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EXPERIMENTAL STUDY

# Effect of leucine on NF-κB pathway in liver regeneration after partial hepatectomy in rats, as determined by miniarray analysis

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**ABSTRACT**
BACKGROUND: Although some amino acids are recognized to have favorable effects on the liver regeneration after partial hepatectomy (PH), molecular mechanisms underlying these effects are barely known.
OBJECTIVE: Our study was aimed to investigate the effects of valine, glutamine, and leucine amino acids on PH-induced NF-κB signal pathway. The research team studied Leucine in a rat model in vivo. The study took place in the medical and surgical experimental research center at the Eskisehir Osmangazi University in Eskisehir, Turkey. The animals were Wistar albino male rats.
RESULTS: Group I, the sham group, was administered phosphate buffered saline (PBS) after laparotomy. After 70 % PH procedure, group II, III, IV, and V received single intraperitoneal doses of PBS, valine, glutamine, and leucine amino acids, respectively. At hour 6 after PH, expressions of 88 genes involving in NF-κB signal pathway were examined by RT-PCR mini array method in the liver tissue specimen. Fold values below 0.5 and above 2 were regarded as significant.
CONCLUSIONS: Our results suggested that valine, glutamine, and leucine amino acids may alter expressions of the genes of NF-κB signal pathway. In addition, among these amino acids, glutamine and valine proved to be much more effective on NF-κB signal pathway after the PH *(Tab. 1, Ref. 41)*. Text in PDF *www.elis.sk.*
KEY WORDS**:** RT-PCR, leucine, valine, glutamine, gene expression.

## Introduction

Liver cells start to rapidly divide after injury or surgical interventions, such as partial hepatectomy (PH). Cytokines, immune system, metabolic pathways, and matrix, known to be involved in liver regeneration after PH, have complex and incompletely elucidated relations with each other (1, 2)

Regeneration process is initiated with components of immune system and cytokines like TNF-α and IL6 (3). Cytokines, in turn, activate transcription factors such as NF-κB, c-jun, c-fos, and Stat3 (4). Transcription factors are known to be activated within first 30 minutes of regeneration (2), extending up to 4 hours. NF-κB was identified as an important transcription factor in the regeneration after PH (5). It is a dimeric transcription factor playing a role in proliferation, apoptosis, inflammation, and cellular adhesion, regulation of genes, and activation of more than 70 genes (6, 7). It is available at every type of cell in an inactive form in cytosol. Once activated, it is rapidly translocated into the nucleus and activate cascades. It has 5 family members: NF-κB1, NF-κB2, RelA (p65), RelB and c-Rel (8). Many stimuli activate NF-κB via ikb kinase (IKK) dependent phosphorylation and subsequent degradation of ikb (9).

Apart from being major constituents of proteins, amino acids are involved in various cellular functions. Branched-chain amino acids are known to have functions such as: gene expression, cellular metabolism, and amino acid transport (10). In addition, some amino acids and branched-chain amino acids were shown to be important signaling agents for the initiation of regeneration (11, 12, 13).

Administration of amino acids after PH, were demonstrated to be effective on liver regeneration (14). L-glutamine was reported to increase liver regeneration after PH (15). Oral administration of L-glutamine and L-arginine before and after PH was shown to exert regenerative effects (12). Though some amino acids are known to have mitotic effects, underlying cellular mechanisms have not been clearly elucidated (11, 16).

This study examined the effects of leucine, valine, and glutamine amino acids on the NF-κB signal pathway activated during priming phase of regeneration after PH.

## Materials and methods

Our study was performed upon the approval by the Institutional Ethical Committee for Animal Care and Use at the Eskisehir Osmangazi University, Eskisehir, Turkey with approval no 389/2014.

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*Experimental protocol*

Male *Wistar albino* rats having 200–250 g body weight were used in the experiment, which included a total of 5 groups, selected by randomization, ensuring the presence of 7 animals in each group.

Animals in the test groups were anesthetized by intramuscular administration of 10 $mg.kg^{-1}$ xylazine and 70 $mg.kg^{-1}$ ketamin. Animals in the sham group received intraperitoneal (i.p.) PBS following laparotomy procedure. Other experimental groups underwent PH procedure by Higgins and Anderson's (1931) technique (17). After surgical intervention, PBS, L-valine, L-glutamine, and L-leucine was administered as a single dose to Group II, III, IV, and V, respectively. Animals were sacrificed by taking a whole blood via intracardiac puncture after 6 hours of PH. Liver tissue specimens were obtained for RT-PCR array.

*L-valine, L-glutamine, L-leucine administration*

Amino acids used in our experiments were commercially available L-valine 99 % (Merck, Cast No: 72-18-4), L-glutamine 99 % (Biological Inds., Lot No: 110380K03B), and L-leucine 99 % (Sigma, Cat No: BCBM0179V). These amino acids were prepared in a 1 ml 10 mM PBS in a concentration of 1 $g.kg^{-1}$, which were administered through i.p. route just after PH.

