

IN VITRO STUDY OF THE CYTOTOXICITY OF THE MISWAK ETHANOLIC EXTRACT

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تم استخدام المجهر الإلكتروني لدراسة التغييرات التي قد تحدث لخلايا L929 عندما تتعرض إلى تركيز مختلف من سائل الصوديوم هيبوكلورايت وكذلك خلاصة المسواك .
تم تعريض الخلايا إلى السوائل لمدة ساعتين وأربع ساعات .
بينت النتائج انه كلما زاد تركيز السائل كلما كان التغيير في الخلية أكبر .

Scanning electron microscopy was used to examine the morphological cell changes of L929 cell line in tissue culture. The cells were exposed to different concentrations of sodium hypochlorite and Miswak extract. The cells were examined after two and four hour exposure. Results showed that the higher the concentrations of Miswak extract and sodium hypochlorite, the greater the morphological changes of L929 cell line.

Introduction

One of the most important objectives in endodontic therapy is to remove infected pulpal and dentinal tissue debris from the root canal system. This is usually achieved by good mechanical debridement and thorough irrigation with suitable solution.

It has been reported that the residual microorganisms left behind in the root canal system must be considered as an undesirable complication and an important condition affecting the outcome of treatment.^{1,2} Although most antimicrobial agents are potentially toxic in varying degrees, one has to select an antimicrobial irrigant that will support treatment and will not delay healing.³

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The root canal system is normally instrumented and irrigated up to the apical constriction, thus, there is always a risk that the irrigating solution will pass through into the peri-radicular tissues. Therefore, in order to obtain the optimal prognosis for endodontic treatment, the biological effects of the antimicrobial irrigating agents should be evaluated before they are accepted for clinical use. At present, there is no irrigant capable of combining maximal antimicrobial properties with minimal toxicity.

Miswak is a chewing stick made from the aromatic root of a small bush known as the *Arrak* (*Salvadora Persica*). It is commonly used as a brush to clean the teeth.^{4,5} Few studies have indicated that Miswak contains substances that possess plaque inhibiting and antibacterial properties against several types of bacteria which are frequently found in the oral cavity.^{4,6}

Although the antibacterial activity of Miswak has been reported, its toxicity must be considered. In addition, no report has yet been made on the

utilization of its extract as an irrigant solution in endodontic practice. The purpose of this study, therefore, was to evaluate, *in vitro*, the effect of different concentrations of Miswak extract on L929 cell line in tissue culture. Results were compared to sodium hypochlorite (NaOCl) using a scanning electron microscopy.

Materials and Methods

The following concentrations have been prepared for this experiment. These are 5.25% and 1% NaOCl and 25%, 50%, and 100% concentrations of Miswak extract. Full concentration of NaOCl (25%) was obtained from the commercial household bleach 'chlorox'. The 1% NaOCl was prepared by adding 5.25% NaOCl to 1% sodium bicarbonate in a ration of 1:4.

Preparation of the Miswak Extract:

Miswak extract was prepared at King Saud University's College of Pharmacy, in the following method:

Fresh roots of the *Arrak* (*Salvadora Persica*, L. Salvadoraceae) were procured from the local market in Taif City, Saudi Arabia. They were collected from Wadi Allaith. The roots were then cut into small pieces and powdered. An extract (percolate) was prepared from 1 kg of the *Arrak* sample using 96% ethanol by percolation method (6 times, each time 2.5 liter of 96% ethanol was used). The exhaustive extraction was continued until the color of the material used disappeared. The resulting extract (percolate) was then concentrated and the solvent (ethanol) was completely removed at low temperature and reduced pressure. The yield of the extract obtained was found to be 6% (W/W). The extract was stored at 4°C in a tightly closed container to preserve it from any contamination, deterioration and/or decomposition.

