

Effect of microcystin-LR on cultured rat endothelial cells

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Introduction: Microcystin-LR (MCLR), a cyclic heptapeptide synthesised by the cyanobacterium *Microcystis aeruginosa* [1], induces hepatotoxicity in many species including man [2]. After administration to laboratory rodents, MCLR rapidly induces severe liver haemorrhage which is associated with centrilobular hepatocyte necrosis [3]. MCLR not only induces the rapid onset of liver damage in rodents, but also induces necrosis of cultured rat hepatocytes [4, 5]. The mechanism by which MCLR induces hepatotoxicity is not known. MCLR does, however, induce early changes in cultured hepatocytes, such as deformation of cells (blebbing) rapid rise in intracellular calcium, increased phosphorylase-a activity, depletion of glutathione [4, 5], and the release of arachidonic acid metabolites [6]. These early events are followed by the leakage of adenine nucleotides and cytosolic enzymes and eventually, the loss of cell viability [7].

Although MCLR toxicity to cultured hepatocytes has been well documented, relatively little is known about its effect on other non-parenchymal liver cells - that is, sinusoidal endothelial and Kupffer cells. In the present study, we investigated the effects of MCLR on cultured primary liver endothelial cells. We also investigated whether hepatocytes treated with a cytotoxic dose (4 μM) of MCLR released factors (mediators) that could induce changes, or cytotoxicity in endothelial monolayers.

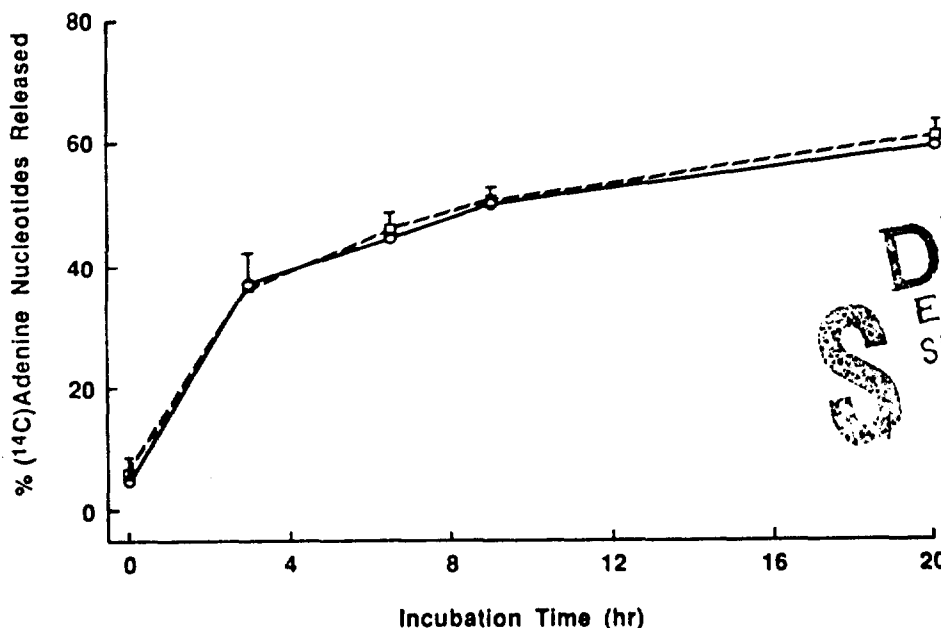
Some endothelial cells were pre-treated with the anti-

oxidants, dithioerythritol (DTE), or silymarin (SM) [8], in order to determine if these agents could prevent changes induced by supernatants derived from MCLR treated hepatocytes. [¹⁴C]adenine nucleotide release was used to monitor cell damage [9]. While the release of adenine nucleotides from healthy endothelial cells is relatively high, it is still used as a method for detecting small perturbations or disturbances in endothelial cells [10]. Microscopy and cell density assays were also used to detect morphological changes in endothelial cells.

