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The Effects of Tissue Culture Medium of Different Concentrations in Sutural Growth

Angel H. Vargas
Loyola University Chicago

THE EFFECTS OF TISSUE CULTURE MEDIUM OF DIFFERENT CONCENTRATIONS IN SUTURAL GROWTH

BY

ANGEL H. VARGAS D.D.S.

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For their assintance whenever I needed during my Orthodontic training and for have given me the encouragement to be alive .

LIFE OF AUTHOR

Angel H. Vargas Armienta was born in Culiacán Sinaloa México in January 31 of 1952 .

His secondary education was received in Escuela Preparatoria de Mazatlán from 1963 to 1966, the college education was obtained in Escuela Preparatoria No. 2 Universidad de Guadalajara from 1966 to 1968 .

In Sept. 1969 he began dental studies at Facultad de Odontologia Universidad de Guadalajara , in 1974 he was teaching in Centro Vocacional de Actividades Medico Biológicas Universidad de Guadalajara, he graduated in 1974 and after 1 year of social service he received his D.D.S.degree in 1975 .

In July 1976 he began a two year graduate program at Loyola University School of Dentistry Chicago Ill. leading to a master of science degree in Oral Biology and a post-graduate courses in Orthodontics .

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INTRODUCTION

Initially, tissue culture methods were designed to study cell anatomy. Later they were used to determine the essential nutrients for growth and development of the cells .

The application of this area of scientific effort became valuable with the observations of the effect of drugs and materials on the living cells in vitro . This was followed by the wide use of natural and synthetic media promoting and supporting the stimulation of cell proliferation in vitro but rarely used for research in vivo, altough there are some studies which determine that the tissue culture medium has stimulating effects on growth effect on DNA synthesis in vivo .

The purpose of this study is to determine the "in vivo" effects of the local infiltration of different concentrations of artificial tissue culture medium on the growth of the periosteal cell cell population of the interparietal suture demonstrated by autoradiographic and cell density analysis .

REVIEW OF LITERATURE

Composition of the suture and its importance"

The connective tissue of the sutures, the growth of the cartilages at the base of the skull and the replacement of these growing tissues by bone are the main factors in the growth of the human skull. In a suture the bones are joined to each other by a thin layer of dense connective tissue which is continuous with the periosteum on the periosteal and endosteal surfaces of the bone.

Sicher and Dubrul[1] in 1965 state that there are three zones of differentiation of the sutural connective tissue. The two zones adjacent to the bone consist of bundles of fibers arranged parallel to the bone and continuing into the bone as Sharpey's fibers, and an intermediate zone which consists of an irregular feltwork of fibers which is rich in cells and in which the straight bundles of fibers from either side seem to end.

Graber[2] in 1961 states that the cranium grows because the brain grows. The increase in size of the cranium under the influence of an expanding brain is accomplished primarily by proliferation of sutural connective tissue and by appositional growth and ossification of the individual bones that make up the cranial vault.

Enlow[3] in 1968 states that the connective tissues within the sutures contain combinations of collagenous and pre-

collagenous fibers as found in periodontal ligament which enter the bone in this form to serve as attachment fibers (Sharpery's Fibers). Binding together sutural bone maintains cranial morphology during growth.

Progressive growth in many of the bony elements in the face and calvaria involves new additions of bone at sutural margins. These surfaces represent one of the "growth sites" in the various craniofacial bones.

Enlow[3] in 1968 states further that sutural bone growth is considered to be a secondary response, however, to other expansive growth forces responsible for the actual displacement of the bones involved. Thus sutural bone additions are passive and do not in themselves produce the movement of the bony elements in directions away from each other. Instead, growth expansion within brain muscle nerve blood vessels like associated with the bones such as the brain is believed to represent the primary source of the expansive force.

INVESTIGATIONS IN THE RATS

Massler and Schour[4] in 1951 studied the growth patterns of the cranial vault in albino rats. They were trying to determine the sites of growth, the bony growth at these sites, the chronology, and the rates of growth at the different sites.

They concluded that the growth of the cranial vault

occurs primarily by rapid deposition of bone at the approximating margins of the bones which comprise the various cranial sutures. The mode of growth of the cranium (by trabeculation) and the rapid rate are the response in the sutures to the rapidly expanding encased brain. The sutures are so arranged in relation to one another as to permit them to contribute to increase the skull width, skull length, and cranial height. Also deposition of bone occurs upon the endosteal and periosteal surfaces to increase the thickness of the vault bone.

The rat as an experimental animal continues to hold its preeminent position for use in laboratory investigation. Its characteristics make it suitable for research intended for application to human biology. The accepted view that the similarities between mammals having the same feeding habits tend to be close, and that in some instances at least, by the use of equivalent ages, the results obtained with one form can be very precisely extrapolated to the other. If the life span of three years in the rat is taken as equivalent to 90 years in man, it is found that the growth changes in the nervous system occur within the same fraction of life span. This is also probably true for the other systems, and makes possible the inference of the data obtained from the two species, with a high degree of precision.

Another study of the growth in the calvaria in the rat

was made by Moss[6] in 1954. He states that when a calvarial bone of an infantile rat was removed, together with the periosteal layer of the underlying dura, regeneration of this bone does not occur. In his studies of the effect of this removal on the morphology of the adjacent growing bones, he concludes that the normal calvarial morphology depends on the osteogenic activity of all the periosteal tissues and that the shape of the calvarial bones is not predetermined by the location of the sutures. It is possible to produce overgrowth of calvarial bones by many methods with displacement of sutural position. This phenomenon appears to depend on the period of development of the osseous tissue. Calvarial sutures mark the plane of articulation between adjacent bones. However the neurocranial sutures are not the chief sites of growth, as extirpation of the sutures singly or severally is not followed by any decrease in either width or length of the calvaria.

He states that the periosteal osteogenic tissues, ectocranial as well as endocranial appear to be the primary growth sites.

Another study of the growth of the longitudinal growth changes in the craniofacial complex in the rat was made by Cleall, Jacobson and Berker[11] in 1971. They state that the growth of the cranium and nasomaxillary region enlarges in both vertical and lateral dimensions by appositional growth

on both the endosteal and periosteal surfaces.

Compensation for increase in intracranial volume is made at the sutures and by differential growth at the centers and edges of the bone to reduce the curvature.

NUTRITIONAL STATUS OF THE GROWING RAT.

Examination of rat's blood for 100 ml. shows" Uric acid 2 mg., non protein nitrogen 38 mg., and urea 2 mg., the proportional amount of serum proteins in 60 days old albino rats is 5.3% total proteins with 3.0% albumins, 2.3% globulins. The percentage of water in the entire blood of 60 days old rat is approximately 80% 5.

The growing rat requires essential nutrients to maintain normal growth of the skull. Table 1 illustrates the dietary requirements of the rat.

TABLE I

Dietary requirements of the rats, reported by the Wistar Institute of Anatomy and Biology, Philadelphia 1924 .

				Daily intake
Calcium	0.5-0.6 %			40-50 mg.
Phosphorus	0.5-0.6 %			35-45 mg.
Potassium				15 mg. Male 8 mg. Female
Sodium	0.5 %			
Chlorine				5 mg.
Copper				0.1 mg.
Iodine				2 micrograms
Magnesium				4 mg./Kg.
Manganese				0.5 mg.
Zinc				40 micrograms
Protein	25-30 % Aminoacids	Lysine	1 %	
		Tryptophane	0.2 %	
		Histidine	0.4 %	
		Phenylalanine	0.7 %	
		Leucine	0.9 %	
		Isoleucine	0.5 %	
		Threonine	0.6 %	
		Methionine	0.6 %	
		Valine	0.7 %	
		Arginine	0.2 %	
Fatty Acids (Methyl linolate)				25 mg.

	Daily intake
Vit. A	4 micrograms
Carotene	15-20 micrograms p/Kg.
Thiamine	10 micrograms
Riboflamin	10 micrograms
Pyridoxine	40 micrograms
Vit. E(Alpha tocopherol)	1 mg.

TISSUE CULTURE NUTRITION

Animal tissue culture as an area of scientific endeavor is considered to have begun with the pioneer experiments of Harrison[9] (1906).

The early tissue culture methods were designed primarily for studies on cell morphology and development and were based on the observation that many tissues could be propagated almost indefinitely in media derived from the animal body, particularly plasma, serum, ascitic fluid and tissue extracts. It has long been recognized that the term "tissue culture" is itself a misnomer. Although tissue derived from many animal species provide the basic material for cultivation studies, it is constituent cells of the tissue that are propagated.

