# University of Alberta

**Enhancement of menadione cytotoxicity by bicarbonate: redox cycling and a possible role for the carbonate radical in quinone cytotoxicity**

**By**

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**Abstract**

We investigated the effect of bicarbonate on quinone redox cycling and cytotoxicity. A cell-free system utilized menadione and ascorbic acid to catalyze a redox cycle, and we utilized murine hepatoma (Hepa1c1c7) cells for in vitro experiments. Experiments were performed using low (2 mM) vs physiological (25 mM) bicarbonate levels in buffer equilibrated to physiological pH. We found that menadione redox cycling was enhanced by bicarbonate using oximetry and ascorbic acid oxidation. Furthermore, we treated Hepa1c1c7 cells with menadione and found that cytotoxicity and oxidative stress (dichlorofluorescin oxidation) was significantly increased with physiological bicarbonate-containing media. Interestingly, the inhibition of superoxide dismutase (SOD) showed a protective effect against menadione cytotoxicity. Using isolated BSA protein, we found a significant increase in protein carbonyls with menadione/ascorbate/SOD with physiological bicarbonate levels; low bicarbonate or SOD-omitted reactions produced less protein carbonyls. In conclusion, these findings suggest that the hydrogen peroxide generated by menadione redox cycling together with bicarbonate are substrates for SOD peroxidase activity that leads to carbonate radical which enhances cytotoxicity. These findings may represent an additional mechanism of quinone-induced toxicity.

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**List of abbreviations**

**ASC** ascorbate

**$ASC^{\bullet-}$** ascorbyl radical

**ATP** adenosine triphosphate

**BSA** bovine serum albumin

**$CO_3^{\bullet-}$** carbonate radical anion

**DCFH-DA** dichlorodihydrofluorescein diacetate

**DDC** diethyldithiocarbamate

**DHA** dehydroascorbic acid

**DMEM** Dulbecco's modified Eagle's medium

**DMPO** 5,5-dimethyl-1-pyrroline-N-oxide

**DPBS** Dulbecco's phosphate-buffered saline

**DTPA** diethylene triamine pentaacetic acid

**EBSS** Earle's Balanced Saline Solution

**EPR** electron paramagnetic resonance

**Glut** glucose transporters

**GSH** glutathione

**GSSG** glutathione dimer (oxidized)

**$HCO_3^-$** bicarbonate

**$HCO_4^-$** peroxymonocarbonate

**HEPES** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid )

**$HO^{\bullet}$** hydroxyl radical

**$HO_2^{\bullet}$** hydroperoxyl radical

**$H_2O_2$** hydrogen peroxide

**mV** millivolts

**NQO** NAD(P)H:Quinone oxidoreductase

**$^{\cdot}NH_2$** amine

**$O_2$** oxygen

**$O_2^{\bullet-}$** superoxide anion radical

**$^1O_2$** singlet oxygen

**$pH_e$** extracellular pH

**$pH_i$** intracellular pH

**Q** quinones

**$QH_2$** hydroquinone

**ROS** reactive oxygen species

**-SH** nucleophilic thiol

**SODs** superoxide dismutases

**SOD1** copper,zinc- superoxide dismutase

**SOD2** manganese- superoxide dismutase

**SOD3** an extracellular- superoxide dismutase

**$SQ^{\bullet}$** semiquinone radical

**TEPA** tetraethylenepentaamine

**UV-Vis** ultraviolet-visible

**V** volts

# Chapter 1 Introduction

## 1.1 Quinone

### 1.1.1 Quinone structure and formation

Quinones are a general term for aromatic compounds that have a fully conjugated cyclic dione structure. The basic structure of quinone are depicted in Figure 1.1.1.

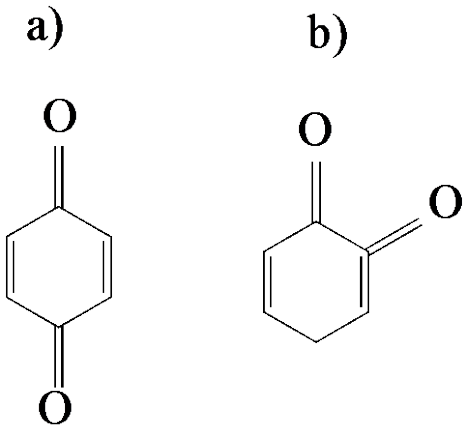



**Fig 1.1.1 Basic structure of quinone molecules.** Quinones exist as a) *para*-(1,4)-benzoquinones, or b) *ortho*-(1,2)-benzoquinones.

It has been reported that quinones are mainly produced via oxidation of aromatic compounds such as phenols, catechols and hydroquinone by monooxygenase or peroxidase, metal ions and molecular oxygen (discussed further in 1.1.3) [1]. The ability of quinones to accept and of their reduced forms to donate electrons is considered as a common feature in this family of compounds. Enzymatically and non-enzymatically, quinones can accept one-electron to form semiquinone radicals that subsequently reduces oxygen molecules to generate superoxide anion radicals. Consequently, forming the

latter radical leads to formation of hydrogen peroxide (discussed further in 1.1.3 and 1.1.4) [1]

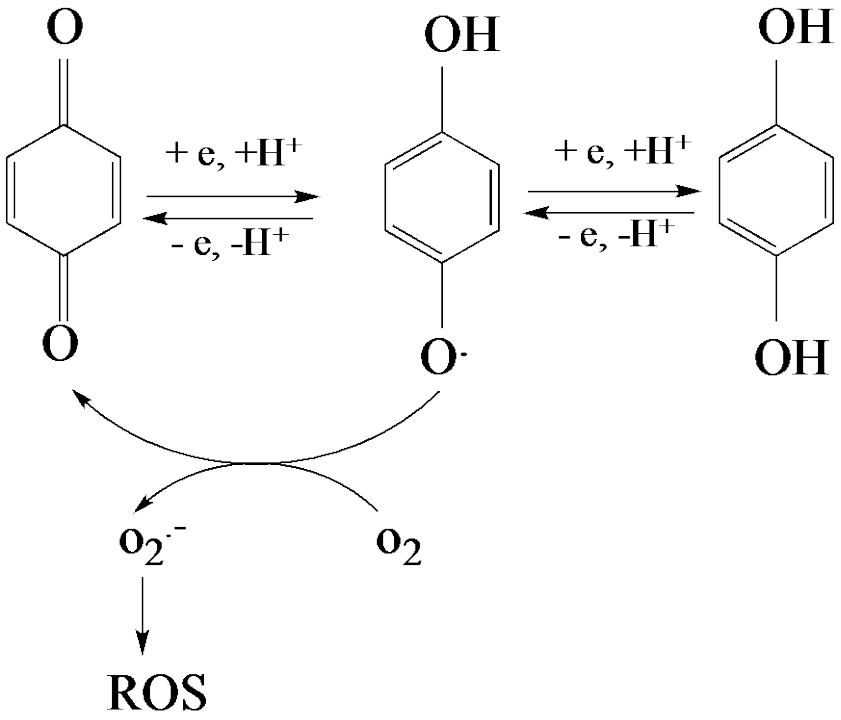



**Fig 1.1.2** **Basic scheme of a quinone redox cycle**. A quinone molecule can accept an electron from a reductant producing a typically unstable semiquinone radical. That latter can undergo further reduction to form a relatively stable hydroquinone, or reduce oxygen to produce superoxide anion radical ($O_2^{\bullet-}$) which can lead to reactive oxygen species (ROS).

### 1.1.2 Quinone sources

There are different sources for quinones that could be environmental and endogenous sources. Quinones occur as environmental pollutants. For example, benzene, which is used as an industrial solvent as well as a constituent of gasoline and cigarette smoke, is metabolized to p-benzoquinone that causes its toxicity (aplastic anemia, acute myelogenous leukemia) via forming a DNA adduct [2]. Tetrachloro-para-benzoquinone (chloranil) is a metabolized product of pentachlorophenol, a fungicidal wood preservative, which inflicts its toxicity through forming 8'-hydroxy-deoxyguanosine, an oxidation product of DNA [2]. It has been reported that naturally occurring naphthoquinones, including juglone (5-hydroxy-1,4-naphthoquinone) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) enhanced lipid peroxidation likely due to the presence of hydroxyl groups in the benzene moiety. Although these compounds exhibit their toxicological effects through their ability to undergo redox cycling and covalent binding to DNA and protein, some naphthoquinones such as lawsone (2-hydroxy-1,4-naphthoquinone) and lapachol (2-hydroxy-3-(3-methylbut-2-enyl)-1,4-naphthoquinone) did not stimulate lipid peroxidation, and would not form adducts with DNA [3].

The endogenous sources of quinones have mainly been shown to be formed from autoxidation of catecholamines (dopamine, noradrenaline, and adrenaline), which are neurotransmitters in the central nervous system, adrenal medulla ,and the sympathetic nervous system. Oxidation of these

catecholamines neurotransmitters results in aminochrome formation, which are cyclized catecholamine derivatives. The ability of aminochrome derivatives to undergo the redox cycling leads to oxidative stress, which was shown to be repressed in the presence of nicotine [4]. Furthermore, there are two major pathways for metabolism of estrogen that can lead to quinone formation. The hydroxylation of estrogen forms 2-and 4-catechol estrogens (CE) and another pathway is at the16 α-position. Further oxidation of catechol estrogens leads to form semiquinone and quinone molecules. In the presence of these structures with molecular oxygen and cytochrome P-450 reductase, the redox cycling could be completed and consequently, ROS could be formed [5,6].

### 1.1.3 Molecular mechanism of quinone toxicity

The molecular mechanisms of quinone cytotoxicity are comprised of two main components: a) covalent binding to macromolecules (protein, DNA) via Michael addition, and b) formation of ROS through redox cycling, resulting in oxidative stress that can oxidize lipid, protein and DNA. Alkylation by quinones (Q) via Michael 1,4-addition can be prevented by glutathione (GSH), which is present in millimolar amounts in the cell:

$$Q + GSH \longrightarrow QH_2 - GSH \qquad (1)$$

The nucleophilic thiol (-SH) moiety (Eq. 1) of GSH resembles protein cysteine; thus, GSH conjugation prevents the alkylation of other protein or DNA nuclophiles (e.g., amine ($^{-}NH_2$) groups of protein lysine, or guanine bases). Certain quinones, such as menadione, however, can oxidize

glutathione to thiyl radicals that dimerize to form oxidized glutathione dimers (GSSG):

$$Q + GSH \longrightarrow QH^{\bullet} + GS^{\bullet} \quad (2)$$

$$GS^{\bullet} + GS^{-} \longrightarrow GSSG^{\bullet -} \xrightarrow{O_2} GSSG + O_2^{\bullet -} \quad (3)$$

It should be noted that quinones that bind GSH can still become reoxidized and consume more GSH or participate in redox cycling reactions. A basic redox cycling reaction is shown in equations 4 and 5:

$$Q \xrightarrow{1e^-, H^+} QH^{\bullet} \text{ (semiquinone)} \quad (4)$$

$$QH^{\bullet} + O_2 \xrightarrow{-H^+} Q + O_2^{\bullet -} \quad (5)$$

It should be pointed out that $O_2^{\bullet -}$ (superoxide) formation can be regarded as a first step in the induction of "oxidative stress." As mentioned above, ROS may oxidize important cellular molecules. Although $O_2^{\bullet -}$ may be harmful itself, it is through its conversion to more reactive ROS that it can also induce oxidative stress. ROS are generally comprised of three main oxygen containing compounds (in order of increasing reactivity): hydrogen peroxide ($H_2O_2$), superoxide anion radical or hydroperoxyl radical ($O_2^{\bullet -}$, $HO_2^{\bullet}$, respectively), and hydroxyl radical ($HO^{\bullet}$). These species can oxidize protein, cleave the protein backbone, and cause DNA strand breaks. A biomarker for $HO^{\bullet}$ reacting with DNA is 8-deoxyguanosine modification, which can be readily analyzed. The $O_2^{\bullet -}$ is detoxified by superoxide dismutase, which results in the formation of $H_2O_2$ (discussed in section 1.5). This reaction also

occurs non-enzymatically, or can also be carried out by an antioxidant such as quercetin, found in onions [7].

The source for the $1e^-$ shown in eq. 4 can be ascorbate or ascorbic acid (Vitamin C), GSH, or enzymatic. Such enzymes that can carry out this one electron reduction include Complex I or NADH:ubiquinone reductase and NADPH cytochrome P-450 reductase [8,9]. However, a generally protective two electron reductase, NAD(P)H:Quinone oxidoreductase (NQO), can form the complementary hydroquinone ($QH_2$) metabolite of the benzoquinone (Eq. 6) that can be subsequently conjugated (to glucuronic acid and/or sulfate) and eliminated:

$$Q + NAD(P)H + H^+ \xrightarrow{NQO} QH_2 + NAD(P)^+ \qquad (6)$$

$QH_2$ + UDP-Glucuronic acid (UDPGA) → $QH_2$-glucuronic acid + UDP

Although two-electron reduction of hydroquinones has been reported to be the bioactivation step for reductively activated quinone drugs, it is primarily a protective enzyme [10].

### 1.1.4 Reduction potential of quinones

The capability to accept electrons from a donor can be assessed by the half-cell reduction potential, which is expressed in volts (V) or millivolts (mV) relative to the donors. The reduction of a quinone occurs in two one-electron steps and each step has a distinct reduction potential. There are various factors that have a significant influence on the reduction potentials of quinone/semiquinone/hydroquinone triad. The redox potential of quinone moieties is decreased by the presence of aromatic rings. Thus, the reduction

potential of anthraquinone moiety is lower than benzo- and naphthaquinone moieties. Also, the different substituents on the quinone rings have a significant effect on the redox potential. For example, a substituent of electron-donating groups would result in lowering the redox potential of quinone moieties (e.g $CH_3$, OH, $NH_2$). On the other hand, the redox potential of quinones would increase by the presence of electron-withdrawing groups (e.g $SO_3$, Cl, Br). When quinones accept an electron from a donor and transfer it to another compound, the second process cannot be carried out unless the redox potential of quinones is lower than the target compound [11]. Generally, the reduction potential must be high enough to allow the reduction of quinone by cellular reductase but not so high that it reduces the transfer of electron to molecular oxygen. It has been shown that quinones that have redox potential between -250 and + 50 mV are able to undergo redox cycling and consequently generate the ROS through oxidizing ascorbate. For example, the reduction potentials of 1,4-benzoquinone and duroquinone to form benzosemiquinone and durosemiquinones are +99 and -240 mV, respectively. Thus, the reduction of 1,4-benzoquinones by accepting one electron is easier than for duroquinones. However, transferring the electrons from benzosemiquinone to oxygen molecule is more difficult than for durosemiquinone [6,12,13].

