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AVALIAÇÃO *IN VITRO* DA CITOTOXICIDADE DO FORMOCRESOL, DO TRICRESOL FORMALINA E DO FORMALDEÍDO EM TRÊS DIFERENTES LINHAGENS CELULARES

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"Meu filho, tudo o que fizeres, faz com doçura, e mais que a estima dos homens, ganharás o afeto."
(Eclesiástico, 3, 19)

"...não haveria frutos se as flores não caíssem."

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LISTA DE ABREVIATURAS

d - dias

DMEM – Meio essencial mínimo modificado por Dulbecco

DMSO – Dimetil sulfóxido

DNA – Ácido Desoxirribonucléico

DPBS – Solução tampão salina fosfato modificada por Dulbecco

EDTA – Ácido dietilaminotetracético

FA – Formaldeído

FC – Formocresol

h - hora

HeLa – Linhagem Celular Estabelecida (células epiteliais originárias de câncer cervical humano)

Hep2 – Linhagem Celular Estabelecida (células epiteliais originárias de carcinoma de laringe humano)

mg - miligrama

mL – mililitro

mM - milimolar

MTT – brometo de 3-(4,5-dimetilazol-2-il)-2,5-difenil tetrazolio

NIH3T3 – Linhagem Celular Estabelecida (Fibroblastos de camundongo)

SFB – Soro fetal bovino

t – tempo

TC – Tricresol formalina

µg- micrograma

µL - microlitro

RESUMO

A avaliação da citotoxicidade dos materiais dentais é de grande importância na Odontologia. O formocresol (FC) e o tricresol formalina (TC) são duas medicações utilizadas na odontologia para desinfecção do canal radicular, que contém formaldeído na sua formulação. Estes produtos são considerados bons antimicrobianos, mas pesquisas a respeito da toxicidade destes medicamentos ainda são necessárias.

O presente estudo avaliou *in vitro* a citotoxicidade do formaldeído, do FC e do TC, utilizando três linhagens celulares estabelecidas, as células HeLa, NIH3T3 e Hep2, cultivadas em condições padrão. As células foram deixadas em contato com cada produto durante 1, 2, 3, 4 ou 5 minutos, sendo então incubadas por 24 horas, 48 horas ou 7 dias. O teste de citotoxicidade utilizado foi o ensaio MTT.

Os resultados deste estudo mostraram que os produtos testados foram tóxicos às diferentes linhagens celulares estabelecidas, em todas as condições testadas. O formocresol foi o produto que apresentou menor citotoxicidade, sendo este resultado estatisticamente significante quando comparado com o tricresol formalina e o formaldeído.

APRESENTAÇÃO DO TEMA

Citotoxicidade dos materiais

Numerosos e variados agentes químicos são usados como anti-sépticos tópicos na prática dental. Como eles são administrados diretamente na cavidade oral, estes agentes deveriam ter baixa toxicidade e alta segurança para a saúde do paciente [1].

A necessidade da utilização de materiais biocompatíveis na Odontologia implica que sejam realizados testes de citotoxicidade. A toxicidade de um material dental pode ser avaliada por testes *in vitro*, testes *in vivo*, incluindo experimentação em animais, e estudos clínicos em humanos. Estudos *in vitro* são principalmente realizados para avaliar a citotoxicidade ou a genotoxicidade de um material dental [2].

A biocompatibilidade pode ser definida como a capacidade de um material de exercer sua função, na aplicação específica, na presença de uma resposta apropriada do hospedeiro. Uma reação adversa pode ser devido à toxicidade de um material dental ou a outros fatores, como a acumulação de bactérias sobre os materiais, causando inflamações. Portanto, a toxicidade pode ser considerada como somente uma reação de não compatibilidade de um material dental [2].

Testes de citotoxicidade

As vantagens dos testes de toxicidade *in vitro*, quando comparado com experimentos em animais e estudos clínicos em humanos, é o controle das

condições experimentais, baixos custos e rápida realização, além de não envolver questões éticas [2].

Os testes *in vitro* podem ser realizados utilizando linhagens celulares permanentes ou culturas primárias (por exemplo, gengiva, mucosa e fibroblastos de polpa) [3]. Alguns autores afirmam que as culturas primárias refletiriam de forma mais precisa as situações *in vivo*, porém apresentam dificuldades no cultivo [2, 3]. Outros, afirmam que o uso de linhagens celulares estabelecidas oferece vantagens no cultivo, pois as condições de cultura são definidas, evitando as variações individuais e a interferência do complexo mecanismo homeostático que ocorre *in vivo* [4, 5].

O Comitê Europeu para Padronização (CEN) é o órgão competente que avalia e recomenda normas para a utilização de materiais biológicos, incluindo os empregados na odontologia. Este Comitê recomenda o desenvolvimento e uso de métodos *in vitro* que possam ser adotados como padrões, minimizando a necessidade de avaliações *in vivo* [6].

A viabilidade celular pode ser avaliada através de vários métodos, porém é aconselhável que a pesquisa utilize um processo que envolva menor tempo e menor variação na análise das amostras [7].