*RNA isolation*

RNA isolation was performed by using pure link RNA mini kit Ambion Life Technologies Cat. No. 12183-018 kit protocol.

*cDNA synthesis*

For cDNA synthesis, high capacity cDNA reverse transcription kits for 200 reaction Cat. No. 4368813 kit were used via the protocol of the manufacturer. Isolated RNA specimens and RNA concentrations measured by Quibit 2.0 invitrogen device were equalized to be 200 ng/ml.

*Miniarray analysis*

Expression analyses of 88 genes of NF-κB pathway through mini array method was performed with ABI Step One Plus RT-PCR, for which SsoAdvanced™ Universal SYBR® Green Supermix Cat. No: 172-5274 was used in accordance with manufacturer's protocol.

*Statistics*

$C_T$ values obtained by RT-PCR were uploaded to Qiagen Data Analysis Center (http://www.qiagen.com), which gave fold regulation values. The software performed its analyses using the Student's t-test. Fold values above 2 and below 0.5 and $p \leq 0.05$ were considered as significant.

**Results**

The expressions of 88 genes of NF-kB signal pathway were compared against 5 housekeeping genes (Gapd, Gusb, Hprt1, Pgk1, Ppia). Differences of gene expressions in the Group III, IV, and V relative to the Group II were shown in Table 1, as stratified by values above fold 2 and $p < 0.05$.

We examined the effects of 3 different amino acids on expressions of 88 genes related to NF-κB pathway involving a regeneration after PH. The increases of expression were compared between the Group I and Group II, which revealed increased expression of $Agt^{+}$, $Bcl2l1^{+}$, $Cflar^{+}$, $Edg2^{+}$, $Irak2^{+}$, $Nfkbia^{+}$, Csf1, Egr1, F2r, Il10, Nod1, Raf1, Tlr1, Tnfaip3, and Tnfrsf10b genes.

The comparison of a decreased genetic expression between the Group I and Group II showed a decreased expression of $Akt1^{+}$, $Casp8^{+}$, $Ikbke^{+}$, $Irak1^{+}$, $Stat1^{+}$, $Ticam2^{+}$, $Tlr8^{+}$, Ikbkg, Il1b, Myd88, Nfkb1, Nlrp12, Rela, Sell, Ticam1, Tlr3, Tlr4, Tlr6, Tnf, Tnfsf10, and Tradd genes in the latter.

Among PH groups, Group III, IV, and V, which received amino acids were compared to the Group II, which received only solvent material in terms of increased genetic expressions. This showed an increased expression of Agt, Akt1, Bcl10, Bcl3, Casp8, Cd40, Cflar, Chuk (Ikkα), Csf1, Csf3, Fadd, Fasl, Fos, Gja1, Hmox1, Icam1, Ifna2, Ikbkg (Ikkγ), Il12b, Il1a, Il1b, Il6, Ltbr, Mmp7, Nfkb1, Rela, Relb, Rhoa, Ticam1, Ticam2, Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, Tlr7, Tnf, Tnfaip3, Tnfrsf10b, Tnfrsf1a (Tnfr1), Tnfsf10, Tnfsf14, and Tradd genes in Group III; $Agt^{+}$, $Atf1^{+}$, $Cflar^{+}$, Chuk $(Ikk\alpha)^{+}$, $Fasl^{+}$, $Gja1^{+}$, Ikbkb $(Ikk\beta)^{+}$, $Il1r1^{+}$, $Ltbr^{+}$, $Raf^{+}$, $Ticam1^{+}$, $Ticam2^{+}$, $Tlr1^{+}$, Tnfrsf1a$(Tnfr1)^{+}$, Akt, Bcl10, Birc, Ccl2, Csf2, Csf3, Egr1, Fadd, Htbr2b, Ifna2, Ifng, Ikbke (Ikkε), Ikbkg (Ikkγ), Il12a, Il1a, Il6, Lta, Ltb, Mapkkk, Nfkb1, Nlrp12, Rela, Relb, Rhoa, Sell, Tlr2, Tlr5, Tlr6, Tlr7, Tlr8, Tnfsf10, and Tnfsf14 genes in Group IV; and $Atf1^{+}$, Akt, Chuk (Ikkα), Il1a, Il6, Ltbr, Nfkbia (Ikbα), Nlrp12, Ticam2, Tlr6, and Tlr7 genes in the Group V ($^{+}$ implies $p \leq 0.05$).

The comparison of the Group III, IV, and V to the Group II in the terms of a decreased genetic expression showed a downregulation of Atf1, Edg2, Htbr2b, Irak2, Nfkbia (Ikbα), Ripk1, and Tmed4 genes in the Group III, Csf1, Fos, Myd88, and Tmed4 genes in the Group IV, and Csf1, Fos, Hmox1, Ifna1, Jun, Myd88, Nfkb2, and Tmed4 genes in the Group V (Tab. 1).

**Discussion**

Even after serious tissue losses, the liver continues hepatic regeneration up to its original size (18). Nutritional and metabolic supplementation like amino acids is critical for the hepatic regeneration (19). Besides being involved in protein synthesis and energy metabolism, amino acids have also a function as signal molecules (10, 12).

