

Effects of Boron Compounds in Rabbits Fed High Protein and Energy Diet: A Metabolomic and Transcriptomic Approach

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Abstract—Current research is targeting new molecular mechanisms that underlie non-alcoholic fatty liver disease (NAFLD) and associated metabolic disorders like non-alcoholic steatohepatitis (NASH). Forty New Zealand White rabbits have been used and fed a high protein (HP) and energy diet based on grains and containing 11.76 MJ/kg. Boron added to 3 experimental groups' drinking waters (30 mg boron/L) as boron compounds. Biochemical analysis including boron levels, and nuclear magnetic resonance (NMR) based metabolomics evaluation, and mRNA expression of peroxisome proliferator-activated receptor (PPAR) family was performed. LDL-cholesterol concentrations alone were decreased in all the experimental groups. Boron levels in serum and feces were increased. Content of acetate was in about 2x higher for anhydrous borax group, at least 3x higher for boric acid group. PPAR α mRNA expression was significantly decreased in boric acid group. Anhydrous borax attenuated mRNA levels of PPAR γ , which was further suppressed by boric acid. Boron supplementation decreased the degenerative alterations in hepatocytes. Except borax group other boron groups did not have a pronounced change in tubular epithels of kidney. In conclusion, high protein and energy diet leads hepatocytes' degenerative changes which can be prevented by boron supplementation. Boric acid seems to be more effective in this situation.

Keywords—High protein and energy diet, boron, metabolomic, transcriptomic.

I. INTRODUCTION

OBESITY is a major risk factor for the development of NAFLD. NAFLD affects up to a third of the human population in many developed countries. Between 10% and 30% of patients with NAFLD have NASH that can progress to cirrhosis. There are metabolic risk factors common to both NAFLD and cardiovascular disease, so patients with NASH

have an increased risk of liver-related and cardiovascular death [1], [2].

The frequency of canine and feline obesity is at least 20 % in industrialized countries. Many diseases are associated with obesity in both species [3].

The influence of the dietary nutrients is important and can help to treat NAFLD and associated metabolic comorbidities. In NAFLD/NASH patients, elucidation of excessive nutrients by careful investigation of their dietary intake leads to better nutrition therapy [4], [5]. In general, hypercaloric diet, high dietary saturated fatty acid and cholesterol, and soft drinks seem to increase stimulate hepatic lipid accumulation and progression into NASH, whereas reducing total caloric intake, increasing soy protein and whey consumption, and the supplements of MUFA, omega-3 fatty acids, and probiotics have preventive and therapeutic effects. Therefore, nutrition serves as a major route of prevention and treatment of NAFLD [6]. Feeding a HP diet prevented the development of NAFLD by enhancing lipid secretion into VLDL particles and a less efficient use of ingested calories [7]. No effective drugs against NASH have yet been developed [8].

The peroxisome proliferator-activated receptors, PPAR α , PPAR β , and PPAR γ , are a family of transcription factors activated by a diversity of molecules including fatty acids and fatty acid metabolites. PPARs play pivotal roles in energy homeostasis and metabolism, and regulate the transcription of a large variety of genes implicated in metabolism, inflammation, proliferation, and differentiation in different cell types [9], [10].

Metabolomics is a tool with the potential for major influence on public health preventive strategies since it provides background knowledge of the underlying mechanisms of obesity and associated diseases such as insulin resistance and type 2 diabetes mellitus [11].

Nutritional amounts of boron fed to animals and humans consuming a diet low in boron induce numerous biochemical and functional responses considered beneficial for bone growth and maintenance, brain function, and perhaps cancer risk reduction [12]. According to our previous studies boron contributed to reducing fat infiltration of the liver and visceral fat amount and seemed to be effective on energy metabolism in rabbit fed with high energy diet [13], and NMR-based metabolomics become a tool for better understanding obesity, liver and boron metabolism [14].

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The aim of the present study was to investigate whether chronic consumption of a HP and energy diet, alters the response of the liver and kidney tissues to boron supplementation in drinking water.

