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ACETAMINOPHEN; FROM LIVER TO BRAIN: NEW INSIGHTS INTO DRUG PHARMACOLOGICAL ACTION AND TOXICITY

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Abstract

Acetaminophen (APAP) is a well-known analgesic and antipyretic drug. It is considered to be safe when administered within its therapeutic range, but in cases of acute intoxication, hepatotoxicity can occur. APAP overdose is the leading cause of acute liver failure in the northern hemisphere. Historically, studies on APAP toxicity have been focused on liver, with alterations in brain function attributed to secondary effects of acute liver failure. However, in the last decade the pharmacological mechanism of APAP as a cannabinoid system modulator has been documented and some articles have reported “in situ” toxicity by APAP in brain tissue at high doses. Paradoxically, low doses of APAP have been reported to produce the opposite, neuroprotective effects. In this paper we present a comprehensive, up-to-date overview of hepatic toxicity as well as a thorough review of both toxic and beneficial effects of APAP in brain.

Graphical Abstract

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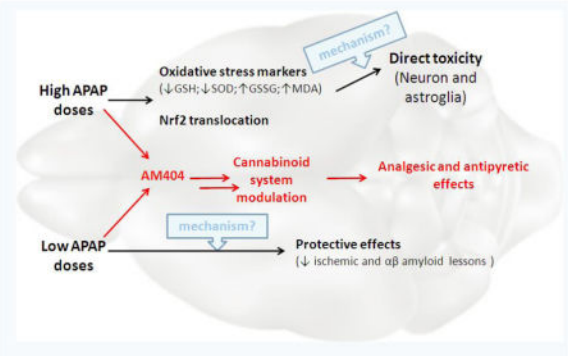

Keywords

Acetaminophen; hepatic toxicity; trpv1; necroptosis; caspase; DAMPs

1. Introduction

Acetaminophen (N-acetyl-para-aminophenol, paracetamol, APAP) was originally synthesized in 1878 by Morse (1) and first used clinically by von Mering in 1887 (2). During that period, phenacetin was the most widely used analgesic in clinical practice. In the 1950s, the analgesic and antipyretic properties of APAP were re-discovered by Brodie and Axelrod (3) and they demonstrated that APAP was in fact the active metabolite of phenacetin. At that time, APAP was introduced in the U. S. market as a replacement drug for phenacetin, whose use was discontinued due to its nephrotoxic potential. Owing to its widespread acceptance as a safer alternative to phenacetin, APAP became one of the most popular and widely used over-the-counter analgesic-antipyretic drugs in the world, and the most commonly prescribed medication in children (4; 5). Also, since the 1980's APAP has become the first drug of choice for the treatment of pain and fever in children because of the high incidence of Reye's syndrome associated with pediatric use of aspirin (6). In the U.S., approximately 79% of the general population consume APAP regularly (7). Many prescription and nonprescription formulations contain APAP alone or in combination with other drugs. Excessive self-medication is a prevalent practice responsible for many cases of APAP intoxicantion.

2. An Update on the Mechanism(s) of Pharmacological Action

Historically, APAP was initially categorized as a nonsteroidal anti-inflammatory drugs (NSADs). Multiple investigations compared its mechanism of action to that of classical NSAIDs, such as acetyl salicylic acid which inhibits the cyclooxygenase (COXs) pathway (8). However, APAP was proven to be ineffective as an anti-inflammatory drug. It is well established that NSAIDs inhibit COX-dependent production of prostaglandins (9), while APAP largely lacks peripheral anti-inflammatory properties, suggesting that its site of pharmacological action is within the central nervous system. Indeed, APAP crosses the blood brain barrier with ease and is distributed homogeneously throughout the central nervous system (CNS) (10; 11) after either low (12), therapeutic (10) or toxic doses (13; 14).

Clinical studies have demonstrated the presence of APAP in cerebrospinal fluid (11; 15; 16). In the brain, APAP has been shown to produce selective inhibition the COX pathway, while this inhibition by APAP is absent in peripheral tissues such as the stomach (17; 18). This tissue selectivity for COX inhibition explains the contrasting adverse effects produced by classical NSAIDs, including gastric ulceration and alterations in hemostasis which are not seen with either short- or long-term therapeutic use of APAP. The mechanism of COX inhibition centrally is not through direct binding of APAP to the active site of the enzyme. On the contrary, APAP seems to reduce the active form of COX, rendering it catalytically deficient. This mechanism of inhibition also explains why COX inhibition by APAP only occurs in areas where peroxides levels are very low, as what is seen in the brain (19). This peroxide-mediated inhibition of COX activity can also explain why APAP is not active in peripheral sites of inflammation, where the concentration of peroxide is greater than that encountered in brain. Similarly, low doses of APAP has been suggested to reduce the oxidative stress (20; 21) that has been implicated in prostaglandin release and fever (22; 23).

In addition to its antipyretic properties, APAP also produce analgesia. Several studies indicate that these two pharmacological outcomes occur through distinct mechanisms. Recently, a newly proposed mechanism of action for the analgesic properties of APAP has been described, which involves modulation of the endogenous cannabinoid system. This mechanism may provide an explanation for the “relaxation” or “calming” effect that has been reported in some patients that consume APAP (24; 25). This effect is characterized by a peculiar sense of well-being, relaxation and tranquility. These manifestations are also shared with other members of the family of “aniline analgesics”. The current thought is that APAP, after becoming de-acetylated to p-aminophenol (Figure 1, Panel A) undergoes conjugation with arachidonic acid by fatty acid amide hydrolase (FAAH) enzymes to form N-arachidinoyl-phenolamine (AM404) in the brain, spinal cord, and dorsal root ganglia (26). The structures of AM404, anandamide and *N*-arachidonoylethanolamine (AEA), the latter being the endogenous cannabinoid neurotransmitter, are shown in **Panel B** of Figure 1. Because of structural similarities, AM404 is a weak agonist of cannabinoid receptors type 1 and 2 (CB1 and CB2), and an inhibitor of the anandamide membrane transporter (AMT), which leads to increased levels of endogenous cannabinoids (27–29). AM404 is also a potent activator of the vanilloid subtype 1 receptors (TRPV1) (30). In conclusion, APAP directly activates TRPV1 receptors, and the cannabinoid CB1 receptors indirectly by increasing endogenous anandamide levels, both mechanisms present in thermoregulatory and pain centers in the brain (29; 31–33). In summary, in addition to a centrally-mediated COX inhibition mode of action, vanilloid and cannabinoid signaling pathways are also implicated in pharmacological actions of APAP (34).

