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ACUTE TOXICITY OF ZINC PYRITHIONE TO HUMAN SKIN CELLS *IN VITRO*

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Abstract. Zinc pyrithione introduced into cultures of rapidly proliferating NCTC 2544 human skin epithelial cells and normal human skin fibroblasts had a rapid cytotoxic effect even at very low concentrations (0.1–0.5 μg/ml); there was no dose-dependent suppression of cell proliferation and no apparent interference with mitosis. Sodium pyrithione had a similar effect. Zinc oxide and zinc sulphate were at least 100 times better tolerated than zinc pyrithione, but no stimulatory effect on cell growth was detected with low concentrations of either compound. These results suggest that zinc pyrithione's action against dandruff is more likely to arise from a non-specific toxicity for epidermal cells than by an anti-mitotic effect or by remedying a local zinc deficiency.

Key words: Zinc pyrithione; Toxicity; Skin fibroblasts

Zinc pyrithione is the active ingredient in several shampoos used to control dandruff. There is convincing evidence that such shampoos are effective (6) but the mechanism of their action is obscure. Two possibilities seemed worth investigating; first, that zinc pyrithione has a direct anti-mitotic effect on epidermal cells, like selenium disulphide (13), another agent used in antidandruff shampoos; and second, that exogenous zinc applied to the scalp remedies a local deficiency of that trace element, since zinc deficiency produces a range of abnormalities, including scaling, in various keratinizing epithelia (3, 9, 14). We therefore added zinc pyrithione and two other zinc compounds to cultures of rapidly proliferating epithelial cells and fibroblasts from normal human skin, so that any inhibitory or stimulating effects on proliferation could be identified and studied further.

METHODS

Two strains of human skin fibroblasts, HSF8 and HSF10, at passages 7–17, were those used in previous work (18). NCTC 2544 human skin epithelial cells were obtained from Flow Laboratories, Irvine, Scotland. Zinc pyrithione was provided as a 48% aqueous suspension and sodium pyrithione as a 40% aqueous solution (K & K-Greef Fine Chemicals Limited, Croydon, UK). Zinc sulphate septahydrate and zinc oxide were of ANALAR grade (British Drug Houses, Poole, UK).

Cells were maintained in Dulbecco–Eagle medium containing 10% foetal calf serum, 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Gibco-Biocult, Paisley, Scotland). Newly seeded cultures were flushed with 5% CO_2/air and incubated at 37°C. Medium was replaced three times per week. Cells were released for counting with trypsin-versene and counted electronically in a Coulter Counter Model DN, with an error of less than 1% in duplicate counts. Viability counts were performed in a haemocytometer using a 0.5% solution of nigrosin.

For each proliferation experiment up to 32 replicate cultures were set up in Nunc plastic flasks (growth area 25 cm^2), using aliquots of about 1×10^5 cells (Day 0). Four flasks were used for each concentration of drug, plus two groups of four controls. On Day 1 all media were replaced, and on Day 3 one control group was used for cell counts. The other flasks received media containing the appropriate concentration of drug. Each drug was dissolved or dispersed in distilled water and control cultures received distilled water only. On Day 4 all media were replaced, and on Day 6 cell counts were made on all flasks. The increase in total cells in cultures receiving drugs was expressed as a percentage of the mean increase in controls over Days 3–6. About 1.2×10^6 cells were recovered from control cultures, representing a more than ten-fold increase over Day 0.

In two other experiments to test for growth stimulation by zinc sulphate, attempts were made to lower the zinc concentration in the standard control medium, since this might already satisfy the zinc requirements of the cells. As the main source of zinc was thought to be the foetal calf serum, this was reduced from 10% to 3%, or substituted by 10% serum first prepared by dialysis against 2 mM EDTA (21) in phosphate-buffered saline for 48 hours. Zinc analyses were later performed on medium stored in polystyrene tubes at −20°C, using a Spectrascan SM1 111 plasma emission spectrometer.

