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A simple and effective method for the removal of trace metal cations from a mammalian culture medium supplemented with 10% fetal calf serum

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Direct batch addition of sterile Chelex ion-exchange resin to Dubecco's modified Eagle's medium supplemented with 10% fetal calf serum with gentle stirring removed a very wide variety of trace metal ions from the medium to varying extents dependent upon Chelex content (between 0.01 and 4% w/v), exposure time (between 5 min and 10 days) and temperature (4, 25 and 37 °C). Prolonged treatment (10 days) with 4% w/v Chelex at 4 °C reduced the concentration of zinc, strontium, aluminum, copper, manganese, nickel and chromium from 100 to 2.7, 12.1, 7.7, 22.6, 13.0, 14.7 and 53.3% of their original concentrations, respectively. Re-supplementation of the metal depleted medium with a defined cocktail of metals restored the growth potential of the medium which was then capable of supporting growth over at least three subcultures without a decrease in fibroblast cell yield, demonstrating its suitability in cell culture studies on trace metal ions.

Keywords: deficiency, fibroblasts, growth medium, metal

Introduction

Trace metals are of crucial metabolic importance and many are indeed essential elements; however, our knowledge of the roles of trace elements at the cellular level is often limited. Control over trace metal concentrations, particularly the ability to achieve highly reduced metal cation concentrations in media, is of great benefit to cell culture studies on trace metal elements. Thus, investigations into the role of trace metals in growth and disease, the effects of certain drugs on metal-related physiological functions (and *vice versa*), and studies on metal ion inter-relationships would be greatly simplified if metal ion concentrations could be easily manipulated within culture media.

Since addition of metal ions to a medium can be simply performed, we concentrate here on the reduction of trace metal concentrations. We have chosen a 'typical' cell culture medium, Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM/FCS medium), and aim to reduce metal ion levels to well below those found in the original cell culture medium. A technique originally developed successfully for the removal of copper from DMEM/FCS medium in the study of Menkes' disease (Rayner & Suzuki 1994) has been found applicable to the removal of a large number of other trace metals.

The simple methodology behind the metal removal is described in detail together with Chelex concentration, time and temperature dependent metal removal profiles for many physiologically important trace metals over a range of treatment conditions.

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Materials and methods

Chemicals

DMEM (Sigma, St Louis, MO, USA) was supplemented with 10% bovine calf serum (M. A. Bioproducts, Walkersville, MD, USA), 3.7 g l^{-1} $NaHCO_3$ (Wako Pure Chemical Industries, Japan), 100 units ml^{-1} penicillin (Merck, Darmstadt, Germany) and 100 μg ml^{-1} streptomycin (Meiji Seika, Japan). Albumin fraction V was purchased from Sigma. Barnstead quality water was used throughout. The medium, prepared in 1.0 l batches, was sterilized by membrane filtration after adjusting the pH to 7.5.

A metal stock solution prepared from either atomic absorption standard solutions or metal chloride salts (Wako Pure Chemical Industries Osaka, Japan) was made up in 0.1 M HCl to be 1000 times the concentration found

in the complete medium (values in mg l^{-1}: Ca, 70000; Mg, 20000; Zn, 320; Sr, 100; Cu, 20; Mn, 7; Ni, 2; Cr, 1). An appropriate aliquot of metal cocktail solution was added to both metal deficient media stocks before being used to culture cells.

Cell lines

Human skin fibroblasts (control, male, ATCC number CCD-32Sk) purchased from the American Type Culture Collection (Rockville, MD, USA) were cultured in 25 cm^2 Falcons (Becton Dickinson Labware, Franklin Lanes, NJ, USA) as stock cultures with a subculture interval of 7 days. A subculture ratio of 1:3 was used to prepare further stock cultures.

Medium treatment

For the general 10 day treatment of Chelex (Bio-Rad, Hercules, CA, USA), ion-exchange resin (weighed to equal 4% w/v of the medium to be treated) was cold sterilized by the addition of half the equivalent amount of 100% ethanol (w/v) to the beads at room temperature (about 25 °C) followed by an incubation period of 15 min, decanting of the liquid and allowing the beads, spread out in a weighing boat, to dry overnight in a sterile cabinet with the air flow on (UV light off).