II. MATERIAL AND METHODS

A. Animals, Diets and Boron Administration

The experimental design was approved by the Committee on Use of Animals in Research of Selcuk University, Faculty of Medicine. Forty female New Zealand White rabbits, aged 8 months, 2-2.5 kg weighted have been used as animal material. The animals have been individually housed in an air-conditioned room with controlled temperature (20-23°C) and automatic lighting. Next, the animals have been randomly divided into four groups each consisted of ten animals. The average body weights of the groups were equal at the beginning of the experiment. All the groups were fed with a high protein and energy diet based on grains and containing 11.76 MJ/kg (Diet CP 5701, CP Turkey). The diet contained 2,850 kcal/kg metabolizable energy. The protein and cellulose contents were 18% and 12%, respectively. Boron added to 3 experimental groups' drinking waters (30 mg boron/L) as boron compounds: borax decahydrate ($\text{Na}_2\text{O}_4\text{B}_7 \cdot 10\text{H}_2\text{O}$, experimental 1), borax anhydrous ($\text{Na}_2\text{O}_4\text{B}_7$, experimental 2), and boric acid (H_2BO_3 , experimental 3). The diets and drinking waters were provided *ad libitum*.

B. Blood and liver sampling

Blood was drawn from the ear central arteria into heparin-coated and non-heparinized tubes at the start and after 3 months of treatment. Plasma was obtained by centrifuging the blood at 1000 x g for 15 min at 4°C. The rabbits were sacrificed following overdose pentobarbital sodium. The liver was then removed, rinsed with physiological saline, and weighed. Liver tissue was sampled from the left lobe, and the samples were stored at -80°C.

C. Biochemical Analysis

Plasma was analyzed for lipid peroxidation (LPO), glucose, insulin, glucose-6-phosphate dehydrogenase (G6PD), antioxidant potential (AOP) with commercial ELISA test kits (EAST BIOPHARM). Triglyceride and cholesterol (total, HDL and LDL) were measured by spectrometer (BT 3000 Spectrometer, Italy).

D. NMR Spectroscopy

NMR spectra were measured at 700 MHz using a Bruker Avance spectrometer equipped with a triple resonance (^1H - ^{13}C - ^{15}N) room temperature probe. Prior to the measurement, each sample was kept at room temperature (about 22°C) for one hour. Then, the sample was inserted in the probe and allowed 10 minutes for the temperature to stabilize at 37°C. Prior to the measurement, the probe tuning, shimming and pulse calibration was performed automatically for each sample. T_2 -relaxation filtered spectra were measured using standard Bruker cpmgpr1d pulse sequence with the relaxation delay of 3s, 78 ms T_2 -filter with a fixed echo delay of 403 μs

and acquisition time 3.3 s. Four dummy scans and 32 transients were applied and 64k points were collected. In both types of spectra, water signal was suppressed by presaturation applying 25 Hz irradiation filed during the relaxation delay. NMR spectra were zero-filled to 128k data points and then multiplied with an exponential window function with a 1.0 Hz line broadening prior the Fourier transforms. The first spectra in each series were manually phase corrected and referenced to TSP. The same processing parameters were then applied to the whole series. The signal intensities and integrals for the individual metabolites in the NMR spectra were evaluated automatically by finding the maxima in the predefined regions and fitting the Lorentzian curves to the detected peaks.

E. RNA extraction, cDNA synthesis, and qPCR

Tissues obtained from rabbit liver were immediately snap-frozen in liquid nitrogen and stored at -70°C. At the time of RNA extraction, equal proportions (50 mg) of livers were minced with a scalpel followed by homogenization within TRI Reagent (Sigma, USA). RNA isolation protocol and quality measurements from rabbit liver were performed according to [15]. The RNA pellets in each tube were dissolved in 100 μl of DEPC-treated sterile water. Ten μl of each RNA sample was electrophoresed on 1% agarose gel for quality control. Concentration was measured and 260/280 ratio was calculated to assess purity by using a nanodrop spectrophotometer (Thermo Scientific, USA). One μg of total RNA was treated with RNase free DNase I (Fermentas, USA) to clean gDNA contamination, which was then reversely transcribed in the presence of random hexamer and oligodT primers in equal volume by using Revert Aid First Strand cDNA Synthesis Kit (Thermo SCIENTIFIC, USA) according to the manufacturer's protocol.