Glasstone[10] in 1935 reports of a study to determine if dental tissues would differentiate normally when isolated and cultivated in vitro. He shows in this experiment that isolated dental tissues are self diferentiating. The results were obtained from the cultivation of tooth germs in vitro which by eliminating all the vascular and nervous connections produced a more complete isolation than grafting. The experiment also shows that odontoblasts differentiate in vitro only when ameloblasts are present.

Whole or partial tooth germs cultivated in vitro show remarkable power of physiological differentiation. The den-

tin papilla developed normally forming odontoblasts which deposited normal tubular dentin. Dentin formation could take place in the abscence of the enamel epithelium provided that odontoblasts were present in the explant. He states that one of the functions of the enamel organ is to determine the gross morphological structure of the tooth.

Vogeelar and Erlichman[7] in 1936 report on the significance of aminoacids for the growth in vitro of human fibroblasts in which they give a general survey of the literature. The growth of human fibroblasts from thyroid gland taken from thirteen persons, varying in age from fourteen to forty eight years were used, fibroblasts show a good growth over a period of at least two months in a medium composed of equal parts of Plasma, calcium, ringer, feeding solution and 2.10% glycine solution. The finding was that there is a total abscence of any cell emigration or growth in such medium of equal parts of plasma, calcium, ringer, feeding solution and 2.10% glycine solution.

In vitro, Morgan[8], in 1958, states that many of the more recent tissue culture techniques have been designed specifically for cell nutrition studies. The method which yields a succesful single cell culture is based on the concept that present tissue culture media are inadequate to support the growth of small inoculi, unless the media has previously been altered by the metabolic activity of large

rapidly growing cultures.

IN VIVO SUPPORT OF GROWTH

In vivo, Stanley Dudrick[12] in 1968 tried to determine if tissue maintenance and synthesis and growth could be achieved exclusively by intravenous infussion of basic nutrients for prolonged periods of time. His findings were that with parenteral nutrients the problems of toxicity and availability are accentuated and the additional requirements of sterility and non pyrogenicity are imposed. Furthermore the suthor intravenous fat emulsion for clinical use in this Country, complete parenteral diets in isotonic forms are generally not possible. However with hypertonic solutions all essential nutrients can be provided.

Infusion into the superior vena cava allows rapid dilution and pripheral distrobution of the nutrients at isotonic concentration. Finally development of a sterile technique for catheter placement together with aseptic safeguards in manufacture and administrations of solution, insures sterility during long terms of intravenous infusions. This is the first demonstration that growth, development and positive nitrogen balance can be achieved by long term total parenteral nutrition in animals and man.

IN VIVO GROWTH STIMULATION WITH TISSUE CULTURE MEDIUM

Toto, Black and Sawinski[13] in 1968, report in their publication that protein probably associated with acid muco-

polysacharide is believed to be the active ingredient in extracts of powdered bovine cartilages which when parenterally administrated caused marked acceleration of wound healing in men, mice, dogs and guinea pigs. Also plasma in Tyrode's solution impregnated into polyvinyl sponges when implantated intramuscularily stimulated the production of fibroblasts and increased the rate of formation of collagen fibrils. They also report that the tissue culture media are derived largely from tissue extracts which serve as the substrate for the growth of cells in vitro.

It was only natural to ask whether or not artificially produced media could stimulate growth of cells in vivo. They concluded that the tissue culture medium (T199) stimulates the proliferation of loose connective tissue in the skin of the mouse. The cells of the loose subcutaneous tissues are competent in as much as they can synthesize DNA and differentiate along several lines. Such cells when placed in tissue culture medium are capable of proliferation in vitro, it is not unexpected that such cells would be stimulated to grow when tissue culture was added in vivo.

AUTORADIOGRAPHY

Autoradiography is the term used for the method of employing photographic emulsions to study the occurrence and distribution of radioactive substance in tissues.

Fischer and De Gruyter[14] in 1971 found that the tritia-

ted thymidine has proved to be a very good test substance in experiments for establishing reliability of a method and yields genuine "autoradiographic silver grains". Thymidine is a pyrimidine derivative linked to deoxyribose; as a nucleoside it is a starting material in DNA synthesis and, in connection with mitosis, is predominantly incorporated into cell nuclei. The labeled thymidine is incorporated only in electron tight parts of the nuclear chromatin. The argument about the extent of incorporation of thymidine into cytoplasmic polynucleotides not yet been resolved. Thymidine has thus proved its value not only in cytological studies in light microscopic autoradiography but also in connection with electron-microscopic technique.

AUTORADIOGRAPHY FOR BIOLOGISTS

Graham[15] in 1972, states that a number of procedures fall under the quantitative autoradiography, he suggests that in the counting of the proportion of labeled cells, grain counting over a large area and grain counting over a limited area, such as cell or a nucleus, when working with the light microscope, it is useful to limit the area under observation by using an aperture in the eye piece. For grain counting over relatively large areas, for example, cytoplasm in brain, low grain densities are to be recomended; it is considerably less laborious to count a small number of grains over many areas than large number over a few.

METHOD OF SUBCUTANEOUS OR INTRAPERITONEAL INJECTION OF TRITIATED THYMIDINE

Addison[16] in 1942, he states that subcutaneous injection can be given most easily into the abdominal wall, with the rat held in the left hand, the needle is inserted with a quick thrusting motion just above Poupart's ligament, and the point push upward about an inch in the direction of the head. If it is desired that the injection be intraperitoneal, the needle is inserted, in the same location and direction but more deeply. The needle should not pass much posterior to the abdominal wall nor be inserted high enough to damage the liver. If neccesary as much as 10 to 20 c.c. of fluid may be given in a single injection.

SUTURAL GROWTH STUDIES USING TRITIATED THYMIDINE

Sinclair[18] in 1976, made an investigation to determine the short term histological change of the fascioskeletal complex resulting from rapid palatal expansion by orthodontic appliance in the Rhesus monkey. The histological changes in the sutural areas were determined by histological examination of slides and the labeling index per 1,000 cells and cell density per 11μuM^2. The histological evaluation of the sutural tissues in the experimental animal revealed that there was a decreased in cell density as the sutural connective tissue was abnormally wide and the fibers disorientated. There was an increase in intercellular fluid, vascularize-

tion, bone apposition and remodeling. The labeling index revealed that there was a large numerical increase in cellular proliferation in the nasal sutures of the experimental animal.

Reardon[17] in 1976, reports the reaction of the intermaxillary suture, the maxillopalatine suture and the periodontal ligament to the palate spliting procedures in monkeys. Tritiated thymidine was administrated intravenously on the tenth day of the experiment. The evaluation of the histological changes was mainly judged by means of cell density and labeling index differences. He found after ten days there was an increase in the cellular proliferation and decrease in cell density in the palatal sutures and periodontal ligament in the maxillary dentition of monkeys subjected to palate spliting therapy.

Garcia[19] in 1977, showed increased labeling with tritiated thymidine of the interparietal suture connective tissues in mice using 1% T199 (Tissue culture Medium) or, at least, may have stimulated growth by a mechanism not yet revealed.

The growth stimulating effect of 1% Tissue culture Medium (T199) on the interparietal suture as reported by Bayardo[20] (1977), has led to the question of what effects increased concentration of tissue culture would produce. The study which follows is based upon the model used by Bayardo

but with increased concentration of tissue culture medium in order to answer this question.

MATERIAL AND METHODS

Twenty albino rats * 30 days old, each averaging 50 grms. in weight, were divided into five groups each 4 animals. Three cubic centimeters of air was subcutaneously injected on the periosteal surface of the interparietal suture area of each rat to create a pouch. After 24 hours the control group of 4 rats, was injected with 3 c.c. of buffered physiologic saline solution ** into the little bubble created by the previously injected air, repeating each procedure every third day for two weeks (Table I). The second group which was one of the experimental groups, as the control group was injected 3 c.c. of an artificial isotonic tissue culture medium (T199) *** in a concentration of 3% into the little bubble created by the previously injected air, repeating each procedure every third day during two weeks (Table I). The third experimental group, was injected with 3 c.c. of an artificial isotonic tissue culture medium (T199) *** in a concentration of 5% into the little bubble created by the previously injected air, repeating each procedure every third day during two weeks (Table I). The fourth group was injected 3 c.c. of (T199) *** in a concentration of 10% following the same procedure of the other groups. The fifth group as the other groups was injected 3 c.c. of (T199) in a concentration of 20% following exactly the same procedure of the other four groups.