The stock solution of Miswak extract was prepared by dissolving 1.0 gram of the Miswak extract weighed, using a 4 decimal digital weighing scale*, in 5 ml sterilized Ringer solution. In

order to prepare the 50% Miswak concentration, 2.5 ml of the Miswak stock solution was added to 2.5 ml Ringer solution. The 25% Miswak concentration was prepared by mixing 2.5 ml of the 50% Miswak concentration with 2.5 ml Ringer solution.

Cell Line (L 929 /Mouse Fibroblast)

Three- to five-day-old cultures of L 929 cells was used (Flow Lab). They were prepared in the Tissue Culture Lab of the College of Pharmacy, King Saud University. The cells were suspended in culture medium at a density of 5×10^5 cells per milliliter and grown in a tissue culture flask* The culture medium was changed every other day and the day before performing the experiment. Cells were harvested with 0.02% trypsin in phosphate buffered saline (PBS) by incubation at 37°C and 100% humidity for 5 to 10 minutes.

Culture Medium

Eagle's minimum essential medium (MEM), with Earl's BSS (Flow Laboratory) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 2.2 mg sodium bicarbonate per ml, was used. In addition, 100 IU/ml penicillin and 50 g/ml streptomycin were added to the culture medium.⁷

Experimental Procedures

Cell growth was examined using a wild light microscope¹ before the start of the experiment. The cells were washed three times in PBS solution and harvested with 0.02% trypsin before being suspended in a culture medium. The cell suspension was washed and centrifuged in a culture medium four times at 300x g for five minutes. The experiments were performed in plastic tissue culture clusters¹ containing 24 wells each with an inner diameter of 16 mm. A 5 mm² sterilized glass slide was placed in each culture well and a small scratch prepared on the corner of each glass slide for an easy identification and orientation of the sample during SEM examination. One milliliter of the cell suspension was mixed with 1.0 ml of

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⁵Costar 205, Broadway, Cambridge, MA, USA

⁷Heerugg, Switzerland

various concentrations of the medicaments in the culture well and incubated at 37°C and 100% humidity for two and four hours. At the same time, 1.0 ml of the cell suspension and 1.0 ml of the culture medium were added to culture wells without the tested materials to serve as the control (negative). A total of 30 culture wells were used, 5 for each tested solution and 5 for controls.

Scanning Electron Microscope (SEM)

At the end of the incubation period, the cells were prefixed in 0.1% glutaraldehyde in a tissue culture medium for 5 minutes. The medium was then decanted and replaced with 2.0% glutaraldehyde in 100mM Na-cacodylate buffer (pH 7.2) at room temperature and fixed for half hour. Specimens were dehydrated for 5 minutes each through 50%, 70%, 90% and 100% ethanol. The glass slides were carefully removed using a sterile tweezer for critical-point dried with CO₂ using Samdri-PVT-3B machine*. The glass slides were fixed with silver conducting paint, and gold-sputter coated to a thickness of 5-7µm. Specimens were then examined with scanning electron microscope[§] operated at an accelerating voltage of 25Kv.

Three- to five-day-old cultures of L929 cells were prepared for SEM examinations in the same manner.

Results

The cytological features of L929 cells grown in tissue culture for 5 days are illustrated in Figure 1. The cells appeared in spindle and stellate shapes. Few microvilli are seen. Ruffles and small blebs were observed covering the outer surface of the cells.

A. Two-hour exposure:

Control:

Figure 2 illustrates the normal morphology of L929 cells grown in tissue culture for two hours. The cells were elongated, spindle or fusiform-shaped. Dividing cells appeared round and scat-