RESULTS

The results of several experiments are superimposed in Fig. 1. The curves show a sharp decline in

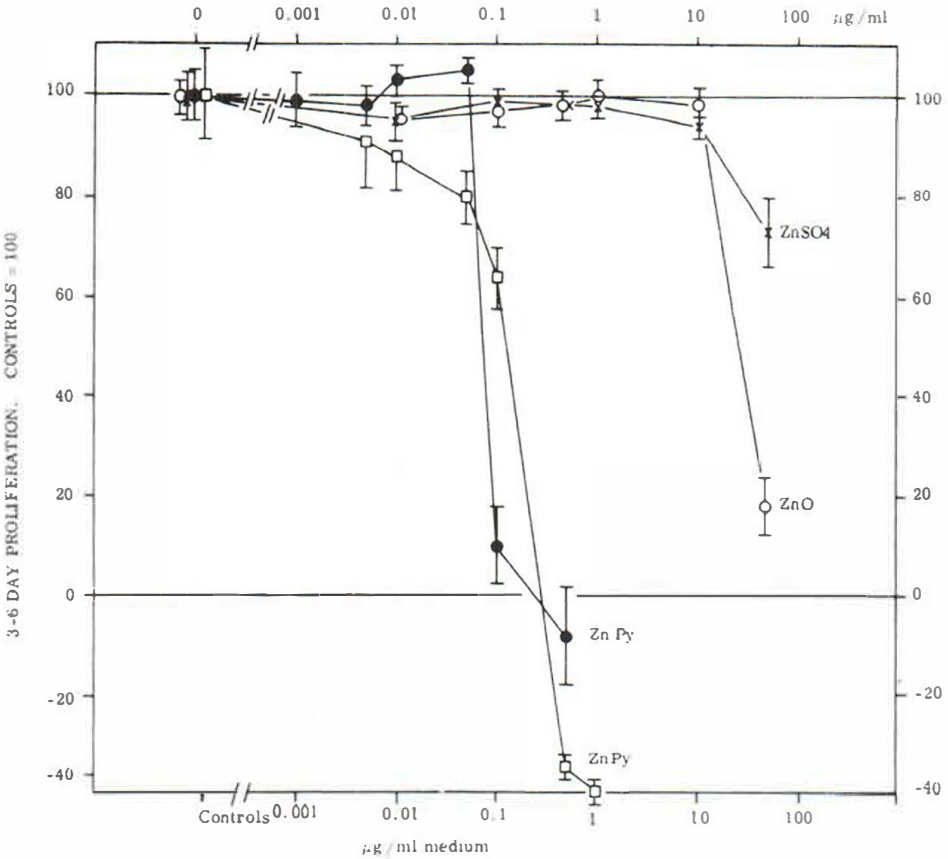

Fig. 1. Effect of zinc compounds on proliferation of HSF8 fibroblasts (round symbols or crosses) and NCTC 2544 epithelial cells (square symbols). Growth of control cultures between days 3 and 6 is represented as 100. Minus values indicate that fewer cells were present at Day 6 than at Day 3. Each value is the mean of four cultures, shown with its standard error. ZnPy, zinc pyrithione; ZnO zinc oxide; $ZnSO_4$, zinc sulphate (heptahydrate).

proliferation with 0.1–0.5 μg/ml zinc pyrithione for both HSF8 fibroblasts and epithelial cells; an experiment with HSF10 fibroblasts gave a similar result. The response was not the graded suppression of proliferation at increasing drug concentrations that we have seen with other drugs (16, 18). Inspection of cultures with phase contrast microscopy suggested that the 1, 10 and 50 μg/ml concentrations were rapidly lethal and at 0.5 μg/ml many cells were irreversibly damaged within 2 hours of adding the drug. Only 60% of the cells recovered on Day 6 after treatment with 0.1 μg/ml were still viable. Cells exposed to still lower concentrations of zinc pyrithione appeared to be unaffected apart from occasional very small foci of damaged fibroblasts at 0.05 μg/ml, which did not diminish the final cell count and viability was 93%, as in controls. In these cultures mitosis appeared to proceed normally, and there was neither inhibition nor stimulation of proliferation. Sodium pyrithione had no certain effect at 0.1 μg/ml but was toxic at 0.5 μg/ml and higher concentrations.

Zinc sulphate and zinc oxide suspension had no effect on HSF8 fibroblasts at even 10 μg/ml, but depressed proliferation significantly at 50 μg/ml. The greater effect of zinc oxide at 50 μg/ml is misleading, since zinc sulphate septahydrate was used, and at equivalent zinc concentrations the inhibition would be more equal. In addition the unstabilised suspension of zinc oxide tended to settle out, increasing the concentration around the cells on the bottom of the flask. Zinc pyrithione, with an aqueous solubility of 15 ppm, would be in solution except at 50 μg/ml.

In three other experiments to test for growth stimulation by zinc, low concentrations of zinc sul-

phate (0.07–0.7 μg/ml) had no perceptible effect on NCTC 2544 cells grown in standard medium (zinc content 0.9 μg/ml), on fibroblasts grown in medium containing 10% dialysed serum (0.35 μg/ml zinc), where growth was only 50% of that in standard medium with 10% whole serum, or on fibroblasts grown in medium containing only 3% complete serum (0.8 μg/ml zinc) where growth was reduced to 40% of that in standard medium.

