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Research Article

DETERMINATION OF MAJOR ORGANIC CONSTITUENTS IN FK 506 TREATED TISSUES OF ALBINO RATS

Chittibabu. B*[1], Ayya Raju. M[2]

1. Department of Zoology, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India
2. Department of Biochemistry, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India

***Corresponding Author: Email** rajuma.svu@gmail.com

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ABSTRACT

FK 506 is an immunosuppressive agent and highly stable 11-amino acid cyclic polypeptide. Present study demonstrated that FK 506 at the dose and time periods (1 mg / kg over 7 or 28 days impair the rat tissue based organic constituents, FK 506 depleted the rat tissue total protein, carbohydrate, lipid and glycogen contents of all rat tissues selected for the present study and whereas the same FK 506 treatment enhanced the rat tissues FAA and FFA contents: compared to saline treated rat control tissues. The overall data it is evident that FK 506 administration in the present study impairs the overall general metabolism of rat tissues.

Keywords: FK 506, Albino rat, Biomolecules.

INTRODUCTION

In the life on an organism, study of major organic constituents like proteins, lipids and their related metabolites provide the basis for an understanding of overall metabolism of the organism, under varied environmental pathological conditions. The organisms, however, they are known to orient their overall metabolism to overcome their own environmental / pathological stress. Proteins have been defined as extremely complex nitrogen containing organic compounds which are found in all animal and vegetable cells, where they constitute a major part of the living protoplasm of these cells. The proteins differ from carbohydrates and fats, not only in their function but also in their elementary composition. Carbohydrates compose a large number of relatively heterogeneous compounds; these are especially prominent constituents of plants. A fact primarily attributable to the abundance of cellulose and starch, but also occur and serve important functions in animals. These serve as the chief source of energy in the food of humans and many other animals, glucose and glycogen are the two free carbohydrate compounds of general utility in mammals. The other carbohydrate materials are, the pentoses, galactose, mannose, the aminosugars, the sugar derived organic acids. These almost exclusively in mammals exist as constituent groups of complex molecules, such as compound proteins or compound lipids, closely related to glucose and glycogen i.e., readily derived from them and convertible into them, are lactic acid and pyruvic acid. The term lipids is applied to a group of naturally occurring substances characterized by their insolubility in water and are soluble in fat solvents like ether, chloroform, benzene etc. Chemically, the lipids are either esters of fatty acids or substances capable of forming such esters. The oxidation of fats leads to the formation of fatty acids and

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these actively participate in energy-generating processes. The main storage of fat is the adipose tissue. Immunosuppressive agents such as FK 506 are well known to interact with membranes of cellular systems in experimental models. FK 506 is a neutral macrolide isolated from a soil fungus, Streptomyces tsukubaensis. FK 506 is 10 to 100 times more potent than CsA and appears to affect the same common early pathway as CsA1. FK 506 is an effective immunosuppressive agent in liver, pancreas, kidney, heart and small bowel transplantation2. Primarily these agents form lipid peroxides, impair glucose tolerance and also interact with proteinaceous parts of cellular systems3. In view of this, the author at the outset studied the impact of FK 506 on rat tissue major organic constituents.

MATERIALS AND METHODS:

Collection and growing Animals

Albino rats weighing 150 ± 10gm were selected for the present study. Animals were Fed ad libitum with commercial rat diet supplied by Kamadhenu Agencies, Bangalore, India and were housed at constant room temperature of 15 ± 5°C. They were allowed to acclimate to laboratory conditions for at least ten days after arrival before use. Prior to experiment, they were fasted for 24 hr with free access to water. They were divided into 4 groups of 7 each.

Treatment of FK 506 with Rats:

Group I & II rats acted as controls, received only saline (Oral) over 7 or 28 days respectively (daily doses). Group III rats were gavaged daily with 1 mg/kg body wt. of FK 506 in 0.5ml of saline / 7 days, (short term) and group IV were gavaged daily with 1 mg / kg body wt. of FK 506 in 0.5ml of saline over 28 days (long term). After 7 or 28 days of FK 506 treatment of rats, they were anaesthetized with pentobarbitone 5 mg / kg and were sacrificed. Major tissues, like brain, heart, liver and kidney, were isolated, quickly blotted on a filter paper, weight frozen in liquid nitrogen and were stored at -800C till used.

Estimation of Total Proteins

Total protein were estimated by the method of Lowry et al., (1951)4 using crystalline bovine serum albumin as standard. The control and experimental rat tissues were individually homogenized (1%w/v) in distilled water. To 1.0ml of the homogenate, 2.0ml of 10% TCA was added to precipitate the proteins and the contents were centrifuged at 2000 xg for 15 min. The residue was dissolved in 1.0 ml 1N NaOH. To 0.1ml of this, 4.0 ml of alkaline copper reagent was added followed by 0.4 ml of folin phenol reagent (1:1 folin phenol: water). The colour that developed was read at 600 nm in a spectrophotometer against a reagent blank. The protein content was expressed as mg/g wet weight of tissue.

Estimation of total carbohydrates

Total carbohydrate content in control and FK-506 treated rat tissues was estimated by the method of Carrol et al., (1956)5. The tissues were individually homogenized in 10% TCA and centrifuged at 3000 rpm for 15 minutes. To 1.0 ml of TCA supernatant 4.0 ml of anthrone reagent was added and the colour was read against a reagent blank at 600 nm in a spectrophotometer. From the optical density, the total carbohydrate content was calculated and compared with the standard. The values were expressed as mg of carbohydrate gm wet wt. of tissue.

Estimation of total lipids

The tissue total lipid content was estimated by the method of Folchh et al.,(1957)6 and as modified by Christie, et al., (1982)7. The individual tissues from control and experimental groups were homogenized in a mixture of chloroform: methanol (2:1) and centrifuged at 3000 rpm for 15 minutes. A small quantity of water was added to the supernatant and the contents were vigorously shaken. The aquatic layer was separated from biphasic solution. A small aluminum boat was weighed and a known volume of the chloroform layer was added and evaporated at 50°- 60°C in a vacuum drying oven. The container was weighed after complete evaporation of the chloroform phase. The differences between initial and final weights gave the total lipid content. The lipid content was expressed as mg of total lipid / gm dry wt. of tissue.

Estimation of free Amino Acids (FAA)

The total FAA content in control and FK 506 treated rat tissues was estimated by the method of Moore and Stein (1954)8. Individual tissue was homogenized (3%) and prepared in 10% TCA and centrifuged at 600xg for 15 minutes. To 0.2 ml of the supernatant, 2ml of ninhydrin reagent was added and was kept in boiling water for 6 minutes and cooled immediately, at room temperature. The samples were made up to 10 ml with distilled water and the

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colour was read at 575 nm in a spectrophotometer and the values were expressed as µM of tyrosine / g wet wt of tissue.

