FIBROBLAST ATTACHMENT TO ROOT-END FILLING MATERIALS

by

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This thesis is submitted by Sylvester Carlton Robinson, D.D.S. and has been approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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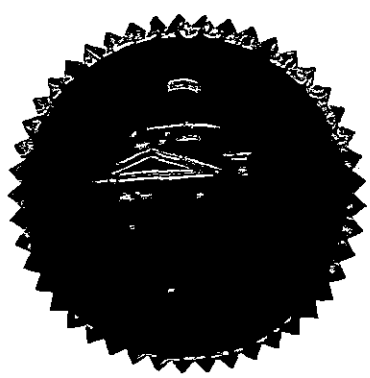

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Statement of the Problem

In an early 1884 description of the tooth root-end resection procedure, Farrar recognized the need to seal the apex of the sectioned root canal[1]. Apical surgery is usually performed in the presence of periapical pathosis, when orthograde endodontic treatment or retreatment is not considered feasible, or these procedures have resulted in failure[2]. It has been thought that the reason for failure in many cases was an inadequate apical seal of the root canal system. Thus, while the apical portion of the root is exposed during surgery, a root-end filling is placed to improve the seal of an existing root canal obturation. It has been proposed by some that all canals, even those that appear well obturated, should be routinely treated with a root-end filling and numerous materials have been advocated for use[3,4]. Whether all should be so treated is debatable; however, a number of characteristics are required for sealing materials when they are used. One of these is the material's biocompatibility, and a second is an ability to maintain its integrity when in contact with tissue fluids.

There have been numerous studies that examined the ability of root-end fillings to seal, but very few have examined the overall cytotoxicity of the materials,

and most have been limited in scope[3,5,6]. Because of this, there is considerable controversy about the preferred material for root-end fillings.

The purpose of this research was to determine the biocompatibility of six different root-end filling materials. In order to do this we examined the following:

1. Whether the materials had any toxic effect on the *in vitro* growth of human cells,

2. Whether the materials inhibited cell metabolism,

3. Whether cells could recover from possible toxic responses to the materials, and

4. Whether leachable substances from the material affected a cell's ability to attach to a surface, in this case, the culture dish.

Research into the ability of materials to either enhance or inhibit cell growth and attachment will assist the clinician in the selection of the appropriate root-end filling material.

Literature Review

Endodontic surgical procedures enhance the retention of many teeth which would otherwise require extraction[7]. The goals of endodontic surgery are to remove any pathological tissue from the periapical bony defect, to evaluate the seal of the root canal system in the apical portion of the root, and to create a good apical seal if one does not exist[8]. The insertion of a root-end filling has been recommended as a routine operation during apical surgery regardless of the apparent technical quality of the root canal obturation, unless orthograde endodontic treatment is performed

in conjunction with surgery. When orthograde treatment is not performed, a root-end filling enhances the prognosis of apical surgery[9]. Thus, the purpose of the root-end filling is to provide an apical seal to an otherwise unobturated root canal or to improve the seal of an existing obturation. In a post-periapical surgery retrospective study by Hirsh *et al.*, the variable of greatest importance was whether or not the root canal was obturated, in this case with a silver amalgam root-end filling[3]. Root-end fillings were recommended in cases with large bony defects, unsatisfactory root canal fillings, and inflamed cysts[10]. Another more recent study reviewed the radiographs of 715 patients six months or more after treatment.[11] Over half the teeth in the sample did not receive root-end fillings during surgery. Amalgam root-end fillings represented 53% of the root-end fillings, while Intermediate Restorative Material (IRM) and Alumina SUPEREBA (EBA) (zinc oxide-eugenol cements) made up 11% and 35%, respectively. It was reported that healing was independent both of the placement of a root-end filling as well as the type of root-end filling placed. Healing in the absence of root-end fillings was reported in about 61% of surgical cases. This compares favorably with the success rate of 69% when a root-end filling is placed. The results suggest that teeth with a root-end filling may have the same prognosis for successful healing as teeth that are unfilled. Thus, the very need for placing a root-end filling in conjunction with a root-end resection is in dispute.

In a high percentage of apicoectomy cases where a root-end filling has been placed, the material of choice has been amalgam because of its availability in the dental office, ease of manipulation, radiopacity, slight bacteriostatic effects, good

seal, and, most importantly, its historical success. Marcotte *et al.*, compared periapical healing after placement of amalgam and gutta percha as root-end fillings in Rhesus monkeys[12]. Using histological evaluation over a 3- to 15-week period, they indicated that both amalgam and gutta percha were equally well tolerated by the periapical tissue. Many clinical studies have shown similar success with amalgam as the root-end filling material[10,13].

At the same time, Kopp and Kresberg reported some disadvantages of amalgam[14]. Mercury can be introduced into the periapical tissues, and amalgam can be scattered into adjacent tissues. In addition, nonsterile material is introduced into the body, and corrosion can possibly occur when contaminated by tissue fluid. Some also claim amalgam can be toxic[15]. Continued concern over amalgam's mercury toxicity warrants the further investigation of its biocompatibility and also increases the interest in alternative materials.

In addition to concerns about mercury, a case reported by Omnell suggested that zinc salt deposited periapically as a result of an electrolytic process was a contraindication for the use of amalgam that contained zinc[16]. From this single case and others[17] that implicated zinc-containing amalgam in the delay in amalgam expansion, zinc-containing amalgam was not recommended for surgical root-end placement. A delay in expansion may have an adverse effect on the seal by creating large defects in the filling-tooth interfaces. These defects could harbor bacteria and possibly be a cause for failure. However, Jorgensen has noted that zinc-free amalgam tends to corrode more readily than amalgam containing zinc[18]. Since the expansion

is the result only of moisture contamination during condensation, no problem of delayed excessive expansion should exist when the operator places the amalgam in the root-end. Martin *et al.* have shown that the histologic inflammatory response of rat connective tissue to zinc-containing amalgam is not significantly different from zinc-free amalgam[19].

Despite the numbers of studies that have shown the histologic response to amalgam to be clinically acceptable, alternative materials using zinc oxide-eugenol are being used in an increasing number of cases. Both of the zinc oxide-eugenol formulations commonly used are reinforced to decrease the solubility of the materials *in vivo*. Intermediate restorative material (IRM, Caulk, Milford, DE) is reinforced with polymethyl methacrylate, and Alumina SUPEREBA cement (Bosworth Co. Skokie, IL) is reinforced with alumina, natural resin, and ortho-ethoxy benzoic acid. EBA cement is reported to have a high compressive strength, a high torsional strength, a neutral pH and low solubility[37]. It is only slightly more radiopaque than a gutta percha root canal filling.

Results of studies on the sealing properties of various zinc oxide-eugenol based compounds have shown that these materials have excellent sealing properties[8,30]. Bondra *et al.* used dye to compare leakage *in vitro* of teeth sealed with IRM, high copper amalgam, and EBA cements, and found that IRM and EBA cements have significantly less leakage at the apex than amalgam placed with copalite cavity varnish[4].

One study most often referenced in determining whether EBA is biologically

acceptable. Oynick and Oynick(1978) published a study using a block section of the apex of a tooth that had been in the mouth for 12 years after placement of the root-end filling[20]. They compared the histologic and scanning electron microscopic results of Staline Super EBA cement with silver amalgam. The EBA showed better adaptation, no expansion, and growth of collagen fibers over the material and into the cracks of the tooth was evident. This image confirmed the favorable clinical impression at the time the block section of the apex of the tooth was removed with the surrounding periapical tissues.

However, Maher *et al.* studied the histologic response to amalgam and IRM root-end filling materials in the root apex of the ferret canine[21]. Their microscopic examination showed a decrease in inflammation and the formation of a fibrous capsule over a 15-week period in the amalgam specimens. IRM specimens showed persistent inflammation and slower healing.

A retrospective study of cases ranging from a minimum of six months to a maximum of ten years after treatment compared the clinical and radiographic success rates of teeth sealed with Super EBA, IRM, and zinc-free high copper spherical amalgam.[22] The results revealed that both Super EBA and IRM significantly improved the success rate when compared with amalgam. The success rates were 75% for amalgam, 91% for IRM, and 95% for Super EBA. Results such as this and the ease of placement have made IRM the choice in 11% of cases receiving root-end fillings and EBA the choice in 36% of cases[23]. There have been no studies that have addressed the biocompatibility of these materials at placement or over time.

When amalgam sets, microleakage occurs because of initial contraction, and use of a cavity varnish may prevent excessive microleakage. IRM and EBA have continued to be suspect due to their solubility in tissue fluids. Against this background, glass ionomer cements were introduced into the market in 1974. Glass ionomers are hybrids of the silicate and polycarboxylate cements which bond physicochemically to dentin and enamel. They possess anticariogenic activity by fluoride release. These cements eliminate microleakage because of their chemical bonding to both enamel and dentin.[24]. Kawahara *et al.* found that the cytotoxic effects of glass ionomer cements on cell cultures were negligible[25]. A similar conclusion was reported by Hanks *et al.*[26]. In an *in vivo* study of implanted pellets of glass ionomer-silver cement and zinc oxide-eugenol in surgically prepared bone cavities in rats, both materials appeared to be well tolerated by the tissue.[27] Bone apposition directly against the cement occurred in a number of glass ionomer samples by 80 days. The zinc oxide-eugenol produced more fibrosis. It appears from the findings of this study that IRM and glass ionomer-silver cements are not totally benign, but are relatively biocompatible. The silver-containing glass ionomer cements are more radiopaque and easier to locate radiographically than nonsilver-containing glass ionomer cements.

Callis and Santini placed non-silver-containing glass ionomer (Ketac-fil) root-end fillings into ferret canines and found that after 28 days bone fill was quite advanced, with intimate contact between the bone and cement [28]. Blackman *et al.* implanted pellets of glass ionomer-silver cement and a zinc oxide-eugenol cement

into the soft tissue and bone of rats[20]. They observed new bone contacting the implanted material and the presence of minimal inflammation. Unfortunately, glass ionomer cement is more difficult to manipulate and is technique sensitive, especially if there is moisture contamination during the initial set. The presence of moisture or blood in the root-end preparation may also prevent proper dentin conditioning and adhesion of the glass ionomer to the dentin walls. However, an *in vitro* dye leakage study comparing the sealing ability of glass ionomer root-end fillings with thermoplasticized gutta percha and amalgam showed no significant difference in apical dye leakage[29]. Another study used a fluid filtration method to measure microleakage and evaluated the seal of cold-burnished gutta-percha, amalgam, Super EBA cement, and a glass ionomer material (Ketac-Silver)[30]. They found that Ketac-Silver produced a significantly inferior apical seal when compared to the other materials.

Thermoplasticized injectable gutta percha was introduced as having advantages over other root-end filling materials because it is the material that is used in the orthograde root canal procedure. Escobar *et al.* compared the apical seal of injectable thermoplasticized low-temperature gutta percha (Ultrafil) without sealer with that of a zinc-free amalgam[31]. Their *in vitro* investigation found that the silver amalgam retrofillings demonstrated no leakage in four roots and slight leakage in the remaining six specimens. Previous research has indicated that the quality of the seal is significantly improved when sealer is used with either orthograde or retrograde high temperature thermoplasticized gutta percha. Because in many cases after a root

resection procedure, exposed gutta percha is left instead of placing a root-end filling, the biocompatibility of gutta percha should be considered and compared with other root-end filling materials.

Gutta percha is relatively nontoxic, although some cytotoxicity has been reported[32]. While the degree of toxicity depends on the composition of a particular brand of gutta percha, the preponderance of the literature indicates that gutta percha is nonirritating to the apical tissue[33,34]. Some antibacterial activity has also been reported, which has been ascribed to the zinc oxide component of gutta percha cones[35]. Thus, studies have advocated one visit root canal treatments coupled with surgery and gutta percha to seal the apex of the root prior to resection.

There is a myriad of materials from which to select in an attempt to find the "perfect" root-end filling. None to date has fulfilled all the criteria of an ideal root-end filling. Therefore, the endodontic surgeon must be cognizant that success of the procedure does not lie in the essence of the apical filling material only. A lack of understanding of the materials used, coupled with improper use, could contribute to ultimate failure.

Since tissue compatibility is one critical factor, the purpose of this investigation was to study the possible toxic effects of six root-end filling materials most likely to be considered for use.

MATERIALS AND METHODS

General Methods and Materials

Root-end Filling Materials:

The composition of the materials tested are listed in Table I. Those selected are presently the subject of the greatest clinical interest. They are: (1) Amalgam (Kerr Contour, Kerr Manufacturing Co., Romulus, MI); (2) Super EBA (Harry J. Bosworth Co., Skokie, IL), a zinc oxide-eugenol type of material reinforced with alumina, natural resin, and ortho- ethoxybenzoic acid that comes as separate powder and liquid and is mixed immediately prior to use; (3) Intermediate Restorative Material (IRM, L.D. Caulk Co., Milford, DE), which is a zinc oxide-eugenol material reinforced with methyl methacrylate polymer, also a separate powder and liquid which are mixed immediately prior to use; (4) Gutta-Percha (Hygenic Co., Akron, OH); and two glass ionomer restorative materials (5) Ketac-Fil and (6) Ketac-Silver (Espe, Norristown, PA). The latter are also powder-liquid combinations mixed at time of use. As a control, dental sticky wax was used because it was also used to lute the materials to the culture dish during the experiments.

MATERIAL	COMPOSITION
Contour amalgam (AM)	Alloy: 31% Tin, 41% Silver, 28% Copper Amalgam: 47% Mercury, 53% Alloy
Gutta percha (GP)	20-30% Natural gutta percha, 65-85% zinc oxide, 1-5% barium or strontium sulfate, 0.1-0.5% color pigments, and minor amounts of waxes, antioxidants, plasticizers, and resins
SUPEREBA cement (EBA)	Powder: 60% Zinc oxide, 35% Alumina, 5% Natural resin Liquid: 62.5% Ortho-ethoxy benzoic acid, 37.5% Eugenol
Glass ionomer cements KETAC-SILVER (KS) KETAC-FIL (KF)	Powder: Calcium fluoroaluminosilicate glass, Barium glass or zinc oxide, silver Liquid: 2:1 Polyacrylic acid/itaconic acid copolymer
Intermediate Restorative Material (IRM)	Powder: 80% Zinc oxide, White rosin, Zinc stearate, Zinc acetate, 20% Polymethyl methacrylate Liquid: 85% Eugenol, 15% Olive oil

Table I. Composition of root-end sealing materials

Fabrication of Samples:

Triplicate samples of each material were fabricated aseptically in a mold 1 cm in diameter by 1 mm thick. Each material was dispensed and mixed according to the manufacturer's directions and allowed to fully set. The amalgam and glass ionomer cement were triturated for 10 seconds. Some materials were removed from the template before fully setting in order to recover the sample without fracture. Three treatment samples were made for each root-end filling material. The materials were allowed to set for an additional 2 hours prior to being sterilized by germicidal ultraviolet irradiation for 30 minutes on each side. The sterile samples were then placed into sterile 35 X 10 mm plastic tissue culture dishes (Falcon Plastics, Becton Dickinson Labware, Lincoln Park, NJ). The specimens were secured to the center of the tissue culture dishes with dental sticky wax. In previous studies, this wax has been shown to have no cytotoxic effects *in vitro*[36]. As a control, similar size dental sticky wax disks were utilized.

Gingival Fibroblast Cell Cultures:

Human gingival fibroblasts (HGF) were obtained as primary cultures from the Medical College of Georgia School of Dentistry, Department of Oral Biology. These cultures were grown from pooled gingival biopsies (three separate samples) taken during routine periodontal surgical procedures on adult patients. The cultures were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air.

Confluent cell monolayers of human gingival fibroblasts were transferred by suctioning the medium from the tissue culture flask, rinsing the cell layer with 5 ml of Tris-HCL buffer (pH 7.4), then adding 1.5 ml of 0.25% Alseviers's ATV trypsin solution. The flask was incubated for 3 minutes to allow the cells to be dislodged from the flask. An equal volume of EMEM was added to the flask to stop the action of the trypsin. If the cells were to be passed, another 30 ml of medium was added to the flask and half the volume of cells transferred to a new flask with 15 ml of additional medium.

If the cells were to be used in an experiment, the cultures were treated as described below.

Cell and Counting and Plating for Experiments:

Confluent cultures were split by decanting the medium from the tissue culture flask, rinsing the cell layer and flask with approximately 30 ml of sterile phosphate-buffered saline (PBS), then adding 2.5 ml of 0.5% trypsin/0.1% EDTA. The trypsin was layered evenly over the cell layer and incubated at 37°C for 5 minutes. The cells were dislodged from the flask, then suspended by mixing on a gyratory mixer. 100 μl of cell suspension was added to 300 μl of 0.5% Trypan blue (1:4 dilution) in a 12 x 75 mm test tube. The cells were mixed well and allowed to sit for 5 to 15 minutes to allow them to take up the Trypan blue. The nonstained (viable) cells were then counted in a Neubauer hemocytometer on an Olympus BH2 microscope. Appropriate dilutions were made in EMEM to obtain the numbers of cells appropriate for plating.

Experimental Methods

Morphologic toxicity assays:

Gingival fibroblasts (2.5 X 10^5 per dish) in 3 ml of medium were seeded into 35 x 10 mm sterile tissue culture dishes containing the sample disks. The cultures were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2 and 95% air, and each culture was examined by inverted phase contrast microscopy 24 hours after plating. Morphologic evaluation of cellular monolayer toxicity used the following standards: (1) cellular rounding, (2) loss of attachment to the dish, (3) confluency, and (4) peripheral cellular inhibition around the disk. Each sample was scored by dividing each culture dish into four concentric regions from the sample disk center to the culture dish wall. A score between 0 (without toxicity) to 4+ (maximal damage to the cell monolayer) was assigned to each culture, dependent upon the distance of the aberrant cells were from the sample disk (Fig. 1).

Metabolic impairment assays:

To determine whether there were subtle changes as a result of toxicity in the absence of morphologic changes, cellular ribonucleic acid (RNA) synthesis was monitored. Tritiated uridine was used to label RNA. After 24 hours of initial incubation with the sample disks, the medium was replaced with medium containing

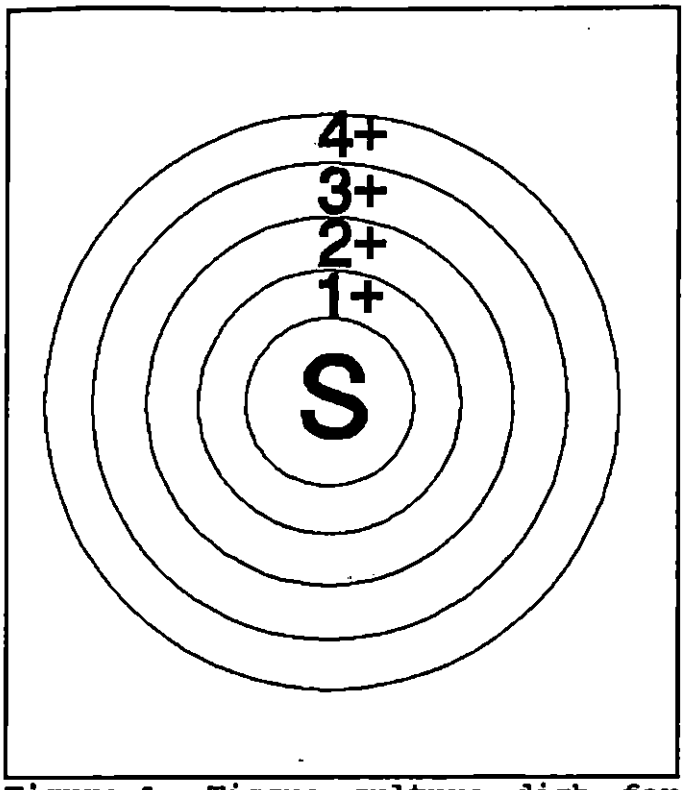


Figure 1. Tissue culture dish for morphologic toxicity scoring. Scores from 0 to 4+ assigned to each culture were based on distance of aberrant cells from sample disk (S).

15 μCi/ml ^{3}H-Uridine (^{3}HUR), (38.5 Ci/mmol, NEN, Boston, MA). The cultures were incubated at 37°C in 5% CO_2 and 95% air for 24 hours to allow the cells to incorporate the isotope. The medium was removed from each dish and the cells rinsed with 1 ml Tris-HCl buffer, pH 7.4, to remove nonviable cells and remaining traces of medium. To each dish 1 ml of 10% (w/v) trichloroacetic acid (TCA) was added to precipitate the radiolabeled nucleic acid. After 1 hour, the cells were washed and rinsed three times with TCA to remove any unincorporated isotope. A volume of 50 μl of 5 N sodium hydroxide was applied to each dish for one hour to neutralize any residual TCA. The cells were placed in solution by adding 950 μl of Laemmli solubilization buffer (LSB) (0.0625 M Tris-HCL, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 5% (w/v) 2-mercaptoethanol, and 10% (w/v) glycerol) and transferred to Eppendorf tubes. Duplicate aliquots (150 μl) of the solubilized cell extracts containing the radiolabeled RNA combined with 5 ml of Scintiverse BD scintillation fluid (Fisher Scientific Inc., Atlanta, GA) and quantitated in a liquid scintillation counter (Beckman Instruments Model #3801, Fullerton, CA).

