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QUINONE-INDUCED DNA SINGLE STRAND BREAKS IN RAT HEPATOCYTES AND HUMAN CHRONIC MYELOGENOUS LEUKAEMIC K562 CELLS

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Abstract—In rat hepatocytes exposed to the quinones menadione and 2,3-dimethoxy-1,4-naphthoquinone (2,3-diOMe-1,4-NQ) a decrease in NAD^+ is observed. DNA damage and activation of poly(ADP-ribose)polymerase are often associated with a decrease in NAD^+. Using rat hepatocytes and human myeloid leukaemic cells (K562), we examined the extent of DNA damage induced by these quinones at non-toxic concentrations, i.e. at concentrations at which the cells completely exclude the dye trypan blue. Both quinones caused significant DNA damage at very low concentrations (5–100 μM). With 2,3-diOME-1,4-NQ (15 μM) or menadione (15 μM) single strand breaks (SSB) were observed at very early time points (<5 min), reaching a maximum between 20 and 30 min. Most SSB were repaired within 45 min of the removal of the quinones. Whilst extensive repair was observed within 4 hr of the removal of 2,3-diOMe-1,4-NQ (15 μM), only partial repair was observed following exposure to menadione (15 μM). SSB induced by 2,3-diOMe-1,4-NQ (15 μM) were completely inhibited by the iron chelator 1,10-phenanthroline (25 μM), whereas in cells exposed to menadione (15 μM) they were only partially inhibited. Finally, although the membrane integrity of K562 cells was unaffected by exposure to high concentrations of both quinones (≤400 μM), cytostasis was observed at much lower concentrations (50 μM). Our results demonstrate that at very low concentrations these quinones induce extensive DNA damage possibly caused by hydroxyl radicals. The DNA damage was accompanied by an early cytostasis but no loss of membrane integrity.

Quinones are both widely distributed in nature and used extensively as therapeutic agents. They may exert their toxicity by a number of mechanisms including oxidative stress caused by redox cycling [1, 2], direct interaction with cellular macromolecules [3] and inhibition of mitochondrial electron transport [4]. Oxidative stress following redox cycling often results from a disproportionate consumption of oxygen and cellular reducing equivalents [NAD(P)H], resulting in the generation of active oxygen species ($O_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$) [1, 2]. Depending on the balance of prooxidant and antioxidant activities, oxidative stress may lead to a number of effects including cell death. Cytotoxicity, measured by the inability of the cell to exclude the dye trypan blue, is usually preceded by a depletion of the pyridine nucleotide NAD^+ [5]. A decrease in this nucleotide may play a role in the later depletion of ATP, observed just prior to cell death [5]. The oxidation of critical sulphydryl groups such as those present in ATP-dependent Ca^{2+} translocases results in their inactivation and a subsequent increase in cytosolic free Ca^{2+} [1, 6–8]. The elevation of intracellular Ca^{2+} has also been associated with the activation of proteases and phospholipases and the early onset of cell death [9, 10]. At low concentrations of the quinones when the cells completely exclude trypan blue, the depletion in NAD^+, the oxidation of sulphydryls and the increase in intracellular Ca^{2+} are small and transient [5, 7].

Depletion of NAD^+ is often associated with DNA damage resulting in the activation of poly(ADP-ribose)polymerase which utilizes NAD^+ as a source of ADP-ribose [11–13]. During oxidative stress, single strand breaks (SSB§) in DNA may be induced by hydroxyl radicals generated during the metabolism of redox cycling quinones [2]. This process involves the reduction of H_2O_2 by divalent metal ions, particularly Fe^{2+} in an iron-catalysed Haber–Weiss reaction [14, 15]. During oxidative stress, DNA damage may also result from the activation of Ca^{2+}-dependent endonucleases possibly leading to DNA fragmentation and apoptosis [16, 17].

