Connexin hemichannel inhibition reduces acetaminophen-induced liver injury in mice

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Abstract

Historically, connexin hemichannels have been considered as structural precursors of gap junctions. However, accumulating evidence points to independent roles for connexin hemichannels in cellular signaling by connecting the intracellular compartment with the extracellular environment. Unlike gap junctions, connexin hemichannels seem to be mainly activated in pathological processes. The present study was set up to test the potential involvement of hemichannels composed of connexin32 and connexin43 in acute hepatotoxicity induced by acetaminophen. Prior to this, *in vitro* testing was performed to confirm the specificity and efficiency of TAT-Gap24 and TAT-Gap19 in blocking connexin32 and connexin43 hemichannels, respectively. Subsequently, mice were overdosed with acetaminophen followed by treatment with TAT-Gap24 or TAT-Gap19 or a combination of both after 1.5 hour. Sampling was performed 3, 6, 24 and 48 hours following acetaminophen administration. Evaluation of the effects of connexin hemichannel inhibition was based on a series of clinically relevant read-outs, measurement of inflammatory cytokines and oxidative stress. Subsequent treatment of acetaminophen-overdosed mice with TAT-Gap19 only marginally affected liver injury. In contrast, a significant reduction in serum alanine aminotransferase activity was found upon administration of TAT-Gap24 to intoxicated animals. Furthermore, co-treatment of acetaminophen-overdosed mice with both peptides revealed an additive effect as even lower serum alanine aminotransferase activity was observed. Blocking of connexin32 or connexin43 hemichannels individually was found to decrease serum quantities of pro-inflammatory cytokines, while no effects were observed on the

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occurrence of hepatic oxidative stress. This study shows for the first time a role for connexin hemichannels in acetaminophen-induced acute liver failure.

Keywords

connexin; hemichannel; gap junction; hepatotoxicity; acetaminophen; inflammation

1   Introduction

Drug-induced liver injury is the leading cause of acute liver failure in Western countries. A majority of the clinical cases of acute liver failure results from either accidental or intentional overdose of acetaminophen (APAP), a readily available analgesic and antipyretic drug (Ichai and Samuel, 2011; Lee, 2008). In case of an APAP overdose, a substantial amount of APAP is metabolized by cytochrome P450 2E1, yielding the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which depletes the glutathione (GSH) pool and that gives rise to deleterious APAP-protein adducts (Dahlin et al., 1984). As a consequence, impaired mitochondrial respiration and oxidative stress are triggered accompanied by the onset of massive hepatocyte cell death as well as the induction of an inflammatory response (Blazka et al., 1995; Cover et al., 2006; Jaeschke and McGill, 2015).

As holds for most cellular processes, cell death and inflammation are driven by the interaction between 3 communication mechanisms, namely extracellular, intracellular and intercellular signaling. The latter can be mediated by gap junctions, a group of cell-to-cell contacts composed of 2 hemichannels of adjacent cells, which in turn are built up by 6 connexin (Cx) proteins (Maes et al., 2014; Vinken et al., 2008). At present, 21 connexin proteins have been identified in humans, while in rodents 20 connexin proteins have been characterized. They all share a similar structure consisting of 4 membrane-spanning domains, 2 extracellular loops, a cytoplasmic loop, cytosolic *N*-terminal and *C*-terminal regions (Oshima, 2014). Connexins are named after their molecular weight. In liver, hepatocytes predominantly express Cx32 and to a lesser extent Cx26, while non-parenchymal cells, including Kupffer cells and stellate cells, mainly harbor Cx43 (Maes et al., 2015a). Nonetheless, hepatic connexin expression patterns undergo drastic changes upon chronic liver disease, such as in liver fibrosis and cirrhosis as well as during acute liver injury (Maes et al., 2015b; Vinken, 2012). In this respect, our and other groups previously reported a switch in RNA and protein production from Cx32 and Cx26 to Cx43 upon APAP intoxication in rodents (Maes et al., 2016a; Naiki-Ito et al., 2010). This upregulation of Cx43 is due to recruitment of Cx43-expressing inflammatory cells, but equally reflects *de novo* production of Cx43 by hepatocytes (Maes et al., 2016a). In fact, although being critical mediators of liver homeostasis (Maes et al., 2015a), connexins and their channels are also frequently involved in liver toxicity (Maes et al., 2015b). A number of studies have shown a role for Cx32-based gap junctions in APAP-triggered hepatotoxicity using genetically modified animals, albeit with contradicting outcomes (Igarashi et al., 2014; Maes et al., 2016b; Naiki-Ito et al., 2010; Park et al., 2013). In a recent study, our group questioned the suitability of genetically deficient rodents for investigating the involvement of Cx32 in APAP-induced hepatotoxicity (Maes et al., 2016b). A possible alternative is the use of

inhibitors of Cx32-based signaling. In this regard, a non-specific inhibitor of gap junctional communication, 2-aminoethoxy-diphenyl-borate (2-APB), was reported to protect against liver failure and death in mice when co-administered with APAP (Patel et al., 2012). However, a follow-up study demonstrated that this protection was only minor or completely lost when 2-APB was administered 1.5 hour or 4-6 hours, respectively, after APAP. In addition, part of the protection could be attributed to solvent effects and inhibition of the metabolic activation of APAP as well as to inhibition of the c-jun-*N*-terminal kinase signaling pathway (Du et al., 2013). On the other hand, our group demonstrated that APAP-related liver injury is increased in $Cx43^{+/-}$ mice (Maes et al., 2016a). In line with this, inhibition of Cx43 after bile duct ligation in mice produced detrimental effects with markedly greater hepatocellular necrosis (Balasubramaniyan et al., 2013). This discrepancy between outcomes after APAP-induced toxicity of mice deficient in Cx32 or Cx43 may be due, at least in part, to opposite actions of gap junctions and connexin hemichannels. The latter not only serve as structural precursors of gap junctions, but also establish as pathway for cellular signaling on their own by connecting the cytosol of individual cells with the extracellular environment. Strikingly, while gap junctions are mainly associated with physiological functions, connexin hemichannels seem preferentially active in pathological conditions (Chandrasekhar and Bera, 2012; Decrock et al., 2009b; Maes et al., 2015b). Research in this area is largely impeded by the lack of inhibitors able to distinguish between both channel types (Bodendiek and Raman, 2010; Evans and Leybaert, 2007). An exception lies with a number of peptides, which reproduce specific amino acid modules of selected connexin proteins. In the present study, Gap24 and Gap19, both linked to a transactivator of transcription (TAT) sequence, were tested for their potential to specifically inhibit Cx32 and Cx43 hemichannels, respectively, in cultures of primary rat hepatocytes, and to reduce the clinical manifestation of hepatotoxicity in a mouse model of APAP-induced acute liver failure.