Three or four experiments with three identical plates for each sample were performed using successive confluent cultures in passages 6-11 for the sequential isotope studies. Three experiments with two plates per root-end filling material treatment were also performed with cell cultures from confluent flasks in passages 6-11 for longitudinal studies. An unpaired t-test was used to test the significance of differences in isotope incorporation in the sequential experiments. A 2-way analysis of variance (ANOVA) was used with a Scheffe post hoc procedure to test

significance differences in RNA synthesis for the longitudinal studies.

Colorimetric Assay Procedure:

A colorimetric assay was used to examine the changes in cytotoxic effects, as a reflection of cell numbers, over time. Such proliferation assays are widely used for the study of growth factors, cytokines, nutrients, and for the screening of cytotoxic or chemotherapeutic agents. The cell Titer 96 (MTT) assay (Promega, Madison, WI) used in these studies is based on the cellular conversion of a tetrazolium salt into a blue formazan product that is easily detected using a spectrophotometer (ELISA plate reader). During the incubation period, living cells convert the tetrazolium component of the dye solution into a blue formazan product that is insoluble in tissue culture medium. The solubilization solution is then added to the culture well to convert the insoluble blue formazan product into a colored solution. The plates are allowed to stand overnight in a sealed container, then the absorbance is read at 570 nm with 650 nm background correction. The formation of formazan is thought to take place via mitochondrial dehydrogenase activity, although other locations with dehydrogenase activity may contribute to total formazan production.

Preparation of eluates:

Sample disks were prepared and placed in autoclaved screw-cap glass vials containing 9 ml of EMEM with 10% FBS. Six samples disks were sealed into each vial with Parafilm and placed in a refrigerator for 24 hours. Each day for ten days, the samples disks were transferred to a new vial containing fresh EMEM with 10% FBS and resealed with Parafilm. In all studies utilizing eluates, fresh medium and

medium treated like that in which the disks were soaked were used as controls.

Effect of Material on Attachment of HGF:

The purpose of this experiment was to determine if the leachable substances affected fibroblast attachment to the tissue culture dish. A solution of 50 μl of medium, 5,000 cells and 50 μl of eluate from each day were pipetted into columns 1-10 of a 96-well tissue culture plate. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 4 hours to allow the cells to attach. At the end of the incubation period, the plate was emptied by inversion and blotting onto plastic-backed absorbent paper. The unattached cells were removed by washing twice with 200 μl of sterile PBS. 100 μl of fresh medium and 15 μl of sterile MTT dye solution were added to each well. The plate was incubated for 4 hours at 37°C in 5% CO_2 to allow the cells to convert the yellow MTT dye to insoluble blue formazan crystalline product. At the end of the incubation period, 100 μl of solubilization solution was added to each well. The plate was sealed by placing a plate size piece of Parafilm between the plate and the cover and incubated overnight at 37°C to solubilize the formazan product. The next morning the plate was read in the microplate reader at 570 nm with 650 nm background correction.

Effect of Material on Attached HGF after 4 hours and 48 hours of exposure:

The purpose of these experiments was to determine the toxic effects of the eluates of the materials by measuring cell metabolism after 4 hours and 48 hours of exposure. 96-well tissue culture plates containing 5,000 cells per well were incubated overnight at 37°C in 5% CO_2 to allow the cells to become attached. The plates were

emptied by inversion and blotting onto plastic-backed absorbent paper and then washed once with 200 μl of sterile PBS. 50 μl of medium and 50 μl of eluate for each of the ten days were pipetted into the wells of ten columns. The plates were incubated for 4 or 48 hours at 37°C in 5% CO_2, 95% air. After the incubation period, the cells were washed twice with 200 μl of sterile PBS and then 100 μl of fresh medium was added to each well. The plates were incubated at 37°C in 5% CO_2 overnight, and the MTT assay was performed as previously described.

RESULTS

Morphologic Toxicity:

Microscopic evaluation of gingival fibroblasts exposed to amalgam showed a zone of growth inhibition around the sample. This material was given scores from 1.0+ to 2.5+ (Table II, Figure 2). There was a complete lack of cell attachment within the zone of inhibition. Outside that zone, the fibroblasts showed normal morphologic characteristics.

Gutta percha samples produced a small inhibitory zone that was given a score of 0 to 0.5+; there were areas that showed a slight cellular toxicity. A few fibroblasts adjacent to the sample disk demonstrated pyknosis, swelling, cellular rounding, and loss of attachment, whereas in other areas the cells showed normal morphology adjacent to the sample disk (Fig 3).

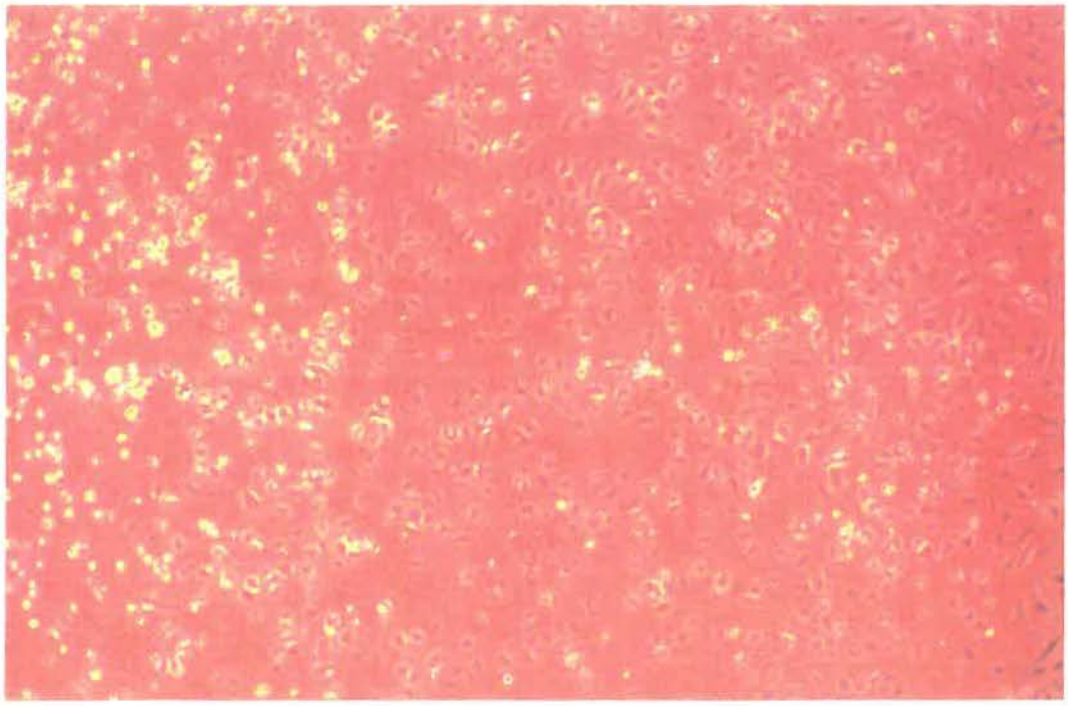
IRM and EBA were both scored 4+, which represents cell destruction over the entire plate. All the features of abnormal cells previously described were evident across the entire tissue culture dish. EBA containing cultures had scattered areas where a few fibroblasts with normal spindle shapes were seen (Fig 5). However with IRM, there was a total absence of normal appearing fibroblasts (Fig 4).

Material	Score
Amalgam	1.0+ - 2.5+
Gutta percha	0.0+ - 0.5+
IRM	4+
EBA	4+
Ketac-fil	0
Ketac-silver	0

Table II. Morphologic evaluation of toxicity of root-end filling materials for gingival fibroblasts. Values for scores determined in Methods.

Fig. 2. *Morphology of HGF exposed to amalgam*

a. *Top - Amalgam sample disk edge at left, showing zone of abberant cells.*
b. *Bottom - Same sample showing normal morphology as distance from sample disk increases.*