Estimation of free fatty Acids (FFA)

In the control and experimental tissues are extracted with chloroform: methanol mixture (2:1) ratio and FFA present were determined by suitably modifying the procedure of Schmidt et al., (1974)9 as given by Bergmeyer et al., (1974)10. The samples were treated with chloroform and copper reagent and the contents were thoroughly shaken for 20 min. Finally the chloroform phase was carefully separated with the help of a separator funnel. The chloroform phase so collected was taken and 0.2 ml of sodium diethyl dithiocarbanate was added to develop the colour. The colour was read at 440nm against a reagent blank. Stearic acid was used as standard. The values of FFA were expressed as µM of stearic acid/gm wet wt. of tissue.

Estimation of Glycogen content

Glycogen content in control and experimental tissues was estimated by the method of Carrol et al., (1956)5 using anthrone reagent. Homogenates 5% (w/v) of tissues were prepared in 10% trichloroacetic acid. The homogenates were centrifuged for 10 minutes and the supernatant were taken for the estimation of glycogen. To known aliquots of supernatant, 5ml of 95% ethanol was added and the contents were kept in the refrigerator overnight for complete precipitation of glycogen. The contents were centrifuged for 15 min at 1500 rpm and the residue was dissolved in 2ml of distilled water. To this, 4 ml of anthrone reagent was added and kept for 15 min. in boiling water bath. After cooling, the colour was measured at 620nm in a spectrophotometer against a reagent blank. The blank received 1ml of 10% trichloroacetic acid in the place of homogenate. The amount of glycogen is expressed in terms of mg glycogen/gm wet wt. of tissue.

Statistical Analysis

For each parameter, the mean of individual observations (for both control and experimental groups) were taken into consideration statistical significance of the data was analyzed through one way ANOVA.

RESULTS

The data presented in Table 1,2 and Fig 1 & 2 depicts that FK 506 at a dose of 1mg / kg in both short (7 days) and long term (28 days) treated rats decreased the rat brain, heart, liver and kidneys total protein content and the changes were found to be statistically significant ($p < 0.01$) for most of the tissues over the control. The decrease in the total protein content in FK 506 treated rat tissues was found to be time-dependent. The percent depletion of FK 506 treated rat tissue proteins was greater for the long term (28 days) FK 506 treated group of tissues compared to short term (7 days) FK 506 treated ones, and the percent depletion was more for liver (51.20%) followed by heart (46.34%) > kidney (35.83%) and for brain (29.08%) (Table 2 & Fig 2). Identical trends were also obtained for FK 506 treated rat tissues carbohydrates, lipids and glycogen contents (Table 1, 2 and Fig.3-8).

The FAA and FFA of FK 506 treated rat brain, heart, liver and kidney like tissues showed elevated trends over their corresponding control values and the changes were found to be statistically significant ($p < 0.01$) (Table-1, Fig.9-12). The changes were found to be in a time and dose dependent manner.

DISCUSSION

FK 506 is available for oral administration as capsules (Tacrolimus capsules) containing the equivalent of 0.5 mg/1mg or 5 mg of anhydrous capsules. Letko et al., (2001)11, have suggested a dose 1 mg or 5 mg for clinical trials. Inamura et al., (2004)12 have preferred an FK 506 dose of 0.32mg/kg in rats. Nicoleth; have chosen an FK 506 dose of 2.5 kg over 27 to 120 days in their experimental studies on rats. Kawashima et al., (1990)13 preferred an FK 506 dose of 0.1 or 1 mg/ kg in their studies in rats as cited by Kahan et al., (1991)14 various authors have preferred 0.5 mg/kg/day or 1mg/kg or 2 mg/kg over 1 or 2 weeks in their experimental studies. Thus multi center FK 506 liver study group (1994) preferred a dose of 0.5-2 mg of FK 506 in their experiments. From the above it is evident that various authors have selected varied doses of FK 506. To study the toxic effects of FK 506 in their experimental models. The author of the present study has conveniently selected 1mgkg/wt of FK 506 to examine its in vivo effect on rat tissue oxidative metabolic parameters in the tissues of albino rats and the time periods selected were 7 days as short term and 28 days as long term. Decrease in rat tissue total protein was observed in FK 506 treated rat tissues in the

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Table 1: Effect of FK 506 on 7 days rat brain, heart, liver and kidney Total proteins (mg/g wet et), Total carbohydrate (mg/g wet wt), Total lipid (mg/g wet wt), Glycogen (mg/g wet wt), Free amino acids (mg/g wet wt) and Free Fatty acid (μM stearic acid/g wet wt) levels *in vivo*.

Name of the Metabolite	7 days (1mg FK 506/kg wt)							
	Brain		Heart		Liver		Kidney	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
Proteins Av±SD	77.30 ± 1.58	62.00 ± 1.80	72.16 ± 1.33	55.74 ± 1.32	88.69 ±1.3	59.14 ±0.642	105.85 ±1.20	84.14 ±0.541
PC		-19.79%*		-22.75%*		-33.31%*		-20.51%*
Carbohydrates Av±SD	36.53 ± 0.306	25.96 ± 0.224	39.09 ± 0.405	28.47 ± 0.360	57.69 ± 0.964	34.94 ± 0.361	38.06 ± 0.422	24.07 ± 0.221
PC		-28.93%*		-27.16%*		-39.43%*		-36.75%*
Lipids Av±SD	292.67± 2.93	266.09± 1.96	267.62± 2.84	248.35± 1.67	506.32± 4.72	434.78± 6.73	451.67± 3.64	321.91± 5.66
PC		-9.08%*		-7.20%*		-14.12%*		-28.72%*
Glycogen Av±SD	2.86± 0.210	2.32± 0.096	2.43± 0.315	2.07± 0.083	12.32± 0.424	6.76± 0.364	2.60± 0.032	2.04± 0.154
PC		-18.88%*		-14.81%*		-45.12%*		-27.18%*
Free amino acids Av±SD	70.26± 1.32	79.06± 0.961	62.99± 0.842	79.77± 1.031	77.76± 0.914	92.46± 2.63	56.99± 0.882	73.55± 1.34
PC		12.52%		26.63%*		18.90%*		29.05%*
Free fatty acids Av±SD	241.56± 3.69	253.08± 2.16	161.08± 1.72	173.67± 2.49	170.42± 1.37	181.27± 2.08	105.18± 1.94	157.79± 2.64
PC		4.76%*		7.81%*		6.36%*		50.01%*

Table 2: Effect of FK 506 on 28 days rat brain, heart, liver and kidney Total proteins (mg/g wet et), Total carbohydrate (mg/g wet wt), Total lipid (mg/g wet wt), Glycogen (mg/g wet wt), Free amino acids (mg/g wet wt) and Free Fatty acid (μM stearic acid/g wet wt) levels in vivo.