Materials and methods: The following materials were obtained commercially from the indicated sources: silymarin (SM) (Aldrich, Milwaukee, WI), [¹⁴C]adenine (60 mCi mmol⁻¹, New England Nuclear, Boston, MA), tissue culture media and fetal bovine serum albumin (GIBCO, Grand Island, NY), tissue culture ware (Becton-Dickinson Labware, Lincoln Park, NJ), collagen, collagenase type IV, dithioerythritol (DTE), endothelial growth factor and heparin (Sigma, St. Louis, MO). Male W.F. LEW, inbred rats (G. Anderson, USA-MRIID, Fort Detrick, Frederick, MD) weighing between 250-300 g were used. MCLR (> 95% purity by HPLC) was obtained from Dr W. Carnichael, Wright State University, Dayton, Ohio.

Rat hepatocytes were isolated and cultured according to the method of Elliget and Kolaja [11]. Hepatocytes were separated from non-parenchymal cells by low speed centrifugation and the number of viable cells was determined by trypan blue exclusion. Hepatocytes were suspended at half-a-

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Figure 1: Effect of microcystin-LR on the percent release of [¹⁴C]adenine nucleotides from primary sinusoidal endothelial monolayers (means ± SD; n = 3). [¹⁴C]adenine labelled endothelial cells were incubated in medium containing 4 μM microcystin-LR (□-□) or medium alone (○-○). There was no difference (AOV, α = 0.05 for F-test) between treatments.

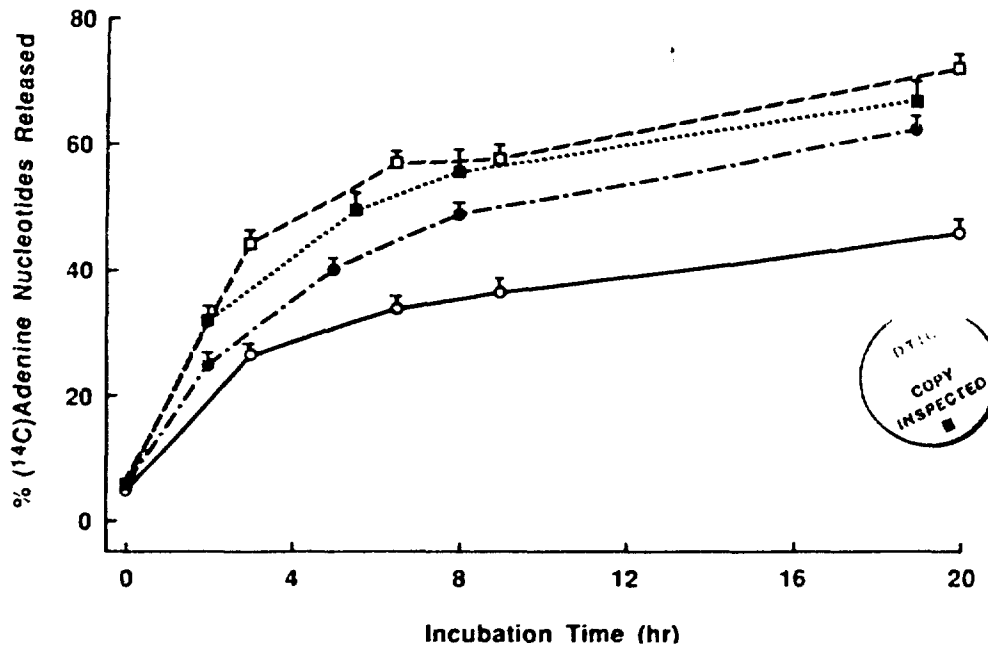


Figure 2: Effect of supernatants derived from microcystin-LR-treated hepatocytes on the percent release of $[^{14}\text{C}]$ adenine nucleotide from primary sinusoidal endothelial monolayers (means \pm SD; $n = 3$). $[^{14}\text{C}]$ adenine loaded endothelial cells were incubated with supernatants collected from hepatocytes incubated with $4\ \mu\text{M}$ microcystin-LR (□) or medium alone (○). Some endothelial cells were treated with either $2.5\ \text{mM}$ dithioerythritol (■) or $0.2\ \text{mM}$ silymarin (●) 30 min before receiving supernatants from microcystin-LR treated hepatocytes. Silymarin treatment, but not dithioerythritol, was statistically significant (AOV, $\alpha = 0.05$) from microcystin-LR treatment. All treatments were statistically significant from medium control.

million viable cells per mL in Leibovitch's (L15) culture medium containing 17% fetal bovine serum (FBS) and seeded in collagen-coated, 35 mm, 6-well-plates.