2 Materials and methods

2.1 Chemicals and reagents

TAT-Gap24 (YGRKKRRQRRRGHGDPLHLEEVKC) and TAT-Gap19 (YGRKKRRQRRRKQIEIKKFK) were synthesized by Thermo Fisher (Germany) with a purity of at least 90%. All other chemicals were commercially available products of analytical grade and were supplied by Sigma (USA) unless specified otherwise.

2.2 Rat hepatocyte isolation and cultivation

Male outbred Sprague-Dawley rats (Charles River Laboratories, France) weighing 250-275 g were kept under controlled environmental conditions with food and water *ad libitum*. Hepatocytes were isolated by use of a 2-step collagenase method and cell viability was assessed by trypan blue exclusion (Papeleu et al., 2006). Viable (85 %) hepatocytes were seeded on a plastic surface at a density of 0.57 x 10^5 cells/cm^2 in William's medium E (Thermo Fisher, USA) supplemented with 7 ng/ml glucagon, 292 mg/ml *L*-glutamine, antibiotics (7.33 IU/ml sodium benzyl penicillin, 50 μg/ml kanamycin monosulphate, 10 μg/ml sodium ampicillin, 50 μg/ml streptomycin sulphate) and 10 % v/v fetal bovine serum (Thermo Fisher, USA). Cell culture plates were placed in an incubator (37 °C, 5 % CO_2) and

after 4 hours, cell culture media were removed and replaced by serum-free medium supplemented with 25 μg/ml hydrocortisone sodium hemisuccinate and 0.5 μg/ml insulin. Hepatocyte isolation and cultivation procedures for the housing of rats as well as for the isolation and cultivation of hepatocytes were approved by the local Ethical Committee of the Vrije Universiteit Brussel, Belgium (project number 12-210-3).

2.3 Measurement of extracellular adenosine triphosphate release

Connexin hemichannel activity was tested by measuring extracellular release of adenosine triphosphate (ATP) using a commercial luciferin/luciferase assay kit as previously described (Vinken et al., 2010). Briefly, 24 hours after cell seeding, cultured rat hepatocytes were exposed during 30 minutes to 20 μM TAT-Gap24, 20 μM TAT-Gap19, 50 μM carbenoxolone (CBX) or solvent control. Subsequently, cultured hepatocytes were washed with Hank's balanced salt solution (HBSS) buffered with 25 mM Hepes (Thermo Scientific, USA), considered as the baseline condition, and divalent-free buffer in order to trigger connexin hemichannel opening. After 2.5 minutes incubation at room temperature, ATP assay mix was added and luminescence was measured spectrophotometrically. Results were expressed as percentage of ATP released in divalent-free medium after 30 minutes exposure to the solvent control.

2.4 Fluorescence recovery after photobleaching

Gap junction activity was tested by fluorescence recovery after photobleaching (FRAP) analysis as described previously (Vinken et al., 2010). Briefly, 24 hours after cell seeding, cultured hepatocytes were exposed during 24 hours to 20 μM TAT-Gap24, 20 μM TAT-Gap19, 50 μM CBX or solvent control. Thereafter, cells were loaded with 10 μM calcein-acetoxymethyl ester in HBSS-Hepes for 40 minutes at room temperature in the absence of light. The tracer solution was replaced by HBSS-Hepes and cultured cells were maintained for 30 minutes in the dark at room temperature. Fluorescence within a single cell was photobleached by 1 second spot exposure to 488 nm Argon laser light, and dye influx from neighboring cells was recorded over the next 6 minutes with a water immersion objective (Nikon Eclipse Ti, Japan). Fluorescence in the bleached cell was expressed as the percentage recovery relative to the prebleach level. At least 4 cells per culture dish were examined.

2.5 Administration of APAP and connexin hemichannel inhibitors to mice

For *in vivo* testing purposes, 8-week to 10-week old male C57BL/6 mice (Jackson Laboratories, USA) were used and housed in the animal facility of the Department of Pathology at the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil. The animals were kept in a room with ventilation (16-18 air changes/hour), relative humidity (45-65 %), controlled temperature (20-24 °C) and light/dark cycle 12:12, and were given water and balanced diet (NUVILAB-CR1, Nuvital Nutrientes LTDA, Brazil) *ad libitum*. Mice were starved 15-16 hours *prior* to APAP administration. APAP was dissolved in saline (0.9 % NaCl), slightly heated and injected (30-37 °C) intraperitoneally at 300 mg/kg body weight, after which animals regained free access to food (Maes et al., 2016c). 1.5 hour after APAP injection, mice were additionally administered either 10 mg/kg TAT-Gap19, 10 mg/kg TAT-Gap24 or a combination of both dissolved in saline, or only saline through retro-orbital injection of volumes not exceeding 150 μl. The conditions for

administration of both compounds were optimized during preliminary testing. No overt signs of organ toxicity were observed upon gross necropsy and histopathological examination (data not shown). Mice were euthanized at the start of the experiment and 3, 6, 24 and 48 hours after APAP injection by exsanguination during sampling under isoflurane-induced anesthesia. Blood, collected by cardiac puncture, was drawn into a heparinized syringe and centrifuged for 10 minutes at 1503xg, and serum was stored at -20 °C. Livers were excised and fragments were fixed in 10 % phosphate-buffered formalin or snap-frozen in liquid nitrogen with storage at -80 °C. This study has been approved by the Committee on Bio-ethics of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (protocol number 9999100314), and all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals".

2.6 Histopathological liver examination

For microscopic evaluation, formalin-fixed liver fragments were embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin and eosin for blinded evaluation of liver damage as described elsewhere (Gujral et al., 2002). The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the cross-sectional areas over the entire section. Pictures were taken with an inverted Axio Vert A1 microscope (Carl Zeiss, Germany).

2.7 Analysis of serum alanine aminotransferase

Alanine aminotransferase (ALT) activity was measured with an automated spectrophotometric Labmax 240 analyzer (Labtest Diagnostica, Brazil) after appropriate dilution of the collected serum samples. Values were expressed in IU/L.

2.8 Analysis of hepatic protein adducts

APAP-protein adducts were measured by high-pressure liquid chromatography with electrochemical detection as described elsewhere (Muldrew et al., 2002) with some modifications (McGill et al., 2013). Briefly, low molecular weight compounds were removed *via* Bio-spin 6 columns (Bio-Rad, USA) and the protein fraction was subsequently digested with proteases to liberate APAP-cysteine conjugates. The protein-derived APAP-cysteine conjugates were quantified and normalized to protein concentration in the original samples.

2.9 Analysis of serum cytokines

Liver tissue was homogenized in Complete Lysis-M buffer with protease inhibitors (Roche, Germany). Homogenates were centrifuged at 14000xg for 15 minutes at 4 °C and protein concentrations in supernatants were determined according to the Bradford procedure (Bradford, 1976) using a commercial kit (Bio-Rad, USA) with bovine serum albumin as a standard. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure levels of mouse interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)α (BD Biosciences, USA) as previously described (Maes et al., 2016a; Maes et al., 2016b).