The preparation of the air pouches and the injections of saline solution or T199 were done with great care in order to minimize the injurious effect of such administrations.

* Abrams Animal Supply

** Fisher Scientific Company, Fair Lawn , New Jersey.

*** T199, Grand Island Biological Supply, Grand Island, N.Y.

At the end of two weeks the akin overlying the injection sites was inspected. Groups 4 and 5 showed erythema and ulceration of the skin. This obviously inflammatory reaction precluded further study using tritiated thymidine to label cells reacting to the T199. Therefore, only the control and groups 2 and 3 were continue in the study.

Approximately 8 hours after the last injection, the animals were weighed and 1μ ci of tritiated thymidine$^{\phi}$ (sp.act. 1.8 Ci/mM) per gram of body weight was administred intraperitoneally to each one of the rats in the control and 3 % and 5 % T199 groups. One hour post injection the animals were sacrificied using diethyl ether, and 1 x 1 cmblock of the interparietal suture area was excised. The tissues were fixed in cold neutral formalin and then prepared for microscopic examination by cutting paraffin embedded blocks into sections 6μ thick. Autoradiogram of the sections were prepared with liquid emulsion$^{\phi\phi}$ and placed in light-tight boxes . After 5 week exposure at 4 degrees C., the sections were developed, washed, fixed in the dark room and subsequently

with nuclear fast red and indigo carmine dyes .

The slides were placed under a microscope, and with the aid of an eyepiece reticular of 100μ^{2}@ , the nuclei counts were determined utilizing a blood cell counter@@ .

The nuclei of the cells, excluding endothelial cells, in the interparietal suture area of randomly selected preparations were counted in each the control and experimental groups , the labeled nuclei incidence observed during the counts were separately recorded .

The total numbers of nuclei per 100μ^{2}observed in all the cells in each of six sections from each specimen and reported as the cell density were summed, and the mean number of cells per 100μ^{2} was calculated. The frequency of tritiated thymidine labeled cells similarly was summed and reported as an index calculated as the number of nuclei per 100μ^{2} ,as well as the "T" test for significant differences between the means .

¢ New England Nuclear

¢¢ NTB_3, Eastman Kodak Company, Rochester N.Y.

@ American Optical Corporation, Bufalo N.Y.

@@ Clay Adams, Parsippany, N.J.

TABLE II

Schedule of frequency of administration of saline solution and "T 199" in 30 days old albino rats .

Date	Control	Exp.3 %	Exp.5 %	Exp.10 %	Exp.20 %
May-23-76	3 c.c.Air	3 c.c.Air	3 c.c.Air	3 c.c.Air	3 c.c.Air
May-24-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199	3 c.c.T199	3 c.c.T199
May-26-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199	3 c.c.T199	
May-28-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199		
May-30-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199		
Jun-01-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199		
Jun-03-76	3 c.c.S.S.	3 c.c.T199	3 c.c.T199		

TABLE III

COMPOSITION OF THE TISSUE CULTURE MEDIUM

COMPONENT	mg/L
Inorganic Salts	
$CaCl_2$ (Anhyd)	140.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.72
KCl	400.00
KH_2PO_4	60.00
$MgSO_4 \cdot 7H_2O$ (Anhyd)	97.72
NaCl	8000.00
$Na_2HPO_4 \cdot 7H_2O$(Anhyd)	47.70
OTHER COMPONENTS	
Adenin sulfate	10.00
Adenosinetriphosphate	1.00
Cholesterol	0.20
Adenylic acid	0.20
Alpha tocopherol phosphate	0.01
Deoxyribose	0.50
Glucose	1000.00
Glutahione	0.05
Guanine HCl(free base)	0.30
Hypoxanthine Na salt	0.35
Phenol red	20.00
Ribose	0.50
Sodium acetate	50.00
Thymine	0.30
Tween 80 (TM)	20.00
Uracil	0.30
Xanthine	0.34

COMPONENT	mg/L
Aminoacids	
DL-Alpha-Alanine	50.000
L-Arginine HCl	70.000
DL-Aspartic acid	60.000
L-Cysteine HCl-H_2O	0.110
L-Cysteine 2HCl	26.000
DL-Glutamic acid.H_2O	150.000
L-Glutamine	100.000
Glycine	50.000
L-Histidine(HCl.H_2O)	21.880
L-Hydroxyproline	10.000
DL-Isoleucine	40.000
DL-Leucine	120.000
L-Lysine HCl	70.000
DL-Methionine	30.000

COMPONENT	mg/L
DL-Phenylalanine	50.000
L-Proline	40.000
DL-Serine	50.000
DL-Thereonine	60.000
DL-Tryptophan	20.000
L-Tyrosine 2Na	57.880
DL-Valine	50.000
Vitamins	
Ascorbic acid	0.050
d-Biotin	0.010
Calciferol	0.100
Ca Pantothenate	0.010
Chlorine chloride	0.500
Folic acid	0.010
i-Inositol	0.050
Menadione	0.010
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.050
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.010
Thiamine HCl	0.010
Vitamin A (acetate)	0.140

FINDINGS

HISTOLOGIC:

Examination of the overlying skin and the periosteum, suture and endosteum of the parietal bone of the rat injected twice each week with saline or various concentrations of T199 revealed acute inflammation and proliferation of fibroblasts.

Skin: The skin showed edema, increased numbers of dilated capillaries, increased numbers of fibroblasts and varying diffuse infiltration of the polymorphoneuclear leukocytes.

Saline Control Skin: The subcutaneous connective tissues show foci of leukocytes and fibroblasts showing active fibrogenesis and patent dilated capillaries. Also seen were few particles of embedded hair.

T199 Skin: The subcutaneous connective tissues injected with 3% and 5% of T199 showed edema, separation of collagen fibers, increased numbers of fibroblasts and active fibrogenesis. There were focal and diffuse polymorphonuclear leukocytes infiltrating the connective tissue.

No differences in the quantity of the leukocytes could be made. However, they appeared to be slightly greater than the saline injected skin.

T199 10% and 20% produce severe necrosis of the skin

showing edema, atrophy and desquamation with a fibrinopurulent exudate. The subcutaneous tissues show granulation tissue composed of dilated capillaries and dense fibroblastic proliferation, fibrogenesis and polymorphonuclear leukocytic infiltration (Fig. 7). 10% and 20% of T199 is severely irritating to the skin as compared with either saline or 3% and 5% of T199.

BONE:

The periosteum shows edema, increased thickening of fibroblasts and slight infiltration with polymorphonuclear leukocytes both with saline and T199.

Saline: The periosteum is edematous and shows dilated capillaries, and moderate polymorphonuclear leukocytic infiltration (Fig. 1). There is evidence of new bone formation as indicated by a basophilic apposition line separating the old from newly apposed bone. The suture appears free from inflammatory exudate. It is composed of fibrovascular tissue showing osteoblasts lining the newly forming bone (Fig. 2).

T199 3% appears similar to the saline control. However, there is evident greater thickness of new bone as measured by the basophilic apposition line separating old from newly apposed bone. Moreover, the new bone is thicker both on the endosteal as well as the periosteal surfaces of the parietal bone. The suture area does not show evidence an inflammatory infiltrate, although both the endosteal and periosteal

connective tissues show edema and slight infiltration by polymorphonuclear leukocytes (Fig. 3).

T199 5% shows new bone formation on the periosteal, endosteal and sutural surfaces of the parietal bone as indicated by the basophilic apposition line separating old from newly formed bone. The bone appears in a mosaic pattern suggesting evidence of resorption followed by apposition forming an irregular pattern of bone formation (Fig. 4). The suture connective tissues appear free of any inflammatory exudate, while the periosteum and endosteum shows only slight polymorphonuclear leukocytic infiltration. However, both endosteum and periosteum show edema and increased fibroblastic proliferation.

T199 10%: The periosteum shows increased cellularity with fibroblasts; the suture space is widened and filled with fibroblasts and shows many patent capillaries. There is evidence of thick, new bone formation on the periosteal and endosteal surfaces after osteoclastic resorption as indicated by the reversal basophilic apposition lines. The suture connective tissue appears free from an inflammatory exudate while the periosteum shows slight polymorphonuclear leukocytic infiltration (Fig. 5).

T 199 20%: The periosteum is thickened, shows many dilated capillaries, increased fibroblasts and moderate infiltration with polymorphonuclear leukocytes. The bone shows se

vere resorption with loss of preexisting bone on the periosteal sutural and endosteal surfaces. Polymorphonuclear leukocytic infiltration is seen in the sutural and endosteal connective tissues as well as the periosteum. There is evidence of some new bone formation in the sutural connective tissues and upon the old bone on the periosteal surface (Fig. 6).