Cytokines such as TNF-α and IL-6 and the activation of NF-κB by cytokines were shown to be required for the initiation of liver regeneration (20).

NF-κB activation may be mediated through TNF signal pathway, for which cytoplasmic Tradd and Rip molecules were activated via Tnfr1, receptor of TNF molecule, to activate Ikk. This activation leads to a proteolytic degradation of Ikb, inhibitor of NF-κB, to liberate NF-κB (21).

TNF-mediated NF-κB activation was known to be initiated within 30 minutes after PH and maintained up to 4–5 hours (22). The expression of NF-κB inhibitor, Ikb, was reported to be elevated since the 3rd hour after PH. (23). NF-κB may limit its activation by increasing the expression of Ikb, its own inhibitor (24).

**Tab. 1. Differences of gene expressions in the Group III, IV, and V relative to the Group II.**

| | Gene Symbol | Group III | | Group IV | | Group V | |
|---|---|---|---|---|---|---|---|
| | | Fold Change | p-value | Fold Change | p-value | Fold Change | p-value |
| 1 | Agt | 2.90** | 0.177453 | 2.65** | 0.025824$^{+}$ | 1.32 | 0.282214 |
| 2 | Akt1 | 7.35** | 0.052919 | 2.66** | 0.114277 | 2.12** | 0.063030 |
| 3 | Atf1 | 0.45* | 0.283783 | 2.64** | 0.025615$^{+}$ | 2.11** | 0.028864$^{+}$ |
| 4 | Bcl10 | 2.88** | 0.273720 | 2.09** | 0.138814 | 1.04 | 0.934594 |
| 5 | Bcl2 | 1.14 | 0.411367 | 1.67 | 0.226964 | 1.67 | 0.070231 |
| 6 | Bcl2l1 | 1.15 | 0.582771 | 1.05 | 0.678727 | 0.84 | 0.780562 |
| 7 | Bcl3 | 2.29** | 0.145561 | 1.66 | 0.149259 | 1.32 | 0.289323 |
| 8 | Birc2 | 0.92 | 0.856259 | 1.67 | 0.170651 | 0.83 | 0.558095 |
| 9 | Birc5 | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 10 | Casp1 | 0.56 | 0.984800 | 1.32 | 0.382031 | 0.83 | 0.551887 |
| 11 | Casp8 | 4.58** | 0.062699 | 1.66 | 0.249227 | 1.32 | 0.278070 |
| 12 | Ccl2 | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 13 | Cd40 | 2.88** | 0.244934 | 1.32 | 0.485726 | 1.32 | 0.413672 |
| 14 | Cflar | 3.65** | 0.077259 | 2.64** | 0.025615$^{+}$ | 1.04 | 0.808848 |
| 15 | Chuk (Ikkα) | 3.66** | 0.077166 | 2.65** | 0.025598$^{+}$ | 1.67 | 0.026857$^{+}$ |
| 16 | Csf1 | 4.54** | 0.199091 | 0.16* | 0.431447 | 0.05* | 0.104155 |
| 17 | Csf2 | 1.86 | 0.554143 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 18 | Csf3 | 4.68** | 0.245880 | 2.64** | 0.197976 | 0.85 | 0.684897 |
| 19 | Edaradd | 0.72 | 0.432284 | 0.84 | 0.611185 | 0.66 | 0.437387 |
| 20 | Edg2 | 0.26* | 0.059347 | 0.66 | 0.816445 | 0.83 | 0.752723 |
| 21 | Egr1 | 0.73 | 0.490823 | 3.32** | 0.136667 | 1.05 | 0.686320 |
| 22 | Elk1 | 1.82 | 0.190418 | 1.32 | 0.271958 | 1.32 | 0.280189 |
| 23 | F2r | 0.58 | 0.526455 | 1.05 | 0.900655 | 0.84 | 0.590071 |
| 24 | Fadd | 2.29** | 0.246490 | 2.08** | 0.204005 | 1.32 | 0.517994 |
| 25 | Fasl | 2.91** | 0.242346 | 3.34** | 0.022722$^{+}$ | 1.67 | 0.182061 |
| 26 | Fos | 7.31** | 0.327866 | 0.41* | 0.318699 | 0.16* | 0.222833 |
| 27 | Gja1 | 2.90** | 0.095988 | 2.09** | 0.043454$^{+}$ | 1.33 | 0.367852 |
| 28 | Hmox1 | 3.62** | 0.174942 | 0.52 | 0.228299 | 0.41* | 0.243628 |
| 29 | Htbr2b | 0.45* | 0.611591 | 2.64** | 0.118568 | 1.67 | 0.183597 |
| 30 | Icam1 | 3.66** | 0.223831 | 1.66 | 0.356859 | 1.32 | 0.607146 |
| 31 | Ifna1 | 0.90 | 0.608085 | 1.28 | 0.757878 | 0.42* | 0.145689 |
| 32 | Ifna2 | 7.08** | 0.371684 | 2.14** | 0.406406 | 0.83 | 0.676331 |
| 33 | Ifng | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 34 | Ikbkb (Ikkβ) | 1.14 | 0.399769 | 2.09** | 0.006577$^{+}$ | 1.04 | 0.808848 |
| 35 | Ikbke (Ikkε) | 1.15 | 0.449155 | 2.09** | 0.104109 | 1.32 | 0.145187 |
| 36 | Ikbkg (Ikkγ) | 2.90** | 0.429992 | 3.35** | 0.292777 | 1.67 | 0.941383 |
| 37 | Il10 | 0.58 | 0.393695 | 0.83 | 0.909643 | 0.52 | 0.540129 |
| 38 | Il12a | 1.82 | 0.557493 | 2.09** | 0.428026 | 0.65 | 0.422334 |
| 39 | Il12b | 2.34** | 0.881171 | 1.35 | 0.640898 | 0.53 | 0.399237 |
| 40 | Il1a | 3.67** | 0.187531 | 4.19** | 0.189792 | 2.67** | 0.086894 |
| 41 | Il1b | 2.90** | 0.388955 | 1.64 | 0.511465 | 0.52 | 0.291038 |
| 42 | Il1r1 | 1.45 | 0.457404 | 3.34** | 0.021926$^{+}$ | 1.32 | 0.422711 |
| 43 | Il6 | 7.57** | 0.131460 | 2.69** | 0.183919 | 2.14** | 0.401134 |
| 44 | Irak1 | 0.58 | 0.494929 | 1.33 | 0.443896 | 1.67 | 0.192787 |
| 45 | Irak2 | 0.36* | 0.107999 | 0.83 | 0.408843 | 0.53 | 0.029195$^{+}$ |
| 46 | Jun | 1.15 | 0.577982 | 0.83 | 0.985008 | 0.33* | 0.139474 |
| 47 | Lta | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 48 | Ltb | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 49 | Ltbr | 3.62** | 0.132229 | 3.32** | 0.019143$^{+}$ | 2.11** | 0.010892$^{+}$ |
| 50 | Malt1 | 1.82 | 0.260868 | 1.04 | 0.671396 | 0.66 | 0.221175 |
| 51 | Mapkkk | 1.45 | 0.920793 | 2.10** | 0.440796 | 0.66 | 0.430483 |
| 52 | Mmp7 | 5.83** | 0.117434 | 1.61 | 0.548509 | 1.67 | 0.511533 |
| 53 | Myd88 | 1.13 | 0.390021 | 0.32* | 0.743709 | 0.20* | 0.275001 |