O ensaio MTT é um teste usado para avaliar a viabilidade celular, de execução rápida e objetiva, baseado em uma reação colorimétrica. O sal MTT (brometo de 3-(4,5-dimetilazol-2-il)-2,5-difenil tetrazólio) entra na mitocôndria da célula viável e é clivado pela enzima succinato desidrogenase, produzindo cristais formazan, de coloração azul escuro. A quantidade de cristais formada é

diretamente proporcional ao número de células viáveis. Assim, quanto mais escura a coloração ao final da reação, maior é a viabilidade celular. A densidade óptica resultante do teste MTT é determinada em espectrofotômetro [7].

Ação antimicrobiana do formocresol e do tricresol formalina

A principal proposta do tratamento de canal radicular de dentes com necrose pulpar é eliminar as bactérias e seus produtos, responsáveis pela inflamação local, antes de obturar o conduto radicular (Figura 1). Os microorganismos têm um papel fundamental na etiologia das doenças periapicais e pulpares [8], e seu controle e eliminação são importantes durante o tratamento endodôntico [9].

A eliminação dos microorganismos do canal radicular é almejada usando soluções irrigadoras durante a instrumentação e usando medicações intracanais. É esperado que estes tratamentos possam atingir as ramificações do canal radicular e outras áreas inacessíveis ao clínico, auxiliando no tratamento endodôntico (tratamento de canal) [10].

Formocresol

Nas últimas cinco décadas, a substância mais amplamente usada e aceita no tratamento de dentes primários, na pulpotomia (tratamento conservador da polpa radicular dos dentes decíduos), tem sido o formocresol. Estudos têm demonstrado uma propriedade de fixação tecidual adequada, proporcionando sucesso radiográfico e clínico [11, 12]. Porém, muito interesse tem surgido sobre o

potencial mutagênico e carcinogênico de produtos contendo formaldeído, dos efeitos tóxicos do formocresol, e da possível difusão desta substância para os tecidos sistêmicos e circunvizinhos do dente. Em contraste com outros aldeídos, o formaldeído é uma molécula pequena que penetra através do canal radicular. Estudos em animais têm confirmado que o formocresol aparece sistemicamente após seu uso em pulpotomias e que injúria celular pode ocorrer nos tecidos sistêmicos [13].

O agente ideal da pulpotomia deveria exercer efeito máximo sobre os microorganismos locais, enquanto sua citotoxicidade deveria ser mínima sobre a polpa remanescente e os tecidos circunvizinhos. A quantidade de tempo necessária para a concentração antimicrobiana matar os microorganismos é também clinicamente relevante. É de pequena importância para o clínico o uso de concentrações específicas da substância se sua ação não puder ocorrer numa quantidade de tempo clinicamente razoável (2 – 3 dias). Além disso, é importante que a substância exiba uma atividade antimicrobiana no local a ser desinfectado [14].

Atualmente, na rotina endodôntica, o formocresol é usado por 5 minutos na aplicação clínica. No estudo de Hill, 1991, o formocresol foi usado a 1,5%, e o tempo máximo necessário para matar todos os microorganismos foi de 2 minutos. Portanto, uma aplicação de 5 minutos de formocresol foi mais longa que o necessário para matar os microorganismos em um dente cariado [14].

O formocresol é um agente antibacteriano efetivo quando testado contra bactérias anaeróbias selecionadas (*Peptococcus magnus, Proprionibacterium acnes, Veillonella parvula, Lactobacillus fermentum, Porphyromonas gingivalis e*

Fusobacterium nucleatum). Quando comparado com outros medicamentos, o formocresol produziu significantemente largas zonas de inibição sobre as bactérias. Pesquisas fortalecem que o formocresol tem demonstrado boas propriedades antimicrobianas sobre bactérias anaeróbias facultativas [15, 16].

Tricresol formalina

O tricresol formalina é usado como curativo de demora na câmara pulpar de dentes permanentes com necrose pulpar e preparo químico-mecânico incompleto do canal. Este medicamento propicia a desinfecção do canal, dando condições para reparar as lesões periapicais. Ocorre também uma ação bactericida à distância, pela liberação de vapores do formaldeído [17].

O tricresol formalina é um composto à base de formaldeído e cresol. O formaldeído é um gás produzido pela incompleta combustão do metanol, é solúvel em água, apresentando solução aquosa de aproximadamente 38% a 40% de formaldeído em peso, chamado formalina [18]. O tricresol diminui as propriedades irritantes do formaldeído. Este material é derivado do “carvão de breu”, sendo um potente anti-séptico e considerado menos tóxico que o formaldeído [12].

Formaldeído

O formaldeído é um reagente muito reativo, e é conhecido como potencialmente citotóxico, mutagênico e carcinogênico [19].

Estudo *in vitro* realizado em células epiteliais da mucosa bucal humana sugeriu que o formaldeído tem a capacidade de causar numerosos efeitos citopatológicos [20].

A exposição da mucosa oral ao formaldeído pode ocorrer através da liberação de certos materiais dentais e por inalação pela boca [21]. Uma das formas de inalação do formaldeído é através de vários materiais odontológicos, como ionômero de vidro, materiais de base de dentadura acrílica, pastas e cimentos endodônticos [22].

