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Oxidative Stress Induced by Iodoacetamide (An Emerging Disinfection By-Product) on HepG-2 Cells

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Abstract

Iodoacetamide (IAcAm) is a type of emerging nitrogenous disinfection by-product (N-DBP) with high health risk. Up to now, several studies have been carried out on the toxicity of IAcAm, but the study on oxidative damage of IAcAm on human cells was not available. In this study, the oxidative stress and damage induced by IAcAm on HepG-2 cells were investigated. Results showed that superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity exhibited a decreasing trend; but nuclear factor erythroid 2-related factor 2 (*NRF2*) and glutamate cysteine ligase catalytic subunit (*GCLC*), both the mRNA and protein level, generally appeared an increasing trend with the increase of IAcAm concentration. These results suggested that IAcAm-exposed cells produced excessive reactive oxygen species (ROS) and initiated the compensation mechanism of NRF2 to deal with oxidative stress. Malonaldehyde (MDA), an index for oxidative damage, had no obvious change at 24 h ($p>0.05$) but significantly increased at 48 h ($p<0.05$). This result indicated that HepG-2 cells could protect themselves from ROS attack by consuming antioxidant enzyme (e.g., SOD and GSH-Px) and upregulating the genes related to antioxidation after 24 h exposure of IAcAm; yet, at 48 h, the antioxidant defense system could no longer prevent oxidative damage of ROS, causing severe damage of the lipid membrane.

Introduction

To prevent the occurrence of water-borne diseases and ensure the safety of drinking water, the use of disinfectants (such as chlorine, chloramines, and ozone) is indispensable in the water treatment process. However, disinfectants tend to react with organic matter in water to generate disinfection by-products (DBPs) (Krasner *et al*., 2006; Zhou *et al*., 2019; Xu *et al*., 2022), which have carcinogenic, teratogenic and mutagenic effects (WHO 2000; Richardson

Abbreviation List (in alphabetical order)	
Full name	**Abbrev.**
disinfection by-products	DBPs
glutamate-cysteine ligase catalytic subunit	GCLC
glutathione	GSH
glutathione peroxidase	GSH-Px
haloacetamide	HAcAm
iodoacetamide	IAcAm
iodinated haloacetamides	I-HAcAms
malonaldehyde	MDA
nitrogenous disinfection by-products	N-DBPs
nuclear factor erythroid 2-related factor 2	NRF2
optical density	OD
phosphatic buffer solution	PBS
reactive oxygen species	ROS
superoxide dismutase	SOD
tris-buffered saline and tween 20 solution	TBST

et al., 2007; Li and Mitch 2018). Up to now, more than 600 kinds of DBPs have been found in drinking water (Cortes and Marcos 2018). Among them, trihalomethanes and haloacetic acids are the most abundant, and have been regulated by many countries and regions (Richardson *et al.*, 2007; Ding *et al*., 2013; Lin *et al.*, 2018; Zheng *et al.*, 2020; Weng *et al.*, 2022). Nitrogenous and iodinated DBPs such as haloacetamide (HAcAms) are usually in low levels in drinking water and have not been regulated (Krasner *et al*., 2006; Ding *et al*., 2013; Dong *et al*., 2019), yet it has attracted much attention due to its extremely high cytotoxicity and genotoxicity (Richardson *et al.*, 2007; Wagner and Plewa 2017; Hong *et al*., 2023). Many studies have been carried out on the toxicity of haloacetonitriles since the 1980s (Lin *et al*., 1986; Ahmed *et al*., 1989; Ahmed *et al*., 1991; Lipscomb *et al*., 2009; Komaki *et al*., 2014; Dong *et al*., 2018b), but the toxicology research on HAcAms, especially on iodinated haloacetamides (I-HAcAms), remained sparse. This may be because commercial standards for I-HAcAms have not been available until recently.