| | Gene Symbol | Group III | | Group IV | | Group V | |
|---|---|---|---|---|---|---|---|
| | | Fold Change | p-value | Fold Change | p-value | Fold Change | p-value |
| 54 | Nfkb1 | 2.31** | 0.233735 | 4.22** | 0.135455 | 1.32 | 0.858515 |
| 55 | Nfkb2 | 1.45 | 0.423108 | 0.66 | 0.653060 | 0.42* | 0.130159 |
| 56 | Nfkbia (Ikbα) | 0.45* | 0.341405 | 1.05 | 0.631853 | 0.83 | 0.545166 |
| 57 | Nlrp12 | 0.72 | 0.586729 | 2.63** | 0.227015 | 2.63** | 0.223709 |
| 58 | Nod1 | 1.45 | 0.401434 | 0.83 | 0.413195 | 0.84 | 0.760535 |
| 59 | Ppm1a | 1.44 | 0.403539 | 1.65 | 0.076832 | 0.83 | 0.948844 |
| 60 | Raf1 | 1.14 | 0.568916 | 2.09** | 0.006776$^{+}$ | 1.32 | 0.286889 |
| 61 | Rel | 1.82 | 0.258608 | 1.66 | 0.254159 | 1.32 | 0.407666 |
| 62 | Rela | 5.87** | 0.114688 | 2.11** | 0.117564 | 1.33 | 0.573765 |
| 63 | Relb | 3.67** | 0.134630 | 2.11** | 0.057190 | 1.05 | 0.817494 |
| 64 | Rhoa | 2.89** | 0.243751 | 2.09** | 0.058194 | 1.06 | 0.982010 |
| 65 | Ripk1 | 0.22* | 0.616376 | 1.66 | 0.168215 | 1.04 | 0.837471 |
| 66 | Sell | 1.44 | 0.682606 | 4.27** | 0.095179 | 0.84 | 0.772210 |
| 67 | Selp | 1.44 | 0.485435 | 1.04 | 0.913896 | 1.04 | 0.926805 |
| 68 | Stat1 | 0.91 | 0.889355 | 1.66 | 0.277887 | 1.32 | 0.415825 |
| 69 | Tbk1 | 1.44 | 0.472772 | 0.52 | 0.226474 | 0.52 | 0.232255 |
| 70 | Ticam1 | 4.59** | 0.138449 | 2.63** | 0.035703$^{+}$ | 1.66 | 0.198663 |
| 71 | Ticam2 | 4.61** | 0.195329 | 2.09** | 0.036330$^{+}$ | 2.10** | 0.068098 |
| 72 | Tlr1 | 2.30** | 0.337655 | 2.10** | 0.037894$^{+}$ | 1.05 | 0.740540 |
| 73 | Tlr2 | 3.65** | 0.194652 | 3.34** | 0.123152 | 1.32 | 0.988961 |
| 74 | Tlr3 | 2.31** | 0.552481 | 1.32 | 0.860757 | 1.33 | 0.816866 |
| 75 | Tlr4 | 2.35** | 0.407341 | 1.31 | 0.892203 | 0.84 | 0.998547 |
| 76 | Tlr5 | 3.67** | 0.134534 | 2.10** | 0.058263 | 1.32 | 0.489474 |
| 77 | Tlr6 | 1.44 | 0.788154 | 6.75** | 0.182145 | 3.37** | 0.967725 |
| 78 | Tlr7 | 2.32** | 0.389797 | 3.36** | 0.122806 | 2.12** | 0.460053 |
| 79 | Tlr8 | 0.71 | 0.696847 | 2.11** | 0.197133 | 1.66 | 0.026120$^{+}$ |
| 80 | Tlr9 | 0.72 | 0.540917 | 1.32 | 0.467436 | 1.32 | 0.445833 |
| 81 | Tmed4 | 0.11* | 0.216281 | 0.13* | 0.220841 | 0.07* | 0.203175 |
| 82 | Tnf | 2.90** | 0.336586 | 1.66 | 0.298188 | 1.66 | 0.248659 |
| 83 | Tnfaıp3 | 2.90** | 0.282540 | 1.32 | 0.638992 | 0.66 | 0.388715 |
| 84 | Tnfrsf10b | 2.90** | 0.236509 | 1.67 | 0.217003 | 0.84 | 0.538167 |
| 85 | Tnfrsf1a (tnfr1) | 2.89** | 0.152560 | 2.64** | 0.025615$^{+}$ | 1.32 | 0.287757 |
| 86 | Tnfsf10 | 2.29** | 0.267714 | 2.64** | 0.089001 | 1.66 | 0.295509 |
| 87 | Tnfsf14 | 2.90** | 0.237460 | 2.02** | 0.495206 | 0.65 | 0.423712 |
| 88 | Tradd | 2.88** | 0.139188 | 1.66 | 0.348725 | 1.05 | 0.839023 |