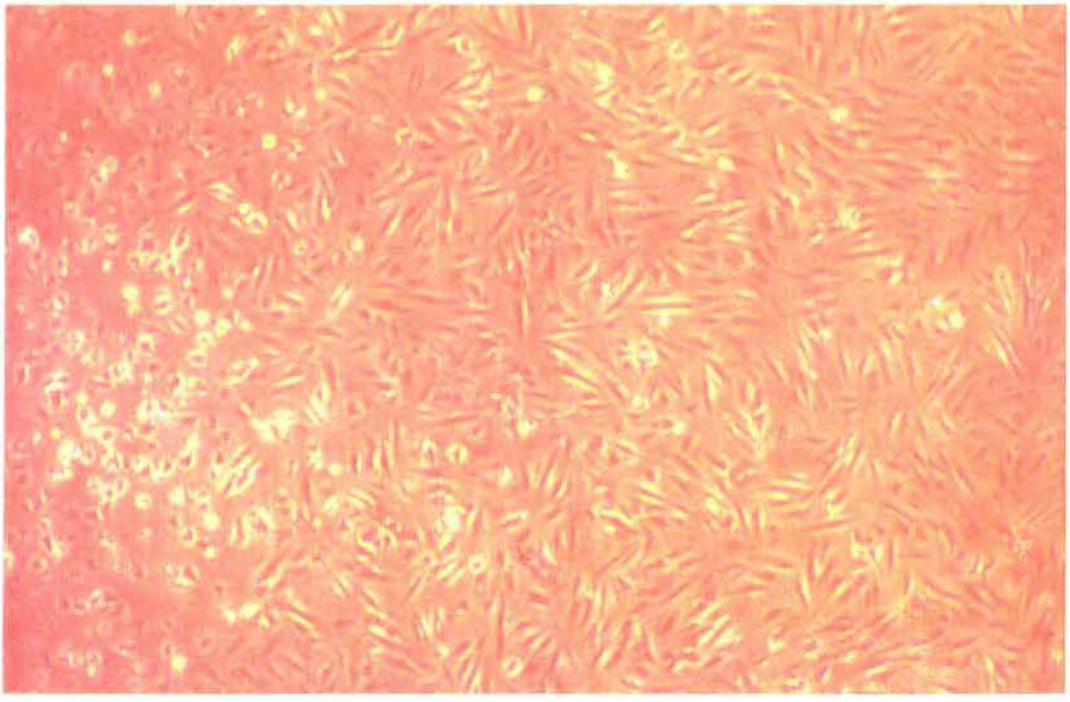


Fig. 3. *Morphology of HGF exposed to gutta percha*

Fig. 4. *Morphology of HGF exposed to IRM*

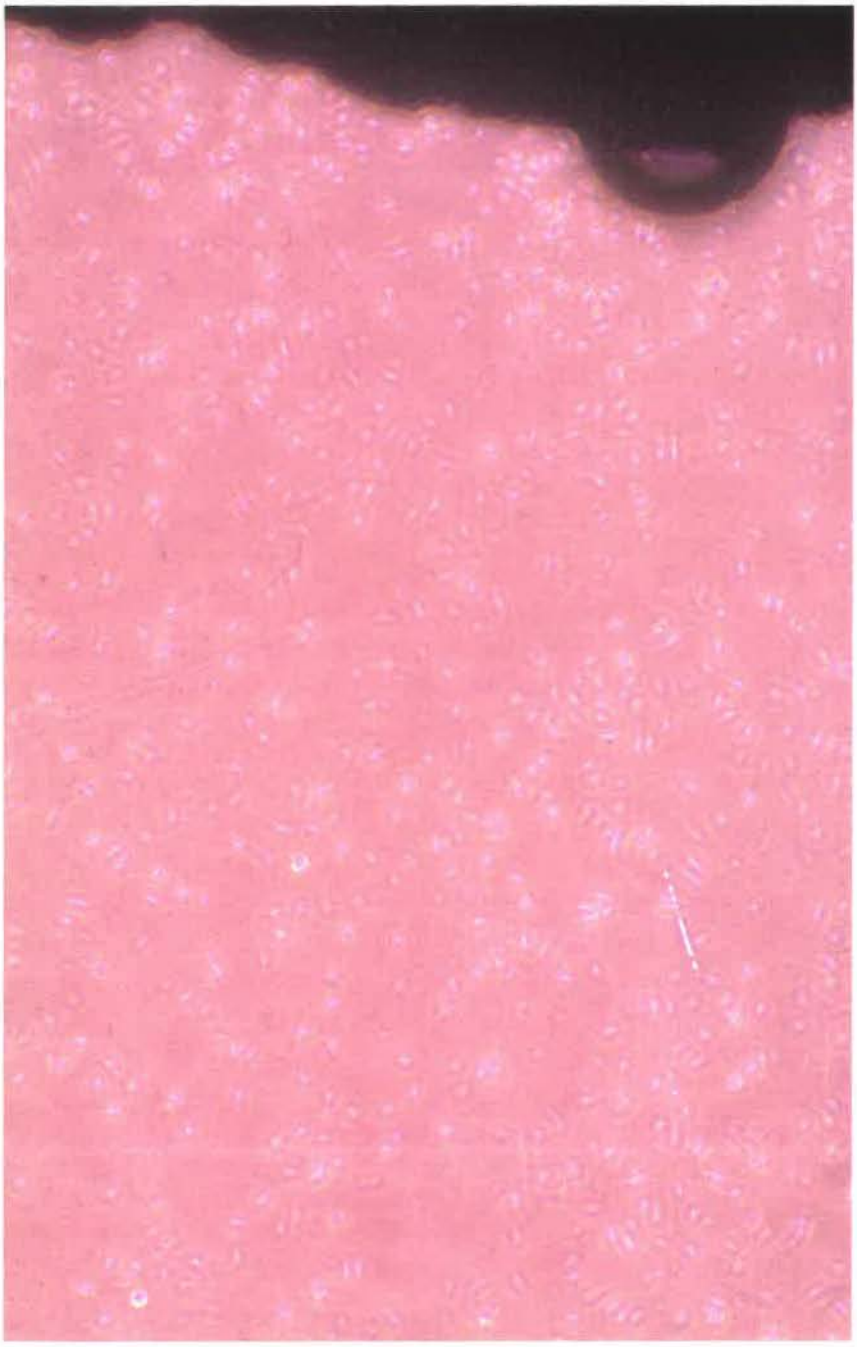


Fig. 5. *Morphology of HGF exposed to EBA*

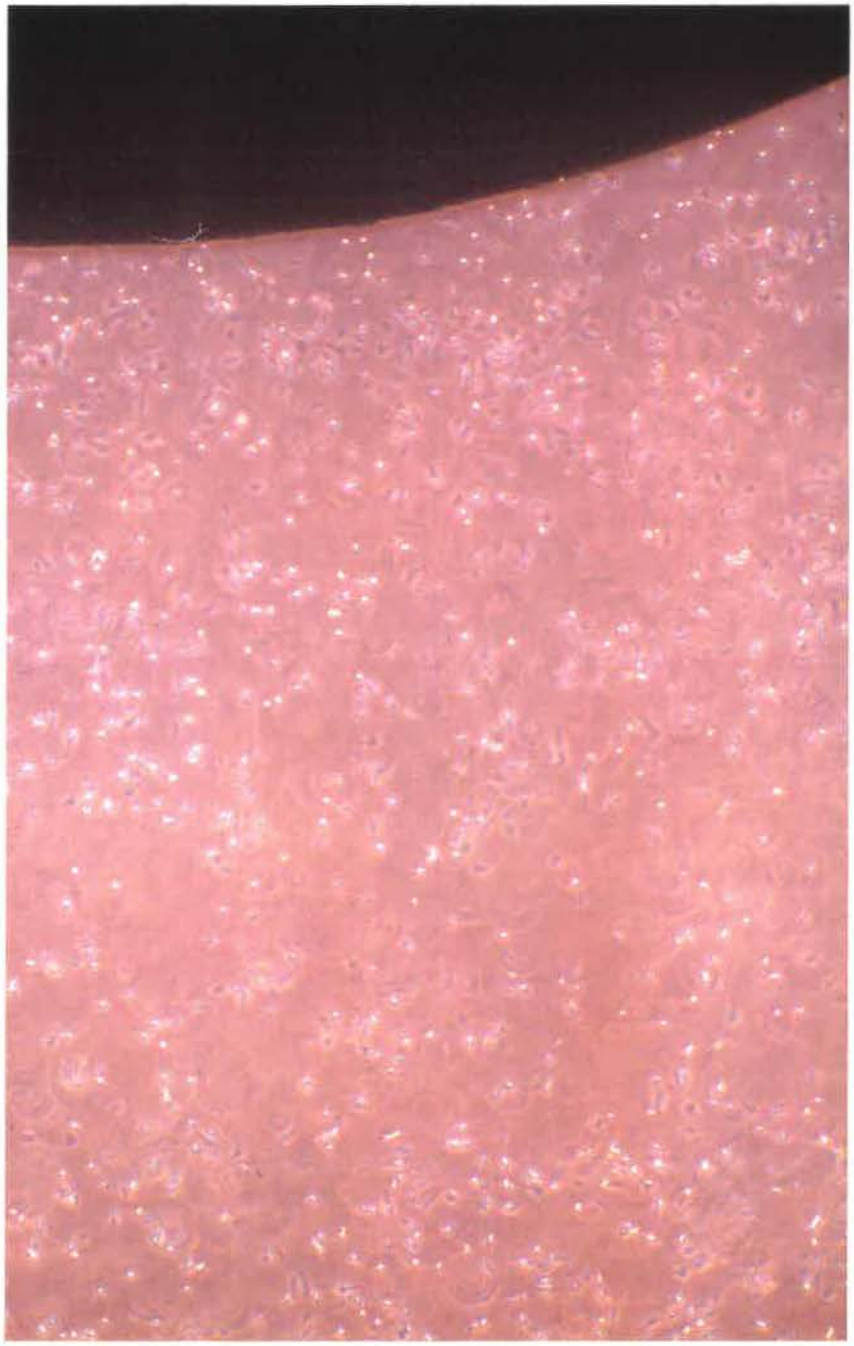


Fig. 6. *Morphology of HGF exposed to Ketac-fil*

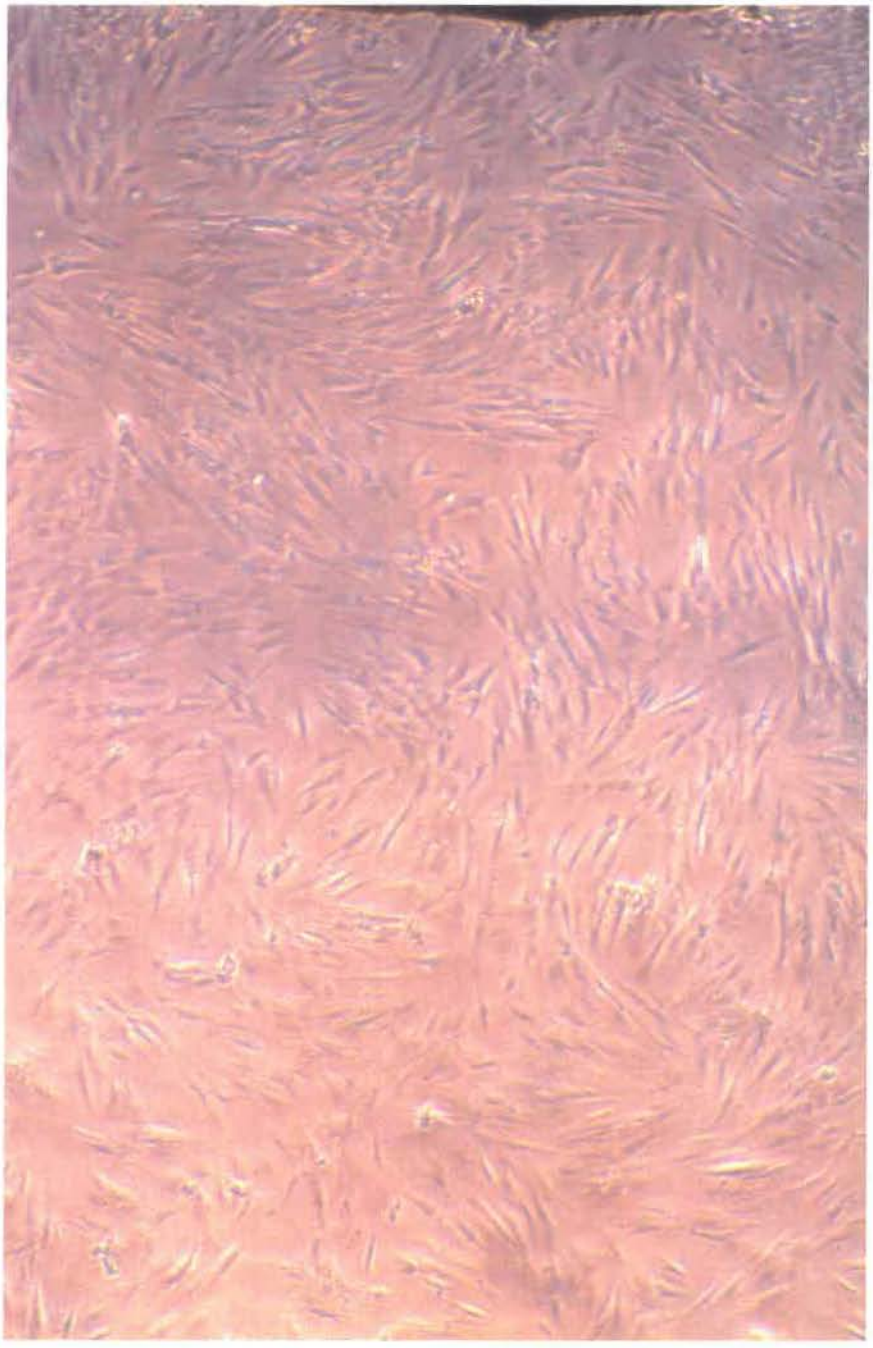


Fig. 7. *Morphology of HGF exposed to Ketac-silver*

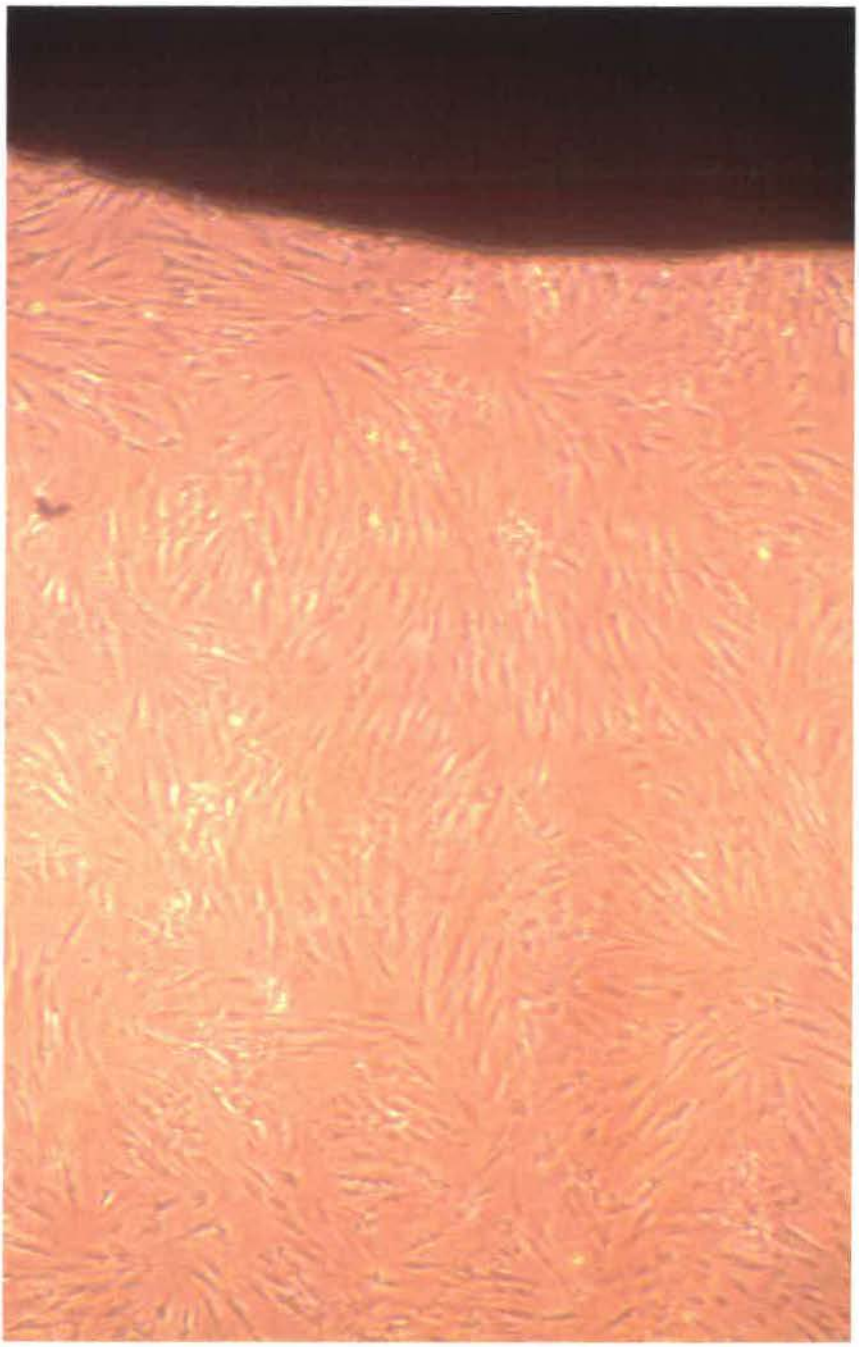


TABLE III. Incorporation of ^{3}H-Uridine Into Cells Exposed to Amalgam. Values are the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P value
Amalgam	255248	82476	$p > 0.05$ Not significant
Control	193626	49998	

Experiment #2

Material	Mean CPM	Std Deviation	P value
Amalgam	101908	13736	$p < 0.05$ Significant difference
Control	13251	135	

Experiment #3

Material	Mean CPM	Std Deviation	P value
Amalgam	141508	17203	$p < 0.05$ Significant difference
Control	102346	5196	

Experiment #4

Material	Mean CPM	Std Deviation	P value
Amalgam	120641	21555	$p > 0.05$ Not significant
Control	92790	6475	

Fig. 8. *^{3}H-Uridine incorporation into RNA of HGF exposed to Amalgam compared to control.*

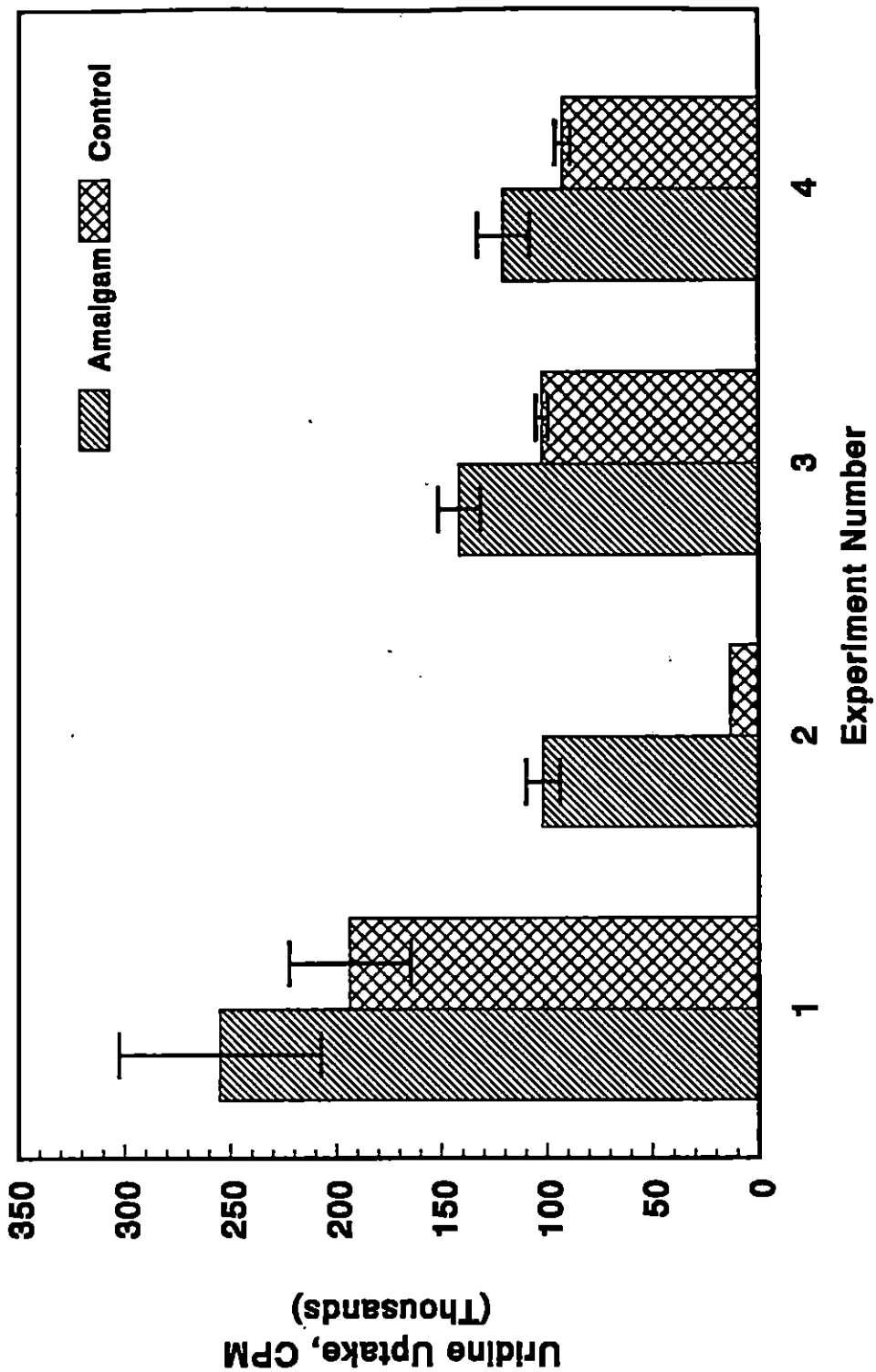

Amalgam
Control
350
300
250
200
150
100
50
0
1
2
3
4
Experiment Number
Uridine Uptake, CPM
(Thousands)

TABLE IV. Incorporation of ^{3}H-Uridine Into Cells Exposed to Gutta Percha. Values are the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P value
GP	125352	2262	$p > 0.05$ Not significant
Control	87640	17352	

Experiment #2

Material	Mean CPM	Std Deviation	P value
GP	11630	12319	$p > 0.05$ Not significant
Control	108583	36656	

Experiment #3

Material	Mean CPM	Std Deviation	P value
GP	42452	21894	$p > 0.05$ Not significant
Control	81763	17372	

Fig. 9. *^{3}H-Uridine incorporation into RNA of HGF exposed to gutta percha compared to control.*

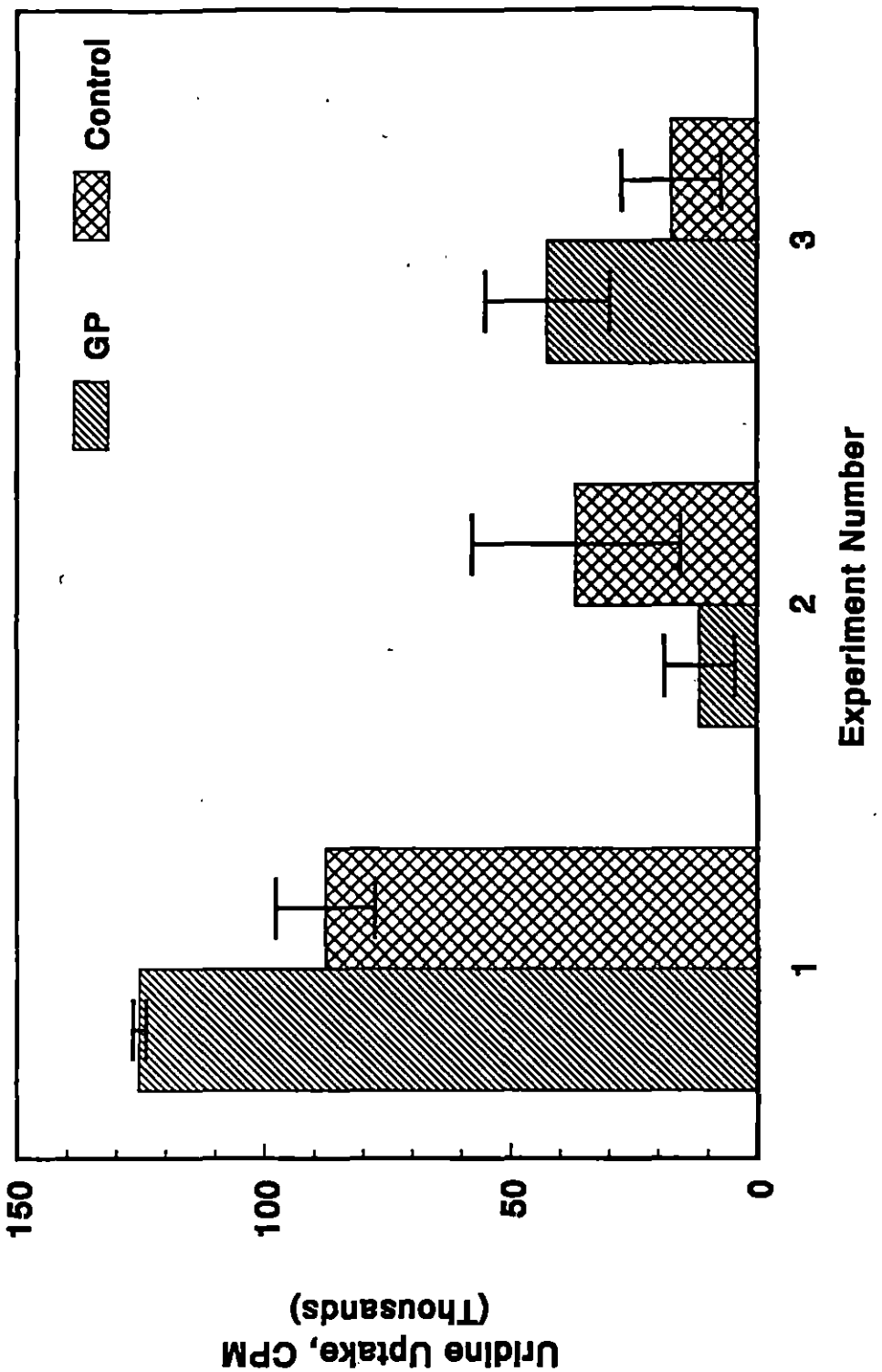

Control
GP
150
100
50
0
1
2
3
Experiment Number
Uridine Uptake, CPM
(Thousands)

TABLE V. Incorporation of ^{3}H-Uridine Into Cells Exposed to IRM. Values are the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P value
IRM	1015	40	$p < 0.05$ Significant difference
Control	271707	86347	

Experiment #2

Material	Mean CPM	Std Deviation	P value
IRM	1448	224	$p < 0.05$ Significant difference
Control	229504	60757	

Experiment #3

Material	Mean CPM	Std Deviation	P value
IRM	734	268	$p < 0.05$ Significant difference
Control	88025	29776	

Fig. 10. *^{3}H-Uridine incorporation into RNA of HGF exposed to IRM - Levels are so low that graphic representation is level with the bottom line of the graph - See Table V.*

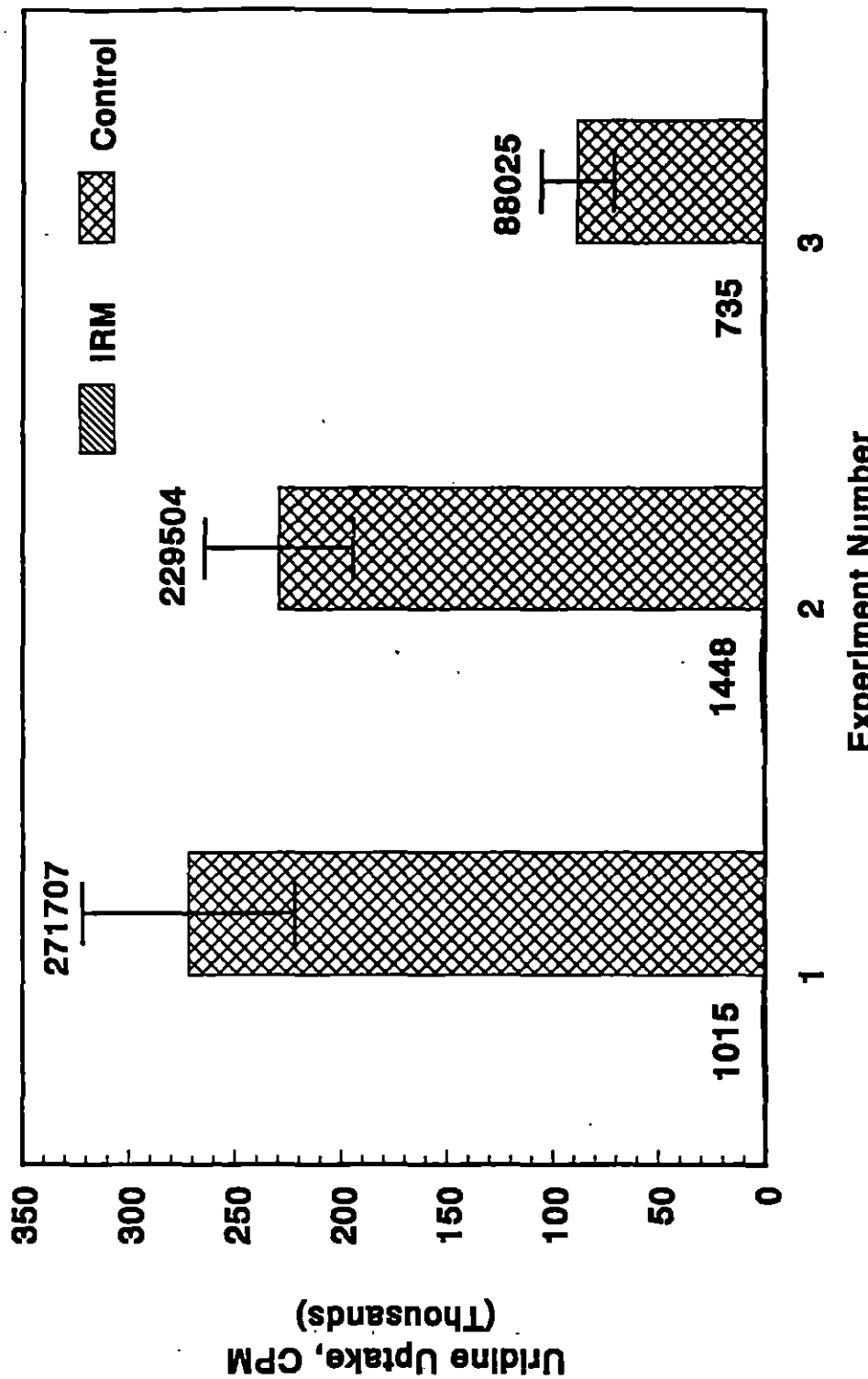

Urdine Uptake, CPM
(Thousands)
350
300
250
200
150
100
50
0
Control
IRM
271707
1015
229504
1448
88025
735
1
2
3
Experiment Number

TABLE VI. Incorporation of ^{3}H-Uridine Into Cells Exposed to EBA. Values of the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P value
EBA	1848	659	$p < 0.05$ Significant difference
Control	57019	6513	

Experiment #2

Material	Mean CPM	Std Deviation	P value
EBA	4281	3530	$p < 0.05$ Significant difference
Control	86276	14011	

Experiment #3

Material	Mean CPM	Std Deviation	P value
EBA	12629	10116	$p < 0.05$ Significant difference
Control	112545	32733	

Fig. 11. *^{3}H-Uridine incorporation into RNA of HGF exposed to EBA compared to control.*