2.10 Hepatic glutathione and glutathione disulfide analysis

GSH and glutathione disulfide (GSSG) levels in liver tissue were measured using a modified Tietze assay (Jaeschke and Mitchell, 1990). Briefly, frozen liver tissue was homogenized in 3 % sulfosalicylic acid/ethylenediaminetetra-acetic acid and centrifuged at 18000xg for 5 minutes at 4 °C to remove precipitated proteins. After further dilution with potassium phosphate buffer, samples were assayed with a cycling reaction utilizing GSH reductase and dithionitrobenzoic acid. Measurement of GSSG was performed using the same method after trapping GSH with N-ethylmaleimide and removal by solid phase extraction (Jaeschke and Mitchell, 1990). GSSG content was expressed as GSH equivalents.

2.11 Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). The number of repeats (n) for each analysis varied per experiment and/or assay are specified in the figure legends. Results were statistically processed by 1-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni tests using GraphPad Prism6 software with probability (p) values of less than or equal to 0.05 considered as significant.

3 Results

3.1 TAT-Gap24 and TAT-Gap19 block extracellular release of ATP

Exposure of cells to lowered extracellular divalent ion conditions is a well-known procedure to potentiate or trigger the opening of connexin hemichannels (Stout et al., 2002; Ye et al., 2003). Therefore, monitoring extracellular ATP release during the exposure of cells to divalent ion-free medium is indicative for connexin hemichannel activity. As a positive control in these experiments, CBX was used, which is a general inhibitor of connexin-based channels. CBX suppresses gap junctional and connexin hemichannel signaling at concentrations of 3 to 200 μM (Davidson et al., 1986; Goldberg et al., 1996; Wang et al., 2013a). In the present study, 30 minutes exposure of cultured primary rat hepatocytes to 50 μM CBX reduced ($p < 0.0001$) extracellular ATP release to 40.85 ± 17.03 %. Exposure of the cells for 30 minutes to 20 μM TAT-Gap24 or 20 μM TAT-Gap19 significantly decreased ($p < 0.01$) ATP release triggered by divalent ion-free medium to 69.23 ± 9.97 % or 65.79 ± 4.44 %, respectively (Fig. 1.). These results thus demonstrate that TAT-Gap24 and TAT-Gap19 efficiently inhibit connexin hemichannels in hepatocytes in the concentration range that was previously used in other cell types (De Vuyst *et al.*, 2006; Wang *et al.*, 2013b).

3.2 TAT-Gap24 and TAT-Gap19 do not affect gap junction activity

In order to confirm the specificity of TAT-Gap24 and TAT-Gap19, especially with respect to their actions on channels built up by the same proteins as their target channels, their effects on gap junctions were monitored in cultures of primary hepatocytes using FRAP analysis. Similar to the ATP release assay, CBX was used as a positive control in the FRAP experiments. 24 hours exposure of the cells to 50 μM CBX indeed decreased ($p < 0.05$) the fluorescence recovery signal, representative for the gap junction activity, to 35.86 ± 17.03 % compared to solvent control. Exposure of the cultured hepatocytes to 20 μM TAT-Gap24 or 20 μM TAT-Gap19 for 1 day resulted in fluorescence recovery of 96.77 ± 10.61 % and 87.14

± 29.07 %, respectively (Fig. 2.). These results further substantiate the target specificity of TAT-Gap24 and TAT-Gap19 when applied to hepatocytes. Their specificity towards hemichannels can be partly attributed to the intracellular location of the target of TAT-Gap19 and TAT-Gap24. Indeed, other connexin peptides mimicking sequences in the extracellular loop region inhibit hemichannel activity within minutes, but also prevent the docking of 2 facing hemichannels and thus affect gap junctional communication when applied for periods of several hours (Decrock et al., 2009a; Desplantez et al., 2012; Evans and Leybaert, 2007).

3.3 Connexin hemichannel inhibition diminishes liver damage in APAP-induced hepatotoxicity

A single dose of 300 mg/kg APAP is well known to cause severe hepatocellular injury, which can be detected by massive necrosis and extensive increased serum ALT activity (Jaeschke et al., 2012b; Maes et al., 2016c). In this study, liver damage became manifested about 3 hours after APAP injection, with culminating serum ALT activity up to 24 hours (Fig. 3A.). To evaluate the effect of connexin hemichannel inhibition *in vivo* in this model of acute hepatotoxicity, 10 mg/kg of TAT-Gap24 and/or 10 mg/kg TAT-Gap19 were administered 1.5 hour after the APAP overdosing. These doses were extrapolated from the *in vitro* testing outcome and correspond to a concentration of 20 μM, thereby assuming distribution in the blood volume that is approximately 8 % of the body weight (Wang et al., 2013b). Upon treatment of APAP-overdosed mice with TAT-Gap24, TAT-Gap19 or a combination of both compounds, modest yet non-significant differences in the necrotic areas were observed after APAP administration (Fig. 3B-F.). Blockage of Cx32 hemichannels attenuated serum ALT activity after 24 hours ($p < 0.05$), while TAT-Gap19 only slightly decreased ALT values after 24 and 48 hours. Simultaneous treatment of APAP-overdosed mice with TAT-Gap24 and TAT-Gap19 revealed an additive effect by drastically lowering ALT serum activity ($p < 0.001$) after 24 hours (Fig. 3A.).

3.4 Connexin hemichannel inhibition does not affect hepatic protein adduct formation in APAP-induced hepatotoxicity

The mechanism of toxicity of APAP, and thus the mouse model as such, relies on the formation of the deleterious reactive metabolite NAPQI. In case of APAP overdosing, NAPQI reacts with protein sulfhydryl groups, which leads to the formation of noxious liver protein adducts (Mitchell et al., 1973). To confirm that the observed diminished liver damage after treatment with TAT-Gap24 and/or TAT-Gap19 occurs independently of NAPQI formation, APAP-protein adducts were measured 3 and 6 hours after APAP administration. APAP-protein adducts were detectable in all mice with no significant differences between the different experimental groups (Fig. 4.). These data show that TAT-Gap24 and TAT-Gap19 do not influence hepatic APAP adduct formation.

3.5 Connexin hemichannel inhibition differentially affects serum cytokine levels in APAP-induced hepatotoxicity

Connexin hemichannels have been identified as key mediators of inflammatory responses (Willebrords et al., 2016). Accordingly, a number of cytokines, namely IL-1β, IL-6, IL-10, and TNFα, which are considered of relevance for controlling hepatic injury-associated inflammation (Blazka et al., 1995; Cover et al., 2006), were evaluated. Serum cytokine

levels of mice sacrificed after 6 and 24 hours were measured by ELISA analysis. Increased serum quantities of the pro-inflammatory cytokines IL-1β and TNFα were noticed in APAP-treated mice after 24 hours. Treatment with TAT-Gap24 and TAT-Gap19 separately decreased ($p < 0.05$) serum amounts of these pro-inflammatory cytokines 24 hours after APAP overdosing ($p < 0.05$) (Fig. 5A-B.). In addition, TAT-Gap19 reduced IL-6 serum levels (Fig. 5C.). No statistically significant differences in serum cytokines were measured when TAT-Gap24 and TAT-Gap19 were administered together to APAP-overdosed mice (Fig. 5A-D.). Effects on serum IL-10 serum levels remained absent in all experimental conditions (Fig. 5D.). These results show that closing of Cx32 or Cx43 hemichannels counteracts inflammation following APAP intoxication.

