Vascular Endothelial Growth Factor and Hepatocyte Regeneration in Acetaminophen Toxicity

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Abstract

Vascular endothelial growth factor (VEGF or VEGF-A) is a major regulator of angiogenesis and has been recently shown to be important in organ repair. The potential role of VEGF in acetaminophen (APAP)-induced hepatotoxicity and recovery was investigated in B6C3F1 male mice. Mice were treated with APAP (300 mg/kg IP) and sacrificed at various time points that reflect both the acute and recovery stages of toxicity. VEGF-A protein levels were increased 7 fold at 8 hrs and followed the development of hepatotoxicity. VEGF receptor 1, 2, and 3 (VEGFR1, VEGFR2, and VEGFR3) expression increased throughout the time course, with maximal expression at 48, 8, and 72 hrs, respectively. Treatment with the VEGF receptor inhibitor, SU5416 (25 mg/kg IP at 3 hrs), had no effect on toxicity at 6 or 24 hours. In further studies, the role of SU5416 on the late stages of toxicity was examined. Treatment of mice with APAP and SU5416 (25 mg/kg IP at 3 hrs) resulted in decreased expression of proliferating cell nuclear antigen (PCNA), a marker of cellular proliferation. Expression of platelet endothelial cell adhesion molecule (PECAM), a measure of small vessel density, and endothelial nitric oxide synthase (NOS), a downstream target of VEGFR2, were increased at 48 and 72 hrs following toxic doses of APAP, and treatment with SU5416 decreased their expression. These data indicate that endogenous VEGF is critically important to the process of hepatocyte regeneration in APAP-induced hepatotoxicity in the mouse.

Key words: angiogenesis, liver injury, liver repair, hepatocytes, acetaminophen
INTRODUCTION

Acetaminophen (APAP) overdose is the most prevalent cause of drug-induced acute liver failure. Recent studies have reported the upregulation of a number of cytokines and chemokines in APAP toxicity. \(14-16, 21, 22, 24, 29\) Several cytokines have been shown to have hepatoprotective properties, such as promoting hepatocyte regeneration or controlling pro-inflammatory cytokine expression. For instance, our laboratory previously reported that interleukin 6 (IL-6) knock out (KO) mice had delayed hepatocyte regeneration following the administration of toxic doses of APAP. \(22\) In addition, a number of chemokines such as macrophage inhibitory protein 2 (MIP-2) and interferon gamma inducible protein (IP-10) have also been shown to be important in hepatocyte regeneration following APAP toxicity. \(3, 16, 21\)

Growth factors are small molecular weight proteins that are closely related to cytokines. Growth factors have received little study in drug-induced liver toxicity. Previous studies have shown that vascular endothelial growth factor A (VEGF-A) is upregulated in several models of hepatic injury, such as partial hepatectomy, carbon tetrachloride toxicity, and ischemia reperfusion injury. \(17, 35\) VEGF is a potent mitogen for endothelial cells and is well known for its effects on angiogenesis in physiological and pathological conditions. The following study was conducted to examine the role of VEGF in APAP-induced hepatotoxicity and to examine the effect of VEGF neutralization on toxicity and hepatocyte regeneration in our model.
METHODS & MATERIALS

Reagents. Acetaminophen (APAP, paracetamol) and carboxymethylcellulose (CMC) were obtained from Sigma Chemical Co. (St. Louis, MO). Universal DAKO LSAB + (Labeled Streptavidin-Biotin) Peroxidase kit and DAKO protein block (serum free) were purchased from DAKO Corporation (Carpinteria, CA). Immunopure Peroxidase Suppressor and Coomassie Plus Protein Assay Reagent were obtained from Pierce Chemical Co. (Rockford, IL). Gills Hematoxylin II and Permount were both acquired from Fisher Scientific, Inc. (Pittsburgh, PA). The VEGF inhibitor, SU5416, was obtained from CalBiochem (Spring Valley, CA).