Tausimis, Rockville, MD, USA

[§]Jeol JSM-T330, Japan

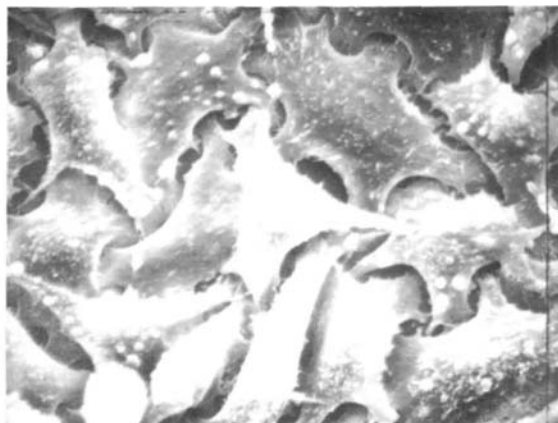


Figure 1. Scanning electron micrograph of L929 cells grown in tissue culture for 5 days. The cells are elongated and stellate in shape. Ruffles and small blebs are covering the cell surface. Few microvilli are seen (X2000).

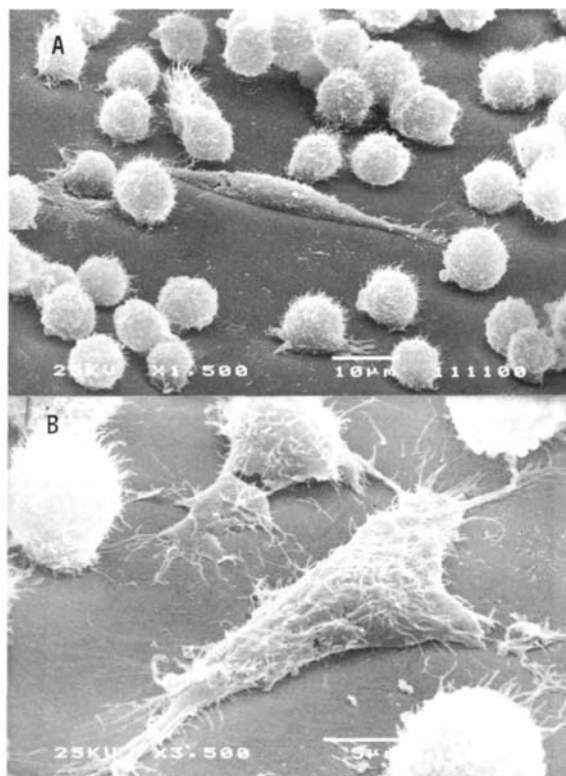


Figure 2. Scanning electron micrograph of L929 cells grown in tissue culture for 2 hours: (A) Round cells are scattered indicating mitosis; some start to form normal spindle-shaped morphology; (B) Stellate-shaped cells are covered by microvilli.

tered throughout the culture. Some cells appeared stellate in shape and covered with ruffles and numerous microvilli. Transformation of some cells from round to the polarized stage was also noticed.

L929 cells exposed to various Miswak concentrations:

The L929 cells exposed to full Miswak concentration : appeared round in shape and scattered throughout the culture [Fig. 3A]. The cell wall showed an irregular surface covered with numerous large irregular blebs. No microvilli or ruffles were seen. Remnants of damaged cells were also seen.

Cells exposed to 50% Miswak concentration were round in shape and the cell wall appeared