3.6 Connexin hemichannel inhibition does not alter the liver oxidative status in APAP-induced hepatotoxicity

In healthy cells, more than 99 % of the total glutathione pool occurs in reduced form (GSH) and less than 1 % exists in the oxidized form (GSSG) (Jaeschke and Mitchell, 1990). However, in APAP toxicity, the GSH pool becomes depleted due to excessive NAPQI production (Jaeschke et al., 2012a). In this study, a gradual recovery of the total GSH pool became evident after 6 and 24 hours (Fig. 6A.). Although no significant differences were found in the recovery after 24 hours, the combined treatment with both mimetic peptides postponed the recovery, as significant lower ($p < 0.05$) total GSH levels were found 6 hours after APAP administration (Fig. 6A.). GSH acts as a critical anti-oxidant and is converted to its oxidized GSSG form upon reaction with reactive oxygen species. For all experimental groups, GSSG levels were elevated after 6 hours and peaked at the 24 hour time point (Fig. 6B.). Administration of TAT-Gap19 reduced ($p < 0.01$) tissue GSSG levels 6 hours after APAP overdosing. However, a tendency for higher GSSG levels was observed after 24 hours. Hence, upon normalization of GSSG values as a percentage of the total GSH amount, no differences were found 24 hours after APAP toxicity (Fig. 6C.). This indicates that connexin hemichannel closure does not affect the oxidative stress status after APAP-induced hepatotoxicity.

4 Discussion

Given their ubiquitous and well-acknowledged involvement in tissue functioning, gap junctions have been extensively explored as drug targets over the past decades. As a result, a wide variety of gap junction modulators have been successfully tested as pharmacological tools for a plethora of diseases (Bodendiek and Raman, 2010). Historically, connexin hemichannels have been considered as merely structural precursors of gap junctions. Nevertheless, a large body of evidence now points to independent roles for connexin hemichannels in cellular signaling by connecting the intracellular compartment with the extracellular environment, particularly in pathological conditions (Contreras et al., 2002; Sáez and Leybaert, 2014; Wang et al., 2013b). Research in this direction is, however, still surrounded by controversy (Spray et al., 2006), which is partly due to the lack of tools to distinguish between gap junction and hemichannel signaling. In recent years, peptides mimicking sequences in the cytoplasmic loop region of connexins, including Gap24 and Gap19, were found to act as specific connexin hemichannel inhibitors (De Vuyst et al., 2006;

Wang et al., 2013b). Gap19 has been proven to bind to a sequence in the *C*-terminal tail of the connexin structure, thereby interfering with the intramolecular interaction between the cytoplasmic loop and *C*-terminal end, which is critical for connexin hemichannel opening (Bouvier et al., 2009; Hirst-Jensen et al., 2007; Iyyathurai et al., 2013; Ponsaerts et al., 2010; Wang et al., 2013b). As the targets of both peptides are located within the cell, they were linked to a HIV-1 TAT sequence in order to improve cytosolic uptake (Lindgren et al., 2000). The efficacy and specificity of Gap24 or Gap19 to suppress the opening of Cx32 and Cx43 hemichannels, respectively, has been demonstrated previously in cell lines expressing Cx32 (De Vuyst et al., 2006), astrocytes (Abudara et al., 2014) and cardiomyocytes (Wang et al., 2013b). The results of the present study show that this also holds true for hepatocytes, as evidenced by reduced extracellular release of ATP, indicative of connexin hemichannel opening, and unmodified gap junction activity. Subsequently, both peptides were applied to test the involvement of Cx32 and Cx43 hemichannels in APAP-induced hepatotoxicity *in vivo*, which is known to be accompanied by pronounced cell death and inflammatory responses. It should be stressed that the *in vitro* results obtained in rat hepatocytes are comparable to the *in vivo* model in mice, as there are only marginal differences between both species in Cx32 and Cx43 amino acid sequences (Beyer et al., 1987; Miller et al., 1988). In addition, rat hepatocytes were selected to set up cell cultures because connexin expression and their channel activities were previously fully characterized in this *in vitro* setting (Vinken et al., 2006, 2010, 2012). In contrast, mice were preferred over rats for *in vivo* testing purposes, since the latter are known to be poor *in vivo* models for APAP-induced toxicity (Maes et al., 2016c; McGill et al., 2012). Furthermore, alterations in connexin production have been studied in detail in this mouse model of APAP-induced liver injury (Maes et al., 2016a). In the present study, intoxicated mice were treated with TAT-Gap24 and/or TAT-Gap19 1.5 hour after APAP overdosing. This 1.5 hour timeframe is necessary for completion of APAP biotransformation and hence for formation of the deleterious NAPQI metabolite (McGill et al., 2013), and our results show that this process is not affected by TAT-Gap24 or TAT-Gap19. In case of TAT-Gap19, only minor changes in liver injury were recorded. In contrast, a significant reduction in serum ALT values was found upon treatment of APAP-overdosed animals with TAT-Gap24. Importantly, simultaneous treatment of mice with both mimetic peptides revealed an additive effect, as significantly lower ALT activity was found after co-treatment with TAT-Gap24 and TAT-Gap19 (Fig. 3A.). However, this was not reflected in the histopathological quantification of the necrotic areas. This could be explained by the fact that these areas are estimated per grades of 5 % and thus might overlook subtle changes between different experimental groups. Overall, these results suggest the involvement of connexin hemichannel signaling in APAP-induced acute liver failure. Connexin hemichannels hereby could facilitate the uptake of toxic substances and may contribute to the loss of essential metabolites. In this regard, ATP released through connexin hemichannels is known to decrease intracellular ATP content, as such favoring cell death through necrosis and inflammation (Decrock et al., 2009a; Kalvelyte et al., 2003; Leist et al., 1997). This corresponds with observations of the present study, since blockage of Cx32 or Cx43 hemichannels reduced serum levels of IL-1β and TNFα. Of note, co-administration of both peptides did not alter serum levels of these cytokines. The relevance of this finding remains unclear. Alternatively, cytosolic GSH may be liberated through Cx32 and Cx43 hemichannels, thus jeopardizing anti-oxidant cellular

defense (Keleva et al. 2013; Rana and Dringen, 2007; Tong et al., 2015). However, the results of the current study do not seem in line with this scenario, as no differences were found in GSH amounts between the different experimental groups after 3 and 24 hours (Fig. 6A.). Interestingly, Cx32 and Cx43 have been identified in mitochondria (Boengler et al. 2013; Fowler et al. 2013) and mitochondrial Cx43-based hemichannels have been shown to contribute to cardiomyocyte cell injury and cell death (Gadicherla et al. 2017). Moreover, TAT-Gap19 administration inhibited necrotic cell death induced by hypoxia/reoxygenation (Gadicherla et al. 2017). This suggest that the beneficial effect of connexin hemichannel inhibition might not only rely on paracrine/autocrine signaling, but also on direct effects on mitochondria during the initiation of necrosis. Future studies should elucidate whether this situation also may take place in liver.