Beads thus treated were added directly to freshly prepared medium containing a sterile Teflon coated magnetic stirrer. The bead suspension was then stirred in a cold room at 4 °C for 10 days after which the medium was decanted and re-filtered.

Medium treated for 1 h with 4% w/v Chelex was similarly prepared but stirring was performed at room temperature (about 25 °C). To determine the metal removal profile over 1 h, 2000, 500, 200, 50, 20 and 5 mg Chelex were each added to 50 ml medium. A further 50 ml medium without Chelex served as a control. At regular time intervals (before Chelex addition, 5, 10, 20, 30, 40, 50 and 60 min) stirring was temporarily stopped to allow removal of 0.5 ml aliquots of resin-free medium for metal analysis.

Metal removal profiles using 4% w/v Chelex (sterilized as above) at 4 °C for 10 days were performed using 3 × 50 ml medium volumes. Stirring was stopped and 0.5 ml samples were aseptically removed at the times indicated. Samples, in Eppendorf tubes, were centrifuged (20000 *g* for 60 s at 4 °C) to remove slight turbidity which developed after about 1 day of incubation.

Metal analysis

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was performed with an ICP spectrometer Model JY48 PVH (Seiko Instruments and Electronics, Japan). Samples were introduced diluted with water. Metal concentrations were determined by standard addition of metal standard solutions to the medium.

Graphite furnace atomic absorption spectrometry (GF-AAS) was performed with a simultaneous multi-element atomic absorption spectrometer Model Z-9000 with Zeeman background correction (Hitachi, Japan). Samples were prepared for analysis by addition (1:1 v/v) of a matrix modifier containing 0.5 M HNO_3, 0.5% Triton X-100 and 10% NH_4NO_3 in water. Aliquots (10 μl) of samples and standards were analyzed using minor modifications of the standard furnace temperature settings recommended by the manufacturer. The elements nickel and chromium were measured after concentration (5 × 10 μl) within the graphite cuvette. Concentrations were checked against standards and by standard addition to ensure linear relationships of absorption/emission with concentration.

Fibroblast culture

Cells were harvested by trypsin treatment, centrifuged and resuspended in 3 volumes of untreated fresh medium (1:3 split). Suspended cells were subcultured into one 24-well and one 6-well microtiter plate. For the first subculture only, after 2 h incubation to allow fibroblast adherence medium was aseptically removed by aspiration. Untreated medium, 1 h treated medium (metals re-supplemented) and 10 day treated medium (metals re-supplemented) were then pipetted into the wells (0.3 and 1.2 ml) for the 24- and 6-well plates, respectively. Further subcultures were performed with the fibroblasts only in contact with (un)treated medium relevant to each well. The 6-well plate served for subculturing purposes only.

Cells were then incubated for 7 days at 37 °C with 5% CO_2 after which the 24-well plate was placed on ice, the media were removed by aspiration and each well was washed three times with Hank's solution (0.5 ml). Protein was determined as follows using albumin fraction V (Sigma) as standard. After removing the last Hank's solution wash by aspiration, cell protein was solubilized in 0.25 M NaOH/0.025% Triton X-100 (0.2 ml $well^{-1}$) at 80 °C for 5 min. An aliquot (20 μl) of five times diluted Bradford Reagent (Bio-Rad) was added and the absorbance was measured at 600 nm using a Bio-Rad Model 2550 EIA Reader after thorough mixing.

Results

All metal cations investigated displayed time and Chelex concentration dependent depletion curves (Figures 1 and 2). The cations calcium and magnesium were the most readily removable followed by zinc. The metal concentration values measured in a typical batch of treated medium (4% w/v Chelex) prepared for further use in cell culture studies are shown summarized in Table 1.

Manganese, copper and strontium showed a clear temperature dependence with respect to metal sequestration into the ion-exchange resin; higher physiological temperatures increasing the rate of metal removal (Figure 2). Copper appeared most sensitive to this effect. Nearly twice as much copper remained at 4 °C than at 37 °C (4% w/v Chelex resin) when incubated for 8 h.

