino acid in biological systems and is involved in protein synthesis and is metabolized in mammals along tryptophan-niacin or kynurenine pathway[1]. Petzke, et al.[2], have reported that chronic ingestion of high protein diets well above requirements (25.7 or 51.3% casein) does not lead to oxidative stress in adult rats when diets are adequate in antioxidants, in contrast to chronic feeding of an adequate protein diet (13.8% casein). And there were also studies indicating apoptotic tissue changes associated with depletion of essential nutrients[3].

Numerous investigations have been reported that the supplementation of essential nutrients such as methionine[4], zinc[5], riboflavin[6] etc. restored the intact protein synthesis as well as redox cycle in protein restricted or deficient animals which help in retarding several pathological states.

It is well documented that deficiency or accumulation of tryptophan has been implicated in various age-related complications like renal-insufficiency[7], cataract[6] and Aids dementia complex[8] etc.

Bel and Artigas[9] have reported that the administration of tryptophan-free diet to rats resulted in a drastic reduction in the synthesis of 5-hydroxy tryptophan. Force-fed feeding of rats with elevated level of tryptophan (1%) with respect to control (0.2% tryptophan) showed a significant rise in hepatic protein synthesis, cytochrome P-450 and b_5 activity[10].

In this study we have investigated the effect of marginal tryptophan deficiency and adequate tryptophan administration on the antioxidant enzymes of hepatic and neuronal tissues. We have also observed behavioral, morphological and physiological alterations due to tryptophan deficiency. The data related to ocular disorders and tryptophan-kynurenine and tryptophan-serotonin pathways will be discussed elsewhere.

Materials and methods

Materials: L-Tryptophan, 2-thiobarbituric acid (TBA), 1, 1, 3, 3-tetraethoxy propane (TEP), O-pthaldehyde (OPT), reduced glutathione (GSH), 2,4-dinitrophenylhydrazine, guanidine hydrochloride and Bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solutions are of analytical grade.

Animals and diet: Male Wistar-NIN rats of weanling age (21-24 days) having an average bodyweight of 35-40 g, were obtained from National Centre for Laboratory Animal Sciences (NCLAS) NIN, Hyderabad and randomly assigned into three groups with twelve to fifteen animals in each group. The dietary composition was in accordance with John and Bhat[6] with necessary modifications as per the experimental protocol.

Diet composition of each group is as follows: group-A: control group (n = 12); group-B: adequate-tryptophan group (0.05% trp. /100gm diet) (n = 15); group-C: marginal tryptophan-deficient group (n = 20). The diet composition of each group was tabulated in table I.

Table I
Composition of diet supplemented to the each group. Dietary components in (g%)

S. No	*Dietary components*	*Control*	*Tryptophan administered (0.05%)*	*Tryptophan Deficiency (0%)*
1.	Sucrose	71.6	71.6	71.6
2.	Vitamin Free casein	20	6	6
3.	Gelatin	–	12	12
4.	Refined Pea nut oil	5	5	5
5.	Salt mixture*	4	4	4
6.	Vitamin Mixture#	0.6	0.6	0.6
7.	Choline chloride	0.4	0.4	0.4
8.	L-Methionine	0.3	0.3	0.3
9.	L-Alanine	–	0.022	0.022
10.	L-Tryptophan	–	0.05	–
11.	Vitamin-A	10,000 IU	10,000 IU	10,000 IU
12.	Vitamin-D	800 IU	800 IU	800 IU
13.	Vitamin-E	1 mg	1 mg	1 mg

* Wesson LG. A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. *Science* 1943; 75:339-40.
# Bliss CL, Gyorgi P. The animal vitamin assays. In: Gyorgi P. ed. Vitamin Methods Vol. 2, New York: Academic Press, Inc., 1951: 41-275.

Marginal-tryptophan deficiency was created by feeding the rats with 6% Casein and 12% gelatin. Casein consists of 0.15% L-tryptophan which is about half the minimal requirement reported for growing rats[11-13]. Gelatin comprises the entire range of amino acids in the form of peptides except tryptophan[14].

The Departmental Animal Ethics Committee has approved the animal care. The experiment was carried out for three months. All the animals had free access to food and water and were caged separately with similar ambience and the diurnal rhythm. Food intake and body weights were monitored daily and weekly respectively.