Experimental Animals. Six-week old male B6C3F1 mice (mean weight, 24.4 grams) were obtained from Harlan Sprague Dawley (Indianapolis, IN). All animal experimentation was in accordance with the criteria of the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by University of Arkansas for Medical Sciences Animal Care and Use Committee. Mice were acclimatized one week prior to the planned experiments. Mice were fed ad libitum and were housed in individual cages on a 12 hr light/dark cycle. On the day prior to experiments, mice were fasted overnight and dosing studies began at 0800 the following morning. In initial experiments, mice were dosed with APAP (300 mg/kg i.p. in saline) and sacrificed at 1, 2, 4, and 8 hrs after APAP (n = 5-6 mice per time point). In another experiment, mice were dosed with APAP (300 mg/kg i.p. in saline) and sacrificed at 24, 48, 72, and 96 hrs after APAP (n = 5-6 mice per time point). Control mice received saline only. In other experiments, animals received SU5416 (25 mg/kg, sq) 3 hr after APAP and were sacrificed at 6, 24, 48, or 72 hrs after APAP (n = 5-6 per treatment group). The SU5416 was administered in 200 uL of a solution containing carboxymethylcellulose (CMC; 0.5%), NaCl (0.9%), polysorbate 80 (0.4%), and benzyl alcohol.
(0.9%) in deionized water as previously described. Control mice in this experiment received APAP plus vehicle. At the indicated time, animals were anesthetized with CO₂. Blood was removed from the retro-orbital plexus and allowed to coagulate at room temperature. Blood was centrifuged and the serum was removed for measurement of alanine aminotransferase (ALT). Mice were then euthanized with CO₂ and the livers were removed. The livers were weighed and a portion was preserved in formalin for histological sections. The remaining livers were snap frozen in liquid nitrogen and stored at -80°C for additional analyses.

**Toxicity Assays.** Serum ALT was determined with a spectrocolorimetric diagnostic kit (Teco Diagnostics, Anaheim, CA).

**Growth factor protein receptor assays.** Snap-frozen liver samples were thawed, weighed, and homogenized in solutions containing 1 ml of protease inhibitor cocktail (Complete; Boehringer Mannheim, Indianapolis, IN). The resulting supernatants were analyzed in duplicate and standardized to the weight of the homogenized liver sample. VEGF-A and other growth factors were measured in the supernatants of liver homogenates by a Luminex 100 analyzer (Applied Cytometry Systems, Dinnington, United Kingdom) using a Mouse Growth Factor Four-Plex Antibody Bead kit (BioSource International, Inc, Camarillo, CA) as per the manufacturer’s instructions. The limits of detection for growth factors were as follows: VEGF-A (15 pg/mL), fibroblast growth factor (30 pg/mL), platelet-derived growth factor (15 pg/mL), and granulocyte colony stimulating factor (15 pg/mL).

**Western Blot Analysis.** VEGF Receptor 1 (VEGFR1 or Flt-1), VEGF Receptor 2 (VEGFR2 or Flk-1), VEGF Receptor 3 (VEGFR3 or FLT4), platelet endothelial cell adhesion molecule (PECAM), and endothelial nitric oxide synthase (eNOS) protein levels were measured by western blot. Liver tissue was homogenized on ice in 25 mM Tris-HCL buffer, containing 1 mM
EDTA, 1 mM EGTA, 0.1 % (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. Homogenates were centrifuged at 1500 x g at 4°C for 10 minutes to remove cell debris. Liver homogenates (50 µg) were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting. Membranes were blocked overnight at 4°C in blocking buffer (5% nonfat dry milk) followed by a 2 hr incubation at room temperature with a monoclonal anti-Flt-1 antibody (1:400), a monoclonal anti-Flk-1 antibody (1:400), a polyclonal anti-flt-4 antibody (1:250), a polyclonal anti-PECAM antibody (1:200), or a monoclonal anti-eNOS antibody (1:750). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After treating the membranes with the primary antibodies, a peroxidase-conjugated goat anti-mouse IgG secondary antibody was used at a 1:2000 dilution for 1 hr at room temperature. Band detection was performed using ECL Plus detection (Amersham, Piscataway, NJ).