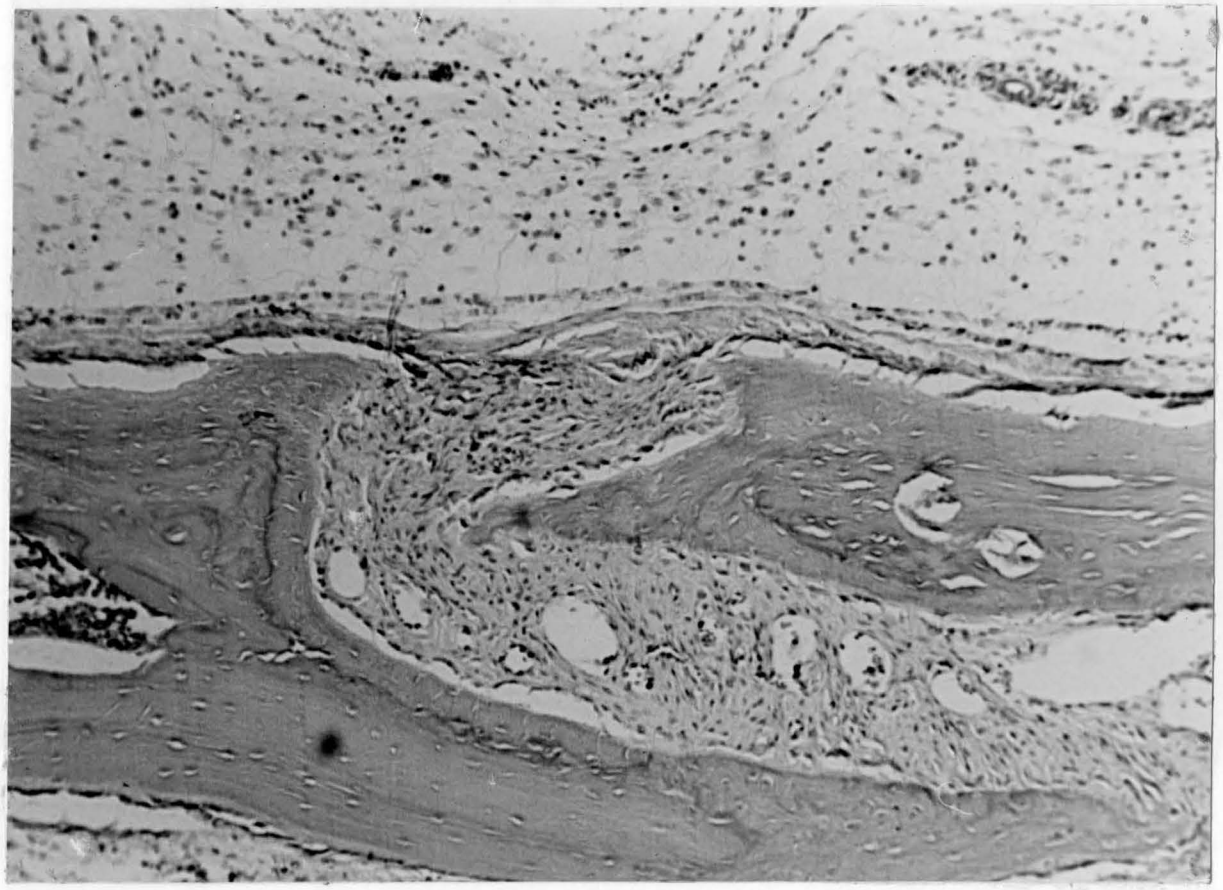

Figure 1 : Frontal view of the interparietal suture of albino rat 42 days old treated with saline solution .
Hematoxylin eosin . Low magnification.

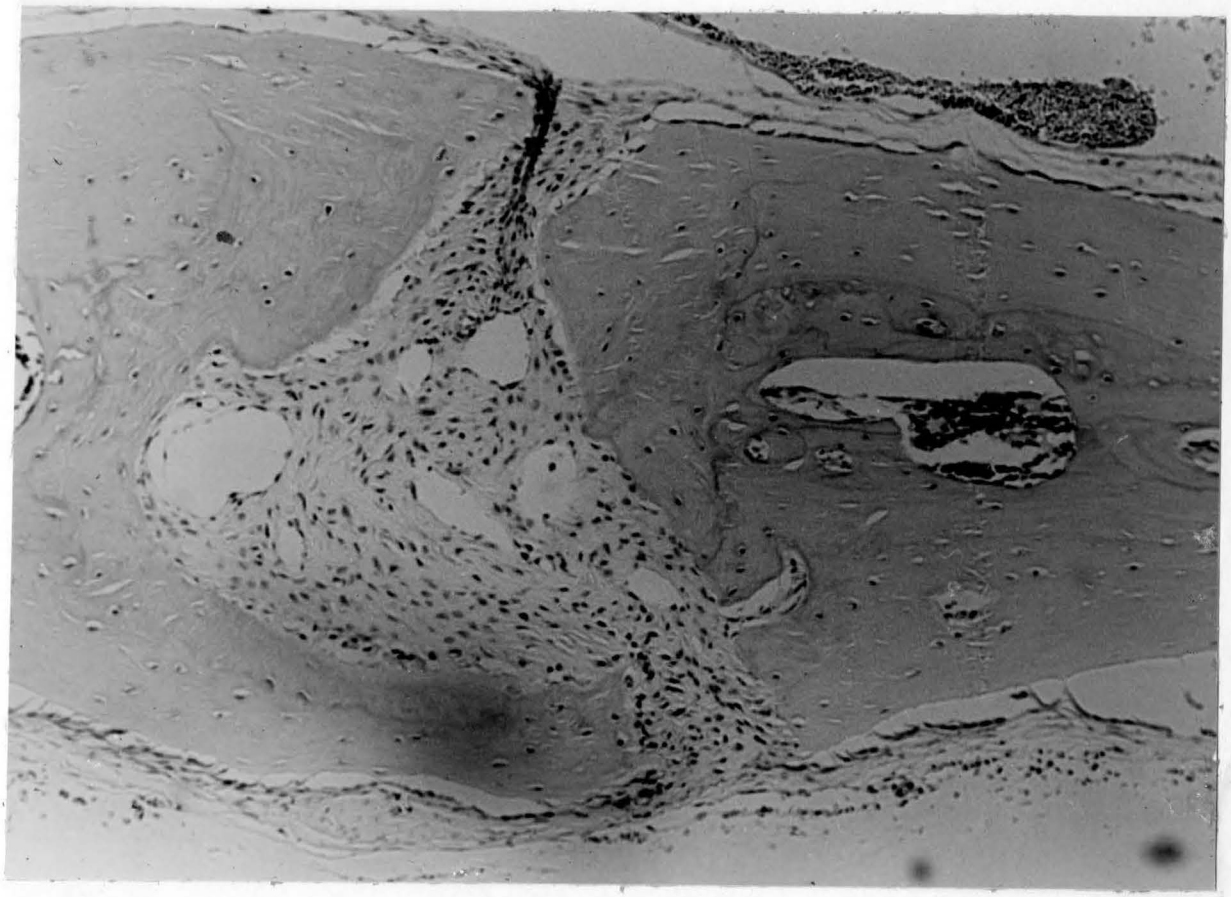

Figure 2 : Frontal view of the interparietal suture of albino rat 42 days old treated with saline solution .
Hematoxylin eosin . High magnification .