Primers were either obtained from published sequences [16] or derived from rabbit sequences by using IDT Primer Quest Tool program. The expression of GAPDH mRNA was employed as a reference gene selected as being the best-fit housekeeping gene in the experimental model in this study. Real Time PCR reactions were performed on a Light Cycler Nano Real Time PCR instrument (Roche Diagnostics, GERMANY) in 25 μl reaction volumes including 12.5 μl Maxima SYBR Green/ROX qPCR Master Mix (Thermo SCIENTIFIC, LITHUANIA), 10 pMol each primer and 2 μl isolated RNA sample (cDNA) as template. Real Time PCR profile was an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing beginning at 60°C for 30 sec and extension at 72°C for 30 sec. In addition, Roche Light Cycler Nano Tm calling operation was performed for melting temperature analysis. For the confirmation of Real Time PCR products, they were separated by electrophoresis on 2% agarose gels and were visualized after ethidium bromide staining.

F. Histopathological Analyses

Liver and kidney samples were fixed in Formalin solution, neutral buffered, 10%. Paraffin blocks were obtained after routine follow up processes. Thin sections (5 μm) were cut

from each sample, stained with Hematoxylin and Eosin (H&E), and examined under light microscopy. Lesions were scored as mild (+1), moderate (+2) and severe (+3).

G. Boron Analysis

Using reference material 8414 (National Institute of Standards and Technology) boron concentrations were measured in serum, consumed water and feed, liver and feces samples by ICP AES (VARIAN VISTA AX CCD) method.

H. Statistical Analyses

All laboratory and histopathological data were presented as the mean \pm SE. The data were evaluated by one-way ANOVA-Duncan using the SPSS 13 program (for Windows/SPSS® Inc, Chicago, USA), and the differences between the means assessed using Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

For transcriptomics, before statistical analysis, the efficiencies of amplification of our target genes and internal control (GAPDH) were examined using qPCR amplification

of serial dilutions of cDNA. On the basis of confirmation that the amplification efficiencies of the target and reference genes are nearly the same, data normalization process was performed according to Livak and Schmittgen [17] using $2^{-\Delta C_T}$ method, where $\Delta C_T = C_{T, \text{target}} - C_{T, \text{reference}}$ (where $C_{T, \text{target}}$ and $C_{T, \text{reference}}$ are the threshold cycles for the target and reference genes amplifications, respectively).

III. RESULTS

Cumulative food intake, body weight gain, visceral fat accumulation and body mass index did not differ between the groups.

A. Biochemical Parameters

Decreased LDL-cholesterol concentrations alone were observed in all the experimental groups in which boron levels in serum were increased compared to the control (Tables I and II). Boron was more extracted through feces in experimental groups 2 and 3.

TABLE I
BIOCHEMICAL PARAMETERS

Parameters	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
LPO, $\mu\text{M}/\text{mg}$ protein	1.55 \pm 0.12	1.49 \pm 0.08	1.45 \pm 0.11	1.86 \pm 0.07
Glucose, mg/dL	123 \pm 14.7	139 \pm 13.2	135 \pm 10.6	136 \pm 13.3
Insuline, $\mu\text{IU}/\text{L}$	9.52 \pm 0.92 ^a	11.0 \pm 1.01 ^{ab}	10.3 \pm 0.68 ^{ab}	13.6 \pm 0.63 ^{ab}
G6PD, U/g Hb	3.72 \pm 0.47	4.25 \pm 0.27	4.61 \pm 0.82	3.86 \pm 0.40
AOP, $\mu\text{M}/\text{mg}$ protein	1.07 \pm 0.04	1.18 \pm 0.07	1.20 \pm 0.02	1.09 \pm 0.04
Total-C, mmol/L	36.1 \pm 6.43	33.3 \pm 4.14	47.0 \pm 8.89	42.3 \pm 7.73
HDL-C, mmol/L	21.7 \pm 2.44	22.3 \pm 1.76	27.2 \pm 2.85	23.3 \pm 2.20
LDL-C, mmol/L	12.0 \pm 2.59 ^a	7.55 \pm 1.91 ^b	7.66 \pm 2.36 ^b	6.9 \pm 2.87 ^b
TAG, mg/gr liver	33.0 \pm 4.38	34.2 \pm 3.32	35.4 \pm 5.39	38.0 \pm 5.34