**Immunohistochemistry.** Fresh liver tissues, previously trimmed to approximately 2-mm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 hr. Paraffin-embedded tissue sections were deparaffinized with xylene (2 x 5 min, 25°C), then rehydrated in a series of graded ethanol washes and deionized water. The sections were then placed in Pierce Immunopure peroxidase suppressor for 30 minutes to quench endogenous peroxidase activity. Next, DAKO protein block (serum-free) was added to each tissue section for 30 minutes to block nonspecific binding. After washing in PBS, the sections were incubated with a mouse monoclonal VEGF-A (1:50) antibody for 60 min at room temperature. For color development the protocol described in the DAKO LSAB+ kit was used. The slides were counterstained with Gill's Hematoxylin II for 2 minutes and following rinsing in deionized water were immersed in ammonia blue for 2 min. The slides were dehydrated and mounted with
Permount. Sections of pancreatic parenchyma served as the positive control for VEGF immunohistochemistry. Normal mouse liver tissue with the primary antibody replaced by mouse serum were used as a negative control. Immunohistochemical analysis with proliferating nuclear cell antigen (PCNA) was performed using the DAKO ARK peroxidase kit (DAKO Corporation; Carpinteria, CA) at an antibody concentration of 1:75. PCNA is a commonly used assay of cellular proliferation that correlates with DNA synthesis and liver thymidine kinase activity.

**Statistical Analysis.** Results are expressed as means ± SE. Comparisons between multiple groups were by one-way analysis of variance followed by the Tukey HSD post-hoc test; p ≤ 0.05 was considered statistically significant. Comparison of differences in PCNA immunohistochemistry was performed using a Mann Whitney non-parametric test. SPSS Version 10.0 (SPSS Inc, Chicago, IL) was used for statistical analyses.
RESULTS

VEGF Upregulation in APAP Toxicity

To determine if VEGF was upregulated in APAP-induced hepatic necrosis, mice were treated with APAP (300 mg/kg ip). The mice were sacrificed at 1, 2, 4 and 8 hrs and in a separate experiment the mice were sacrificed at 24, 48, 72 and 96 hrs. Consistent with previous data from our laboratory,(24) significant hepatotoxicity (elevation of serum ALT and histological necrosis [data not shown]) was apparent at 4 hrs (Fig 1A), peaked at 8 to 24 hrs, remained high for 48 hrs, and resolution occurred between 48 and 96 hrs (Fig 1B). As shown in Figure 1C, a significant increase in VEGF-A protein was detected in liver homogenates at 8 hrs. This increase followed the onset of toxicity (Fig 1A). By 24 hrs, VEGF-A levels were extremely high (a 34 fold elevation over controls) and levels peaked at this time point and subsequently declined (Fig 1D).

The upregulation of VEGF-A in APAP treated livers was also examined by immunohistochemical analysis of tissue sections. These data showed that VEGF-A staining was present in the hepatocytes of the periportal and mizonal areas of the APAP-treated liver (Fig 2B; arrows). The immunohistochemical expression of VEGF-A in hepatocytes is consistent with data reported in other models of liver injury.(4, 17)

Levels of other growth factors (fibroblast growth factor, platelet-derived growth factor, granulocyte colony stimulating factor) were not significantly elevated in the APAP treated mice (data not shown).

VEGF Receptor Upregulation in APAP Toxicity

The protein expression of the three VEGF receptors (VEGFR1, VEGFR2, and VEGFR3 (42)) was examined by immunoblots (Fig 3). Minimal expression of all three receptors was
present in the saline treated mice. Increased expression of VEGFR1, VEGFR2, and VEGFR3 protein (Figs 3A-C) was apparent throughout the time course, with peak expression for VEGFR1 at 48 hrs, VEGFR2 at 8 hrs, and VEGFR3 at 72 hrs.

**Effect of SU541 on APAP Toxicity**

To examine the role of VEGF in the early stages of APAP toxicity, mice were treated with APAP, followed by SU5416, a synthetic VEGF inhibitor that is highly specific for VEGF receptor signaling. The mice received SU5416 3 hours after APAP, a time point beyond the metabolic phase of toxicity. The mice were sacrificed at 6 or 24 hrs after APAP. Both biochemical evidence of toxicity (ALT values; Fig 4A) and histological examination of H & E stained slides (Fig 4B) showed comparable degrees of toxicity in the two groups of mice at both time points. Thus, treatment with SU5416 did not alter APAP toxicity.