O risco aparente dos efeitos adversos sobre a saúde quando da exposição ao formaldeído e pela possibilidade de efeitos potencialmente tóxicos sobre o tecido oral, através de materiais dentários, tem preocupado o profissional da odontologia. A citotoxicidade do formaldeído foi investigada no estudo de Nilsson et al. [20], por meio de cultura de células orais humanas, e o resultado demonstrou que o formaldeído foi mais tóxico sobre os fibroblastos do que sobre as células epiteliais [20].

OBJETIVOS

Geral

O objetivo deste estudo foi avaliar a citotoxicidade do formocresol, do tricresol formalina e do formaldeído.

Específicos

- Avaliar a citotoxicidade do formocresol, do tricresol formalina e do formaldeído em diferentes tempos de exposição (1, 2, 3, 4, e 5 minutos), nas linhagens celulares:

  Hep2 (células epiteliais originárias de carcinoma de laringe humano)

  NIH3T3 (fibroblastos de camundongos)

  HeLa (células epiteliais originárias de câncer cervical humano)

- Avaliar o efeito citotóxico do formocresol, do tricresol formalina e do formaldeído após 24 horas, 48 horas e 7 dias de incubação após o tratamento com os produtos citados, nas linhagens celulares Hep2, NIH3T3, HeLa

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SCIENTIFIC ARTICLE

Evaluation *In Vitro* of Formaldehyde, Formocresol and Tricresol Formalin Cytotoxicity in Three Different Established Cell Lines.

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ABSTRACT

The cytotoxicity evaluation of dental material is of great importance in dentistry. Formocresol (FC) and tricresol formalin (TC) are two medicines used in dentistry to disinfect the root canal, both containing formaldehyde (FA) in their formulation. These products are considered good antimicrobial agents, but determine the toxicity of these products is still necessary. The present study evaluates the cytotoxicity *in vitro* of FA, FC and TC, using three established line cells, HeLa, NIH3T3 and Hep2, cultivated under standard conditions. Cells were left in contact with each product during 1, 2, 3, 4 or 5 minutes, being after that incubated for 24 hours, 48 hours or 7 days. The cytotoxicity test used was the MTT assay. Our results showed that the test products were toxic to the different cell lines used, in all assayed conditions. Formocresol was the product which presented the lowest cytotoxicity, with a statically significant result when compared with tricresol formalin and formaldehyde.

Key words: cytotoxicity, cell culture, formocresol, formaldehyde, tricresol formalin.

INTRODUCTION

A great number of dental materials remain in contact with oral tissues for a long period of time. The properties and the long exposure of these dental materials to the oral cavity can be important factors concerning toxicity. For this reason, biochemical assays to evaluate the cytotoxic effect of these dental materials are relevant, considering the potential risk for both patient and clinician [1].

The endodontics materials evaluated in this study, formocresol (FC) and tricresol formalin (TC), have an extent history of use. These materials have a strong antimicrobial activity on disinfecting the root canal [2, 3]. However, literature data regarding their cytotoxic effect is still relatively modest when compared to data on antibacterial effects.

International standards for preclinical *in vitro* screening of dental materials allow the use of relevant primary cells; however, the use of established cell lines is recommended [4]. The use of primary cells does not assure that the observed results will be reproductive, once primary cells may have specific metabolic potentials, which does not occur in permanent cell lines [5].

The well established toxic and carcinogenic potentials of formaldehyde have aroused great interest in dentistry. The knowledge that some dental materials can liberate formaldehyde created the need of evaluating the toxicity degree of dental materials [6, 7].

The aim of this study was to evaluate the toxicity of formaldehyde, formocresol and tricresol formalin in three different established cell lines, exposed

to the products in different time intervals, and kept afterwards in different incubation times. The cytotoxic effect was evaluated by measuring cell viability.

MATERIALS AND METHODS

Cell Culture

The established cell lines used were NIH3T3 (mouse fibroblasts), Hep2 (epithelial cells from human larynx carcinoma) and HeLa (human cervical cancer epithelial cells) (American Type Culture Collection, Rockville, MD).

Cell lines were cultivated in $25cm^2$ culture flasks (TPP, Switzerland, Europe), with Dulbecco Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY), containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and gentamycin (10μg/mL) (GIBCO, Grand Island, NY), and were incubated at 37°C, in 5% CO_2 atmosphere (SANYO MCO-15A, Japan).

Formaldehyde, formocresol and tricresol formalin *in vitro* cytotoxicity test

Cytotoxicity of formaldehyde (FA) (Nuclear, Diadema, SP, Brazil), formocresol (FC) (Biodinâmica, Ibiporã, PR, Brazil) and tricresol formalin (TC) (Diodinâmica, Ibiporã, PR, Brazil) was tested in NIH3T3, Hep2 and HeLa cells (Table 1). The formaldehyde was diluted in DPBS (Dubecco's phosphate-buffered saline solution) (GIBCO, Grand Island, NY) to a concentration of 19%, in order to be used in the same concentration as it is found in the original formulation of the test products.