Name of the Metabolite	28 days (1mg FK 506/kg wt)							
	Brain		Heart		Liver		Kidney	
	Control	Expt	Control	Expt.	Control	Expt.	Control	Expt.
Proteins Av±SD	78.01 ±0.624	55.32±0.536	75.00±1.05	40.24±0.436	90.17±1.39	44.00±0.365	105.91±1.47	67.96±0.522
PC		-29.08%*		-46.34%*		-51.20%*		-35.83%*
Carbohydrates Av±SD	38.22± 0.942	20.18± 0.761	42.32± 1.23	23.61± 0.647	59.19± 1.08	22.06± 1.149	34.42±0.442	18.31± 0.699
PC		-47.20%*		-44.21%*		-62.73%*		-46.80%*
Lipids Av±SD	293.11± 1.36	252.24± 3.22	270.34± 1.04	232.08± 2.91	510.36± 4.30	305.94± 2.16	460.30± 4.19	241.20± 3.942
PC		-16.20%*		-14.15%*		-40.05%*		-47.59%*
Glycogen Av±SD	2.92± 0.136	2.06± 0.022	2.45± 0.044	1.91± 0.052	11.96± 0.36	5.12± 0.27	2.63± 0.036	1.73± 0.046
PC		-29.45%*		-22.04%*		-57.19%*		-34.22%*
Free amino acids Av±SD	74.24± 1.26	83.14± 2.041	63.34±0.96	87.22± 0.74	79.31± 1.36	105.23± 2.491	57.23± 0.78	82.35± 0.88
PC		11.98%*		37.70%*		32.68%*		43.89%*
Free fatty acids Av±SD	243.41± 2.41	262.08± 3.04	160.11± 0.982	253.08± 2.16	174.32± 1.23	193.05± 2.37	139.67± 1.02	163.24±2.64
PC		7.67%*		58.06%*		10.74%*		16.87%*

Each value is the mean ±SD of 7 samples. AV: Average SD: Standard Deviation PC: percent change over control Expt: Experimental $p<0.01$

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Fig. 1: Effect of FK 506 on rat tissue Total Protein content *in vivo*

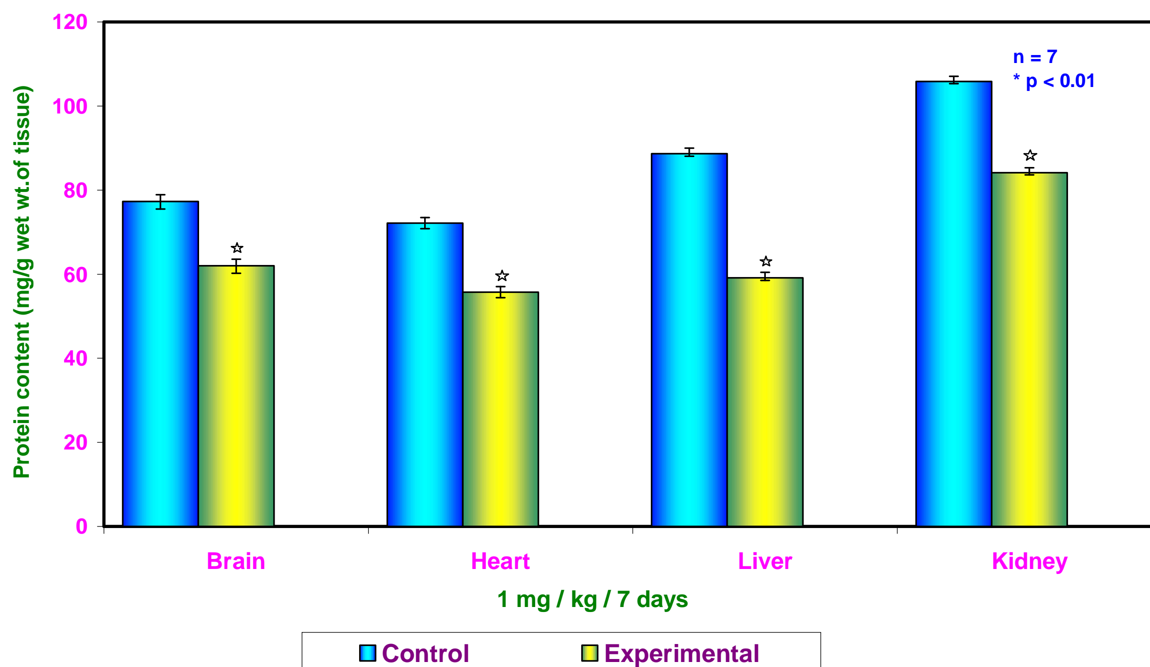
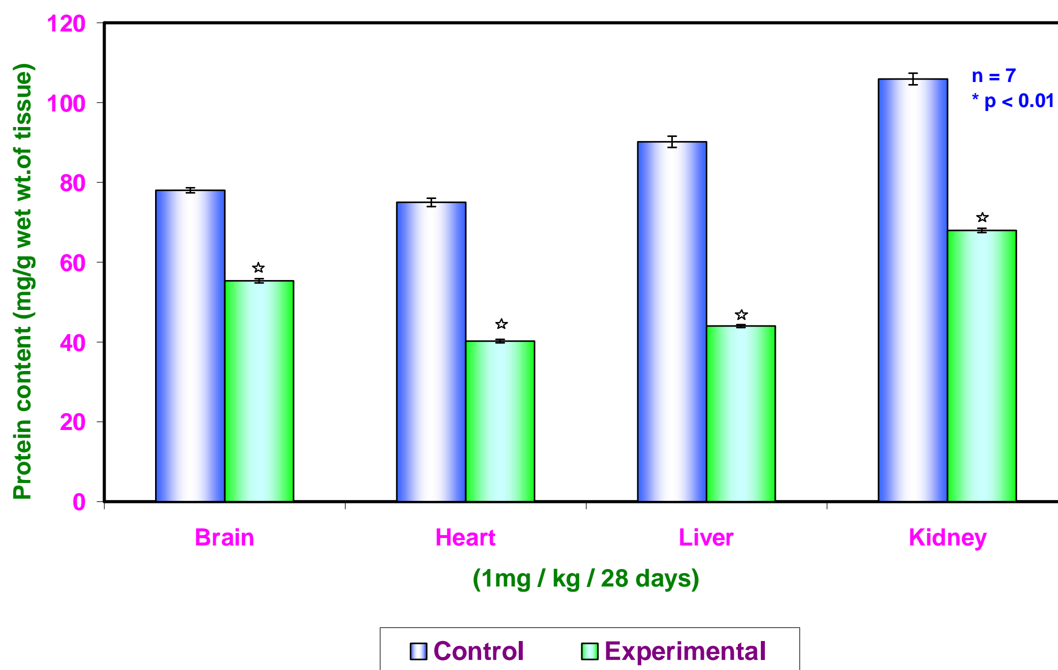