**Effect of SU5416 on Hepatocyte Regeneration following APAP Toxicity**

To elucidate the role of endogenous VEGF-A in the later stages of toxicity, mice were treated with SU5416 and were sacrificed at 48 or 72 hrs after APAP. Another group of mice received APAP followed by vehicle only. At 48 hrs, ALT values were significantly higher in the mice that received APAP plus SU5416, compared to the APAP plus vehicle group (Fig 5). However, histological evaluation of relative necrosis indicated that there was not a significant difference between the toxicity in the APAP plus SU5416 group and the APAP plus vehicle group. To examine the effect of SU5416 on hepatocyte regeneration, immunohistochemical analysis for PCNA was performed. PCNA is a 36kD acidic nuclear protein expressed in the G1 and S phase of mitosis and is commonly used as a measure of hepatocyte regeneration in liver injury models. In previous studies, our laboratory reported that PCNA staining was significantly increased by 48 hrs in APAP toxicity in the mouse. Minimal
PCNA staining was present in the vehicle treated mice (Fig 6C). Prominent staining for PCNA was apparent at the junction of the midzonal and centrilobular regions of the livers of the APAP plus vehicle groups at 48 hrs (Fig 6A; arrows). In contrast, minimal staining for PCNA was present in the mice treated with APAP plus SU5416 (Fig 6B). Quantitative image analysis showed that the intensity of PCNA staining was significantly increased in the APAP treated mice compared to the vehicle treated mice (132.5 ± 7.5 vs. 95.8 ± 6.8; p=0.009) and significantly reduced in the SU5416 treated mice at 48 hrs (104.3 ± 8.2; p=0.041). These data suggest that VEGF receptor signaling is critical to the process of hepatocyte regeneration in APAP toxicity.

**Effect of APAP Treatment on PECAM and eNOS Expression**

A number of proteins have been reported to be induced during angiogenesis. These include platelet endothelial cell adhesion molecule (PECAM) and endothelial nitric oxide synthase (eNOS). PECAM expression is a commonly used relative measure of microvessel density (neoangiogenesis).(11, 44, 51). eNOS is a constitutive endothelial protein important in the control of portal blood pressure by regulating nitric oxide (41) and has been shown to be induced by VEGF.(9) Figure 7 shows that both PECAM and eNOS were induced at 48 and 72 hrs in the livers of APAP treated mice. The anti-angiogenic effect of SU5416 was assessed by measuring the relative expression of these proteins. As shown in Figure 7A, marked reduction in PECAM expression was present in the SU5416 treated mice, as compared to the APAP treated mice at both 48 and 72 hrs. Similarly, decreased expression of eNOS was found at 48 and 72 hrs (Fig 7B).
DISCUSSION

VEGF-A is a homodimeric glycoprotein of relative molecular mass 45,000 daltons and is the only mitogen that specifically acts on endothelial cells. It is well known as a pro-angiogenic factor in the developing vascular system and is also important in the pathogenesis of tumors, diabetes and prematurity-induced retinopathy. VEGF-A is secreted by hepatocytes, as well as many other cell types, and has also been shown to be important in wound healing in bone and skin injury. In the present study, the time course of VEGF-A protein and receptor expression was examined in a mouse model of APAP toxicity. VEGF-A protein and receptor levels were significantly increased in mice treated with APAP (Figs 1-3) and this upregulation followed the onset of biochemical and histological toxicity, suggesting a role for endogenous VEGF-A upregulation in the recovery stages of toxicity. In addition, immunohistochemical studies demonstrated VEGF-A in the healthy regions of the liver (Fig 2), known to be important in the process of hepatocyte regeneration.

To further examine the relationship of VEGF-A to recovery following toxicity, mice were treated with SU5416, an anti-angiogenic compound that specifically inhibits VEGF mediated signaling. SU5416 is a hydrophobic small molecule that has prolonged pharmacodynamic effects in mice, and was developed as an anti-tumor agent due to its inhibitory effects on angiogenesis. This compound is a tyrosine kinase inhibitor that inhibits VEGF receptor mediated phosphorylation and subsequent signal transduction. SU5416 is thought to primarily have effects on VEGFR2, although one study found that it may also have effects on VEGFR1 mediated signaling. In the present study, treatment with SU5416 after APAP had no effect on toxicity at either 6 or 24 hrs. In further studies, treatment with SU5416 after APAP was associated with a significant reduction in cellular proliferation as
measured by immunohistochemical assays for PCNA. Thus, treatment with SU5416 slowed recovery in APAP treated mice.