Among the I-HAcAms species, the earliest commercially available standard was for iodoacetamide (IAcAm), which was widely used as a typical thiol reagent in proteomic study in addition to its use as a DBP standard (Young 1980; Shau and Dawson 1985; Fuentes *et al*., 1994; Sarkany *et al*., 2000; Marchand *et al*., 2006; Schmidt and Dringen 2009). Therefore, IAcAm had been the subject of more toxicology studies as compared to other I-HAcAms species. Previous studies have shown that IAcAm causes extremely high cytotoxicity and genotoxicity to Chinese hamster ovary cells (Plewa *et al*., 2008), and apoptosis or necrosis in porcine kidney cells of LLC-PK1 (van De Water *et al*., 1999). Chen and Stevens (1991) reported that IAcAm caused lipid peroxidation to LLC-PK1 cells (as indicated by leakage of lactate dehydrogenase), but this damage can be reversed by addition of exogenous antioxidants. A study from Deng *et al*., (2014) showed that after mice were exposed to IAcAm for 30 days, the activity of antioxidant enzyme, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase were reduced; nevertheless the level of 8-hydroxy-2-deoxyguanosine, an indicator for DNA oxidative damage, increased. These studies provide important information about the toxicity of IAcAm, and indicate that oxidative stress may be an important mechanism driving IAcAm toxicity. But, these studies were mainly focused on animals or animal cells. Since toxicity and the related mechanism may vary by species (Kong 2012), it is necessary to investigate the toxicity and the mechanism of IAcAm using human cells, which may help to better understand its health risk to the people who may be exposed.

Though there are several studies available on the toxicity of IAcAm to human cells (e.g. HepG-2, CCD 841 CoN cell and MCF7), they are mainly focused on cell proliferation rates, apoptotic pathways and the reactivity of IAcAm with cellular proteome thiols (Sayess *et al*., 2017; Hong *et al*., 2018; Hall *et al*., 2020). Considering that oxidative stress has been considered the central mechanisms responsible for harmful effects caused by pollutants/toxicants including IAcAm (Deng *et al*., 2014; Sun *et al*., 2019; Zhang *et al*., 2021), and our previous study also detected significant reactive oxygen species (ROS) signal in IAcAm exposed HepG-2 cells, it can be inferred that oxidative damage may occur within human cells exposed to IAcAm (Hong *et al*., 2018). But, there is a data gap regarding the direct evaluation of oxidative stress of IAcAm on human cells.

Based on the above information, the HepG-2 cell line was used to investigate the oxidative stress on human cells induced by IAcAm. The parameters of SOD and GSH-Px, two important antioxidant enzymes, as well as malonaldehyde (MDA), an indicator for oxidative damage, were included. The expression of *NRF2* (nuclear factor erythroid 2-related factor 2, a main regulatory master of cell redox homeostasis) and *GCLC* (catalytic subunit of glutamate cysteine ligase, a rate-limiting enzyme during GSH synthesis) were also evaluated. It is hoped that the present study will provide a better toxicological basis for the health risk assessment of IAcAm and the management of drinking water.

2. Materials and Methods

Reagents and Kits. IAcAm (≥ 98.0%) was purchased from CanSyn (German); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, non-essential amino acids, penicillin-streptomycin, 0.25% trypsin, and phosphatic buffer solution (PBS) were obtained from Gibco USA; Dimethyl sulfoxide was purchased from American sigma company; Phenylmethanesulfonyl fluoride, Western and IP Lysate and BCA protein kit were provided by Shanghai Beyotime Biotechnology Company; SOD and MDA kits were bought from Nanjing Jiancheng Bioengineering Institute.

Cell culture and exposure. The HepG-2 cell line was pro-

vided by the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). The reason to choose HepG-2 cells in this study is as follows: the liver is an important organ of detoxification in the human body, so it makes sense to use the liver cells to study the toxic effects of foreign chemicals, including IAcAm. However, the enzymatic activity of the normal human primary hepatocyte is unstable and will be weakened during cell passage/cell proliferation. HepG-2 cells, originating from human liver cancer tissue, contain biotransformed metabolic enzymes with homology to human normal cells, and the enzyme activity is stable and easy to measure, facilitating toxicology studies. HepG-2 cells have been widely used as an in vitro model for the study of human toxicity to pollutants (Leekumjorn *et al*., 2008; Sohn *et al*., 2013; Tsai *et al*., 2017; Wen *et al*., 2020).

