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Review Article

New potential biomarkers of acetaminophen-induced hepatotoxicity

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ABSTRACT

Acetaminophen (APAP) is one of the most common antipyretic and analgesic drugs. Despite various precautions patients use APAP in amounts exceeding acceptable daily doses. APAP overdosing contributes to APAP intoxication, which leads to acute liver injury or necessity of exigent liver transplantation. Biomarkers that can be helpful in early diagnosis of liver injury during APAP overdosing are studied worldwide. This review presents recent reports on new potential biomarkers and their prospective application in clinical practice.

Contents

1. Introduction

Acetaminophen (N-acetyl-p-aminophenol, APAP, paracetamol) is one of the most commonly used antipyretic and analgesic drugs. Taking it in therapeutic doses, is harmless, however, APAP overdosing may cause hepatotoxicity. Many pharmaceutical products contain APAP and are available without any prescription. Their easy availability results in constantly-increasing rate of APAP poisoning cases. For this reason, the safety of APAP is still widely discussed.

APAP has similar properties (analgesic and antipyretic properties) to nonsteroidal anti-inflammatory drugs (NSAIDs), but it does not possess any anti-inflammatory activity. Applied in recommended doses, APAP does not cause gastrointestinal side effects, which is common for NSAIDs. After ingestion, about 90% of APAP is metabolized in the liver, where it is conjugated with glucuronic

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acid (50–60%) and sulfuric acid (25–35%). A small amount of this drug (3%) binds to cysteine. The products of these conjugations are pharmacologically inactive and are eliminated by the urinary system. About 5% of APAP is eliminated by the kidneys in an unchanged form. Another 5% of APAP undergoes N-hydroxylation by cytochrome P450 enzymes, producing a toxic metabolite i.e. N-acetyl-*p*-benzoquinone imine (NAPQI, *N*-acetylimidoquinone). In therapeutic APAP doses, reactive NAPQI is sufficiently deactivated by conjugation with sulfhydryl groups of glutathione (GSH). The final product of APAP metabolism is mercapturic acid, which is eliminated with urine. The situation changes if APAP is overdosed. Metabolic reactions cause hepatic GSH depletion and NAPQI binds to various hepatocyte macromolecules such as proteins, lipids and DNA. It leads to metabolic disturbances and cell death. N-acetylcysteine (NAC) restores GSH resources, therefore administration of NAC plays an essential role in medical care of patients with APAP overdose. However, NAC is fully hepatoprotective only when distributed within 8 h after APAP ingestion [1].

After APAP overdose, NAPQI reacts with protein sulfhydryl groups forming adducts with them [2]. Protein adducts are formed primarily in mitochondria causing their dysfunction and leading to oxidative stress. It begins from the activation of various MAP kinases [3], which after chain reactions, causes phosphorylation of c-jun-N terminal kinase (P-JNK). P-JNK translocates to the mitochondria which enhances formation of reactive oxygen species, mainly peroxynitrite [4,5]. It leads to the opening of the membrane permeability transition (MPT) pores. Consequently, the membrane potential collapses and the ATP synthesis is inhibited. Another consequence of the MPT opening is mitochondrial matrix swelling and the resulting rupture of the outer membrane. The rupturing of the membrane triggers leakage of various intermembrane proteins (for instance endonuclease G) into cytosol. The proteins are transported to the nucleus where they initiate DNA fragmentation [6]. The end result is a hepatocyte necrosis [7].

The mitochondrial dysfunction that occurs in liver damage can be assessed trough measuring the mitochondrial matrix enzyme glutamate dehydrogenase (GDH) or mitochondrial DNA (mtDNA) in patient's serum [8]. Furthermore, protein adducts can also be observed in serum after APAP overdose [9]. Numerous studies on APAP toxicity have been carried out in recent years. Researchers discovered several novel potential diagnostic biomarkers of liver necrosis. The markers belong to the cell death markers group and were observed both in mouse and in human serum: cytokeratin-18, microRNA-122, high mobility group box 1 protein (HMBG-1), nuclear DNA (nDNA) fragments or argininosuccinate synthetase (ASS) [8,10–12].