In addition to a reduction in immunohistochemical staining for PCNA, treatment with SU5416 was associated with a reduction in PECAM and eNOS expression. Previous studies have examined the relationship of VEGF and eNOS.(37) The angiogenic effects of VEGF are mediated by eNOS in vascular endothelial (reviewed in (27)) and sinusoidal endothelial cells.(41) Autophosphorylation of VEGFR2 results in eNOS activation.(9) Kawachi et al.(26) found that mice genetically deficient in eNOS had increased liver injury in a model of post-ischemic liver injury. Fukumura et al.(12) reported that eNOS KO mice had reduced angiogenesis. Collectively, the data from the present study suggest that endogenous VEGF is important in the recovery stages of APAP toxicity and that neutralization of VEGF slows the recovery process and is associated with a reduction of proteins previously implicated in the process of angiogenesis.

Previous studies in animal models of liver injury have supported a role for VEGF in the recovery stages following liver injury.(17, 45) Ishikawa et al.(17) showed that mRNA levels of VEGF and its receptors were increased in a rat model of carbon tetrachloride toxicity. Similar findings have been reported in studies of partial hepatectomy in the rat.(35, 45) In vitro, VEGF induced the proliferation of endothelial cells, including hepatic sinusoidal endothelial cells.(10) Increased expression of mRNA for VEGF was shown in isolated cellular fractions including hepatocytes, Kupffer cells, and stellate cells in rats treated with carbon tetrachloride.(17)

Previous data suggest that VEGF receptors may have differential effects (reviewed in (43)). VEGF is best known for its pro-angiogenic effects, most of which are mediated by VEGFR2.(31) Comparatively less is known about the specific effects of VEGFR1, while
VEGFR3 is important in lymphangiogenesis. Using receptor specific mutants in a co-culture model of endothelial cells and hepatocytes, selective activation of VEGFR1 on endothelial cells increased mRNA levels of hepatocyte mitogens (interleukin 6 [IL6] and hepatocyte growth factor [HGF]). It was thus proposed that VEGFR1 mediates “cross-talk” between parenchymal and non-parenchymal cells, causing the release of IL6 and HGF by endothelial cells leading to pro-survival effects on hepatocytes. Previous data have shown that endothelial cells are metabolically active, are targets of APAP toxicity, and that microcirculatory dysfunction involving endothelial cells precedes parenchymal cell injury in APAP toxicity. Both HGF and IL6 have been shown to be important in hepatocyte regeneration in several models of hepatic injury, including APAP toxicity. We previously showed that IL6 KO mice treated with toxic doses of APAP had delayed hepatocyte regeneration. Thus, cumulatively the data support a mechanism where both VEGF and IL6 are critical mediators of hepatocyte regeneration following APAP toxicity.

One of the observations of this study was that serum ALT levels were significantly higher in the APAP plus SU5416 treated mice than the APAP only treated mice at 48 hrs (Fig 5). Examination of liver tissues from the mice revealed comparable degrees of necrosis, despite this apparent difference in ALT data between the treatment groups at 48 hrs (Fig 5). Interestingly, we previously observed discrepancies between ALT values and histology in other studies of APAP toxicity. From a pathologic perspective, histological examination of liver tissue is considered to be the gold standard for the assessment of toxin mediated liver injury. Thus, other factors, possibly relating to the rate of leakage of ALT from hepatocytes into the circulation or the clearance of ALT from the circulation, may be responsible for the observed differences between ALT and histological examination of liver tissues.
In closing, we found that VEGF protein and receptor expression was markedly increased in the late stages of toxicity in APAP treated mice. Inhibition of VEGF mediated signaling with SU5416 significantly delayed hepatocyte regeneration and this intervention was associated with reduced expression of PECAM and eNOS. Further study will be required to examine the possible protective effects of exogenous VEGF (36, 48) as a novel therapeutic approach for stimulating hepatocyte regeneration following APAP overdose.
Acknowledgements

This work was supported by grant DK 02971 from the National Institute for Diabetes and Digestive Diseases (LPJ) and by a grant-in-aid from the Arkansas Biosciences Institute, the major research component of the Tobacco Settlement Proceeds Act of 2000.
REFERENCES


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Figure Legends

**Figure 1. Time course of serum ALT and hepatic VEGF-A protein in APAP Toxicity.**
Mice were treated with APAP (300 mg/kg IP) and sacrificed at 1, 2, 4 and 8 hrs (A) after APAP.
In a second time course study (B), mice were treated with APAP and sacrificed at 24, 48, 72, and 96 hrs after APAP (300 mg/kg IP). ALT values (A, B) were significantly elevated at 4, 8, 24, and 48 hrs, compared to saline treated controls (p < 0.05). VEGF-A levels (C, D) were significantly elevated at 8 and 24 hrs, compared to saline treated controls (p < 0.05).

