**Baswilic Acid Disable Signal Transduction of IL-6/STAT-3 in Ehrlich Tumor Bearing Irradiated Mice**

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| **Keyword:** | Baswilic acid, gamma radiation, IL-6R, STAT-3 |
Baswilic Acid Disable Signal Transduction of IL-6/STAT-3 in Ehrlich Tumor Bearing Irradiated Mice

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Abstract

Background. Baswilic acid (BA) was claimed for its capability of triggering apoptosis as well as inhibiting angiogenesis in tumor tissue.

Purpose. In the present study, the effect of BA on the IL-6/STAT-3 signal was investigated in irradiated mice bearing ehrlich ascites carcinoma solid tumor (E).

Methods. 25 mg/kg/day (i.p) of BA were administrated to mice bearing E and exposed to 4Gy gamma radiation.

Results. Data analysis revealed a specific impact of BA on the IL-6R mRNA and survivin mRNA in E and irradiated E mice. Also, significant improvements were observed in the protein expression of JAK-1, P-JAK-1, STAT-3, P-STAT-3 and Caspase-3 as well as VEGF and IL-6 levels.

Conclusion. It could be suggest that, BA interfered with IL-6/STAT-3 signal transduction that prevent the activation of Caspase-3 and subsequently trigger the process of apoptosis. However, the alternative angiogenesis pathway, include the over expression of VEGF level, which depend on IL-6/STAT-3 signalling was inhibited by the action of BA. Here it is possible to recommend that cancer therapeutic strategies have to include BA administration.

Keywords: Baswilic acid, gamma radiation, IL-6R, STAT-3,
1. Introduction

Signal-transducer-and-activator-of-transcription (STAT) is a family of six different transcription factors which play major roles in cytokine signalling. STAT-3 is one of the major mediators of tumorigenesis. The oncogenic significance of activated STAT-3 molecules is due to their effects on numerous parameters of the development and progression of malignancy, such as apoptosis, cell proliferation, angiogenesis, and immune system evasion (Aggarwal et al. 2006). It was initially regarded as an acute-phase response factor activated by interleukin-6 (IL-6). IL-6 first binds to the IL-6 receptor (IL-6R), and this complex then associates with gp130, inducing dimerization and the initiation of signalling STAT-3 phosphorylation by Janus kinase (JAK) (Mitsuyama et al. 2007). The caspases, key players in the apoptotic pathway, are a family of cysteine proteases. They are synthesized as proenzymes and activated by proteolytic cleavage. Caspase 3 is considered to be the key effector caspase. Survivin is a member of the inhibitor of the apoptosis protein family. It inhibits apoptosis by directly or indirectly suppressing the caspase 3 function (Poomsawat et al. 2014). Angiogenesis is an essential step in the development of tumors. Vascular endothelial growth factor (VEGF) plays fundamental role in the fluid accumulation and tumor growth in ascites tumor (Agrawa et al. 2011).

Cancer continues to be one of the major devastating diseases. Cancer is a class of disease in which a cell or a groups of cells displays uncontrolled growth, invasion and metastasis (Dolai et al. 2012). Experimental tumors have great importance in modeling, and Ehrlich ascites carcinoma (EAC) is one of the commonest tumors. It is a spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in outbred mice by serial intraperitoneal (i.p.) passages. EAC has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor-specific transplantation antigen (TSTA). EAC is used as ascites or a solid form due to these purposes,
that is, if ascites fluid contains the tumor cell that injects i.p., the ascites form is obtained, but if it injected subcutaneous (s.c.) or intramuscular (i.m.), a solid form is obtained (Ozaslan et al. 2011 and El-Bahy et al. 2012). Since the description of Ehrlich ascites, researchers exploit it for chemotherapeutic studies (Jaganathan et al. 2010).