3. Incidence of hepatic toxicity

Although APAP was initially approved for clinical use in the 1950s, concerns about its safety did not become prominent until the 1970s. In the 1980s, APAP sales exceeded those of aspirin in many countries. Hepatotoxicity and liver failure by APAP intoxication were not recognized until the first cases of fatal APAP poisoning were reported in the mid-1980s.

According to the American Association for the Study of Liver Diseases, the incidence of APAP-related liver toxicity has been increasing significantly over the past decades. APAP intoxication is now the most common cause of acute liver failure (ALF) in the United States, Great Britain and several countries in Europe (35). More than 100,000 calls to Poison Control Centers, 56,000 emergency room visits, 2,600 hospitalizations and nearly 500 deaths are attributed to APAP use in United States every year (36).

Cases of APAP liver toxicity can be classified as intentional and unintentional. Intentional APAP overdosing involving single ingestion is a common form of suicide attempt due to its low cost and accessibility. Unintentional overdoses, which are common in adults and children, account for more than 50% of all cases and is primarily due to therapeutic misuse and excessive dosing over a period of time; usually more than 3 days. Moreover, these “therapeutic misadventures” are also attributed to the use of multiple non-prescription combined formulations containing APAP.

The problem with these over-the-counter products is that most consumers are not knowledgeable or do not bother to read which of these formulations contain APAP. Others patient may not be informed or do not understand the concept of maximum recommended daily dose, or are not aware of the potential for hepatotoxicity with excessive dosing, despite the strong warnings and dosing recommendations in the label or package inserts.

Within prescription drugs, the use of Vicodin (hydrocodone 5mg; APAP 300 mg) is a prominent form of excessive APAP consumption, particularly in individuals that develop tolerance and dependence on the hydrocodone component of this medication. The recommended dose is one to two tablets every 4 to 6 hours. With such prescription regimen, a patient could easily exceed the recommended maximal daily dose of 3g of APAP.

Under certain conditions, APAP hepatotoxicity can occur even at therapeutic doses. There are a number of well documented risk factors that lower the dose threshold for APAP toxicity. These include, chronic alcohol use, co-administration of another drugs that induce the activity of cytochrome P450 (CYP 450) enzymes, and malnutrition which reduces the stores of protective thiols in liver.

4. Hepatic Metabolism and Mechanism(s) of Toxicity

Following therapeutic administration of APAP, approximately 25% of dose is undergoes “first pass” metabolism, with the liver playing a primary role (37). In the adult, APAP is mainly conjugated with glucuronic acid (40–67%) and sulphate (20–46%) (38; 39). A minor fraction (5–15%) undergoes oxidative metabolism by CYP450, particularly the CYP2E1, CYP1A2, CYP3A4, and CYP2A6 isoforms. Such enzymatic reaction generates a reactive intermediate known as N-acetyl-p-benzoquinoneimine (NAPQI) (40) (Figure 2). NAPQI is normally neutralized by combination with reduced glutathione (GSH), which subsequently is converted to cysteine or mercapturic conjugates. These two metabolites are non-toxic and readily eliminated in urine and bile (41).

With excessive APAP dosing, the Phase II conjugative pathway become saturated and more of the APAP metabolism is shunted into the CYP450 pathway, resulting in greater NAPQI

formation (42–44). One of the earliest events associated with APAP intoxication and excessive NAPQI generation is depletion of GSH stores, both the cytosolic and mitochondrial pools (45). Hepatocyte injury is triggered when GSH stores are depleted to less than 30% of normal values. After GSH is exhausted, residual, un-neutralized NAPQI is then free react with alternative targets, such as nucleophilic macromolecules, proteins, DNA and unsaturated lipids. This sets in motion a series of cellular events that ultimately results in hepatocellular death (46). Cell death by APAP has been attributed, in part, to the formation of NAPQI-adducts with specific critical targets in the cell. Selective covalent binding of NAPQI to key targets proteins has been determined to be more relevant to liver injury than total protein binding (47). Furthermore, mitochondrial GSH depletion seems to be more important that cytosolic depletion, since 3′-hydroxyacetanalide (AMAP), an APAP isomer, produces a similar degree of cytosolic GSH depletion to that produced by APAP, but without producing hepatotoxicity (48). This indicates that overdosing with APAP triggers mitochondrial dysfunction as a consequence of NAPQI binding to mitochondrial proteins, which is also associated with ROS generation and inhibition of mitochondrial respiration (49–51), and a decrease in ATP levels (52; 53). After the initial formation of NAPQI adducts, several other sequential as well as in tandem events lead to the amplification and propagation of cellular death resulting in tissue damage, as is shown in figure 3 (54).

In addition to decrease in mitochondrial function, induction of oxidative stress by APAP is observed. Primary hepatocytes in culture exposed to a toxic concentration of APAP accumulate reactive oxygen species (ROS), which oxidize 2′,7′-dichlorodihydrofluorescein, a marker of cellular oxidative stress (55). Also, the concentrations of glutathione disulfide (GSSG), a marker of intracellular ROS formation, increase substantially with toxic APAP administration to mice (52; 53). All these events precede cell death by several hours.

Although the production of ROS has been reported to play an important role in the pathophysiology of APAP intoxication, experimental results have shown that potentially, the most important reactive species are nitric oxide (NO) derivatives. Experimental data showed that superoxide anion can react with NO to form peroxynitrite (56) which is generated predominantly in mitochondria. This event was demonstrated by sub-cellular fractionation of liver tissue after APAP intoxication in mice (57), which is consistent with the increased formation of superoxide in this organelle (52; 53). The importance of peroxynitrite formation was also demonstrated by immunohistochemical staining for nitrotyrosine protein adducts in cells undergoing necrosis after APAP overdose (58; 59). The source(s) of NO after APAP treatment remain unclear. iNOS is an important, but not the only possible source of NO in APAP liver toxicity. In addition to GSH depletion and induction of oxidative stress by APAP, mitochondrial membrane permeability transition (MPT) also occurs (55; 60). The MPT leads to mitochondrial dysfunction involving organelle swelling and uncoupling of oxidative phosphorylation. The opening of pores in the inner mitochondrial membrane allows the passage of solutes with a molecular weight size of 1.5 kDa, such as cytochrome c (cyt c) (61). Upon its release, Cyt c activates caspase-9 either directly or through APAF-1 in an ATP-dependent manner, resulting in the binding of APAF-1 to caspase-9, a complex known as the apoptosome (62–64). Activated caspase-9 then activates caspases-3 and -7, thus leading to apoptosis. Also during APAP toxicity, the magnitude of the decreased in cellular ATP content can lead to inhibition of the apoptosomes activation, and necrosis

