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RELATIONSHIP OF CELLULAR GLUTATHIONE CONCENTRATION WITH THE CYTOTOXICITY OF ACETAMINOPHEN IN DIFFERENT CELL LINES

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ABSTRACT

Acetaminophen is used clinically as an analgesic and antipyretic drug. Although acetaminophen is safe at therapeutic doses, it produces hepatic injury in both human and experimental animals, and also induces morphological transformation in cultured cells when given in excessive doses. The hepatic injury produced by acetaminophen is generally thought to be initiated by a reactive metabolite of acetaminophen, N-acetyl-p-benzo-quinoneiniine (NAPQI), formed by cytochrome P450. NAPQI is initially detoxified by conjugation to reduce glutathione (GSH). A primary cellular target of Acetaminophen is GSH, which is an extremely important cellular antioxidant. A marked cellular GSH level change following the exposure to arsenic has been reported, which is inversely related to the intracellular accumulation of arsenic. In this study, we have investigated the effects of acetaminophen on the cellular total GSH level in different tumor and normal cell lines. Five different cell lines of human hepatic carcinoma (HePG2), human lung adenocarcinoma (A549), human ovarian carcinoma (SKOV3), dog kidney (LLCPK1) and Chiness hamster ovary (CHO) cell lines were exposed to the IC_{50} concentrations of acetaminophen for two hours. Acetaminophen cytotoxicity was measured using clonogenic assay, and the total cellular GSH level were analyzed using a photo metrically assay. Our results showed that acetaminophen had different degrees of cytotoxicities on different cell lines as shown by IC_{50} values; 18.6 for HepG2, 4.16 for A549, 5.01 for SKOV3, 5.6 for LLCPK1, and 16.7 for CHO cell lines. According to our results, GSH level alterations after exposure to acetaminophen were also different for different cell lines; 24.21 for HepG2, 778.21 for A549, 977 for SKOV3, 1367.3 for LLCPK1, and 312.43 for CHO cell lines. It is concluded that acetaminophen undergoes different metabolic pathways in different cell lines that produces various species which might or might not bind to GSH. Although cells would increase their cellular GSH content after exposure to acetaminophen, but the superiority of either pathway in each cell line would determine the GSH consumption and therefore the cellular protection against the cytotoxicity of acetaminophen in each cell line.

Keywords: Acetaminophen, glutathione, HepG2, A549, SKOV3, LLCPK1, CHO

INTRODUCTION

Acetaminophen or paracetamol is a widely used analgesic and antipyretic with very few side effects at its usual therapeutic doses (Spooner *et al.*, 1976). However, when taken at high doses (Chiu *et al.*, 1978) or in combination with other drugs such as alcohol (Slattery *et al.*, 1996), acetaminophen is known to cause hepatic necrosis (Spooner *et al.*, 1976; Chiu *et al.*, 1978) and in the most severe of cases can cause liver failure and death (Multimer *et al.*, 1974). The metabolism of acetaminophen has been widely studied (Multimer *et al.*, 1974; Spooner *et al.*, 1976; Chiu *et al.*, 1978; Reed *et al.*, 1990 Slattery *et al.*, 1996).

At therapeutic doses acetaminophen is primarily detoxified by glucuronidation, sulphation and renal excretion (Savides and Oehme, 1983). However, as the dose of acetaminophen increases, more of the compound is metabolised by cytochrome P450 (Patten *et al.*, 1993) to produce N-acetyl-p-benzoquinoneimine (NAPQI) (Miner and Kissinger, 1979; Dahlin *et al.*, 1984). This metabolite is a highly reactive compound that acts as both an oxidising agent that converts reduced glutathione (GSH) to oxidised glutathione (GSSG), and an electrophile that is able to bind covalently to intracellular molecules, most importantly GSH, to form the corresponding GSH adduct (Buckpitt *et al.*, 1979). However, at very high doses of acetaminophen, and subsequent production of high levels of NAPQI, GSH levels are sufficiently depleted to allow NAPQI to react with macromolecules, including proteins and DNA, and initiate events that ultimately lead to hepatotoxicity (Moore *et al.*, 1985; Huggett and Blair, 1983).