Morphological and Behavioral studies

During the study period daily food intake and weekly body weights were recorded. Morphological (hair loss) and behavioral changes were observed periodically.

Tissue extraction and processing: At the end of the experiment animals were sacrificed by CO_2 asphyxiation and tissues were harvested and stored at -80°C till further analysis. The liver and neuronal tissues were extracted from each animal and liver was perfused with normal saline (0.9% NaCl) to reduce red blood cell contamination. A 10% homogenate of hepatic and neuronal tissues was prepared and MDA levels were estimated in total homogenate. The rest of the biochemical parameters were carried out with 25,000x g supernatant and necessary centrifugations depending on the assays.

Biochemical estimation of oxidative stress markers and antioxidant enzymes: Malonadialdehyde (MDA) production was estimated by thiobarbituric acid-reactive substances (TBARS) as described by Buyan et al.[15]. Reduced glutathione was estimated by the spectrofluorometric method of Hissin et al.[16]. Total Superoxide dismutase (SOD, E.C 1.15.1.2) activity was assayed by monitoring the rate of inhibition of pyrogallol reduction[17]. One unit of SOD represents the amount of enzyme required for 50% inhibition of pyrogallol reduction/min. Catalase (CAT, E.C 1.11.1.6) activity of tissues was measured by the method of Aebi[18] by monitoring the disappearance of H_2O_2. One unit of catalase represents the decrease of 1 µmol H_2O_2-/min. The activity of Glutathione-s-transferase (GST, E.C 2.5.18) was assayed spectrophotometrically using CDNB (1-Chloro-2, 4-dinitrobenzene) as substrate[19]. Protein carbonyl content of soluble protein of tissues was measured spectrophotometrically using the 2, 4-dinitrophenyl-hydrazine[20].

Advanced glycation related fluorescence: The Maillard reaction products i.e. advanced glycation end products (AGE) fluorescence was measured in soluble protein (0.3 mg/2 ml in 0.05 M sodium phosphate buffer, pH 7.4). Fluorescence spectra were obtained from 400-500 nm with excitation at 370 nm in a spectrofluorometer[21].

Tryptophan fluorescence

Tryptophan florescence was measured in the soluble protein fraction (0.15 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4) to determine the protein oxidation and conformational changes in different groups of animals. The fluorescence spectra were obtained at excitation 295 nm and emission between 310-400 nm[22].

Protein estimation: The protein content of tissues was estimated by the method of Lowry et al.[23], using BSA as the standard.

Statistical analysis: The differences between the control and treated groups were analyzed using one-way ANOVA, followed by Post Hoc test (multiple comparison). The differences were considered significant if p was at least < 0.05.

Results

Body weights and Food intake

Significant loss in body weight was observed in the animals that received tryptophan deficient diet (group-C) than that of group-A&B animals (fig. 1). The daily food consumption was significantly less in the animals, which received tryptophan deficient diet (group-C) than the groups A and B (data not shown). The food consumption in tryptophan deficient rats was reduced considerably in relation to adequate tryptophan and control groups by the end of 1.5-2 months and, deficiency became life threatening by this time. The mortality rate was 50% in tryptophan deficient group while there was no mortality in group A & B respectively.

Behavioral and morphological studies

Throughout the course of our study, animals of control and adequate tryptophan groups were healthy. Interestingly, tryptophan deficient group showed severe retardations in growth and development of muscular

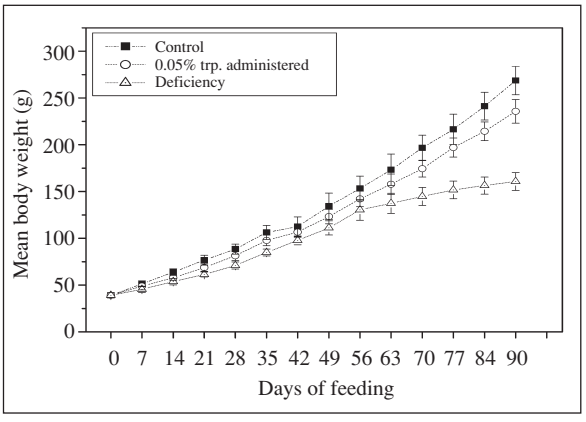


Fig. 1.—Body weights of all the experimental animals during the course of study. The values are Mean ± SD.