Cells were grown in 96-well microplates (TPP, Switzerland, Europe) at a density of $5X10^4$ cells, with 100μl of DMEM containing 10% FBS and 10μg/mL gentamycin. After 24 hours incubation, culture media was discarded and the test

product was added (50µL). Incubated cells were exposed to the test products during 1 (t1), 2 (t2), 3 (t3), 4 (t4) and 5 (t5) minutes. After the exposure time, the product was aspirated and culture media was added. Cells were incubated at 37°C and 5% of CO_2 during test times. Cell cultures treated with no product were used as controls. After incubation for 24 hours (24h), 48 hours (48h) and 7 days (7d), cells were observed under optical microscopy to evaluate cell morphology.

MTT Assay

Cell viability was evaluated by the MTT assay. It is based on the ability of the mitochondrial enzyme succinate dehydrogenase to convert the yellow water-soluble tetrazolium salt 3-[4,5-dimethyl-2-thiazolyl]–2,5–diphenyl-2H-tetrazolium bromide (MTT) into formazan crystals in metabolically active cells. This water-insoluble, dark-blue colored product is stored in the cytoplasm of cells, and is solubilized afterwards, generating a blue color, which is directly proportional to the amount of metabolically active cells.

After the appropriate incubation period (24h, 48h, 7d), 200µg of MTT (Sigma, St. Louis, USA) was added to each well of tested cells, followed by 4 hours incubation at 37°C and 5% CO_2. Medium was than removed and formazan crystals were solubilized with 120µL per well of dimethyl sulfoxide (DMSO, Henrifarma, São Paulo, SP, BR), generating a blue colour. Optical density was read at a wavelength of 550nm (microplate reader, BIORAD, Japan) [8].

A variety of assays can be used to evaluate cellular viability, each of them with its own properties. In this study, the MTT test was used due to its simple

execution, accessible costs and objective results. The reading of optical density in the end of the test is proportional to the cellular viability: the bigger the optical density, the bigger the number of viable cells, and, therefore, smaller toxicity of tested product [8].

Statistical analysis

Statistical analysis was performed using Statistical Analysis System statistical (SAS) software, version 6.08. Results were analyzed by one-way ANOVA and Tukey test. Statistical significance was considered for values of $P < 0.05$ (95%CI).

RESULTS

The analysis of cellular viability for Hep2 cell line, when treated for 1, 2, 3, 4 or 5 minutes with formocresol (FC), tricresol formalin (TC) or formaldehyde (FA) at different post treatment incubation times (24h, 48h or 7days), showed a reduction in cell viability in all tested conditions, when compared to the non treated controls (Table 2). The results observed in different treatment times showed distinct effects on cell viability (Figure 2). Comparing the effect of different products in different times of treatment and post treatment incubation on Hep2 cells, a statistically significant lower toxic effect was observed for FC ($p<0.005$) (Table 2).

The analysis of FA treatment on Hep2 cells in different times showed a difference of cytotoxic effect depending on the post treatment incubation time. When incubation was 24 hours, there was a direct proportional increase in cytotoxic effect with time exposition; in 48h, an important, but not significant, difference was observed in the toxic effect (Figure 2).

When NIH3T3 cells were tested, also a reduction in cell viability in all tested conditions was observed, when compared to the non treated control (Table 3).

A different effect on cell viability was found for the treatment times used (Figure 3). The analysis of cell treatment with TC on NIH3T3 cells showed a significant difference in toxicity on cells incubated 24 hours post treatment, with the lowest toxicity observed at 1 minute time of cells contact with TC. All other conditions tested showed no significant differences (Figure 3).

The experiments performed with NIH3T3 cells, showed a statistically significant lower cytotoxic effect for FC in relation to TC and FA, in all tested

conditions. The cytotoxic effect of TC was significantly smaller than FA effect only at 1 minute exposure time (Table 3).

Formaldehyde treatment over NIH3T3 cells showed different results. When cells incubated 24 hours or 7 days post treatment were analyzed, no significant difference in cytotoxicity was observed at different treatment times. however, with cells incubated for 48 hours after treatment, a significant difference was observed i.e. being 3 minutes the lowest toxic time period and 1 minute the most toxic period (Figure 3).

Treatment of HeLa cells with test products also resulted in a reduction of cell viability in all conditions, when compared to the non treated control (Table 4). Cell viability was differently affected by distinct treatment times (Figure 4).The experiment with HeLa cells did not show significant difference in cytotoxicity with FA and TC in the different tested conditions.

However, the treatment with FC resulted in significant difference regarding toxicity, among treatment times in all post treatment incubation conditions, being 1 minute the most toxic time. The less cytotoxic groups were: for 24 hours incubation post treatment the 3 minutes time, for 48 hours the 4 minutes time and for 7 days the 4 and 5 minutes time. The toxic effect observed for FC on HeLa cells was the lowest among the three test products (Table 4, Figure 4).

DISCUSSION

According to Osorio et al, the toxicity of materials used in dental practice is accessed using a three-step approach. A first step is to screen a candidate material using a series of *in vitro* cytotoxicity assays. Then, if the material is determined not to be cytotoxic *in vitro*, it can be implanted in subcutaneous tissue or muscle and the local tissue reaction evaluated. Finally, the *in vivo* reaction of the target tissue *versus* the test material must be evaluated in human subjects or animals [9].