TABLE II
BORON LEVELS (PPM)

Parameters	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
Serum	0.17 \pm 0.26 ^a	1.31 \pm 0.04 ^b	1.61 \pm 0.06 ^b	1.37 \pm 0.01 ^b
Liver	0.33 \pm 0.08	0.46 \pm 0.004	0.42 \pm 0.03	0.47 \pm 0.03
Feces	1.779 \pm 0.01 ^a	2.179 \pm 0.1 ^{ab}	3.423 \pm 0.03 ^b	3.179 \pm 0.02 ^b
Feed	3.8807	3.8807	3.8807	3.8807
Water *	0.0091	0.0091	0.0091	0.0091

* before boron supplementation

TABLE III
ABSOLUTE CONCENTRATIONS OF PLASMA ENDOGENOUS METABOLITES [$\mu\text{MOL}/\text{ML}$]

Parameters	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
Tyrosine	0.062 \pm 0.02	0.071 \pm 0.012	0.083 \pm 0.008	0.055 \pm 0.006
Choline	0.003 \pm 0.003	0.003 \pm 0.024	0.004 \pm 0.025	0.002 \pm 0.003
Creatine	0.05 \pm 0.001	0.06 \pm 0.02	0.07 \pm 0.041	0.032 \pm 0.004
Citrate	0.057 \pm 0.018	0.041 \pm 0.06	0.052 \pm 0.056	0.035 \pm 0.0027
Glutamate	0.002 \pm 0.0003	0.003 \pm 0.002	0.005 \pm 0.001	0.006 \pm 0.04
Glutamine	0.026 \pm 0.004	0.031 \pm 0.03	0.023 \pm 0.011	0.033 \pm 0.002
Acetate	0.026 \pm 0.01 ^a	0.025 \pm 0.012	0.049 \pm 0.013 ^b	0.075 \pm 0.005 ^c
Alanine	0.010 \pm 0.002	0.008 \pm 0.003	0.013 \pm 0.005	0.012 \pm 0.004
Leucine	1.47 \pm 0.54	1.18 \pm 3.3	1.08 \pm 0.010	1.18 \pm 0.001
Mannose	0.004 \pm 0.009	0.003 \pm 0.004	0.173 \pm 0.002	0.15 \pm 0.008
Valine	0.12 \pm 0.033	0.13 \pm 0.132	0.18 \pm 0.021	0.14 \pm 0.005

TABLE IV
PPAR α , B/ δ , AND γ STEADY STATE LEVELS IN RABBIT LIVER EXPOSED TO DIFFERENT DIETS

PPAR family	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
PPAR α	1.040 \pm 0.07 ^{ab}	1.220 \pm 0.13 ^a	0.870 \pm 0.07 ^{ab}	0.730 \pm 0.17 ^b
PPAR γ	0.035 \pm 0.004 ^a	0.029 \pm 0.001 ^{ab}	0.023 \pm 0.001 ^{bc}	0.018 \pm 0.003 ^c
PPAR β/δ	0.053 \pm 0.006	0.057 \pm 0.006	0.042 \pm 0.006	0.049 \pm 0.008

TABLE V
PRIMERS OF GENES FOR PPAR α , B/ δ , AND γ USED IN REAL-TIME PCR ANALYSIS

Locus	Primer sequence	PCR product (bp)	Accession number
PPAR α	ACATGGAGACGCTGTGTATG TGGCAGCAGTGAAGATG ATCAGGCTCCACTACGGTGT	103	AF013264
PPAR β/δ	TCA CTGGCACTTGTTGCGGTTCTT CTT	136	XM_001498870
PPAR γ	TTCTGTCAAGATCGCCCTCG TGGGGATGTCTCATAATGCCA GCTGAACGGGAACTCACT	193	
GAPDH	CCTGTTCAACCCTTCTT	125	NM_001082253

B. Metabolomic Evaluation

There were no significant differences in the content of tyrosine, choline, creatine, citrate, glutamate, glutamine, alanine, valine, leucine among the groups. Content of acetate compared to the control group was in about 2x higher for

experimental group 2, at least 3x higher for experimental group 3 (Table III and Fig. 1).