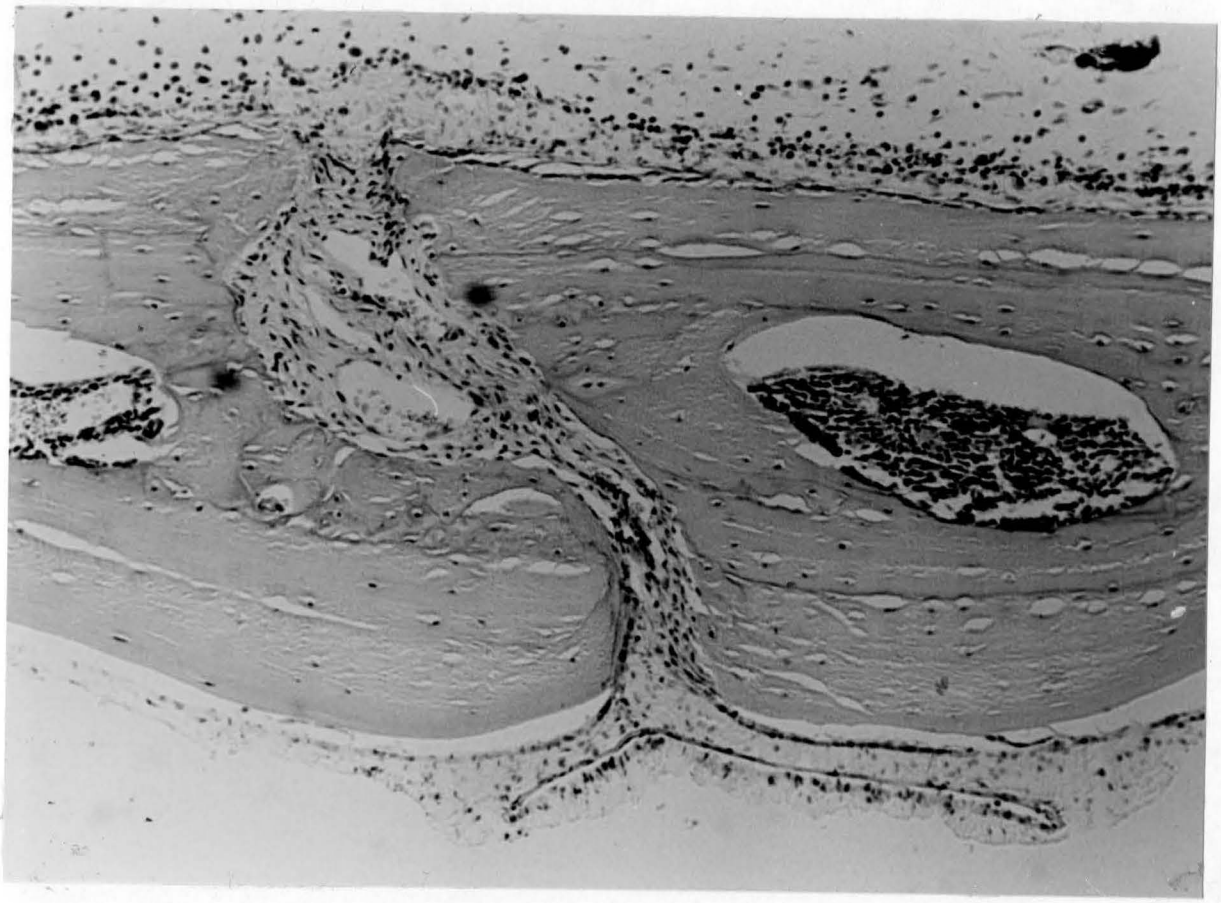

Figure 3 : Frontal view of the interparietal suture of 42 days old albino rat treated with 3 % T199.
Hematoxylin eosin .

Figure 4 : Frontal view of the interparietal suture of 42 days old albino rat treated with 5 % T199 .
Hematoxylin eosin .

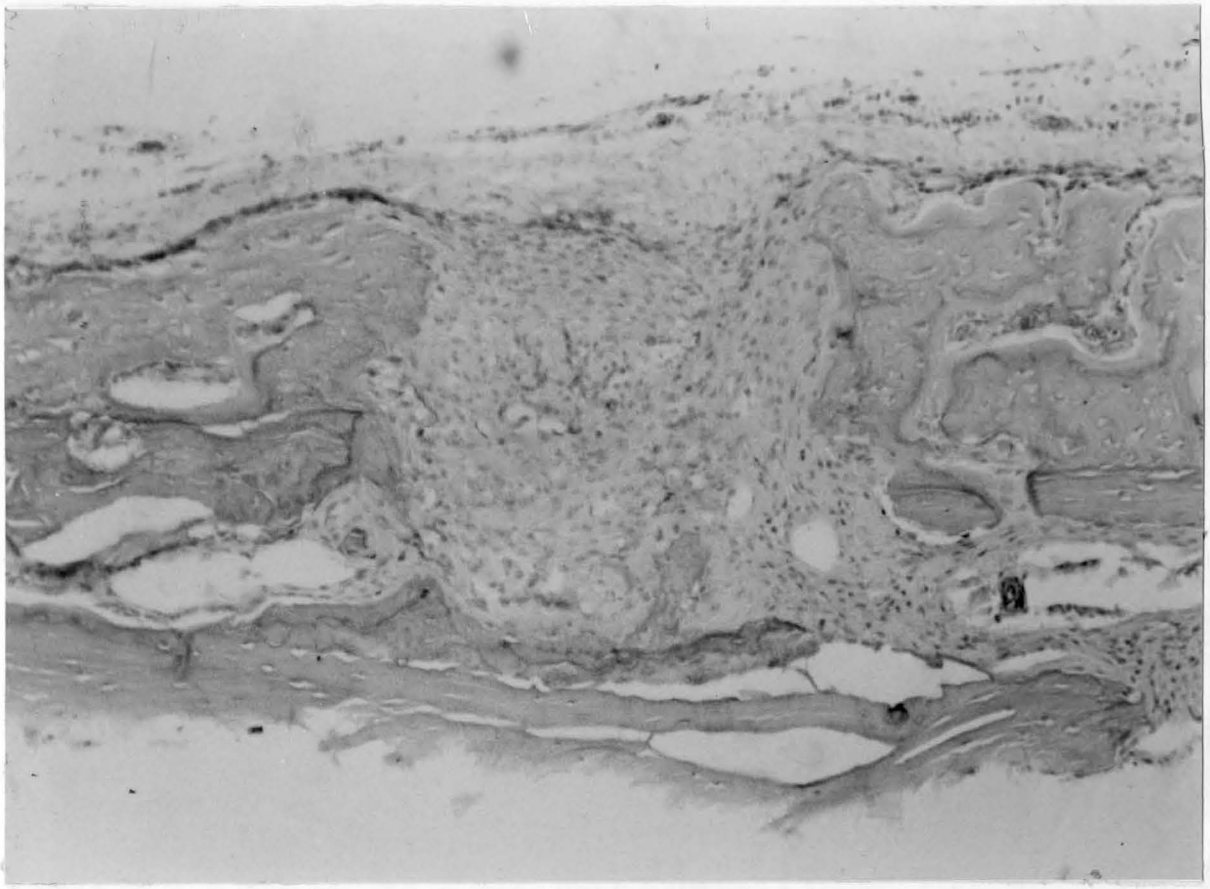

Figure 5 : Frontal view of the interparietal suture of 42 days old albino rats treated with 10 % T199 .
Hematoxylin eosin .

Figure 6 : Frontal view of the interparietal suture of 42 days old albino rat treated with 20 % T199 .
Hematoxylin eosin .

Figure 7 : Frontal view of the skin of the interparietal suture area of 42 days old albino rat treated with 20 % T199 .
Hematoxylin eosin .