There has been much discussion about the mode of action of NAPQI in causing hepatic necrosis (Moore *et al.*, 1985; Huggett and Blair, 1983; Tee *et al.*, 1986). Huggett & Blair have suggested that when interacting with thiol groups, nucleophilic addition along with arylation may occur leading to the formation of protein thiol-NAPQI adducts (Huggett and Blair, 1983). Others have suggested that because of the oxidising properties of NAPQI, thiol groups in important enzymes might be oxidised to disulphides, thereby altering their ability to function normally (Tee *et al.*, 1986). In this respect, the membrane bound ATP-dependent Ca2+ translocases have been suggested as critical targets of NAPQI oxidation (Weis *et al.*, 1991). Their loss of activity would result in an increase in intracellular Ca^{2+}, and cause disruption of the cytoskeleton and plasma membrane, leading to cell blebbing and eventually death.

Glutathione (L-y-glutamyl-L-cysteinyl-glycine; GSH) is the key component of a ubiquitous antioxi-dant system that defends the cell against the toxic effects of reactive oxygen species. GSH maintains thiol groups of soluble and structural

proteins in the proper redox state, and also functions in a scavenging system similar but complementary to enzymes like super oxide dismutase (Blum and Fridovich, 1985). GSH has several important cellular functions. GSH participates as coenzyme and is involved in amino acid transport. It is involved in metabolism and the maintains of the thiol moieties of proteins and low molecular weight compounds, such as cysteine and coenzyme A. (Clerici *et al.*, 1996).

GSH is an important mediator of Ace metabolism/detoxification. Increasing GSH with n— acetylcysteine resulted in resistant cell lines. It is shown that GSH appears to be a key player in determining the consequences of ace exposure including whether apoptosis will occur. The sensitivity of various tumor cell lines to Acet-induced growth inhibition and apoptosis has been shown to be inversely related to intracellular GSH Concentration (Anderson, 1998; Lauterburg *et al.*, 1983).

Whether GSH is the principle parameter in the Acetaminophen cytotoxicity is the main question of our research. If it is so, the intracellular amount of initial GSH, or its consumption after exposure to Acetaminophen should present the degree of cellular resistance. Variations in cellular GSH content and consumption after the exposure to Acetaminophen should then represent the degree of sensitivity and resistant to this drug in different cell lines. In this study, we have measured the cellular toxicity of Acetaminophen in four different cell lines from different tissues, as well as the cellular GSH levels before and after the exposure to acetaminophen.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisen- hofen, Germany). Standard GSH solutions were prepared freshly for each experiment in 6% (v/v) met phosphoric acid and diluted in phos-phate-EDTA buffer (pH 7.4).

Cell lines and clonogenic assay

Human hepatoma cell (HepG2) was purchased from the Pasteur institute in Tehran (Iran). Human lung carcinoma (A549), Human Ovary carcinoma (SKOV3), Human renal normal cell (LLCPK1) and Chiness hamster ovary (CHO) were as generous gift from Dr. Rakesh Goel, Ottawa Regional Cancer Center, Ottawa, Canada.. All cells were grown in DMEM/ F12 media (Gibco BRL, USA) supplied with 10% fetal bovine serum and penicillin (100IU/ml)/streptomy-cin (100IU /ml), Cells were maintained in a humidified 37°C incubator with 5% CO2 for three passages before the start of experiments. Cells were exposed to the different concentrations of Acetaminophen in a range of 0.5 to 20 µM for 2 hours. Clonogenic assay was carried out after the cell exposure to acetaminophen based on Von Hoff method (Von Hoff *et al.*, 1985; Conney, 2003).

GSH assay

GSH analysis was performed spectrophotometrically according to the enzymatic method. In brief, different cell lines (HepG2, A549, SKOV3, LLCPK1and CHO) were grown to 80% confluences as was described above, in 25 mm plates. Experiment and control cells were harvested and collected in 1 ml of PBS , Comnted and subsequently stored at -20°C. Determination of GSH was performed by a modification of the method of Cohn and Lyle. To 0.5 ml of the 100,000g supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0 was added. The final assay mixture (2.0 ml) contained 100 µl of the diluted cell lines supernatant, 1.8 ml of phosphate- EDT A buffer and 100 µl of the OPTsolution. Containjng 100 µg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to aquartz cuvette. Fluorescence at 420 nm was determined with the activationat350 nm (21, 22).