Sinusoidal endothelial cells were isolated according to the procedure of Smedsrød and Pertoft [12] using two-step Percoll gradient. The number of viable endothelial cells was determined by trypan blue exclusion. Endothelial cells were resuspended at one-half million viable cells per mL in RPMI 1640 containing 10% FBS and 15 mg endothelial growth factor and seeded on collagen-coated, 35 mm, 6-well-plates.

Cells were incubated overnight at 37°C with 5% CO_2 and 90% humidity. After incubation, the majority of cells had attached to the well surface and established a monolayer. Non-attached cells were removed by aspiration and an additional 1 mL of culture medium was added to each well.

One mL of L15 medium containing $4\ \mu\text{M}$ MCLR or 1 mL of medium alone was added to hepatocytes, then incubated for 16 h (overnight) at 37°C in a humidified incubator in the presence of 5% CO_2 . After incubation, cell supernatants were removed from both toxin-treated and control (no toxin) cells, centrifuged at $500\ g$ for 4 min in an Eppendorf centrifuge, Model-5414, to remove cell debris, and stored at 4°C for a maximum of 2 h before use.

After endothelial cells were incubated overnight, the culture medium from each well was replaced and cells were labelled with $[^{14}\text{C}]$ adenine ($1.4\ \mu\text{M}$, $0.082\ \mu\text{Ci}$) as described by Shirhatti and Krishna [9]. Labelled cells were then incubated with 1 mL of L15 medium containing $4\ \mu\text{M}$ MCLR, medium alone, or 1 mL of supernatants from control or MCLR-treated hepatocytes for a total of 20 h. In addition, some endothelial cells were pre-treated for 30 min with $2.5\ \text{mM}$ DTE or $0.2\ \text{mM}$ SM followed by the addition of

supernatants derived from MCLR-treated hepatocytes or L15 medium.

The amount of $[^{14}\text{C}]$ adenine nucleotides released from cells into supernatant were counted (Beckman scintillation counter, model LS5800, Fullerton, CA). Cells were lysed with 1 mL of 0.05% digitonin and an aliquot of cell lysate was analysed for radioactivity and protein content (Pierce protein reagent, Rockford, IL). Control and treated cells were examined under phase contrast microscope (Nikon Diphot inverted phase contrast microscope) for morphological changes. Photographs of cells ($25 \times 20\ \text{cm}$) were subjected to quantitative analysis to describe morphological changes. Total cell length (L) including projection(s), cell body length (b) and cell body width (w) were measured manually. The photograph was divided into five equal areas, where 10 cells were randomly selected and measured.

Data were analysed for statistical difference using *t*-test ($\alpha = 0.05$) between two population means.

In conducting the research described in this report, the authors adhered to the "Guide for Care and Use of Laboratory Animals", NAS/NRC.

Results: The release of $[^{14}\text{C}]$ adenine nucleotides from endothelial cells treated with $4\ \mu\text{M}$ MCLR was not significantly different from control cells incubated with L15 medium alone (Figure 1). Concentrations of up to $50\ \mu\text{M}$ MCLR did not induce any significant release of nucleotides than control levels (data not shown). Endothelial cells treated with supernatants from control (no toxin) hepatocytes (Figure 2) released less $[^{14}\text{C}]$ adenine nucleotides than cells incubated in L15 medium alone (Figure 1), that may be due to the release of "conditioning factors" which were beneficial to endothelial