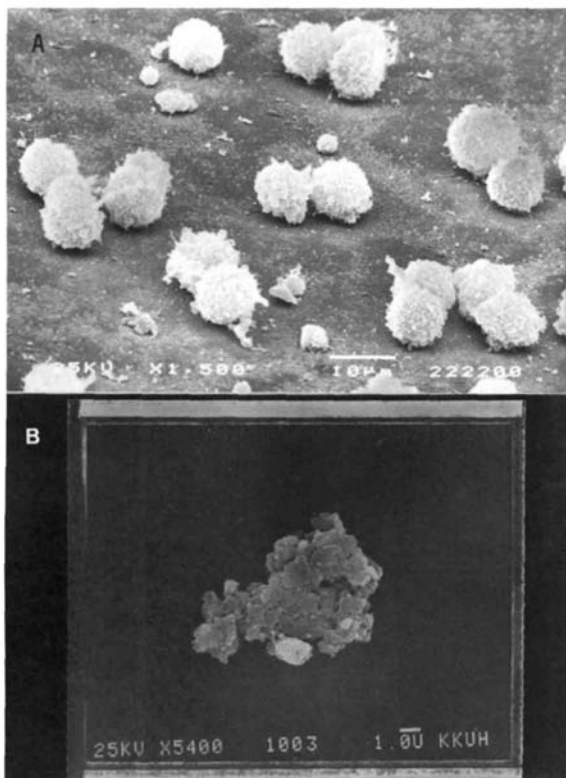


Figure 3. Scanning electron micrograph of L929 cells exposed for 2 hours to (A) full Miswak concentration showing round cells in shape and remnants of damaged cells; (B) to 5% NaOCl concentration with the cells irregular in shape indicating total damage.

irregular covered with numerous large blebs. Some tissue debris of damaged cells were scattered throughout the culture.

Those cells exposed to 25 % Miswak concentrations were round, forming aggregates throughout the culture. Some cells were covered with numerous small blebs some of which were covered with numerous microvilli. Tissue debris was also seen.

L929 cells exposed to NaOCl concentrations:

Very few round cells covered with numerous small blebs were found on cells exposed to 5% NaOCl. Debris of damaged cells was scattered throughout the culture. Some cells appeared irregular and totally damaged [Fig. 3B].

Those exposed to 1 % NaOCl were round and covered with numerous small blebs. No microvilli, ruffles or tissue debris was seen.

A. Four-hour exposure:

Control:

When the L929 cells were grown in tissue culture for four hours, numerous round cells were seen. Cell wall appeared generally smooth and covered with numerous microvilli [Fig. 4]. However, no blebs could be seen and some cells exhibited a discoid profile. The lamellar cytoplasm forms a ring surrounding the endoplasm and pseudopods extended along its edge.

L929 cells exposed to various Miswak concentrations:

L929 cells exposed to full Miswak concentration were round and covered with numerous small blebs. Some cells were covered with microvilli. Remnants of damaged cells were seen [Fig. 5A].

Those cells exposed to 50% Miswak concentration appeared round and some were forming aggregates. Its cell wall contained numerous small blebs or ruffles. Extruded cytoplasm was also noticed.

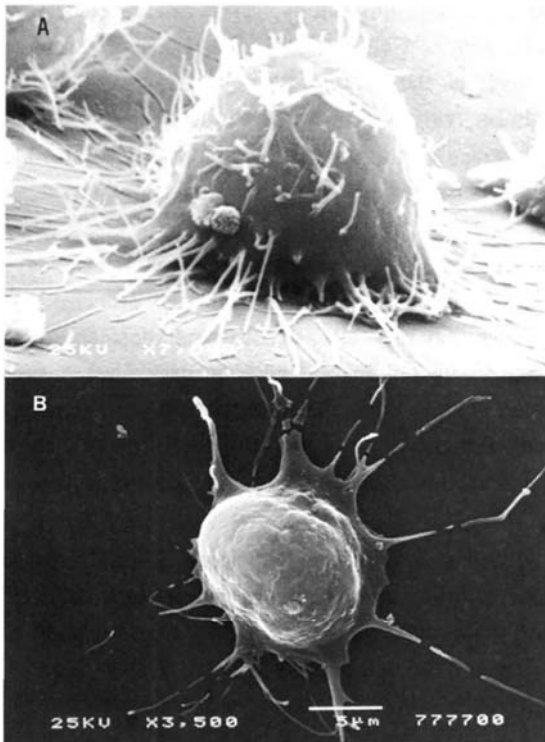


Figure 4. Scanning electron micrograph of L929 cells grown in tissue culture for 4 hours: (A) The cell is covered with microvilli which allows it to attach itself to the substrate.; (B) A cell in a radially distributed state and is discoid in shape. Note the amellipodia and ruffles around the smooth cell body.

Those cells exposed to 25 % Miswak concentration were round in shape. Its cell wall appeared smooth and porous covered with few ruffles. No blebs appeared on the cell surface.