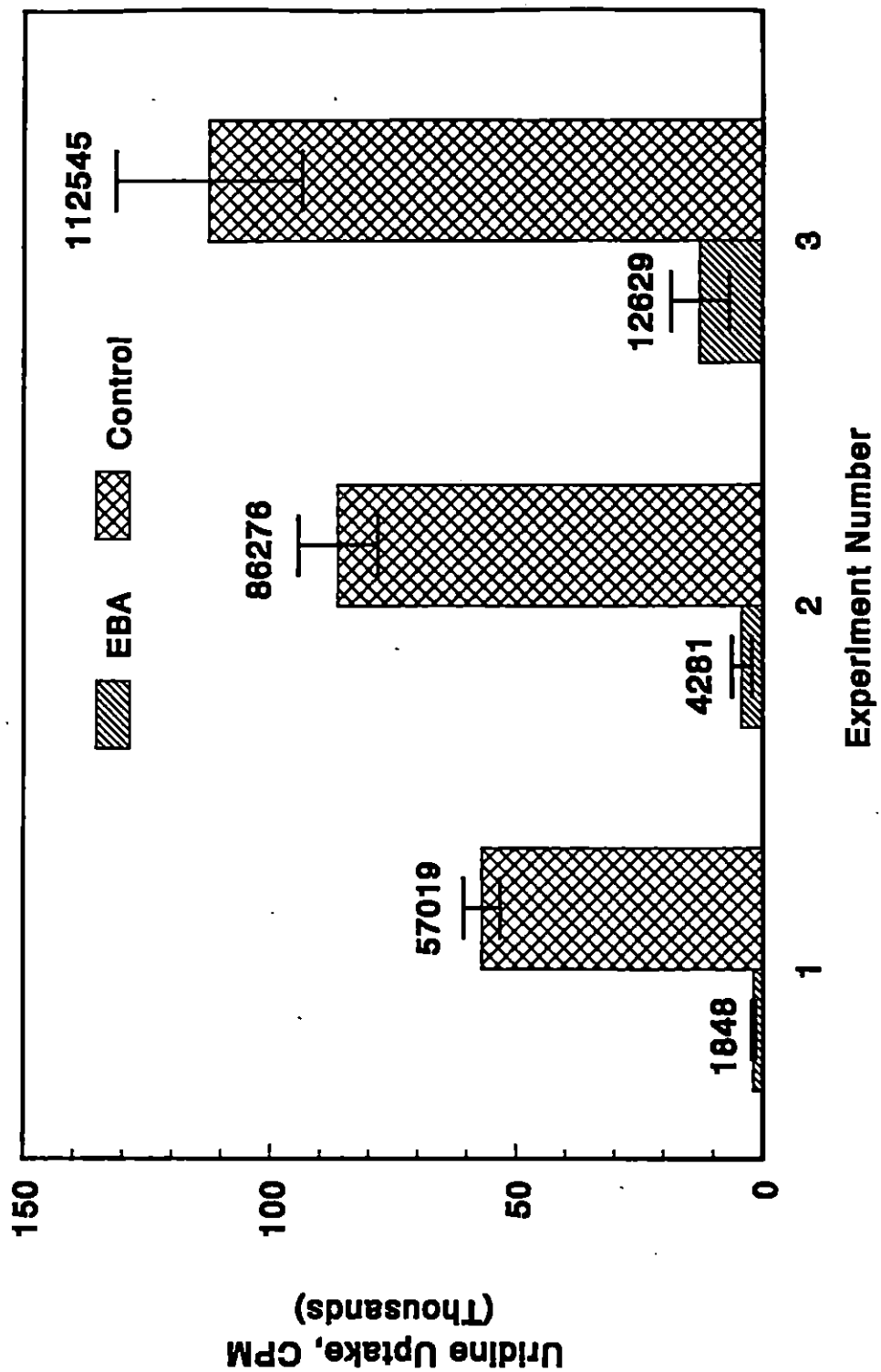

Uridine Uptake, CPM
(Thousands)
150
100
50
0
EBA
Control
1848
57019
4281
86278
12629
112545
1
2
3
Experiment Number

TABLE VII. Incorporation of ^{3}H-Uridine Into Cells Exposed to Ketac-fil. Values are the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P value
KF	114683	19196	p > 0.05 Not significant
Control	84777	8459	

Experiment #2

Material	Mean CPM	Std Deviation	P value
KF	97700	84520	p > 0.05 Not significant
Control	97333	6429	

Experiment #3

Material	Mean CPM	Std Deviation	P value
KF	53315	50042	p > 0.05 Not significant
Control	46202	7005	

Experiment #4

Material	Mean CPM	Std Deviation	P value
KF	24276	6269	p > 0.05 Not significant
Control	16970	3216	

Fig. 12. *^{3}H-Uridine incorporation into RNA of HGF exposed to Ketac-fil compared to control.*

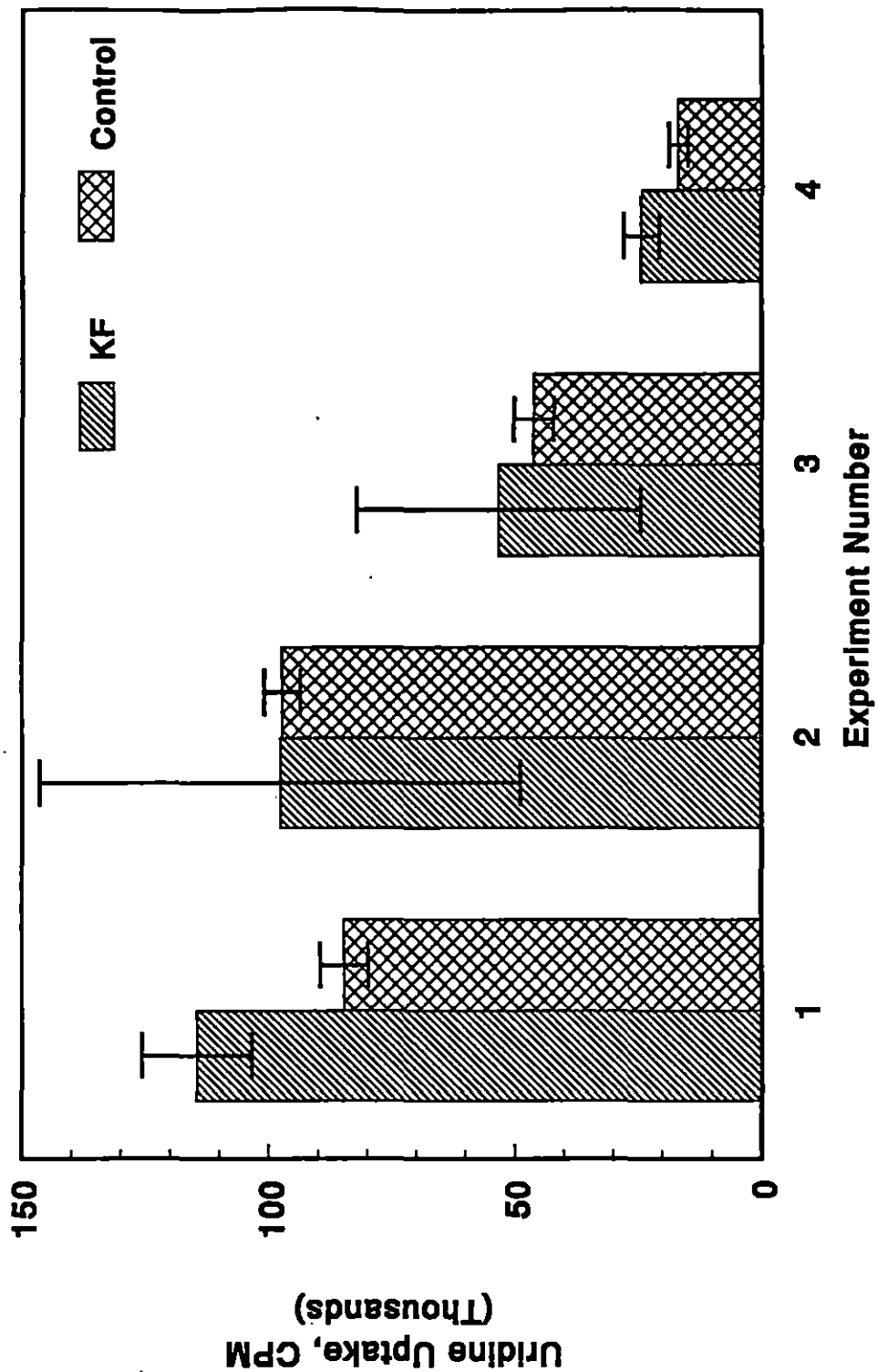

Control
KF
150
100
50
0
1
2
3
4
Experiment Number
Uridine Uptake, CPM
(Thousands)

TABLE VIII. Incorporation of ^{3}H-Uridine Into Cells Exposed to Ketac-silver. Values are the mean of three plates.

Experiment #1

Material	Mean CPM	Std Deviation	P values
KS	94484	34433	$p < 0.05$ Significant difference
Control	381410	45175	

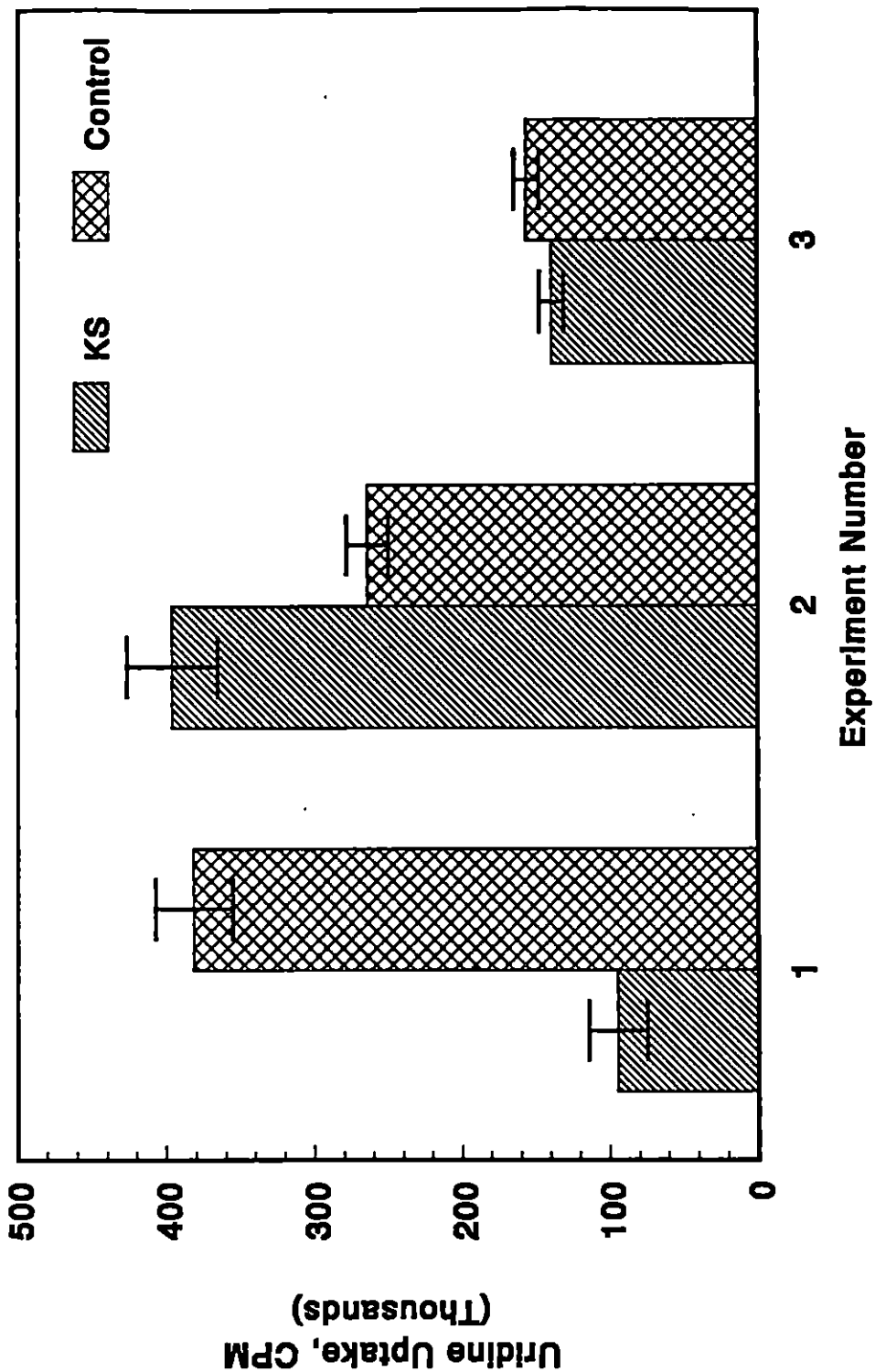
Experiment #2

Material	Mean CPM	Std deviation	P value
KS	396166	52930	$p < 0.05$ Significant difference
Control	264242	24418	

Experiment #3

Material	Mean CPM	Std Deviation	P value
KS	138870	14686	$p > 0.05$ Not significant
Control	156138	16997	

Fig. 13. *^{3}H-Uridine incorporation into RNA of HGF exposed to Ketac-silver compared to control.*


Uridine Uptake, CPM
(Thousands)
500
400
300
200
100
0
KS
Control
1
2
3
Experiment Number

The morphology of the cells seeded in culture dishes with Ketac-fil and Ketac-silver were normal and given a score of 0, (indicating no evident toxicity)(Figs. 6 and 7). Cells can be seen growing adjacent to the disks in all samples.

Metabolic assays:

Toxic effects on cell metabolism were assessed as inhibition of ^{3}H-uridine incorporation into total RNA. The mean counts per minute (CPM) of incorporated radioisotope from the sequential experiments are shown in Tables III-VIII. Each root-end filling material was first tested independently and compared to control. An unpaired t-test was used to compare mean CPM for statistical differences for each experiment.

Table III and Fig. 8 demonstrate the responses to amalgam in the various experiments. Generally there appeared to be a stimulation of incorporation of ^{3}HUR into cellular RNA despite the morphologic evidence of some toxicity. The stimulation was statistically significant in two of the four experiments. It is also evident that there was less overall incorporation into the cells in the three latter experiments as compared to the first.

Gutta percha produced no effects as compared to control cultures although overall incorporation into cells was less in the later than the early cultures (Table IV and Fig. 9).

IRM and EBA, both zinc oxide-eugenol containing materials, showed virtually complete cytotoxicity in the morphologic assays and this response is evident in the metabolic assays. Incorporation of ^{3}HUR into cells in cultures exposed to IRM was

negligible compared to control cultures; all were significantly less than comparable control cultures (Table V and Fig. 10). Overall incorporation into cells also was less in the two later experiments as compared to the first. Similarly, EBA produced significantly less incorporation of ^{3}HUR into RNA than control cultures, but incorporation during the later experiments was increased compared to the early experiment (Table VI and Fig. 11). When RNA synthesis of cells exposed to Ketac-fil was studied, the labeled RNA counts show that the cement had CPM values that were slightly higher than the control. There was no morphologic evidence of inhibition, and results of the RNA synthesis assays revealed no statistical difference in RNA synthesis between this glass ionomer cement and the wax control (Table VII and Fig. 12).

Results using Ketac-silver suggested that the synthesis of RNA was either stimulated or about the same as the control. In experiment #1, the counts for the Ketac-silver were significantly lower. Experiment #2 showed a reverse result with the counts for the glass ionomer higher than the control. The counts from experiment #3 were not significantly different from the control (Table VIII and Fig. 13). Since there was no inhibition of growth seen in the microscopic scoring, the results of experiment #1 may reflect some experimental error.

Effects of Cell Age:

Because the data suggested that the growth/passage stage of the cells could have an effect on the isotope incorporation response, studies were conducted wherein all materials were examined in the same experiment at different (8-11) cell passages.

Analysis of variance with a Scheffe post hoc procedure was used to determine which means were significantly different for the longitudinal studies. The results varied, depending upon experiment, root-end filling material and cell passage. It appears that as the cell passage number increased, especially at the later passages, the uptake of isotope and the sensitivity of the cells decreased as evidenced by the continual decrease in total isotope incorporation. The values obtained from later experiments appeared less different from each other (Table IX, Fig. 14).

However, the patterns of cytotoxicity seen in the sequential studies were also evident in the longitudinal studies. The counts recorded for the zinc oxide-eugenol materials (IRM and EBA) were significantly different from the other materials studied ($p = 0.05$). The other root-end filling materials were not statistically different from each other.

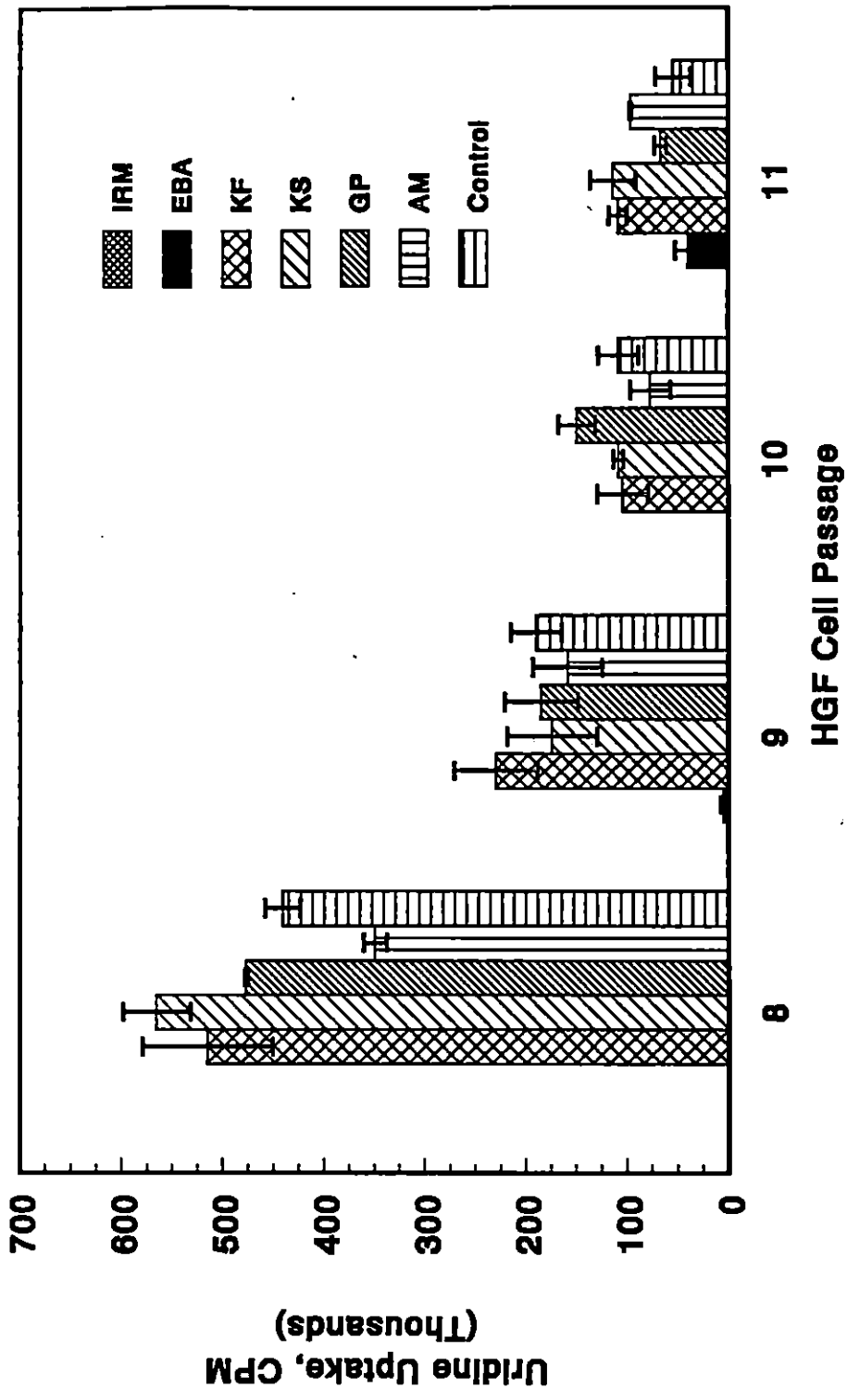
MTT Assays of HGF Attachment:

To assess the extent of toxic substances which leach out of the materials and the time over which this occurred, samples were soaked in medium and the eluates tested for their ability to inhibit attachment and growth as compared to growth and attachment in fresh medium and medium incubated in the same

TABLE IX . Results of Longitudinal Studies - Mean Counts per Minute (CPM)

Cell Passage	Material	Mean CPM	Std Deviation
#8	Amalgam	349,207	16104
#9	Amalgam	158,311	48296
#10	Amalgam	76,660	9611
#11	Amalgam	92,264	1630
#8	Gutta percha	476,595	2185
#9	Gutta percha	184,780	51034
#10	Gutta percha	149,031	25543
#11	Gutta percha	66,264	8461
#8	IRM	423	11
#9	IRM	560	85
#10	IRM	139	13
#11	IRM	140	77
#8	EBA	536	29
#9	EBA	5,257	3702
#10	EBA	497	429
#11	EBA	39,852	17244
#8	Ketac-fil	514,938	90752
#9	Ketac-fil	229,518	57831
#10	Ketac-fil	104,050	35325
#11	Ketac-fil	108,740	12641
#8	Ketac-silver	562,208	46716
#9	Ketac-silver	173,938	63042
#10	Ketac-silver	108,048	6848
#11	Ketac-silver	113,984	31528

Fig. 14. *^{3}H-Uridine incorporation into RNA of HGF exposed to all materials at the same time and at different cell passage number.*


IRM
EBA
KF
KS
GP
AM
Control
700
600
500
400
300
200
100
0
8
9
10
11
HGF Cell Passage
Uridine Uptake, CPM
(Thousands)

manner as that which contained the sample. Due to the large number of samples to be assayed we only assayed each sample once, so we were unable to do statistical analyses. Only trends are described. Cells exposed to eluates of amalgam from days one, two and three showed some inhibition of attachment to the tissue culture plates. There was no apparent inhibition of attachment from eluates of days four through ten, as the MTT absorbance values for amalgam were similar to both the incubated control medium and the fresh control medium (Fig. 15).