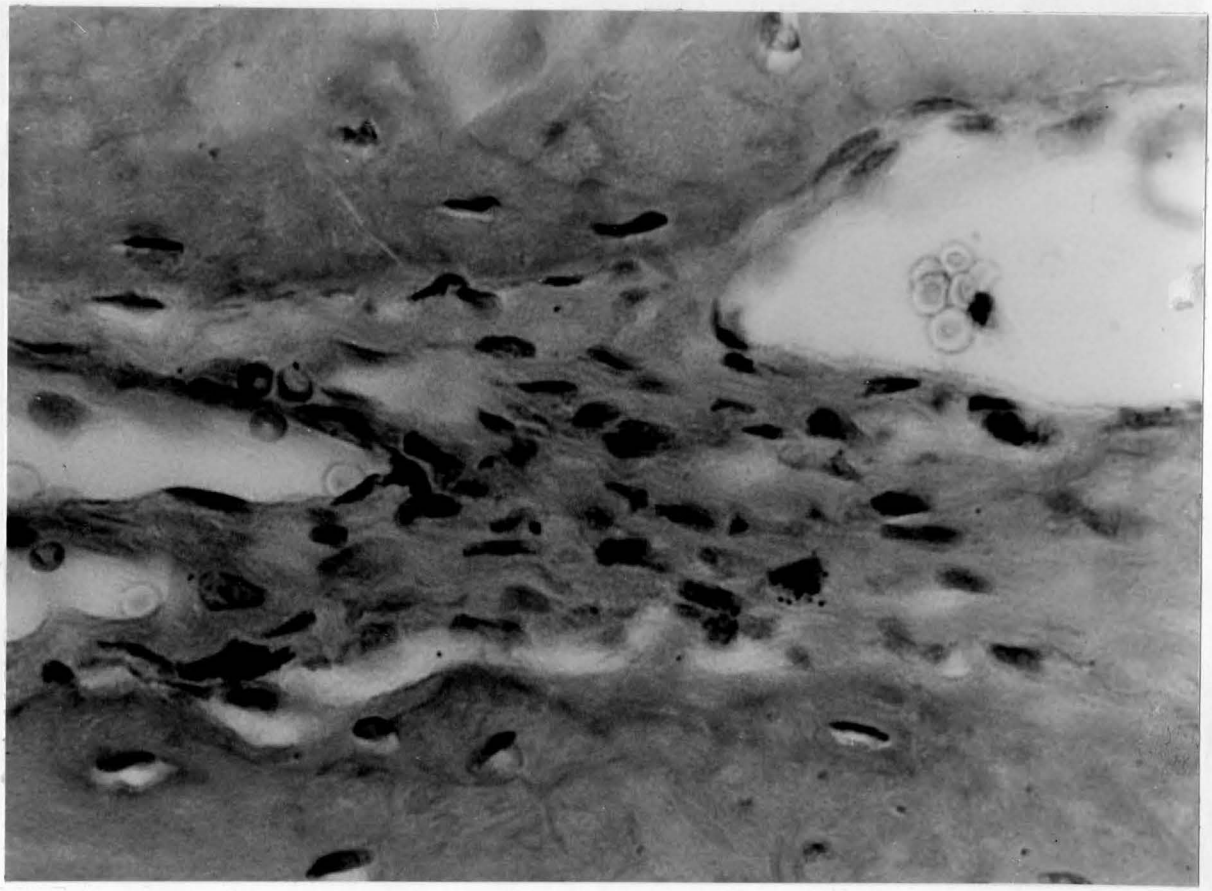

Figure 8 : Autoradiogram of the interparietal suture of 42 days old albino rat treated with saline solution .

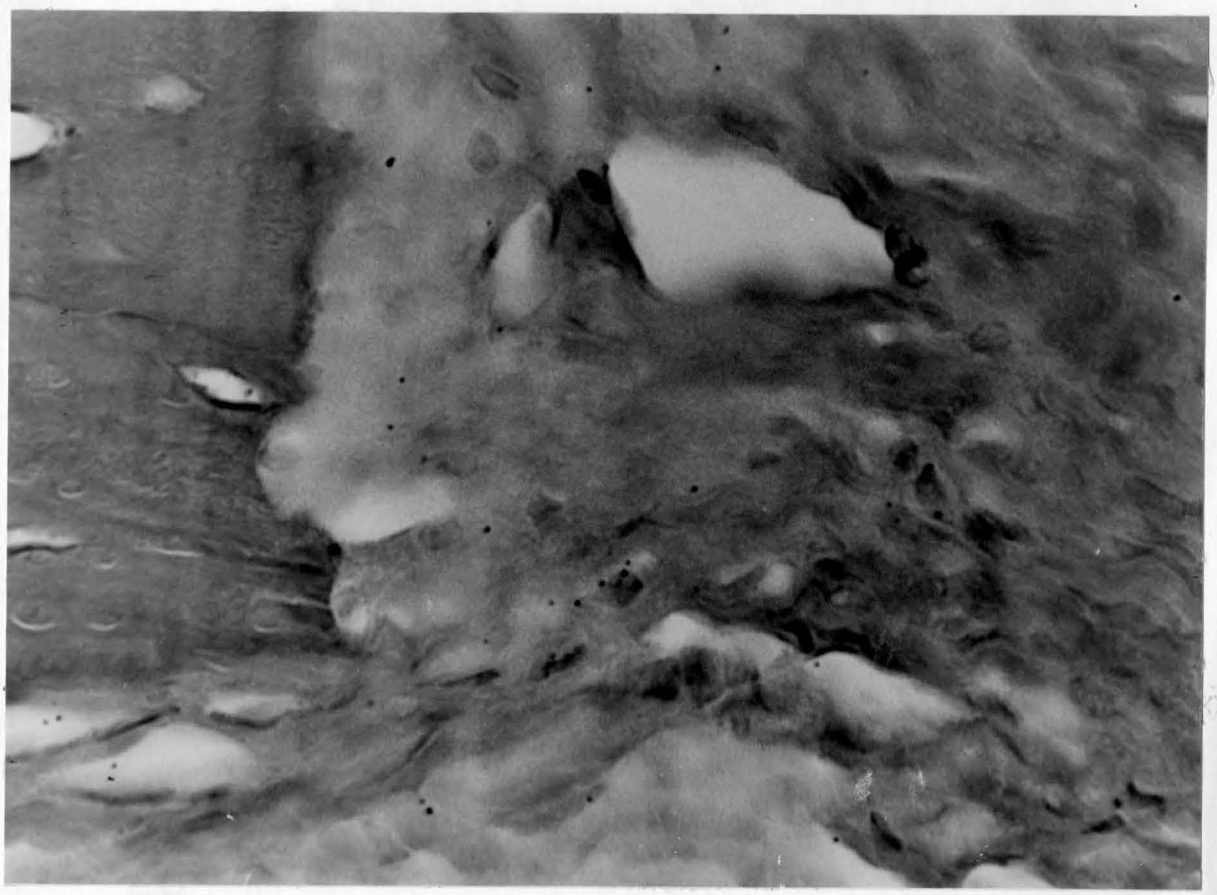

Figure 9 : Autoradiogram of the interparietal suture of 42 days old albino rat treated with 5 % T199 .

TABLE IV

CELL DENSITY IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH SALINE SOLUTION .

slide	suture	reticular	number of nuclei	progressive No.of nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	1	1	115	115	16.5	272.25
1	1	2	102	217	3.5	12.25
1	2	3	100	317	1.5	2.25
1	2	4	77	394	-21.5	462.25
1	3	5	108	502	9.5	90.25
1	3	6	102	604	3.5	12.25
1	4	7	77	681	-21.5	462.25
1	4	8	70	751	-28.5	812.25
1	5	9	92	843	- 6.5	42,25
1	5	10	103	946	4.5	20.25
1	8	11	111	1057	12.5	156.25
1	8	12	83	1140	-15.5	240.25
2	2	13	113	1253	14.5	210.25
2	2	14	99	1352	0.5	0.25
2	4	15	107	1459	8.5	72.25
2	4	16	85	1544	-13.5	182.25
2	6	17	108	1652	9.5	90.25
2	6	18	86	1738	-12.5	156.25
2	2	19	124	1862	25.5	650.25
3	2	20	104	1966	5.5	30.25
3	4	21	110	2076	11.5	132.25
3	4	22	97	2173	- 1.5	2.25
3	9	23	94	2267	- 4.5	20.25
3	9	24	97	2364	- 1.5	2.25

TABLE V

CELL DENSITY IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH 3 % T199 .

Slide	suture	reticular	number of nuclei	progressive No.of Nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	1	1	93	93	-18.8	353.44
1	1	2	135	228	23.21	538.7
1	3	3	84	312	-27.8	772.84
1	3	4	114	426	2.20	4.84
1	5	5	100	526	-11.79	139.00
1	5	6	120	646	8.20	67.24
1	7	7	92	738	-19.79	391.64
1	7	8	117	855	5.20	27.04
1	8	9	157	1012	45.20	2043.04
1	8	10	103	1115	- 8.79	77.27
2	1	11	127	1242	15.20	231.04
2	1	12	87	1329	-24.79	614.54
2	3	13	136	1465	24.20	585.64
2	3	14	101	1566	-10.79	116.42
2	4	15	123	1689	11.20	125.44
2	4	16	108	1797	- 3.79	14.36
2	5	17	115	1912	3.20	10.24
2	5	18	104	2016	- 7.79	60.69
3	1	19	132	2148	20.20	408.04
3	1	20	102	2250	- 9.79	95.84
3	4	21	124	2374	12.20	148.84
3	4	22	95	2469	-16.79	281.9
3	5	23	108	2577	- 3.79	14.36
3	5	24	106	2683	- 5.79	33.52

TABLE VI

CELL DENSITY IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH 5 % T199 .