## 1.2 Menadione (vitamin K3)

The most abundant forms of vitamins K are phylloquinones (vitamin K1), menaquinones (vitamin K2) and menadione (vitamin K3) as shown in figure 1.2.1. For instance, phylloquinones (vitamin K1), which have 3-phytyl substituent at the 3 position, are synthesized by plants and considered as a major source for vitamin K. Menaquinones (vitamin K2), which contain polyunsaturated isoprene units, are produced by bacteria, for example, bacteroides fragilis strains that are in the colonic flora of humans. The other form of vitamin K is 2-methyl-1,4-naphthoquinone (vitamin K3), which lacks substitution at position 3, is synthesized by selective oxidation of 2-methylnaphthalene. Although K vitamins play an essential role in a number of life's key biochemical processes, including blood coagulation, and bone metabolism and cell growth, reduced K vitamins may trigger oxidative stress in colonic epithelial cells [14-17]. This suggests that the naphthoquinone core in vitamin K1, K2 and K3 is critical key for generating ROS and oxidative stress.

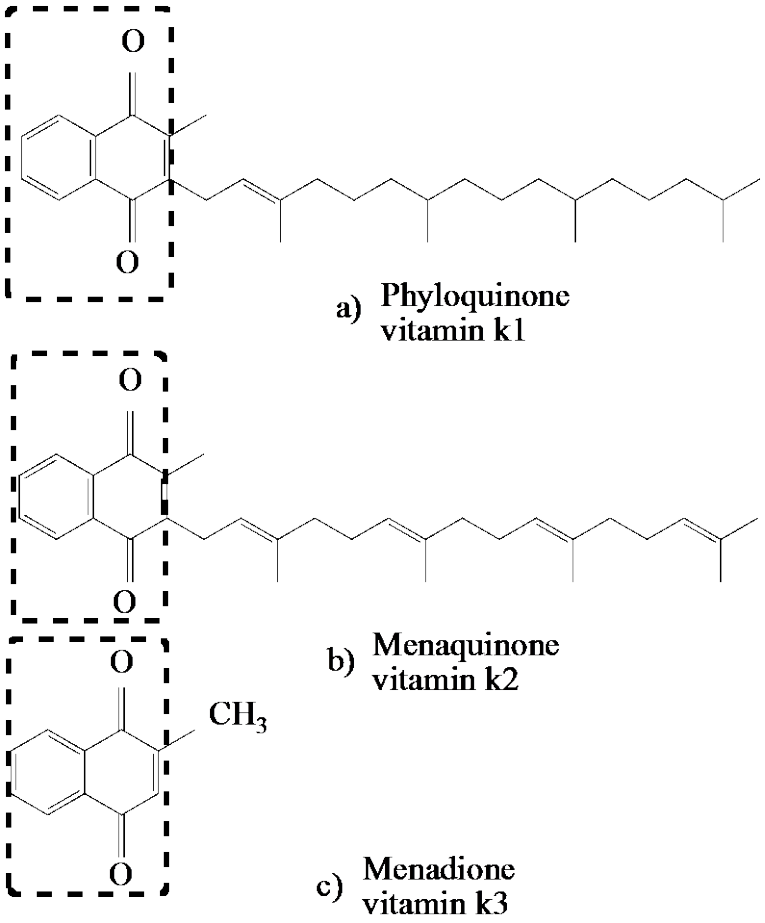



**Fig. 1.2** Structure of vitamin K compounds that are naturally occurring (a) and (b); and synthetically occurring (c).

Naphthoquinones derived from naphthalene are widely distributed in plants and can be used as chemotherapeutic drugs, antibacterial agents, antimalarials and fungicides. Compounds containing naphthoquinone such as juglone (5-hydroxy-1,4-naphthoquinone), plumbagain (5-hydroxy-2-methyl-1,4-naphthoquinone), lawsone (2-hydroxy-1,4-naphthoquinone) and menadione, a synthetic quinone, exert their pharmacological and toxicological effects through their ability to undergo enzymatic redox cycling to damage macromolecules, such as DNA, lipids and proteins. The structure of naphthoquinone compounds plays a critical role in their cytotoxicity. For example, juglone (5-hydroxy-1,4-naphthoquinone) is more toxic to hepatocytes than lawsone (2-hydroxy-1,4-naphthoquinone) because of inducing electron-attracting/electron-donating group. In juglone, the presence of the hydroxyl group on the benzene ring resulted in an increase of the reduction potential; however, the presence of a hydroxyl group on the quinone moiety led to a decreased reduction potential for lawsone. [7]

It has been reported that menadione readily diffuses in different cell types such as HepG2 cells, MCF-7 cells, and yeast without the assistance of transport proteins and pumps as it has an amphiphilic character; that is, menadione is both hydrophilic and hydrophobic to be soluble in water and plasma membrane. Menadione, 2-methyl-1,4-naphthoquinone, an artificial, synthetic, and water soluble naphthoquinone, has been shown to induce cell death by either apoptosis or autoschizis/ necrosis in a concentration-and time-dependent manner. For instance, a loss in cellular thiols, extensive

production of reactive oxygen species, such as $O_2^{\bullet-}$ and $H_2O_2$, DNA damage, the disturbance of intracellular calcium ion homeostasis, ATP depletion and cell death induced by a concerted mechanism of menadione on isolated rat hepatocytes [18-20].

Menadione is a well-studied quinone that is known for its ability to generate free radicals and hydrogen peroxide through redox cycling reactions and alkylation of macromolecules [21-23]. It has been reported that the redox potential of menadione is -203 mV. This indicates that menadione is able to undergo the redox cycling and consequently generate reactive oxygen species through oxidizing ascorbate. Menadione is reduced by ascorbate to form semidehydroascorbate and the menadione semiquinone free radical. Enzymatically, the semiquinone radicals are generated in vivo by one-electron reduction of menadione through presence of flavin-protein enzymes, such as, microsomal NADPH cytochrome p-450 reductase, microsomal NADH $b_5$ reductase, and NADH dehydrogenase. Consequently, generation of semiquinone radicals results in formation of ROS and quinone via their reactions with molecular oxygen [24]. However, menadione is also able to undergo two-electron reduction by NADPH: quinone oxidoreductase. The latter reduction would result in formation of a hydroquinone rather than production of semiquinone radicals. The futile redox cycling of menadione is depicted in Figure 1.2.2.

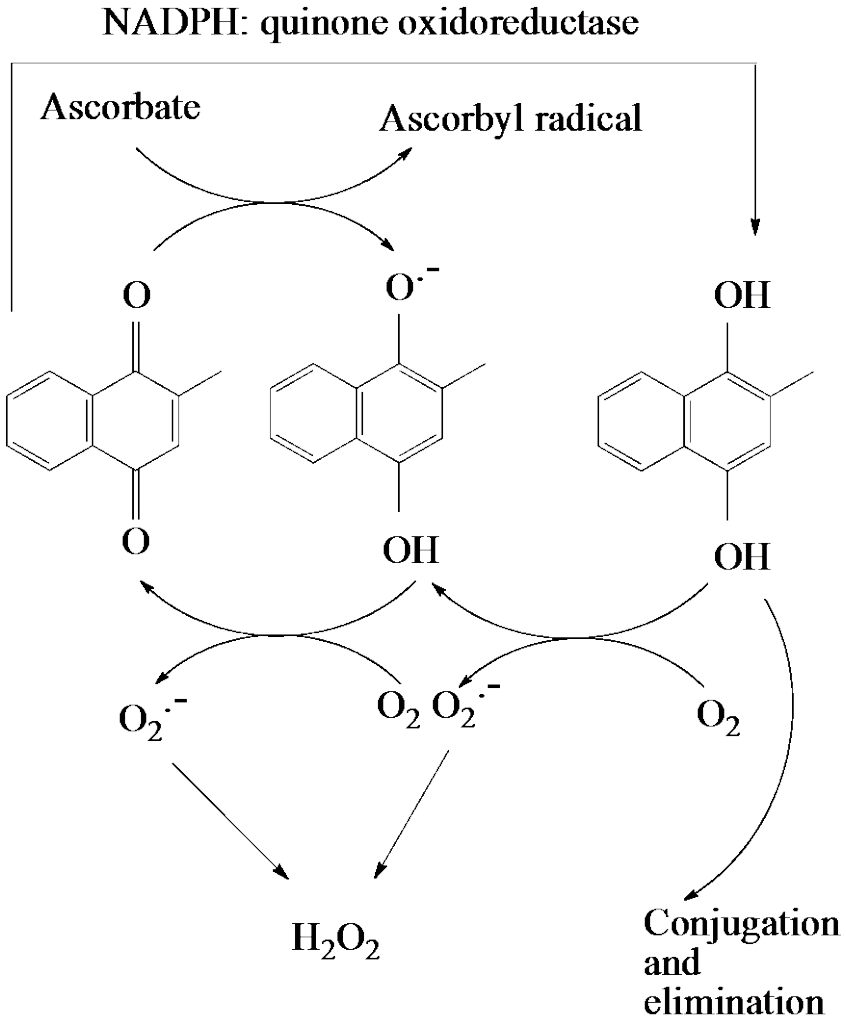



**Figure 1.2.2** The menadione redox cycle enzymatically and non-enzymatically, generates $H_2O_2$.

In addition, there is another chemical character for quinones, which is their ability to undergo a 1,4-Michael addition or arylation to form adducts with sulfhydryl and primary amines as it consists of a naphthoquinone moiety with a double bond α to a ketone group [1]. It has been reported that menadione induce loss of cellular thiols through its ability to conjugate with glutathione that consequently forms oxidized glutathione dimers (GSSG). The interaction of menadione with GSH resulted in the generation of superoxide anion radicals, which are dismutated rapidly in the presence of SOD to form $H_2O_2$ and $O_2$ [20].

Because of menadione activity, it has been used as a model toxicant in various studies, including apoptotic pathways in mammalian cells [25], yeast and bacterial macromolecules damage [26-28] and has been evaluated as a potential chemotherapeutic agent in conjunction with other compunds ( mitomycin, ascorbic acid) [29,30].

**1.3 Ascorbic acid**

In most mammalian cells**,** the intracellular concentrations of ascorbic acid, or vitamin C, are much higher than in plasma concentration, which is variable depending on the dose and route of ascorbic acid administration. This concentration gradient results from the uptake of dehydroascorbic acid (DHA), the two-electron oxidized form of ascorbate. Once DHA is transported via Glut 1 glucose transporters inside the cell, it reduced to ascorbate by GSH, 3 alpha-hydroxysteroid dehydrogenase, and thioredoxin reductase. Thus, the concentration of ascorbate would increase as it is a poor substrate for the

Glut 1 glucose transporter, it could leave cells only by diffusion. Inside the cell, free radicals that are generated from mitochondrial metabolism or proteins that contain iron such as hemoglobin which could oxidize ascorbate by one-electron to form ascorbyl free radicals. Dismutation of these radicals or oxidation of ascorbate by two-electrons lead to form DHA that will be lost from the cell via the Glut 1 glucose transporter or will be converted to 2,3-diketo-1-gulonic acid [31].

It has been reported that ascorbate, at pharmacologic concentrations (0.3-20 mM), killed some cancer cell types. The mechanistic killing of these cells was shown to be dependent on $H_2O_2$ formation and independent of metal chelators. It has been shown that there is a linear relationship between ascorbate concentration and generation of $H_2O_2$. It was proposed that pharmacologic ascorbate concentration in extracellular fluid generates ascorbyl radical and releasing electron would reduce a protein-centered metal that consequently resulted in formation of $O_2^{\bullet-}$, which dismutated to $H_2O_2$. Thus, pharmacologic ascorbate concentration results in cell death through generation of $H_2O_2$ that easily diffuses into the cells [32].

Ascorbic acid (vitamin C) is a particularly synergistic molecule that significantly catalyzes the redox cycling process and likely plays a role in anticancer activity. For instance, ascorbate and menadione, when used in combination, exhibit a synergistic cytotoxic effect on murine hepatoma cell lines. Because Glut 1 transporters are overexpressed in cancer cells, the concentration of ascorbate is very high within these cells. The ability of

ascorbate to donate an electron to menadione results in formation of semidehydroascorbyl and semiquionone radicals, and lead to redox cycling. It has been shown that the activity of catalase and glutathione peroxidase in hepatomas are not expressed highly enough to detoxify $H_2O_2$. Thus, presence of $H_2O_2$ generates oxidative stress, which is lethal for cancer cells [33-35].

**1.4 Hydrogen peroxide, bicarbonate and carbonate radical**

It is believed that $H_2O_2$ is an important product of the redox cycle that can mediate a portion of the anti-cancer activity. $H_2O_2$ is known to modulate caspase activity directly, but is also involved in the generation of the highly reactive hydroxyl radical ($HO^{\bullet}$), which can oxidize macromolecules at a diffusion limited rate [36,37]. $H_2O_2$ is produced through the dismutation of two molecules of superoxide anion radical, a process that is rapidly catalyzed by superoxide dismutase (SOD) (discussed further in 1.5)[38].

In cooperation with carbonic acid anhydrase, bicarbonate ($HCO_3^-$) that is present at 25 mM in biological fluids plays a major role in regulation of pH in vivo [39]. It has been reported that oral bicarbonate therapy plays an essential role in reducing the metastases in experimental models of breast and prostate cancers. Its effect mainly appears on enhancing the $pH_e$ that consequently reduced the release of lysosomal proteases. However, this effectiveness will be reduced with larger tumors [88]. The role of $HCO_3^-$ as a mediator of oxidative stress can be traced back to studies carried out using superoxide dismutase (SOD). The first prooxidant incidence of $HCO_3^-$ was the

finding that the presence of $HCO_3^-$ in buffer enhanced the oxidation of luminol [40]. A prominent role for $HCO_3^-$ has been suggested to involve the peroxidase activity of SOD, where $H_2O_2$ is oxidized in the active site of the enzyme, forming a transient $Cu^{2+}$- $HO^\bullet$ species, which then oxidizes $HCO_3^-$ to the carbonate radical anion ($CO_3^{\bullet-}$) [41,42].

Furthermore, the $CO_3^{\bullet-}$ has been detected with electron spin resonance after trapping with DMPO (5,5-dimethyl-1-pyrroline-N-oxide, a spin trap agent) [43]. The spin adduct usually detected is actually DMPO-OH which is due to the breakdown of the unstable DMPO-$OCO_2$ adduct, or possibly via DMPO oxidation to a radical cation that reacts with hydroxyl from solution [44,45]. Evidence also suggests that the $CO_3^{\bullet-}$ can diffuse to oxidize distal targets [46] and has been suggested to be involved in amyotrophic lateral sclerosis, where a gain of function mutation in SOD may enhance the peroxidase activity producing more oxidative stress [42-47]. The $CO_3^{\bullet-}$ has a high one-electron oxidation potential $E^{o\prime}(CO_3^{\bullet-}, H^+/ HCO_3^-$ = 1.78 V), which is relatively close to the hydroxyl radical $E^{o\prime}$ ($HO^\bullet$, $H^+$/H2O = 2.3 V) [48,49]. Similar to the $HO^\bullet$, the $CO_3^{\bullet-}$ can oxidize many different targets, including amino acid residues such as protein tryptophan and tyrosine, histidine [50], and causes protein aggregation [51-53].