2. Review

2.1. Circulating acylcarnitines

Acylcarnitines are conjugates of carnitine and fatty acids. Long-chain fatty acids cannot pass through the mitochondrial membrane, but conjugation with carnitine facilitates their penetration into the mitochondria. The presence of these fatty acids in mitochondria is necessary for β-oxidation. Accumulation of acylcarnitines (palmitoylcarnitine, myristoylcarnitine, oleoylcarnitine and palmitoleoylcarnitine) was observed in mouse serum after APAP treatment and was linked to β-oxidation impairment [13], although it may be a result of NAPQI binding to carnitine-acylcarnitine translocase (CACT) or carnitine palmitoyltransferase II (CPT II) – enzymes involved in acylcarnitines metabolism [14]. Disturbances to the activity of these enzymes could initiate the accumulation of acylcarnitines in cytosol. It is also possible that the increased levels of acylcarnitines proceed from β-oxidation in mitochondria and after mitochondrial MPT, the acylcarnitines are released to plasma. On the contrary, McGill et al. [15] did not notice a significant difference of acylcarnitines levels in human serum after APAP overdose, compared to the control group. These findings were explained by the effect of NAC treatment that supports mitochondrial function. Another study of human serum after APAP treatment was performed by Bhattacharyya et al. [16]. They examined serum collected from children with APAP exposure (low dose and overdose) and compared to controls. They measured acylcarnitines regardless of alanine transaminase (ALT) levels and checked the influence of time-dependent NAC treatment. Moreover, the study group included more patients compared to the McGill et al. [14] research. Bhattacharyya et al. [16] reported that long-chain circulating acylcarnitines levels, especially palmitoyl- and oleoyl-carnitines, were significantly elevated in children with APAP exposure compared to controls. Whereas there was no significant difference in acylcarnitines levels between both APAP exposure groups. However, in children with APAP overdose receiving delayed NAC treatment (more than 24 h after overdose), acylcarnitines were at a higher level than in early NAC treatment group. These findings confirm that acylcarnitines levels are influenced by the time of NAC treatment after APAP exposure. In summary, the results of the studies discussed above proved that circulating acylcarnitines levels can be evaluated as potential biomarkers of mitochondrial injury and APAP hepatotoxity. Their concentration can be easily measured by applying ultraperformance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS).

2.2. Liver-specific blood proteins

Hu et al. [17] identified five liver-specific blood proteins as markers of APAP-induced hepatotoxicity in humans using proteomic technologies (label-free antibody microarrays, quantitative immunoblotting, and targeted iTRAQ mass spectrometry). Betaine–homocysteine *S*-methyltransferase 1 (BHMT), dihydropyrimidinase (DPYS), fumarylacetoacetate hydrolase (FAH) and fructose-1,6-bisphosphatase 1 (FBP1) showed higher concentrations than ALT–which is the gold standard in diagnosing hepatotoxicity. 4-hydroxyphenylpyruvatedioxygenase (HPD) concentration was comparable with ALT. S-adenosyl-L-methionine (SAMe) is a precursor of GSH. Its concentration in the liver decreases dramatically in the case of APAP hepatotoxity. Hu et al. [17] linked the decreasing SAMe with the change in the levels of three enzymes involved in metabolism of SAMe (MAT1A, GNMT and BHMT)–they decreased in the liver but were elevated in the blood. It was suggested that these liver-specific blood proteins might be useful in GSH and SAMe levels' assessment and therefore might have prognostic value in hepatotoxicity. Hu et al. [17] also observed interesting changes in the concentration of membrane-bound catechol-O-methyltransferase (MB-COMT) in mouse plasma and liver tissue after APAP intoxication. Catechol-O-methyltransferase (COMT) is an enzyme that catalyzes the transfer of a methyl group and has two isoforms–soluble and membrane-bound. The western blot assay of liver tissue showed that after APAP overdose, the soluble catechol-O-methyltransferase (S-COMT) level decreased whereas MB-COMT increased. It was suggested that there may be a specific mechanism in the liver that allowed the translation of S-COMT into MB-COMT after overdosing APAP. The mechanism of COMT regulation requires further investigation (Fig. 1).

Another potential new biomarker is the mitochondrial protein, carbomoyl phosphate synthetase 1 (CPS1). This protein level increases faster than aspartate transaminanse (AST) and ALT within the first 3 h after APAP ingestion [18,19]. CYPS1 levels were