**Figure 2. Immunohistochemical detection of VEGF in mouse liver after APAP.** Mice were dosed with APAP (300 mg/kg IP) and sacrificed at the indicated times (Fig 1). VEGF staining (arrows) was apparent in the midzonal and periportal regions of the liver at 24, 48, and 72 hrs.

**Figure 3. Time course of VEGFR1, VEGFR2, and VEGFR3 protein expression in mice treated with APAP.** Mice were dosed with APAP (300 mg/kg IP) and sacrificed at the indicated times. Minimal expression of VEGFR1, VEGFR2, and VEGFR3 was present in the saline treated mice. VEGFR1, VEGFR2, and VEGFR3 expression increased throughout the time course with peak expression of VEGFR1 at 48 hrs (A), and VEGFR2 at 8 hrs (B) and VEGFR3 at 72 hrs (C).

**Figure 4. Effect of SU5416 on Toxicity.** A. Mice were treated with SU5416 3 hrs after APAP (300 mg/kg IP) and sacrificed at 6 or 24 hours. ALT values were comparable in the mice that received SU5415, compared to the mice that received APAP plus vehicle; *p < 0.05, compared to mice treated with vehicle alone. B. H & E sections of representative mice. Top panel shows mouse treated with APAP plus vehicle, sacrificed at 6 hrs. Bottom panel shows mouse treated with APAP plus SU5416, sacrificed at 6 hrs. The mice had comparable degrees of hepatocellular necrosis.
Figure 5. Effect of SU5416 on serum ALT levels in APAP treated mice. Mice were treated with SU5416 3 hr after APAP (300 mg/kg IP). ALT levels were significantly higher at 48 hrs in the mice that received SU5416, compared to the mice that received APAP plus CMC vehicle. (*indicates significant difference from APAP plus inhibitor group, p < 0.05). No difference was present in the two groups of mice sacrificed at 72 hrs.

Figure 6. Effect of SU5416 on PCNA immunohistochemistry in APAP treated mice. PCNA staining (arrows) was present in the midzonal and centrilobular regions of the livers of mice treated with APAP and sacrificed at 48 and 72 hrs (A). In contrast, PCNA was significantly reduced in the mice treated with SU5416 (B). No to minimal staining for PCNA was present in the vehicle treated mice (C).

Figure 7. Effect of SU5416 on PECAM and eNOS expression. Representative immunoblots of PECAM (A) and eNOS (B) expression in mice treated with APAP and SU5416. Both PECAM and eNOS were reduced in the SU5416 treated mice. (A). Lane 1, CMC vehicle control; Lane 2, APAP treated mouse sacrificed at 48 hrs; Lane 3, APAP plus SU5416 treated mouse, sacrificed at 48 hrs; Lane 4, APAP treated mouse, sacrificed at 72 hrs; Lane 5, APAP plus SU5416 treated mouse, sacrificed at 72 hrs. B. Lane 1, CMC vehicle control; Lane 2, APAP treated mouse sacrificed at 48 hrs; Lane 3, APAP plus SU5416 treated mouse, sacrificed at 48 hrs; Lane 4, APAP treated mouse, sacrificed at 72 hrs; Lane 5, APAP plus SU5416 treated mouse, sacrificed at 72 hrs.
VEGF and acetaminophen toxicity

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A

- **OD mm²**
- 0
- 5
- 10
- 15

- **Time**
  - 48 hrs
  - 72 hrs

- **Conditions**
  - Vehicle
  - APAP
  - APAP + SU5416
  - APAP + SU5416

B

- **OD mm²**
- 0
- 2
- 4
- 6

- **Time**
  - 48 hrs
  - 72 hrs

- **Conditions**
  - Vehicle
  - APAP
  - APAP + SU5416
  - APAP
  - APAP + SU5416