L929 cells exposed to NaOCl concentrations:

For the L929 cells exposed to 5 % NaOCl concentration, few of them were seen covered with large blebs or small hair-like projections. Some cells showed an irregular, rough and porous cell wall surface [Fig. 5B]. Debris of damaged cells was also seen.

For those cells exposed to 1% NaOCl concentration, many of them were seen as rounded and covered with irregular blebs. Other cells were covered with ruffles and microvilli.

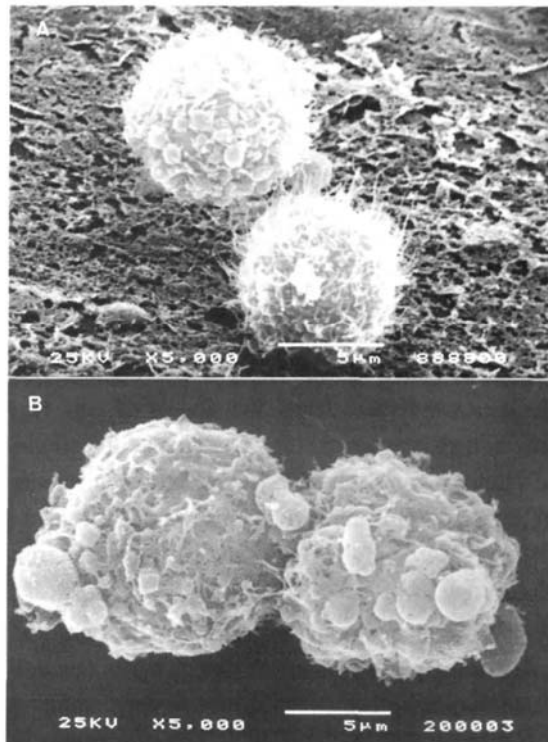


Figure 5. Scanning electron micrograph of L929 cells exposed for 4 hours to (A) full Miswak concentration showing round cells covered with small blebs and short microvilli. Tissue debris of damaged cells are well scattered through the culture; (B) 5% NaOCl concentration showing round cells with porous cell wall covered by numerous large blebs and ruffles.

Discussion

Tissue culture techniques have several practical and economical advantages. Kawahara, et al⁸ have found the tissue culture method as an extremely favorable technique for testing all types of dental filling materials. Endodontic filling materials and medicaments are usually in close proximity to the periradicular tissue for which biological responses usually resulted from the migration of the chemical components of the root canal filling material to the adjacent tissue. The inherent toxic nature at the cellular level will determine the injurious nature of the material.⁹ When evaluating endodontic material in tissue culture, the adequate

contact between the tested material and the responding cells is crucial. Therefore, in this study the tested material and cells were in direct contact with each other. The method used was similar to that of Spangberg⁷ and Al Nazhan and Spangberg¹⁰. Due to its toxic reaction, only the morphological cell changes, *in vitro*, have been described using a phase contrast microscope.^{8,11} Scanning electron microscopy results, describing the morphological changes in the target cells, have only been investigated by Al Nazhan and Spangberg¹⁰ which enabled them study the details of cell surfaces at high magnification. The change in cellular morphology is a particularly valid criterion for the evaluation of cell damage. This was followed up in the present study by using the scanning electron microscopy.

A 2- and 4-hour experimental period had been considered optimum to detect early reaction which may have been caused by the tested solution as recommended by the FDI.¹² The culture condition was suitable for L929 cells to grow and examination of the 5-day-old culture cells had clearly demonstrated the general shape of fibroblasts as reported by other investigators.^{10,13,14}

The L929 cells exposed to the control culture medium exhibited a round or spindle-like shape. Cells were covered with microvilli sprouting from various parts of the cell body and attached to the glass (substrate) providing a good diffusion. The presence of microvilli was a characteristic feature of the diffusion of L929 cells. It is important in providing sufficient mechanical stability that maintained the structural integrity of the fibroblasts in culture.¹⁵ As the cells began to diffuse, the microvilli shortens and decreases in number.^{13,16} The degree of diffusion varies with time and varies according to the cell cycle stage. Thus, the shape of the diffusing cells varied from one cell to another.