The response to gutta percha was similar to that of amalgam. An initial small inhibition of fibroblast attachment was seen during the first two days, however by day three the levels of dehydrogenase activity measured in the assay appeared to be at the control level. Enzyme activity continued to increase until day eight (Fig. 16). Studies with IRM, EBA, and Ketac-fil suggested that cell attachment slightly decreased during the first two days, when material components were leaching into the medium, but by day four there appeared to be no effect (Figs. 17, 18, and 19). Eluates from day one of Ketac-silver appeared to have a slight inhibitory effect on attachment during the experimental period. However, by the second day the levels of dehydrogenase activity were about the same as both control media (Fig. 20). None of the materials tested revealed any sustained effect on HGF attachment.

Effects of material after 4 and 48 hours of exposure to preplated cells:

After 4 hours of exposure to amalgam eluates, there appeared to be no difference in dehydrogenase activity in preattached cells as compared to controls (Fig. 21). After 48 hours, toxicity was present for eluates of days one through three. However,

by day four the values were very close to the control medium (Fig. 22). The initial absorbance recorded after 48 hours for gutta percha eluates followed the same general patterns as amalgam (Fig 23). Exposure for 48 hours showed an apparent prolonged toxicity through day eight (Fig. 24).

Measurement of dehydrogenase activity in the presence of eluates of the zinc oxide-eugenol materials, EBA and IRM, showed no toxic effect after 4 hours of exposure (Figs. 25 and 27). However, after 48-hours of exposure, enzyme activity was severely decreased by eluates from day one and toxic effects were apparent from samples through the ten days. Cell enzyme activity recovered but never reached the levels of the control as was seen with other materials (Fig. 26, 28).

Neither of the glass ionomer eluates caused much inhibition after four hours of exposure (Figs. 29 and 31). After a 48 hour incubation, Ketac-fil eluate from day one produced a large decrease in enzyme activity. By day two, activity had returned closer to control levels and reached the levels of the control at day six (Fig. 30). Eluate from Ketac-silver exposed for 48 hours to HGF, did not have an initial acute effect as great as that of Ketac-fil. Cells seemed to recover from toxicity by leachable components by the fourth day, when the response was the same as that to the control media (Fig. 32).

Summary of Results:

In summary, the results indicate that (1) IRM and EBA produced a toxic response evident morphologically; this was more extensive than the response to amalgam and gutta percha. (2) Glass ionomer cements produced little or no detectable toxic

response. (3) RNA synthesis was significantly impaired by IRM and EBA but not by amalgam, gutta percha, Ketac-fil or Ketac-silver; (4) Although there appeared to be some initial growth inhibition with some materials,after four days, with the exception of IRM and EBA, the levels of cytotoxic materials eluted from the various material did not appear to inhibit cell growth.

Fig. 15. *Effect of elutes of amalgam on HGF attachment*

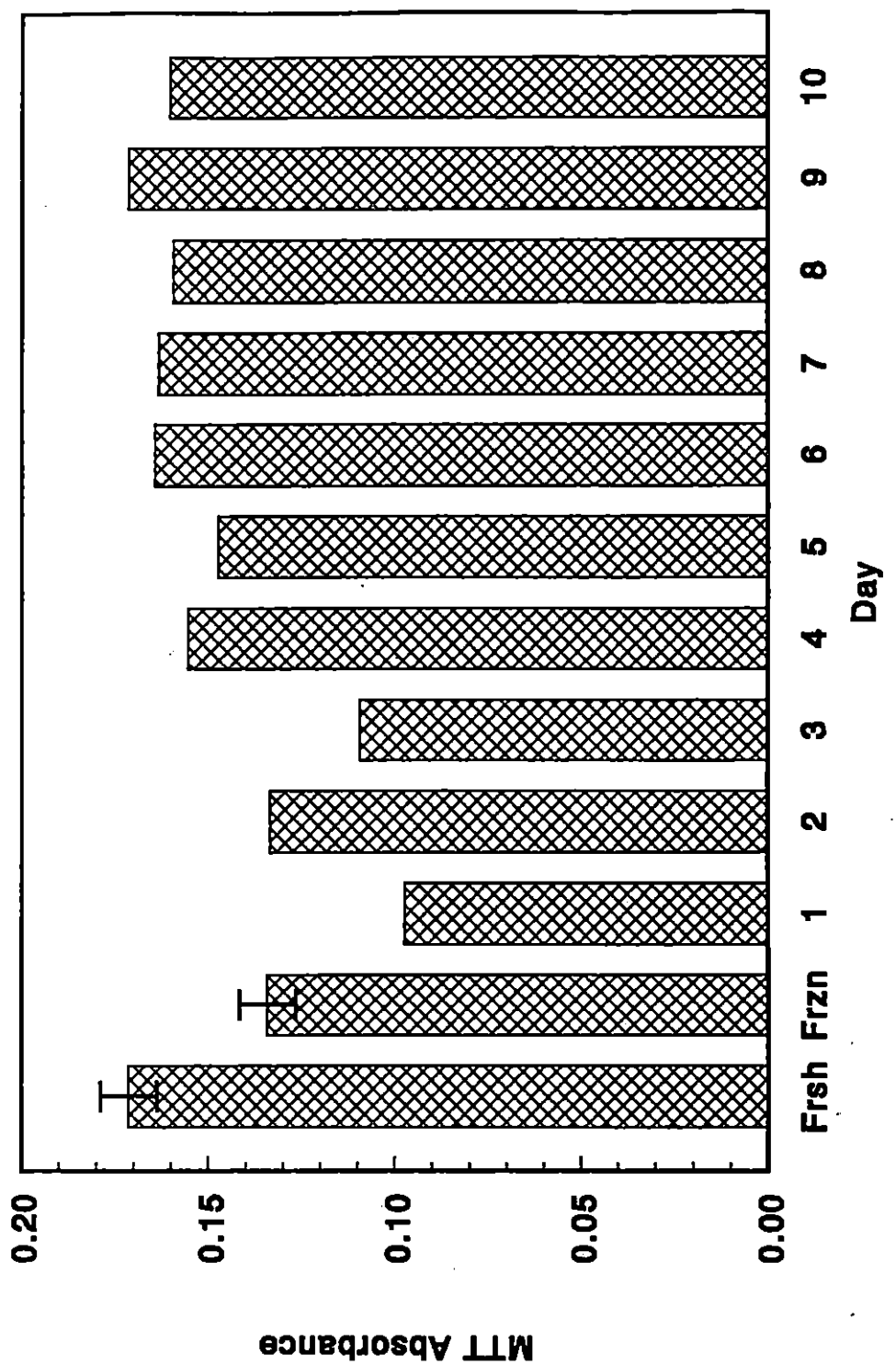

MTT Absorbance
0.20
0.15
0.10
0.05
0.00
Frsh
Frzn
1
2
3
4
5
6
7
8
9
10
Day

Fig. 16. *Effect of elutes of gutta percha on HGF attachment*

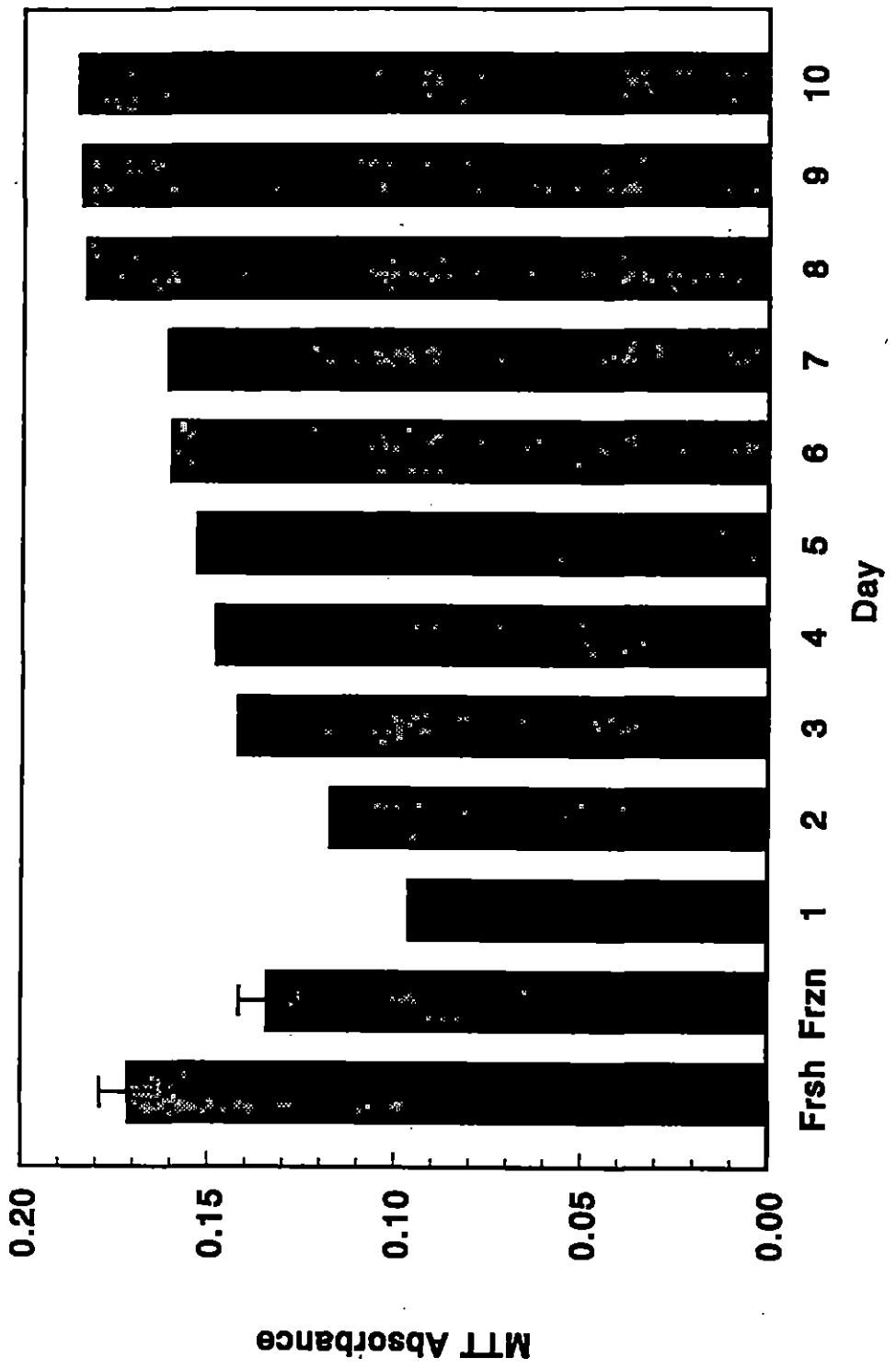

MTT Absorbance
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 17. *Effect of elutes of IRM on HGF attachment*

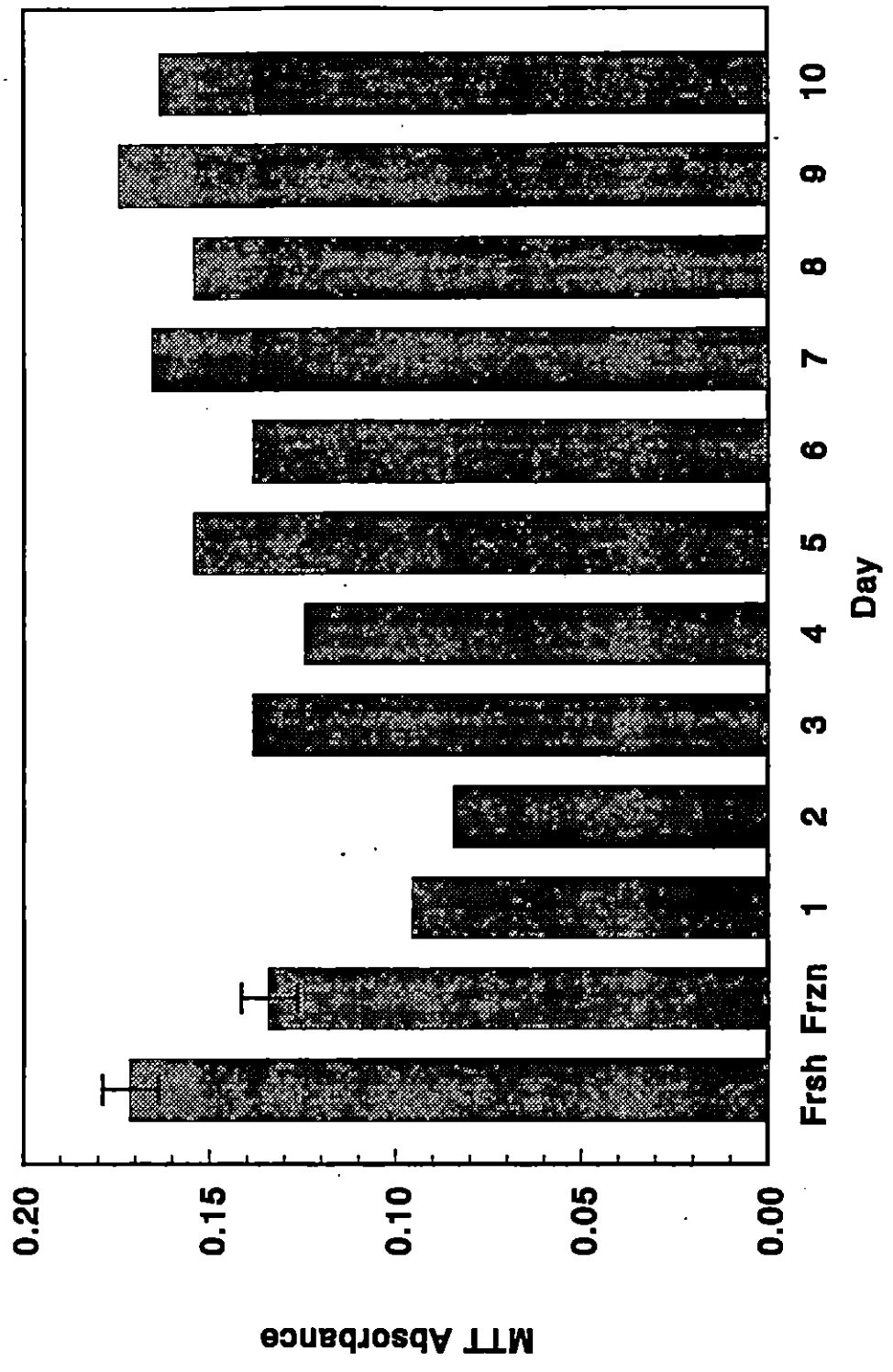

MTT Absorbance
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 18. *Effect of elutes of EBA on HGF attachment*

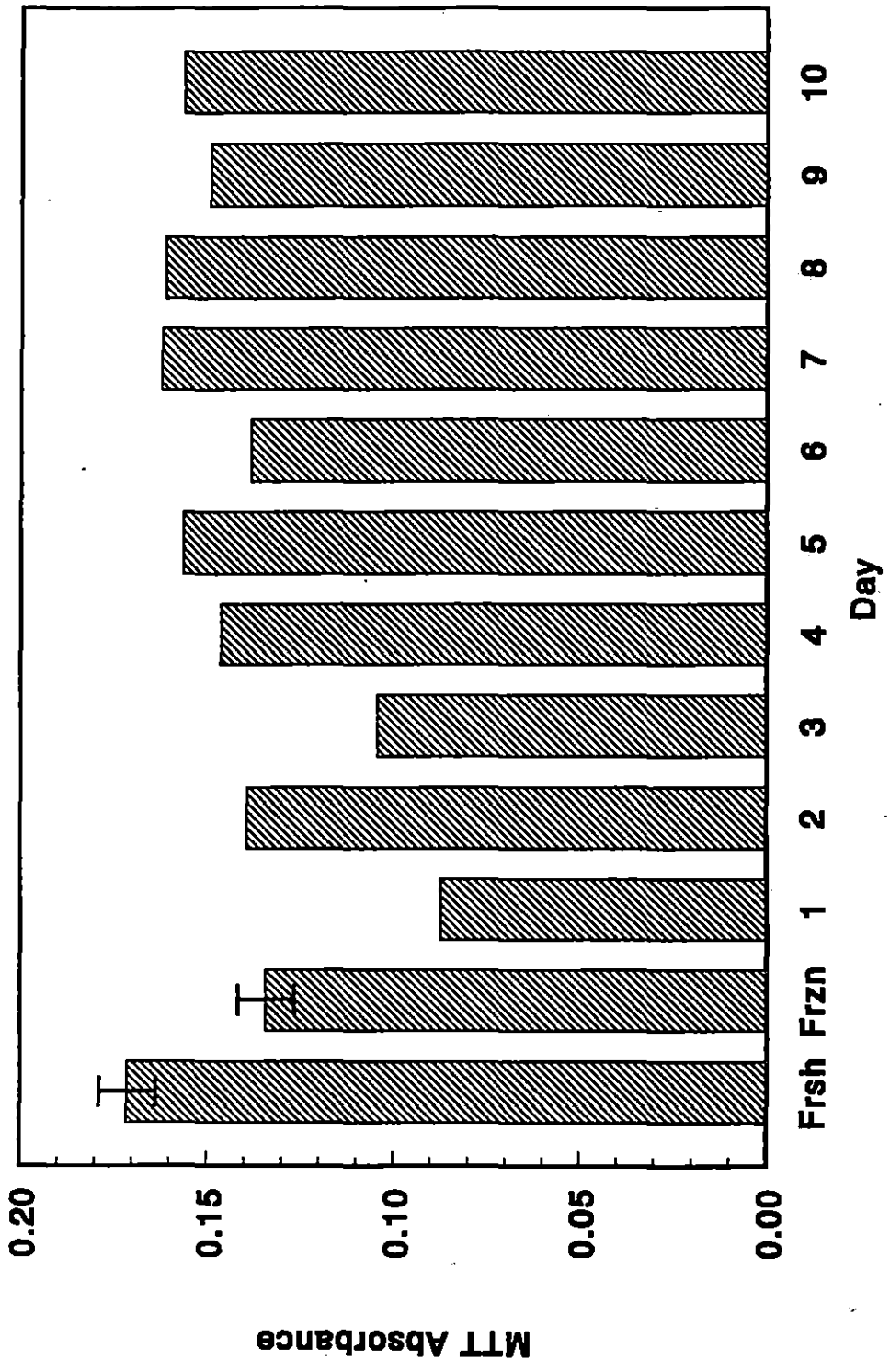

MTT Absorbance
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 19. *Effect of elutes of Ketac-fil on HGF attachment*