HepG-2 cells were cultured in DMEM supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids and 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HepG-2 cells in log phase were inoculated into a 6-well plate with a density of 5×10^5/well. After the cells attached to the wall, IAcAm exposure (5 μM, 10 μM, 15 μM, 20 μM) was performed. Solvent treatment (dimethyl sulfoxide ≤ 1%) was used as the control. When the exposure time reached 24 h and 48 h, the cells were harvested for further analysis.

Preparation of protein extracts. Cells treated with IAcAm were washed softly with pre-cold PBS twice and then all the PBS was discarded. After that, 200 μL of cell lysis buffer (190 μL cell lysis buffer+10 μL Phenylmethanesulfonyl fluoride) was added. The whole cell lysis buffer was transferred to the tubes on ice and was treated by ultrasonic wave. Then the tubes were centrifuged at 4°C for 20 min at 12000 rpm. The supernatant was measured by the BCA protein kit.

Detection of MDA and SOD. SOD was measured using xanthine oxidase assay in this study. Xanthine oxidase catalyzes xanthine to produce superoxide anion radical (O_2^-), and O_2^- oxidizes hydroxylamine to form nitroso salt. The nitroso salt can react with chromogenic agent (P-aminophenol sulfonic acid and methamamine) to form purple red color, which can be quantified by UV-visible spectrophotometer. When the tested sample contains SOD, the nitrite formation will reduce, the measured absorbance value is lower than the control tube, and the SOD activity of the tested sample can be obtained through formula calculation. The reagents and the specific procedures followed the kit instructions.

The formula for calculation is listed below as Equation 1. In Equation 1, OD means optical density at 550 nm. OD_c means the OD value of the control sample; OD_t means the OD value of the test sample; $V_{reaction}$ means the volume of the reaction solution; V_{sample} means the volume of the test sample; and Con_{prot} means protein concentration.

MDA was measured using thiobarbituric acid (TBA) assay. The condensation reaction between TBA and MDA results in the formation of red products with a maximum absorption peak at 532 nm. The absorbance at 532 nm was measured with an UV-visible spectrophotometer, and then the MDA content was calculated by the formula. The reagents and the specific procedures followed the kit instructions.

The formula used for calculation is listed below as Equation 2. In Equation 2, OD means optical density at 532 nm. OD_t means the OD value of the test sample; $OD_{t\text{-}bk}$ means the OD value of the test blank sample; OD_{st} means the OD value of the standard sample; $OD_{st\text{-}bk}$ means OD of the standard blank sample; Con_{prot} means protein concentration.

RT-PCR assay. Cells were cultured in a series of 6-well plates at an initial density of 3×10^5/well. After exposure to IAcAm for 4 h, total RNA was isolated with the RNA prep pure Cell/Bacteria Kit. The concentration, as well as the purity of RNA, was measured with Nano Drop. Reverse transcription was performed following the steps of kit instructions. The obtained cDNA was stored at -80°C. A two-step method was used to determine the gene expression. The primer sequence of *NRF2*, *GCLC* is presented in Table 1. The sequence.18S, was used as the internal control. PCR procedures were carried out following the steps below: set temperature at 95°C to make the cDNA denaturate, then amplification through 39 cycles (95°C for 5 seconds, annealing at 60°C for 30 seconds, extension at 72°C).