We have shown previously that in hepatocytes exposed to non-toxic concentrations of either 2,3-dimethoxy-1,4-naphthoquinone (2,3-diOMe-1,4-NQ) (a pure redox-cycling quinone) or menadione (a quinone which acts mainly by redox cycling but in part by arylation of macromolecules) the dye trypan blue is completely excluded but a decrease in NAD^+ was observed [5], suggesting that some DNA damage may have occurred. In this study, we have used rat hepatocytes and human chronic myelogenous leukaemic cells (K562) [18] [which possess NADPH-cytochrome *c* (cytochrome P450) reductase [19] one of the major enzymes involved in the one electron reduction of redox cycling

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§ Abbreviations: 2,3-diOMe-1,4-NQ; 2,3-dimethoxy-1,4-naphthoquinone; SSB, single strand breaks; DMSO, dimethyl sulphoxide.

quinones] to investigate the extent of DNA damage induced by these quinones. Our initial studies were carried out with isolated hepatocytes [5], which only remain viable for a short period of time (4–6 hr), making it difficult to distinguish between the effects of DNA damage and other causes of cell death. In order to overcome this difficulty we have extended the study to K562 cells, which provide a more suitable model to assess the long term effects of DNA damage [20].

Our results suggest that at low non-toxic concentrations, when the cells completely excluded trypan blue, the quinones induce extensive DNA damage in the form of SSB which are probably induced by hydroxyl radicals. SSB are observed within 5 min of exposure to the quinones, and extensive repair is observed within 1 hr of the first signs of damage. We also found that at concentrations of the quinones at which the cells totally exclude trypan blue, a significant reduction in cell proliferation was observed.

MATERIALS AND METHODS

Materials. Collagenase Type 1, HEPES and proteinase-K were obtained from the Sigma Chemical Co. (Poole, U.K.). [^{14}C]Thymidine (50–60 mCi/mmol) was obtained from Amersham International (Rickmansworth, U.K.). Polyvinyl chloride filters were purchased from Millipore. Menadione (2-methyl-1,4-naphthoquinone) was obtained from the Aldrich Chemical Co. (Gillingham, U.K.) and 2,3-diOMe-1,4-NQ was synthesized as described previously [1]. All other chemicals were obtained from BDH (Poole, U.K.).

Isolation and incubation of rat hepatocytes. Hepatocytes were prepared as described previously [5, 21] by collagenase perfusion on the livers of adult male Wistar rats (220–260 g). Cell viability (85–95%) was determined by their ability to exclude trypan blue, 0.4% (w/v) final concentration. The isolated cells (10^6 cells/mL) were suspended in Krebs–Henseleit buffer containing 12.5 mM HEPES under an atmosphere of 95% O_2/5% CO_2 (pH 7.4) and incubated at 37° in continuously rotating round bottom flasks. Following isolation the cells were allowed to equilibrate by preincubation at 37° for 30 min prior to treatment.

The quinones were dissolved in DMSO and added at a final DMSO concentration of 0.1% (v/v). The control incubations had a final DMSO concentration of 0.1% (v/v) alone. After exposure to the quinones, 0.8-mL aliquots of cell suspension (10^6 cells/mL) were removed and spun at 500 rpm for 1 min in a bench centrifuge (4°). The supernatant solution was removed by aspiration and the cells resuspended in 2 mL of ice cold Krebs–Henseleit buffer and kept on ice for 30 min prior to determination of DNA damage as described below.

Cell culture and treatment. Human chronic myelogenous leukaemic cells (K562) [18], from the Department of Oncology, University College and Middlesex School of Medicine, were cultured in RPMI 1640 supplemented with 5% heat-inactivated foetal calf serum and L-glutamine (2 mM) at 37°. In order to determine damage to DNA, cells in exponential growth phase were labelled with 0.015 μCi/mL [^{14}C]thymidine for 20 hr, then incubated in the absence of radiolabel for 1 hr to chase all the radioactivity into high molecular mass DNA prior to treatment. Cells (10^6/mL) were then exposed to quinones [in DMSO 0.1% (v/v)] and incubated for various times at 37°. Aliquots (0.8 mL) of the cell suspension were removed and made up to 10 mL with ice-cold phosphate-buffered saline and immediately assayed for DNA damage as described below.

Measurement of SSB in DNA. SSB were assayed by alkaline elution as described previously [22]. Briefly the cells (8×10^5) were lysed on 2-μm pore, 25-mm diameter polycarbonate filters with a sodium dodecylsarcosine-NaCl-EDTA lysis solution at pH 10. Elution was carried out at a rate of 2 mL/hr for 15 hr in a tetrapropylammonium hydroxide-EDTA solution at pH 12.1 in the presence of proteinase K. Samples were collected every 3 hr and assayed for ^{14}C radioactivity. As the DNA of isolated hepatocytes could not be radioactively labelled, the eluted DNA was determined fluorometrically [23].