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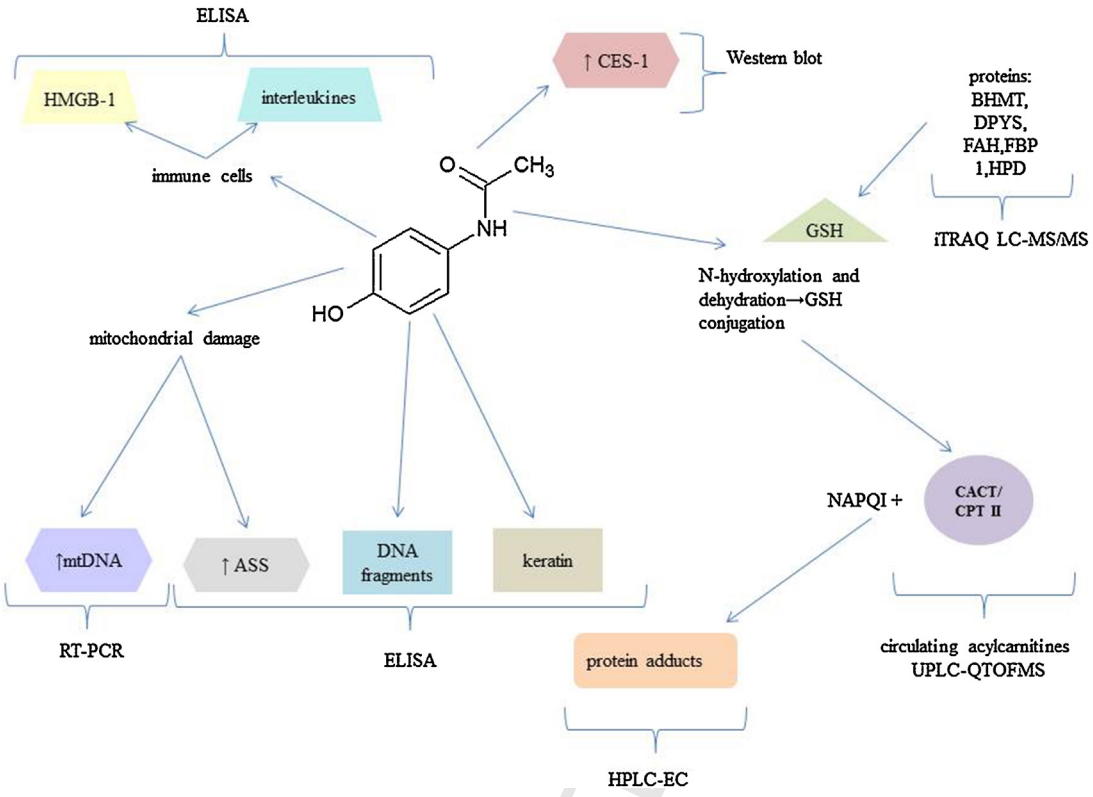

Fig. 1. Summary of new potential biomarkers connected with APAP overdosing and suggested method of their detection.

elevated after APAP treatment in mouse and human blood, as well as in hepatocytes culture medium.

2.3. Protein adducts

APAP forms adducts with hepatocytes proteins. It had been a paradigm that GSH depletion caused increasing levels of NAPQI, simultaneously binding proteins and creating adducts [2]. Recent studies show that the effect was not dependent on doses of APAP [20] nor GSH depletion [8]. Evaluation of the proteins, which can bind to APAP, revealed that APAP has high ability to react with mitochondrial proteins [21]. McGill et al. [15] described the APAP-protein adducts as potential new biomarkers that can be measured in patients' serum or in liver tissues. It was noticed that the protein adducts reach the highest level about 0.5–1 h after administration and then the level decreases. Higher doses of APAP could cause a peak of these adducts sooner. In addition, the effect was not dependent on the level of GSH. Protein adducts have a 1–2 days half-life [22] and supposedly are released predominantly from necrotic hepatocytes [7,9,11]. Additionally, it was noticed that adducts appear in plasma even before ALT peak [20]. It was suggested that protein adducts could be more useful for diagnosing APAP overdose than measuring APAP levels. Therefore, it was proposed to measure APAP-CYS and ALT levels in patients' serum to diagnose hepatotoxicity after APAP overdosing [22,23]. Moreover, it may be beneficial to assess other conjugates like APAP-GSH [24]. However, the studies by McGill et al. showed that protein adducts could appear in plasma even without liver injury.

In summary, measurement of various acetaminophen-protein adduct could be conducted within using high-performance liquid chromatography with electrochemical detection (HPLC-EC) in both serum and liver tissues, for instance Heard et al. applied this method to measure concentration of paracetamol-cysteine adducts.