### 1.5 Superoxide dismutase

In mammalian cells, there are three major families of superoxide dismutases (SODs) that include a cytoplasmic copper,zinc-enzyme (CuZn-

SOD or SOD1) , a manganese containing mitochondrial enzyme (MnSOD or SOD2) , and an extracellular form of CuZnSOD (SOD3). CuZn-SOD and Mn-SOD are considered as a first-line defence against superoxide radicals that are produced during normal intracellular metabolism. The main function of SODs is that it dismutases superoxide anion radicals, to form $O_2$ and $H_2O_2$..

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad (1)$$

It has been reported that cell killing, increased lipid peroxidation, and hemolysis are associated with elevation of intracellular SOD activity [54]. Although there are different toxicity mechanisms of $H_2O_2$, most notably the Fenton reaction which leads to hydroxyl radical ($HO^{\bullet}$) catalyzed by the presence of trace metals, a potential mechanism of $H_2O_2$ biotoxification could be through a backreaction of SOD known as SOD peroxidase activity that was first noted in 1975 [55]. The back reaction between CuZn-SOD and its own product $H_2O_2$ resulted in inactivation and fragmentation of the enzyme and production of $\cdot OH$, a highly reactive oxidant. It has been reported that the reaction between $H_2O_2$ and CuZn-SOD could generate a strong bound oxidant, SOD-Cu(II)-$\cdot$OH that reacts with bicarbonate to form carbonate anion radical ($CO_3^{\cdot -}$) as in reaction 4, a diffusible oxidant that was detected using spin trapping methods [41,42,55].

In reaction 2 and 3, excess $H_2O_2$, a product of the dismutation in reaction 1, results in a rapid reduction of Cu (II) in the Cu,Zn SOD that consequently

leads to formation of a strong bound oxidant. The latter product reacts with bicarbonate anion to generate $CO_3^{\bullet-}$ ( reaction 4).

$$\text{SOD- Cu(II)} + H_2O_2 \longrightarrow \text{SOD- Cu(I)} + O_2^{\cdot-} + 2H^+ \qquad (2)$$

$$\text{SOD- Cu(I)} + H_2O_2 \longrightarrow \text{SOD- Cu(II)-}\dot{O}H + OH^- \qquad (3)$$

The reaction of bicarbonate with the strong bound oxidant form of SOD was not realized until 1999 [41] (reaction 4).

$$\text{SOD- Cu(II)-}\dot{O}H + HCO_3^- \longrightarrow \text{SOD- Cu(II)} + CO_3^{\cdot-} + H_2O \qquad (4)$$

The presence of bicarbonate or carbon dioxide with hydrogen peroxide would lead to the formation of peroxymonocarbonate [56] (reaction 5).

$$H_2O_2 + HCO_3^- \rightleftharpoons HOOCO_2^- \qquad (5)$$

Furthermore, the reaction mechanism has evolved to suggest that $H_2O_2$ is not involved, per se, but rather a combination of $H_2O_2$ with $HCO_3^-$, which leads to peroxymonocarbonate is kinetically a more favourable reactant for SOD peroxidase activity [56]. However, there has been some controversy over the particular reactants, which lead to the $CO_3^{\bullet-}$ [57] (reactions 4 and 6).

$$\text{SOD- Cu(I)} + HOOCO_2^- + H^+ \longrightarrow \text{SOD- Cu(II)} + CO_3^{\cdot-} + H_2O \qquad (6)$$

# Chapter 2 Rationale, Hypothesis and Objectives

## 2.1 Rationale

There are many different roles for quinone molecules from a biochemical, industrial, pharmaceutical and environmental standpoint. This study is focused on specific mechanisms by which quinones may mediate damage to biological tissues. The toxicity of quinones is initiated from two basic initiating steps: redox cycling (oxygen activation, leading to $H_2O_2$ formation) and/or essential macromolecule alkylation. The end result of these processes is usually either cellular death or DNA damage potentially leading to carcinogenesis. As many authors have discussed this subject previously, the novelty in this study lies in the specifics of the mechanisms that are involved. The carbonate radical ($CO_3^{\bullet -}$) was found to be produced from SOD through a backreaction that involved $H_2O_2$. We have described above that redox cycling of menadione produces oxidative stress through formation of $H_2O_2$. If $H_2O_2$ produced from menadione is oxidized by SOD peroxidase activity, $CO_3^{\bullet -}$ formation is possible. This mechanism could represent an additional mechanism of quinone toxicity in general.

## 2.2 Hypothesis

We hypothesize that the cytotoxicity of menadione, 2-methyl-1,4-naphthoquinone, is enhanced by the presence of bicarbonate/ carbon dioxide, a biological buffer, either enzymatically or non-enzymatically (according to figure 2.2).

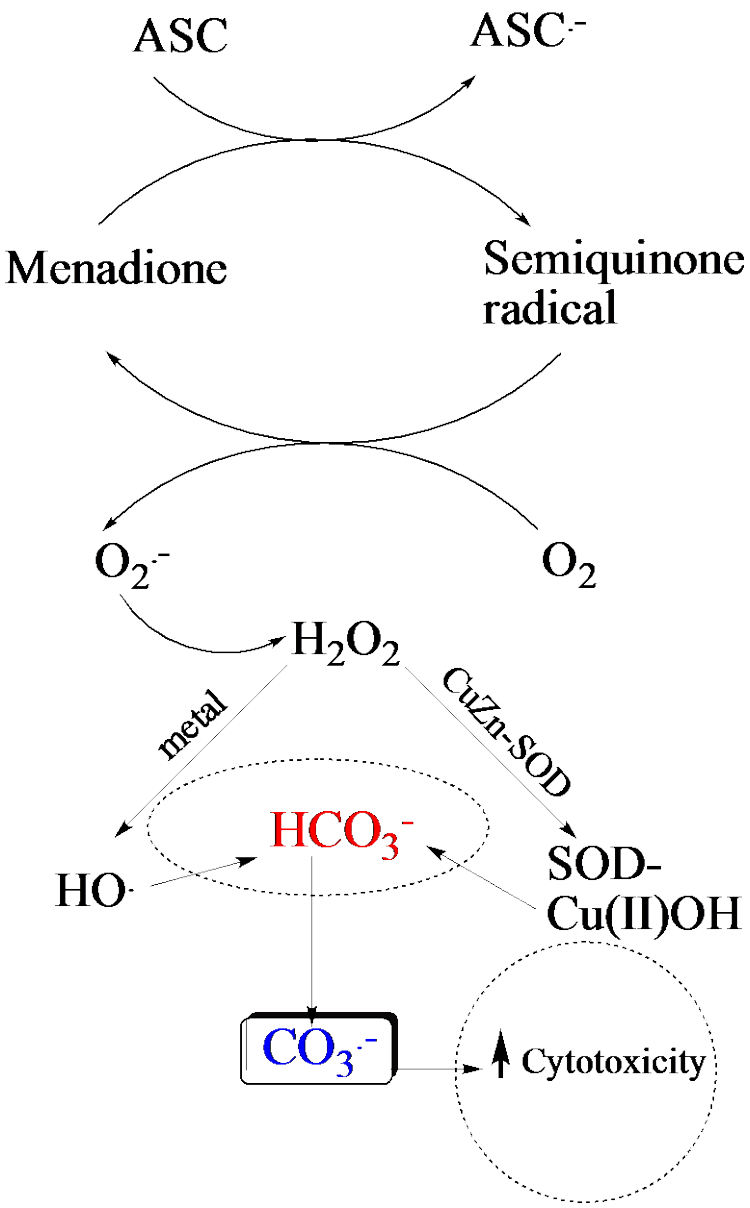



**Figure 2.2** hypothetical scheme for the role bicarbonate in menadione cytotoxicity.

## 2.3 Objectives

To understand the interplay between quinones and bicarbonate, we used menadione as a representative of quinone-containing drugs as its ability to undergo futile redox cycle as well as alkylation macromolecules. Our objectives are:

- Determining the effect of bicarbonate on menadione redox cycling, enzymatically and non-enzymatically.
- Detect the effect of bicarbonate on ROS generated during menadione redox cycling.
- To determine the effect of SOD inhibition on menadione cytotoxicity.

# Chapter 3 Materials and Methods

## Materials and Methods

### 3.1 Reagents

Menadione, ascorbic acid, Dulbecco's phosphate-buffered saline (DPBS), sodium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide, potassium chloride, magnesium sulphate, D-glucose, hydrogen peroxide, diethyldithiocarbamate (DDC), $NaHCO_3$, acetazolamide, bovine serum albumin (BSA), diethylene triamine pentaacetic acid (DTPA), tetraethylenepentaamine (TEPA), ferric chloride and 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich (Oakville, ON). AlamarBlue reagent, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES, Trypsin and CellTiter-Glo$^R$ were purchased from Fisher Scientific Company (Ottawa, ON). The Oxyblot protein oxidation kit was purchased from EMD (Billerica, MA). Earle's Balanced Saline Solution (EBSS) was prepared by addition of different concentration of $NaHCO_3$ to 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 138 mM NaCl, 1.0 mM $Na_2HPO_4$, 5.5 mM D-glucose and 20 mM HEPES [58]. The pH measurements were recorded on a pH meter electrode Accumet$^R$ (Fisher scientific).

### 3.2 Oxygen Analysis

Oxygen consumption was measured by utilizing a Free Radical Analyzer (Model TBR 4100) connected to an oxygen sensor and interfaced to a desktop PC for data acquisition (World Precision Instruments, Sarasota, FL,

USA). 1 mM Ascorbic acid was used to initiate the redox cycling of 50μM menadione. After exporting the data to MS Excel, initial rates of oxygen consumption were calculated by taking initial slope after the addition of ascorbate (from 77-140 s). The number of moles of $O_2$ present in 1ml was calculated by utilizing ideal gas law (PV=nRT) and consequently the concentration of $O_2$ was calculated according to World Precision Instruments TBR4100/1025 page 37. All the reactions were carried out EBSS buffers that contained different concentrations of bicarbonate and 20 mM HEPES, pH 7.4, at room temperature.

### 3.3 Electron paramagnetic resonance for the detection of the semidehydroascorbyl radical

EPR experiments were performed on a Bruker Elexys E-500 spectrometer. After measuring the pH, 200 μl of the reaction was transferred to a flat cell and the scan was started immediately. The semidehydroascorbyl radical was detected directly in the presence of 50 μM menadione and 1 mM ascorbic acid. All reactions were performed in EBSS buffer containing 20 mM HEPES and different concentration of bicarbonate pH 7.4 at room temperature.

### 3.4 Spectrophotometric assay of ascorbic acid

All UV-Vis absorption spectra were acquired on a Spectra Max M5 Multi-Mode Microplate Reader. Absorbance of ascorbic acid and kinetic spectra of its degradation were performed by utilizing 1 cm path length quartz cuvette.

The optical density of ascorbic acid was monitored at 260 nm and kinetic scans were recorded for 15 min. All reactions were initiated by the addition of 300 μM $H_2O_2$ in EBSS containing 15 μM ascorbic acid and different concentrations of $NaHCO_3$ in the presence and absence of 100 μM diethylenetriaminepentaacetic acid (DTPA) pH 7.4 at room temperature.

### 3.5 Cell culture

Cells of murine Hepatoma cell line (Hepa 1c1c7) were kindly provided by ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10 % fetal bovine serum Hyclone$^R$ (Thermo scientific) and 100 units/ml antibiotic antimycotic (10000 u penicillin, 10000 ug streptomycin and 25 ug fungizone) Gibco$^R$ (Invitrogen) at a density of 5 X$10^5$ cells/ml and incubated at 37 $^oC$ in a humidified atmosphere with 5 % $CO_2$ for 48 hours. The medium was replaced with a fresh one and the cells were seeded in 96-well plates at 37 $^oC$ in a humidified atmosphere with 5 % $CO_2$ for another 24 hours, prior to the experiment.

### 3.6 Menadione, bicarbonate and diethyldithiocarbamate treatment

Twenty-four hours after seeding murine hepatoma (Hepa 1c1c7) cells in clear bottom 96 well plates and after washing the cells with Dulbecco's phosphate buffered saline (DPBS), the culture medium was replaced with EBSS buffers. One buffer contained different concentrations of menadione ( 1,

3, 10, 30, 100 μM) and 25 mM bicarbonate; another buffer contained different concentrations of bicarbonate (1, 2, 5, 12.5 and 25 mM) and 30 μM menadione. Both EBSS buffers contained 20 mM HEPES, pH 7.4. Hepa1c1c7 cells were incubated at 37 $^{o}$C in a humidified atmosphere with 5 % $CO_2$ for three hours. Cells treated with 0.04 % dimethyl sulfoxide (vehicle) in EBSS buffer containing 25 mM bicarbonate and in EBSS buffers containing different concentration of bicarbonate (1, 2, 5, 12.5 and 25 mM) were used for controls. For diethyldithiocarbamate (DDC) treatment, twenty-four hours after seeding murine hepatoma (Hepa 1c1c7) cells in 96 well clear bottom plates, the cells were incubated with different concentrations of DDC (1, 10, and 100 μM) in DMEM free of FBS at 37 $^{o}$C in a humidified atmosphere with 5 % $CO_2$ for 60 min. The cells were washed twice by DPBS and incubated in EBSS buffer containing 25 mM bicarbonate and 30 μM menadione at 37 $^{o}$C in a humidified atmosphere with 5 % $CO_2$ for three hours. The cells treated with in the presence of 0.04% DMSO were included as controls.

**3.7 cell viability assay**

Cytotoxicity of menadione was determined by colorimetric assay based on the alamarBlue$^{R}$ assay where viable cells catalyzed the reduction of resazurin, a non-fluorescent indicator dye, to resorufin, a highly red fluorescent compound. After treatment of murine hepatoma Hepa 1c1c7 cells with test compounds (menadione, bicarbonate, and DDC) and after washing with DPBS, the cells were incubated with 100 μl of AlamarBlue solution

(diluted 1:10 with DPBS), at 37 $^{o}$C in a humidified atmosphere with 5 % $CO_2$ for six hours. Fluorescence intensity was measured by exposure of plates to an excitation wavelength at 570 nm and an emission wavelength 590 nm. All measurements were performed by utilizing Spectra Max M5 Multi-Mode Microplate Reader at room temperature. Cytotoxicity was alternatively measured by a luminescent assay CellTiter Glo$^{R}$ (Promega, Corp., Madison, WI) a luminescence based on quantitation of the ATP content present in the cells.

### 3.8 Reactive oxygen species (ROS) determination

#### 3.8.1 DCFH assay:

Twenty-four hours after seeding murine hepatoma (Hepa 1c1c7) cells in 96 well clear bottom plates, medium was replaced by EBSS buffers containing different concentrations of menadione (20, 40 and 80 μM) and different concentrations of bicarbonate (2, 25 mM). 10 μM DCFH was added to the cells and incubated for 30 min. DCF fluorescence was measured by SpectraMax M5 Multimode Microplate reader at an excitation wavelength 485 nm and an emission wavelength of 520 nm. The relative fluorescence was used as a non-specific measure of relative oxidative stress induced by the treatments.