Determination of NAD$^+$ in K562 cells. NAD$^+$ was assayed as described previously [5]. Briefly, following exposure to the quinones, 1.0-mL aliquots (10^6 cells/mL) of cell suspension were sedimented by bench centrifugation at 1000 rpm for 3 min and the supernatant fraction aspirated. NAD$^+$ was extracted by additon of 0.5 M perchloric acid and the acid extract stored at −80° prior to analysis.

Determination of growth inhibition. K562 cells (10^6 cells/mL) were exposed to various concentrations of the quinones for 15 min after which they were suspended in fresh medium and diluted to 10^5 cells/mL. The cells were then incubated for 72 hr at 37° during which time cell number was determined at 24-hr intervals.

RESULTS

Quinone-induced toxicity and SSB in rat hepatocytes

Neither menadione (50 μM) nor 2,3-diOMe-1,4-NQ (50 μM) was toxic to isolated hepatocytes as assessed by trypan blue exclusion in agreement with previous studies [5]. In contrast, a brief exposure (15 min) of hepatocytes to either quinone caused significant SSB (Fig. 1) as assessed by alkaline elution [22]. Under these conditions, the extent of SSB was greater in cells exposed to menadione than to 2,3-diOMe-1,4-NQ (Fig. 1). In preliminary experiments, the SSB induced by both quinones were partially inhibited by preincubating the hepatocytes for 10 min with the iron chelator 1,10-phenanthroline (25 μM) [24] before exposure to the quinone. A significant number of SSB was also observed in control hepatocytes incubated in the absence of quinone. This was possibly related to the presence of approximately 15–20% dead cells in the incubation. In order to reduce this background value and also to utilize the more sensitive radiometric assay for DNA SSB, we investigated the effects of the quinones in human myeloid leukaemic cells (K562).

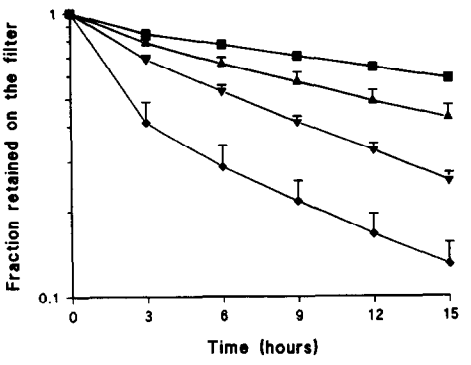

Fig. 1. The effect of quinones on the elution kinetics of DNA from rat hepatocytes. Hepatocytes (10^6 cells/mL) were exposed to DMSO alone (■), menadione 50 μM (◆) 2,3-diOMe-1,4-NQ 50 (▲) or 100 μM (▼) for 15 min at 37°. Cells (8×10^5) were then diluted (×10) in ice-cold phosphate-buffered saline, washed and lysed on the filter before alkaline elution. Values represent the means ± SEM of three hepatocyte preparations.

Quinone-induced toxicity, SSB and pyridine nucleotide changes in K562 cells

In K562 cells exposed to either menadione (15–400 μM) or 2,3-diOMe-1,4-NQ (15–400 μM) for 15 min, no loss of membrane integrity was observed during the following 4 hr. However, in K562 cells exposed to either quinone (15 μM), SSB could be detected by 5 min and within 10 min DNA damage was extensive (Fig. 2). Within the first 10 min of exposure there was greater DNA damage in cells exposed to 2,3-diOMe-1,4-NQ (Fig. 2a) than in cells exposed to menadione (Fig. 2b). Maximum DNA damage was observed between 20 and 30 min with no further increase in SSB between 30 and 60 min. Both quinones induced a concentration-dependent increase in SSB (Fig. 3). SSB were detected at concentrations as low as 5 μM. At concentrations ≤10 μM, the extent of SSB induced by menadione was less than that induced by 2,3-diOMe-1,4-NQ (Fig. 3).