DISCUSSION

Zinc pyrithione was originally added to dandruff shampoos for its antimicrobial properties, but a microbial aetiology for dandruff is now thought unlikely (see reviews (7, 19)). Selenium disulphide, another ingredient of shampoos for dandruff, suppresses mitosis in the epidermal cells of the scalp (13), and it seemed possible that zinc pyrithione might act in the same way. Unfortunately, despite recent advances in keratinocyte culture (8, 20) proliferation tests on serially cultured human epidermal cells are still impracticable. Our substitute, NCTC 2544, an established line of aneuploid epithelial cells derived from normal human skin (12) gave the same result as two unquestionably normal strains of human skin fibroblasts. While fibroblasts are not directly relevant to dandruff aetiology, their responses to several drugs resemble those of epidermal cells, e.g. growth of both *in vitro* is stimulated by hydrocortisone (15, 20) and DNA synthesis is inhibited by methotrexate (26); however, fibroblasts can be grown and serially maintained without difficulty. Zinc pyrithione's effect on both cell types was one of gross cytotoxicity, quite different from the dose-related suppression of cell proliferation caused by sodium salicylate, for instance, in the same fibroblasts (Priestley, unpublished), and without the mitotic arrest seen in human fibroblast and NCTC 2544 cultures treated with griseofulvin (16). The toxicity occurred at concentrations below those inhibiting microorganisms (24), and our failure to reproduce it with two other zinc compounds indicated that the pyrithione moiety was responsible. This was confirmed when sodium pyrithione had a similar toxic effect.

The second possible action of zinc pyrithione was to correct a zinc deficiency in the dandruff scalp, since zinc deficiency can cause malfunctioning of both epithelial and mesenchymal skin components (5, 9, 14). Clinical symptoms of zinc deficiency in acrodermatitis enteropathica are associated with a reduction in plasma zinc of about 50% (9). Yet all three zinc compounds failed to improve cell growth. Even when the zinc concentration of the medium was reduced over 50% by dialysis, supplements of zinc sulphate had no effect. So far, without such extreme measures as adding a chelating agent to the culture medium (21), it has been impossible to duplicate the skin's undoubted zinc requirement in cell culture (25). However, the severity of the cytotoxicity produced by zinc pyrithione makes the correction of a hypothetical zinc deficiency seem far less likely in dandruff.

From our results, and if cellular reactions to zinc pyrithione *in vitro* resemble those *in vivo*, we would expect any zinc pyrithione reaching living skin cells to be toxic. Yet in toxicological studies (1, 23) applications to normal and even abraded skin produced little harmful effect, and long-term use of 1–2% zinc pyrithione shampoos has raised no specific problems. The explanation must be that exposure to shampoos is so brief, and the affinity of zinc pyrithione for skin so low (1% or less of material applied is retained (22, 23)) that absorption is negligible. Application of 10% suspensions of zinc pyrithione with dimethyl sulphoxide to rabbit skin, however, caused hind limb paralysis, lung necrosis and rapid death, with skin irritation at the application site (2). Some constituent of the shampoos, or of the skin itself may block percutaneous absorption or reduce zinc pyrithione's cytotoxicity; sebum reduces its antibacterial effect (11), while mucin, saliva and traces of serum decreased the antibacterial action of the parent compound 1-hydroxy-2-pyridinethiol (10). The toxicity to mammalian and microbial cells *in vitro* and *in vivo* appears to come from the heterocyclic pyrithione constituent: percutaneous absorption of zinc from zinc oxide tape and dressings is harmless (4).

The result of this work, therefore, is that neither a specific antimitotic action of zinc pyrithione, like that of colchicine, nor the more general suppression of cell proliferation seen with anti-inflammatory drugs, nor correction of a local zinc deficiency, have received support from tests on skin cells *in vitro*. Instead the data show that zinc pyrithione could act by reducing the number or mass of scalp epidermal cells by a non-specific toxicity, perhaps related to the compound's high activity against bacteria and fungal cells (24), but this would only occur if it penetrated through the stratum corneum. A

different possibility, so far untested, is that zinc pyrithione may suppress dandruff by reacting through its sulphur moiety with the keratin proteins of the dead epidermal squames; this reaction in itself might block further penetration into the epidermis and protect living cells from damage.

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