Fig. 2: Effect of FK 506 on rat tissue Total Protein content *in vivo*



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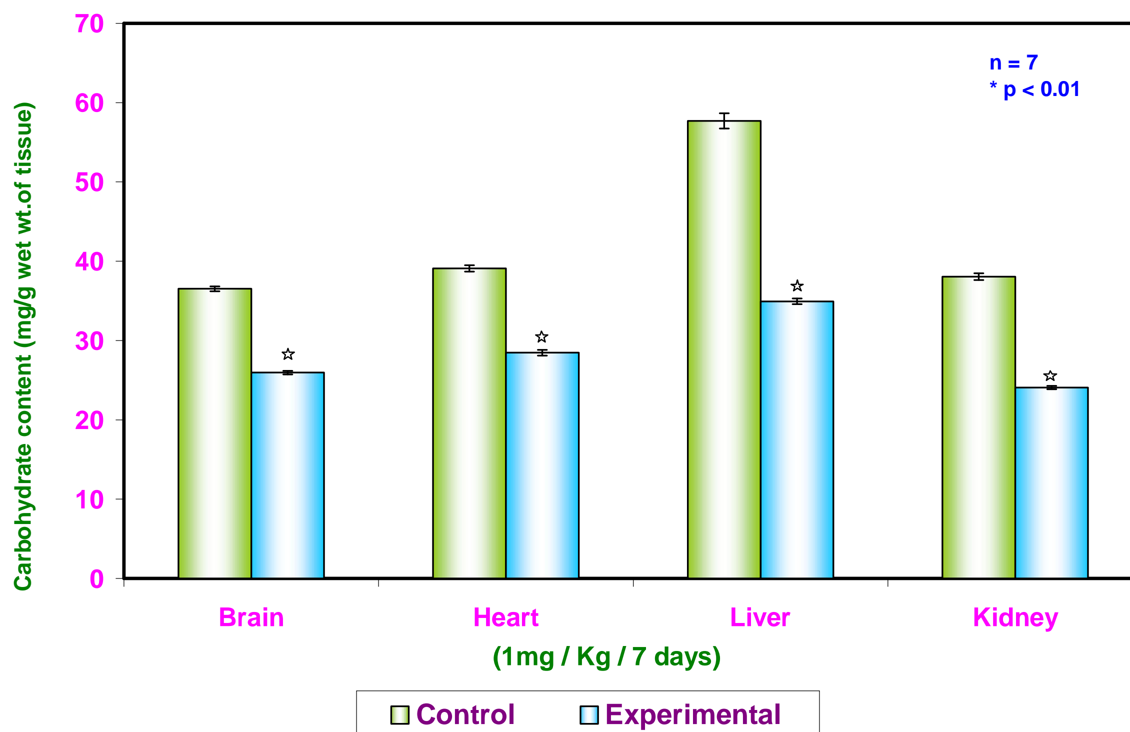

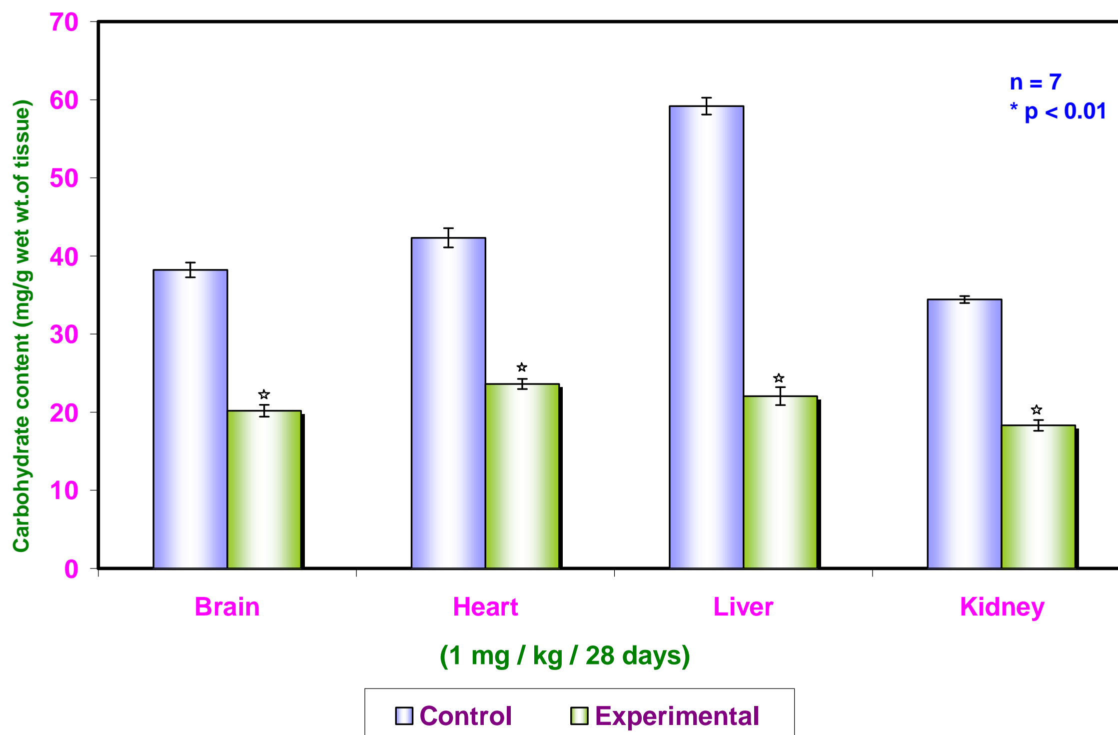

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Fig. 5: Effect of FK 506 on rat tissue Total Lipid content *in vivo*