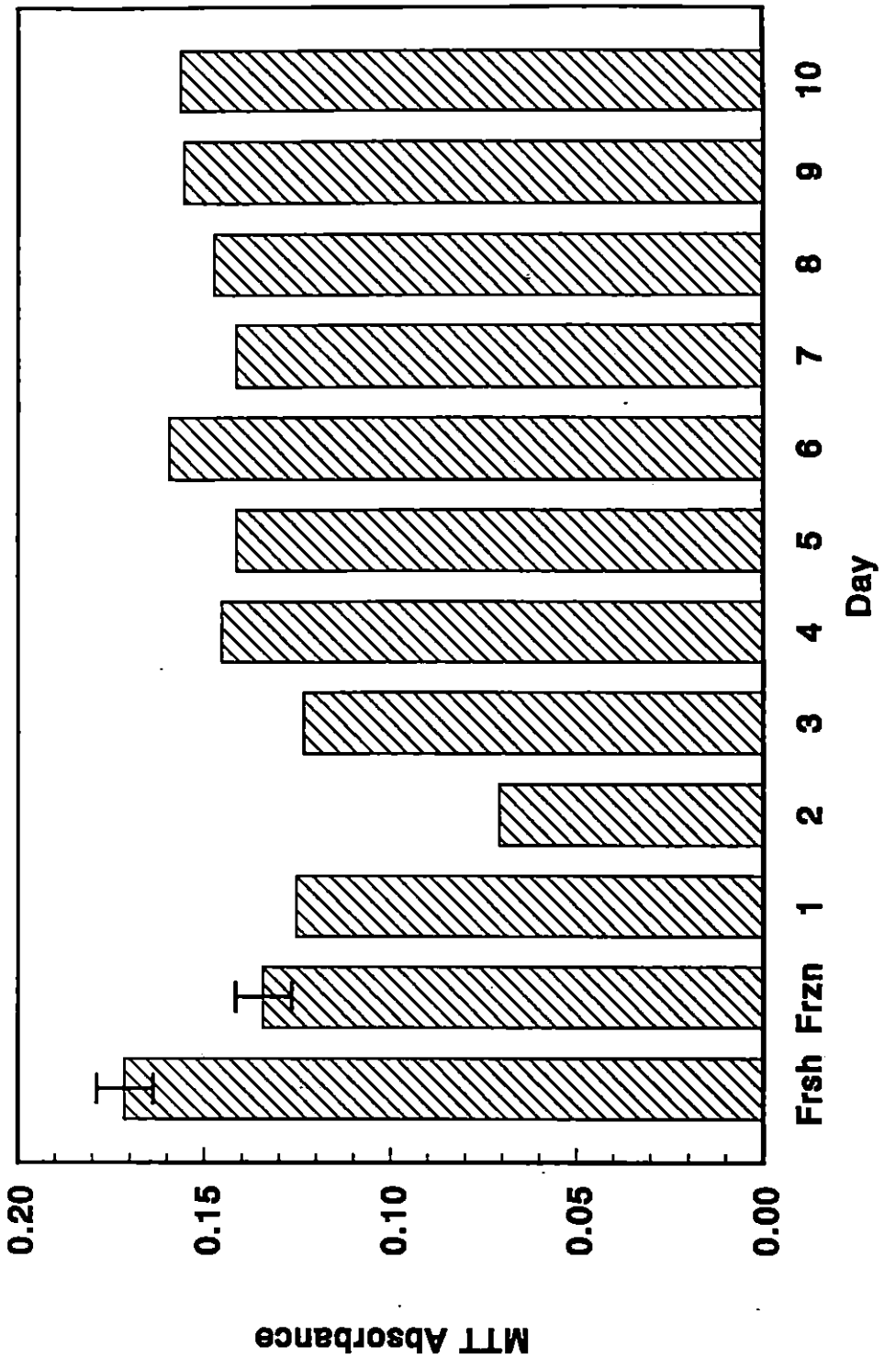

MTT Absorbance
0.00
0.05
0.10
0.15
0.20
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 20. *Effect of elutes of Ketac-silver on HGF attachment*

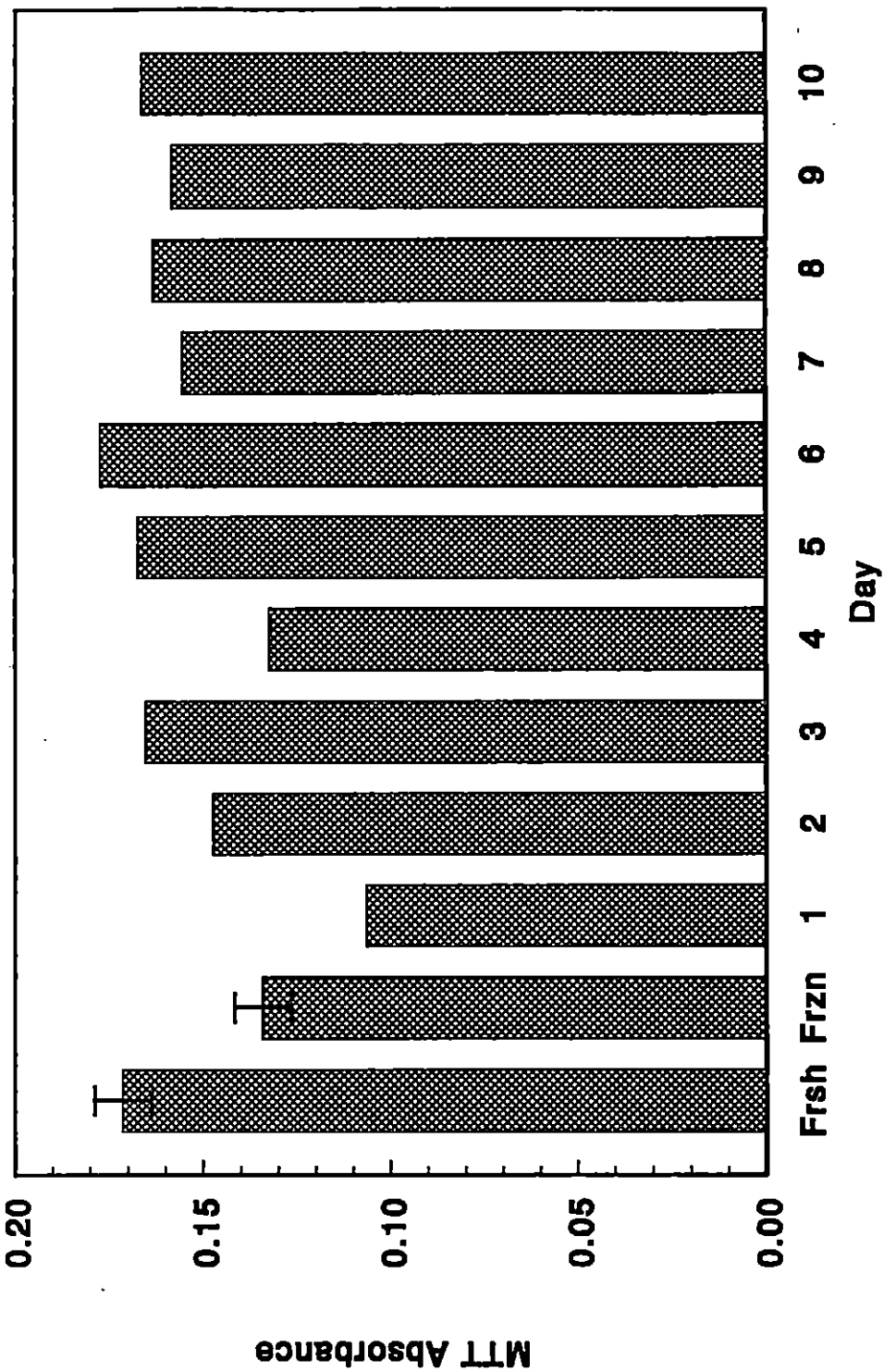

MTT Absorbance
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 21. *Toxicity to HGF exposed to amalgam eluates for 4 hours*

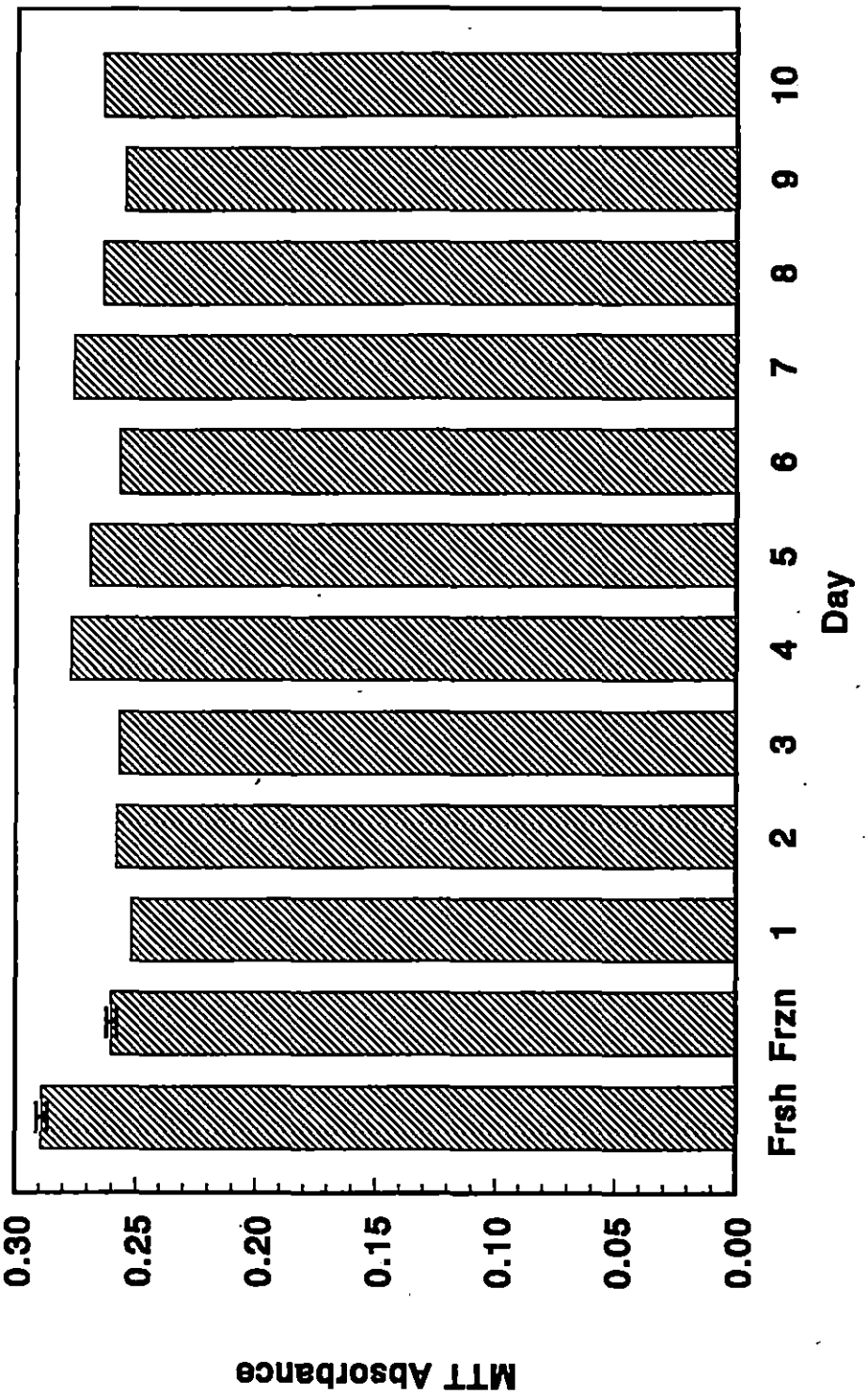

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 22. *Toxicity to HGF exposed to amalgam eluates for 48 hours*

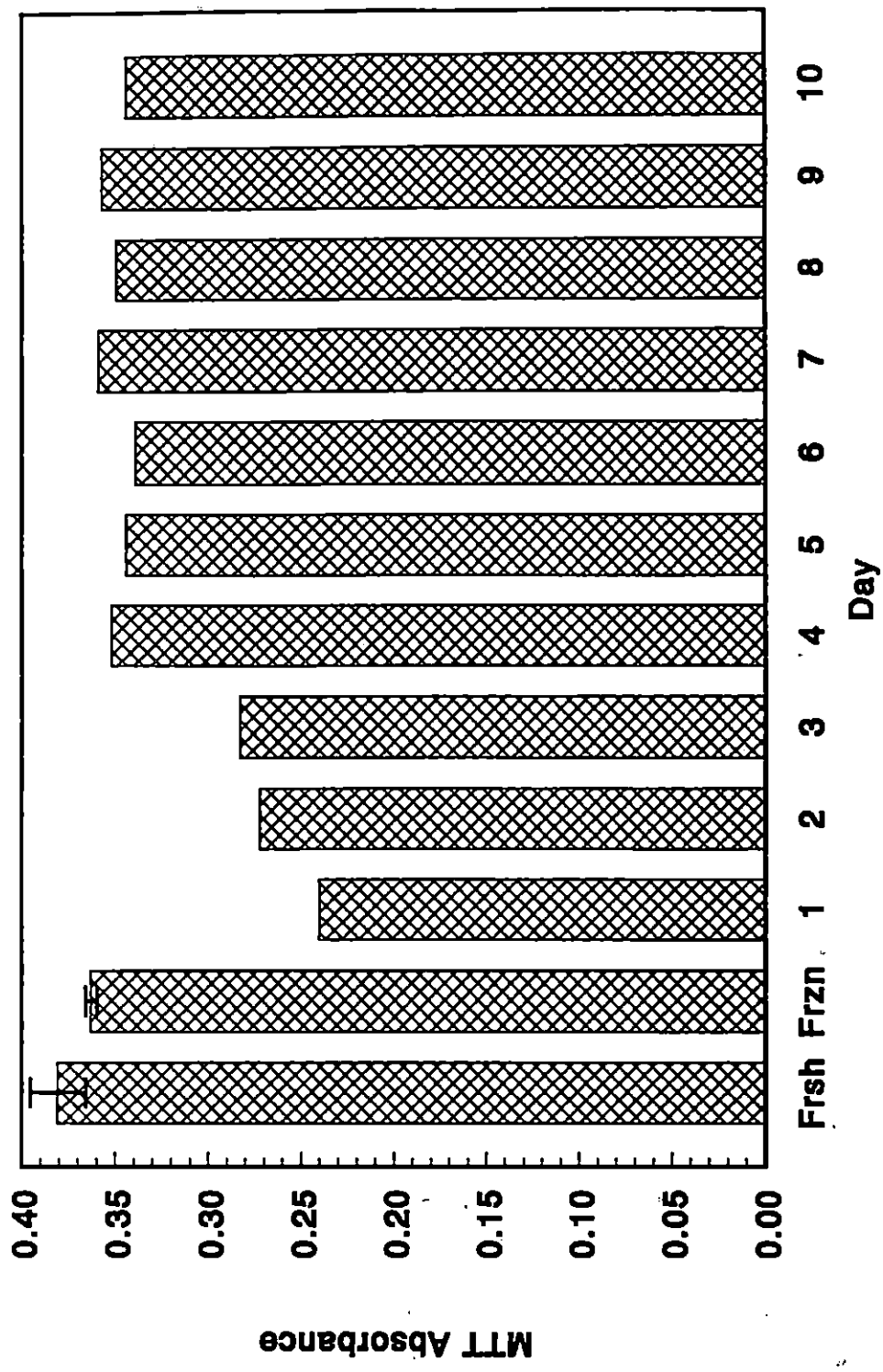

MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 23. *Toxicity to HGF exposed to gutta perch eluates for 4 hours*

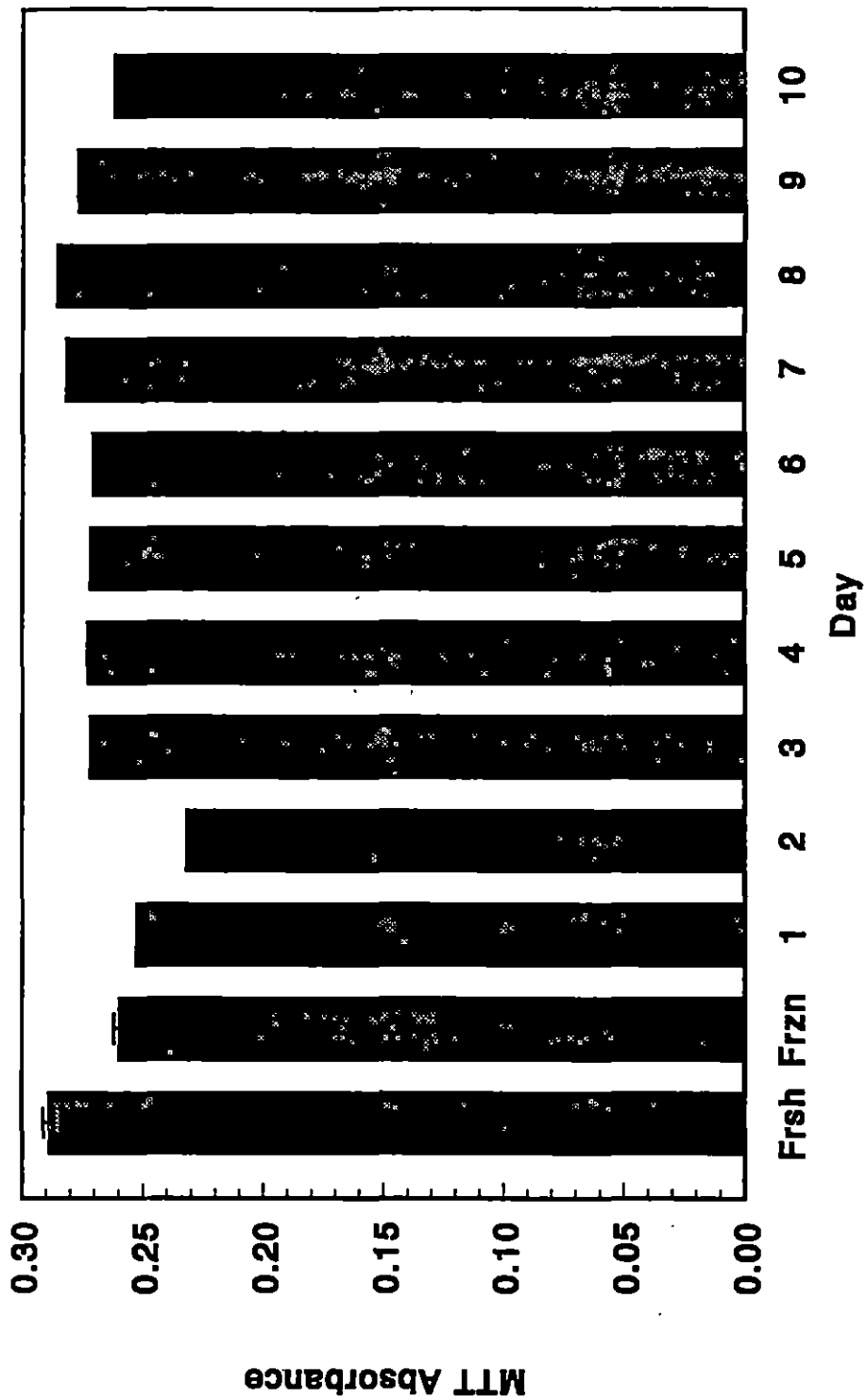

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 24. *Toxicity to HGF exposed to gutta percha eluates for 48 hours*