Analysis of the metals in solution in the medium during Chelex treatment over a 10 day period showed a steady removal of zinc, copper and manganese from

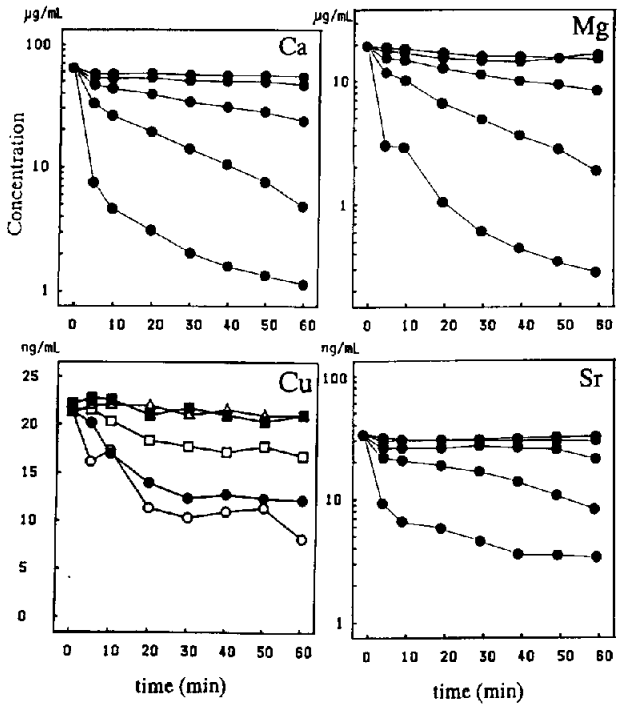

Figure 1. Removal profiles of calcium, magnesium, copper and strontium from medium during ion-exchange treatment (60 min). Of the four metals investigated under the conditions employed (see Materials and methods), copper proved by far the most resistant to removal. Amounts of Chelex, 4 (○), 1 (●), 0.4 (□), 0.1 (■) and 0.04 (△) w/v Chelex were each added to 50 ml fresh medium with stirring and incubation at room temperature. A further 50 ml medium without Chelex served as a control.

Table 1. Metals in DMEM supplemented with 10% FCS after Chelex treatment (4% w/v)

Metal (ng ml $^{-1}$)	Complete medium	Chelex treated 1 h (room temp.)	Chelex treated 10 days (4°C)
Ca[a]	68 200 ± 600	4200 ± 160	1660 ± 290
Mg[a]	22 600 ± 1500	1840 ± 130	700 ± 10
Zn	294.2 ± 3.2	13.76 ± 0.20	7.84 ± 0.28
Sr[a]	105.2 ± 1.7	41.30 ± 0.70	12.70 ± 1.04
Al	39.38 ± 1.60	5.58 ± 0.34	3.02 ± 1.44
Cu	21.32 ± 0.34	13.64 ± 1.28	4.82 ± 1.00
Mn	6.94 ± 0.24	3.08 ± 0.10	0.90 ± 0.08
Ni	1.50 ± 0.28	1.64 ± 0.10	0.22 ± 0.14
Cr	0.90 ± 0.04	0.74 ± 0.04	0.48 ± 0.04

Three samples each were measured to provide the standard deviation.
[a]Element determined by ICP-AES; other elements determined by GF-AAS.

the medium into the Chelex; the metal removal profiles differ significantly between metals. The remaining metals in the medium become increasingly more difficult to remove with time.

Three subcultures in 10 day treated medium (4% w/v Chelex at 4 °C) after re-supplementation of metals demonstrate a medium capable of sustaining growth of fibroblast cultures over at least three subcultures without noticeable effect on final growth after 7 days for any of the three subcultures (see Table 2).

Initial investigations in to the metal removal capability of Ca^{2+}/Mg^{2+}-Chelex, in place of Na^{+}-Chelex otherwise employed here, were discontinued because the relative speed of trace metal removal was extremely slow compared with the Na^{+}-Chelex form supplied by the manufacturers (result not shown).