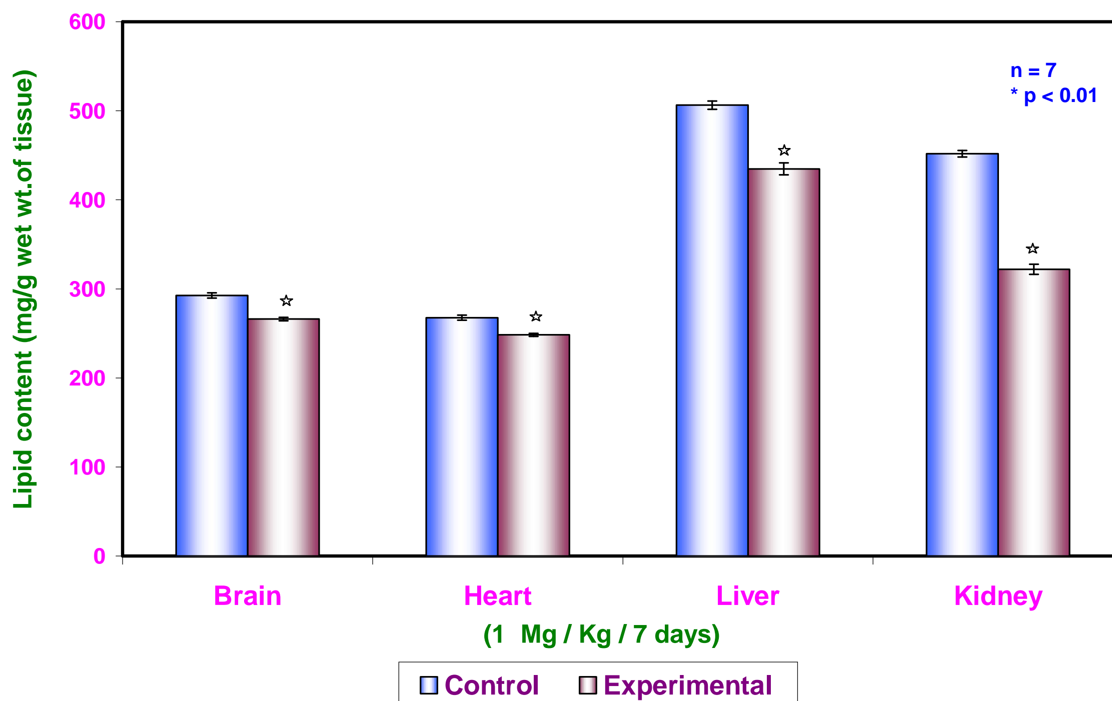

Fig. 6: Effect of FK 506 on rat tissue Total Lipid content *in vivo*

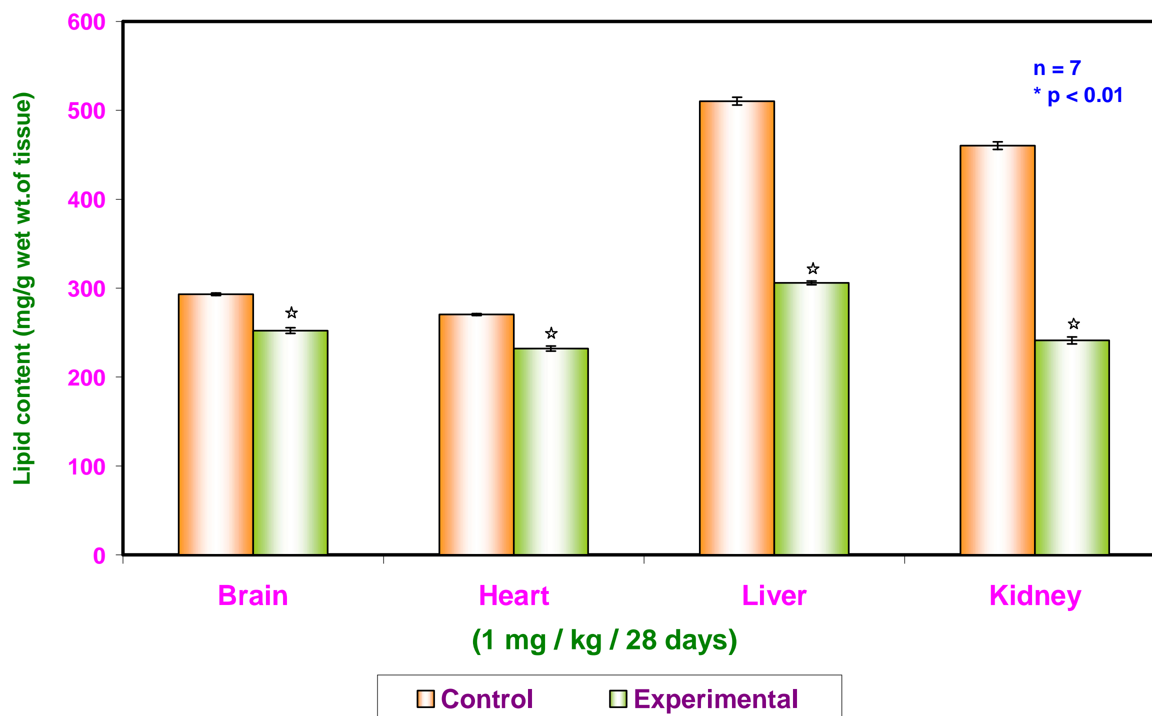

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Fig. 7: Effect of FK 506 on rat tissue Glycogen content *in vivo*