The results on *in vitro* cytotoxicity tests of materials used in dentistry may reflect the effect observed in living tissues [9]. Cell cultures have advantages over animal experimentation since they afford highly defined culture conditions thereby avoiding the complex homeostatic mechanisms that occur *in vivo* [10]. The European Committee for Standardization is a competent organ for the adoption of harmonized standards for biological devices, including dental materials. The committee encourages the increasing study and use of *in vitro* methods so that these can be adopted as standards, thereby minimizing the need for *in vivo* assessments [11]. Studies using established cell lines and primary cell cultures concluded that the use of established cellular lines for cytotoxicity tests is recommended, as they are more resistant to culturing and treatment with cytotoxic agents [12, 13].

In present study it was evaluated the *in vitro* cytotoxicity of 3 antiseptical products used routinely in root canal procedure, FA, TC and FC, in three different established cell lines (NIH3T3, Hep2 and HeLa) with the MTT cytotoxicity assay.

NIH3T3 cells are a fibroblast lineage similar to cells present in dental pulp and surrounding tissues. Hep2 cells are of epithelial origin from larynx carcinoma and they regard oral mucosa cells. The HeLa cells come from cervix carcinoma, and also resemble oral mucosa. Therefore, cell lines used in this experiment are representative of cells exposed to the process of root canal asepsis. Toxicity profile of different test products varied among each other and among tested cell lines. This variation could be explained by the different characteristics of cells, from biologic origin to ability to grow *in vitro*.

In the endodontics practice, the FC and TC are used directly into the root canal, with the aim of asepsis, due to their excellent antimicrobial activity. The TC is used as a disinfectant in the therapy of root canal with pulp necrosis and incomplete biomechanical instrumentation. The FC acting as antiseptic and cell fixative agent is maintained in contact with the pulp of the primary teeth during 5 minutes. However, the formaldehyde present in the formulation of these products (19%) exerts a toxic effect on cells of the pulp and surrounding tissues [14]. Formaldehyde is a very reactive chemical, and its cytotoxic, mutagenic, carcinogenic and pro-allergenic potentials are well known and have been of concern also in dentistry [6, 7].

A clinical study conducted in dogs' teeth by Garcia-Godoy suggested that the activity of formocresol during a shorter exposure (one minute) could be satisfactory and even superior to the so far recommended 5 minutes [15]. In the present study, TC, FA and FC were tested at 1, 2, 3, 4 and 5 minutes of contact *in vitro* with 3 established cell lines, and kept in culture for 24h, 48h and 7 days. When cell viability was tested, among all products and all conditions, the 1 minute

exposure times revealed the lowest number of cells. Considering that cells directly affected with formaldehyde toxic effect would dye and thus not recover the proliferation ability, we could speculate that cells surviving after FA treatment could suffer important alterations, maybe mutations, which would result in a higher proliferation rate. This could be a reasonable explanation, which remains to be investigated, once the carcinogenic potential of FA is well known.

The cytotoxic effect observed in all tested conditions for FC on Hep2 cells was lower than the observed for FA and TC. This data is in accordance with the proposal of FC formulation, which is based on the reduction of toxicity with the addition of orto-cresol [16].

In a study conducted by Nilsson et al., the result showed a significantly higher FA toxicity in fibroblasts than in epithelial cells [17]. In our study, a similar results regarding cytotoxicity was observed for cells of fibroblast and epithelial origin. These conflicting results are probably related to the fact that Nilsson et al. used fibroblast primary culture and HeLa cells in their study, while in our study we used a fibroblast established cell line (NIH3T3) and HeLa cells.

Cytotoxicity observed for treatment with FC on NIH3T3 cell was the lowest among test products. Besides, in all incubation times there was a significant difference between treatment times, with the highest toxicity observed at 1 minute contact. The presence of orto-cresol in the formulation of TC and FC might have contributed for this decreased toxic effect, especially in the case of FC (Figure 3).

In our study, all test products were cytotoxic for the different cell lines tested, in all conditions. Previous studies, investigated toxicity of formaldehyde and cresol (compounds present in the formulation of FC and TC) in mammal cells, testing cell

viability immediately after 24 hours exposure time, and reported a toxic effect of both products [18, 19]. Besides the different conditions used, their results are in accordance with ours.

The cytotoxicity of FC on established cell lines was investigated in other studies which observed a high rate of cell death [20, 21]. In our study, we also detected a cytotoxic effect of FC, even in the shorter exposure times, but lower than those observed for TC and FA.

For each cell line and culture time (24h, 48h, 7d) mock controls were used, consisting of cells submitted to all manipulations, but treated with no product. Cell viability of mock was considered as reference for products cytotoxicity effect. The 24h and 48h mock cells showed much higher cell viability than treated cells, FC being the least toxic product. In the 7 days mock cells, a high rate of cell death was observed, probably due to inhibition contact or lack of nutrients, once incubation media was not replaced nor subcultures performed during the incubation period.