instead of apoptosis ensues. APAP overdose not only depletes mitochondrial GSH levels, induces formation of peroxynitrite in mitochondria (57), moreover it causes intracellular Ca^{2+} accumulation. The mechanisms involved in Ca^{2+} accumulation includes its release from mitochondria and through inhibition of the Ca^{2+}-Mg^{2+}-ATPase located in the plasma membrane (65). This increase in Ca^{2+} levels can activate calpains which in turn mediate protein degradation. Increased proteolytic degradation acting on structural proteins also contributes to hepatocyte necrosis (66). Additionally, proteolytic enzymes can be released from the necrotic hepatocytes damaging neighboring cells (67). Finally, nuclear Ca^{2+} levels have been shown to increase with toxic doses of APAP, which lead to the activation of Ca^{2+}-dependent endonuclease (68). It has been demonstrated that endonuclease inhibition decreases cell lethality (69), suggesting that DNA fragmentation has a permissive role in APAP liver toxicity. Additionally, the absence of nuclear nitrotyrosine adducts in APAP treated mice suggests that the DNA damage observed during APAP intoxication is not linked to nitrite formation (57). Overall, there is substantial evidence establishing a connection between mitochondrial dysfunction and nuclear DNA damage. Therefore, in parallel with cytoplasmic and mitochondrial events associated with APAP toxicity, nuclear DNA damage is also involved (68; 70).

Some of the previously stated observations indicate that during APAP liver toxicity, some components of the apoptotic machinery become activated. However, liver histopathology shows primarily focal necrotic areas that spread throughout the parenchyma. The most affected area is the centrilobular zone (zone III) due to the greater distribution and content of CYP450, and thus greater bioactivating capacity of hepatocytes in that region. This regioselectivity is also dose-dependent. Toxicity and spread of necrosis of can also extend to zones I and II, which is commonly seen with extremely toxic doses of APAP.

The considerable overlap between the series of events that are associated with both apoptosis and necrosis during APAP intoxication, provides the basis of new speculations regarding how APAP actually induces cell death. Among them, two new pathways have been implicated in APAP toxicity, namely, c-Jun N-terminal kinase (JNK) and a “hybrid” cell death pathway described as necroptosis. Apparently, intracellular ROS increments activate the Mitogen-Activated Protein Kinases (MAPK) cascade resulting in sustained JNK phosphorylation (71–74), which sensitizes the mitochondria to JNK signaling. A consequence of p-JNK translocation into the mitochondria is that it provides a second hint that amplifies oxidative stress and promotes MPT formation (75). More specifically, P-JNK binds to Src homology 3 domain binding protein 5 (Sh3bp5 or Sab) on the outer mitochondrial membrane, which then promotes mitochondrial ROS production by a mechanism not yet fully understood. This results in production of ROS to levels that are sufficient to induce the MPT (76). In support of the mechanistic involvement of this pathway, inhibition of either pathway JNK or Sab has been shown to protect against APAP hepatotoxicity (71; 74; 76; 77).

Necroptosis, a recently described form of cell death is mediated by the necroptosome, a protein complex. This complex is involved in the initiation of necroptosis (78) and contains Receptor Interacting Protein Kinase-1 (RIPK1) and RIPK3 among its proteins. Necroptosis is inhibited by necrostatins (Nec), including Nec-1. These substances act as necrosis

inhibitors targeting RIPK1. Their use as experimental tools provided evidence that TNF-induced necrosis is a kinase-regulated process (79; 80). In the absence or inhibition of caspase-8 (81–85) through genetic manipulation or caspase inhibitors (such as z-VAD) the interaction between RIPK3 and its substrate Mixed Lineage Kinase Like (MLKL) is promoted, which results in cell membrane disruption and cell death (86). In fact, recent studies showed that the RIPK1 inhibitor, Nec-1, protects against APAP hepatotoxicity *in vivo* in mice and also *in vitro* (87–89), strongly suggesting that necroptosis is mechanistic contributor to APAP toxicity.

As previously reported RIPK3$^{-/-}$ mice and hepatocytes in culture from these knockouts are protected from APAP toxicity (87). A very recently publication (90) contradicts these findings. The discrepancy between studies were primarily attributed to the different genetic backgrounds of the knockout mice used [(C57Bl/6N in the Dara et al. (2015) study, versus C57Bl/6J used in Ramachandran et al. (2013)]. Beyond the genetic background issue, Dara et. al. (90) pointed out that non-specific detection of RIPK3 protein employing different commercial sources of anti-RIPK3 antibodies may be another factor contributing to the contrasting outcomes between studies. Dara et al. utilized a monoclonal specific antibody against RIPK3 generated by Genentech that is not commercially available. They reported low levels of RIPK3 expression in primary mouse hepatocytes under basal conditions, after APAP treatment and also in RIPK3$^{-/-}$ mice. Additionally, they observed that the RIPK3$^{-/-}$ mice were not protected from APAP, but that RIPK1 knockdown does protects RIPK3 $^{-/-}$ mice from APAP toxicity. In order to rule out the involvement of necroptosis, they also demonstrated that MLKL$^{-/-}$ mice, where the ultimate mediator of necroptosis is knocked down, were not protected from APAP toxicity. Collectively, they concluded that RIPK1 participates in APAP toxicity upstream of JNK, whereas RIPK3 and MLKL seem to be dispensable, suggesting that the classical necroptosis pathway is not involved in APAP-induced necrosis, but RIPK1 is essential for this process. These conflicting findings on the role of RIPK3 in APAP hepatotoxicity is extensively discussed in a commentary advocating that the classical necroptosis pathway is not the major mechanistic form of cell death mediating APAP liver injury, but at the same time it also raises several important points about the role or RIPK1 and RIPK3 in APAP hepatotoxicity (91).

A consequence of hepatocyte necrosis is the release of a series of cellular constituents, such as high mobility group box-1 (HMGB1) protein (92), ATP (93), mitochondrial DNA (94) and nuclear DNA fragments (91). A number of these molecules have been shown to exacerbate inflammation through various mechanisms, such as via binding to DAMP receptors like TLR4 (92). It has been proposed that APAP toxicity manifest a second phase of tissue injury that is independent of drug metabolism and mediated by activation of the innate immune system (95–98). The role of Kupffer cells and macrophages in APAP hepatotoxicity continues to be a controversial subject. For a complete review on this see Woolbright and Jaeschke (99).