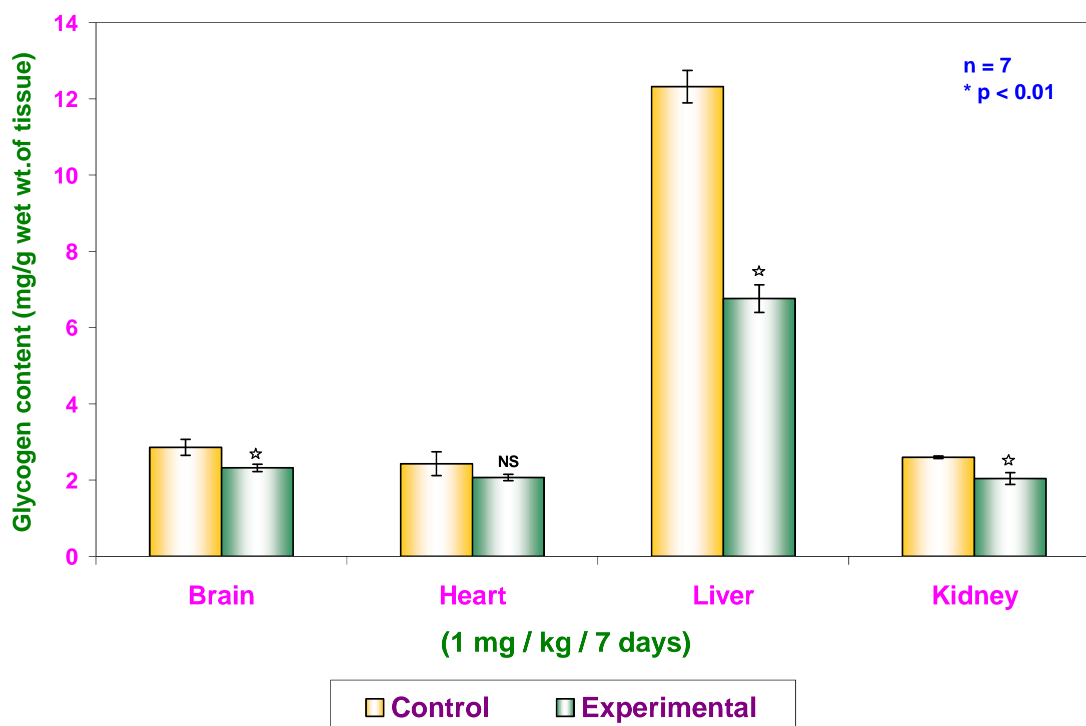

Fig. 8: Effect of FK 506 on rat tissue Glycogen content *in vivo*

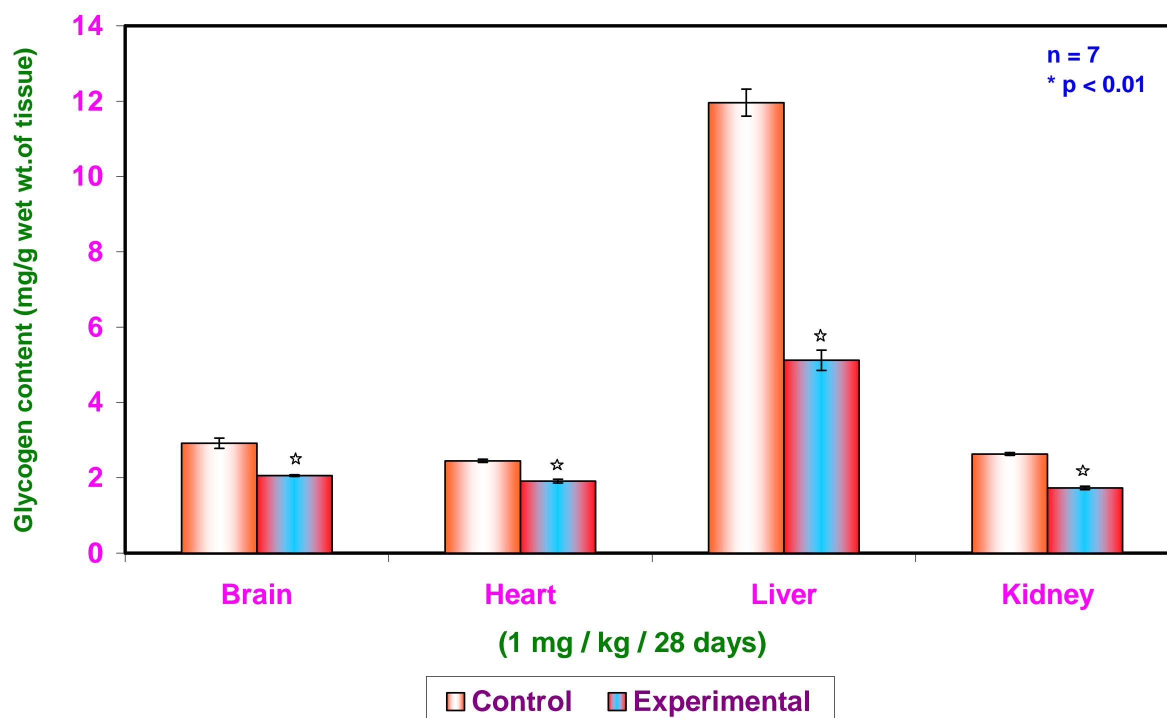

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Fig. 9: Effect of FK 506 on rat tissue Total Free Aminoacid levels *in vivo*