Within 45 minutes of the removal of the extracellular quinone, extensive repair of SSB was detected (Fig. 4). Almost complete (>95%) repair was observed within 4 hr of the removal of 2,3-diOMe-1,4-NQ (15 μM) (Fig. 4a). In the presence of menadione (15 μM) initial repair was extensive; however, little further repair was observed and at 4 hr only 85% of SSB were repaired (Fig. 4b).

In K562 cells preincubated for 30 min with 1,10-phenanthroline (25 μM) and then exposed to 2,3-diOMe-1,4-NQ (15 μM) for 15 min, no SSB were observed (fig. 5a). In cells exposed to menadione (15 μM) for 15 min, the SSB were significantly reduced but were not inhibited completely (Fig. 5b). Higher concentrations of 1,10-phenanthroline (50 μM) failed to reduce further the SSB in menadione-treated cells suggesting an additional mechanism of DNA damage (Fig. 5b).

We have reported previously a decrease in the pyridine nucleotide NAD^+ in hepatocytes exposed to either 2,3-diOMe-1,4-NQ or menadione [5]. In K562 cells exposed to either quinone (15 μM) for 15 min, a significant decrease in NAD^+ was observed (Fig. 6).

The effect of the quinones on the replication of K562 cells

In order to obtain some indication of the longer term consequences of SSB on cell growth, we examined the rate of proliferation of K562 cells following exposure to the quinones. At low concentrations of quinone (15 μM), the rate of proliferation of cells exposed for 15 min was identical to controls (Fig. 7). A reduced rate of growth was observed in cells exposed to higher concentrations of 2,3-diOMe-1,4-NQ (50–100 μM) (Fig. 7a). No

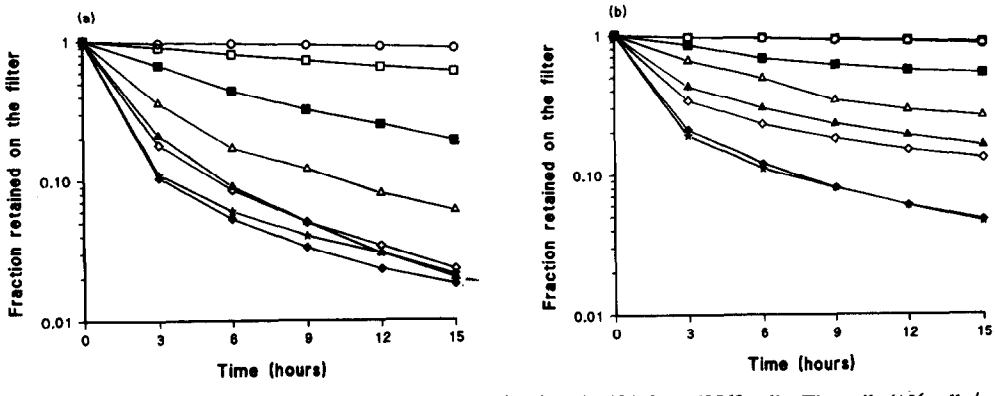

Fig. 2. The effect of incubation time on the elution kinetics of DNA from K562 cells. The cells (10^6 cells/mL) were exposed to (a) 2,3-diOMe-1,4-NQ 15 μM and (b) menadione 15 μM for 0 min (○), 1 min (□), 5 min (■), 10 min (△), 15 min (▲), 20 min (◇), 30 min (◆) and 60 min (*). At the indicated times, cells were removed and elution kinetics determined. The results represent the mean of at least two incubations.