In summary, this study substantiates the role of connexin hemichannels as "pathological pores". This may open perspectives for the establishment of novel therapeutic strategies, *in casu* of acute APAP-induced liver toxicity.

Acknowledgements

List of abbreviations

2-APB	2-aminoethoxy-diphenyl-borate
ALT	alanine aminotransferase
ANOVA	analysis of variance
APAP	acetaminophen
ATP	adenosine triphosphate
CBX	carbenoxolone
Cx	connexin
ELISA	enzyme-linked immunosorbent assay
FRAP	fluorescence recovery after photobleaching
GSH	glutathione
GSSG	glutathione disulfide

HBSS	Hank's balanced salt solution
IL-1β/6/10	interleukin 1β/6/10
n	number of repeats
NAPQI	*N*-acetyl-*p*-benzoquinone imine
p	probability
SEM	standard error of the mean
TAT	transactivator of transcription
TNFα	tumor necrosis factor α

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Highlights

- Inhibition of connexin hemichannels by connexin mimetic peptides.

- No effects of connexin mimetic peptides on gap junctions.

- Cx32 hemichannel inhibition reduces ALT activity and inflammation in APAP-induced liver injury.

- Cx43 hemichannel inhibition reduces inflammation in APAP-induced liver injury.

- Additive effects of Cx32 and Cx43 hemichannel inhibition on APAP-induced liver injury.
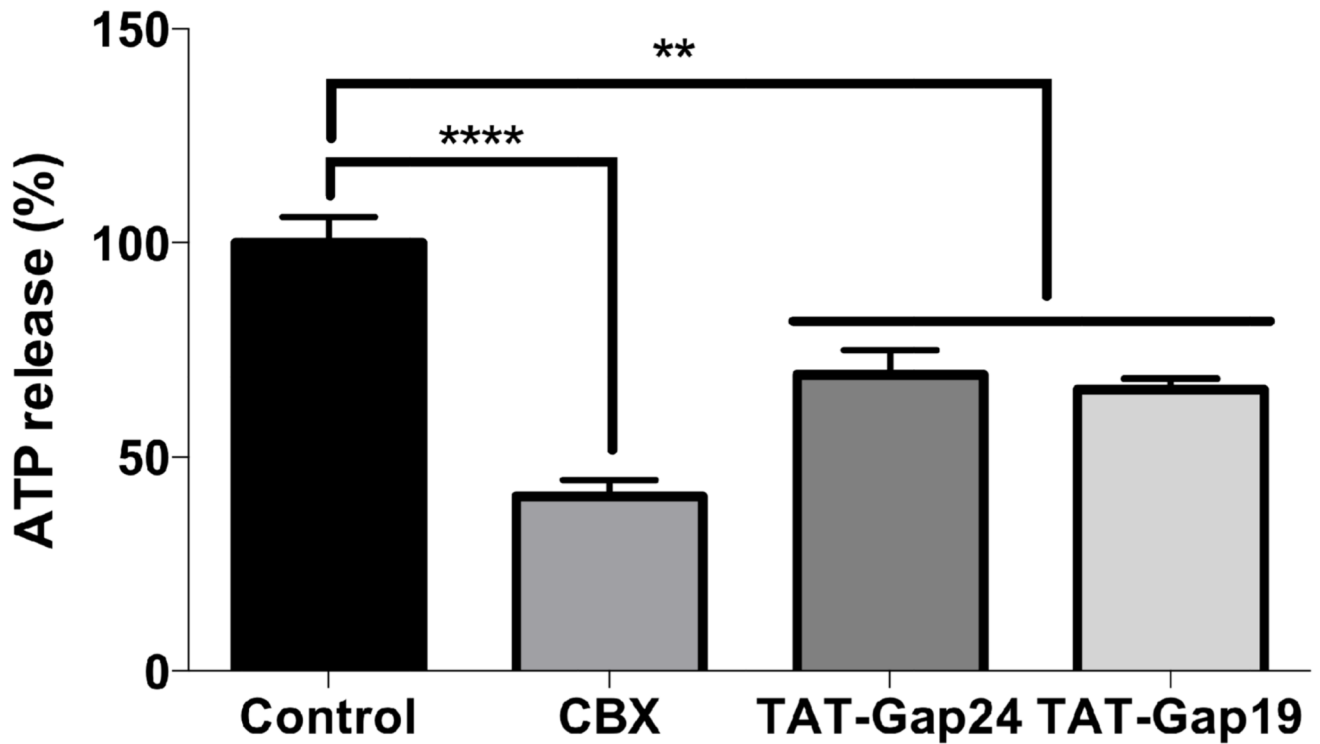
Figure 1. TAT-Gap24 and TAT-Gap19 block extracellular release of ATP.
Primary rat hepatocytes were isolated and cultivated as specified in "Materials and Methods". 