Slide	suture	reticular	number of nuclei	progressive No. of nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	1	1	104	104	- 3.875	15.01
1	1	2	74	178	-33.875	1147.51
1	3	3	110	288	2.125	4.51
1	3	4	121	409	13.125	172.27
1	5	5	121	530	13.125	172.27
1	5	6	116	646	8.125	66.02
1	9	7	116	762	8.125	66.02
1	9	8	85	847	-22.875	523.27
1	13	9	129	976	21.125	446.27
1	13	10	100	1076	- 7.875	62.02
2	1	11	107	1183	- 0.875	0.77
2	1	12	72	1255	-35.875	1287.02
2	4	13	80	1335	-27.875	777.02
2	4	14	111	1446	3.125	9.77
2	8	15	117	1563	9.125	83.27
2	8	16	96	1659	-11.875	141.02
2	9	17	111	1770	3.125	9.77
2	10	18	72	1842	-35.875	1287.02
3	1	19	127	1969	19.125	365.77
3	1	20	130	2099	22.125	489.52
3	4	21	116	2215	8.125	66.02
3	4	22	123	2338	15.125	228.77
3	5	23	138	2476	30.125	907.52
3	5	24	113	2589	5.125	26.27

"t" TEST $$t = \frac{(\bar{X}_1 - \bar{X}_2) - 0}{\sqrt{S^2\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

$$S^2 = \frac{\Sigma(X_1 - \bar{X}_1)^2 + \Sigma(X_2 - \bar{X}_2)^2}{(N_1 - 1) + (N_2 - 1)} = \frac{4134 + 7215.92}{(24-1)+(24-1)} = \frac{11349.92}{46} = 246.73739$$

$$t = \frac{(98.5 - 111.79) - 0}{\sqrt{246.73\left(\frac{1}{24} + \frac{1}{24}\right)}} = \frac{13.29}{\sqrt{246.73(.0832)}} = \frac{13.29}{\sqrt{20.5279}} = \frac{13.29}{4.5307} = 2.9333$$

THE CRITICAL "t" VALUE WITH 45 DEGREES OF FREEDOM FOR P= .01
IS 2.69 SO THAT 2.9333 IS JUDGED TO BE STATISTICALLY SIGNIFICANT .

TABLE VIII

COMPARISON OF TWO SAMPLE MEANS OF CELL DENSITY IN SALINE AND 5 % T199 TREATED ALBINO RATS BY THE "t"TEST .

CONTROL GROUP MEAN :

$$\bar{X} = \frac{\Sigma X}{N} = \frac{2364}{24} = 98.5$$

EXPERIMENTAL GROUP MEAN :

$$\bar{X} = \frac{\Sigma X}{N} = \frac{2589}{24} = 107.875$$

CONTROL STANDARD DEVIATION :

$$S^2 = \sqrt{\frac{\Sigma (X-\bar{X})^2}{N-1}} = \sqrt{\frac{4134}{23}} = \sqrt{179.7391} = 13.4066$$

EXPERIMENTAL STANDARD DEVIATION :

$$S^2 = \sqrt{\frac{\Sigma (X-\bar{X})^2}{N-1}} = \sqrt{\frac{8354.71}{23}} = \sqrt{363.2482} = 19.0590$$

"t" TEST

$$t=\frac{(\bar{X}_1-\bar{X}_2)-0}{\sqrt{S^2\left(\frac{1}{N_1}+\frac{1}{N_2}\right)}}$$

$$S^2=\frac{\Sigma(X_1-\bar{X}_1)^2+\Sigma(X_2-\bar{X}_2)^2}{(N_1-1)+(N_2-1)}=\frac{4134+8354.71}{(24-1)+(24-1)}=\frac{12488}{46}=271.4782$$

$$t=\frac{(98.5-107.875)-0}{\sqrt{271.4782\left(\frac{1}{24}+\frac{1}{24}\right)}}=\frac{9.375}{\sqrt{22.5869}}=\frac{9.375}{4.7525}=1.9726$$

THE CRITICAL "t" VALUE WITH 45 DEGREES OF FREEDOM FOR P= .10
IS 1.68 SO THAT 1.9726 IS JUDGED TO BE STATISTICALLY SIGNIFICANT.

TABLE IX

LABELLED CELLS IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH SALINE SOLUTION .

slide	suture	reticular	number of nuclei	progressive No.of nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	1	1	4	4	1.125	1.26
1	1	2	4	8	1.125	1.26
1	3	3	4	12	1.125	1.26
1	3	4	3	15	0.125	.01
1	4	5	1	16	-1.875	3.51
1	4	6	5	21	2.125	4.51
2	1	7	3	24	0.125	0.01
2	1	8	1	25	-1.875	3.51
2	3	9	3	28	0.125	0.01
2	3	10	5	33	2.125	4.51
2	4	11	2	35	-0.875	0.76
2	4	12	2	37	0.875	0.76
3	2	13	4	41	1.125	1.26
3	2	14	1	42	-1.875	3.51
3	4	15	1	43	-1.875	3.51
3	4	16	1	44	-1.875	3.51
3	9	17	0	44	-2.875	8.26
3	9	18	2	46	0.875	0.76
4	2	19	4	50	1.125	1.26
4	2	20	3	53	0.125	0.01
4	4	21	3	56	0.125	0.01
4	4	22	2	58	0.875	0.76
4	6	23	6	64	3.125	9.76
4	6	24	5	69	2.125	4.51

TABLE X

LABELLED CELLS IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH 3 % T199.

Slide	suture	reticular	number of nuclei	progressive No.of nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	1	1	7	7	2.96	8.76
1	1	2	7	14	2.96	8.76
1	2	3	3	17	-1.05	1.10
1	2	4	3	20	-1.05	1.10
1	3	5	4	24	-0.05	0.0025
1	3	6	4	28	-0.05	0.0025
1	4	7	6	34	1.96	3.84
1	4	8	3	37	-1.05	1.10
1	5	9	3	40	-1.05	1.10
1	5	10	3	43	-1.05	1.10
1	7	11	7	50	2.96	8.76
1	7	12	3	53	-1.05	1.10
2	1	13	4	57	0.05	0.0025
2	1	14	3	60	-1.05	1.10
2	3	15	6	66	1.96	3.84
2	3	16	2	68	-2.05	4.21
2	4	17	4	72	0.05	0.0025
2	4	18	3	75	-1.05	1.10
2	5	19	1	76	-3.05	9.30
2	5	20	3	79	-1.05	1.10
2	7	21	5	84	0.96	0.92
2	7	22	1	85	-3.05	9.30
2	8	23	5	90	0.96	0.92
2	8	24	7	97	2.96	8.76

TABLE XI

LABELLED CELLS IN THE INTERPARIETAL SUTURE OF 42 DAYS OLD ALBINO RATS TREATED WITH 5 % T199.

Slide	suture	reticular	number of nuclei	progressive No.of nuclei	$(X-\bar{X})$	$(X-\bar{X})^2$
1	4	1	3	3	-0.125	0.015
1	4	2	5	8	1.875	3.51
1	5	3	4	12	0.875	0.76
1	5	4	1	13	-2.125	4.51
1	6	5	2	15	-1.125	1.26
1	6	6	2	17	-1.125	1.26
2	1	7	4	21	0.875	0.76
2	1	8	5	26	1.875	3.51
2	3	9	4	30	0.875	0.76
2	3	10	5	35	1.875	3.51
2	5	11	3	38	-0.125	0.015
2	5	12	2	40	-1.125	1.26
3	1	13	6	46	-2.875	8.26
3	1	14	2	48	-1.125	1.26
3	4	15	2	50	-1.125	1.26
3	4	16	2	52	-1.125	1.26
3	5	17	2	54	-1.125	1.26
3	5	18	0	54	-3.125	9.76
4	1	19	2	56	-1.125	1.26
4	1	20	3	59	-0.125	0.015
4	3	21	4	63	0.875	0.76
4	3	22	5	68	1.875	3.51
4	7	23	4	72	0.875	0.76
4	7	24	3	75	-0.125	0.015

TABLE XII

COMPARISON OF TWO SAMPLE MEANS OF LABELLED CELLS IN SALINE AND 3 % T199 TREATED ALBINO RATS BY THE "t" TEST .