The biological effects of radiation affect both neoplastic and normal tissues. The nature and extent of such effects, however, depend on selected biological parameter and can be modified by chemical agents such as chemotherapeutic agents. A precise control of the mode of action of the radiation is important in order to achieve the maximum effect on tumor tissue, while minimizing the effect on normal tissue. The combination therapy of treatment of tumors with radiation and different anticancer drugs has been tried clinically. The herbal drugs have gained attention and popularity because of their negligible toxicity and possibly with a ray of hope that they may replace some of the available anti-neoplastic drugs that are highly toxic (Jagetia and Venkatesha 2005).

Baswilic acid (BA) is isolated from Boswellia serrata. BAs have been used to treat Crohn disease, ulcerative colitis, bronchial asthma, endotoxin induced hepatitis and arthritis. BA is a mixture composed of four major pentacyclic triterpene acids: beta-boswellic acid, 3-acteyl beta boswellic acid, 11-ketobeta-boswellic acid and 3-acetyl-11-keto-beta-boswellic acid (AKBA), are reported to be effective as anti-inflammatory, immune-modulator and anti-asthmatic agent (Agrawa et al. 2011).

This study looks at the possible beneficial effect of BA in cancer treatment via studying IL-6/STAT-3 pathway in ehrlich bearing irradiated mice. Among these, mRNA expression of IL-6 receptor/IL-6 level, JAK-1/phospho-JAK-1 (P- JAK-1), STAT-3/phospho-STAT-3 (P- STAT-3), in addition to apoptotic (survivin and Caspase-3) proteins as well asangiogenic (VEGF) protein were assayed.
2. Materials and methods

2.1. Materials:-

Baswilic acid was obtained from a Nature way product, Inc. Springville, Utah 84663 USA, while other Chemicals and reagent were obtained from Sigma-Aldrich Chemical Co., USA.

2.2. Radiation Facility:-

Whole body $\gamma$-irradiation of mice was performed with a Canadian gamma cell-40, ($^{137}$Cs) at the National Center for Radiation Research and Technology, Cairo, Egypt at a dose rate of 0.46 Gy /min.

2.3. Experimental animals

Adult female Swiss albino mice weighing (22-25g) obtained from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Cairo) were used in this study. The animals were acclimatized for one week and during this period they maintained on a commercial standard pellet diet and water ad libitum. In vivo, studies were conducted under national research centre guidelines for the use and care for laboratory animals and were approved by an independent ethics committee of the national centre for radiation research and technology.

2.4. Methods:-

2.4.1. Tumor transplantation:

A cell line of Ehrlich Ascites Carcinoma (EAC) was used in this study. The parent line was supplied by Egyptian National Cancer Institute (NCI), Cairo University. EAC cells is originated from human breast cancer which modified to grow in female Swiss albino mice and maintained by i.p. of 2.5 million carcinoma cells in the mice. The EAC cells were counted before i.p. using the bright line haemocytometer and dilution was done using
physiological sterile saline solution. To assess Ehrlich Solid Tumor (EST) in thigh, 0.2 ml EAC cells (2.5x 10^6 cells /mouse) were inoculated i.m. in the right thigh of the lower limb of female mouse (Gupta et al. 2004 and El-Bahy et al. 2012).

2.4.2. Experimental design:

In the present study, 50 mice were categorized randomly into 5 equal groups (10 mice each) as follows: Control Group (C): Mice of this group neither treated nor irradiated just received saline by gavages. Ehrlich Group (E): EAC cells were implanted i.m. into the right thigh of the hind limb of mice at zero time of experiment. Group (E+BA): Mice of this group received 7 successive dose of BA (25 mg/kg/day) (i.p.) (Agrawa et al. 2011) started from 13th day of tumor implantation and continued to 20th day. Group (E+R): Mice of this group inoculated with EAC and exposed to radiation (4Gy) on 13th day of the experiment. Group (E+ BA+R): Mice were received EAC, exposed to radiation and administered with BA. At the end of BA treatments, the animals were sacrificed. The skeletal muscle (normal control) and tumor tissues E groups were collected for biochemical and histopathological examinations.