Discussion

Although chemically defined culture media would be far superior for trace metal ion studies compared with complex media of biological origin, 'defined' media support the growth of very few cell lines, show poor growth characteristics, since they lack many unknown/ ill-defined growth factors, and are not fastidiously defined with respect to trace metals. The necessary addition of FCS complicates determination of trace element bioavailability and metal removal, but is necessary to support good growth in many mammalian culture studies.

The aim of this study was to develop a simple and efficient technique to significantly reduce the concen-tration of a broad range of physiologically important metal cations

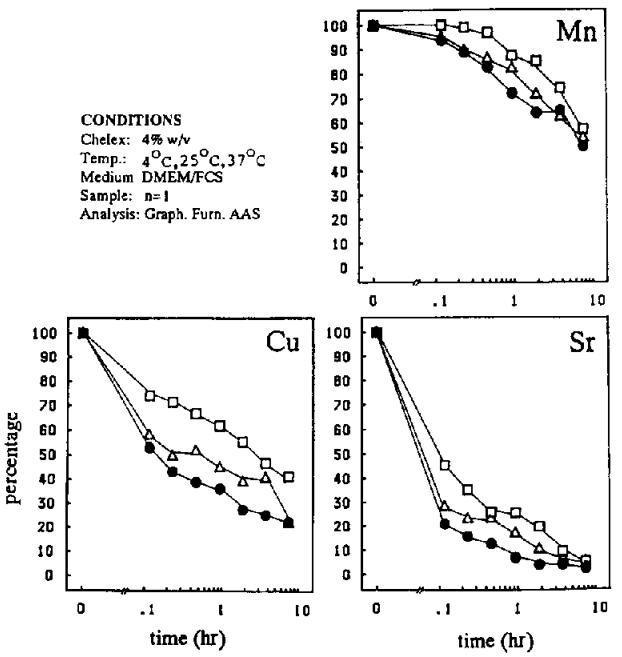

Figure 2. Removal profiles of copper, manganese and strontium from medium during ion-exchange treatment (10 h). Metal removal profiles with 4% w/v Chelex at 4 (□), 25 (△) and 37 (●) °C for 10 h were performed using 50 ml medium volumes. Stirring was stopped and 0.5 ml samples were aseptically removed at the times indicated. Increases in the time of medium contact with ion-exchange resin and increases in temperature effect a greater efficiency in metal removal. Note the logarithmic nature of the *x*-axis.

Table 2. Effect of Chelex treatment (10 days with 4% w/v) on fibroblast growth after re-supplementation of metals with a defined metal cocktail

Medium Chelex treatment	Source culture	Subculture 1	Subculture 2	Subculture 3
Untreated	18.2 ± 0.6	15.2 ± 0.6	15.0 ± 3.1	17.1 ± 2.5
1 h: metals re-supplemented		16.1 ± 0.9	19.8 ± 0.3	18.0 ± 0.3
10 day: metals re-supplemented	—	20.1 ± 1.3	19.1 ± 0.6	22.0 ± 1.0

from a standard mammalian cell culture medium whilst satisfying the following conditions: (i) no addition of potentially toxic low molecular weight metal chelating compounds, (ii) minimum removal of proteins, low molecular weight growth factors and/or organic substances, and (iii) maintenance of the growth potential of the medium. In principle, batch addition of Chelex to culture medium fulfills these criterion and has the additional advantages that the beads are biologically inert and readily removable from treated medium.

The method developed here incorporates direct contact of the gel beads with the medium and therefore maximizes the speed at which metal removal will occur compared, for example, with separating the ion-exchange matrix from the medium with a dialysis membrane.