### 3.8.2 Carbonyl assay:

According to the manufacturer's protocol, detection of carbonyl groups was performed by utilizing Oxyblot oxidized protein detection kit. Before SDS-PAGE separation, 2,4-dinitrophenylhydrazine was incubated with samples containing 5 μg BSA, 16 μM SOD, 5 μM menadione, 1 mM ascorbic acid, and 2 mM or 25 mM sodium bicarbonate for 15 min at room temperature. After SDS PAGE and transfer onto nitrocellulose membrane the blots were treated with anti-dinitrophenylhydrazone antibodies, followed by secondary antibodies to reveal carbonyl-containing BSA. Blots were developed with chemiluminescence HRP substrate.

### Statistical analysis

The results were expressed as the mean ± SD, and statistical differences were evaluated by t-test and one-way ANOVA followed by student-Newman-Keuls for pairwise comparisons.

# Chapter 4 Results

## Results

### 4.1 Bicarbonate enhanced menadione redox cycling.

Non–enzymatically, $O_2$ and ascorbic acid could elicit the effect of bicarbonate on menadione redox cycling. To test our hypothesis, we monitored $O_2$ consumption and $ASC^{\bullet-}$ intensity and ASC oxidation by utilizing oxygen electrode, EPR and UV-Vis spectrometry.

### $O_2$ analysis

The effect of bicarbonate (2 and 25 mM) on the initial rate of $O_2$ consumption is shown in Figure 4.1. In EBSS buffer containing 2 and 25 mM bicarbonate, we showed that menadione (50 μM) was reduced by ascorbic acid (1 mM) to generate semiquinone radical ($SQ^{\bullet}$) and semidehydroascorbyl radical ($ASC^{\bullet}$). $SQ^{\bullet}$ can then be oxidized back to the parent menadione subsequently generating $O_2^{\bullet}$. Thus, this process is known as redox cycling and is not dependent on bicarbonate [75,76]. Simultaneously, dismutation of $O_2^{\bullet}$ either in the presence or absence of SOD resulted in $H_2O_2$ formation. Interestingly, with $H_2O_2$, we found that there was significant increase in the initial rate of $O_2$ uptake with 25 mM bicarbonate as compared with 2 mM bicarbonate.

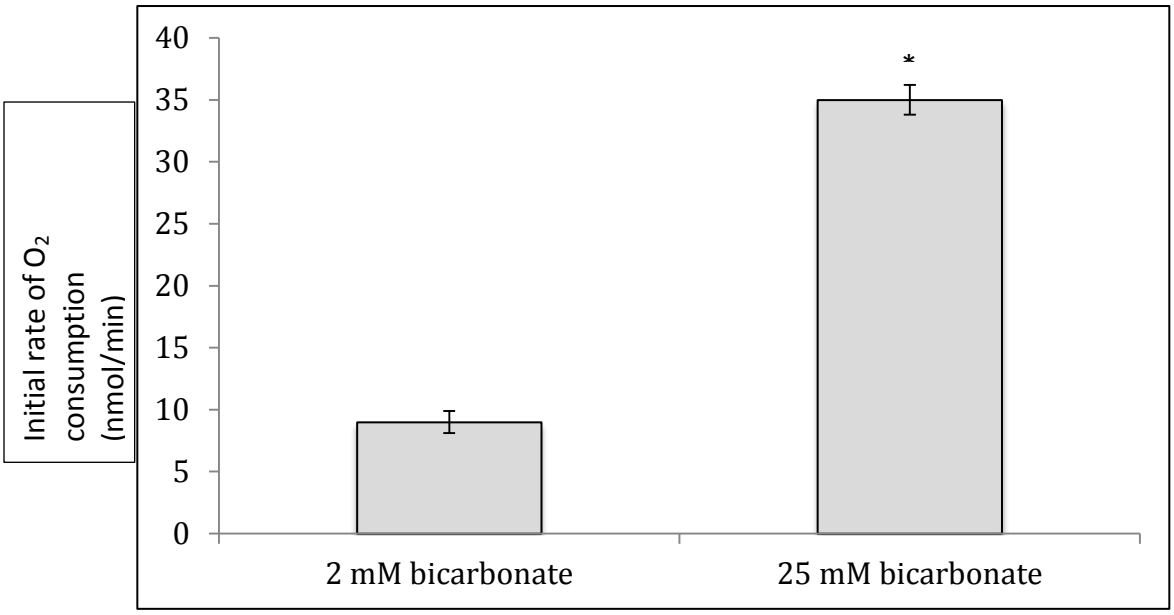
**Semidehydroascorbyl radical monitoring**

To further elaborate the effect of bicarbonate on menadione/ ascorbate redox cycle, we performed EPR experiment to monitor the signal intensity of semidehydroasorbyl radical. Figure 4.1.1 shows the effect of bicarbonate (2 and 25 mM) on the signals of semidehydroascorbyl radical, the first oxidation product of ascorbic acid oxidation. We detected the characteristic (at g=2) doublet signals by addition of ascorbic acid (1 mM) and menadione (50 μM) to EBSS buffer containing 2 and 25 mM bicarbonate. Interestingly, the intensity of EPR signals, corresponding to an increase in semidehydroascorbyl radical was increased by 25 mM bicarbonate compared to 2 mM bicarbonate..

**Ascorbic acid oxidation**

$O_2^{\bullet-}$, $HO^{\bullet}$, $SQ^{\bullet-}$and possibly $CO_3^{\bullet-}$ radicals that resulted from menadione/ascorbate redox cycle, reacted with ascorbate to generate semidehydroascorbyl radical. To further evaluate the effect of bicarbonate on ascorbic acid oxidation and to confirm our finding in EPR experiment, we utilized UV-Vis spectrophotometry to monitor the oxidation of ascorbate by $HO^{\bullet}$ and $CO_3^{\bullet}$. (i.e. without menadione). Figure 4.1.2 shows absorbance kinetic scans spectra of ascorbic acid. Using EBSS buffer containing different

concentrations of bicarbonate (0,2 and 25 mM), reactions were initiated by addition of $H_2O_2$ (300 μM), to exclude the effect of radicals that initiated from menadione/ascorbate redox cycle, (300 μM) to ascorbic acid (15 μM). Although there was oxidation of ascorbic acid in the absence of bicarbonate, it was increased by two folds with the presence of 25 mM bicarbonate as compared with absence of bicarbonate or presence of 2 mM bicarbonate. However, the oxidation of ascorbic acid that increased with 25 mM bicarbonate was abolished with addition of DTPA (100 μM). Thus, the reaction $H_2O_2$ with EBSS buffer metal ions resulted in formation of $HO^{\bullet}$ that subsequently reacted with bicarbonate to generate $CO_3^{\bullet}$, a strong oxidizing agent. The latter radical and $HO^{\bullet}$ appear to both be responsible for ascorbic acid oxidation.




**<u>Figure 4.1</u> Oxygen consumption during redox cycling of menadione with ascorbic acid using different concentrations of $NaHCO_3$ in EBSS buffer**. Data were shown as the mean ± SD (n=3). * $p < 0.05$ for 30 μM menadione + 1 mM ascorbate + 25 mM $NaHCO_3$ vs. 30 μM menadione + 1 mM ascorbate + 2 mM $NaHCO_3$.

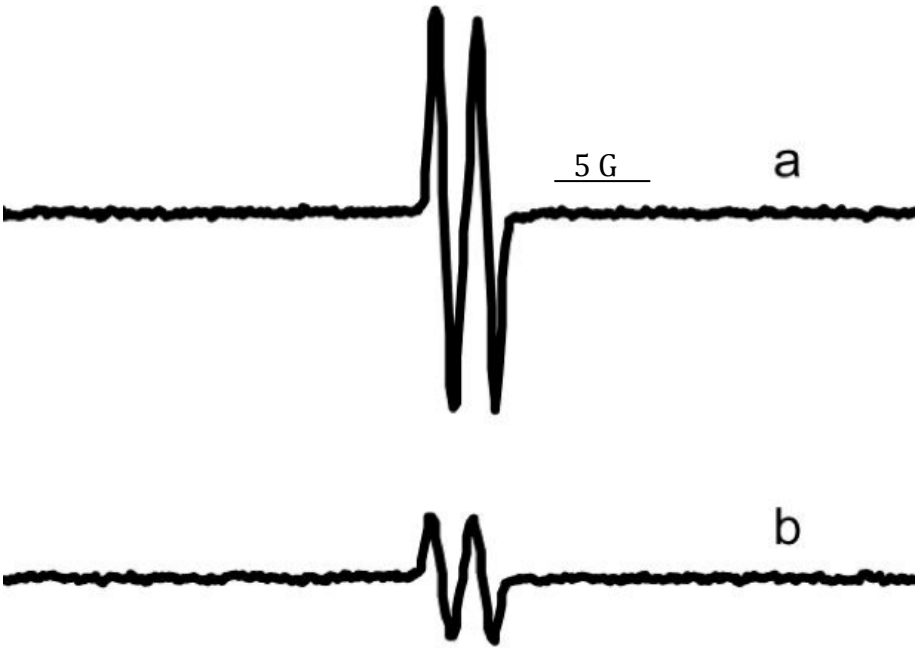



**Figure 4.1.1 EPR spectrometry for detection of the semidehydroascorbate radical.** EPR spectrometry was used to record the spectra shown (described in Materials and Methods). Briefly, menadione (50 μM) and ascorbic acid (1 mM) were mixed in EBSS pH 7.4, and transferred to a flat cell for recording EPR spectra. The reaction containing 25 mM $NaHCO_3$ (a) or 2 mM $NaHCO_3$ (b) both demonstrated detection of the characteristic doublet (g = 2) of the semidehydroascorbate radical, with the amount of the latter in (a) being greater than three-fold than in (b). Scans are representative of three separate experiments. EPR settings: modulation amplitude = 1 G, Sweep time = 84 s, microwave power = 20 mW. Intensity of the spectra were scaled to the more intense spectrum (a).

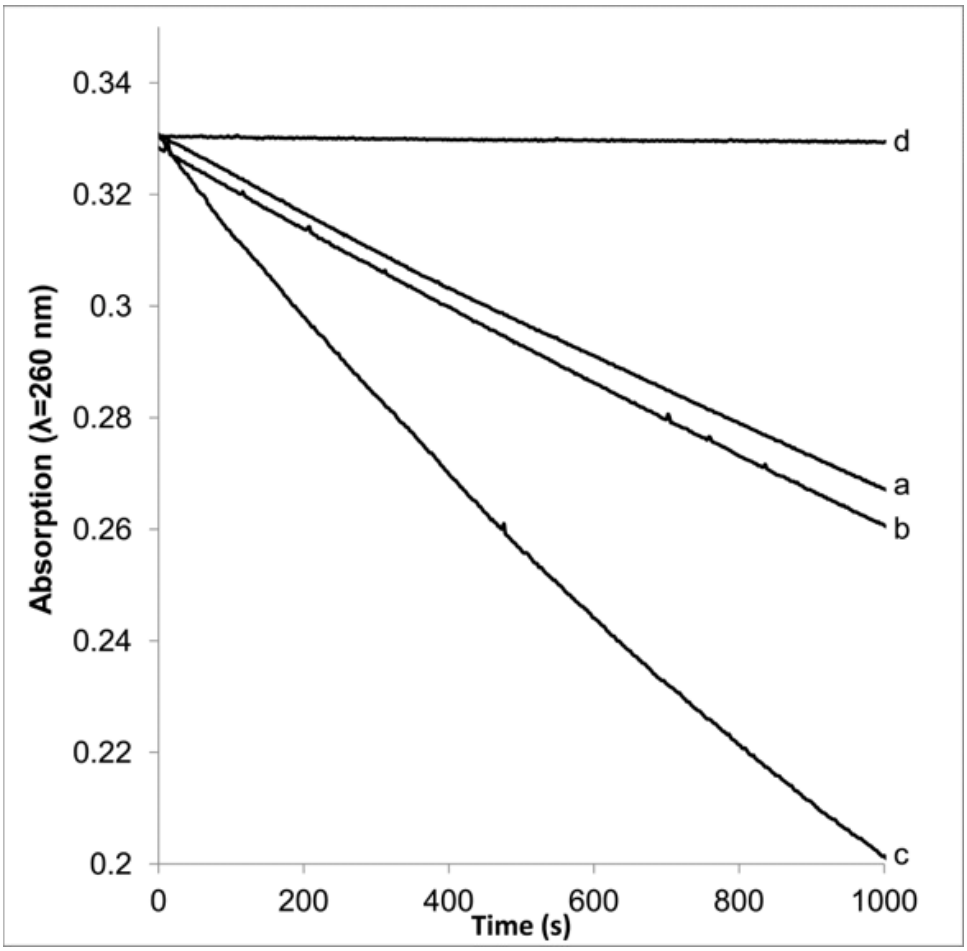
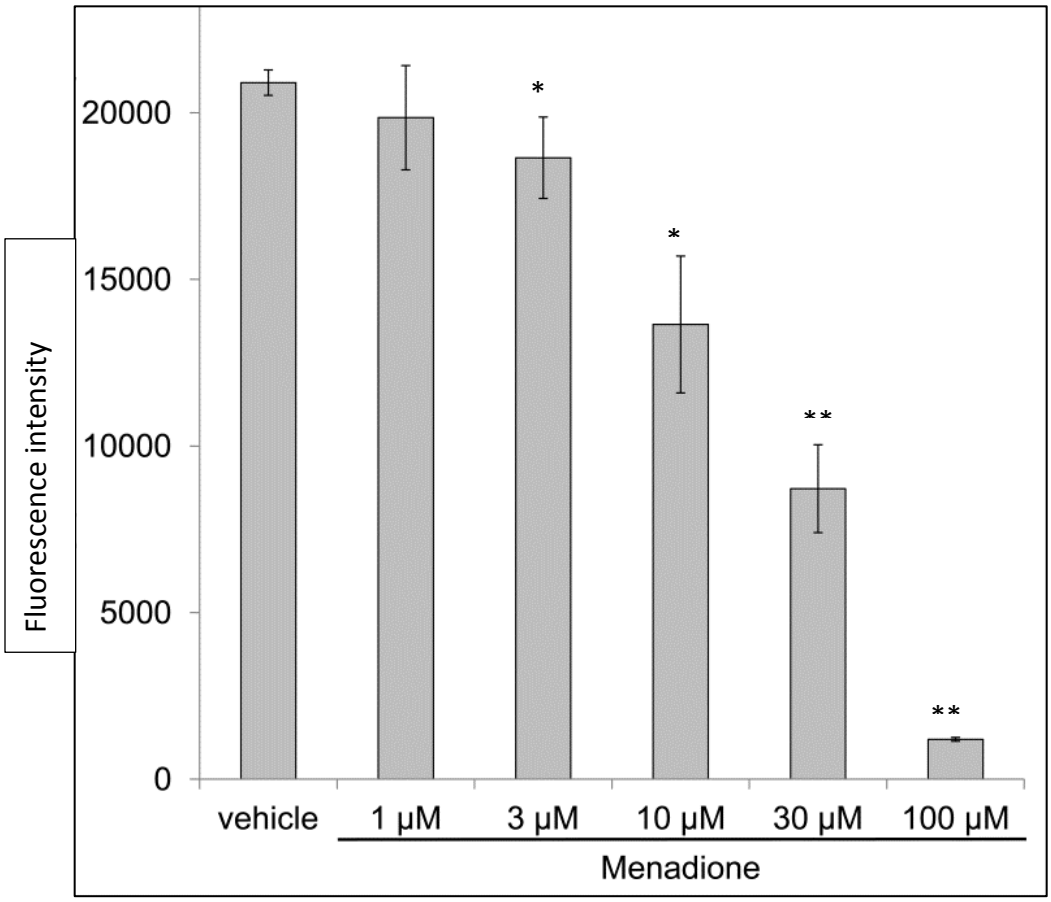



**Figure 4.1.2 Kinetic UV-Vis spectrophotometry of ascorbic acid.** All scans were recorded using a spectrophotometer set to read in kinetic mode at λ=260 nm as described in Materials and Methods. Reactions were carried out in EBSS containing either 0, 2, or 25 mM $NaHCO_3$, pH 7.4 at room temp and contained 15 μM ascorbic acid and 300 μM $H_2O_2$. In all reactions $H_2O_2$ was added last to initiate the reaction. The scans show the rate of oxidation of ascorbic acid by monitoring the ascorbic acid maximum in the presence of 0 mM $NaHCO_3$ (a), 2 mM $NaHCO_3$ (b), 25 mM $NaHCO_3$ (c) and 25 mM $NaHCO_3$ + 100 μM DTPA (d). Scans shown are representative three separate reactions (n=3).