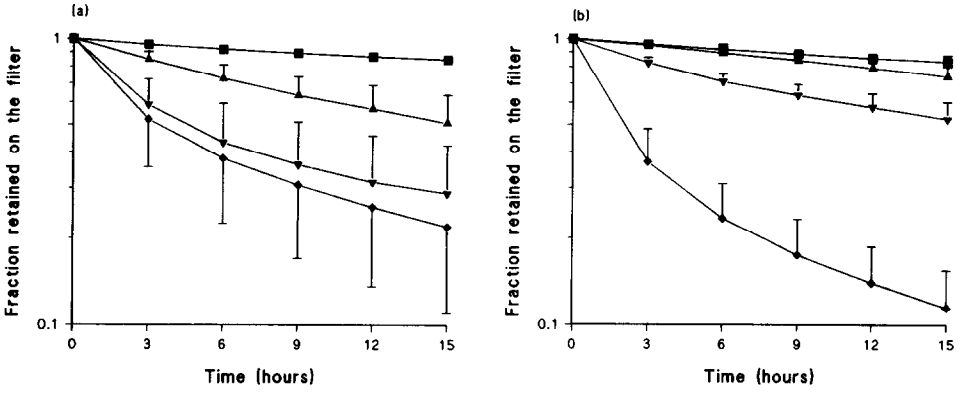

Fig. 3. The effect of quinone concentration on the elution kinetics of DNA from K562 cells. Cells were exposed to (a) 2,3-diOMe-1,4-NQ and (b) menadione at the following concentrations: 0 (■), 5 (▲), 10 (▼), 15 μM (◆), for 15 minutes before being removed for alkaline elution. The results represent the means ± SEM of at least four incubations.

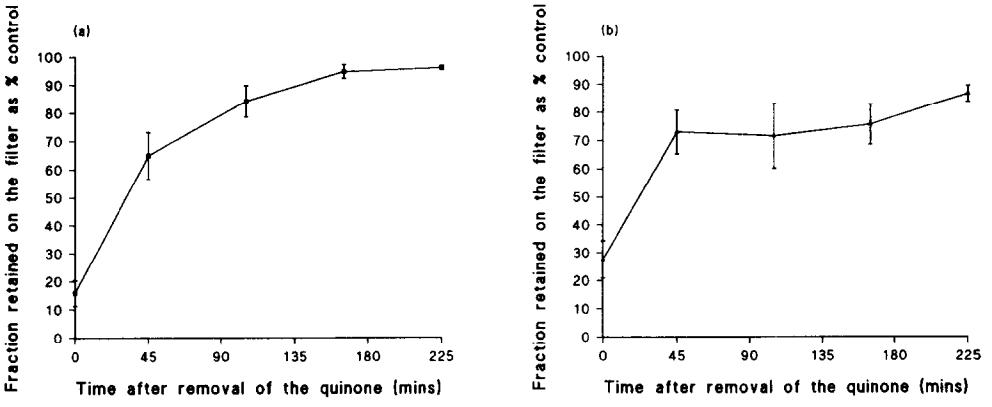

Fig. 4. The repair of SSB induced by quinones in K562 cells. Cells were incubated with either (a) 2,3-diOMe-1,4-NQ (15 μM) or (b) menadione (15 μM) for 15 min then resuspended in fresh medium. At the specified times, 0.8-mL aliquots were removed and elution kinetics determined. Values represent means ± SEM of at least five incubations. Control cells were treated with DMSO for 15 min and then resuspended in fresh medium. The fraction retained on the filter in control cells was always above 83%.

proliferation was observed in cells exposed to higher concentrations of menadione (50–100 μM) (Fig. 7b).

DISCUSSION

We had reported previously a decrease in NAD^+ in rat hepatocytes exposed to either 2,3-diOMe-1,4-NQ or menadione at non-toxic concentrations as assessed by trypan-blue exclusion [5]. In order to ascertain if this was linked with DNA damage, we investigated the extent of SSB in rat hepatocytes exposed to these quinones. In hepatocytes both quinones induced significant SSB (Fig. 1), which in preliminary studies was partially inhibited by 1,10-phenanthroline, pointing to the involvement of hydroxyl radicals. The presence of SSB in hepatocytes exposed to either quinone suggested that the previously reported decrease in NAD^+ [5] was due primarily to the activation of the enzyme poly(ADP-ribose)polymerase [11, 12], although previously we were unable to inhibit the decrease in NAD^+ with 3-aminobenzamide, an inhibitor of the polymerase [5].

Exposure of K562 cells for 15 min to either menadione (10–400 μM) or 2,3-diOM3-1,4-NQ (10–400 μM) induced extensive SSB (Fig. 2) without any loss of membrane integrity during the ensuing 4 hr. This observation is in agreement with previous studies which suggested that the presence of SSB did not automatically lead to acute toxicity [25]. SSB were detected within 5 min of exposure to either quinone and correlated well with the oxidation of NADPH and other signs of redox cycling [1, 2]. These observations implicate the process of redox cycling in the early appearance of quinone-induced SSB.