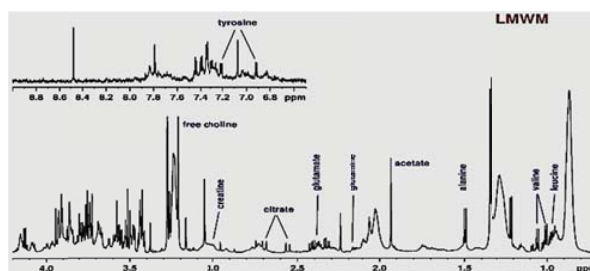


Fig. 1 The NMR spectral characteristics: A pulse sequence that suppresses the macromolecule signals is applied, thus, enhancing the detection of smaller solutes

C. Transcriptomic Evaluation

PPAR α , β/δ , and γ mRNA expressions were detected in the rabbit liver by the primers given in Table IV. PPAR α mRNA expression was significantly decreased by diet in boric acid group. Anhydrous borax attenuated mRNA levels of PPAR γ , which was further suppressed by boric acid. However, PPAR β/δ mRNA expression level was not affected by the boron compounds given to the rabbits in this study (Table IV and V).

TABLE VI
HISTOPATHOLOGICAL LESIONS IN LIVER

Parameters	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
Hepatocyte degeneration	1.000 \pm 0.645	0.929 \pm 0.450	0.786 \pm 0.756	0.500 \pm 0.289
Focal necrose	0.714 \pm 0.756	0.357 \pm 0.476	0.286 \pm 0.567	0.143 \pm 0.378
MNH inf. in portal area	1.143 \pm 0.690	0.857 \pm 0.627	1.071 \pm 1.397	0.643 \pm 0.476

TABLE VII
HISTOPATHOLOGICAL LESIONS IN KIDNEY

Parameters	Control (n=10)	Borax (n=10)	Anhydrous borax (n=10)	Boric acid (n=10)
Dilatation (in tubules)	0.571 \pm 0.345	0.643 \pm 0.627	0.500 \pm 0.289	0.571 \pm 0.450
Degeneration (in tubular epithels)	0.929 \pm 0.345	1.143 \pm 0.627	0.714 \pm 0.267	0.714 \pm 0.393
Hyaline cylinders	0.286 \pm 0.567	0.429 \pm 0.535	0.714 \pm 0.267	0.357 \pm 0.378
MNH inf.	0.286 \pm 0.488	0.714 \pm 0.809	0.429 \pm 0.60	0.371 \pm 0.240

D. Histopathological Findings

The distribution of histopathological changes for groups in liver and kidney samples are shown in Tables VI and VII. Even if there is no statistically difference, it has been observed that boron supplementation decreased the degenerative alterations in hepatocytes. Except borax group other boron groups did not have a pronounced change in tubular epithels of kidney (Figs. 3 A, C, D). The observed lesions were less than control group (Table VII). Borax group had mild degenerative alterations in tubular epithels (Figs. 2 B, 3 B). A mineralization in one sample and amyloidosis in another one sample were found in boric acid group.

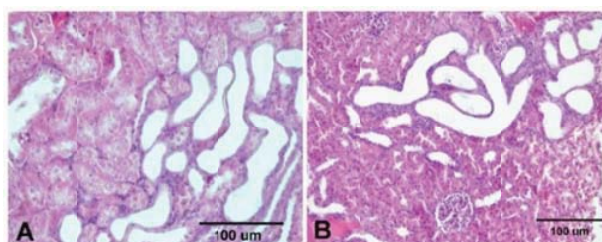


Fig. 2 Tubular degeneration and dilatation in kidney: H&E.
A. Control group, B. Borax group

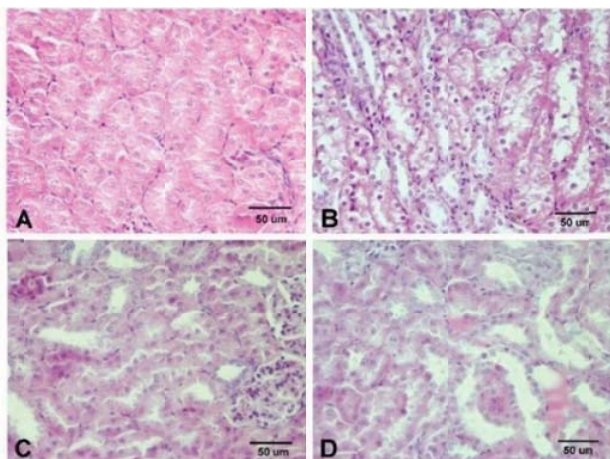


Fig. 3 Tubular degeneration in kidney: H&E. A. Control group, B. Borax group, C. Borax anhydrous group, D. Boric acid group

IV. DISCUSSION

We evaluated the effects of boron compounds by an omics approach in rabbits fed high HP and energy diet.