**4.2 Bicarbonate enhanced menadione cytotoxicity in Hepa 1c1c7 cells**.

Dose-dependente cytotoxicity assessed by AlamarBlue of menadione on Hepa 1c1c7 cells is shown in Figure 4.2.1 In the presence of 25 mM bicarbonate, the cell viability was decreased by increasing the concentration of menadione. We selected to use 30 μM menadione to study the effect of bicarbonate on modulating menadione cytotoxicity. Figure 4.2.2 shows dose-dependent effects of different concentrations of bicarbonate (1, 2, 5, 12.5 and 25 mM) with menadione on hepa1c1c7 cells. In the presence of menadione, the cell viability was decreased with increasing the concentration of bicarbonate. However, the bicarbonate itself did not demonstrate significant diferences in viability when used in this range. In order to confirm the results that resulted from utilizing AlamarBlue assay and to provide further evidence of the role of bicarbonate on menadione cytotoxicity, the cell viability was determined by utilizing ATP assay Figure 4.2.4. It has been reported that menadione per se depleted ATP through glycolysis inhibition. This is shown in figure 4.2.3.In both assays we found that the cytotoxicity of menadione was significant increase with 25 mM bicarbonate as compared with 2 mM bicarbonate concentrations, suggesting a role of bicarbonate in menadione cytotoxicity.




**<u>Figure 4.2.1</u> Effect of different concentrations of menadione and bicarbonate on murine hepatoma cells (Hepa 1c1c7) survival.** The dose-dependent cytotoxicity of menadione was determined by incubating different concentrations of menadione in Hepa 1c1c7 cells for 3 hours in EBSS buffer containing 25 mM bicarbonate. Cell viability was measured by utilizing the AlamarBlue assay. Data were shown as the mean ± SD (n=6). * $p < 0.05$; ** $p < 0.001$ vs. vehicle.

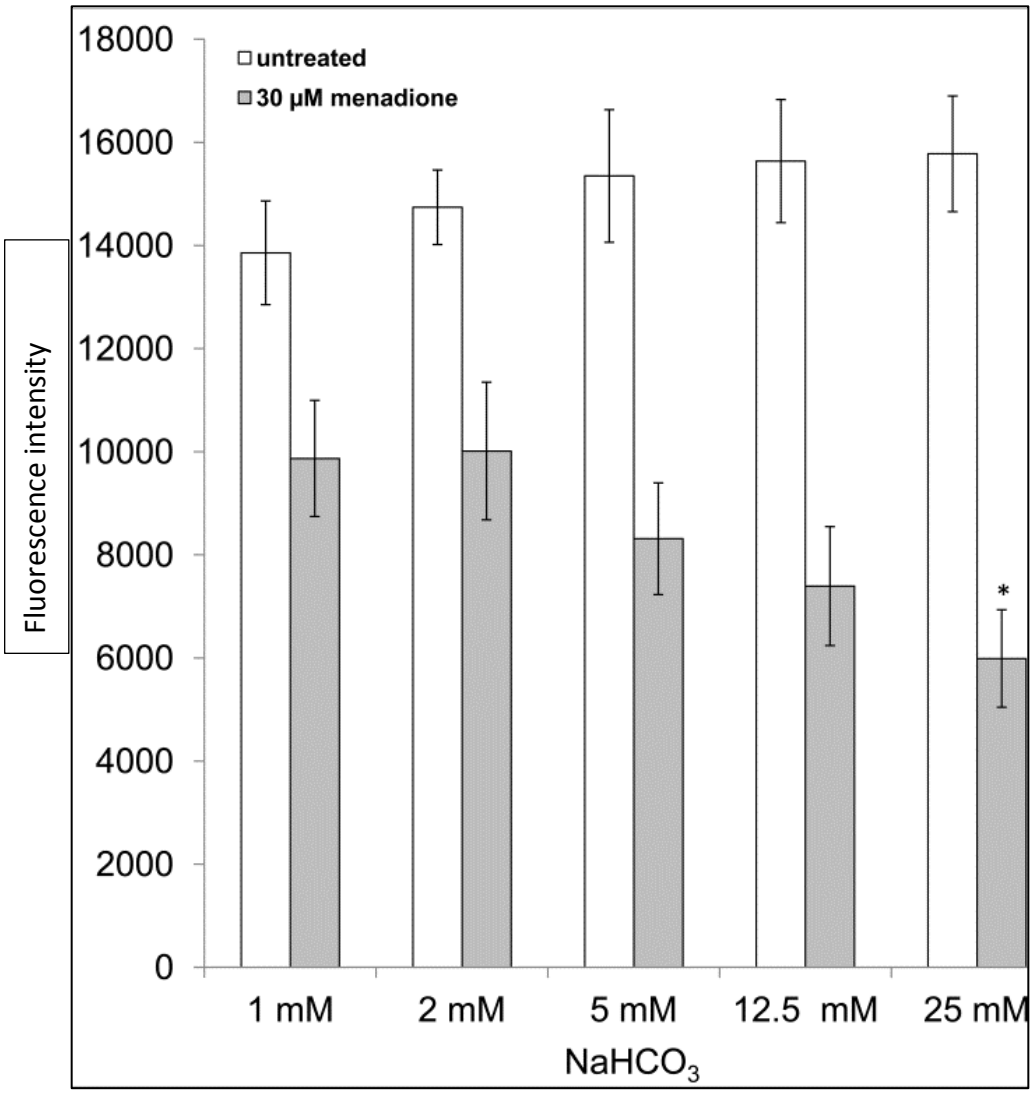



**Figure 4.2.2 Effect of different concentrations of bicarbonate and fixed concentration of menadione on murine hepatoma cells (Hepa 1c1c7) survival.** The effect of $NaHCO_3$ on the cytotoxicity of menadione was determined by using a fixed concentration of menadione with variable concentrations of $NaHCO_3$. Cell viability was measured by utilizing the AlamarBlue assay. Data were shown as the mean ± SD (n=6). * $p < 0.001$ for 30 μM menadione + 25 mM $NaHCO_3$ vs. 30 μM menadione + 2 mM $NaHCO_3$.

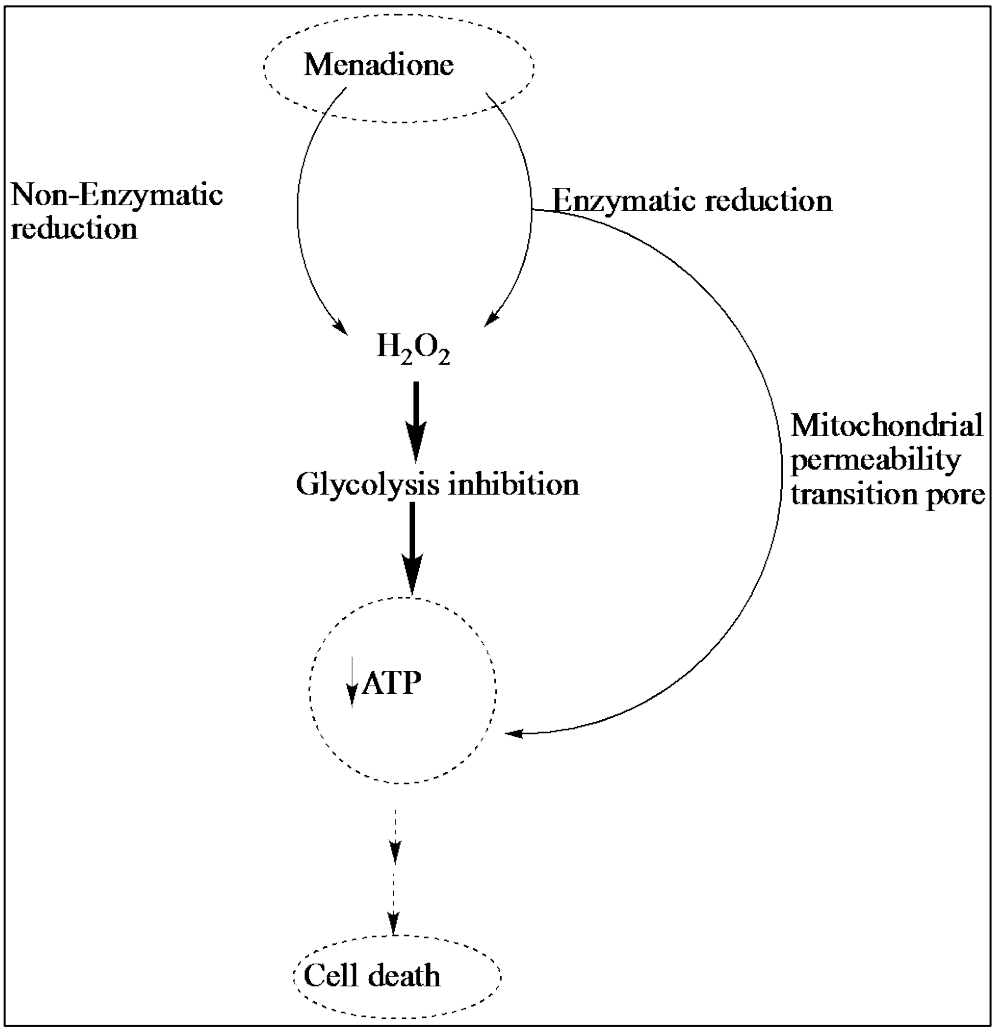
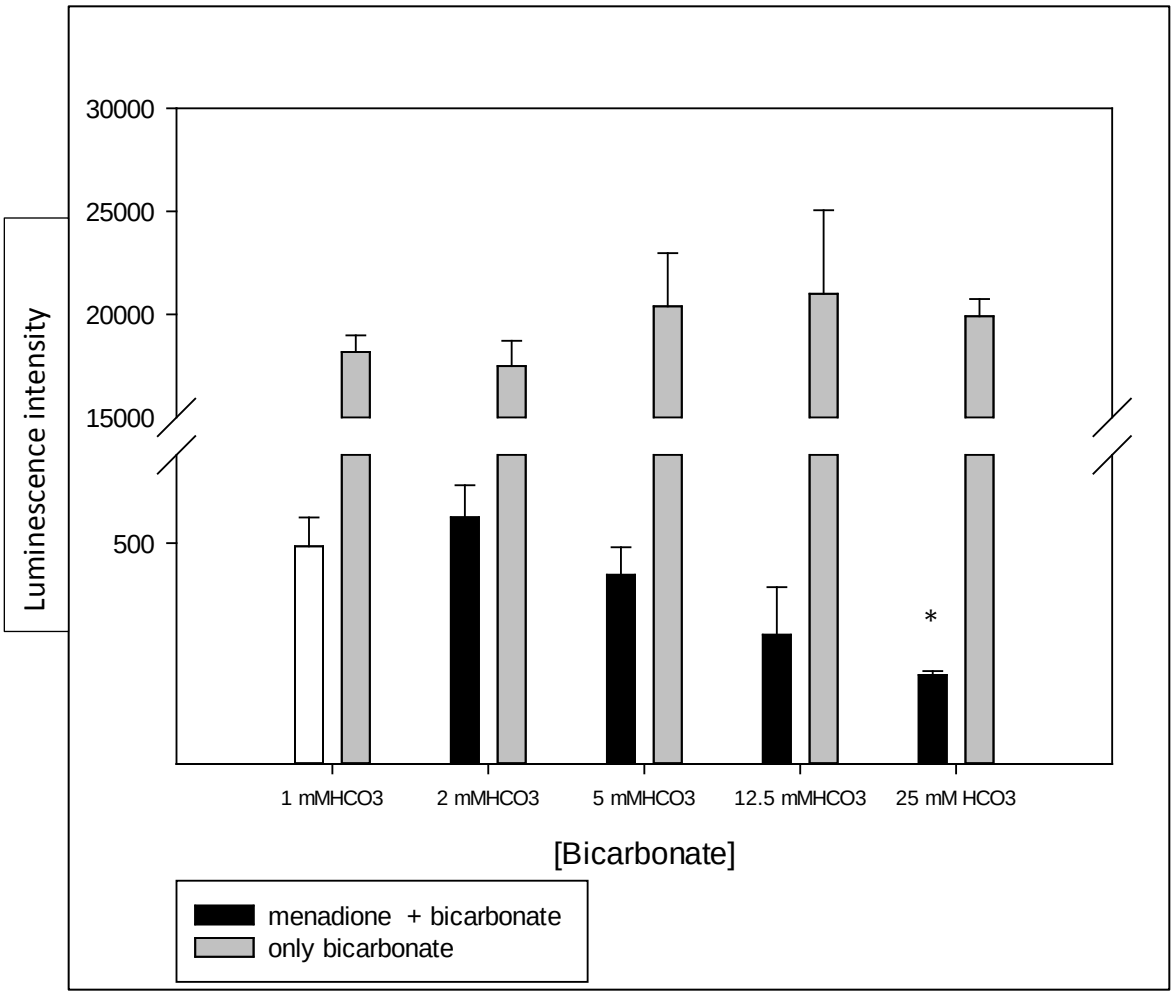



**Figure 4.2.3 Schematic Effect of menadione on ATP.** Enzymatically or non-enzymatically, redox cycling of menadione is known to generate superoxide that dismutates into $H_2O_2$. The latter can directly or indirectly inhibit glycolysis and also via affecting MPT pore that subsequently resulted in depletion of ATP [87].