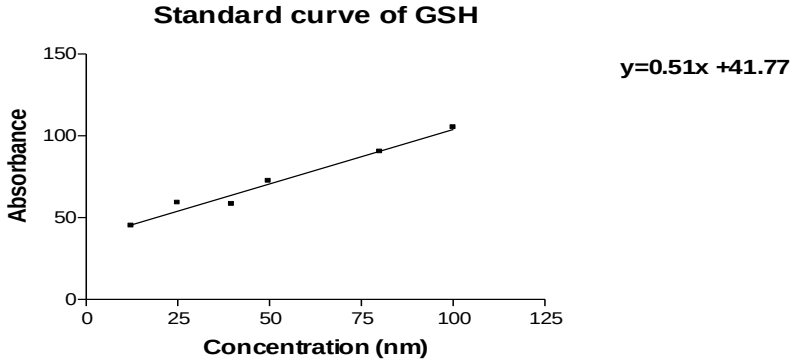

Fig.1. Standard Curve of GSH.

The total amount of glutathione in the samples was determined as GSH (µmol) Per million cells using a standard curve obtained by plotting the known amounts of GSH (100; 80; 50; 40; 25 and 12.5µm), incubated under the same experimental conditions, versus the rate of change of absorbance at 420nm [r =0.989] .

Statistical Analysis

The results are expressed as mean± S.E.M. Differences between means were elaborated by one way analysis of variance or the kraskal – wallis method for multiple comparison. Differences at $P<0.05$ were considered to be significant. IC_{50} s were calculated using Graph pad prism software.

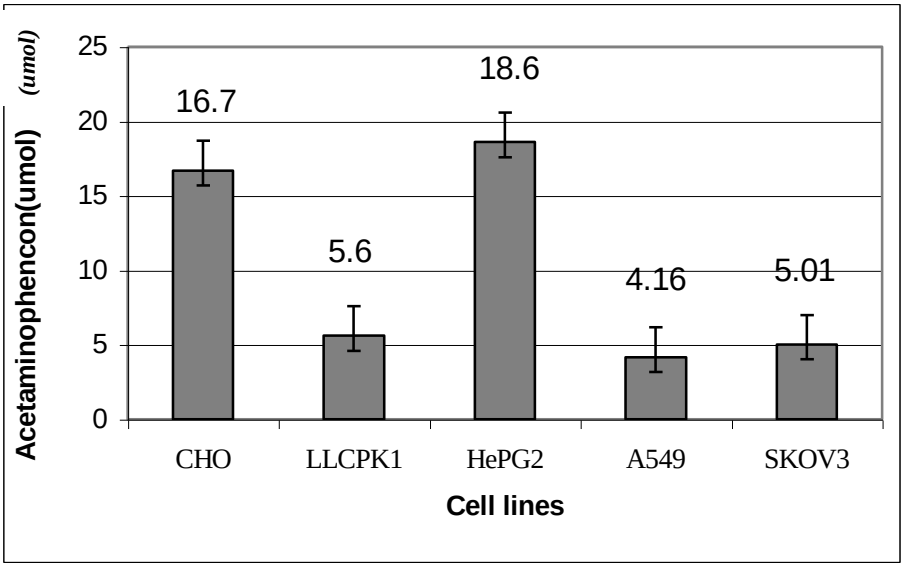

Fig. 2. Comparison of acetaminophen IC_{50} (μmol) on different under study cell lines.

RESULTS

Fig. **2** represents the IC50 amounts of acetaminophen in different under-investigation cell lines(HepG2, A549, SKOV3 ,LLCPK1 and CHO). The IC_{50}s of acetaminophen in all under-investigation cell lines are A549: 4.16 ± 0.35, HepG2: 18.60 ± 1.29, SKOV3: 5.01 ± 0.36, LLCPK1: 5.60±0.87 and CHO: 16.70±1.06

As is shown in this figure, the rank order of IC_{50}s is A549 < SKVO3< LLCPKl<CHO< HepG2. Interestingly, there is a statistically significant difference ($P<0.05$) between the IC_{50} of acetaminophen in (CHO and HepG2) versus other cell lines (A549, SKVO3 and LLCPK) but no significant differences between CHO and HepG2 and/or between A549, SKVO3 and LLCPK. However,

Fig. **3** represents the concentrations of GSH in these cell lines in nmol/g, before and after the 2 hours exposure to the IC_{50}s of acetaminophen for each cell line. Based on this figure, the rank order of total GSH concentration for under investigation cell lines is as CHO< LLCPKl in normal cell lines and HepG2 < A549 < SKOV3 in tomur cell lines. After the exposure to acetaminophen, the rank order of GSH concentration is similar to previous . As is shown in this figure, the maximum and minimum cellular levels of GSH after the exposure to acetaminophen are belong to LLCPKl in normal cell lines and HepG2 in cancer cell lines with the GSH concentrations of 1367.30±113.10 and 24.21±1.00 nmol/g, respectively.