Generally, toxic drugs can attack the cell, especially the cytoplasm or the nucleus, which

alters the cell morphology. They can act on resting cells, too.¹⁷ Cell surfaces undergo a progressive change as they move through interphase.¹⁸ In addition, variation in metabolic activity during the different stages of the cell cycle may be responsible for the variable cell's susceptibility to toxic materials.¹⁹ Therefore, caution must be exercised when coming to conclusion from SEM which takes a random static view of a dynamic process. It appears that the cells' density and their ability to make contact with one another may have a profound influence on their morphology.²⁰ The idea behind suggesting the use of Miswak extract as an irrigating solution is due to its good antimicrobial activity.

After a two-hour exposure to full Miswak concentration, changes in the general cell morphology were observed. The cells became round in shape and the cell surface was covered with blebs. These changes were due to the cytoplasmic shrinkage causing the cells to detach from the substrate. Cell detachment has been reported as a method of recording cell injury.²¹

Upon exposure of the cultured cells to lower concentrations of Miswak extract solution, the toxic material responsible for the previous morphological changes have been reduced. The blebs, therefore, became smaller in size and few microvilli covered the smooth cell wall surface.

Mohammed and Turner²² were the only one who studied the cytotoxic potential of the Miswak using the agar overlay method. No cytotoxic effect had been noticed, however, cytotoxicity became evident after 24 hours. This was because the agar overlay method depends on the diffusion of the medicament through the agar material. In addition, it did not provide direct contact between the cells and the tested solution.

When cells were exposed to 5.25% NaOCl for two hours, much cell debris and few cells were observed throughout the culture. This indicates that total cell lysis had taken place and most of the

cells were seriously damaged. This was due to the strong action of sodium hypo-chlorite which breaks the peptide links upon contact with tissue proteins.²³

Previous *in vitro* studies of the toxicity potential of 5.25% NaOCl, using cultured cells, were in agreement with our findings. Spangberg et al²⁴ and Koskinen et al²⁵ found that 5.25% NaOCl is extremely toxic to HeLa cells, human fibroblasts and lymphocytes.

When the exposure time of cultured cells to the tested medicaments was increased to 4 hours, some morphological cell changes could still be observed. However, microvilli were observed when the cells were exposed to the three Miswak concentrations (full, 50% and 25%). This indicates that cellular changes which were observed in the two-hour exposure may be reversible. Ruffles seen on cells exposed to the 50% and 25% Miswak and the 1% NaOCl concentrations could also be explained in the same manner. The morphological cell changes after 2- and 4-hour exposures to 5.25% NaOCl were similar. Results obtained from the 4-hour exposure showed that the 25% Miswak is the least irritant. This was clearly demonstrated microscopically.

Conclusion

Based on the results obtained from this study, it can be concluded that:

1. Morphological changes of L929 cell-line were noticeable when exposed to different concentrations of Miswak extract and NaOCl. This indicated that some of its chemical components may be harmful to the periradicular tissue.
2. The higher the concentration of Miswak extract and NaOCl are, the greater the morphological changes of the L929 cell.
3. Reappearance of microvilli and ruffles on the surface of L929 cells after a 4-hour exposure period to different Miswak extract concentrations may be an early sign of cell recovery.

4. However, in order to reach a final conclusion concerning toxicity of the tested medicaments, the recovery of cells should be further studied.