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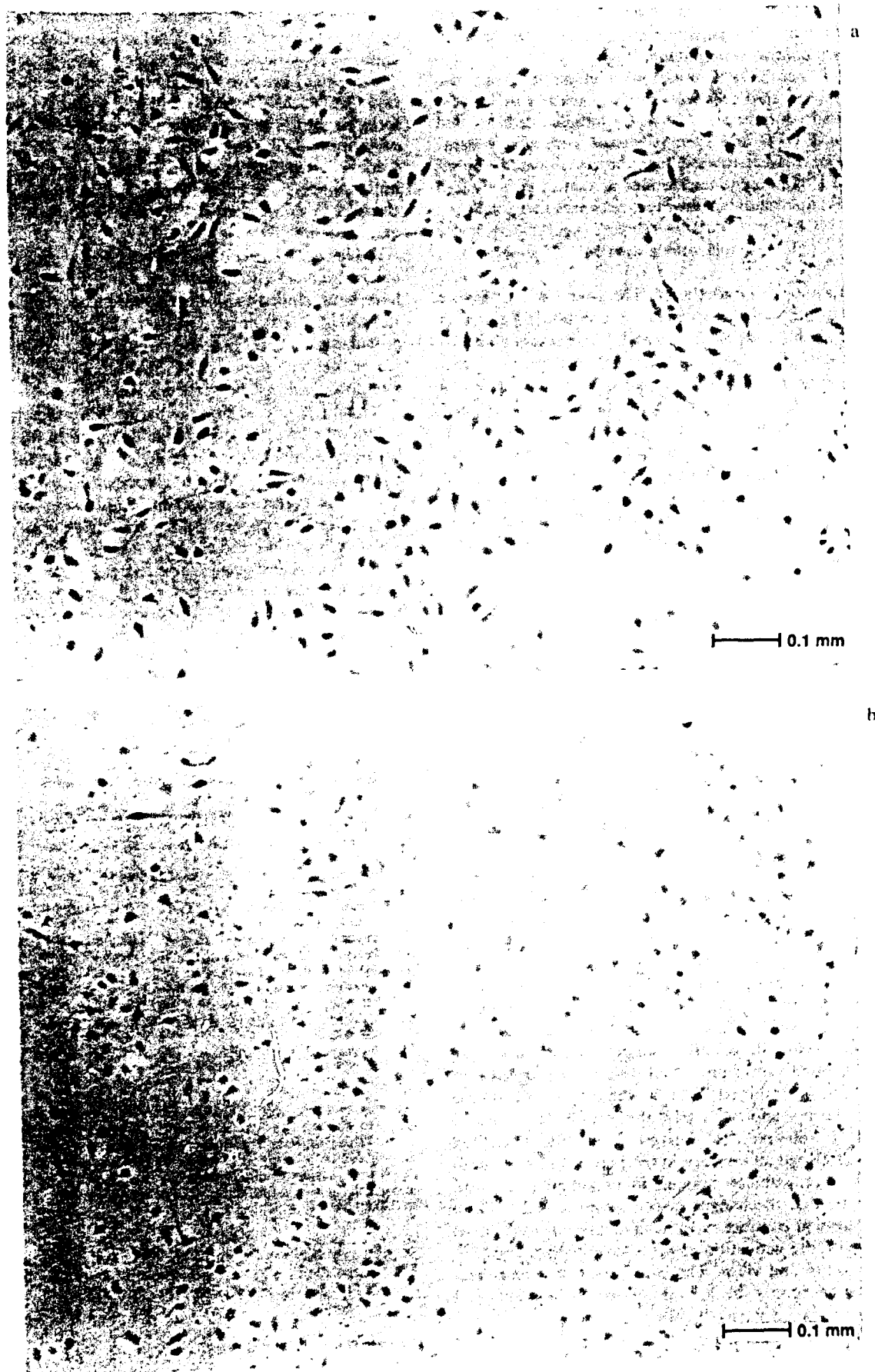


Figure 3: Phase contrast micrograph representative of rat primary sinusoidal endothelial cells 4 h after exposure to (a) 4 μM microcystin-LR, (b) supernatants derived from 4 μM microcystin-LR-treated hepatocytes. Endothelial cells exposed to medium alone or to supernatant derived from control hepatocytes were similar to that in (a)

cells. However, endothelial cells incubated with supernatants derived from hepatocytes treated with 4 μ M MCLR released significantly more [14 C]adenine nucleotides than cells incubated with control supernatants (Figure 2).

The difference in [14 C]nucleotide release between the treated and untreated cells was observed as early as 3 h post exposure and continued for 20 h. Endothelial cells treated with 2.5 mM DTE and then incubated with supernatants derived from MCLR-treated hepatocytes released similar amounts of [14 C]adenine nucleotides as that of cells treated with MCLR supernatants. However, cells pretreated with SM then with MCLR supernatants released significantly lower levels of [14 C]adenine nucleotides than MCLR supernatants (Figure 2).