2.4. Mitochondrial DNA, nDNA fragments and glutamate dehydrogenase

In another study, McGill et al. [9] evaluated patients' serum after APAP intoxication by measuring levels of selected parameters. The real-time PCR was conducted to assess mitochondrial DNA (mtDNA) concentration, whereas the ELISA assay enabled detection of nDNA. The study results showed, not only an increasing level of glutamate dehydrogenase (GDH) – the mitochondrial matrix enzyme, but also an increasing level of mtDNA and nDNA fragments, which come from nuclear DNA fragmentation by endonucleases [9,25]. As previously mentioned, APAP overdosing leads to the rupturing of mitochondrial membranes and leakage of different proteins, including endonucleases [6].

A fact, supporting the hypothesis that the main mechanism of hepatotoxicity caused by APAP is extensive mitochondrial damage, is the presence of GDH in serum. GDH is a high-molecular-weight enzyme which cannot pass the mitochondrial membranes in physiological conditions [25]. Similar effects of mitochondrial damage were observed in the HepaRG cell line culture after APAP treatment [8]. It is important to note that different drugs, which also cause centrilobular necrosis, such as furosemide, did not reveal mitochondrial damage. Because of these findings McGill et al. [25] suggested measuring mtDNA, nDNA and GDH as a part of a novel biomarkers panel.

2.5. Argininosuccinate synthase

Argininosuccinate synthase (ASS) is a mitochondrial enzyme that catalyzes biosynthesis of argininosuccinate from the citrulline and the aspartate. It was observed that the ASS level increased intimately as a consequence of liver injury caused by endotoxin or APAP intoxication in both, humans and rodents, serum [12,26]. The

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measurement of ASS was performed in the plasma using a sandwich ELISA. However, an increased level of ASS was also noticed in the case of furosemide intake. Therefore, it is not specific only for mitochondrial damage [12].

2.6. DNA fragments

As was described in an earlier section, after APAP overdosing endonucleases from mitochondria are transported to the nuclei, where they cause fragmentation of DNA. Nuclear fragments of DNA may be measured via ELISA assay in patients' serum. The method requires application of a secondary antibody against DNA fragments and a primary antibody against histones [9].

2.7. Keratin 18

One of the filament phosphoglycoproteins are keratins 8 and 18 (K8 and K18). Their expression occurs mainly in simple-type epithelia and they play an important role in cell integrity and shape. Ku et al. [27] showed that expression of K18 influenced the APAP-induced hepatotoxicity. It was found that mice with mutated K18 were more susceptible to liver injury compared to a group with normal K18. Ku and Omary [28] noticed that apoptosis mediated by Fas is accompanied by phosphorylation of K18. Furthermore, Caulin et al. [29] proved that one of the first events during disturbances of apoptosis is a segmentation of K18 mediated by caspases (mainly caspase-3, -7, -9).

Shutte et al. [30] presented a distinction between two forms of K18. According to the obtained results, full-length K18 is released from necrotic cell death, whereas fragmented K18 occurs during apoptosis. Both full-length and fragmented keratin concentrations can be measured with ELISA kit in patients' serum [11,30].Cummings et al. [31] also proposed that K18 (full-length and fragmented) can be a marker for assessing the effectiveness of treatment in patients with APAP intoxication. Another experiment was performed by Antoine et al. [32] who studied mice serum after treatment by various doses of APAP. The levels of K18 as a cleaved form and unmodified form were measured by LC-MS method. In the untreated group the authors could not find neither the fragmented form nor the full-length form. However, in serum from treated mice, both forms of K18 were identified. Furthermore, it was proved that an elevated level of full-length K18 is correlated with an increasing amount of damaged hepatocytes. In another study, Antoine et al. [11] investigated the levels of both forms of K18 in patients' serum and observed a correlation between ALT, prothrombin time and full-length K18 activity. Moreover, the authors found that the elevated full-length K18 levels were related to worse patient prognosis. Strnad et al. [33] studied the influence of genetic predisposition on liver injury development. The study results showed a link between two genes – KRT8 and KRT18 that are involved in keratin expression, and susceptibility to acute liver failure (ALF). It is expected that patients with mutations in these two genes can have a worse course of ALF.

2.8. Carboxylesterase-1

Another potential new biomarker whose level increases during APAP-induced hepatotoxicity, is a carboxylesterase (CE). It belongs to the family of enzymes that are involved in the hydrolysis of different compounds including drugs and prodrugs. Because of this activity, carboxyloesterases (CES) are classified as phase-I-metabolizing enzymes. Furthermore, the CES are divided into five main groups: CES-1,CES-2, CES-3, CES-4 and CES-5. Expression of CES-1 is observed mainly in the liver [34]. It has also been reported that the expression of CES-1 was significantly increased in the HepG2 cells after incubation with paracetamol. The assessment of the CES-1 level was conducted using the western blot method [35]. The main limitation of these studies arises from using the HepG2 cell line culture. HepG2 cells have extremely low CYP450 enzymes expression in comparison to human hepatocytes. CYP450 enzymes are crucial in APAP metabolism, therefore the HepG2 is not a good model to study APAP toxicity [8]. The assessment of CES-1 expression needs to be validated by studying other cell line types.