Our results indicated that all antimicrobial endodontics products evaluated were toxic in all different tested cell lines, with FC presenting the lowest cytotoxicity. Therefore, care must be taken when using these chemical agents in dental practice to minimize their possible adverse effects on human health [18]. The ideal endodontic drug should be bactericidal to all organisms found in the root canal but must be nontoxic to the periapical tissues. Since the ideal drug has not been discovered, a drug that most nearly approaches the ideal should be selected [20]. Reports on *in vitro* cytotoxicity testing of materials used in endodontics are relatively poor in the literature, and sometimes may not reflect the *in vivo* situation. Therefore, further investigations on endodontics materials are still necessary to find

the ideal material that would fulfill all the properties needed for endodontics materials.

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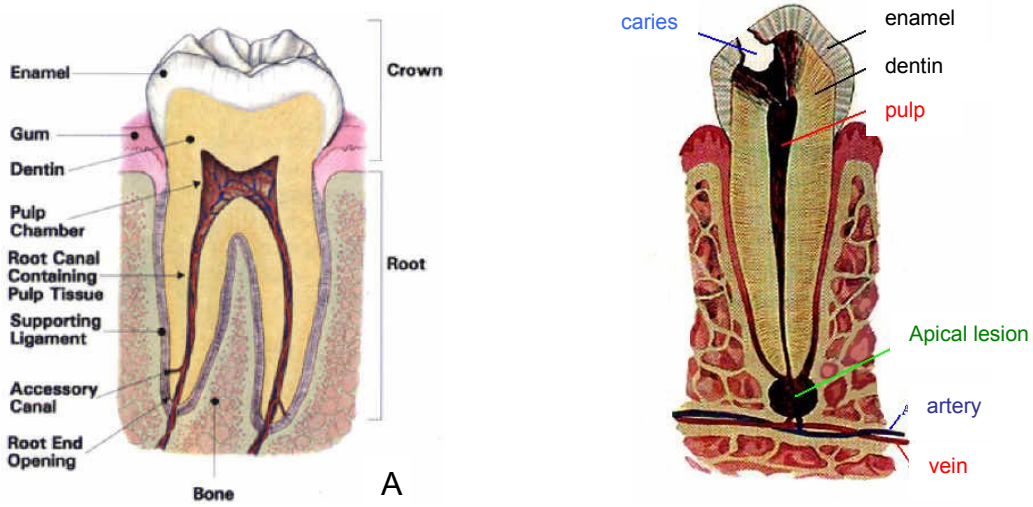
Table 1 – Composition and manufacturers of the tested products

	Test products		
	FC: formocresol	TC: tricresol formalin	FA: formaldehyde
Manufacturer	Henrifarma, Ibiporã, PR, BR	Henrifarma, Ibiporã, PR, BR	Nuclear, Diadema, SP, BR
Presentation	Liquid	Liquid	Liquid
Ingredients	Formaldehyde 19% orto-cresol 35% Glycerin Absolute ethyl alcohol	Formaldehyde 19% orto-cresol 17% Bidistilled water Absolute ethyl alcohol	Formaldehyde 38% solution P.A.



Picture 1: Schematical drawings showing to the diverse structures of the tooth (A) and a apical injury (B)

Table 2 – Cellular viability of Hep2 cell line incubated for 24 hours, 48 hours and 7 days after treatment with FA, TC and FC

Treatment time (minutes)	Test product (OD)								
	24 horas			48 horas			7 dias		
	FA	TC	FC	FA	TC	FC	FA	TC	FC
1	^{B} 0,023	^{B} 0,024	^{A} 0,907	^{B} 0,029	^{B} 0,043	^{A} 0,099	^{B} 0,049	^{B} 0,045	^{A} 0,433
2	^{B} 0,020	^{B} 0,036	^{A} 0,812	^{B} 0,030	^{B} 0,049	^{A} 0,091	^{B} 0,043	^{B} 0,045	^{A} 0,522
3	^{B} 0,016	^{B} 0,036	^{A} 0,880	^{B} 0,024	^{B} 0,043	^{A} 0,095	^{B} 0,044	^{B} 0,049	^{A} 0,477
4	^{B} 0,015	^{B} 0,037	^{A} 0,714	^{B} 0,063	^{B} 0,053	^{A} 0,110	^{B} 0,048	^{B} 0,050	^{A} 0,473
5	^{B} 0,016	^{B} 0,093	^{A} 0,942	^{B} 0,062	^{B} 0,051	^{A} 0,108	^{B} 0,051	^{B} 0,062	^{A} 0,507

FC: Formocresol; TC: tricresol formalin; FA: formaldehyde; OD: optical density. OD of controls: 24h=1.609, 48h=1.109, 7d=0.682; *p*=0.0001 (Tukey Test). Numbers represent optical density average of different experiments. Letters (A and B) reefer to statistical significance of cytotoxic effect observed for all tested products (FA, TC, FC) in each incubation time (24h, 48h, 7d). Same letter indicates no statistically significant difference of toxicity between treatment times in each product; different letters indicate statistically significant difference.