** Fold-change in values greater than 2, *Fold-change in values less than 0.5, $^{+}$ Statistically significant at $p < 0.05$ by Student t-test

In our study, Group II showed an increased expression of Ikbα and decreased expression of Tnf, Tradd, and Ikk-γ compared to the sham group, which showed a tendency of TNFα-mediated NF-κB activation towards an inhibition at 6th hour after PH.

Compared to the Group II, Group III which was administered valine had an increased expression of Tnf, Tnfr1, Tradd, Ikkα, Ikkγ, and Nfkb1 that positively affectsedNF-κB activation and decreased the expression of inhibitor Ikb. Tnf, Tradd, Rıp1, Ikbα, and Nfkb2 expression was not altered in glutamine-receiving Group IV, which revealed an increased expression of Tnfr1, Ikkα, Ikkβ, Ikkγ, and Nfkb1. Leucine-administered Group V had an unchanged Tnf, Tnfr1, Tradd, Rıp1, Ikbα, Nfκb1, Ikkα, Ikkβ, and Ikkγ expression, but a reduced expression of Nfκb2. These findings suggested that valine appeared to increase NF-κB expression via tnf at 6th hour after PH. In addition, the expression of NF-κB inhibitor was also suppressed. Although, glutamine increased the expression of Tnfr1 receptor at 6th hour after PH, no alteration was observed in the Tnf expression. Yet, Tnfr1 is recognized to be also activated via different molecules (lymphotoxin alpha). Moreover, deficiency of Tnfr1 was reported to be associated with defects in liver regeneration, which could be sustained without Tnf (2). This led us to suggest that without altering Tnf and Ikb expressions, glutamine had an increasing effect on Ikk units, Tnfr1, and Nfkb1 expressions at 6th hour after PH, hence causing an increased NF-κB expression. On the other hand, leucine did not appear to have much influence on NF-κB signal pathway at 6th hour after PH.

IL-6, the target gene of Tnf/Tnfr1-mediated NF-κB signal pathway, is one of the cytokines, whose expression is elevated during the early phase of liver regeneration and known to have positive effects in this continuum (2). In our study, the expression of IL-6 increased 7-fold, 2-fold, and 2-fold in the Group III, IV, and V, respectively compared to the Group II. Despite having no effect on NF-κB signal pathway, leucine increased IL-6 expression 2-fold. This may imply leucine to act via another signaling pathway.

On the other hand, double-edged nature of Tnf in liver regeneration should not be underestimated as it may also trigger apoptosis besides regeneration (25). Upon Tnfr1 receptor trimerization, Tnf binds to Fadd molecule, pro-caspase8, which contains death domain in cytoplasmic part, inducing caspase-8 activation (9). Caspase-8, in turn, activates effector caspases, which initiate apoptotic cascade. NF-κB prevents apoptosis in liver regeneration and has positive effects on regeneration (18). Several studies showed that inhibition of NF-κB triggered apoptosis (26, 27).