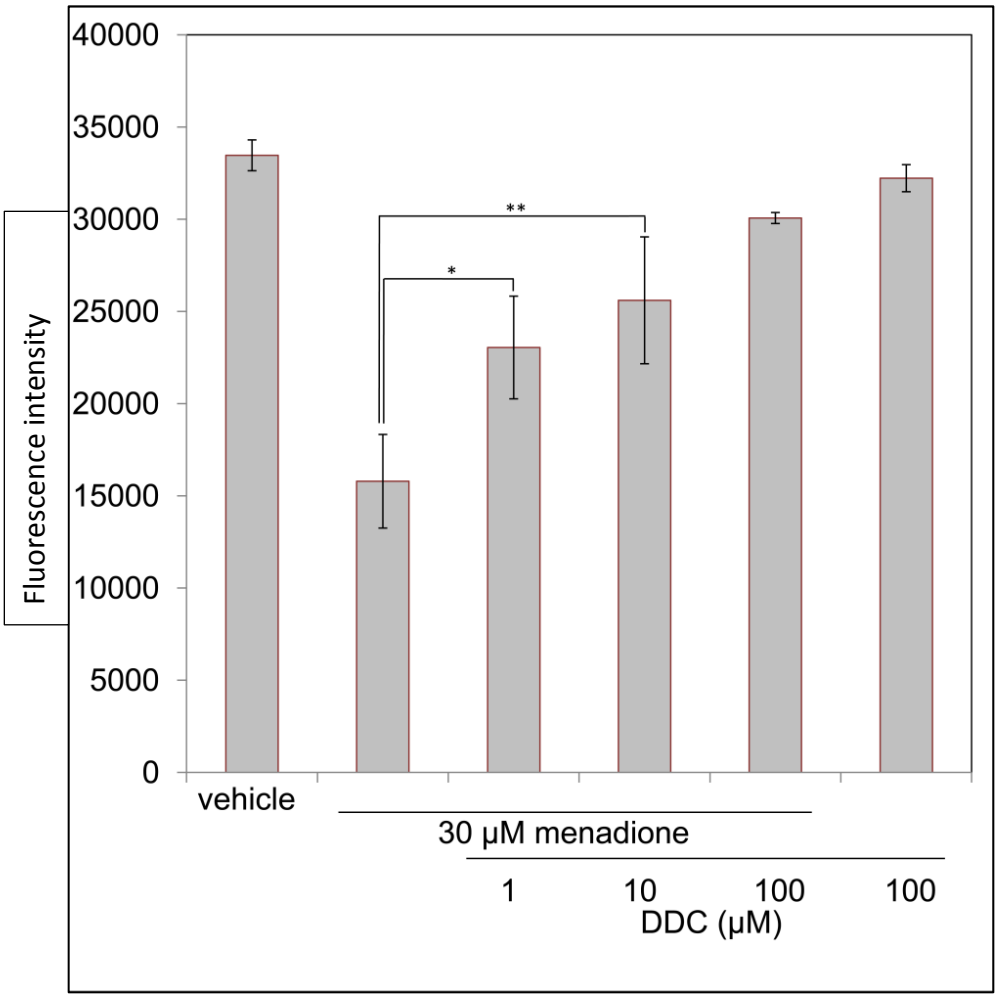



**<u>Figure 4.2.4</u> Effect of different concentrations of bicarbonate and fixed concentration of menadione on murine hepatoma cells (Hepa 1c1c7) survival.**

The effect of NaHCO3 on the cytotoxicity of menadione was determined by using a fixed concentration of menadione with variable concentrations of $NaHCO_3$. Cell viability was measured by utilizing the ATP assay. Data were shown as the mean ± SD (n=3). * $p < 0.05$ for 30 μM menadione + 25 mM $NaHCO_3$ vs. 30 μM menadione + 2 mM $NaHCO_3$.

**4.3 Effect of SOD peroxidase activity on menadione cytotoxicity.**

We demonstrated that bicarbonate enhanced menadione/ascorbate redox cycle through forming carbonate radical that was generated by hydroxyl radical oxidation of bicarbonate. This suggested that hydrogen peroxide, a product of menadione redox cycling, reacted with metal ions to generate hydroxyl radical. On the other hand, we showed that bicarbonate increased the cytotoxicity of menadione. To correlate these pathways, we utilized DDC, an SOD inhibitor. The effect of different concentrations of DDC on menadione cytotoxicity is shown in Figure 4.3.1. In DMEM (without FBS), Hepa 1c1c7 cells were incubated with different concentration of DDC for 60 min at 37 C. After washing the cells (twice) , the medium was replaced with EBSS buffer containing 25 mM bicarbonate and 30 μM menadione. The cytotoxicity of menadione was significantly attenuated by increasing concentrations of DDC, suggesting that SOD peroxidase activity could play a role in menadione cytotoxicity. Even at 1 μM DDC, significant attenuation of menadione cytotoxicity was observed. To confirm our finding, we utilized TEPA, a chelating agente. The effect of TEPA on menadione cytotoxicity was shown in figure 4.3.2.




**<u>Figure 4.3.1</u> Effect of DDC on menadione cytotoxicity.** Murine hepatoma (Hepa 1c1c7) cells were incubated in DMEM media with different concentration of the SOD inhibitor DDC for one hour. Cells were incubated for 3 hours in EBSS buffer containing 25 mM bicarbonate and 30 μM menadione. The cytotoxicity was measured by utilizing alamarBlue assay. Data were shown as the mean ± SD (n=6). * $p < 0.005$ vs 1 μM DDC + 30 μM menadione; ** $p < 0.001$ vs. 10 μM DDC + 30 μM menadione.

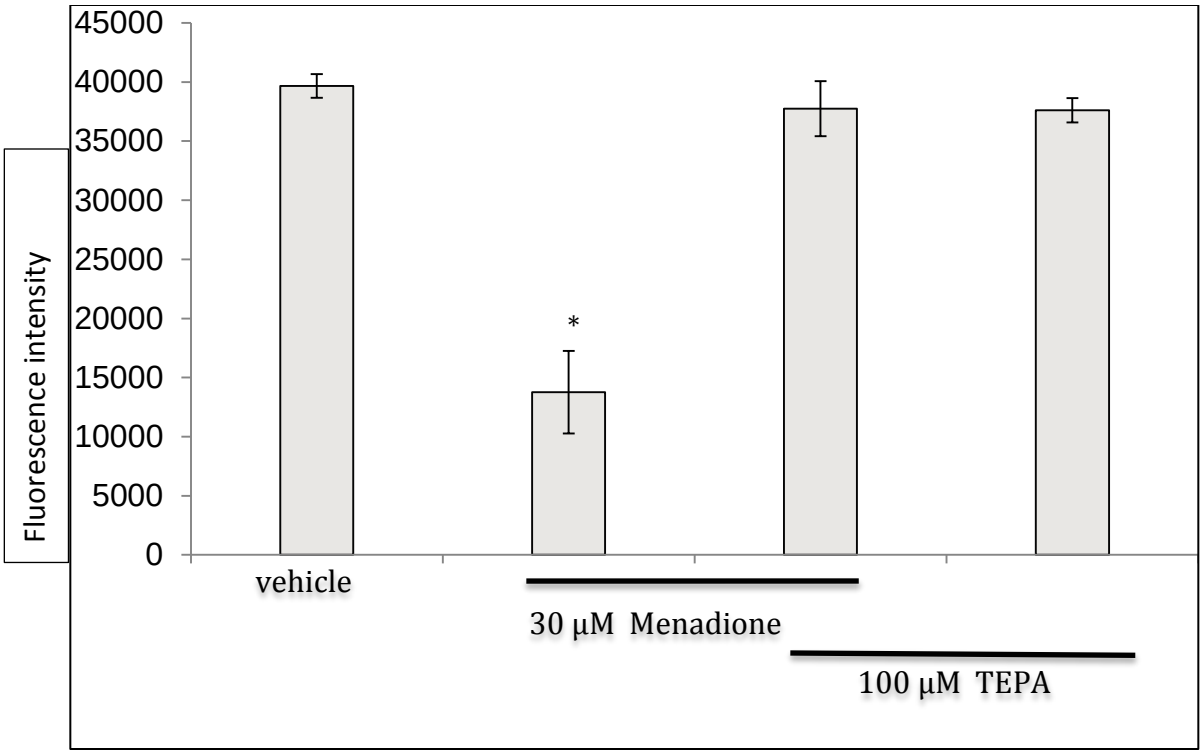



**Figure 4.3.2 Effect of TEPA on menadione cytotoxicity.** Murine hepatoma (Hepa 1c1c7) cells were incubated in DMEM media with 100 µM of the SOD inhibitor TEPA for one hour. Cells were incubated for 3 hours in EBSS buffer containing 25 mM bicarbonate and 30 µM menadione. The cytotoxicity was measured by utilizing alamarBlue assay. Data were shown as the mean ± SD (n=6). * $p < 0.05$ vs 100 µM TEPA + 30 µM menadione.

**4.4 Bicarbonate enhanced menadione-induced ROS formation-DCFH oxidation.**

After incubation the Hepa 1c1c7 cells with EBSS buffer contains different concentrations of bicarbonate (2 and 25 mM) and different concentrations of menadione , the effect of bicarbonate on oxidation of DCFH is shown in Figure 3.4. DCFH, a non specfic probe that reacts with many oxidants including carbonate radical, produced DCF which was monitored fluorescently. Interestingly, the oxidation of DCFH was significantly increased with 25 mM bicarbonate as compared with 2 mM bicarbonte, suggesting that the bicarbonate enhanced menadione-induced oxidative stress.

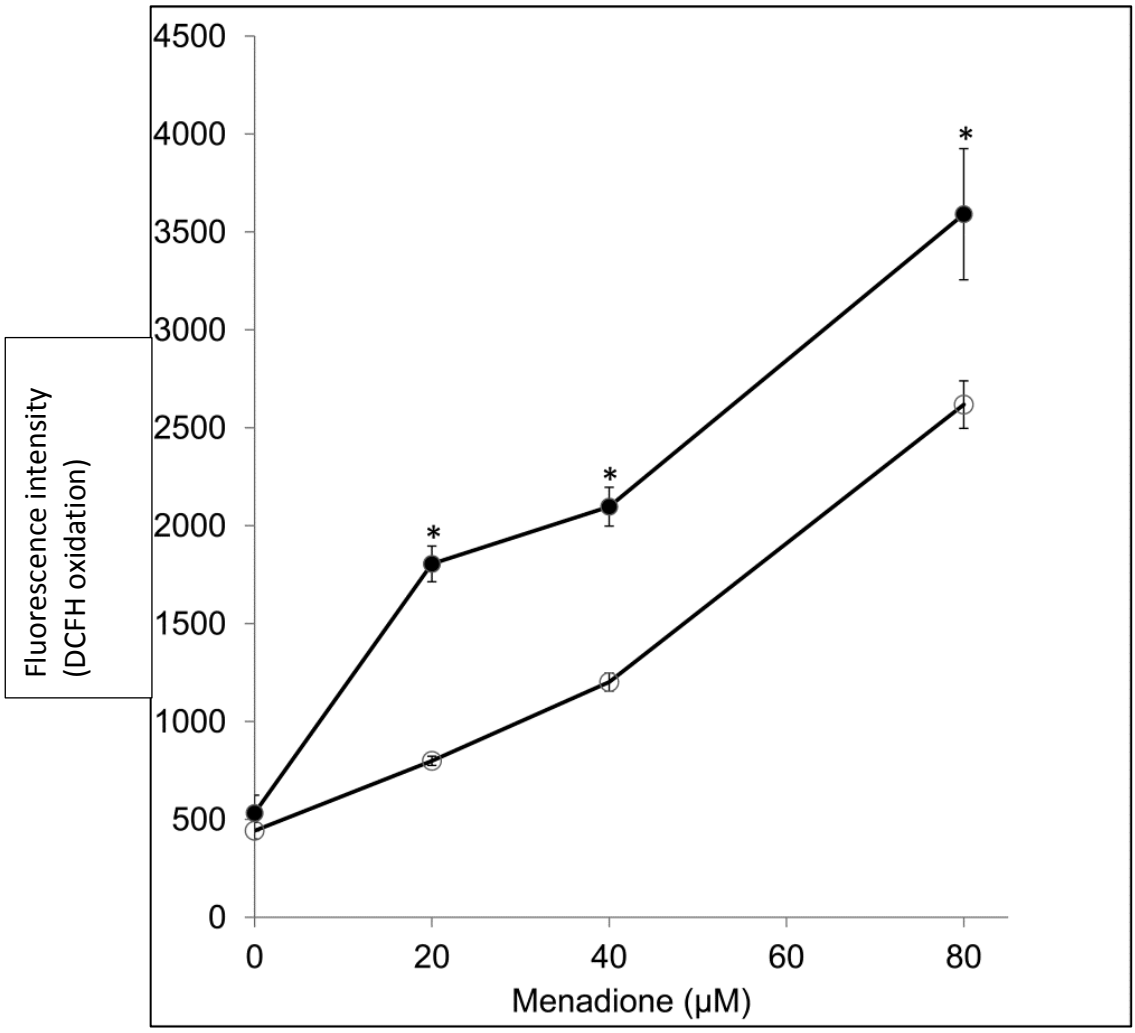



**Figure 4.4 Effect of bicarbonate on menadione–induced DCFH oxidation.** Murine hepatoma (Hepa 1c1c7) cells were incubated in EBSS buffer containing 2 mM (open circles) or 25 mM (filled circles) bicarbonate with different concentration of menadione and 10 μM DCFH-DA. DCF fluorescence, and indicator of ROS formation, was measured on a fluorescent plate reader at an excitation wavelength 485 nm and emission wavelength of 520 nm. Data were shown as the mean ± SD (n=8). * $p < 0.001$ as compared with 2 mM bicarbonate at the corresponding menadione concentration.

**4.5 Bicarbonate enhanced protein carbonylation.**

The effect of different concentrations of bicarbonate on BSA carbonylation is shown in Figure 4.5. In the presence of SOD, protein carbonyl detection was increased with 25 mM bicarbonate as compared with 2 mM bicarbonate. In the absence of SOD, BSA carbonylation was decreased even though BSA was incubated with 25 mM bicarbonate. Taken together, SOD and bicarbonate enhanced menadione-induced oxidative stress as evidenced by protein carbonylation as a marker of oxidative damage.