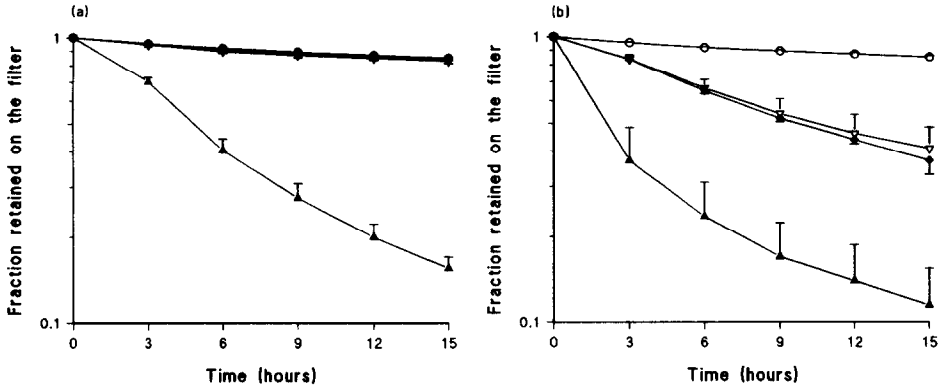

Fig. 5. Prevention of SSB by 1,10-phenanthroline in K562 cells. Cells were exposed for 15 min to (a) 2,3-diOMe-1,4-NQ (15 μM) or (b) menadione (15 μM), either alone (▲), or with the quinone (15 μM) and either 25 (◆) or 50 (▽) μM phenanthroline. Cells treated with phenanthroline were preincubated for 30 min before exposure to the quinone. Control cells (○) were incubated with phenanthroline (50 μM) alone. The results represent the means ± SEM of at least five incubations.

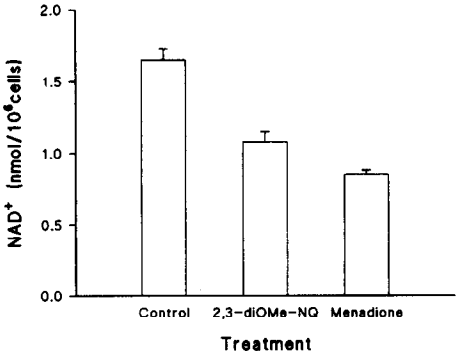

Fig. 6. The effect of quinones on the level of NAD^+ in K562 cells. Cells were exposed to either 2,3-diOMe-1,4-NQ (15 μM) or menadione (15 μM) for 15 min after which NAD^+ was determined as described in the materials and methods. Values represent the means ± SEM of four individual incubations.

In mammalian cells exposed to redox cycling quinones both enzyme-mediated DNA fragmentation [17] and hydroxyl radical-induced SSB [25] have been reported. Whereas DNA fragmentation is observed after several hours of exposure [16], hydroxyl radical-induced SSB may be seen within minutes [11]. The early detection of DNA damage ($\leq$5 min) with alkaline elution kinetics consistent with the formation of SSB points to the generation of hydroxyl radicals as the probable cause of DNA damage in the present study. The generation of hydroxyl radicals is additionally implicated by the inhibition of SSB induced by 2,3-diOMe-1,4-NQ by the iron chelator 1,10-phenanthroline [24, 25, 27] (Fig. 5). 1,10-Phenanthroline has been shown to enter cells and to inhibit hydroxyl radical formation possibly by chelating iron on chromatin [26, 27]. As discussed earlier the availability of Fe^{2+} is crucial to the formation of hydroxyl radicals during redox cycling [2]. Although hydroxyl radicals are very reactive they are unable to diffuse over long distances; hence, if generated outside the nucleus they are unlikely to cause significant DNA damage. The extensive SSB observed at very low concentrations of both quinones may be due to the binding of iron to DNA [14, 20], so allowing the generation of hydroxyl radicals in close proximity to the DNA and maximizing the DNA damage.

Previously, it had been demonstrated that menadione bound directly to DNA causing SSB [28]. The persistence of some SSB in cells preincubated with 1,10-phenanthroline and exposed to menadione (Fig. 5b) may be attributed to this effect. The ability of menadione to arylate nucleophiles [1] would also decrease its effective concentration available to redox cycle and thus reduce its ability to cause SSB at lower concentrations. This may partially explain the observation that SSB in K562 cells exposed to 2,3-diOMe-1,4-NQ $\leq$10 μM occurred to a greater extent than those induced by menadione (Fig. 3).