Equation (1)

$$SOD(U/mgprot) = \frac{(OD_c - OD_t) \times V_{reaction}}{OD_C \times 50\% \times V_{sample} \times Con_{prot}}$$

Equation (2)

$$MDA\left(\frac{nmol}{mgprot}\right) = \frac{(OD_t - OD_{t-bk}) \times 10}{(OD_{st} - OD_{st-bk})\, Con_{prot}}$$

Primer		Sequences of primers (5' to 3')
NRF2	F	AACCAGTGGATCTGCCAACTACTC
	S	CTGCGCCAAAAGCTGCAT
GCLC	F	GATGCTGTCTTGCAGGGAATG
	S	AGCGAGCTCCGTGCTGTT
18S	F	CAGCCACCCGAGATTGAGCA
	S	TAGTAGCGACGGGCGGTGTG

Table 1. Sequences of primers used in quantitative RT-PCR

Western blotting. Western blotting was carried out after HepG-2 cells were exposed to IAcAm for 24 h. The protein was quantified with the BCA Protein Assay Kit. After adjusting the protein of each treatment to the same level, they were separated by electrophoresis on SDS-PAGE gel. The protein was then transferred to nitrocellulose membrane and blocked with 5% BSA at room temperature for 1 h. Antibodies of NRF2 (1:1000), GCLC (1:1000) and β-actin (internal control,1:1000) were incubated overnight at 4°C. Mouse and anti-rabbit IgG (1:5000) were washed with tris-buffered saline and tween 20 solution (TBST) extensively, and then used as the secondary antibodies and incubated for 1 h, and washed with TBST again (3 times,10 min/time). The images were obtained through Kodak 4000 MM (Kodak, Japan).

3. Results

3.1 Effect of IAcAm on antioxidant enzymes

SOD activity in HepG-2 cells (*Fig. 1a*) showed no significant change when treated with low concentration of IAcAm (5 μM) ($p>0.05$). But when the dose of IAcAm increased to 10~20 μM, the activity of SOD decreased significantly ($p<0.05$) as compared with solvent control (*Fig. 1a*). Moreover, from 5 to 20 μM IAcAm, SOD activity decreased in a concentration-dependent manner (*Fig. 1a*). The trend of GSH-Px activity (*Fig. 1b*) also showed a similar pattern to that of SOD (decreasing with IAcAm concentration),yet appeared less sensitive as compared to SOD. The significant reduction of GSH-Px activity was observed at 15 (48 h) or 20 μM (24 h and 48 h).

3.2 Effect of IAcAm on MDA level

The MDA level showed no significant change in HepG-2 cells at 24 h treatment as compared with the control (*Fig. 2*). However, as the exposure time prolonged to 48 h, the MDA levels increased significantly ($p< 0.05$) in all IAcAm treatment groups (5-20 μM), and that increase occurred in a dose-dependent pattern (*Fig. 2*). MDA levels increased by 53%, 59%, 76% and 76% at 5, 10, 15 and 20 μM concentrations, respectively.

3.3 Effect of IAcAm on NRF2 and GCLC gene expression

IAcAm exposure significantly influenced the *NRF2* transcriptional levels (*Fig. 3*). The expression of *NRF2* was firstly inhibited at 5 μM IAcAm (66% of the control), and then slowly recovered at 10 μM (80% of the control) and 15μM IAcAm (103% of the control). At 20μM, the mRNA expression of *NRF2* increased markedly (231% of the control).