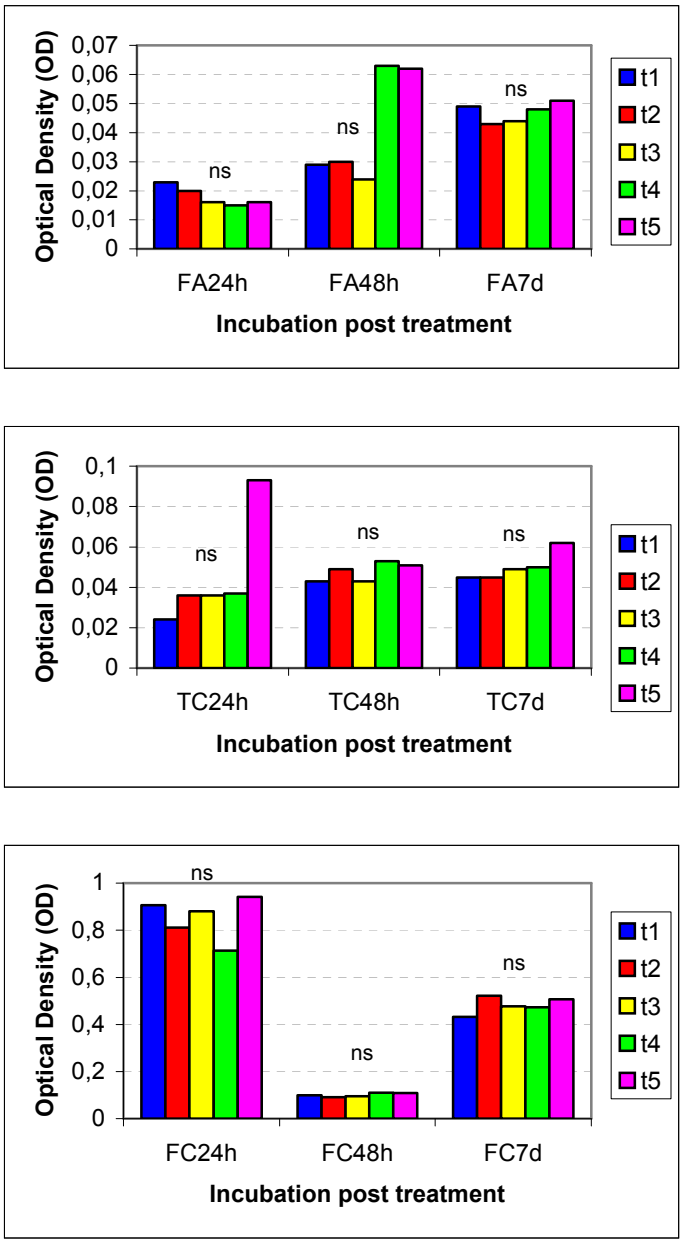


Figure 2 – Cell viability of Hep2 cell line in different times of treatment (1, 2, 3, 4 and 5 minutes) for each test product: FA (formaldehyde), TC (tricresol formalin) and FC (formocresol), and incubated for 24 hours, 48 hours and 7 days post treatment. *p*=0,999 (Tukey Test). ns: statistically non significant difference.

Table 3 - Cellular viability of NIH3T3 cell line incubated for 24 hours, 48 hours and 7 days after treatment with FA, TC and FC

Treatment time (minutes)	Test product (OD)								
	24 horas			48 horas			7 dias		
	FA	TC	FC	FA	TC	FC	FA	TC	FC
1	^{C} 0,026	^{B} 0,057	^{A} 0,328	^{B} 0,017	^{B} 0,051	^{A} 0,160	^{B} 0,021	^{B} 0,027	^{A} 0,269
2	^{B} 0,022	^{B} 0,052	^{A} 0,515	^{B} 0,018	^{B} 0,042	^{A} 0,360	^{B} 0,019	^{B} 0,030	^{A} 0,381
3	^{B} 0,021	^{B} 0,042	^{A} 0,704	^{B} 0,024	^{B} 0,054	^{A} 0,476	^{B} 0,018	^{B} 0,027	^{A} 0,536
4	^{B} 0,026	^{B} 0,051	^{A} 0,679	^{B} 0,024	^{B} 0,045	^{A} 0,593	^{B} 0,019	^{B} 0,029	^{A} 0,491
5	^{B} 0,050	^{B} 0,050	^{A} 0,588	^{B} 0,023	^{B} 0,055	^{A} 0,602	^{B} 0,028	^{B} 0,025	^{A} 0,464

FC: Formocresol; TC: tricresol formalin; FA: formaldehyde; OD: optical density. OD of controls: 24h=0.952, 48h=0.660, 7d=0.059; *p*24h=0.001, *p*48h=0.0001, *p*7d=0.0001 (Tukey Test). Numbers represent optical density average of different experiments. Letters (A, B and C) reefer to statistical significance of cytotoxic effect observed for all tested products (FA, TC, FC) in each incubation time (24h, 48h, 7d). Same letter indicates no statistically significant difference of toxicity between treatment times in each product; different letters indicate statistically significant difference.