A large proportion of the quinone-induced SSB was repaired within 45 min of the removal of the quinone (Fig. 4), in accordance with previous studies [29]. A complete repair of SSB induced by 2,3-diOMe-1,4-NQ was observed within 4 hr, in contrast to those induced by menadione which were still detectable after this time (Fig. 4). This observation together with the data with 1,10-phenanthroline strongly suggests that the SSB induced by menadione were only partially mediated by the generation of hydroxyl radicals. A depletion in NAD^+ was observed in K562 cells exposed to 2,3-diOMe-1,4-NQ (15 μM) and menadione (15 μM) (Fig. 6). As in other cell systems, a depletion in NAD^+ below a critical level may lead to a depletion in ATP and cell death as part of a suicide response [30].

The growth inhibition assay provided the opportunity to examine some long term effects of DNA damage. At a low concentration (15 μM), neither quinone affected the rate of cell proliferation (Fig.

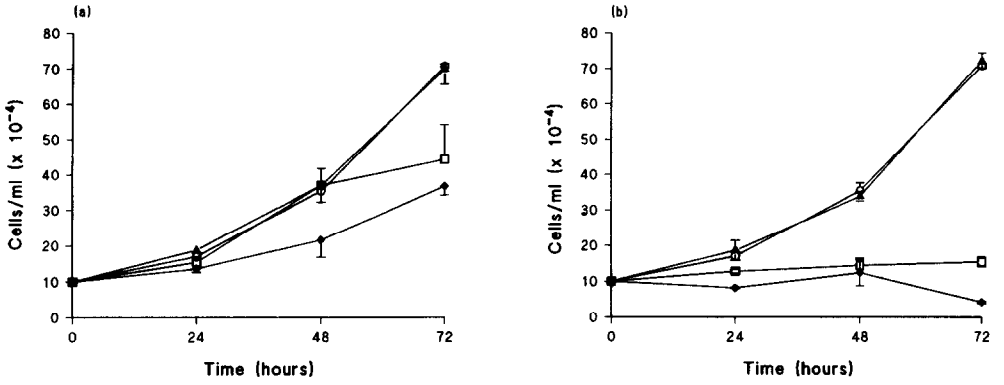

Fig. 7. The effect of quinones on the proliferation of K562 cells. Cells were exposed to (a) 2,3-diOMe-1,4-NQ or (b) menadione at the following concentrations: 0 (○), 15 (▲), 50 (□) or 100 (◆) μM of the quinones for 15 min, then resuspended in fresh medium and diluted to 10^5 cells/mL. The cells were then incubated at 37° for 72 hr and cell number determined at times indicated. The results represent the means ± SEM of three incubations.

7), in agreement with the data on SSB repair when substantial repair was observed (Fig. 4). At higher concentrations of 2,3-diOMe-1,4-NQ (50–100 μM) proliferation was significantly reduced (Fig. 7); this may be accounted for by the extent of DNA damage and the rate of SSB repair above 15 μM. Menadione (50 μM) completely inhibited cell proliferation (Fig. 7), which may have been due to several factors including the extent of DNA damage, a slower rate of DNA repair, a low fidelity of repair as well as the binding of menadione to DNA [28]. These results highlight the importance of the marker used for assessing toxicity in cellular studies [31]. Thus using exclusion of trypan blue as a marker of cellular viability, cells exposed to either quinone at concentrations of up to 400 μM were non-toxic. However, both quinones induced extensive DNA damage at 15 μM and marked cytostatic properties were observed at 50 μM.

In conclusion, in rat hepatocytes and K562 cells, menadione and 2,3-diOMe-1,4-NQ induced extensive DNA damage manifested in the form of SSB and possibly mediated by hydroxyl radicals generated during redox cycling. Despite the high levels of SSB, most were rapidly repaired without the loss of membrane integrity; however, following prolonged incubation a significant reduction in cell proliferation was observed. At the higher concentrations, cell death may be caused by double strand breaks resulting from the formation of more than one single strand break in close proximity. These results highlight again the importance of the marker used to assess cytotoxicity.

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