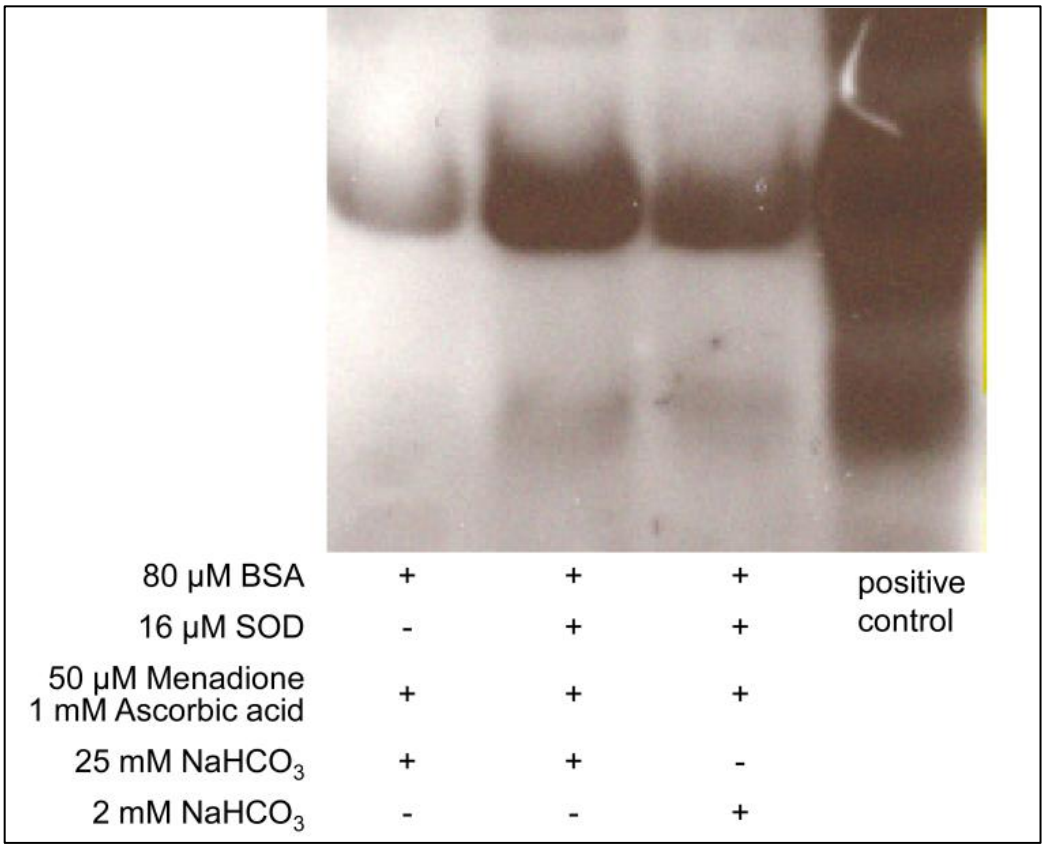



**Figure 4.5 Effect of menadione, NaHCO3, and SOD on protein carbonyl formation.** Protein carbonyls were determined by using the anti-DNP Western blot assay. The positive control (lane 4) was carried out as per the manufacturer's protocol to generate high yield of BSA-carbonyls through metal catalyzed oxidation. All samples were derivatized with DNPH in order to generate the antigen. In the presence of 25 mM bicarbonate, menadione (50 µM), and ascorbic acid (1 mM), BSA-DNP was detected (lane 1); however, the detection was enhanced in the presence of SOD (lane 2) and the latter was attenuated when a lower concentration of NaHCO3 was used (lane 3). All the reactions carried out in EBSS buffer, pH 7.4 at room temp for 30 minutes.

# Chapter 5 Discussion, Conclusion and Future studies

**Discussion**

To test our hypothesis, we investigated the effect of bicarbonate on menadione redox cycling, biochemically and biologically. The proposed effect of bicarbonate on menadione redox cycling is depicted in Figure 5.1**.** In the presence of reducing agents and quinones, the rate of oxygen consumption is known to be catalytically increased [59] and this effect was enhanced by increasing the one-electron reduction potential of quinone. The reduction potential of oxygen/superoxide anion radical ($O_2$/ $O_2^{\bullet}$), menadione/semiquinone (SQ/$SQ^{\bullet}$) and semidehydroascorbyl radical/ ascorbate ($ASC^{\bullet-}$/ASC) are -325 mV, -203 mV and 300 mV, respectively [60]. It has been suggested that when the reduction potential of quinones is between - 250 and + 50 mV that means that those quinones are able to oxidize ascorbate and subsequently generating a redox cycling and reactive oxygen species. It was fortunate that the halfwave-redox potential of menadione was lower than – 250 mV, which was able to oxidize ascorbate. The reaction of ascorbic acid, an excellent antioxidant, with a wide spectrum of radicals leads to formation of ascorbyl radicals that easily dismutase to give ascorbate and dehydroascorbate. Namely, $O_2^{\bullet-}$, $HO^{\bullet}$, $^{\bullet}NO_2$, $CO_3^{\bullet-}$, $SQ^{\bullet-}$and $PhO^{\bullet}$ are scavenged by GSH and ascorbate that subsequently generating GSH redox cycling and ascorbyl radicals, respectively [61-63]. Among these radicals, $HO^{\bullet}$ that generated by the reaction of $H_2O_2$ with reduced transition metal ions [64], equation (1), such as copper (I) and iron (II), oxidizes bicarbonate, equation (2), and carbonate, equation (3), to

generate $CO_3^{\bullet -}$ [65,66].

$$H_2O_2 + Fe^{+2} \longrightarrow HO^{\cdot} + HO^{-} + Fe^{3+} \quad (1) \quad k = 4 \times 10^2\ M^{-1}S^{-1}$$

$$HCO_3^{-} + HO^{\cdot} \longrightarrow CO_3^{\cdot -} + H_2O \quad (2) \quad k = 8.5 \times 10^6\ M^{-}S^{-1}$$

$$CO_3^{-} + HO^{\cdot} \longrightarrow CO_3^{\cdot -} + HO^{-} \quad (3) \quad k = 3 \times 10^8\ M^{-1}S^{-1}$$

Furthermore, the reaction of $CO_3^{\bullet}$ with itself, equation (4), by transferring oxygen anion results in generation of $CO_2$ [67]. $H_2O_2$ would directly react with $CO_2$, equation (5), to generate peroxymonocarbonate ($HCO_4^{-}$) that can be also generated by reaction $HO^{\cdot}$ with $CO_3^{\bullet -}$equation (6), [68].

$$CO_3^{\cdot -} + CO_3^{\cdot -} \longrightarrow CO_2 + CO_4^{-2} \quad (4) \quad k = 1.5 \times 10^7\ M^{-1}S^{-1}$$

$$CO_{2\,(d)} + H_2O_2 \longrightarrow HCO_4^{-} + H^{+} \quad (5) \quad k = \sim 10^{-2}\ M^{-1}S^{-1}$$

$$CO_3^{\cdot -} + HO^{\cdot} \longrightarrow HCO_4^{-} + HO^{-} \quad (6) \quad k = 6 \times 10^9\ M^{-1}S^{-1}$$

Moreover, it has been reported that peroxymonocarbonate, an ionic peracid, is a potent oxidant in aqueous solution [69]; in the presence of transition metal ions or enzyme-metal centers, it reduces to generate $CO_3^{\bullet -}$ [70]. Although the formation of peroxymonocarbonate by reaction of $H_2O_2$ with $HCO_3^{-}$ or $CO_2$ is slow process, the presence of proteins, lipids and mimetics of carbonic anhydrase might enhance the formation of peroxymonocarbonate

via gaseous carbon dioxide/ dissolved carbon dioxide/ bicarbonate, equation (7) [71-73].

$$CO_{2\,(g)} \rightleftharpoons CO_{2\,(d)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \qquad (7)$$

In biochemical assays, we non-enzymatically monitored $O_2$ and ascorbic acid in order to reveal the effect of bicarbonate on menadione redox cycling. During menadione redox cycling that is initiated by the presence of ascorbic acid, the quinone moiety of menadione is reduced to semiquinone radicals which subsequently reduces $O_2$ to generate $O_2^{\bullet}$ and $H_2O_2$ [74-76]. Interestingly, we demonstrated that there was a significantly increase in the initial rate of oxygen consumption with 25 mM as compared with 2 mM $NaHCO_3$ (Figure 4.1). We propose that $NaHCO_3$ enhanced menadione redox cycling through formation of oxidative stress such as $CO_3^{\bullet-}$. For instance, $H_2O_2$, a product of menadione redox cycling, reacted with EBSS buffer metal ions to form $HO^{\bullet}$ via Fenton reaction (Eq. 1). Reaction $HO^{\bullet}$ with $HCO_3^-$ (Eq. 2) led to generate $CO_3^{\bullet-}$ a strong oxidizing agent. Generation of this radicals resulted in oxidation of ascorbic acid, to form semidehydroascorbyl radical, which was simultaneously dismutated to ascorbate and dehydroascorbate. Nevertheless, increased bicarbonate concentration resulted in ascorbate regeneration by formation of $CO_3^{\bullet-}$ that oxidize ascorbate to ascorbyl radicals. The latter dimutated rapidly to ascorbate which increased the redox cycling of menadione via reduction of menadione to semiquinone and consequently increased the consumption of $O_2$.

To further evaluate the effect of bicarbonate on menadione redox, we performed further studies by utilizing EPR spectrophotometry to monitor semidehydroascorbyl radical. We found that physiological bicarbonate concentrations appeared to increase the intensity of the semidehydroascorbyl radical compared to low bicarbonate concentrations (Figure 4.1.1). We speculate that presence of $H_2O_2$ that generated from menadione redox cycle and EBSS buffer trace metal ions could generate $HO^{\bullet}$ that subsequently reacted with bicarbonate to form $CO_3^{\bullet-}$ k= 1.1 x $10^9$ $M^{-1}S^{-1}$. Thus, generation of the latter radicals can enhance semidehydroascorbyl intensity. In order to further evaluate the effect of bicarbonate on the intensity of semidehydroascorbyl radical via involved metal ions and $H_2O_2$ that generated by menadione/ascorbate redox cycle, we curried out further studies by using UV-Vis spectrophotometry. We directly added $H_2O_2$ instead of that generated by menadione redox cycle to exclude the effect of semiquinone and superoxide anion radicals, a products of menadione redox cycle, on ascorbic acid oxidation. We demonstrated that the rate of ascorbic acid oxidation was increased by physiological $NaHCO_3$; however, this effect was abrogated by addition of DTPA, a metal chelator (Figure 4.1.2). We propose that $H_2O_2$ that generated by menadione/ascorbate redox cycle reacted with EBSS buffer metal ions to generate $HO^{\bullet}$; although the latter can oxidize ascorbate itself (i.e., see 0 mM $NaHCO_3$), it also appeared to react with $NaHCO_3$ to generate $CO_3^{\bullet-}$ (Eq. 2).

Taken together, the presence of $H_2O_2$ menadione/ascorbate redox cycling and the presence of trace metals, resulted in $HO^{\bullet}$ formation. The formation of $HO^{\bullet}$ in the presence of $NaHCO_3$ led us to speculate that ascorbic acid was likely being oxidized by $CO_3^{\bullet-}$. The presence of $CO_3^{\bullet-}$ can account for the increase in the formation of semidehydroascorbyl radical (Figure 4.1.1) that dismutated to ascorbate. The reformed ascorbate led to continual reduction of menadione and consequently increased the reduction of $O_2$ by menadione semiquinone radical. Alternatively, we cannot exclude the possible effect of peroxymonocarbonate ($HCO_4^-$), another strong oxidant, which results from the reaction of $CO_3^{\bullet-}$ with $HO^{\bullet}$ (Eq. 6) and the equilibrium of $H_2O_2$ with $CO_{2\ (d)}$ (Eq. 5), or $HCO_3^-$, on enhancement the menadione/ascorbate redox cycle. It was suggested that presence of transition metal ions or enzyme metal centers resulted in generation $CO_3^{\bullet-}$ via reduction of peroxymonocarbonate that also decomposes and subsequently generate $^1O_2$ (singlet oxygen) [77]. Thus, peroxymonocarbonate may catalyze the oxidation 2-methyl-1,4-hydrquinone (menadiol) to 2-methyl-1,4-benzoquinone (menadione), although this was not investigated. We showed that there was an increase in the initial rate of $O_2$ consumption by increasing the concentration of bicarbonate. Therefore, we speculate that the rate of $O_2$ consumption was increased by increasing $NaHCO_3$ concentration through generating of oxidative stress, which assumed is $CO_3^{\bullet-}$.

In order to investigate the biological relevance of the effect of $NaHCO_3$ on menadione redox cycling, we utilized murine hepatoma (Hepa 1c1c7) cells line as a model. In caner cells, intracellular pH ($pH_i$) is neutral-alkaline; however, the extracellular pH ($pH_e$) is acidic [78]. Higher proliferative and glycolytic rate resulted in generation of metabolic acids. $H^+$ extrusion by increasing the activity of $H^+$-ATPases, $Na^+$-$H^+$exchanger and the monocarboxylate-$H^+$ led to elevate the $pH_i$ and to decrease the $pH_e$ [79]. Loading of $HCO_3^-$ with the presence of carbonic anhydrase resulted in formation of $CO_2$. The latter is easily diffused inside the cells and converted by carbonic anhydrase to $HCO_3^-$. Furthermore, it has been reported that $CO_3^{\cdot}$produced from the reaction of $H_2O_2$, $HCO_3^-$ and SOD [80]. However, there is an argument about the substrate for SOD peroxidase activity, either a bicarbonate or carbon dioxide [81]. In presence of 25 mM $NaHCO_3$, a physiological concentration in mammalian tissues, we found that there was a dose dependent effect of menadione on Hepa 1c1c7 cells Figure 4.2.1.

To demonstrate the role of $NaHCO_3$, we utilized EBSS buffer with a fixed concentration of menadione with different concentration of $NaHCO_3$ Figure 4.2.2. We confirmed our findings in Figure 4.2.2 by determining the relative [ATP] as described in Materials and Methods (figure 4.2.4). Interestingly, menadione cytotoxicity was increased by increasing the concentration of $NaHCO_3$ via utilizing two end point assays. Taken together, we can speculate that $H_2O_2$, a product of menadione redox cycling, with $CO_2$, a product of loading $NaHCO_3$, permeate into the cells and in the presence of carbonic

anhydrase, a metalloenzyme that facilitate hydrate/dehydrate $CO_2$, bicarbonate would be formed to regulate $pH_i$. Consequently, the presence of bicarbonate with SOD- Cu(I), a reduction product by $H_2O_2$, would have resulted in formation of oxidative stress, including the carbonate radical. Although there are various studies that demonstrated different mechanisms of menadione cytotoxicity, herein we showed that in Hepa 1c1c7 cells and presence of copper bound oxidant (SOD-Cu(I)), bicarbonate enhanced menadione cytotoxicity by formation of oxidative stress. A previous study using millimolar concentrations of menadione (3 orders of magnitude greater than used in this study) showed that 50 mM (not 25 mM) bicarbonate inclusion in the buffer decreased the viability of yeast cells compared to phosphate [82]. The in activation of aconitase followed a similar trend, however, protein carbonyls were not altered and 50 mM (not 25 mM) bicarbonate was required to produce a decrease in total GSH.