2.9. High mobility group box 1

High mobility group box 1 (HMGB-1) is a nuclear protein with a variety of functions. One of its most important features is the control of gene expression through binding to DNA in specific regions. HMGB-1 is secreted mainly by activated macrophages which activation is observed during liver injury [32,36], therefore, HMBG-1 is measured in patients' serum as an inflammatory marker. However, the monocytes recruitment and activation in the recovery phase causes late HMGB-1 increasing in serum. It was also proposed to assess the HMGB-1 level as a biomarker of cell necrosis [37], which is possible with using the ELISA kit [11] or western blotting methods [36]. The results of other studies also showed a correlation between the poor course of liver injury after APAP overdose and the increasing level of HMBG-1 [37].

2.10. Interleukins

Increasing levels of different cytokines can be observed during inflammation occurring in the regeneration phase after APAP overdosing. It is probably more important in the induction of hepatotoxicity than by the drug itself. In the APAP-induced liver injury, various damage-associated molecules, such as mitochondrial and nuclear DNA and even nuclear protein, can be observed in extracellular space. These molecules stimulate immune system cells that start releasing a variety of cytokines, such as monocyte chemoattractant protein 1 or IL-6 and IL-8, which can be assessed in patients' serum using ELISA method [38]. An increase of pentraxin 1, which is secreted by immune cells after interaction with certain cytokines, can be also observed during APAP intoxication. Moreover, recent studies showed that the level of pentraxin 3 is correlated with encephalopathy and the hepatocytes death after APAP overdosing. The level of pentraxins in plasma and liver tissue was measured with ELISA [39]. Furthermore, it was proved that a high level of cytokines in patients' serum is correlated with the presence and activation of immune cells in the liver. During the liver injury after APAP intake, a stimulation of neutrophils in serum is connected with their activation in the liver [40].

2.11. Circulating microRNA

MicroRNAs are about 21–23 nucleotide-long regulatory RNAs. MicroRNAs are specifically enriched in different tissues, can be released from cells and conjugated with proteins. MicroRNAs stable complexes can be detected in body fluids such as blood or urine. Therefore, microRNAs have been studied as new biomarkers of different pathological states [10,41]. It was observed that after APAP overdosing the levels of microRNA-122 and microRNA-192 were elevated. It was suggested that microRNA-122 is liver-specific whereas microRNA-192 is kidney-specific [41,42]. A number of studies confirmed that the level of microRNA-122 increases in patients with APAP overdose, however, it also increases in other liver injuries, such as viruses infections or hepatic ischemia [43,44]. The increasing level of microRNA-122 in hepatocytes causes down-regulation of target genes and as a result enhances liver regeneration [42]. Most importantly, concentrations of these microRNAs can precede the elevation of ALT. In

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addition, after NAC treatment, the microRNAs' levels decrease much faster than the ALT level. Therefore, microRNAs can be sensitive biomarkers to monitor patient recovery from APAP intoxication [44,45].

3. Conclusions

APAP is one of the most common antipyretic drugs that can be obtained without a prescription. It has been recommended as a safe medicine even for women during pregnancy and children. Nevertheless, recent studies showed many disadvantages of this drug including the most serious one – hepatotoxicity, whose pathogenesis is not fully understood. APAP overdosing may even lead to the need for liver transplantation. Investigators continuously search for new indicators of high specificity to APAP-induced liver damage and what can be used in a routine diagnosis. Current research gives a partial understanding of the molecular mechanism of the APAP-induced hepatotoxicity. Potential new biomarkers are proposed and the most promising are those derived from hepatocytes' damage, such as, mRNA-122, HMGB-1 or a cleaved form of K-18. Although the described new potential biomarkers have not been applied in clinical practice yet, there is a possibility of using them in the future diagnostics. Nevertheless, there are some difficulties with comparing the specificity or sensitivity of the discussed biomarkers, due to their divergent time of appearing in biological samples. The most promising methods, that would help researchers find new biomarkers of liver damage caused by APAP, are recently developed 'omics' techniques.

Conflict of interests

The authors declare no conflict of interests.

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