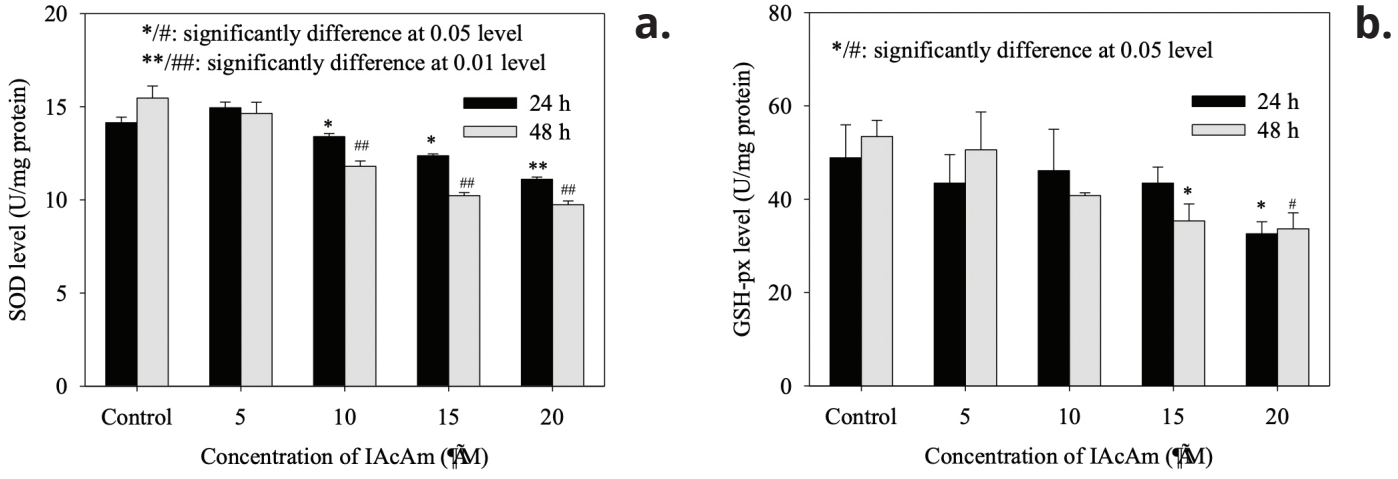

Figure 1. SOD and GSH-px activity after HepG-2 cells exposed to IAcAm (Data were expressed as average ± standard deviation from three replicates; "*" and "#" were used to mark significant difference as compared to the control for 24 h and 48 h treatment, respectively.

The NRF2 protein level expressed in the IAcAm treatment group also showed a similar pattern with mRNA expression (*Fig.4*).

Gene expression of *GCLC* was inhibited (decreased by 56%) significantly ($p<0.05$) at 5 μM, but increased by 31%, 117%, 587% when IAcAm concentration increased to 10, 15 and 20μM, respectively (*Fig. 3*). Western-blot results for protein expression also showed a similar trend (*Fig. 4*), demonstrating the validation of gene expression. Overall, the trend of *GCLC* expression was consistent with *NRF2, i.e.,* inhibited at low IAcAm concentrations, recovered/activated at high IAcAm concentrations.

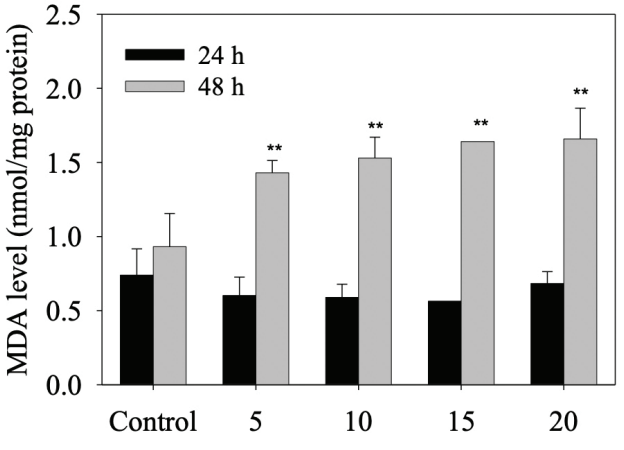

Figure 2. MDA level in HepG-2 cells induced by IAcAm (Data were expressed as average ± standard deviation from three replicates; "**" means significant difference compared to the control at 0.01 level.)