Table II

Effect of tryptophan deficiency on hepatic and neuronal protein content in the rats that received different protein concentrations. The data presented is the mean ± SD (n = 5). The asterisk denotes that the data is significantly different from Group A and B

	Gr-A Control	*Gr-B Tryptophan administered (0.05%)*	*Gr-C Protein-deficient*
Liver (g/gm tissue)	2.28 ± 0.485	1.60 ± 0.266 (70.17%)	1.247 ± 0.445 * (54.69%)
Brain (g/gm tissue)	0.868 ± 0.090	0.608 ± 0.128 (70.04%)	0.397 ± 0.076 * (45.73%)

system. Spinal cord of deficient animals resembled a bow in appearance. Severe hair-loss and skin lesions have been observed. However, there was no indication of dementia in tryptophan deficient animals. Ocular problems have been observed. The data related to ocular problems will be published elsewhere.

Organ development

Retardation in organ development was observed in group C with respect to their controls. However, adequate tryptophan rats (group-B) did not show any retardation of organ development (data not shown).

Protein content

There is a significant decrease in the protein content of tryptophan deficient animals (group-C) with respect to their controls (group-A) (table II). Interestingly, adequate tryptophan administration at 0.05% level to the tryptophan deficient rats (group-B) enhanced the protein levels significantly in relation to controls both in hepatic and neuronal tissues (table II).

Oxidative stress markers: TBARS and protein carbonyls content.

Tissue MDA (measured as TBARS) levels were significantly higher in tryptophan deficient rats (group-C) compared to the control group (table III). A significant inhibition of peroxidation was observed in the group-B (table III).

Carbonyl content: A significant inhibition of carbonyl formation was observed in tryptophan deficient rats (group-C) with respect to controls (table III). However, there is no significant variation in carbonyl formation in group-B with respect to controls (table-III).

Antioxidant enzymes

In tryptophan deficient rats (group-C), reduced glutathione (GSH) level had significantly decreased by 43.53% and 36.26% in hepatic and neuronal tissues respectively with respect to controls (table IIIa, b). However, glutathione content was significantly increased by 70.64% and 67.87% in hepatic and neuronal tissues of group-B animals in comparison to that of controls (group-A) (table IIIa, b).

Tryptophan deficient rats showed a significant decrease in the activity of catalase and glutathione-S-transferase in hepatic and neuronal tissues in relation to groups A and B (table IIIa, b). And there was a significant rise in these enzymes in the tryptophan-administered animals (group-B) in relation to group C (table IIIa, b).

Superoxide dismutase (SOD) activity had significantly increased in tryptophan deficient animals (group-C) compared to adequate tryptophan and control groups (table IIIa). Tryptophan administration at 0.05% had significantly restored the enzyme activity in relation to controls (table IIIa). There was no significant change in SOD activity in neuronal tissue (table IIIb).

Table IIIa

Effect of tryptophan deficiency on lipid peroxidation, reduced glutathione concentration, protein carbonyl content and antioxidant enzyme activities in hepatic tissue of rats. The data presented is the mean ± SD (n = 5). The asterisk denotes that the data is significantly different from Group A and B

Group	*LPO nmol g^{-1} wet weight*	*GSH µmol of GSH g^{-1} wet weight*	*SOD (IU)*	*Catalase µmol H_2O_2 decomposed min^{-1} mg^{-1} protein*	*GST µmol CDNB conjugated min^{-1} mg^{-1} protein*	*Protein Carbonyl Content µmoles mg^{-1} protein*
Gr-A Control	317.48 ± 60.75	7.12 ± 1.05	26.386 ± 2.85	1.67 ± 0.309	205.90 ± 21.36	4.77 ± 1.26
Gr-B Tryptophan administered (0.05%)	507.12 ± 120.26	5.03 ± 0.87 (70.64)	35.948 ± 5.52	1.43 ± 0.085	154.66 ± 14.19	3.84 ± 1.11
Gr-C Protein-deficient	907.41 ± 48.42*	3.10 ± 1.24* (43.53)	57.278 ± 3.70*	1.22 ± 0.247*	89.75 ± 24.27*	2.28 ± 0.41*