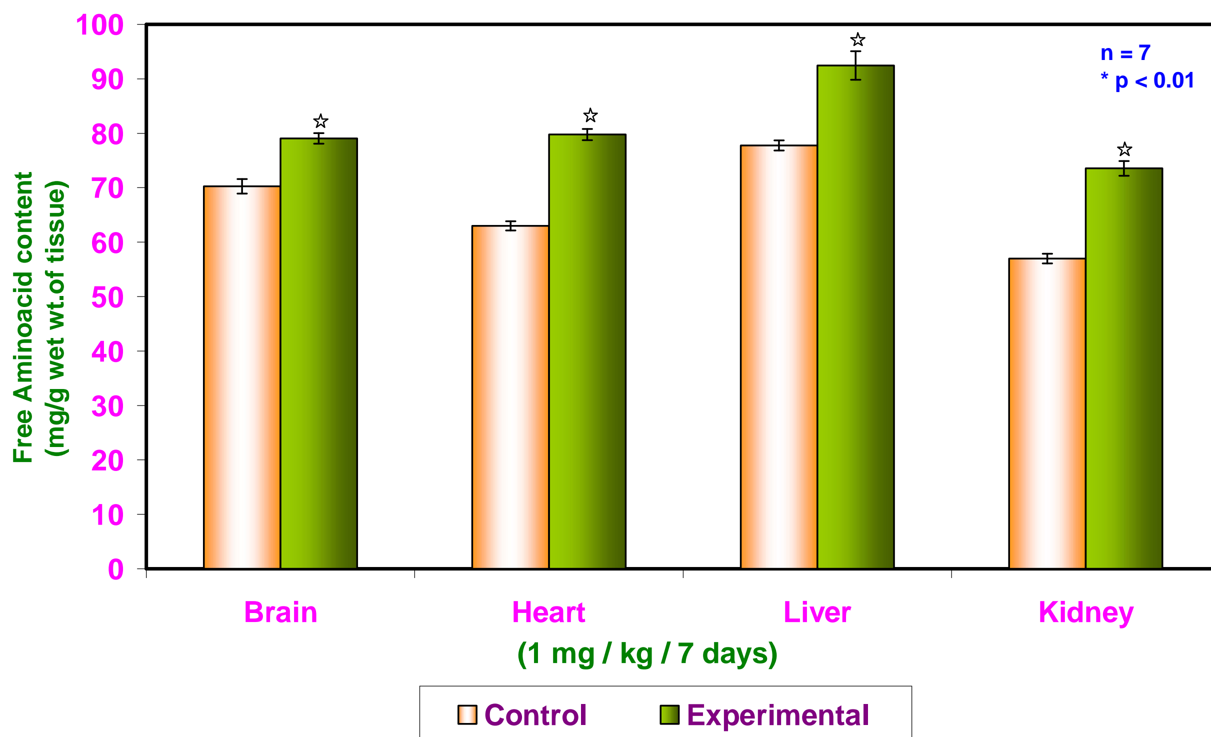

Fig. 10: Effect of FK 506 on rat tissue Free Aminoacid levels *in vivo*

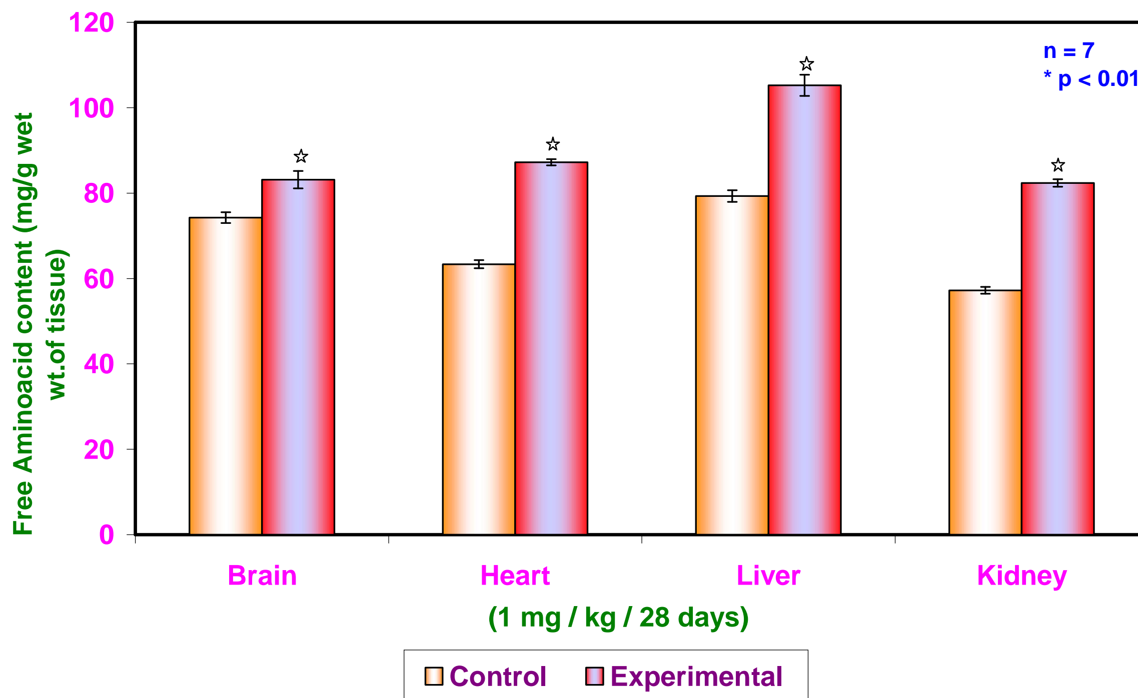

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Fig. 11: Effect of FK 506 on rat tissue Free Fatty acids levels *in vivo*

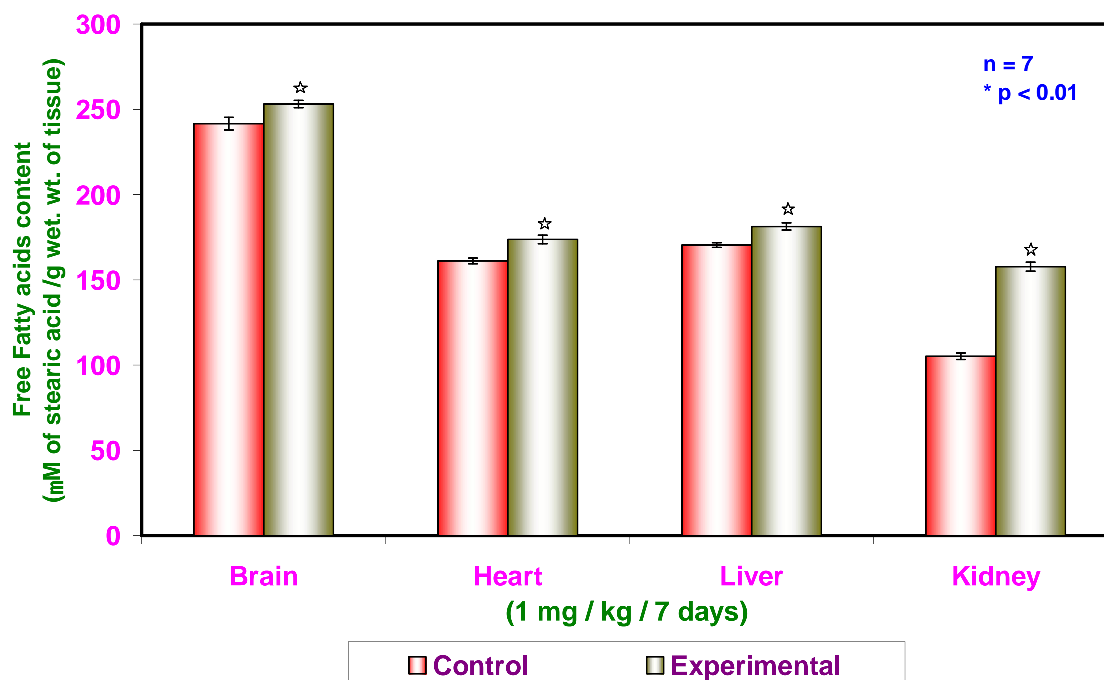
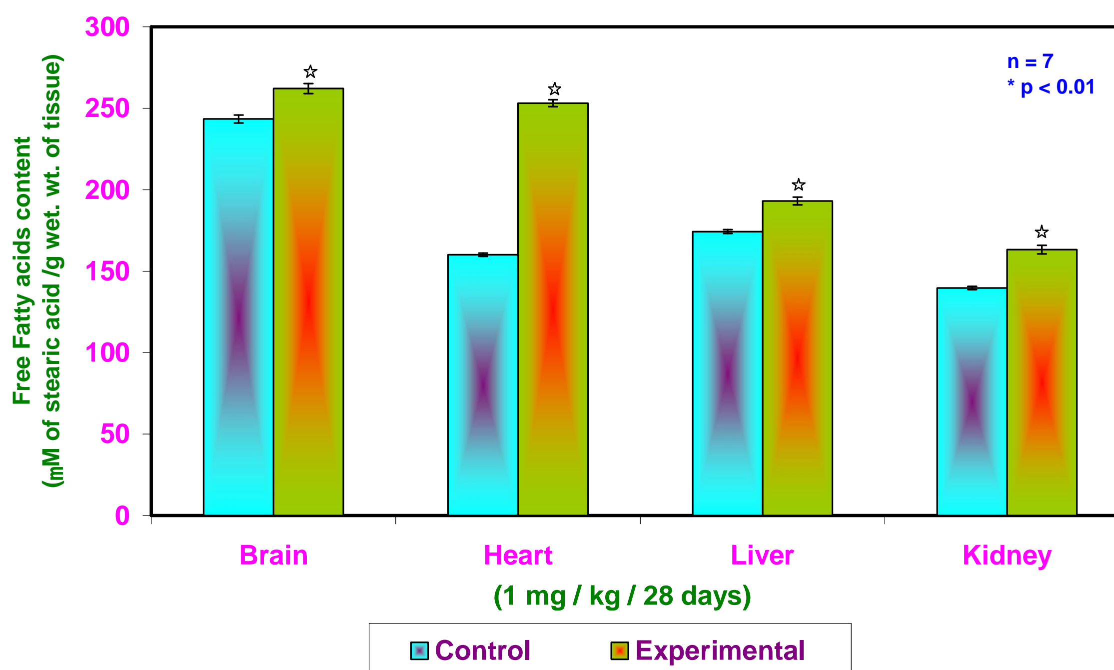