24 hours after cell seeding, cultured hepatocytes were exposed during 30 minutes to 20 μM TAT-Gap24, 20 μM TAT-Gap19, 50 μM carbenoxolone (CBX) or solvent control. Thereafter, the cells were exposed to HBSS-Hepes, considered as the baseline condition, and divalent-free buffer. After 2.5 minutes incubation at room temperature, ATP assay mix was added and luminescence was measured spectrophotometrically. ATP release in the divalent-free buffer were plotted and expressed as percentage of ATP release triggered by divalent-free medium. At least 6 wells per experiment were examined, with a total of 5 experiments (*i.e.* hepatocytes isolated from 5 different rats). Data are expressed as means ± SEM, with **$p < 0.01$ and ****$p < 0.0001$ compared to ATP release triggered by divalent-free medium.
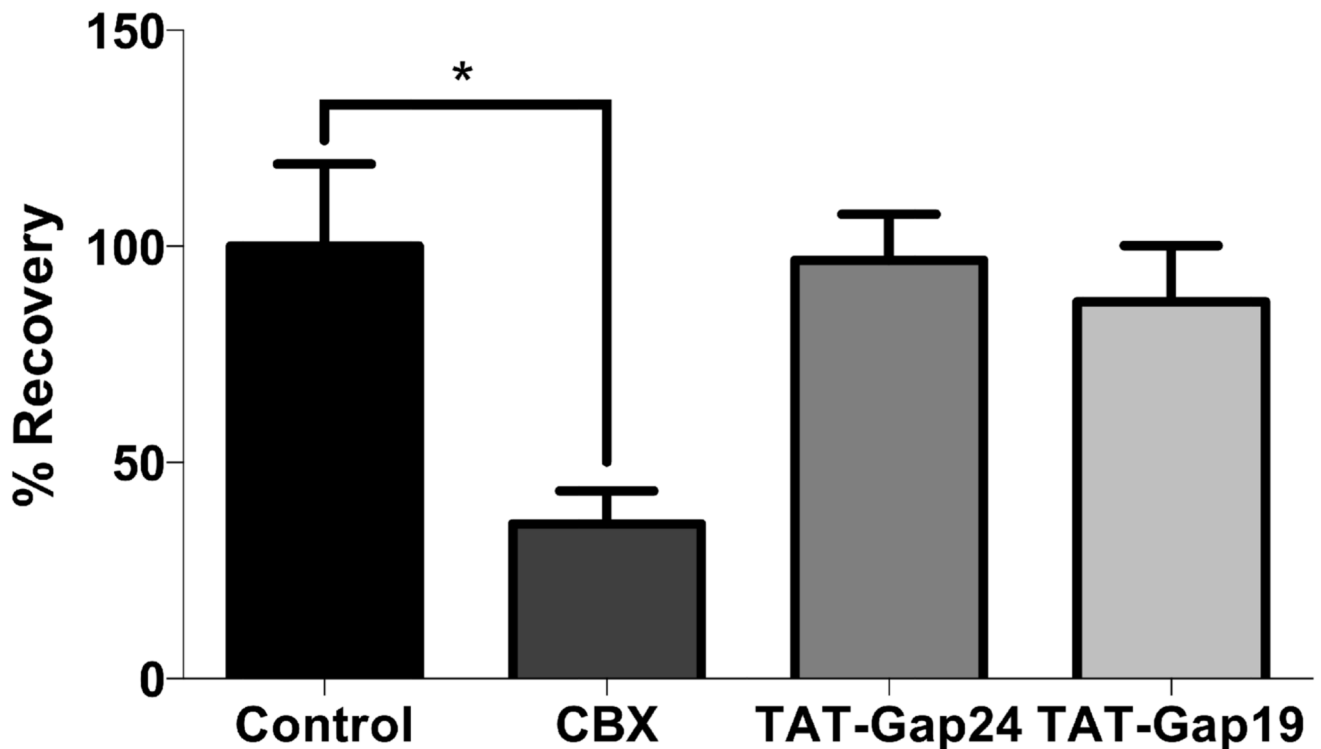
Figure 2. TAT-Gap24 and TAT-Gap19 do not affect gap junction activity.
Primary rat hepatocytes were isolated and cultivated as specified in "Materials and Methods". 24 hours after cell seeding, cultured hepatocytes were exposed during 24 hours to 20 μM TAT-Gap24, 20 μM TAT-Gap19, 50 μM carbenoxolone (CBX) or solvent control. Subsequently, cells were subjected to FRAP analysis. After loading with 10 μM calcein-acetoxymethyl ester, fluorescence within a single cell was photobleached by 1 second spot exposure to 488 nm Argon laser. The dye influx from neighboring cells was recorded over the next 6 minutes. Fluorescence in the bleached cell was expressed as the percentage recovery relative to the prebleach level. At least 5 wells per cell culture dish were examined in each experiment, with a total of 5 experiments (*i.e.* hepatocytes isolated from 5 different rats). Data are expressed as means ± SEM, with *$p < 0.05$ compared to recovery in the solvent control.
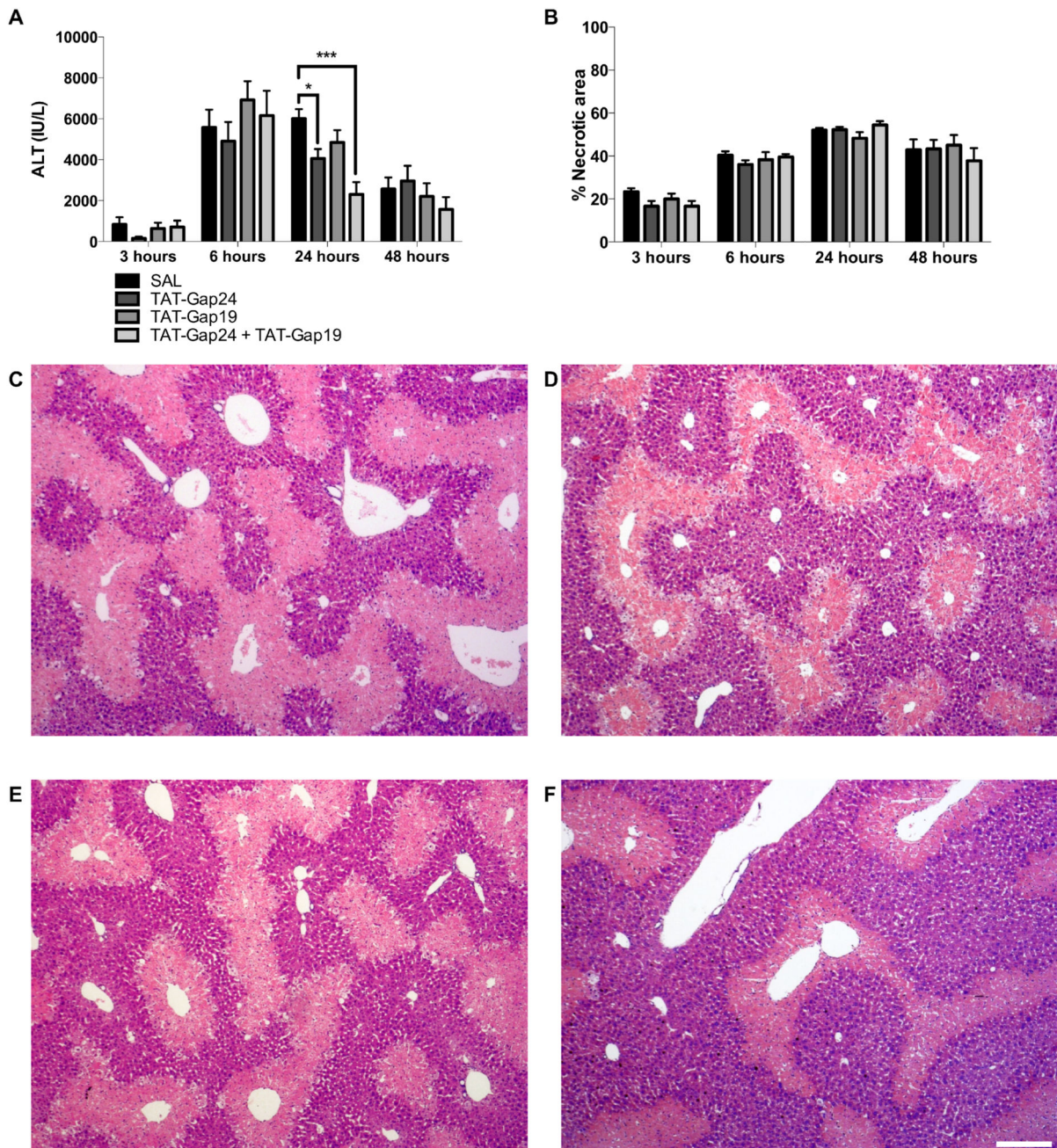
Figure 3. Connexin hemichannel inhibition diminishes liver cell damage in APAP-induced hepatotoxicity.