When we analyzed Fadd and Casp8 expressions, we observed that the Group II had a decreased Casp8 expression with no significant alteration in Fadd expression, compared to the Group I. While the Group III and IV had an increased Fadd expression, leucine-administered Group V could not raise beyond 2-fold compared to the Group II. The expression of Casp8 was only elevated in the valine-administered Group III, which was 4-fold. The study by Zhou et al (2006) reported that Caspase8 activation increased at 168th hour of PH in rats, where no significant change was recorded at hour 6 (28). However, Casp8 expression at 6th hour after PH was 4-fold higher in the valine-administered Group III than that in PBS-administered Group II. In addition, nfkb-mediated gene product was known to inhibit the activation of caspase-8 by directly acting on it (29). It is an interesting finding that the Group III showed both an increased expression of genes activating NF-κB signal pathway and Casp8 gene. Nevertheless, one study revealed that Casp8 might also have proliferative effect apart from its apoptotic effect (30).

Toll-Like-Receptors (TLR) were reported to play an important role in regeneration, to trigger cell proliferation, and cause an impaired liver regeneration in deficient situations (31). Apart from being activated by various structures of bacterial, protozoal, viral, and parasitic pathogens (LPS, flagellin, ssRNA, diacyl protein), TNF may also be activated by endogenous molecules released from damaged cell, tissue, or extracellular matrix proteins, as demonstrated by recent studies (32). It was also reported that cellular injury occurring during hepatectomy might lead to TLR-mediated activation of NF-κB signal pathway, triggering synthesis of various cytokines like IL-6 and Tnf-α (33). Compared to the Group I, the Group II showed an increased Tlr1 expression, decreased Tlr3, Tlr4, Tlr6, and Tlr8 expression, and unchanged Tlr2, Tlr5, Tlr7, and Tlr9 expression. Compared to the Group II, the valine-administered Group III had an increased expression of Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, and Tlr7 with no altered Tlr6, Tlr8 and Tlr9 expressions; glutamine-administered Group IV had an increased expression of Tlr1, Tlr2, Tlr5, Tlr6, Tlr7, and Tlr8 with no altered Tlr3, Tlr4, and Tlr9; and the leucine-administered Group V had an increased expression of Tlr6 and Tlr7 with no change in other genes of TLR. The study by Zorde-Khvalevsky et al (2009) showed a non-uniform and early regeneration period in Tlr3 knockout mice and influence of Tlr3 on IL-6 and NF-κB in liver regeneration after PH was demonstrated (33). On the contrary, Tlr2, Tlr4, and Tlr9 knockout mice were reported to have a normal regeneration (2, 34, 35). However, there have been studies showing a negatively affected regeneration when adaptor protein of TLR signal pathway (except Tlr3), Myd88, was absent (34, 35). In fact, these negative effects were reported to originate from deficient expression of immediate early genes in the synthesis of Tnf-a and IL-6 secondary to reduced NF-κB activation (32). In our study, while Myd88 expression was not altered in the valine-administered Group III, it decreased in the glutamine-administered Group IV and leucine-administered Group V, compared to the Group II. As mentioned above, the expressions of the genes associated with NF-κB activation were increased in the Group III and IV. In addition, IL-6 expression increased in the Group III, IV, and V. Myd88-related signal pathway was implied to be important for the initiation phase of liver regeneration (32).

Angiogenesis is critical during liver regeneration (36). Though the role played by angiotensinogen (AGT) after PH is not well known, it was reported to peak at 6th hour after PH and to maintain its expression up to 24 hours (25). Xu et al revealed a 5-fold increase of AGT expression (37). This is consistent with our finding showing a 5-fold increase of AGT in the Group II than that in the Group I. When valine and glutamine receiving groups were compared to the Group II, a 2-fold increase was observed, whereas ATG expression did not raise beyond 2-fold in the leucine-administered group.

Some amino acids are known as significant agents influencing signal pathways involving the initiation and progression of hepatocyte proliferation (11). However, there were few studies regarding cellular mechanisms underlying these effects. Among them, glutamine was shown to have an impact on liver regeneration after 70 % PH. (38, 39, 40). Leucine was reported to have a proliferative effect on hepatocyte (11). Moreover, in vitro and in vivo studies showed that leucine increased the synthesis of hepatocyte growth factor (HGF), for which valine had no such an effect (13, 41).

In our study, valine administration was associated with an increased expression of 43 genes and decreased expression of 7 genes, glutamine administration was associated with an increased expression of 46 genes and decreased expression of 4 genes, and leucine administration was associated with an increased expression of 11 genes and decreased expression of 4 genes, as detected at 6th hour after PH. While our findings showed that all three amino acids did alter expressions of the genes involved in NF-κB signal pathway of 70 % PH model, these effects were observed to be more prominent with glutamine and valine. Leucine might have a greater influence on another signal pathway.