The morphology of endothelial cells after 4 h of exposure to MCLR, or hepatocyte supernatants is shown in Figures 3a and b. Endothelial cells incubated with L15 medium alone, or MCLR alone (Figure 3a), or supernatants derived from non-treated hepatocytes displayed similarly a spindle-shaped cell with extended, cytoplasmic processes to neighbouring cells ($L = 10.5 \pm 5.5$; $b = 3.5 \pm 1.1$; $w = 1.8 \pm 0.5$ mm). Endothelial cells, however, incubated with supernatants derived from MCLR-treated hepatocytes (Figure 3b), displayed rounded, more contracted cell bodies with fewer extensions ($L = 9.9 \pm 6.3$; $b = 2.9 \pm 1.0$; $w = 2.0 \pm 0.7$ mm). A statistical difference was observed only in the body length of the cells (b).

Endothelial cell densities were determined by protein levels measured 20 h after incubation. There was no statistical difference in protein content (mg mL $^{-1}$) between endothelial cell monolayers treated with L15 medium alone (0.213 ± 0.041), 4 μ M MCLR (0.176 ± 0.052) or supernatants derived from control (0.144 ± 0.019) and treated (0.119 ± 0.008) hepatocytes.

Discussion: The release of [14 C]adenine nucleotides from control endothelial cells incubated in either L15 medium or with supernatants from untreated hepatocytes was similar to that released from porcine aortic endothelial cells labelled with [3 H]adenosine [10]. Endothelial cell monolayers incubated directly with the toxin exhibited the same morphological features, cell density and released the same amounts of [14 C]adenine nucleotides as control cells treated with L15 medium alone. These observations indicate that MCLR does not directly injure primary sinusoidal liver endothelial cells.

Supernatants derived from MCLR-treated hepatocytes, however, did induce significant changes in cultured endothelial cell monolayers. These changes include significant [14 C]adenine nucleotide release, rounded or contracted cell shape and a small reduction in cell density. The release of [14 C]nucleotides combined with a change in cell shape (occurring during approximately the same time period) suggests that supernatants derived from MCLR-treated hepatocytes induced an increase in the permeability of endothelial cells. Permeability changes in endothelial cells are thought to be due to changes in cell shape mediated by a calcium-dependent contraction of actin-myosin microfilaments combined with the activation of actin severing proteins: *i.e.* gelsolin, fragmin and villin. The contraction of cytoskeletal microfilaments induces the loss of cell-to-cell junctions and creates gaps between adjacent cells.

It is possible that supernatants derived from MCLR-treated

hepatocytes contain either active MCLR metabolite(s) or cell products that affect endothelial cells. In fact, MCLR has been shown to induce the release of arachidonic acid metabolites in cultured hepatocytes early after exposure [6]. MCLR-treated hepatocytes have also been shown to release adenine nucleotides (ATP, ADP, AMP) and adenosine [7], which act as local hormones in increasing the permeability of the microvasculature [10]. The release of adenine nucleotides from damaged hepatocytes could therefore play a role in the initiation of liver haemorrhage, followed by permeability changes in endothelial cells, and thereby creating red cell extravasation.

SM lessened the endothelial cell changes induced by supernatant from MCLR-treated hepatocytes. Both DTE and SM have been shown to protect cultured hepatocytes against a variety of hepatotoxic agents including MCLR [13]. SM provides protection possibly through the combined action of scavenging free radicals [14], inhibiting lipoxygenation and, therefore, leukotriene synthesis [15].

It could be argued that the permeability changes produced in endothelial cells by supernatants from MCLR-treated hepatocytes are just the response of endothelial cells to the release of contents of dead hepatocytes. Experiments are now in progress to test this possibility. It is more likely, however, that the factors responsible for producing changes in endothelial cells are induced as a consequence of the interaction of MCLR with hepatocytes. The increase of endothelial cells permeability induced by factors released by MCLR-treated hepatocytes may contribute secondarily to the toxicity of MCLR *in vivo*.

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