Mice (n = 5-20 per group and per time point) were injected 300 mg/kg APAP followed by administration of 10 mg/kg TAT-Gap24, 10 mg/kg TAT-Gap19, a combination of both or saline (SAL) 1.5 hour later. Sampling was performed 3, 6, 24 and 48 hours after APAP overdosing. (A) Serum levels of ALT were measured with an automated spectrophotometer and expressed as IU/L. (B) Liver sections were examined microscopically with quantification of the necrotic areas around the central veins. Data are expressed as means ± SEM, with $*p < 0.05$ and $***p < 0.001$ compared to APAP followed by SAL administration.

Microscopic pictures of the liver tissue 24 hours after APAP administration followed by (C) SAL, (D) 10 mg/kg TAT-Gap24, (E) 10 mg/kg TAT-Gap19 or (F) a combination of 10 mg/kg TAT-Gap24 and TAT-Gap19. The white scale bar represents 200 μm.
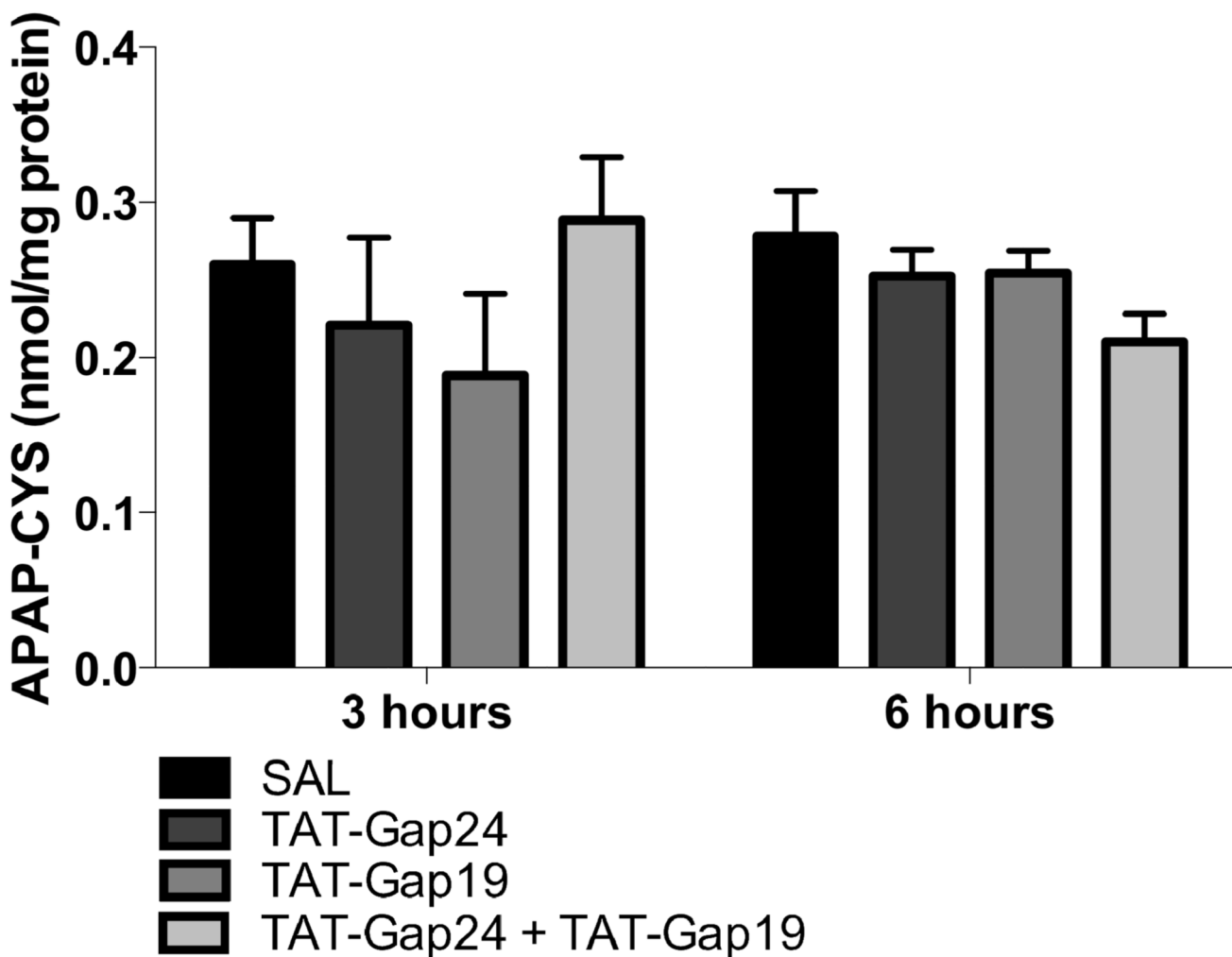
Figure 4. Connexin hemichannel inhibition does not affect hepatic protein adduct formation in APAP-induced hepatotoxicity.