## References

**1. Michalopoulos GK.** Liver Regeneration. J Cell Physiol 2007; 213: 286–300. doi:10.1002/jcp.21172.Liver.

**2. Fausto N, Campbell JS, Riehle KJ.** Liver regeneration. Hepatology 2006; 43: S45–53. doi:10.1002/hep.20969.

**3. Galun E, Axelrod JH.** The role of cytokines in liver failure and regeneration: potential new molecular therapies. Biochim Biophys Acta 2002; 1592: 345–358. www.ncbi.nlm.nih.gov/pubmed/12421677.

**4. Stepniak E, Ricci R, Eferl R, Sumara G, Sumara I, Rath M, Hui L, Wagner EF.** c-Jun / AP-1 controls liver regeneration by repressing p53 / p21 and p38 MAPK activity. Genes Dev 2006; 2306–2314. doi:10.1101/gad.390506.1.

**5. Kurinna S, Barton MC.** Cascades of transcription regulation during liver regeneration. Int J Biochem Cell Biol 2011; 43: 189–197. doi:10.1016/j.biocel.2010.03.013.

**6. Baeuerle PA.** Pro-inflammatory signaling: Last pieces in the NF- k B puzzle? Curr Biol 1998; 19–22.

**7. Mars WM, Liu M, Kitson P, Goldfarb H, Gabauer MK, MichalopoulosG.** Immediate early detection of urokinase receptor after partial hepatectomy and its implications for initiation of liver regeneration. Hepatology 1995; 21: 1695–1701.

**8. Muriel P.** NF- kB in liver diseases : a target for drug therapy. J Appl Toxicol 2009; 29: 91–100. doi:10.1002/jat.1393.

**9. Barkett M, Gilmore TD.** Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6910–6924. doi:10.1038/sj.onc.1203238.

**10. Tomiya T, Omata M, Fujiwara K.** Significance of branched chain amino acids as possible stimulators of hepatocyte growth factor. Biochem Biophys Res Commun 2004: 411–416. doi:10.1016/j.bbrc.2003.07.017.

**11. Kimura M, Ogihara M.** Effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Eur J Pharmacol 2005; 510: 167–180. doi:10.1016/j.ejphar.2005.01.011.

**12. Kurokawa T, An J, Tsunekawa K, Shimomura Y, Kazama S, Ishikawa N, Nonami T, Sugiyama S.** Effect of L-arginine supplement on liver regeneration after partial hepatectomy in rats. World J Surg Oncol 2012; 10: 1. doi: 10.1186/1477-7819-10-99.

**13. Tomiya T, Inoue Y, Yanase M, Arai M, Ikeda H, Tejima K, Nagashima K, Nishikawa T, Fujiwara K.** Leucine stimulates the secretion of hepatocyte growth factor by hepatic stellate cells. Biochem Biophys Res Commun 2002; 297: 1108–1111.

**14. Nelsen CJ, Rickheim DG, Tucker MM, McKenzie TJ, Hansen LK, Pestell RG, Albrecht JH.** Amino acids regulate hepatocyte proliferation through modulation of cyclin D1 expression. J Biol Chem 2003; 278: 25853–25858. doi:10.1074/jbc.M302360200.

**15. Yoshida S, Yunoki T, Aoyagi K, Ohta J, Ishıbashi N, Noake T, Kakegava T.** Effects of glutamine supplement and hepatectomy on DNA and protein synthesis in the remnant liver. J Surg Res 1995; 59: 475–481.

**16. Tomiya T, Nishikawa T, Inoue Y, Ohtomo N, Ikeda H, Tejima K, Watanabe N, Tanoue Y, Omata M, Fujiwara K.** Leucine stimulates HGF production by hepatic stellate cells through mTOR pathway. Biochem Biophys Res Commun 2007; 358: 176–180. doi:10.1016/j.bbrc.2007.04.093.

**17. Higgins GM, Anderson RM.** Experimental pathology of liver. I. Restroration of liver white rat following partial surgical removal. Arch Pathol 1931; 12: 186–202.

**18. Taub RB.** Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 2004; 5: 836–847. doi: 10.1038/nrm1489.

**19. Holecek M.** Three targets of branched-chain amino acid supplementation in the treatment of liver disease. Nutrition 2010; 26: 482–490. doi:10.1016/j.nut.2009.06.027.

**20. Abshagen K, Eipel C, Vollmar B.** A critical appraisal of the hemodynamic signal driving liver regeneration. Langenbeck's Arch Surg 2012; 397: 579–590. doi: 10.1007/s00423-012-0913-0.

**21. Wajant H, Pfizenmaier K, Scheurich P.** Tumor necrosis factor signaling. Cell Death Differ 2003; 10: 45–65. doi:10.1038/sj.cdd.4401189.

**22. Black D, Lyman S, Heider TR, Behrns KE.** Molecular and cellular features of hepatic regeneration. J Surg Res 2004; 117: 306–315. doi:10.1016/j.jss.2003.10.026.