References

1. Engstrom B, Hard, Segerstad L, Ramstrom G, Frostell G. Correlation of positive cultures with the prognosis of root canal treatment. *Odont Revy* 1964;15:275.
2. Engstrom B, Lundberg M. The correlation between positive culture and the prognosis of root canal therapy after pulpectomy. *Odont Revy* 1965; 16:193.
3. Spangberg L. Cellular reaction to intracanal medicaments. In: Grossman LI ed. Transactions of the fifth international conference on Endodontics. Philadelphia, 1973:108.
4. Khoory T. The use of chewing sticks in preventive oral hygiene. *Clin Prev Dent* 1983;5:11-14.
5. Almas K. Miswak (chewing stick) and its role in oral health. *Postgraduate Dent* 1993;3: 214-18.
6. Wolinsky L, Sote E. Isolation of natural plaque: Inhibiting substances from Nigerian chewing sticks. *Caries Res* 1984;18:216-25.
7. Spangberg L. Kinetic and quantitative evaluation of material cytotoxicity in vitro. *Oral Surg Oral Med Oral Pathol* 1973;35:389-401.
8. Kawahara H, Yamagami H, Nakamura M Jr. Biological testing of dental materials by means of tissue culture. *Int Dent J* 1968; 18:443 -67.
9. Autian J. General toxicity and screening tests for dental materials. *Int Dent J* 1974; 24: 235-50.
10. Al Nazhan S, Spangberg L. Morphological cell changes due to chemical toxicity of a dental material: An electron microscopic study on human periodontal ligament fibroblasts and L929 cells. *J Endod* 1990; 16:129-34.
11. Lettre R. Drug action on cells in vitro. *Ann NY AcadSci* 1954;58:1085-88.
12. FDI Commission of Dental Materials, Instruments, Equipment and Therapeutics. Recommended standard practices for biological evaluation of dental materials. *Int Dent J* 1980;30:140-88.
13. Ukena TE, Karnovsky MJ. The role of microvilli in the agglutination of cells by concanavalin A. *Exp Cell Res* 1977;106:309-25.
14. Domnina LV, Ivanova O, Margolis L, Olshevskaja L, Rovensky Y, Vasiliev J, Gelfand IM. Defective

- formation of the lamellar cytoplasm by neoplastic fibroblasts. L cells transformed cells-cell attachment-contact inhibition - scanning electron microscopy-microcinematography. Proc Natl Acad Sci 1972;69:248-52.
15. Vasiliev JM, Gelfand IM. Mechanism of morphogenesis in cell cultures. Int Rev Cytol 1977;50:159-247.
 16. Erickson CA, Trinkaus JP. Microvilli and blebs as sources of reserve surface membrane during cell spreading. Exp Cell Res 1976;99:375-84.
 17. Verne J. Cellular sensitivity to drug action in short term tissue culture. In vitro correlations with sensitivity in vivo. Ann NY Acad Sci 1954;58:1195-201.
 18. Porter K, Prescott D, Frye J. Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. J Cell Biol 1973;57:815-36.
 19. Spangberg L, Al-Nazhan S. The radio-chromium release method for evaluation of cytotoxicity in vitro. Int Endod J 1988; 21:72-8.
 20. Cooper EH, Bedford AJ, Kenny TE. Cell death in normal and malignant tissues. Adv Cancer Res 1975;21:59-120.
 21. Nordling S. Adhesiveness, growth behavior and charge density of cultured cells. Acta Pathol Microbiol Scand 1967;Suppl 192:1 + .
 22. Mohammad AR, Turner JE. In vitro evaluation of Saudi Arabian toothbrush tree (*Salvadora Persica*). Odontostomatol Trop 1983;6:145-48.
 23. Ingle J, Bakland L. Endodontics. 4th ed. Williams &Wilkins, 1994;13:627-37.
 24. Spangberg L, Safavi KE, Kaufman A, Pascon E. Antimicrobial and toxic effect in vitro of a bisdequalinium acetate solution for endodontic use. J Endod 1988; 14(4): 175-78.
 25. Koskinen KP, Rahkamo A, Tuompo H. Cytotoxicity of some solutions used for root canal treatment assessed with human fibroblasts and lymphoblasts. Scand J Dent Rest 1981; 89: 71-8.