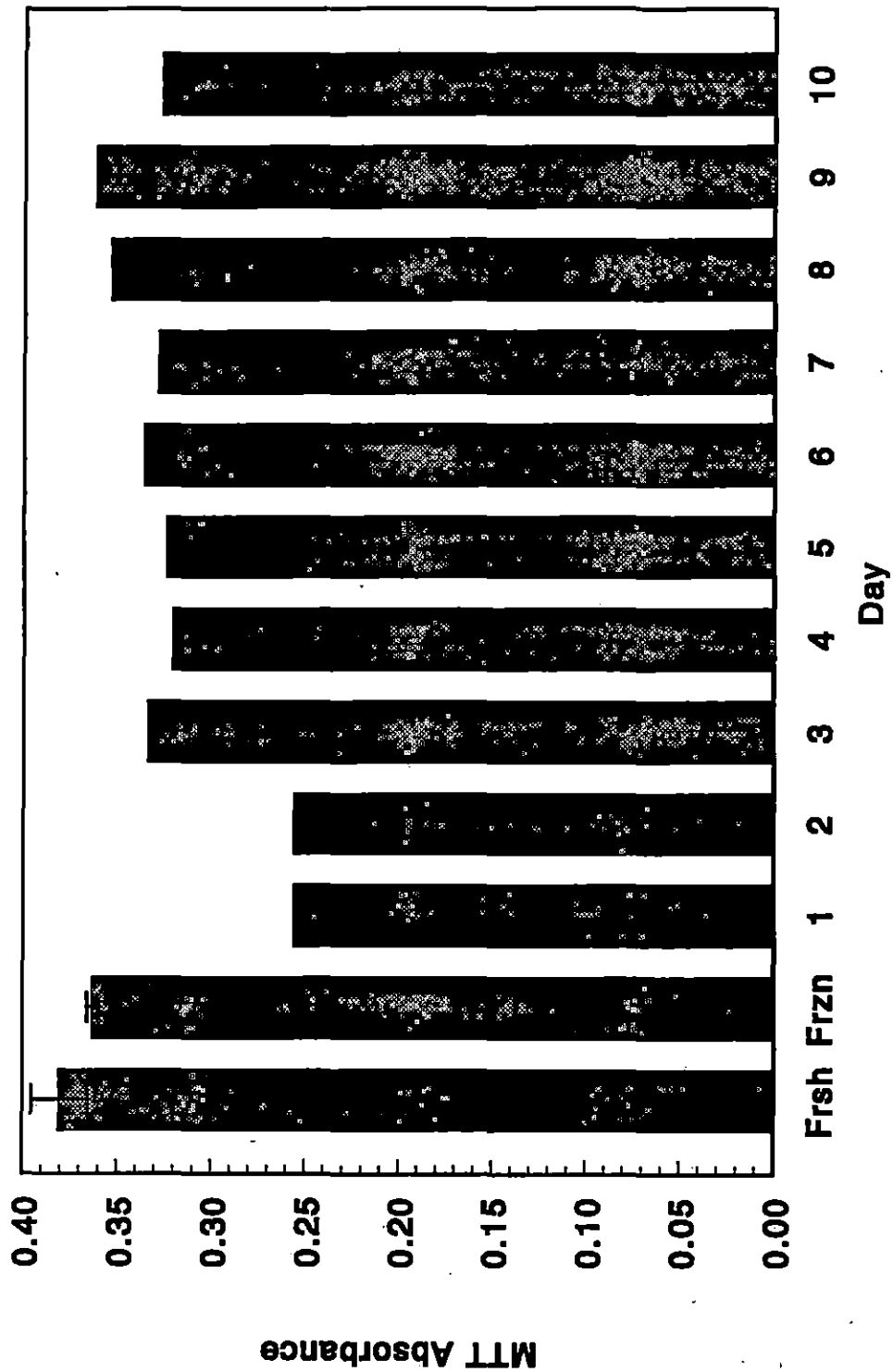

MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn
1
2
3
4
5
6
7
8
9
10
Day

Fig. 25. *Toxicity to HGF exposed to IRM eluates for 4 hours*

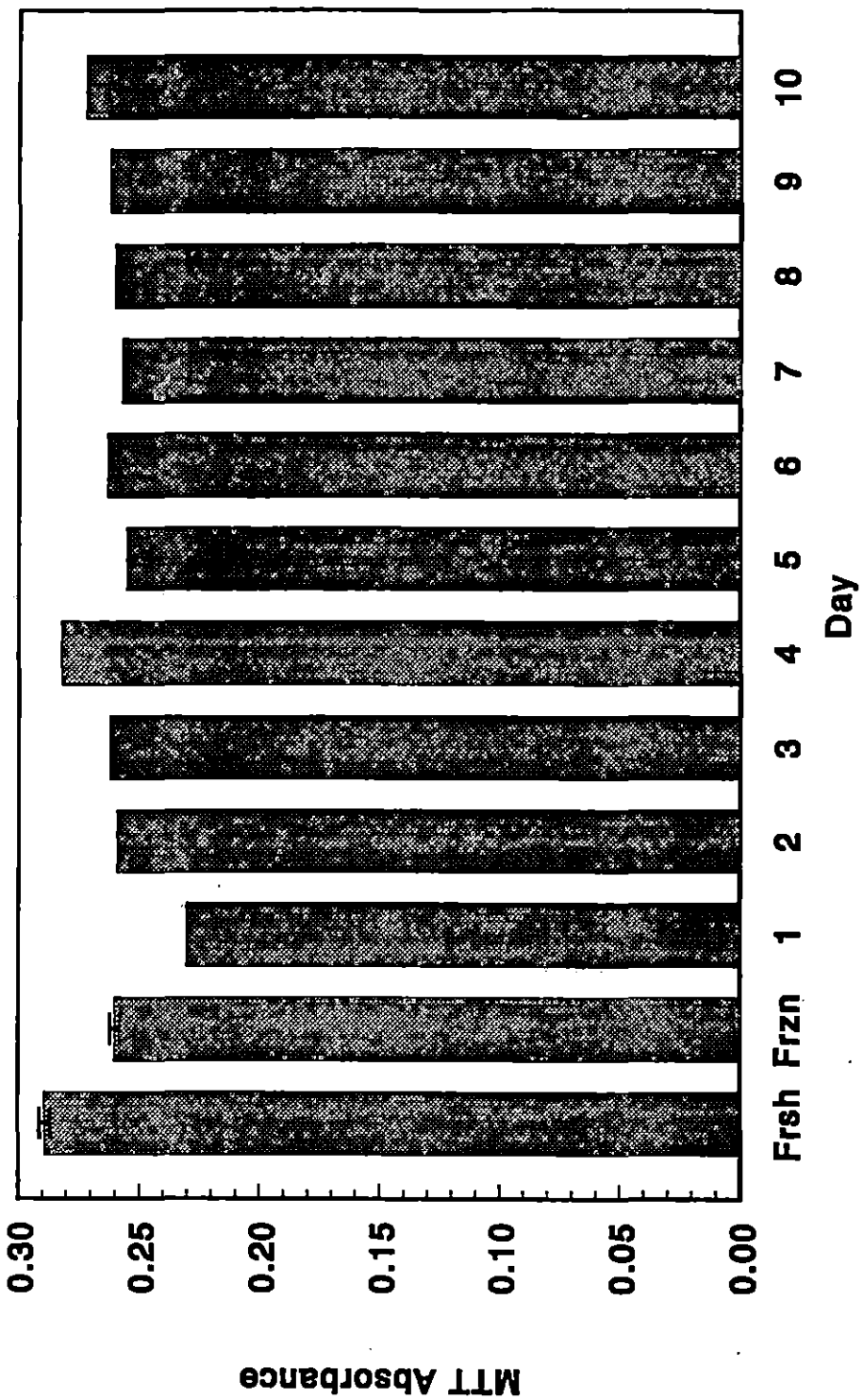

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 26. *Toxicity to HGF exposed to IRM eluates for 48 hours*

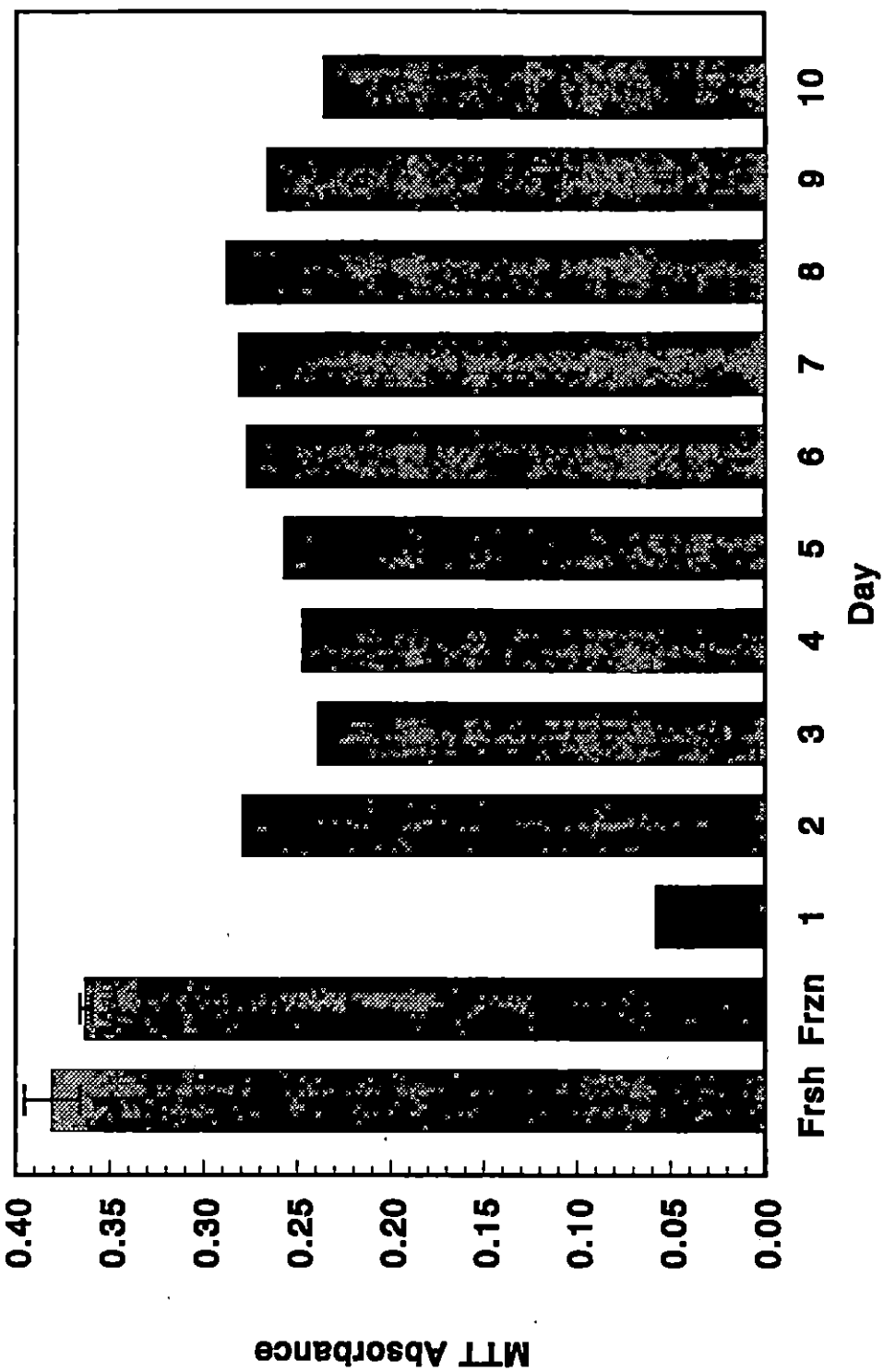

MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn
1
2
3
4
5
6
7
8
9
10
Day

Fig. 27. *Toxicity to HGF exposed to EBA eluates for 4 hours*

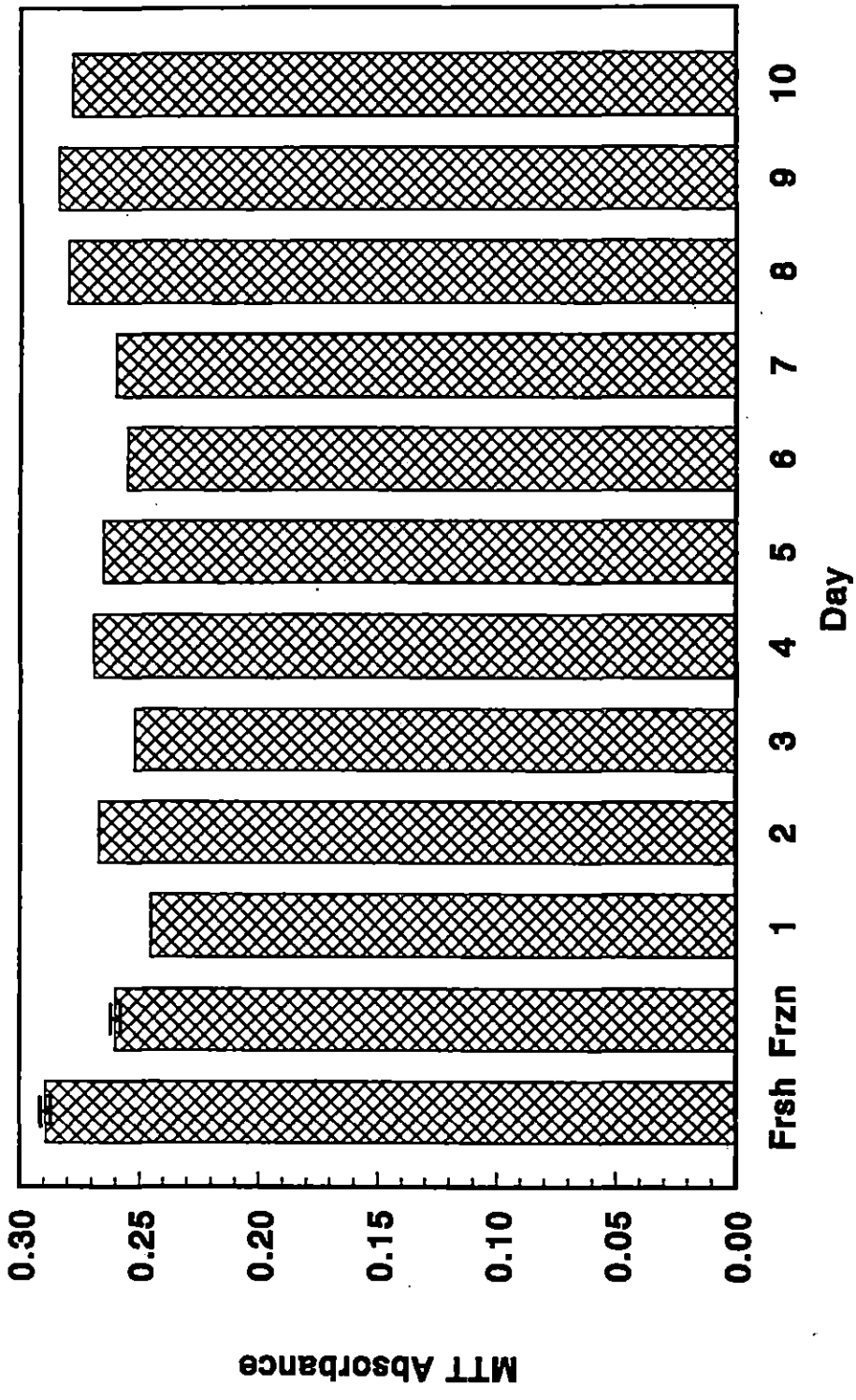

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 28. *Toxicity to HGF exposed to EBA eluates for 48 hours*

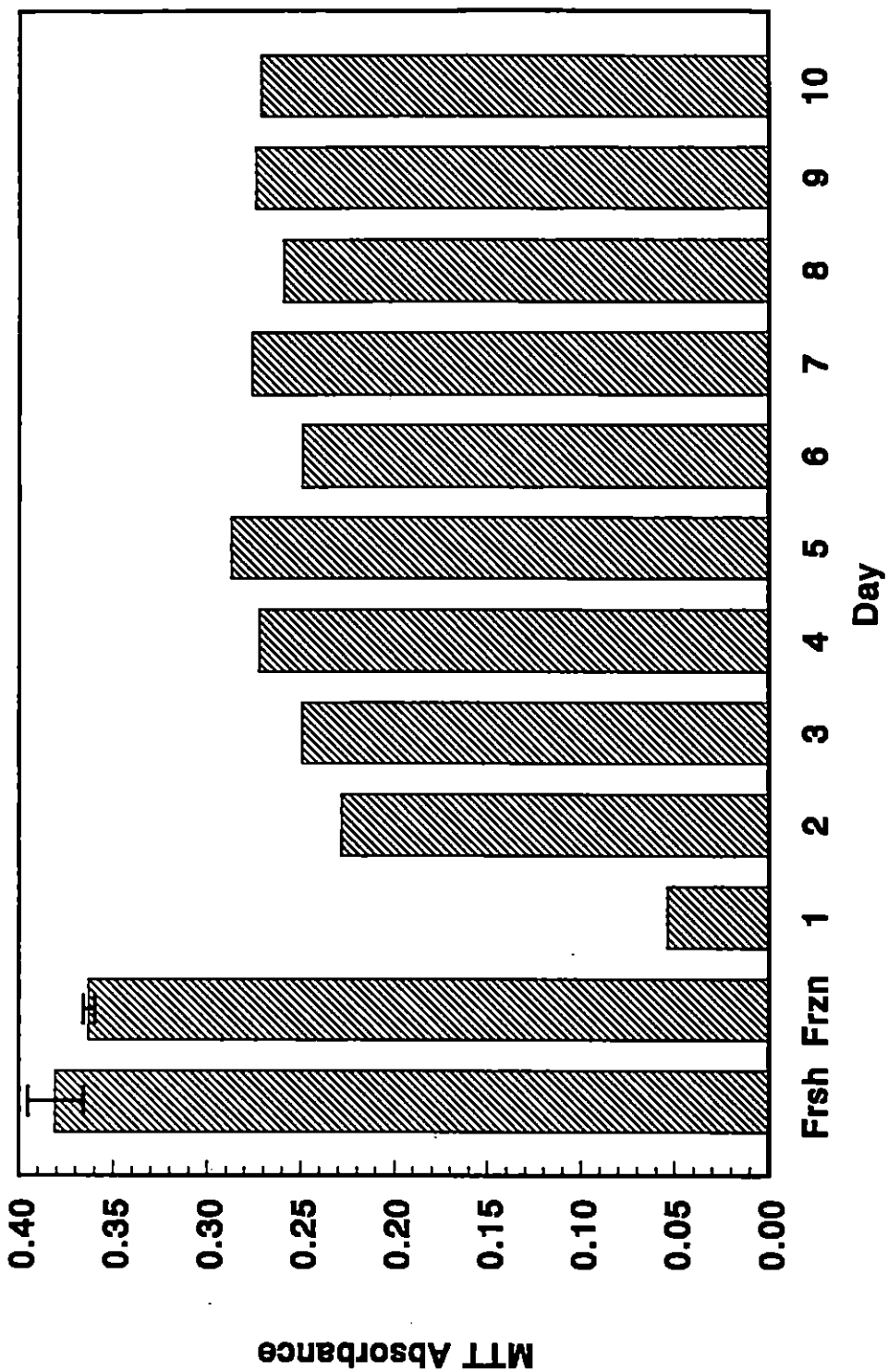

MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 29. *Toxicity to HGF exposed to Ketac-fil eluates for 4 hours*

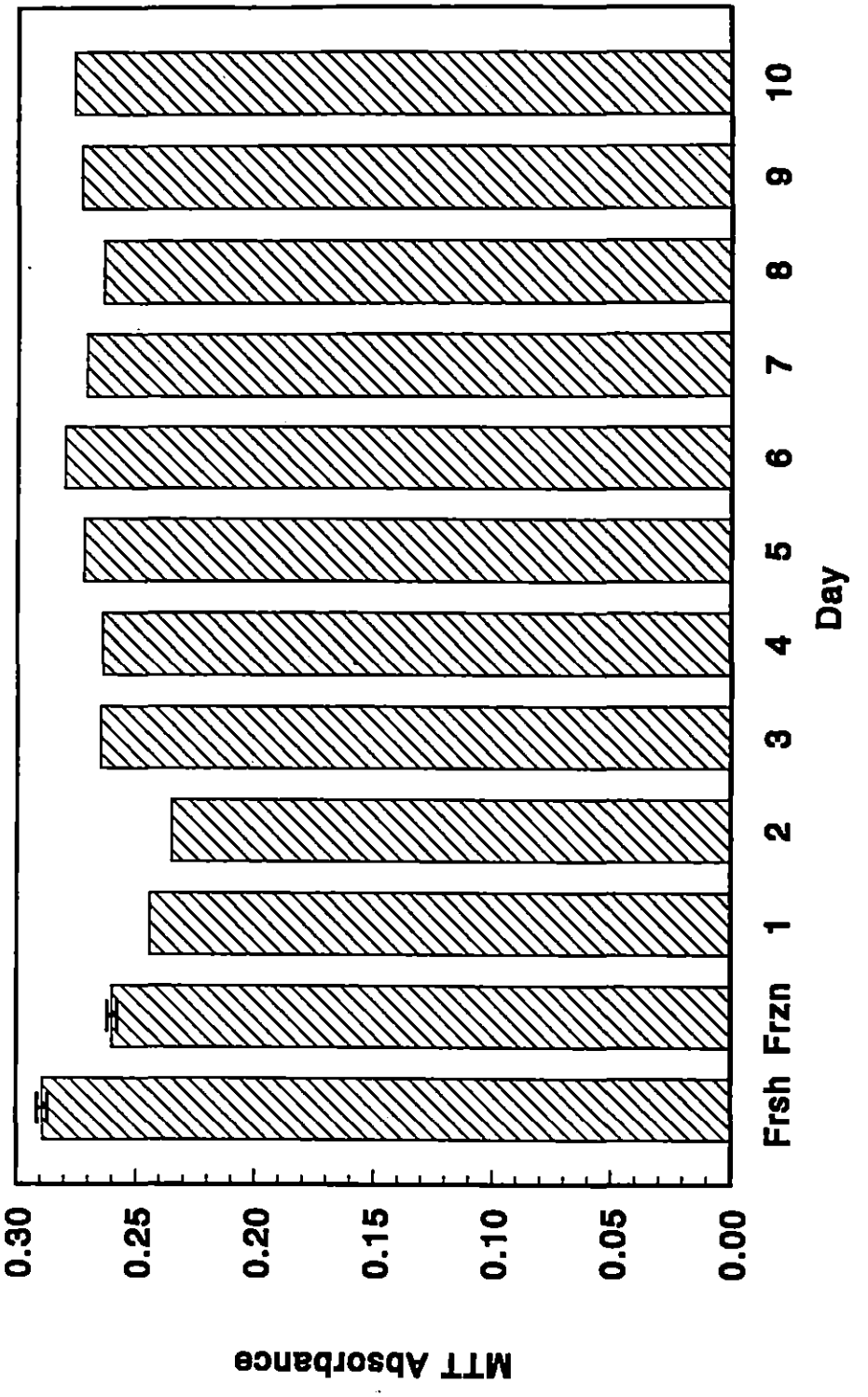

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 30. *Toxicity to HGF exposed to Ketac-fil eluates for 48 hours*