Table IIIb

Effect of tryptophan deficiency on lipid peroxidation, reduced glutathione concentration, protein carbonyl content and antioxidant enzyme activities in neuronal tissue of rats. The data presented is the mean ± SD (n = 5). The asterisk denotes that the data is significantly different from Group A and B

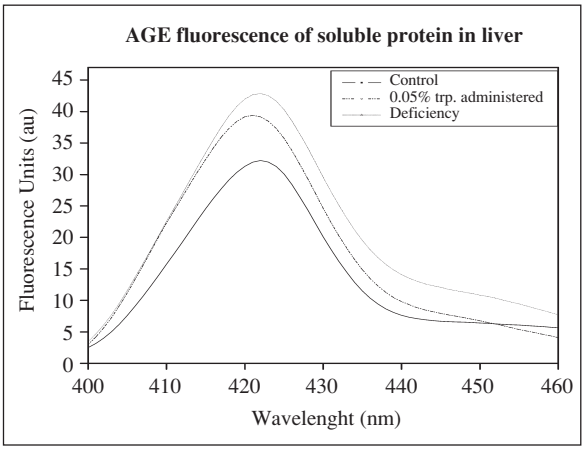
Group	*LPO nmol g^{-1} wet weight*	*GSH µmol of GSH g^{-1} wet weight*	*SOD (IU)*	*Catalase µmol H_2O_2 decomposed min^{-1} mg^{-1} protein*	*GST µmol CDNB conjugated min^{-1} mg^{-1} protein*	*Protein Carbonyl Content µmoles mg^{-1} protein*
Gr-A Control	281.54 ± 64.07	3.86 ± 0.66	4.00 ± 1.99	0.801 ± 0.135	96.91 ± 30.85	2.72 ± 0.34
Gr-B Tryptophan administered (0.05%)	378.22 ± 72.27	2.62 ± 0.85 (67.87)	3.27 ± 1.13	0.618 ± 0.060	68.00 ± 24.86	2.48 ± 0.52
Gr-C Protein-deficient	730.08 ± 54.26 *	1.40 ± 0.60 * (36.26)	2.95 ± 0.75	0.455 ± 0.050 *	33.57 ± 14.43 *	0.90 ± 0.12 *

Advanced glycation end product (AGE) fluorescence: AGE fluorescence (fig. 2) reveals an increase in AGEs formation in tryptophan deficient rats (group-C) compared to controls (group-A). Interestingly, the tryptophan-deficient animals which received 0.05% tryptophan had shown significant inhibition of AGE fluorescence (fig. 2).

Tryptophan fluorescence: Tryptophan fluorescence spectra (fig. 3) showed a decrease in tryptophan fluorescence in tryptophan deficient rats (group-C) in relation to controls (group-A). However, adequate tryptophan administration has been found to inhibit the decrease in tryptophan fluorescence in comparison to group-C (fig. 3).

Discussion

It is well documented that protein deficiency / restriction will affect the morphological, physiological status of the cellular systems possibly through the generation of free radicals / reactive oxygen species, which damage the integrity of biological systems leading to several pathological states[24,25]. In our present study, we have noticed a significant decrease in food intake, body weight, organ development and retardation in muscular system in the rats that were fed on tryptophan deficient diet and this is in agreement with the previous observations of protein deficiency[5,24,26-28]. However, these features were not seen in adequate typtophan group and this is suggestive of a clear protective influence of tryptophan in maintaining the physiological status with respect to controls. In an earlier study carried out by John and Bhat[6] no significant variation in the protein content, tryptophan levels and antioxidant enzymes between the control and pair-fed control groups was reported. In a pilot experiment carried out by us we didn't notice significant variation in the control and pair-fed control groups (data not shown). Therefore, in order to minimize the number of animals due to ethical constraints, we could not maintain pair-fed control groups in our investigation.