5. Therapeutic Approaches to APAP Toxicity

Currently, the only approved antidote for APAP toxicity is oral or intravenous administration of N acetyl-cysteine (NAC). This compound is a precursor in the synthesis of GSH.

Therefore, its administration works in part by restoring the intracellular pool of GSH, and in part by neutralizing any residual NAPQI still present in the liver. This antidote therapy can be quite effective if administered within the first few hours after toxic APAP ingestion. This is not always the case since many patients seek medical care several hours after ingestion when many of the cellular early events previously described are well in motion.

However, new strategies that focus on some of the novel molecular initiating events described earlier as potential therapeutic targets are currently under investigation. Among these, is worth noting that modulation of autophagy has been proposed as a possible therapeutic target in cases of APAP intoxication (100). Related to this process, Parkin is a E3 ubiquitin ligase that under ER-stress conditions translocates into depolarized mitochondria. Inside of mitochondria, Parkin directly interacts with Phosphatase and Tensin Homolog (PTEN)-induced Kinase 1 (PINK1) to induce mitophagy, which is a form of degradation and removal of damaged mitochondria by autophagy. Coincidentally, it has been demonstrated that activation of autophagy protects against APAP liver toxicity (101). From Williams et al. (2015) and Ni et al. (2012) findings (100; 101), it is conceivable that pharmacological up-regulation of PINK1/Parkin-mediated mitophagy could become a potential therapeutic alternative for APAP toxicity.

Additionally, blockers of TLR-4 are also capable of attenuating APAP-induced liver injury (102). Interestingly, activation of TLR-4 promotes activation of X-box Binding Protein-1 (XBP1) (103), which is a well known XBP1 transcription factor associated with endoplasmic reticulum (ER) stress. ER-stress promotes post-transcriptional maturation of XBP1 mRNA resulting in the production of an active form of this transcription factor. Sodium 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid, that has been used in the treatment of several diseases, including liver injury by ischemia/reperfusion (102) and carbon tetrachloride toxicity (104). Among its known pharmacological effects, 4-PBA modulates the function of transcription factors associated with ER-stress, including JNK and XBP1 (105). A recent article evaluated the protective properties of 4-PBA when given prior or after toxic administration of APAP in mice (106). 4-PBA decreased drastically hepatic DNA fragmentation induced by APAP; however, the mechanism was unclear and did not involve Xbp1 mRNA splicing or JNK phosphorylation.

The pharmacological utility of these novel and potentially beneficial therapeutic strategies will be revisited at the end of the next section of this review because both pathways are tightly associated with diseases of the brain. The absence of functional XBP1 pathway produces Parkinson’s Disease (107) and 4-PBA is known to attenuate the severity of several brain diseases, such as Parkinson’s (108), Alzheimer’s (109), and cerebral ischemia (105).

6. Paradoxical effects on the brain: protective vs toxic actions

As previously stated, APAP is a safe drug when administered at therapeutic doses. However, APAP overdosing is the most frequent cause of ALF in USA, England and many European countries (35). During progression of ALF, the appearance of hepatic encephalopathy (HE) is indicative of worsening liver function.

Usually, the HE syndrome produces neuropsychiatric symptoms which manifest themselves from mild to severe, with coma as the ultimate sequelae. HE is characterized by the presence of brain edema and an increment in intracranial pressure which can produce cerebral herniation and compression of bulbar olives, which is the location of the cardiac and respiratory control nuclei. These pathological manifestations are the major cause of HE-mediated death (110). Brain ultrastructural changes during HE have been extensively studied, including edema of endothelial cells, changes in morphology of pericytes and perivascular astrocytes feet, with an increase number of vacuoles and vesicles in both cell types. These changes have been observed during brain necropsies from ALF fatalities produced by viral hepatitis. It is worth noting that no changes in tight junctions have been observed in the endothelial cells of the blood brain barrier but the basal membranes are widened. The mechanism of brain edema is cytotoxic, and to a lesser extent, vasogenic, and also includes signs of inflammation (111).

HE pathophysiology is multifactorial, including a central role for ammonia accumulation, glutamine signaling, oxidative/nitrosative stress and activation of pro-inflammatory mediators (112; 113). Among these, excess blood ammonia ($NH4^+$) levels is one of the most important factor in its genesis. Arrest of the urea cycle produced by extensive hepatocellular damage increases dramatically the $NH4^+$ levels in blood. This small charged molecule is normally balanced with its non-ionized form NH_3 in an aqueous medium. NH_3 is capable of diffusing freely into the CNS. Inside astrocytes, NH_4^+ is conjugated with alpha ketoglutarate to form glutamate. Then, the incorporation of a second NH_4^+ molecule leads to glutamine formation by glutamine synthetase (112; 114). An increment in glutamine concentration inside astrocytes produces osmotic stress, which is cytotoxic to these cells (115).

It is generally accepted that the brain alterations produced by APAP intoxication are secondary to the development of ALF through mechanism(s) already described here. However, APAP readily passes the blood brain barrier and is distributed homogeneously throughout the CNS (10–12; 14). CYP2E1, one of the CYPs isoforms involved in APAP bioactivation by the liver, is also expressed in the brain (116–118). In naïve animals, CYP2E1 is present predominantly in evolutionarily older areas of the allocortex, such as the olfactory bulbs, olfactory cortex, hippocampus, cerebellum and the brainstem (117). This indicate that APAP can be metabolized by brain cells to produce the toxic reactive intermediate NAPQI in situ. However, there are only few published studies that describe the effect of APAP per se on the CNS in the absence of ALF.

It has been demonstrated that GSH content is decreased in different regions of the rat brain after oral administration of APAP (3 g/kg) (119; 120). According to Micheli et al. (1993) the most affected regions showing GSH reductions are the hypothalamus (−20%), raquideal bulb (−14%), and cerebral cortex (−24%). The same authors demonstrated that pretreatment with silymarin at a dose of 200 mg/kg/day for three days prior to APAP dosing, prevents decreases in GSH and ascorbic acid content. Silymarin also prevented APAP-mediated increases in GSSG and malondialdehyde accumulation in brain. Silymarin is an extract of Milk of Thistle (Silybum Marianum) and the authors speculated that its beneficial effects against APAP are probably due to its documented ability to combat lipid peroxidation and to replenish GSH during periods of oxidative stress (120). More recently, it was also

demonstrated that administration of a toxic dose of APAP (600 mg/kg) to mice increases ROS, TBARS (tiobarbituric acid reactive species) and diclorofluorescein oxidation levels in brain homogenates (55). The same work also described mitochondrial swelling, decreased Na^+/K^+ ATPase activity and mitochondrial membrane potential (121).