2.4.3. Biochemical Assay:

Lipid peroxidation was measured by thiobarbituric acid assay, which is based on malonaldehyde (MDA) reaction with thiobarbituric acid forming thiobarbituric acid reactive substances (TBARS), a pink colour complex exhibiting a maximum absorption at 532 nm according to (Yoshioka et al. 1979). Enzyme-linked immunosorbent assay (ELISA) for levels of VEGF and IL6 were determined by using ELISA Kits (R& D systems) according to the manufacturer’s instructions on the supernatants of sample tissue homogenates. In brief, microplates were coated with 100 µl/well of capture antibody, and then they were incubated overnight at 4°C. After washes, the plates were blocked with assay diluent at room
temperature (RT) for 1 h. One hundred microliters of a serum sample was added to each well of the plate, followed by incubation for 2 h at RT. Working detector was added into each well, and the plate was incubated for an additional 1 h at RT before the addition of substrate solution. The reaction was stopped by adding stop solution. The absorbance was read using ELISA reader. The concentrations were calculated from standard curve according to the instructions in the protocol.

2.4.4. Detection of IL-6R and Survivin gene expression by quantitative real time PCR:

RNA isolation and reverse transcription: RNA was extracted from the tumor tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer’s instructions. Genomic DNA was eliminated by a DNase-on-column treatment supplied with the kit. The RNA concentration was determine spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. RNA (1 µg) were used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl₂ (25 mM), RTase buffer (10X), dNTP mixture (10 mM), oligod (t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1 h.

Quantitative real time PCR: qRT-PCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions of 40 cycles of 15 s at 95°C and 60 s at 60°C after an initial denaturation step at 95°C for 10 min. Each 10 µl reaction contained 5 µl
SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5 µl cDNA and 1.9 µl nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analysed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the endogenous control GAPDH (Livak and Schmittgen 2001).

2.4.5. Western blot analysis of JAK-1, P-JAK-1, STAT-3, P-STAT-3 and Caspase-3

Part of tissue was homogenized with RIPA buffer containing 5 M NaCl, 1 M phenylmethylsulfonyl fluoride (PMSF), 10% deoxycholic acid (DOC), 10% SDS, 1 M Tris (pH 8.6). The tissue lysate was centrifuged at 12000 rpm for 20 min at 4°C. The lysate was then collected and protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc., USA). An aliquot of 7.5 µg protein of each sample were denatured then each sample was loaded on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA) using a semidyfer transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were incubated with 5% milk blocking buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and Tris-buffered saline with 0.05% Tween-20 (TBST) at 4°C overnight. The membranes were then washed with TBST and incubated with a 1:1,000 dilution of anti-caspase-3 or anti-JAK-1 antibodies (total and phosphorylated) or anti-STAT-3 (total and phosphorylated) (Thermo Fisher Scientific Inc., USA) for overnight on a roller shaker at 4°C. The filters were washed and subsequently probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham. Life Science Inc., USA). Chemiluminescence detection was performed with the Amersham detection kit according to the manufacturer’s protocols and exposed to X-ray film. The amount of studied protein was
quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer (Biomed Instrument Inc., USA). Results were expressed after normalization for β-actin protein expression (as housekeeping protein) (Mingone et al. 2003).

2.4.6. Histopathological study:

Autopsy samples were taken from the thigh muscle of mice in different groups and fixed in 10% formol saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft et al. 1996).

2.4.7. Statistics:

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan’s Multiple Range test by using statistical package of social science (SPSS) version 15.0 for windows. P ≤ 0.05 were considered as a level of significance.
3. Results

3.1. Biochemical assay:

3.1.1. Impact of BA on IL-6/STAT-3 Pathway in the presence or absent of gamma radiation:

The present study was aimed to investigate the effect of BA on IL6/STAT-3 pathway through assay of IL-6R, IL-6, JAK-1, P-JAK-1, STAT-3 and P-STAT-3.

The control levels of IL-6R and IL-6 were 1.04±0.01 fold and 32.93±0.68 level, respectively. The normal level of JAK-1 and P-JAK-1 were 1.02±0.02 fold and 0.73±0.016 fold, respectively. The STAT-3 and P-STAT-3 were 1.08±0.015 fold and 0.68±0.019 fold, respectively.