Pre-treatment of the Chelex with ethanol had two important functions. Firstly, to ensure the removal of free iminodiacetic acid, which according to the manufacturer's instruction manual may be present in Chelex upon long standing. Secondly, to sterilize the beads; the initial bacterial load of Chelex is very limited (less than 100 organisms per gram resin; manufacturer's specifications) and alcohol is in contact with the beads for several hours. Thus, the beads were subjected to a minimum treatment regime which avoided the necessity to expose the beads to extreme physical handling (e.g. steam sterilization at 121 °C) with a possible concomitant change in resin quality, but was thoroughly adequate for the necessary safeguard against microbial and chemical contamination of the medium from the ion-exchange resin. To this effect bacterial contamination of the medium was never encountered over more than 1 year of intensive use of tens of batches of treated medium.

One possible drawback to the technique could have been the co-removal of important or essential positively-charged species (in addition to metal cations) by the negatively-charged iminodiacetate groups in the ion-exchange resin or by non-specific adsorption. In practice this was not found to be a problem with fibroblasts (see Table 2) under the conditions described, although the use of a minimum of ion-exchange resin is advisable with an extended exposure time being preferable to increased Chelex concentrations.

The removal of metals is extremely dependent upon the amount of Chelex added, the time of contact of the Chelex and the form of Chelex (Na^+ or other metal cation). From Figures 1, 2 and 3 the amount of resin to be added for a pre-determined concentration of metal removal can be estimated. Since most mammalian cell culture media contain significant quantities of FCS the results should provide a guide to the approximate amount of Chelex which could be employed with a wide range of media. For many applications less than 4% w/v Chelex for 60 min at room temperature might be adequate, or a longer contact time and less Chelex. Requirements can be adjusted according to trends apparent from the relevant metal removal curves. Metal removal trends during long incubation periods, up to 10 days, maximize contact time

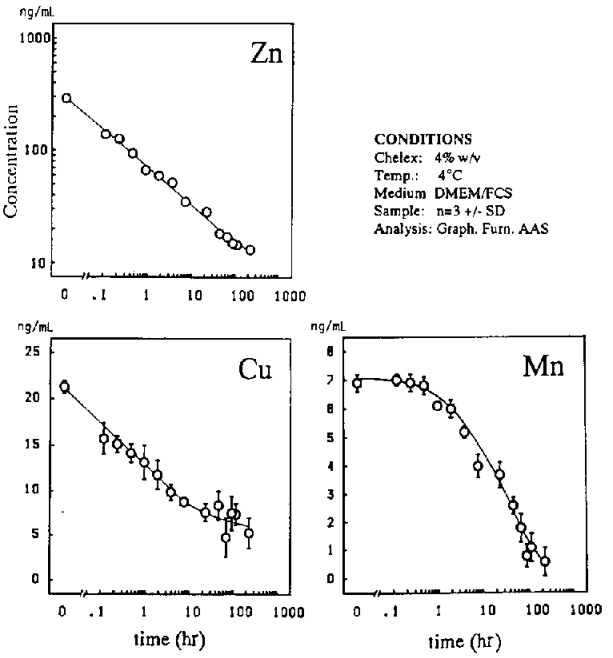

Figure 3. Removal profiles of copper, manganese and strontium. Metal removal profiles using 4% w/v Chelex at 4 °C for 10 days were performed using 3 × 50 ml medium volumes. Stirring was stopped and 0.5 ml samples were aseptically removed at the times indicated. Samples, in Eppendorf tubes, were centrifuged (20 000 ***g*** for 60 s at 4 °C) to remove slight turbidity which developed after about 1 day of incubation. Concentrations were determined by GF-AES after addition of modifier (see Materials and methods).

with the ion exchange resin whilst maintaining resin addition at a minimum and utilize the maximum incubation time at 4 °C, which is unlikely to affect medium growth potential.

Clearly, from Table 1, the limited selection of metals investigated will not represent the complete range of metals removed from the medium. Further investigation with ICP-MS would provide a considerably more complete picture of the trace and ultra-trace metal profiles of the 10 day metal depleted medium with respect to the other important metals not investigated here. However, the growth potential of the medium does not appear to be negatively affected by ultra-trace elements which may have been removed and are not replaced.

Reference

Rayner MH, Suzuki KT. 1994 Effect of medium copper concentration on the growth, uptake and intracellular balance of copper and zinc in Menkes and normal fibroblast cells. *BioMetals* 7, 253–260.