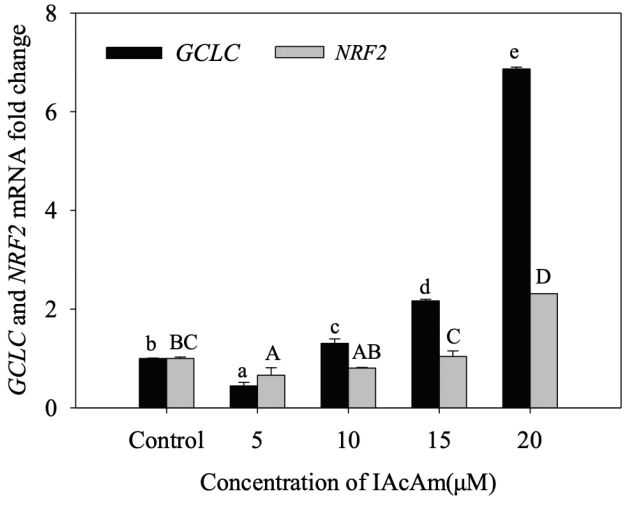

Figure 3. The mRNA expression of NRF2 and GCLC in HepG-2 cells after exposure to IAcAm. (Data were expressed as average ±standard deviation from three replicates; same letter means no significant difference ($p<0.05$) according to Duncan test; lowercase letter for GCLC and uppercase letter for NRF2).

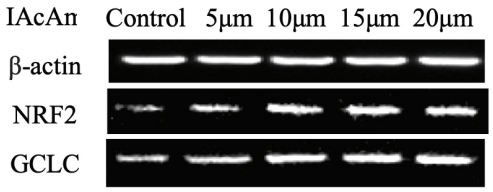

Figure 4. The protein expression of NRF2 and GCLC in HepG-2 cells after exposure to IAcAm.

4. Discussion

SOD and GSH-Px, two important antioxidant enzymes in biological systems, are responsible for removing dangerous ROS (e.g. superoxide anion and peroxide) and protecting cells from oxidative damage (Kong 2012; Mukherjee *et al*., 2015; Dong *et al*., 2018a; Wang *et al*., 2020). Therefore, activity of SOD and GSH-Px can reflect the body's ability to scavenging ROS and are often used to evaluate oxidative stress (Ma *et al*., 2008; Nong *et al.,* 2016; Ma *et al*., 2021). In this study, both the SOD and GSH-Px activity decreased significantly at high IAcAm concentrations (*Fig. 1a*), suggesting that high levels of IAcAm exposure caused oxidative stress, and it increased with IAcAm concentrations. This result was consistent with the previous study on IAcAm, where the ROS level (a direct index for oxidative stress) increased to 1.0, 1.3, 1.4 and 2.0 times of control at 5, 10, 15 and 20 μM of IAcAm, respectively (Hong *et al*., 2018). We theorize that, to deal with the high level of ROS, SOD and GSH-Px were gradually exhausted, and excessive ROS could, in turn, inactivate the activities of SOD and GSH-Px. Furthermore, the variation trends of SOD and GSH-Px activity were in agreement with the cytotoxicity of IAcAm on HepG-2 cells observed in a previous study (Hong *et al*., 2018), where the cell viability reduced in a dose-dependent manner as the IAcAm level increased from 0 to 20 μM, further indicating that oxidative stress may be the cause for the cytotoxicity of IAcAm. The significant reduction of SOD and GSH-Px activity in the IAcAm-treated group suggested that the cells generated ROS, outweighing the self-scavenging capacity, and may cause oxidative damage, which can be seen from MDA levels occurring within the HepG-2 cells.

MDA is the final product of membrane lipid peroxidation and a sensitive diagnostic parameter for oxidative damage of cells (Kong 2012; Sun *et al*., 2020; Gawron-Skarbek *et al*., 2021). In this study, the MDA level in HepG-2 cells showed no significant change at 24 h but enhanced greatly at 48 h (*Fig. 2*). These results suggested that during the first 24 h of exposure, the cells can protect them-

selves from ROS attack through initiating an antioxidant system (such as sacrificing SOD, GSH-Px). Yet, at 48 h, the ROS could not be scavenged completely by the antioxidant system and this caused oxidative damage on the cell membrane. Moreover lipid peroxidation products represented by MDA can further polymerize or cross-link with proteins/enzymes/nucleic acid within the cell, aggravating membrane damage and cell toxicity (Kong 2012).