The antioxidant response element (ARE) is a cis-acting enhancer sequence located in the promoter region of numerous genes involved in detoxification and cytoprotection (122–124). In brain, numerous genes are regulated by this enhancer, such as γ-glutamyl cysteine ligase catalytic subunit, NAD(P)H: quinoneoxidoreductase 1 (Nqo1), heme-oxygenase 1 (Ho-1), and certain members of the family of glutathione S-transferase (GST) isoenzymes. Nuclear factor erythroid 2-related factor 2 (Nrf2), is the putative transcription factor that drives ARE-mediated gene expression (125). Under basal conditions, this transcription factor remains inactive in the cytosol through its association to Kelch-like ECH-associated protein 1 (Keap1) (126). During oxidative or electrophilic stress, this complex is disrupted when susceptible thiols in Keap1 are modified. Then, free Nrf2 translocates into the nucleus where is able to bind to AREs, altering the expression of several cytoprotective proteins. Recently, our laboratories have demonstrated increased accumulation of Nrf2 in brain nuclei during APAP intoxication in mice. This is indicative of in situ oxidative stress and/or generation of APAP's reactive intermediate and a disruption in brain redox balance sufficient enough to be detected by this oxidative stress sensor (Figure 4)(127).

In further support of a Nrf2 response in brain after APAP treatment, the expression of Nqo1 and Ho-1, two known Nrf2 target genes, were significantly increased by APAP. Brain mRNA and protein expression of Nqo1 increased at 24 h after APAP administration, while Ho-1 mRNA levels increased as early as 6h (Figure 5) (127). The different temporal induction profiles of these two genes is likely due to differences in regulatory events. While the up-regulation of Ho-1 gene expression can be mediated by several activators such as, JNK-1, HIF-1α and Nrf2; NQO1 regulation utilizes only Nrf2 (128). In other words, regulation of the Ho-1 gene in models of oxidative stress involved multiple transcriptional activators, including Nrf2, while under the same conditions, Nqo1 undergoes single regulation by Nrf2.

Rats intoxicated with APAP (1000 mg/kg) that exhibit significant increases in blood aminotransferases (indicative of acute liver injury) had no changes in prothrombin time or plasma ammonia concentration (indicative of liver functionality) (129). These results demonstrate the presence of acute liver necrosis without ALF. The same study also analyzed brain glutamate and aspartate concentrations, GFAP expression and its distribution in hippocampus. Lastly, vertical jumping was monitored as an index of behavioral disorders. No modifications in any of these parameters were observed (129). Despite these negative findings, another study by Posadas et. al. showed that rats treated with doses of APAP of 250 or 500 mg/kg had an increased number of TUNNEL positive neurons in a time- and dose-dependent manner (130). In the same study, the plasma APAP levels at 1 hour after treatment were determined to be 1 and 2 mM at 250 and 500 mg/kg, respectively. APAP concentration in cerebrospinal fluid followed a similar time profile pattern, reaching slightly higher concentrations (1 and 3 mM, respectively) (130). Furthermore, this article also

demonstrated a direct toxic effect of APAP in cortical neurons in culture in a time-dose dependent manner at concentrations between 0.5 and10 mM.

Further evidence supporting the potential in situ deleterious effects of APAP in brain comes from studies showing that APAP increases neuronal CYP2E1 enzymatic activity and protein levels, leading to neuronal cell death through mitochondrial-mediated mechanisms that involve cytochrome c release and caspase 3 activation (130). APAP is also known to potentiate staurosporine-mediated cell death in the neuroblastoma cell line SH-SY5Y (131). The postulated mechanism of action here is that APAP induces generation of ROS and decreases GSH levels, sensitizing cells to the toxic effect of staurosporine (131). Additionally, unpublished data from our laboratory shows a direct toxic effect of APAP in mixed primary cultures of astrocytes and oligodendrocytes. Here we observed dose-dependent cell death, as determined by the MTT assay (Figure 6, Panel A). We also observed that the same toxic dose of APAP produces a decrease in cell proliferation, as evidenced by reduced BrdU incorporation, along with increased cell death (Figure 6, Panel B and Panel C, respectively).

In concordance with the previous summarized results, Naziro lu et al. studied the effect of APAP in rat brain using a wide range of single doses (5 – 500 mg/kg). A number of pro-oxidant and anti-oxidant parameters were analyzed (132). Doses of 200 and 500 mg/kg increased total brain microsomal lipid peroxidation and calcium levels, while decreasing vitamin E and microsomal GSH content, and peroxidase and Ca^{2+}-dependent ATPase activities. In contrast, with 5, 10 and 20 mg/kg APAP, increments in microsomal glutathione peroxidase were observed. From these findings the authors concluded that APAP has a dual effect in the brain. At low doses, APAP has a protective effect by preventing ROS accumulation, while doses greater than 100 mg/kg produce brain toxicity (132).

A protective effect in brain at low doses of APAP has also been reported by others. Pretreatment with APAP has been shown to decrease brain toxicity from post-ischemic reperfusion in rats (121) or amyloid β peptide-induced oxidative stress (126). Baliga et al. described that intravenous administration of 15 mg/kg APAP decreases brain damage by hypovolemic hypotension by decreasing mitochondrial swelling, cit c release and caspase 9 activation (133). APAP's protection against ischemic conditions is not an organ-selective effect since APAP has also been demonstrated to be an effective cardioprotective agent during myocardial ischemia–reperfusion injury (134–137). Also, protection by APAP against amyloid β peptide was observed in primary cultures of hippocampal neurons and in the PC12 cell line. This protection was associated with a reduction in oxidative stress status, as evidenced by a reduction in peroxides levels and lipid peroxidation (138). In this study, reduced apoptosis was also described via suppression in NF-kappaB activation (138). APAP pretreatment decreases cell damage induced by quinolinic acid in rat hippocampus (20). Quinolinic acid (2,3-pyridine dicarboxylic acid) is known to produce several neurodegenative diseases by inducing oxidative stress. APAP also decreases oxidative damage produced in neuronal cultures by other neurotoxic compound, such as 6-hydroxydopamine (80). Finally, a recent study using cultured brain endothelial cells showed reductions in inflammatory responses to oxidative stress in the presence of low doses of APAP (21).