The gene expression of IL6R mRNA and IL-6 level in the tumour tissue were significantly higher (P < 0.05) in the E group compared to the control. After administration of BA and exposure to γ-radiation, the data revealed significant modulation in gene expression of IL6R mRNA (Figure 1A) and IL-6 level (Figure 1B) compared to E group.

Also, the protein expression of JAK-1 and P-JAK-1 were significantly increased in tumour tissues of E group compared to the control. But after administration of BA and/or exposed to γ-radiation in E group, a significant decrease (P < 0.05) have been observed in JAK-1 (Figure 1C and 1G) and P-JAK-1 (Figure 1D and 1G) protein expression compared with the E group.

Consequently, significant increase in the protein expression of STAT-3 (Figure 1E and 1G) and P-STAT-3 (Figure 1F and 1G) were observed in E group compared to the control and this result have been significantly improved (P < 0.05) after treatment of BA and/or γ-radiation exposure in E group.
3.1.2. **Impact of BA on Survivin and Caspase-3 in the presence or absent of gamma radiation:**

The control levels of survivin and caspase-3 were about 1.02±0.01 fold and 1.03±0.023 fold, respectively. The gene expression of survivin mRNA was significantly increased (P < 0.05) in E mice when compared to control group (Figure 2A). Meanwhile, protein expression of caspase-3 endured a significant (P < 0.05) decrease (Figure 2B and 2C). In E mice treated by BA and/or exposed to γ-radiation a remarkable modification in gene expression of survivin mRNA and protein expression of caspase-3 was observed (P < 0.05) (Figure 2).

3.1.3. **Impact of BA on VEGF in the presence or absent of gamma radiation:**

The control level of VEGF was 29.03±0.61 pg/mg tissue. The data obtained revealed a significant increase (P < 0.05) in VEGF levels of E mice compared to control (Figure 3A). After administration of BA and/or γ-radiation exposure significant (P < 0.05) modification in VEGF levels was recorded, compared to E mice (Figure 3A).

3.1.4. **Impact of BA on lipid peroxidation in the presence or absent of gamma radiation:**

The control level of MDA (index of lipid peroxidation) was 109.28±2.12 n mole/gm wet tissue. Significant increase (P < 0.05) in MDA level was found in E mice compared to control group. In BA and/or γ-radiation groups significant (P < 0.05) decreased in MDA level was observed compared to E mice (Figure 3B).
3.2. Histopathological study:

The histopathological examination of mice skeletal muscle (thigh muscle) showed no histopathological alteration in the muscle bundle in control mice (Figure 4A). Tumour cells were penetrate muscle bundles and showed cellular pleomorphism and nuclear hyperchromachian E mice group (Figure 4B). In E+R mice, Massive numbers of E tumor cells infiltrating in the muscle bundles were observed (Figure 4C). In E+BA mice, a wide area of necrosis was detected in the muscle bundles associated with apoptosis in the E tumour cells (Figure 4D). Meanwhile, a wide area of necrosis and apoptosis in most of the E tumour cells noticed in the muscle bundles of E+BA+R mice (Figure 4E).