CONTROL GROUP MEAN :

$$\bar{X} = \frac{\Sigma X}{N} = \frac{69}{24} = 2.875$$

EXPERIMENTAL GROUP MEAN :

$$\bar{X} = \frac{\Sigma X}{N} = \frac{97}{24} = 4.0416$$

CONTROL GROUP STANDARD DEVIATION :

$$s^2 = \sqrt{\frac{\Sigma (X-\bar{X})^2}{N-1}} = \sqrt{\frac{58.49}{23}} = \sqrt{2.5430} = 1.5946$$

EXPERIMENTAL GROUP STANDARD DEVIATION :

$$s^2 = \sqrt{\frac{\Sigma (X-\bar{X})^2}{N-1}} = \sqrt{\frac{77.2800}{23}} = \sqrt{3.36} = 1.8330$$

"t" Test

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - 0}{\sqrt{S^2 \left(\frac{1}{N_1} + \frac{1}{N_2} \right)}}$$

$$S^2 = \frac{\Sigma(X_1 - X_1)^2 + (X_2 - X_2)^2}{(N_1 - 1) + (N_2 - 1)} = \frac{58.49 + 77.28}{23 + 23} = \frac{135.77}{46} = 2.9515$$

$$t = \frac{(2.875 - 4.0416) - 0}{\sqrt{2.9515\left(\frac{1}{24} + \frac{1}{24} \right)}} = \frac{1.1666}{\sqrt{2.9515(.0832)}} = \frac{1.1666}{\sqrt{.2455}} = \frac{1.1666}{.4954} = 2.3548$$

THE CRITICAL "t" VALUE WITH 45 DEGREES OF FREEDOM FOR P = .05
IS 2.02 SO THAT 2.3548 IS JUDGED TO BE STATISTICALLY SIGNIFICANT .

TABLE XIII

COMPARISON OF TWO SAMPLE MEANS OF LABELLED CELLS IN SALINE AND 5 % T199 TREATED ALBINO RATS BY THE "t" TEST .

CONTROL GROUP MEAN :

$$\bar{X}=\frac{\Sigma X}{N}=\frac{69}{24}= 2.875$$

EXPERIMENTAL GROUP MEAN :

$$\bar{X}=\frac{\Sigma X}{N}=\frac{75}{24}= 3.125$$

CONTROL GROUP STANDARD DEVIATION :

$$S^2=\sqrt{\frac{\Sigma(X-\bar{X})^2}{N-1}}=\sqrt{\frac{58.49}{23}}=\sqrt{2.5430}= 1.5946$$

EXPERIMENTAL GROUP STANDARD DEVIATION :

$$S^2=\sqrt{\frac{\Sigma(X-\bar{X})^2}{N-1}}=\sqrt{\frac{50.500}{23}}=\sqrt{2.1956}= 1.4817$$

"t" TEST

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - 0}{\sqrt{S^2 \left(\frac{1}{N_1} + \frac{1}{N_2} \right)}}$$

$$S^2 = \frac{\xi(X_1 - \bar{X}_1)^2 + \xi(X_2 - \bar{X}_2)^2}{(N_1 - 1) + (N_2 - 1)} = \frac{58.49 + 50.500}{23 + 23} = \frac{108.990}{46} = 2.3693$$

$$t = \frac{(2.875 - 3.125) - 0}{\sqrt{2.3693 \left(\frac{1}{24} + \frac{1}{24} \right)}} = \frac{.250}{\sqrt{2.3693(.0832)}} = \frac{.250}{\sqrt{0.1971}} = \frac{.250}{0.4439} = 0.5631$$

THE CRITICAL"t" VALUE OF 0.5631 WITH 45 DEGREES OF FREEDOM IS JUDGED TO BE NOW STATISTICALLY SIGNIFICANT .

TABLE XIV

CELL DENSITY

	MEAN	STANDARD DEVIATION	SIGNIFICANCE
CONTROL	98.5	13.4066	P= .01
3 % T199	111.7916	17.7125	

CELL DENSITY

	MEAN	STANDARD DEVIATION	SIGNIFICANCE
CONTROL	98.5	13.4066	P= .10
5 % T199	107.875	19.0590	

LABELING INDEX

	MEAN	STANDARD DEVIATION	SIGNIFICANCE
CONTROL	2.875	1.5946	NON SIGNIFICANT
5 % T199	3.125	1.4817	

LABELING INDEX

	MEAN	STANDARD DEVIATION	SIGNIFICANCE
CONTROL	2.875	1.5946	P= .05
3 % T199	4.0416	1.8330	

DISCUSSION

The introduction of saline or T199 3%, 5%, 10%, 20% twice each week for two weeks in the subcutaneous, supraperiosteal tissues of the interparietal bone causes edema, inflammation, bone resorption and new bone formation.

The evidence of inflammation may be explained by the needle injection administering either saline or T199, impaction of fragments of hair and the concentration of T199. As inflammation was seen in the saline injected animals, and hair particles were observed, it is probable that the irritation to the skin was due to the injury caused by four injections. This injury could be responsible for all of the inflammatory reaction caused by the saline injection, and part of that seen in the T199 treated animals.

The T199 injected skin showed a greater severity of inflammations, and fibrogenesis than did either the saline or 3% and 5% T199 treated animals. This observation strongly suggests that concentrations of T199 cause acute suppurative lesion of the skin with necrosis.

The periosteal, sutural and endosteal connective tissues proliferate and thicken after injection either with saline of T199. However, the injections all are accompanied by bone formation following a period of osteoclastic resorption as indicated by reversal lines seen on the old bone.

The new bone formation seen on the 3% and 5% T 199 injected animals appears substantially thicker than that produced by saline injection alone. On the other hand, 10% and 20% T 199 are accompanied by greater proliferation of the periosteal, endosteal and sutural connective tissues. This proliferative reaction is accommodated by osteoclastic resorption widening the sutural spaces and causing a reduction in the thickness of the parietal bone plate as is evident from resorption of the periosteal and endosteal surfaces. There is new bone formation following the resorption of bone. The greatest quantity of new bone appears in the 3% T 199 treated animals; next the 5%, then 10% and least 20%. In the 20% T 199 treated animals, there is severe bone loss with thinning of the parietal bones.

It can be observed that T 199 causes an increase in the proliferation of periosteum, sutural and endosteal connective tissues. 3% T 199 can increase the amount of bone apposition above that produced by saline alone and apparently equivalent to that produced by 5% T 199.
Furthermore, there is evidently less inflammatory hyperplasia induced with 3% T 199 than either with 10% or 20% of T 199 both of which produce destructive bony osteoclastic reactions before apposition of new bony repair occurs during the period of two weeks.

It would appear that 3% of T 199 is an optimum concen-

tration required to increase bone apposition in the parietal bones in rats as either 10 % or 20 % is destructive while 5 % does not show any significantly greater increase as observed by inspection of the histologic specimens.

Making a comparison between the results of the present study and the one realized by Bayardo[20] using a concentration of 1 % of T199 in albino rats, the comparison was made by the "t" test and the conclusion was that there was no statistically significance between the 1 % and 3 % of T199. Therefore, there was no significant difference between them thus demonstrating that both produce new cell formation .

However, it is possible to prognosticate the value of using tissue culture medium to produce new bone formation and stimulate additional investigations in different kinds of animals and finally try in humans.

SUMMARY AND CONCLUSIONS

Twenty young albino rats were divided into five groups of 4 each. Three cubic centimeters of air was subcutaneously injected into the periosteal surface of the interparietal suture to create a pouch.

After 24 hours, three cubic centimeters of tissue culture medium (T 199) or saline solution was injected into the pouch of the experimental and control groups. The same procedure was repeated every third day for a total period of 2 weeks.

Eight hours after the last injection, the animals were intraperitoneally injected with tritiated thymidine at the rate one microcurie per gram of body weight. One hour after the thymidine administration, all animals were sacrificed .

Histological sections of the interparietal suture areas were prepared for autoradiograms .

Autoradiographic analysis of the periosteal tissue of the interparietal suture showed a slight increase in labeled cells of animals receiving tissue culture medium as compared to the saline control animals, except on those of 5 % T 199.

Cell density analysis of the interparietal suture area showed a high statistically significant increase in the number of cells of the experimental group as compared to

their control.

The results of this study indicate that the artificial medium T199 has a stimulating growth effect on DNA synthesis in vivo when is used in slight concentrations of 3 % T199 because this compared with the 5 % T199 show not a good statistically significance, and the 10 % and 20 % T199 showed necrosis in the skin and the interparietal suture .

The increased number of cells in the suture is evidence of increased sutural growth, and it is strongly suggested that sutural growth may be modified by enhancement with local administration of enriched medium.

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APPROVAL SHEET

The thesis submitted by Angel H. Vargas has been read and approved by the following Committee:

Dr. Patrick D. Toto, Director
Professor and Chairman, Oral Pathology, Loyola

Dr. Milton L. Braun
Professor and Chairman, Orthodontics, Loyola

Dr. Robert J. Pollock
Professor and Chairman, Histology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

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

April 11, 1978
Date

Patrick D. Toto
Director's Signature