7. Conclusion

In summary, while hepatic toxicity by APAP has been extensively studied, the direct toxic effect of APAP in the brain has been received less attention. It is known that high doses of APAP promotes oxidative stress and produces damage to different cell types in the brain. But the structural and functional consequences of these effects is still unknown. This should be the subject of further investigations to clearly discriminate between liver-driven versus true in situ adverse effects of APAP in brain. It is also very important to point out that additional investigations on this subject are needed to define the pathways mediating APAP toxicity in brain. A comprehensive understanding of the mode of toxicant action of APAP in the brain can be instrumental in the development of novel disease-modifying therapeutic approaches. Also, we want to re-emphasize that there is sufficient and convincing evidence that APAP at low doses has a protective effect in the brain. A better and deeper mechanistic understanding of such beneficial effect may similarly lead to new modalities of treatment and management of pathophysiological conditions involving neuro-inflammatory events.

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Abbreviations

AEA	anandamide
AM404	N-arachidinoyl-phenolamine
AMAP	3′-hydroxyacetanalide
AMT	anandamide membrane transporter
ALF	acute liver failure
APAF-1	Apoptosis protease-activating factor-1
APAP	acetaminophen
ATP	adenosine triphosphate
CAR	constitutive androstane receptor
CB	cannabinods receptors
CNS	central nervous system
COXs	cyclooxygenase
CYP	cytochrome P-450 isoenzymesmixed-function oxidase system
DAMPs	damage-associated molecular pattern molecules
DNA	Deoxyribonucleic acid

ER	endoplasmic reticulum
FAAH	fatty acid amide hydrolase
GSH	Glutathione
GSSG	glutathione disulfide
HE	hepatic encephalopathy
HIF-1α	Hypoxia-inducible factors
HMGB1	high mobility group box-1
Ho-1	hemeoxygenase
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like
ECH	associated protein
KO	Knock-out
MAPK	mitogen-Activated
MLKL	mixed lineage kinase like is produce
MPT	membrane permeability transition
NAC	N acetyl-cysteine
NAPQI	N-acetyl-p-benzoquinoneimine
Nec	necrostatins
NO	nitric oxide
Nqo1	NAD(P)H dehydrogenase quinone 1
Nrf2	nuclear factor erythroid 2–related factor 2
NSADs	nonsteroidal anti-inflammatory drugs
PINK1	phosphatase and tensin homolog (PTEN)-induced kinase 1
RIPK	receptor interacting protein kinase
ROS	reactive oxygen species
Sab	Src homology 3 domain binding protein 5
TBP	TATA-binding protein
TRPV1	vanilloid subtype 1 receptors

WT	wild type
XBP1	X-box binding protein-1
4-PBA	Sodium 4-phenylbutyrate

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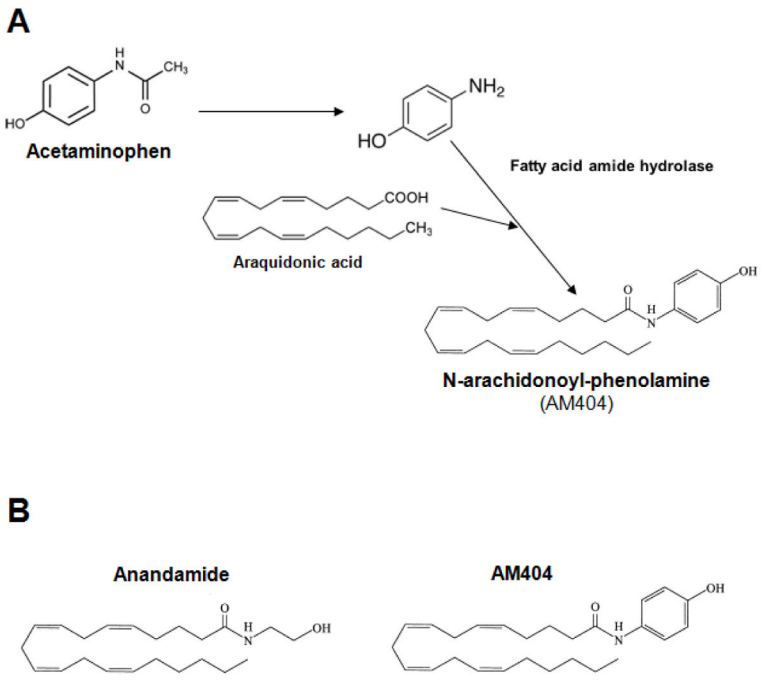

Figure 1. FAAH-metabolite of APAP: Generation of N-arachidonoyl-phenolamine (AM404) in Brain

Panel A: APAP is deacetylated followed by the formation of AM404 by the addition of arachidonic acid, which is catalyzed by fatty acid amide hydrolase (FAAH) in brain. **Panel B**: Structures of AM404 and