DISCUSSION

In this study we have investigated the effects of acetaminophen on cellular total GSH level on tumor cell lines (HePG2, A549, SKOV3) and normal cell lines (LLCPK1, CHO). Our results showed that the cellular total level of GSH in various cell lines after exposure to the corresponding IC_{50} concentrations of acetaminophen in each cell line was altered differently. As is shown in this paper, compare to the matched controls, the cellular GSH level increased in LLCPK1 and A549 cell lines, but decreased in CHO and HepG2 cell lines. However, the cellular total GSH was statistically remains the same in SKOV3 cell line.

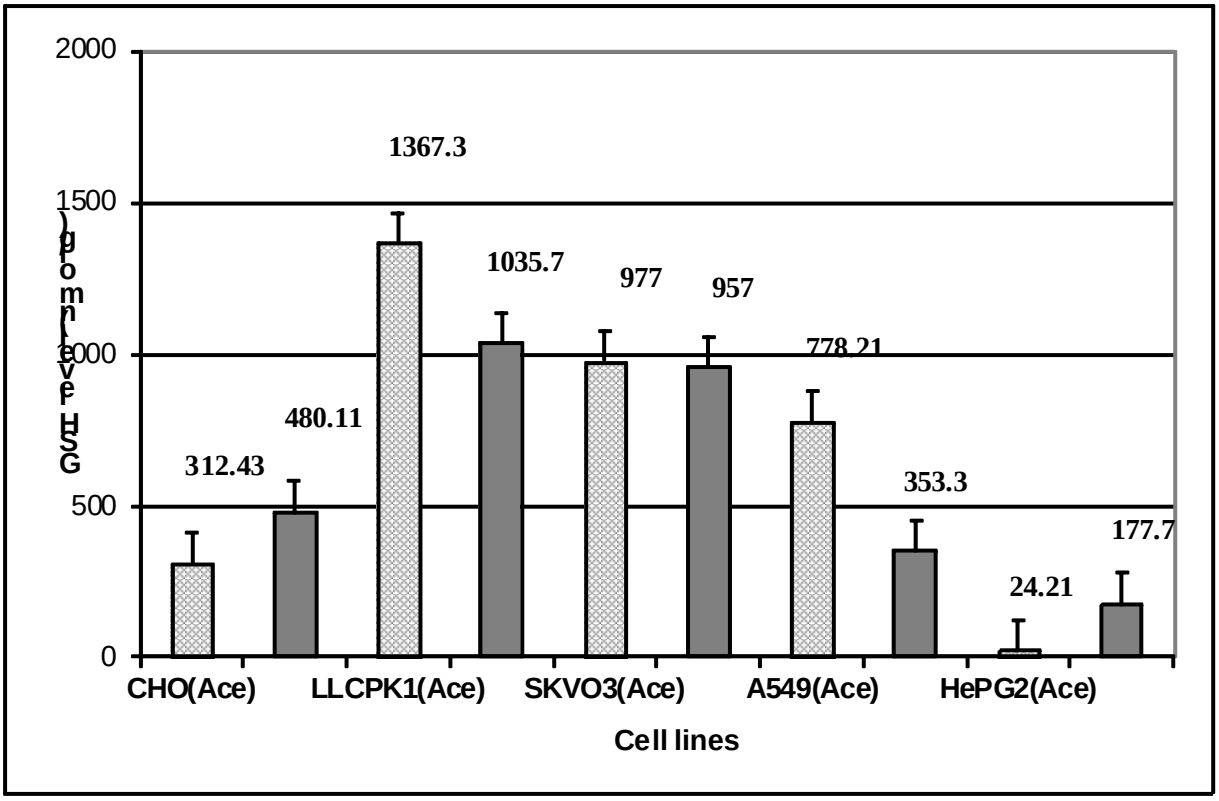

Fig. 3. GSH levels in cell lines.

HepG2 and CHO cell lines are the most resistant cells among the five different cell lines in our study. These are also the two cell lines in which the cellular GSH levels are decreased after the exposure to acetaminophen. On the other hand, A549 and LLCPK1 cell lines, which are the most sensitive cells to acetaminophen in this study, have shown an increase in their cellular GSH levels following the exposure to acetaminophen. The cellular total GSH level in SKOV3 cell line, which is in the middle ranking of sensitivity to acetaminophen cytotoxicty, remains the same after exposure to this drug.