In order to pinpoint the role of Cu, Zn -SOD in menadione cytotoxicity, we used DDC , which chelates the copper of the SOD active site [83]. We found that the menadione cytotoxicity significantly attenuated by DDC Figure 4.3.1. We can speculate that using DDC resulted in attenuation the formation of oxidative stress by preventing the SOD redox cycling via removing essential copper from this enzyme and consequently decreased menadione cytotoxicity. However, DDC, a non specific copper chelating agent, might chelate another cuproenzymes such as cytochrome c [84]. Although the menadione cytotoxicity was also decreased by utilizing another copper

chelating agent, TEPA (figure 4.3.2), it is worthwhile to perform further investigation by utilizing SOD knock out mice in order to notably reveal the effect of SOD on menadione cytotoxicity. Interestingly, it has been reported that menadione increased the activity of SOD. If the activity of SOD was also increased in hepa 1c1c7 cells, it could explain the enhanced cytotoxicity that was inhibited by DDC [82].

We utilized DCFH to broadly detect the role of bicarbonate in menadione cytotoxicity via formation of oxidative stress. DCFH is converted to a fluorescent probe (DCF) in the presence of oxidative stress. An important point to stress is that DCF is easily oxidized by several one-electron-oxidizing species, cytochrome c and $H_2O_2$ in the presence of metal ion and is by no means a specific indicator, but rather a general indicator of oxidative phenomena that are occuring [85]. We showed that DCF fluorescence, a non specific measurement probe, was increased by increasing [$NaHCO_3$] Figure 4.4. We assume that increasing the concentration of $NaHCO_3$ led to enhancement of $CO_2$ diffusion into the cell and consequently increased the generation of oxidative stress, a product of reaction bicarbonate with a strong bound oxidant of SOD. Therefore, the fluorescence probe increased by increasing the generation of oxidative stress.

In order to confirm the role of bicarbonate in generation of oxidative stress, we monitored the carbonylation of protein, a marker for detecting oxidative stress, with utilizing anti-DNP. Interestingly, we found that carbonyl-containing BSA increased by increasing the concentration of

bicarbonate; however, the carbonyl formation was attenuated with absence of SOD Figure 4.5. We presume that reduction of SOD by $H_2O_2$, a product of menadione redox cycling, resulted in formation of a strong bound oxidant of SOD that reacted with bicarbonate to form oxidative stress, which assumed is carbonate radical. Thus, increasing carbonyl-containing BSA was resulted from generating carbonate radical. Our finding are consistent with the previous study that investigated low density lipoprotein damage by iron, ascorbate , and $H_2O_2$ such that bicarbonate was shown to enhance protein carbonyls and lipid peroxidation compared to phosphate ions [86].

## Conclusion

The effect of bicarbonate on menadione cytotoxicity was demonstrated to occur by two different mechanisms as shown in figure 5.1. Both mechanisms were carried out via intermediating bicarbonate. The first mechanism involved non-enzymatic modulation of the redox cycle, which involved free metals, leading to the generation of oxidative stress. The second mechanism is mainly relevant to cells, which appears to involve SOD peroxidase activity, resulting in the generation of oxidative stress, and potentially carbonate radicals. Future studies should be carried out to determine the relative contribution to cytotoxicity of the non-enzymatic pathway compared to the superoxide dismutase peroxidase pathway.

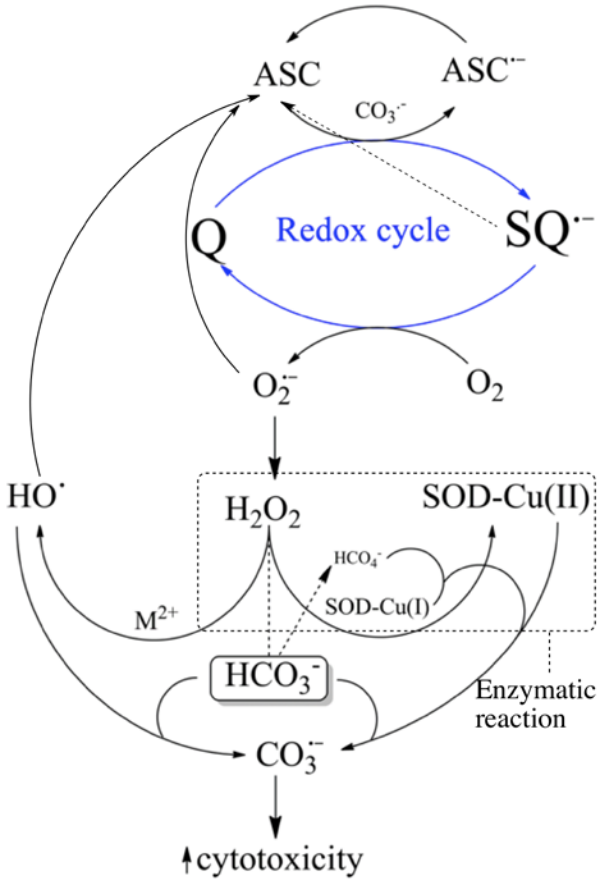



**Figure 5.1 Proposed mechanisms of carbonate radical formation and cytotoxicity from menadione redox cycling.** Redox cycling is known to generate superoxide that dismutates into $H_2O_2$. The latter can interact with bicarbonate ($HCO_3^-$) and generate peroxymonocarbonate ($HCO_4^-$), or undergo Fenton reactions with trace metals to produce hydroxyl radical ($HO^\bullet$). $O_2^{\bullet-}$, $SQ^\bullet$ and $HO^\bullet$ can oxidize ASC to $ASC^{\bullet-}$. $HO^\bullet$ can also oxidize $HCO_3^-$ directly to produce the carbonate radical ($CO_3^{\bullet-}$) or superoxide dismutase can react with $H_2O_2$ (more likely $HCO_4^-$) to also produce $CO_3^{\bullet-}$. We propose that bicarbonate is involved in contributing to the overall cytotoxicity observed due to menadione.

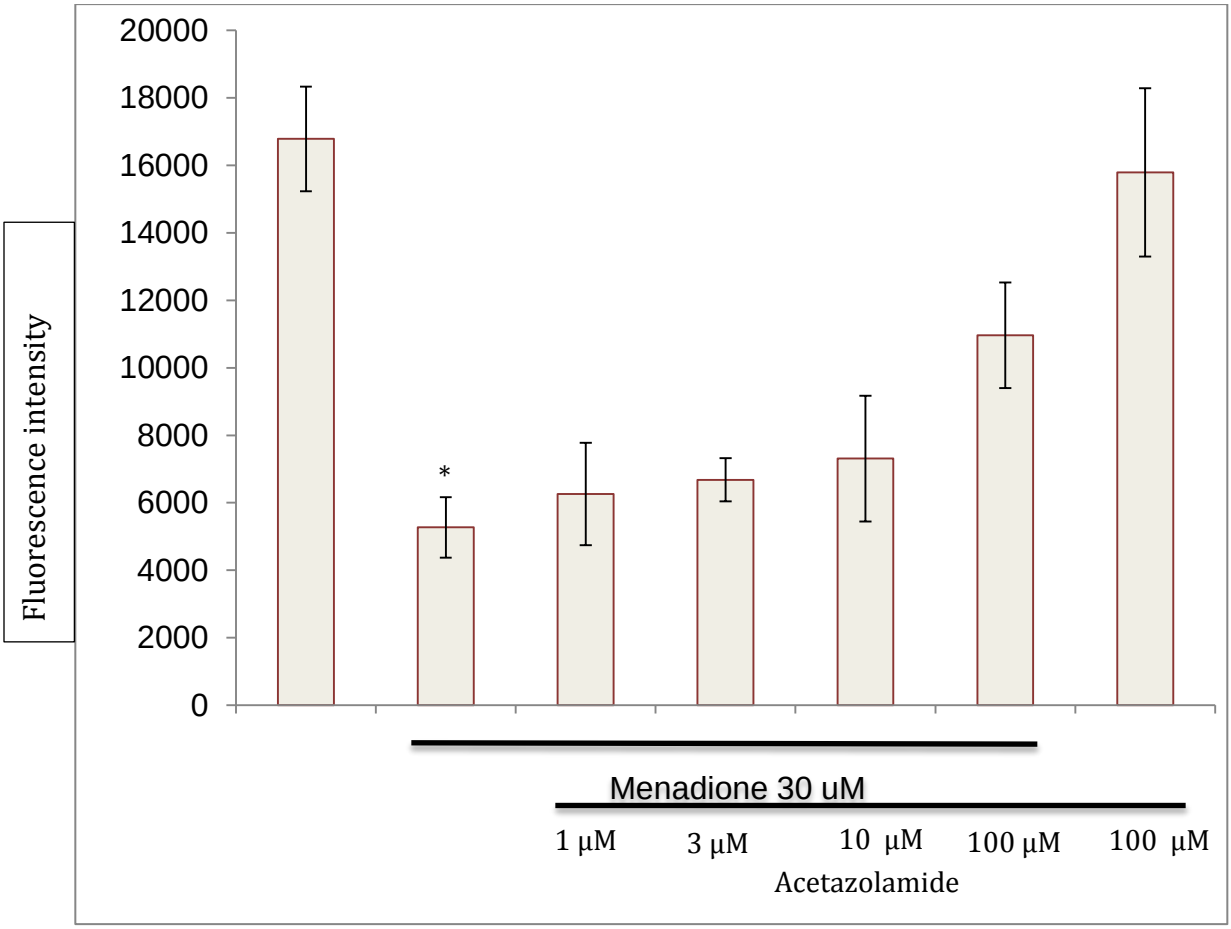
**Future studies**

In order to reveal the role of carbonic anhydrase in menadione cytotoxicity via hydrating/dehydrating carbon dioxide equation (7), we used acetazolamide, a potent inhibitor for these enzymes, to block the formation of bicarbonate. Interestingly, we found that menadione cytotoxicity was significantly attenuated by acetazolamide (100 uM). The effect of acetazolamide on menadione cytotoxicity is shown in Figure 5.2. However, it is worthwhile to utilize carbonic anhydrase knockout mice, as well as and superoxide dismutase knockout mice to confirm our findings using chemical inhibitors. Furthermore, it is also worthwhile to measure the $O_2$ uptake in isolated hepatocytes as well as hepatoma cells line to demonstrate the effect of bicarbonate on menadione redox cycling in different viable biological systems. Taken together, these experiments could determine the relative contribution to cytotoxicity of the non-enzymatic pathway compared to the metalloenzyes pathway, namely superoxide dismutase and carbonic anhydrase.

It has been demonstrated that menadione enhanced ATP depletion (figure 4.2.3), thus, monitoring the cell signals such as P-ERK, P-p38 and PARP is more useful to confirm the effect of bicarbonate on menadione cytotoxicity through generating oxidative stress, carbonate radical by utilizing both normal hepatocytes and hepatoma cells (figure 5.3).

Although there is a challenge to detect carbonate radicals and peroxymonocarbonate in vivo as it is intricate to find a specific spin traps for carbonate and hydroxyl radicals, monitoring different end points, such as tryptophan oxidation, tyrosin oxidation and using spin trapping such as DMPO would indirectly elicit the effect of carbonate radical or hydroxyl radical on menadione cytotoxicity.

Based on the presence of bicarbonate/carbon dioxide a physiological buffer, menadione can be used as a standard because of its ability to undergo redox cycling and arylation. Nevertheless, it is useful to compare the cytotoxicity of quinone redox cycling, such as doxorubicin and 2,6-dimethoxy quinone to alkylating quinones, benzoquinone, tetrachlorobenzoquine, for example, by doing different end points. Because of doxorubicin has half-cell reduction potential lower than -250 mV, which is able to generate reactive oxygen species, and the presence of CuZn-superoxide dismutase in intermembrane of mitochondria, we can envision the role of CuZn-superoxide dismutase in doxorubicin cardiotoxicity. It is worth if we demonstrate that bicarbonate potentiate the quinone (doxorubicin)/ascorbate redox cycle through generating oxidative stress, carbonate radical since tumor cells are preferential uptake of ascorbic acid (vitamin C).




**<u>Figure 5.2</u> Effect of acetazolamide on menadione cytotoxicity.** Murine hepatoma (Hepa 1c1c7) cells were incubated in DMEM media with different concentration of the CA inhibitor acetazolamide for one hour. Cells were incubated for 3 hours in EBSS buffer containing 25 mM bicarbonate and 30 µM menadione. The cytotoxicity was measured by utilizing alamarBlue assay. Data were shown as the mean ± SD (n=6). * $p < 0.005$ vs 100 µM acetazolamide + 30 µM menadione.

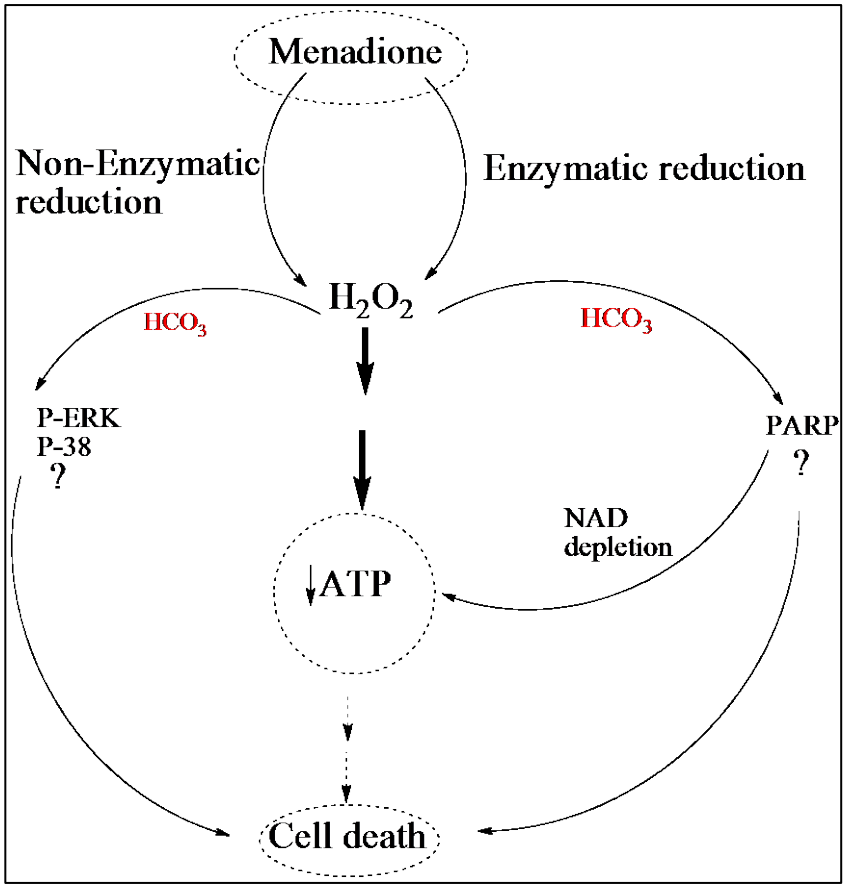



**Figure 5.3 Schematic effect of carbonate radical formation on cell signals during ATP depletion.**

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