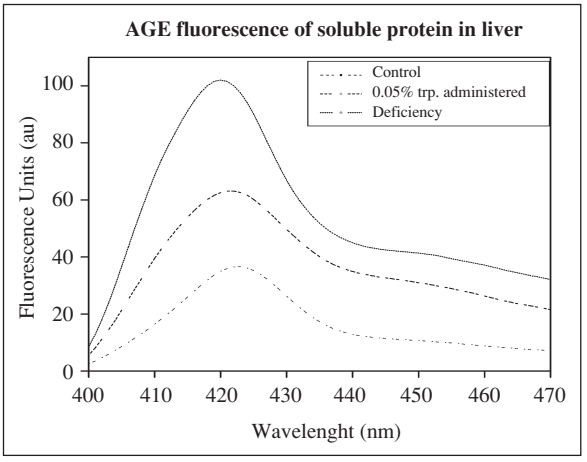
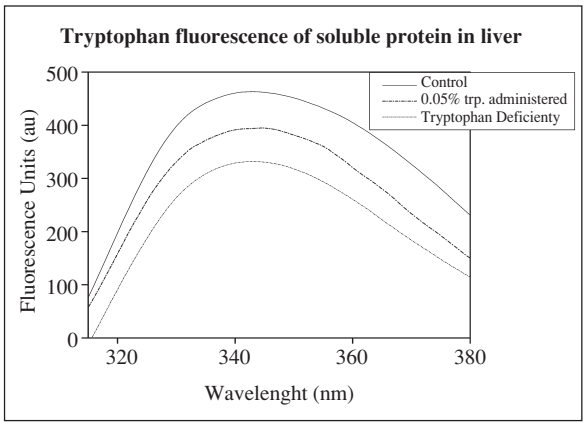


Fig. 2.—AGE fluorescence of hepatic and neuronal tissue, which were fed on different protein concentration with supplementation of tryptophan. Data are average of five values.



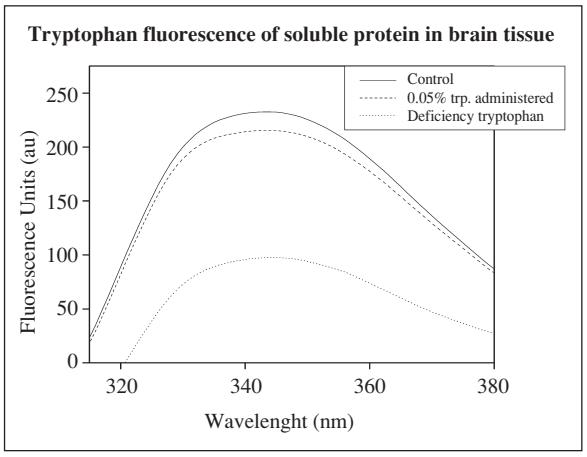


Fig. 3.—Tryptophan fluorescence of soluble protein of hepatic and neuronal tissue in different groups. Data are average of five values.

Tryptophan, a major precursor for many biogenetic and biosynthetic pathways of the physiological systems, plays a vital role in the maintenance of cellular integrity. It is also a major source for the nicotinamide-containing coenzymes, NAD and NADP[1]. The mortality rate was higher in tryptophan deficient animals while there was no mortality in adequate typtophan and control groups indicating the necessity of this amino acid in prolonging life expectancy.

In tryptophan deficient rats there was a remarkable decrease in protein content up to 54.69% and 45.73% in hepatic and neuronal tissues respectively in relation to controls which is in agreement with the earlier reports[2,24]. Interestingly, administration of tryptophan (0.05%) to tryptophan-deficient animals restored the protein content to a moderate extent (70.17% and 70.04%). These findings support that tryptophan, an essential amino acid, plays a crucial role in protein synthesis.

Free radical mediated lipid peroxidation is involved in many pathological processes, and biological systems possess self-defensive mechanisms against these peroxides mediated through enzymatic and non-enzymatic systems. In the present study we have observed significant rise in malondialdehyde products in protein deficient rats which is indicative of severe oxidative stress in these animals. However, adequate tryptophan administration inhibited the peroxidation and thus protecting the tissue from free-radical mediated damage.