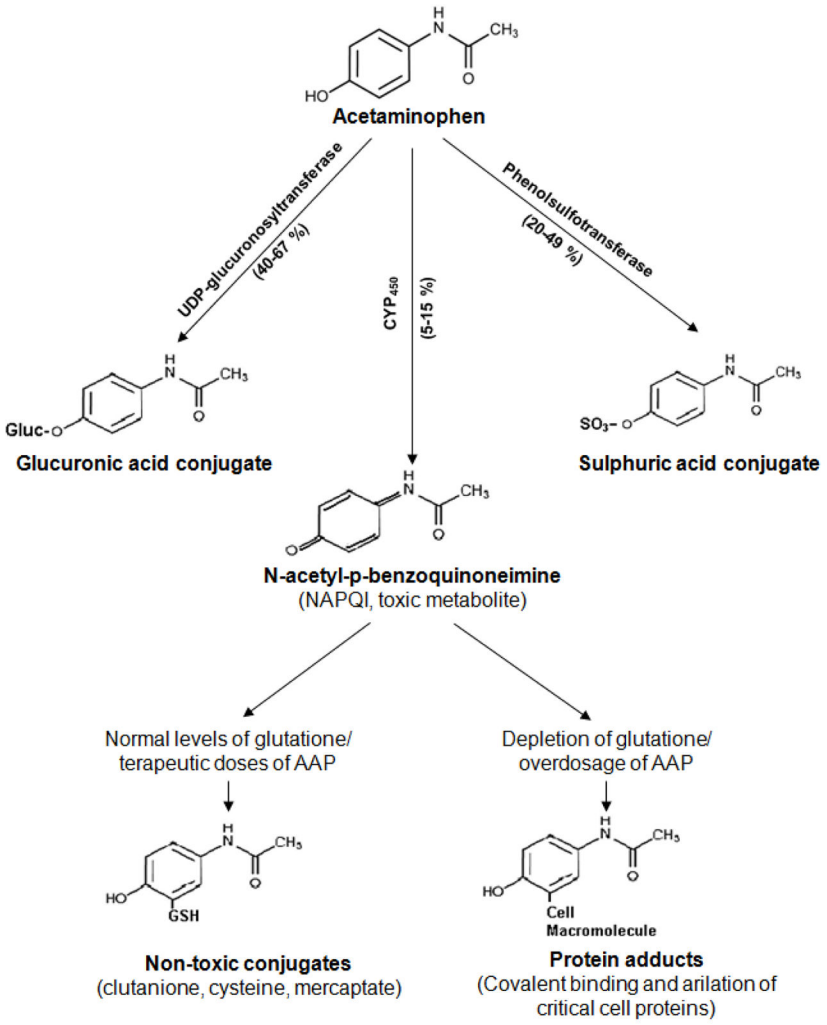

Figure 2. Liver APAP Metabolism

Main metabolic pathways of APAP in liver after administration of therapeutic or toxic doses.

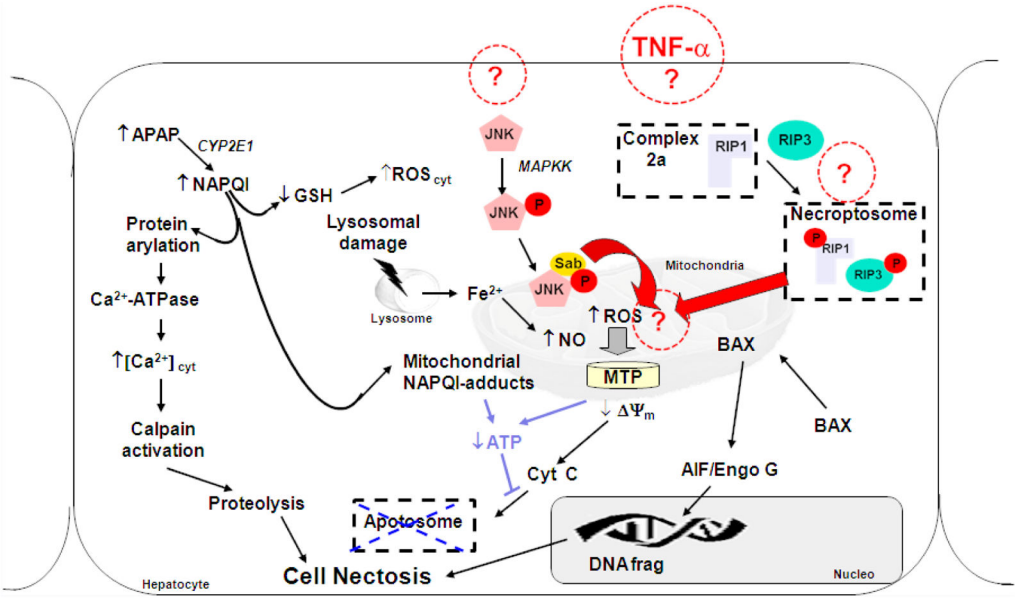

Figure 3. Mechanism of APAP Toxicity in Liver

An Excess of the reactive intermediate NAPQI after GSH stores are depleted is considered to be the molecular initial event in APAP toxicity. Unneutralized NAPQI forms covalent adducts with macromolecules in hepatocytes, increases oxidative stress and inhibits Ca^{2+}-Mg^{2+}-ATPases, which results in intracellular Ca^{2+} dysregulation and accumulation. This increase in Ca^{2+} can activate calpains, mediating proteolytic degradation. Mitochondrial GSH depletion and selective mitochondrial proteins adducts are associated with ROS generation and formation of membrane permeability transition (MPT) pore, which triggers mitochondrial damage and release of cit c. Inhibition of mitochondrial respiration decreases ATP levels, which in terms halt the apoptotic signaling cascade and redirects the cell fate toward necrosis. After initial formation of NAPQI and adducts, several other molecular events contribute to the amplification and propagation of tissue damage. Recently, the phosphorylation of JNK by MAPKK and activation of RIPK1 through necroptosis or by direct interaction with JNK has been also implicated in APAP cytotoxicity. Abbreviations: **APAP,** acetaminophen**; AIF**, apoptosis inducing factor; **ATP,** adenosine triphosphate; **CYP**, cytochrome P-450 isoenzymes mixed-function oxidase system;**Cyt c**, cytochrome c; **DNA**, Deoxyribonucleic acid; **Fe2+** iron, **GSH**, Glutathione**JNK**, c-Jun N-terminal kinase; **MAPK**, mitogen-Activated; **MPT**, membrane permeability transition; **NAPQI**, N-acetyl-p-benzoquinoneimine; **Nec**, necrostatins; **NO**, nitric oxide; **O2-**, superoxide; **RIP**, receptor interacting protein kinase; **ROS**, reactive oxygen species; **Sab**, Src homology 3 domain binding protein 5; Δψ **m**, mitochondrial membrane potential.

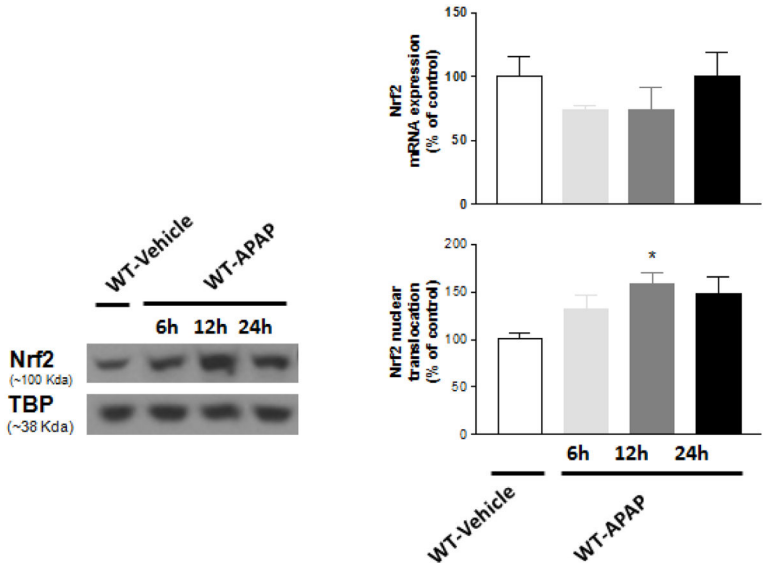

Figure 4. Time-dependent Effect of APAP on Brain Nrf2 Gene Expression and its Nuclear Localization