Combining the results of SOD, GSH-Px, and MDA, we conclude that short term IAcAm exposure is harmful to HepG-2 cells, which produce excessive ROS, not only causing the significant reduction of SOD and GSH-PX, but also damaging the cell membrane and leading to a significant accumulation of MDA. Since the exogenous antioxidant can also be a good quencher for ROS occurring in the organism/cell (Chen and Stevens 1991; Kong 2012), the addition of dietary antioxidants (e.g. N-acetylcysteine, vitamin C) may block the toxicity of IAcAm on human cells.

In response to oxidative stress, the cell will tweak at the gene expression level spontaneously (Kalayarasan *et al*., 2009; Liu *et al*., 2017; Li *et al*., 2021). NRF2 is a vital protein in regulating the process of the antioxidant reaction (Copple *et al*., 2008; Soetikno *et al*., 2013; Choudhury *et al*., 2021). Under normal physiological conditions, NRF2 mainly binds to its inhibitor Keap1 and exists in the cytoplasm in an inactive state. When cells are subjected to oxidative stress, NRF2 will dissociate from Keap1, translocate to the nucleus, and bind to the ARE (anti-oxidation response element) sequence, and activate the expression of a variety of antioxidant enzymes (e.g. SOD, GSH-Px, etc) (Antelmann and Helmann 2011; Yamamoto *et al*., 2018; Patinen *et al*., 2019; Wang *et al*., 2020). In this study, the expression of *NRF2* (both the mRNA and protein level) was firstly inhibited and then slowly recovered, and finally increased markedly. Combining the results of antioxidant enzymes, the trend of *NRF2* expression at 5-20μM IAcAm was opposite to the trend of SOD and GSH-Px activity. This can be attributed to the compensation mechanism of *NRF2*, which can upregulate its transcriptional level to deal with oxidative stress via adjusting antioxidant enzymes (e.g., SOD, GSH-Px, etc). But because of the excessive ROS, the increased SOD/GSH-Px level stimulated by NRF2 was still not enough to scavenge free radicals, and the SOD/GSH-Px activity still reduced significantly as compared to the control (*Fig.1*).

GCLC is one of the downstream target genes of *NRF2*; it is the catalytic subunit of glutamate cysteine ligase, a rate-limiting enzyme during synthesis of GSH (Guan *et al*., 2015; Zhang *et al*., 2019), which can remove hydroxyl radicals and provide reducing power to other antioxidant enzymes (Atkuri *et al*., 2007; Kong 2012). In the present study, the trend of *GCLC* gene expression (both mRNA and protein level) was consistent with *NRF2*, *i.e.,* inhibited at a low exposure level, but recovered/stimulated at a high exposure level, indicating it was strictly regulated by *NRF2*. The significant increase of *GCLC* expression in a high IAcAm concentration reflected the great demand for GSH production, which may be used to 1) resist the excessive ROS induced by IAcAm (Hong *et al*., 2018); and 2) directly react with IAcAm to protect the thiol-containing proteins and enzymes in the cell as IAcAm was a well-known thiol-alkylating reagent (Schmidt and Dringen 2009; Pals *et al*., 2017; Hall *et al*., 2020).

4. Conclusions

At 24 h, HepG-2 cells can marginally resist the ROS caused by IAcAm through consumption of antioxidant enzymes (SOD, GSH-Px) and upregulating the genes related to oxidative stress (*NRF2*, *GCLC*). However, when the exposure time was prolonged to 48 h, the antioxidant defense system could not resist ROS induced by IAcAm anymore, leading to severe oxidative damage and significant accumulation of MDA. The results in this study demonstrated that oxidative stress is an important mechanism for the toxicity of IAcAm on human cells, which suggested that dietary antioxidants may be a good way to prevent the health risk from IAcAm in drinking water.

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