4. Discussion

Several studies targeted the IL-6/STAT-3 signalling due to its pivotal role in tumor proliferation. IL-6 linked with the proliferation of many cancer types (multiple myloma, renal cell, carcinoma, prostate cancer…etc.) has been shown to induce its effect through activation of STAT-3 protein via phosphorylation of its tyrosine units. IL-6 engaged with cell surface cytokine receptor (IL-6 R) activates the janus kinase (JAK) that phosphorylate the cytoplasmic latent STAT-3 proteins (Aggarwal et al. 2006). The data obtained from the present study agreed with the previous viewpoint where the gene expression of IL-6R mRNA/IL-6 level as well as the JAK-1/P-JAK-1 and STAT-3/P-STAT-3 proteins expression are over expressed in E mice group compared to control mice (Figure 1). The observed increases in IL-6R might sustain the present of activated STAT-3 due to increases possibilities of IL-6 cytokine - receptor interaction. Further, over expression of survivin while decreased Caspse3 protein were observed (Figure 2). Also, the staple angiogenic mediator VEGF level increased significantly (Figure 3). Constitutively, active STAT-3 has been implicated in the induction of resistance to apoptosis. Its role in tumorigenesis is mediated
through the expression of various genes that suppress apoptosis, mediate proliferation, invasion, and angiogenesis. These genes include survivin (which crush apoptosis directly or indirectly by suppressing the caspases-3 function) and VEGF (Aggarwal et al. 2006 and Poomsawat et al. 2014). Worthwhile, the increases observed in end product of lipid peroxidation (MDA) in E mice compared to control mice were associate the rising in IL-6/STAT3 signalling (Figure 1). This could be interpreted in the view that the developing tumor stressed housing tissues to release oxygen derived free radicals. The excessive free radicals attacks macromolecules such as lipids initiating lipid peroxidation process ended with the formation of MDA. Agrawa et al. (2011) reported that the MDA concentration in cancer tissue show remarkable increases over its concentration in non-diseased tissue. Further, Vijaya and Balu (2015) showed a strong positive correlation between IL-6 and MDA. Moreover, the accumulation of lipid peroxides moieties during lipid peroxidation steps are involved in the activation of STAT-3 (Mazieére et al. 1999). The histopathological examination of tumor micrograph of E and E+R mice exhibit, a noticeable alteration in muscle bundles and the cells architectures in addition to the occurrence of tumor cell infiltration and penetration when matched with the micrograph of normal control thigh muscle bundles (Figure 4). This results could be attributed to the alteration observed in biochemistry of tumor where the changes in IL-6/STAT-3 signalling and the accumulation of MDA in the cells of muscle bundles destroyed the cellular integrity and pave to tumor proliferation. The MDA accumulation at the end of lipid peroxidation process had deteriorated the intact cellular membrane (Moustafa et al. 2015).

The experimental data reveals noticeable rising in IL-6/STAT-3 signalling turnover in E+R mice compared to control mice however, significant decreases were observed compared to E mice (Figure 1). One could expect that, the rising in IL-6/STAT-3 will be magnified in E+R mice compared to E mice. However, the opposite is happened. This might relate to the
radiation turn suppressive mechanism (senescence effect) that associated with slowing the production of certain cytokines and growth factor (IL-6 and VEGF) (Figure 1 and 3). Yu et al. (2013) found that senescence was induced by 3-6 Gy radiation. Cellular senescence is a permanent cell cycle arrest that was initially described as the terminal phase of primary cell populations that cannot be stimulated to return to the cell cycle by growth factors. Therefore, senescence is viewed as a tumour-suppressive mechanism that prevents cancer cell proliferation. Diverse factors, such as oxidative damage, telomere dysfunction, DNA damage response caused by ionising radiation and several chemotherapeutic drugs can trigger irreversible cellular senescence. Senescent cells remain metabolically active and have undergone widespread changes in protein expression and secretion, ultimately developing senescence-associated secretory phenotypes (SASPs). SASPs include cytokines and chemokines (such as IL-6), growth factors (such as VEGF), several matrix metalloproteinases and nitric oxide (Yu et al. 2013). Freund et al. (2010) reported that after radiation, the DNA damage response is activated immediately, whereas the SASPs develop slowly over several days.