Brain Nrf2 mRNA levels were measured by qPCR. RNA isolation and quantification was performed as detailed in Ghanem et al. (2015) (126). Data are presented as percentage of control and expressed as means ± SE (n=5 mice/group). Nrf2 protein nuclear concentration was determined by Western blotting. Nuclear protein and western-blot was performed as was described in Ghanem et al. (2015) (126). Equal protein (50 μg) was loaded into each lane. TATA-binding protein (TBP) was used as loading control. Densitometric analysis of blot is presented as percentage of control and expressed as mean ± SE (n=5 mice/group. * $p<0.05$ vs Oh).

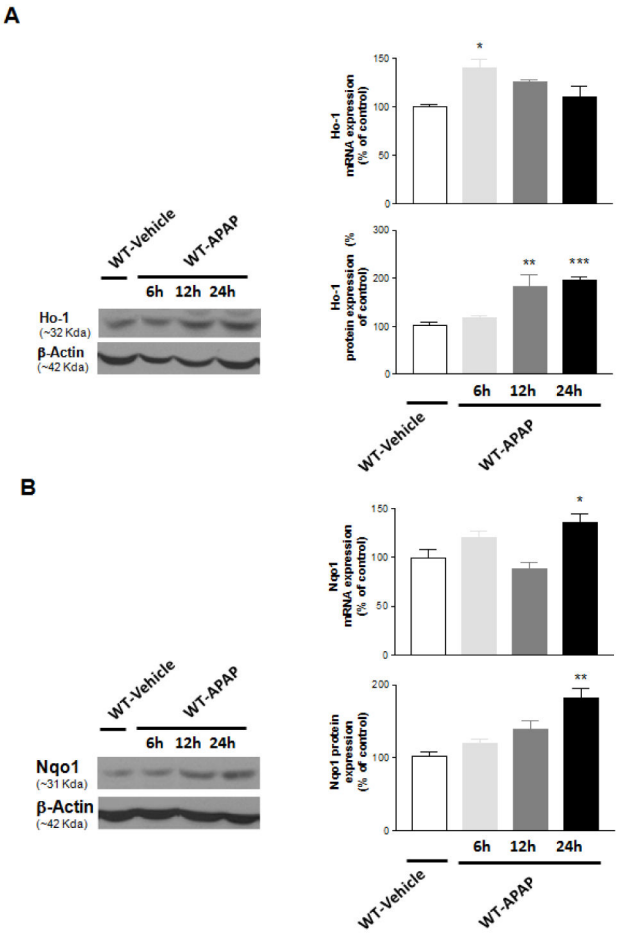

Figure 5. Time-dependent Effect of APAP on the Expression of the Nrf2 Target Genes Ho-1 and Nqo1

Brain Ho-1 **(Panel A)** and Nqo1 **(Panel B)** mRNA and protein levels were measured by qPCR and Western blotting, respectively. Total homogenates were used and the methodology used in RNA and protein studies are detailed in Ghanem et al. (2015) (126). mRNA data are presented as percentage of control and expressed as mean ± SE. For western blotting, equal cytosolic protein (50 μg) was loaded into each lane. β-actin was used as loading control. Densitometric analysis of blots is presented as percentage of control values and expressed as mean ± SE. (n=5 mice/group. *** $p<0.001$; ** $p<0.01$; * $p<0.05$ vs 0h).

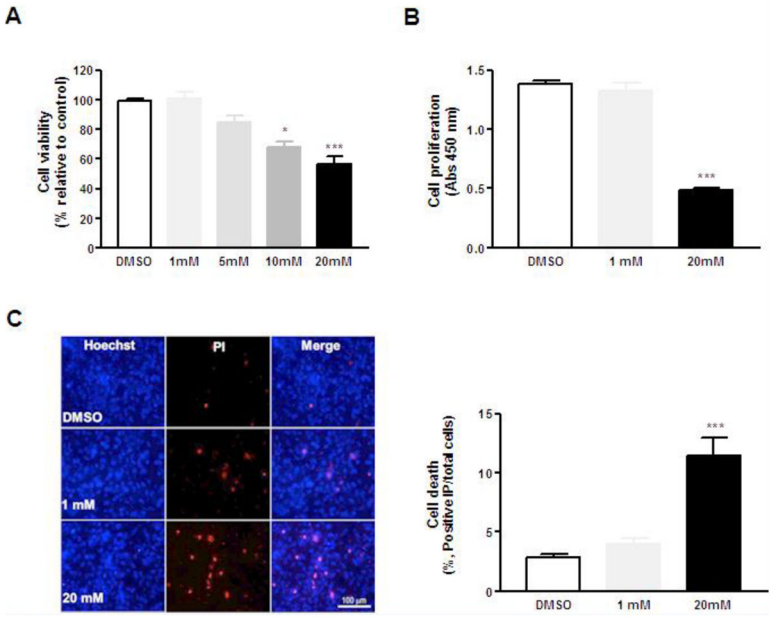

Figure 6. Effect of Different Concentrations of APAP on Primary Culture of Glial Cells

Glial primary cultures from newborn Wistar rats were performed according to Pérez el al (2013) (138). Cell cultures were astrocytes (80–85%) and oligodendrocyte (15–20%). **Panel A: Cell viability.** The cell viability of glial primary culture was measured by the MTT assay (139) after incubation with APAP (1, 5, 10, 20 mM) for 48 h. The absorbance at 570nm was expressed as percentage of control. **Panel B: Cell proliferation.** Proliferation was evaluated in glial primary cultures using 1 and 20mM APAP by Whole-cell BdrU ELISA as was described by Silvestroff et al. (2012) (139). The results were expressed as absorbance at 450nm. **Panel C: Cell death.** Propidium iodine (PI) staining was used to identify dead cells. Total cells were stained with hoechst (139). Data in A, B and C are means±SD of four independent cultures. One-way ANOVA followed by Newman-Keuls multiple comparison test were used in A, B, and C to determine statistical significance; ***P< 0.001, *P< 0.05, symbols above the bar indicate significance compared to corresponding control.