Fig. 12: Effect of FK 506 on rat tissue Total Free Fatty acids levels *in vivo*



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present study. This could have been affected through an indirect stimulation of proteolytic activity which in turn could step up the rat tissues proteolytic activity in different tissues of the FK 506 treated rats causing lysis and break down of proteins. Arise in proteolytic activity could lead to the breakdown of proteins into their constituent amino acids and thinking at be responsible for elevated levels of FAA in short and long term FK 506 treated rat tissues (Table 1 & 2). The pool of FAA in an organ represents the reservoir which is utilized for protein synthesis and to which the products of protein degradation are returned. The increased FAA pools in FK 506 treated rat tissues may actively participate in the tricarboxylic acid cycle to generate more energy to overcome the FK 506 induced toxic stress which is a common feature in most of the animals under varied stress conditions15. These results are in agreement with reports of the earlier author from our laboratory, where for an immunosuppressive agent like CsA at an administered dose of 10mg/kg over 7 or 28 days deplete rat tissues total protein content and enhance the level of rat tissues FAA content. However, the author could not find any citations related to FK 506 interaction of rat tissues total protein & FAA contents excepting to the available reports that FK 506 do interfere with calcineurin – linked proteins in various experimental models (citations were made in the preceeding pages). CsA or FK 506 as soon as they are administered is accumulated by most cells in the body and at high intracellular concentration they are cytotoxic16. Number of investigators has contributed for the understanding of the mechanism of action of CsA or FK 506 most of their experimental studies involves T-cell mediated mechanisms. It appears that less attention was paid to understand the in vivo effect of FK 506 on various basic organic constituents in experimental models. Hence, the author initially attempted to understand the fate of total proteins, carbohydrates, lipids, glycogen, FAA and FFA in short and long term FK 506 treated rat tissues.

Concerning the results of total carbohydrate and glycogen levels in the control and FK 506 rat tissues in this study (table 1,2 fig.2 & 3, 7 & 8), a drop in rat tissue total carbohydrate and glycogen contents was observed and the changes were found to be statistically significant over the control ($p < 0.01$). Under normal metabolic conditions, carbohydrate utilization leads to enhanced levels of glycogen at least in tissues like liver. Glycogen, is the storage polysaccharide of the body and a major source of energy for the metabolic machinery. Proper maintenance of glycogen reserves for energy is one of the important features of normal metabolism. In the present study, glycogen content was decreased in all the major tissues of

FK 506 treated rats (Table 1 & 2). The balance between the demands for supply of energy in the form of glycogen seems to get disturbed upon FK 506 treatment. Corresponding to the decrease in tissue glycogen content, an elevation in serum glucose levels was previously reported by various authors17-20. Gluconeogenesis is the breakdown of glycogen into glucose as a main end product there by decreasing the glycogen content21. FK 506 induced glycogenolysis may lead to hyperglycemia and, in addition a decrease in total carbohydrate content (Table 1 & 2) may be responsible for the reported changes in serum glucose content in immunosuppressive agents like FK 506 treated animals20 and the results of the present study are in agreement with the findings of the earlier authors20. The foregoing confirms that FK 506 treatment may induce a state of hyperglycemia and glycogenolysis in rat tissues and these findings are in agreement with the earlier reports 17-20. Studies were further carried out to know the fate of total lipids and FFA like lipid metabolic profiles in control and FK 506 treated rat tissues in the present study. FK 506 treated rat brain, heart, liver and kidney tissues exhibited lowered levels of their total lipid content and elevated levels of their FFA content over their control group of tissues. The changes were more pronounced for rats treated with FK 506 over 28 days compared to 7 days FK 506 treated group of rats (Table 1 & 2). The decrease in the FK 506 treated rat tissue lipids could either be due to increased lypolysis or due to a reduction in the fatty acid synthesizing enzymes22. Curiously, the FFA increased in all the FK 506 treated rat tissues and this suggests an increased lypolysis. From the data obtained out of the present study, it is evident that FK 506 at the dose and time periods tested may induce a pathological condition like lipolysis. These alterations of FK-506 treated rat tissue total lipid and FFA might further be due to cell disruption of intracellular organelles due to FK 506 stress. Some support is available regarding this hypothesis from the reports of

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earlier authors23-25. Lipid particles from damaged cells or organs may diffuse more rapidly from than normal. As a result, there may be a decrease in tissue total lipids. The elevated FFA with FK 506 might also be due to increased lytic activity of the lipase on triglycerides which releases FFA from parent substances, such as triglycerides, phospholipids, etc. (Bhattacharya and Maity, 1988, Ramani, 2002). From the overall data presented in table 1, it can be concluded that FK 506 by disrupting the overall organic constituents of rat tissue may impair the overall normal metabolism of the rat tissues, and this in part may cause neurotoxicity, myocardial toxicity, hepatotoxicity and nephrotoxicity in rat tissues.

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