These results refined the obvious role of IL-6/STAT-3 signalling in tumorgensis. The management of this pathway may introduce a good opportunities to avoid the development of undesired tumorgenic process. The experimental results obtained upon administration of baswilic acid revealed significant amelioration in the expression of IL-6R mRNA/IL-6 level, JAK-1/P-JAK-1 and STAT-3/P-STAT-3 protein expression as well as the apoptotic markers (caspase-3 and survivin) in addition to angiogenic activator molecules (VEGF) in E and E+R mice compared to normal control (Figure 1). Also, the degree of lipid peroxidation as indices of oxidative stress which exerted due to tumor or radiation exposure or the synergism between the 2 stressors displayed significant decrease after BA administration. Further, the results of histopathological examination for the tumor micrograph of E+BA and E+R+BA
mice groups proportional to E mice reveals noticeable amelioration in cell architecture of the muscle bundle in addition to certain improvement in the state of tumor cells infiltration, penetration and area of necrosis (Figure 4). The results give the impression that BA plays an affordable role in the inhibition of the STAT-3 activation events. The down-regulation of the IL-6R expression and inflammatory cytokine (IL-6) by BA as indicated in our results might be attributed to the suppression of nuclear factor kapa B (NF-κB) (a regulator of gene transcription and gene products) which reported in study of Takada et al. (2006). Kunnumakka et al. (2009) found that AKBA (one of the 4 major baswilic acid families) inhibits the STAT-3 activation process through the suppression of JAK proteins (even the constitutively active form or that induced by the IL-6). Subsequently, the role of active STAT-3 in the inhibition of apoptosis and activation of angiogenesis met many difficulties and appeared inactive. Moreover, AKBA could halt the progression of angiogenic process directly via inhibiting the expression of type-2 receptor for VEGF and subsequently destabilized the linkage of VEGF to the receptors leading to the suppression of gene products involved in proliferation, survival and angiogenesis (Agrawa et al. 2011). Further, BA might promote the apoptotic process directly by cleavage of pro-caspase-3 or indirectly through activation of caspase 8 mediated pathways (Xia et al. 2005). Takada et al. (2006) showed that, BA inhibit the NF-κB regulated gene transcription as well as regulated gene products that involved in antiapoptotic process (like, Survivin) and that involved in angiogenic process like (VGEF). In addition, Agrawa et al. (2011) reported that BA reduced the elevated levels of lipid peroxidation.

Clearly, the treatment by baswilic acid can disrupt the IL-6/STAT-3 pathway signalling in the point of interaction between IL-6 and its receptor. The expected Influence of baswilic acid on the partway of JAK-1 and STAT3 activation not absolute and could be attributed to alteration in the expression of STAT3 and JAK-1 rather than activation of JAK and STAT3.
signaling due to indifference between control and tumor P-JAK-1/JAK-1 and P-STAT3/STAT3 ratios. The combinations of baswelic acid and radiation exposure sustain the antitumorgenic pathways. Therefore, the inclusion of baswelic acid may a good potential therapeutic modality for cancer treatment.
5. Acknowledgement

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6. References


Table Title

Table 1. Primer sequences used for RT-PCR

Figure legends

Figure 1: IL-6/STAT-3 Pathway. A) Gene expression of IL-6R mRNA (real time-PCR), B) IL-6 level, C) Protein expression of JAK-1, D) P-JAK-1, E) STAT-3, F) P-STAT-3 (Western blot analysis). G) PAGE of JAK-1/ P-JAK-1 (130 kDa), STAT-3/ P-STAT-3 (92 kDa) proteins and β-actin (42 kDa) as housekeeping protein. Values are means ± SE (n = 10 mice). Values with (a) superscript significantly different from control, at P≤0.05 and Values with (b) superscript significantly different from E-group, at P≤0.05

Figure 2: Survivin and Caspase-3. A) Gene expression of Survivin mRNA (real time-PCR). B) Protein expression of Caspase-3 (Western blot analysis). C) PAGE of caspase-3 (35 kDa) protein and β-actin (42 kDa) as housekeeping protein. Values are means ± SE (n = 10 mice). Values with (a) superscript significantly different from control, at P≤0.05 and Values with (b) superscript significantly different from E-group, at P≤0.05

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| IL-6R  | Forward 5′-GATGAGTACAAAGTCTGTC-3′  
           Reverse 5′-GTCAGCCTGGTTCTG-3′  |
| Survivin | Forward 5′-GCCACTTGCCAGCTTCC-3′  
             Reverse 5′-GTCACAGCATAGCAAGCCACA-3′  |
| GAPDH  | Forward: 5′- CTCCCATTCTCCACCTTG-3′  
              Reverse: 5′- CTGCTCTCAGTATCCATGC-3′  |