Mice (n = 6-10 per group and per time point) were injected 300 mg/kg APAP followed by administration of 10 mg/kg TAT-Gap24, 10 mg/kg TAT-Gap19, a combination of both or saline (SAL) 1.5 hour later. Sampling was performed 3 and 6 hours after APAP overdosing. The generation of reactive metabolite results in the formation of APAP-cysteine (APAP-CYS) protein adducts, which were quantified by high-pressure liquid chromatography with electrochemical detection using total liver homogenate. Data are expressed as means ± SEM. No significant differences were found between the different experimental groups.
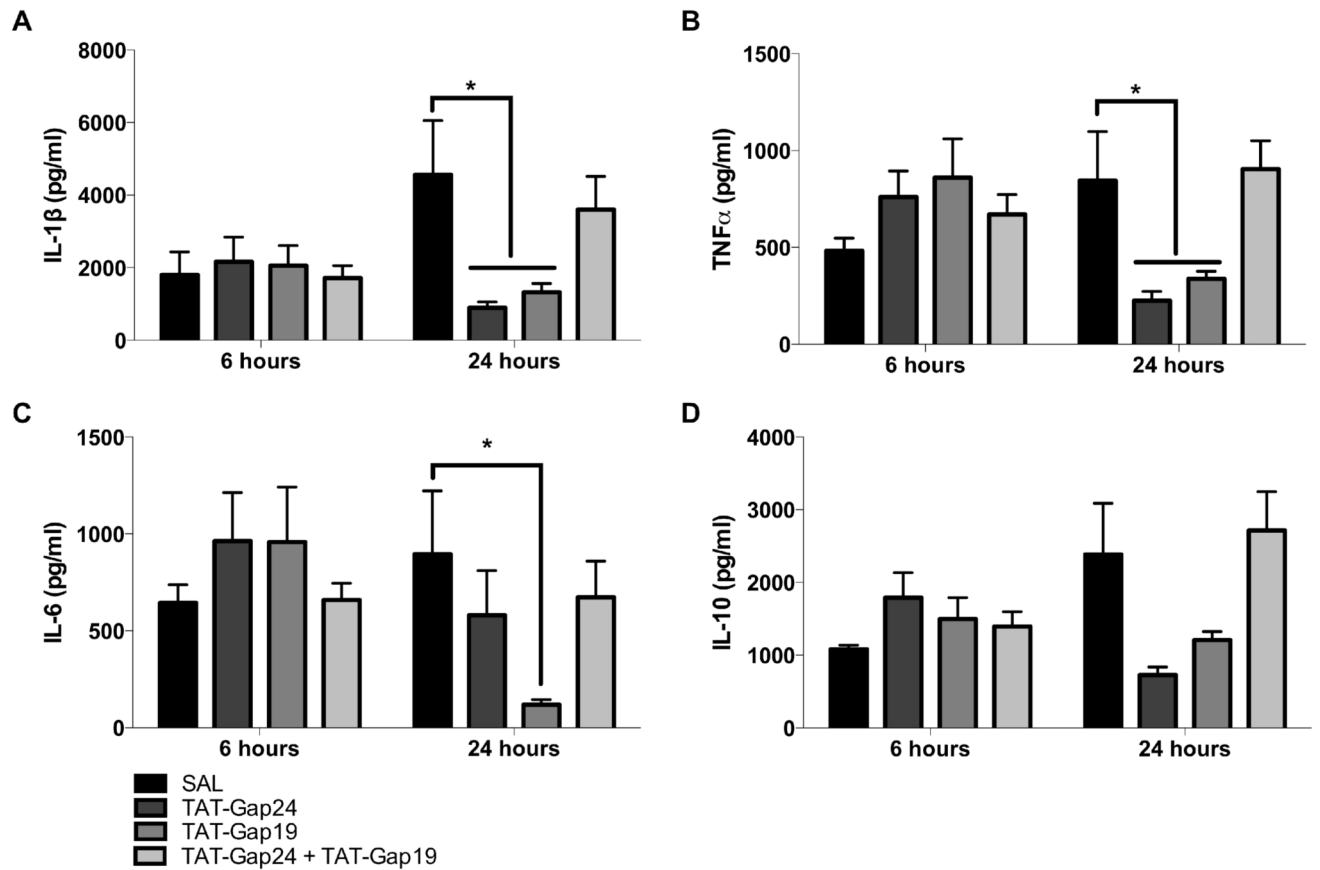
Figure 5. Connexin hemichannel inhibition differentially affects serum cytokine levels in APAP-induced hepatotoxicity.

Mice (n = 6-8 per group and per time point) were injected 300 mg/kg APAP followed by administration of 10 mg/kg TAT-Gap24, 10 mg/kg TAT-Gap19, a combination of both or saline (SAL) 1.5 hour later. Sampling was performed 6 and 24 hours after APAP overdosing. ELISA analysis of serum samples was performed to detect (A) IL-1β, (B) TNFα, (C) IL-6 and (D) IL-10. Data are expressed as means ± SEM, with $*p < 0.05$ compared to APAP followed by SAL administration.
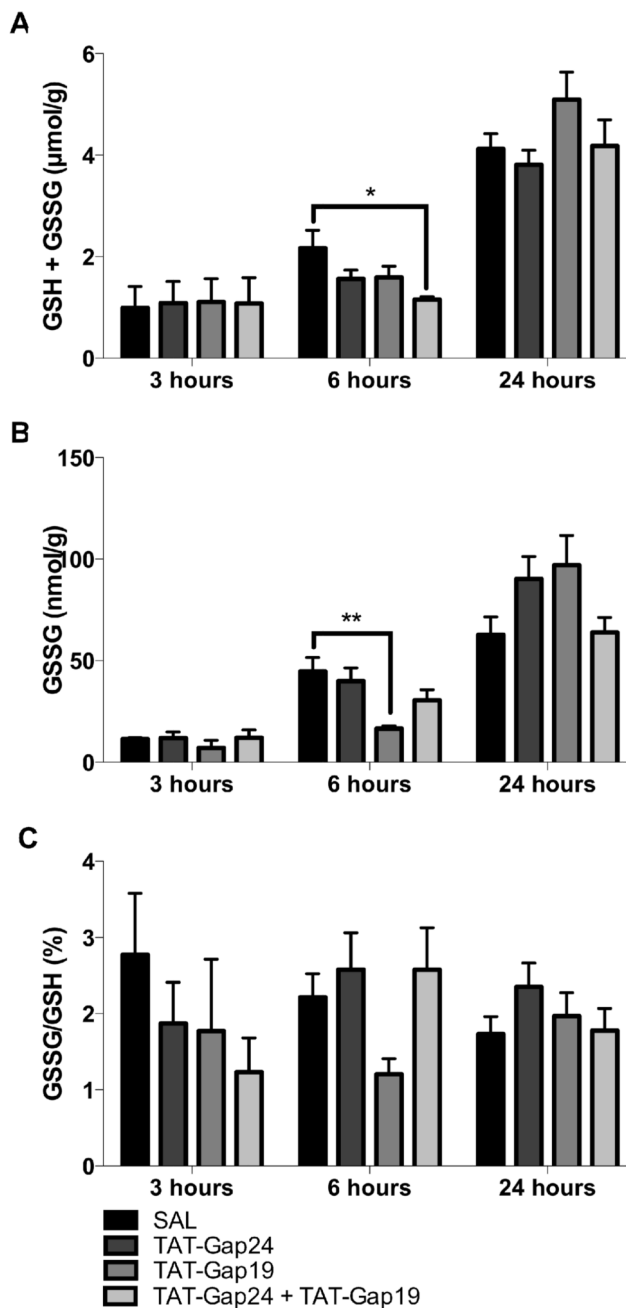
Figure 6. Connexin hemichannel inhibition does not alter the liver oxidative status in APAP-induced hepatotoxicity.

Mice (n = 5-13 per group and per time point) were injected 300 mg/kg APAP followed by administration of 10 mg/kg TAT-Gap24, 10 mg/kg TAT-Gap19, a combination of both or saline (SAL) 1.5 hour later. Sampling was performed 3, 6 and 24 hours after APAP overdosing. Hepatic levels of (A) GSH and (B) GSSG were measured and (D) the GSSG/GSH ratio was calculated. Data are expressed as means ± SEM, with *$p < 0.05$ and **$p < 0.01$ compared to APAP followed by SAL administration.