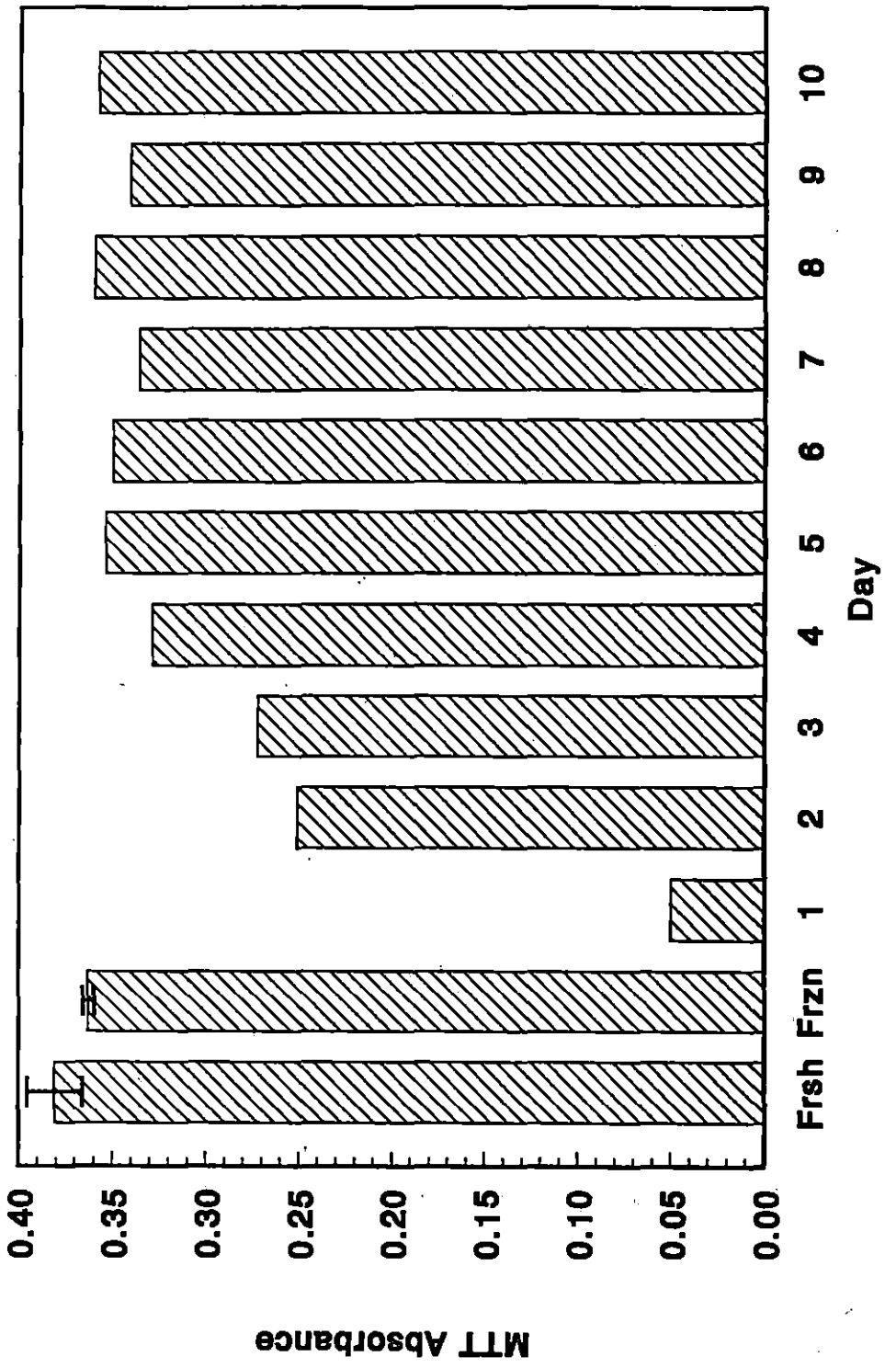

MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh
Frzn
1
2
3
4
5
6
7
8
9
10
Day

Fig. 31. *Toxicity to HGF exposed to Ketac-silver eluates for 4 hours*

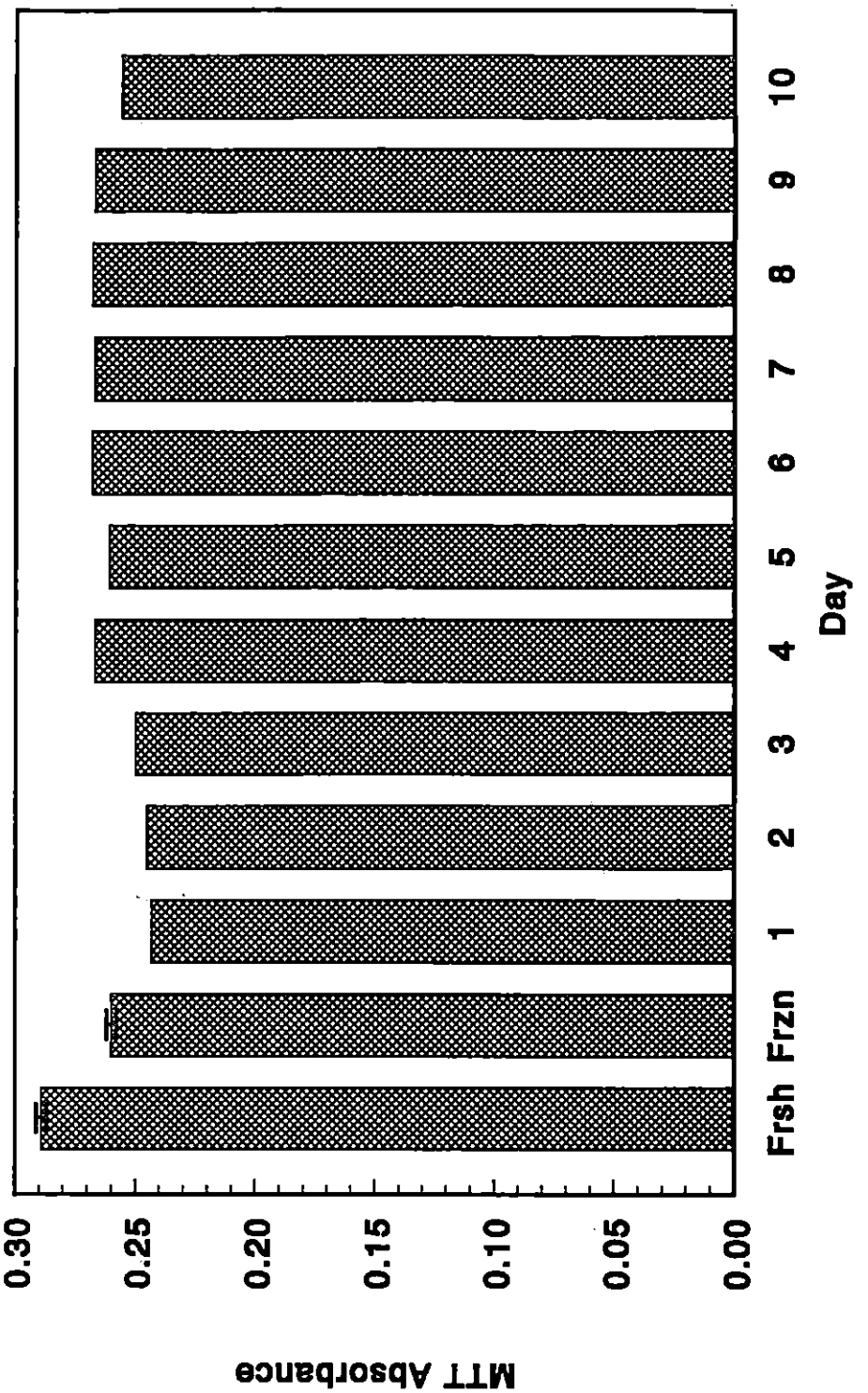
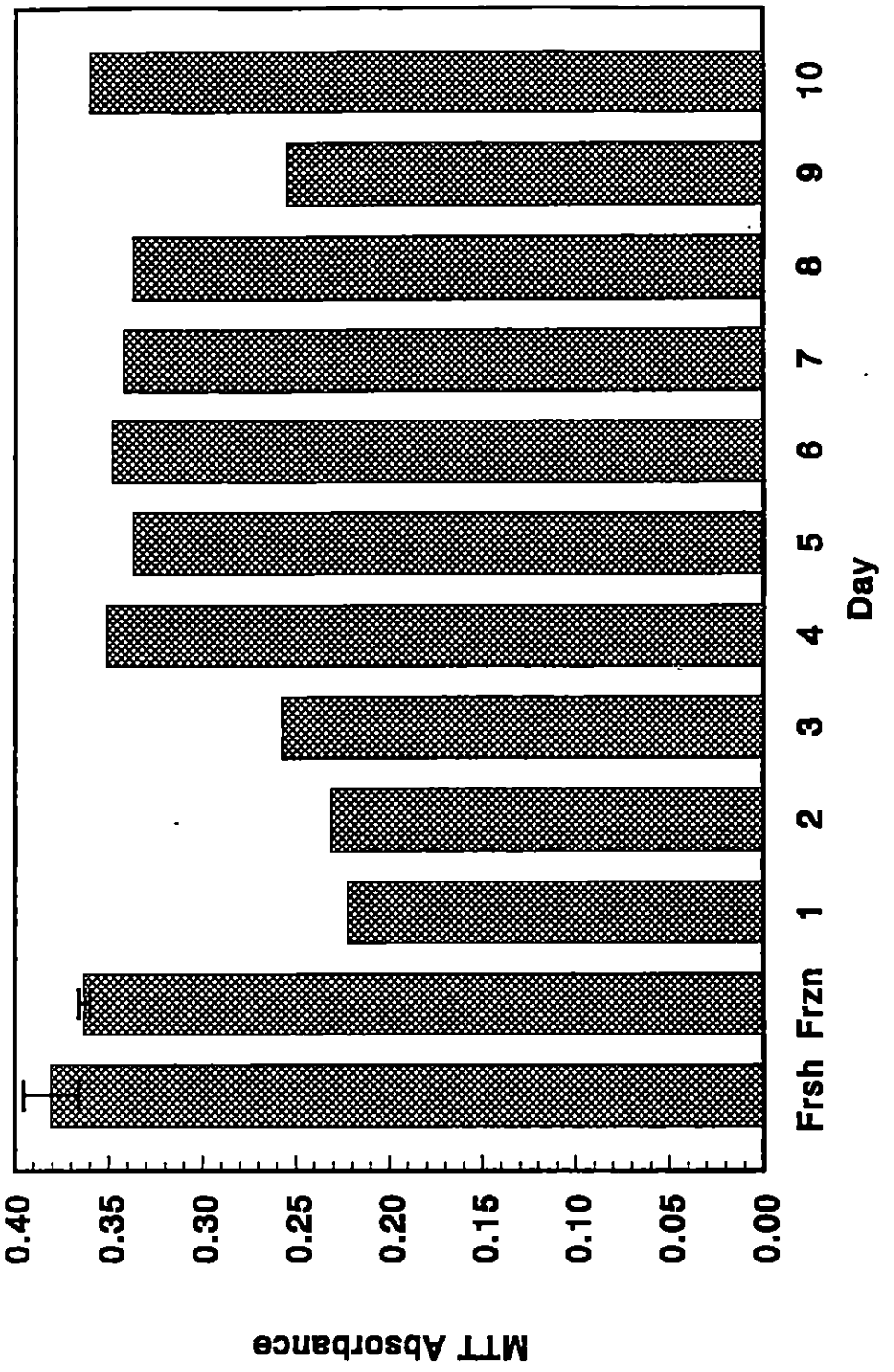

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

Fig. 32. *Toxicity to HGF exposed to Ketac-silver eluates for 48 hours*


MTT Absorbance
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frsh Frzn 1 2 3 4 5 6 7 8 9 10
Day

DISCUSSION

A variety of restorative materials have been used or proposed to serve as root-end filling materials. Several of these have been examined for cytotoxicity in the current study. Multiple parameters were used, since any single measure may not be adequate. Morphologic evaluation of cell responses provides a superficial assessment which can be suggestive of gross toxicity, while metabolic measures that determine the response of several pathways can permit detection of sublethal responses. All were used here.

Amalgam produced a modest zone of inhibition of cell growth by morphologic criteria, suggesting that some cytotoxic component(s) elute from the material. Evaluation of incorporation of ^{3}HUR into RNA in cells exposed to amalgam show it to be equivalent to or greater than that of control cultures. When combined with the obvious toxicity evident morphologically, the results suggest that amalgam can enhance synthesis of RNA. This may be a compensatory response, possibly occurring prior to cell death. If for example, mercury were the cytotoxic component, it could bind to sulfhydryl groups of proteins making them non-functional. The cell would increase protein synthesis as a compensatory mechanism. This suggestion is further supported by the studies using material eluates. Both *in vitro* and in *vivo*

experiments have established that there is a passive dissolution and release of substances from all metals. Thus, even in their passive condition, metals are not inert. Mercury ion release tends to be greatest in the first 24 hours after trituration. It is this release of mercury which is most likely responsible for any growth inhibition seen initially, as well as the enhanced RNA labeling as described above.

Along with the mercury, Contour amalgam has a high copper content (28% of the alloy). High-copper amalgams have an increased release of tin and copper over time. However, toxicity to fibroblasts exposed to eluate for 48 hours suggests that the release of elements occurs for 72 hours following trituration. After 72 hours, there was no difference in cell dehydrogenase activity in cultures exposed to amalgam as compared to the activity of the control. Thus, other elements in addition to mercury may contribute to the responses. The results suggesting that amalgam is initially toxic may not be clinically relevant to the repair process since there is not a significant amount of fibroblast proliferation during the first days of the repair process *in vivo*.

During endodontic therapy, gutta percha is left in contact with periradicular tissue only when a root resection is performed. Many previous studies have shown that gutta percha is biocompatible. Gutta percha was considered in this study because not only is it in tissue contact following resection, but it can also be injected into the root canal from the root end in some clinical situations. Our result lends further credence to the opinion that gutta percha is biocompatible with radicular connective tissues. There was very little growth inhibition evident in the dishes with

gutta percha. Morphologic scores were very close to those of the control, while the metabolic impairment studies also showed that incorporation of ^{3}HUR was not significantly different from the control. There is a high concentration of zinc oxide in gutta percha which could be seen on the surface of the experimental disks during preparation. It is this zinc oxide component which, when in contact with the medium, dissolves and most likely causes the small zone of inhibition.

Other materials recommended as root-end filling materials, such as the zinc oxide-eugenol materials, IRM and EBA, have received favorable comments in recent years. The results of the morphologic and ^{3}HUR incorporation studies demonstrated that the zinc-oxide eugenol materials are the most cytotoxic to human gingival fibroblasts. Growth in dishes with either IRM or EBA showed no normal fibroblasts. RNA synthesis was greatly reduced when compared to the control cultures. As seen in Tables V and VI, the incorporation of isotope during fibroblast RNA synthesis was very low; it was significantly less as compared to the wax control. The counts for Super EBA are only slightly better than those for the IRM, likely a reflection of the few fibroblasts that appear to have some normal morphologic characteristics. The results with these two materials are not surprising when one considers the individual toxicity of eugenol and zinc-oxide. Eugenol that diffuses into the medium, combined with some effects of zinc oxide, can produce a number of toxic responses including cell death/loss or decreased cell attachment, as seen in both the morphologic study and MTT assays.

The particle size of set zinc oxide-eugenol products affects the cement's

strength, and in general, the smaller the particle size, the stronger the cement. Substitution of a portion of the eugenol with ortho-ethoxybenzoic acid (EBA) results in an appreciable increase in strength. It does not, however, decrease the toxicity of the leachable components, most likely the eugenol. Typically the liquid of the EBA contains 62.5% ortho-EBA by weight and 37.5% eugenol by weight. It seems that this amount of eugenol is still sufficient to produce a toxic effect. That which is released from zinc oxide-eugenol cements is toxic to cells in cultures so that they exhibit no mitochondrial enzyme activity[37]. After 10 days, there does not appear to be any difference in the toxic response of fibroblasts exposed to eluates from IRM and Super EBA samples for 48 hours, but the cells do not appear to totally recover from the effects of leachable components, suggesting either a prolonged release of cytotoxic levels of material or a cumulative toxic effect, most likely the former.

The results are very consistent in terms of the overall initial toxicity of IRM and EBA. At the same time, it would be interesting to determine what effect the initial toxic response has on healing time around these root-end fillings, since clinically, these materials have been reported to be highly successful.

The suggestion by Jonck that glass ionomer cements are bioactive and may enhance bone growth has prompted their use as an alternative to amalgam as a root-end filling material[37]. The present study showed that glass ionomers produced no morphologic signs of cytotoxicity when in cell culture. Growth was not inhibited for either of the glass ionomer cements. When incorporation of radioisotope was quantitated, Ketac-fil produced an increase in ^{3}H-uridine incorporation similar to

that seen in the amalgam cultures; however, no zone or areas of growth inhibition were seen. As the cell passage increased during the individual studies, the ^{3}HUR incorporation decreased as the cell passage increased; however, the amount of radioisotope incorporation of the Ketac-fil remained consistent in relation to the control.

In addition, exposure to Ketac-fil and Ketac-silver produced radioisotope incorporation similar to that of the control in the longitudinal studies. Thus, the results of this study also suggest that the glass ionomer cements do not leach significant amounts of toxic components. However, fluoride is known to leach from this material, and fluoride, especially aluminum fluoride, is well known as an activator of G-protein associated cell signalling responses. This may stimulate the cells, resulting in higher RNA labeling.

Glass ionomer cements are supplied as a powder and liquid or as a powder that is mixed with water. Encapsulated Ketac-fil and Ketac-silver have a variety of substances that may leach into the medium, although these may be non-toxic or even stimulatory. There may not be much free material to leach out since these cements harden rapidly. As the initial hardening reaction proceeds, free metallic ions such as Al^{3+}, Ca^{2+}, and Na^{+} disappear due to crosslinking with polyanionic chains of acrylic acid to form the set cement.

Results of the Ketac-silver experiments varied greatly and this may be related to differences in cell passage or to experimental error. Experiment #1 had a large decrease in ^{3}HUR incorporation when there were no morphologic signs of cell

toxicity. Later experiments were more closely related to the results expected from morphologic evaluation and that seen with Ketac-fil, so the first experiment may be aberrant.

Fibroblast attachment did not appear to be affected by the eluates from the glass ionomers. Results indicated that there was a slight decrease in the first two days but this could have been due to residual polyacrylic acid at lower levels.

A comparison between the results of the MTT assays after 48 hours of exposure to eluates from Ketac-silver and Ketac-fil showed that there was a greater initial toxicity from components leaching from Ketac-fil than from Ketac-silver. There may be differences in overall solubility of the two materials.

It should be emphasized that the present results may not be interpreted as suggesting that either of the glass ionomer cements used here are ideal. Other properties, such as glass ionomer's susceptibility to moisture contamination and the need for dentin preparation are much more critical in successful placement of glass ionomers in root-end preparations.

It was noted at several points that the overall responsiveness of the cells decreased with subsequent passages, such that, at later passages, significant difference between treated and control cultures disappeared. This was first evident where multiple studies of a single material were conducted sequentially on a set of cells before beginning a new material. Those sequential studies were conducted on batches of cells held at about the same passage in a frozen state. When we examined the effects of cell passage by examining all materials' effects on a given passage, the longitudinal

studies, it was evident that, for this particular pool at least, there was a rapid decrease in labeling after passage 8. These results suggest that for HGF, a primary cell type, studies should not be conducted using cells beyond passage 8-9. Previously passage 10 had been a more-or-less arbitrary cut-off.

Clinical aspects:

Amalgam had an initial toxic effect but this may not be significant in clinical use. Indeed, the suggestion that amalgam has any significant contraindication for use as an apex sealant after decades of clinical success does not seem to be warranted. Similarly, fibroblast response to gutta percha was minimal. It appeared that when there are no obvious signs of a leaking root canal obturation, a resection to an existing gutta percha seal will leave a nontoxic environment. Within the parameters of this study, IRM and Super EBA appear to be the most cytotoxic. All the samples of IRM and EBA were significantly more inhibitory to cells than the control. It can not be ascertained from this study alone whether there are any long term detrimental effects on healing in the presence of zinc oxide-eugenol materials. Many have demonstrated clinical success when compared to amalgam, particularly with EBA. Glass ionomer cements appear to be biocompatible, however placement may present some clinical difficulties. They have shown low resistance to leakage, are extremely sensitive to moisture, and are very difficult to manipulate. In addition, the need to etch the apical cavity preparation and to coat the filling with varnish may not be practical in the periradicular region. Ketac-fil and Ketac-silver are both injectable materials and show some promise in overcoming the placement difficulties. Finally,

one must always be cautious about that the use of experimental results that provide only relative biocompatibility data. These cannot be used alone to predict clinical success. No one material will meet all the criteria for the ideal root-end filling and each material provides some distinct advantage over the others.

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