Acetaminophen by itself does not react with GSH as is shown in Fig. **4**. Therefore, all changes in GSH absorbencies that have seen in this study are related to the acetaminophen biotransformation differences in different cell lines. Many studies on the mechanisms of acetaminophen-induced liver injury have demonstrated that GSH plays an important role in the detoxification of NAPQI, the reactive and toxic metabolite of acetaminophen, and that liver necrosis begins when GSH stores are almost exhausted (Mitchell *et al.*, 1973). Cysteine pro-drugs, such as N-acetylcysteine, have been reported to protect the liver against acetaminophen-induced injury. These pro-drugs are thought to be metabolized to L-cysteine, thereby supplying a component for hepatic GSH synthesis and consequently protect liver from injury (Corcoram and Wong, 1986; Haz *et al.*, 1986; Lauterburh *et al.*, 1987; Miner *et al.*, 1984; Roberts i*t al.*, 1987). We would therefore conclude that acetaminophen cellular fate might affect its cellular toxicity. It is quite possible that cellular biotransformation of acetaminophen produces various metabolites though different pathways. Different metabolites of acetaminophen might either bind to GSH, and/or signal DNA for more production of GSH. Preference of any of these pathways for the production of various metabolites in different cell lines would indicate the cytotoxicity outcome of acetaminophen in various tissues. As is shown in this study, the cellular metabolism pathway of acetaminophen in CHO and HepG2 cell lines is in favor of the production of NAPQI or some other GSH binding species that neutralize acetaminophen. That is why the cellular GSH in these two cell lines is decreased after the exposure to acetaminophen, and cells are also protected from its cytotoxicity. On the other hand, the superior metabolic pathway in A549 and LLCPK1 cells result non-GSH binding species. Although these two cell lines are also producing higher levels of GSH as a result of exposure to acetaminophen, but are very sensitive to its cytotoxicity, since the cellular GSH in these two cell lines is not able to react with the produced cellular metabolites of acetaminophen. The cellular metabolism of acetaminophen in SKOV3 is neither in favor of the production of GSH binding, nor GSH expression signaling metabolites. Our study shows that there is no unique differential pattern between tumor and normal cells for the cellular metabolism of acetaminophen.

Further studies are required to identify the cellular metabolites of acetaminophen in each of these cell lines, as well

as the generalization of this finding for other chemicals which neutralize by cellular GSH.

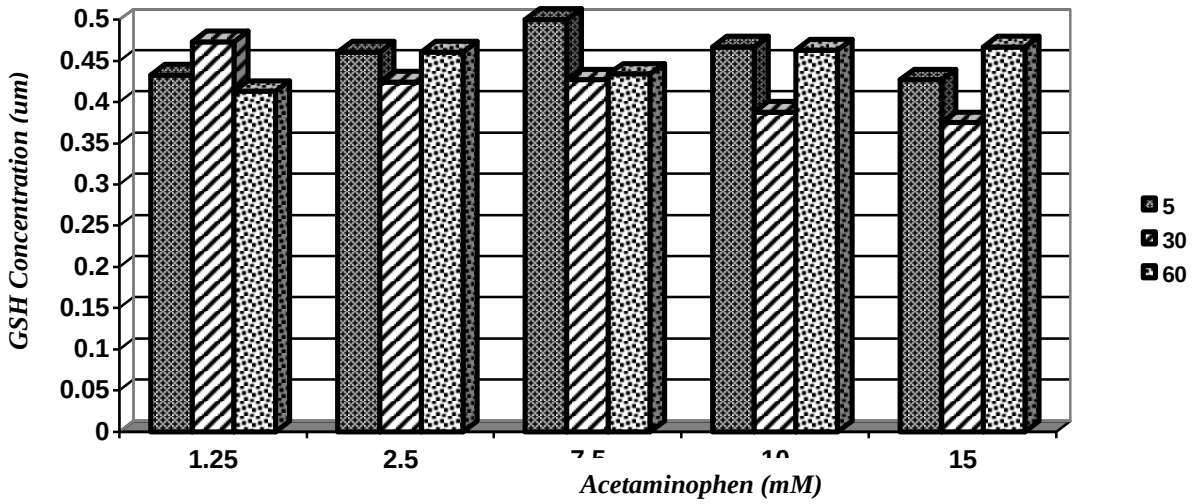

Fig. 4. Kinetic GSH concentration with acetaminophen.

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