Nowadays, new strategies for preventing and treating NAFLD and NASH are needed. The dietary intake of patients with NAFLD is generally characterized by high levels of carbohydrate, fat, and/or cholesterol, and these dietary patterns influence hepatic lipid metabolism in the patients [6]. Mice ingesting high protein as compared to normal protein diets possess a lower body weight, adiposity and hepatic fat due to a combination of modulated processes [8]. Interestingly the present study indicates the degenerative effect of protein on liver hepatocytes. This suggests a specific effect of protein in liver independent of the fat and carbohydrate content of the diet. Increasing protein intake was also reported to reduce liver fat accumulation in rats [18] and mice [19]. This was earlier interpreted as both a direct and indirect action of amino acid. However, the question why energy in the form of amino acids is less efficiently transferred to fat in the liver still remained unsolved [20]. In the present study, apart from the lack of fat infiltration to the liver, degeneration in hepatocytes remains another challenge.

The application of 'omics' techniques, especially metabolomics, represents a promising and needed approach to identify new biomarkers in nutrition assessment, through an integrative application of new technologies in human nutritional research [21]. There have been relatively few metabolomic studies addressing the pathobiology of NASH and its progression from simple NAFLD [22]. A study using NMR, which, unlike mass-spectrometry-based platforms, does not have the power of detecting a large range of molecules [23], contributed raised serum concentrations of glucose, glutamate and taurine [24]. In our previous study, where boron were given to rabbits at 3 different doses and 96 h intervals, the most pronounced findings were significant changes in alanine methionine, pyruvate and creatine [14]. Metabolomic and lipidomic studies of PPAR δ have been relatively rare, as much less work has focused on PPAR δ , in part because of difficulties in developing selective ligands that do not target

the other PPARs as well, and also because of the importance of PPAR δ in normal development, complicating its genetic manipulation in rodent models. However, PPAR δ induced increased citric acid cycle activity, and mobilized free fatty acids, ketone bodies and triglycerides and activity in the linoleic and α -linolenic acid essential fatty-acid pathways. Thus, PPAR δ has a profound role in hepatic and skeletal muscle fatty-acid oxidation [25]. In this present study where doses from 3 different boron compounds were the same, there was significantly difference in only acetate levels of all the experimentals. Acetate is a common anion in biology. It is mainly utilized by organisms in the form of acetyl coenzyme A which represents a central node of carbon metabolism that plays a key role in bioenergetics, cell proliferation, and the regulation of gene expression. The capability of metabolomics to monitor some of the changes in lipid mediators may provide alternative targets either to bypass the PPAR receptor itself, or to provide a more controlled manner of stimulation for these key regulators of cellular metabolism. Among PPARs, PPAR γ is a master regulator of gene expression of metabolism, inflammation, and other pathways in many cell types, especially adipocytes [10]. Down regulation of PPARs, particularly PPAR α in cases of hepatic steatosis may facilitate the activity of hepatic proinflammatory cytokines. Hence, PPAR γ and PPAR α ligands have been considered for administration to prevent the initial inflammatory reactions and render protection to the liver cells [26]. In the present study, down regulation of PPAR α in boric acid group and of PPAR γ in borax anhydrous and boric acid groups were convenient to decreasing degenerative alterations in hepatocytes. Regarding these findings boron seems to be effective on PPARs family inactivation together with hepatic improving.

V. CONCLUSION

In conclusion, high protein and energy diet leads hepatocytes' degenerative changes which can be prevented by boron supplementation. Down regulation of PPAR α and PPAR γ expressions and increased acetate levels were associated with this improving. Even if there is no renal disorder, renal functions should be checked when borax is used long time. Boric acid preceded in this efficiency compared to other boron compounds.

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