In normal physiological state, oxidants generated during metabolism play a significant role in maintaining oxidant-antioxidant ratio. In pathological state, an increase in the reactive free radicals creates an imbalance in this ratio there by making macromolecules vulnerable to oxidative damage. As a result, proteins undergo rapid oxidation leading to the alterations in their structural integrity and in assessing oxidative damage lipid peroxidation and protein oxidation are generally used as biochemical markers[29,30]. In our investigation we have recorded a significant decrease in protein carbonyl content in the tryptophan deficient group with respect to control and adequate tryptophan groups. Lowered damage by free radicals can be expected in tryptophan deficiency with lowered protein content and this could be the underlying mechanism for the observed decrease in carbonyl content in tryptophan deficient rats. This observation is in accordance with the previous report of Youngman et al.[30], who had observed a decrease in carbonyl formation in the aging rats which were fed on protein-restricted diet. However, it cannot be construed that the tryptophan deficient rats are not susceptible to oxidative damage.

The healthy status of living cells can be assessed by the redox cycle tripeptide i.e. reduced glutathione since it is the coenzyme for several enzyme-catalyzed reactions and participates in the detoxification processes. It is well known that the levels of reduced glutathione will decrease in pathological states thereby making the cells prone to oxidative injury[31]. In our study we have observed a significant decrease in glutathione levels in the tryptophan deficient rats which is indicative of pathological state. This observation is in accordance with the previous reports[2,24,32]. However, tryptophan administration at 0.05% raised the levels of glutathione.

The levels of catalase, an ubiquitous hydrogen peroxide detoxifying enzyme, had decreased in tryptophan deficient rats and this indicates a higher level of peroxidation in these rats. Van Pilsum et al.[33], had reported low levels of catalase activity in hepatic tissue in the animals subjected to the deficiency of essential amino acids. There were also reports suggesting that protein deficiency and supplementation of essential amino acid, like, methionine, to the young developing rat brains didn't show significant variation in the enzyme levels[4]. Inclusion of 0.05% tryptophan to tryptophan-deficient animals increased the activity indicating an inhibition or detoxification of H_2O_2.

Glutathione-s-transferases (GSTs), a group of cytosolic enzymes, conjugate with GSH and play a crucial role in a wide variety of substitution reactions and detoxification. In the present study, we have observed a

decrease in the GST level with respect to the controls. This might possibly be due to the high oxidative stress in these rats and due to low concentration of GSH. However, tryptophan administration at 0.05% raised the GST levels perhaps due to increase in GSH level leading to the suppression of free radicals thereby protecting the tissue.

Superoxide dismutase activity was found to increase in hepatic tissue of tryptophan deficient group with respect to tryptophan administered and control groups and this may perhaps be due to the rise in H_2O_2 levels in tissues. Our observations are in accordance with the observations made by Rao et al.[34], who had reported higher m-RNA expression and subsequently higher level of antioxidant enzymes in the rats fed on restricted diet. Administration of tryptophan at 0.05% decreased the SOD levels in relation to tryptophan deficient animals. The probable mechanism in the increase of SOD activity in hepatic tissue of tryptophan deficient animals may be attributed to decreased levels of tryptophan dioxygenase (TDO) and Indoleamine 2,3- dioxygenase (IDO) which scavenge superoxide anion (O_2^-)[1]. We have not observed any significant change in the SOD activity of neuronal tissue and this is in agreement with the findings of Bonatto et al.[4]. Our observation prompts us to draw a tentative conclusion that this could be due to variation in IDO levels as some of the tryptophan metabolites are neurotoxic.

AGE fluorescence has been increased in tryptophan deficient animals indicating the possible glycation, protein unfolding, crosslinking and aggregation which is suggestive of oxidative stress. Decrease in tryptophan fluorescence in these animals further supports the possibility of conformational changes. However, AGE fluorescence was inhibited and tryptophan fluorescence was found to increase in 0.05% tryptophan administered group, which implies the role of tryptophan in stabilization of proteins' native structure.

In summary, our observations reveal that the administration of tryptophan protects the tissues from oxidative damage induced through tryptophan deficiency. Our findings also suggest that administration of tryptophan restores protein content, antioxidant enzyme levels, inhibition of AGE formation and protein aggregation and there by contributing to the well-being of rats in terms of combating oxidative insult.

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