**23. Taub R.** F1-7 Transcriptional control of liver regeneration. Clin Res Forum/International Hepatol Commun 1995; S5–S6. doi: 10.1016/0928-4346(95)90190-I.

**24. Pahl HL.** Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6853–6866. doi:10.1038/sj.onc.1203239.

**25. Lai HS, Lin WH, Lai S, Lin H, Hsu W, Chou C, Lee P.** Interleukin-6 mediates angiotensinogen gene expression during liver regeneration. PLoS One 2013; 8: e67868. doi:10.1371/journal.pone.0067868.

**26. Bellas RE, Fitzgerald MJ, Fausto N, Sonenshein GE.** Short communication murine hepatocytes. Am J Pathol 1997; 151: 891–896.

**27. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW.** NFkB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998; 101: 802–811.

**28. Zhou YH, Miao MY, Li Y, Shi D, Huang H, Wang XM, Jiao BH.** Changes of hepatic caspase-3 and caspase-8 activities during liver regeneration in rats. Acad J Second Mil Med Univ 2006; 5.

**29. Xu Y, Bialik S, Jones BE, Iimuro Y, Kitsis RN, Brenner DA, Czaja MJ, Kuboki S, Okaya T, Schuster R, Blanchard J, Denenberg A, Hector R, Lentsch AB, Physiol AJ, Circ H, Hilgard P, Gerken G, Stockert RJ.** Liver, NF-κB inactivation converts a hepatocyte cell line TNF-α response from proliferation to apoptosis. Am J Physiol Cell Physiol 2011; 275: C1058–C1066.

**30. Bolkent S, Öztay F, Oktayoğlu SG, Baş SS, Karatuğ A.** A matter of regeneration and repair: caspases as the key molecules. Turkish J Biol 2016; 40: 333–352. doi: 10.3906/biy-1507-18.

**31. Zhang Z, Schluesener HJ.** Mammalian toll-like receptors: From endogenous ligands to tissue regeneration. Cell Mol Life Sci 2006; 63: 2901–2907. doi: 10.1007/s00018-006-6189-1.

**32. Seki E, Park E, Fujimoto J.** Toll-like receptor signaling in liver regeneration, fibrosis and carcinogenesis. Hepatol Res 2011. doi:10.1111/j.1872-034X.2011.00822.x.

**33. Zorde-Khvalevsky E, Abramovitch R, Barash H, Spivak-Pohis I, Rivkin L, Rachmilewitz J, Galun E, Giladi H.** Toll-like receptor 3 signaling attenuates liver regeneration. Hepatology 2009; 50: 198–206. doi:10.1002/hep.22973.

**34. Seki E, Brenner DA.** Toll-like receptors and adaptor molecules in liver disease: Update. Hepatology 2008; 48: 322–335. doi:10.1002/hep.22306.

**35. Vaquero J, Campbell JS, Haque J, Mcmahon RS, Riehle KJ, Bauer RL, Fausto N.** Toll-like receptor 4 and myeloid differentiation factor 88 provide mechanistic insights into the cause and effects of interleukin-6 activation in mouse liver regeneration. Hepatology 2011; 54: 597–608. doi:10.1002/hep.24420.

**36. Drixler TA, Vogten MJ, Ritchie ED, Van Vroonhoven TJMV.** Liver Regeneration Is an Angiogenesis-associated Phenomenon. Ann Surg 2002; 236: 703–712. doi:10.1097/01.SLA.0000036265.92985.5F.

**37. Xu C, Shao H, Liu S, Qin B, Sun X, Tian L.** Possible regulation of genes associated with intracellular signaling cascade in rat liver regeneration. Scand J Gastroenterol 2009; 44: 462–470. doi: 10.1080/00365520802495560.

**38. Magalhães CR, Malafaia O, Torres OJM, Moreira LB, Tefil SCDSG, Pinherio MDR, Harada BA.** Avaliação da regeneração hepática com dieta suplementada com l-glutamina: estudo experimental em ratos. Rev Col Bras Cir 2014; 41: 117–121. doi: 10.1590/S0100-69912014000200008.

**39. Ito A, Higashiguchi T.** Effects of glutamine administration on liver regeneration following hepatectomy. Nutrition 1999; 15: 23–28. doi: 10.1016/S0899-9007(98)00133-6.

**40. De Mazza Jesus RP, Bertevello PL, Torrinhas RMDM, Nonogaki S, Alves VAF, Rodrigues JG, Waitzerberg DL.** Effect of Glutamine Dipeptide on Hepatic Regeneration in Partially Hepatectomized Malnourished Rats. Basic Nutr Investig 2003; 19: 930–935. doi: 10.1016/S0899-9007(03)00177-1.

**41. Tomiya T, Inoue Y, Yanase M, Arai M, Ikeda H, Tejima K, Nagashima K, Nishikawa T, Watanabe N, Omata M, Fujiwara K.** Treatment with leucine stimulates the production of hepatocyte growth factor in vivo. Biochem Biophys Res Commun 2004; 322: 772–777. doi: 10.1016/j.bbrc.2004.07.191.

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