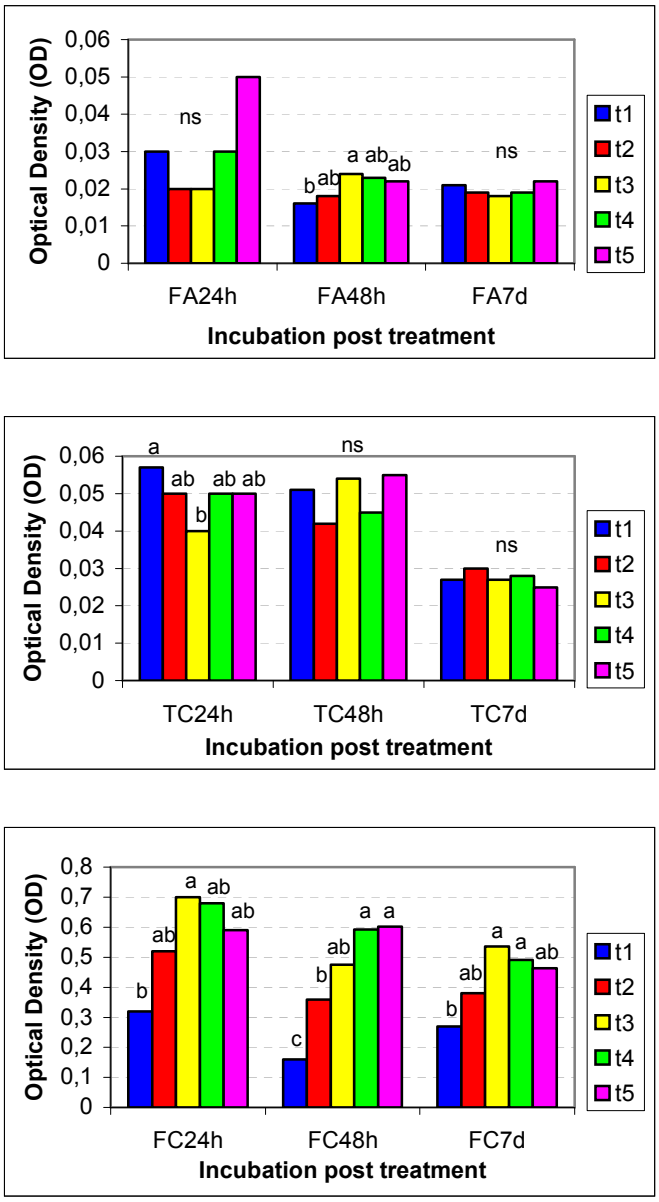


Figure 3 – Cell viability of NIH3T3 cell line in different times of treatment (1, 2, 3, 4 and 5 minutes) for each test product: FA (formaldehyde), TC (tricresol formalin) and FC (formocresol) and incubated for 24 hours, 48 hours and 7 days post treatment. *p*FA24h=0.459; *p*FA48h=0.050; *p*FA7d=0.863; *p*TC24h=0.048; *p*TC48h=0.415; *p*TC7d=0.814; *p*FC24h=0.043; *p*FC48h=0.001; *p*FC7d=0.038 (Tukey Test). ns: statistically non significant difference. Letters (a, b and c) correspond to statistical significance analysis of cytotoxic effect of tested products (FA, TC, FC) in each incubation time (24h, 48h, 7d). Same letter indicates no statistically significant difference of toxicity between treatment times in each product; different letters indicate statistically significant difference.

Table 4 – Cellular viability of HeLa cell line incubated for 24 hours, 48 hours and 7 days after treatment with FA, TC and FC

Treatment time (minutes)	Test product (OD)								
	24 horas			48 horas			7 dias		
	FA	TC	FC	FA	TC	FC	FA	TC	FC
1	^{B} 0,019	^{B} 0,008	^{A} 0,156	^{B} 0,018	^{B} 0,036	^{A} 0,306	^{B} 0,044	^{B} 0,049	^{A} 0,392
2	^{B} 0,026	^{B} 0,006	^{A} 0,234	^{B} 0,017	^{B} 0,044	^{A} 0,390	^{B} 0,038	^{B} 0,048	^{A} 0,501
3	^{B} 0,018	^{B} 0,009	^{A} 0,591	^{B} 0,013	^{B} 0,035	^{A} 0,453	^{B} 0,057	^{B} 0,044	^{A} 0,459
4	^{B} 0,019	^{B} 0,008	^{A} 0,477	^{B} 0,015	^{B} 0,038	^{A} 0,517	^{B} 0,041	^{B} 0,053	^{A} 0,579
5	^{B} 0,021	^{B} 0,005	^{A} 0,553	^{B} 0,012	^{B} 0,035	^{A} 0,347	^{B} 0,038	^{B} 0,054	^{A} 0,621

FC: Formocresol; TC: tricresol formalin; FA: formaldehyde; OD: optical density. OD of controls: 24h=0.922, 48h=0.738, 7d=0.070; *p*24h=0.001, *p*48h=0.0001, *p*7d=0.0001 (Tukey Test). Numbers represent optical density average of different experiments. Letters (A and B) reefer to statistical significance of cytotoxic effect observed for all tested products (FA, TC, FC) in each incubation time (24h, 48h, 7d). Same letter indicates no statistically significant difference of toxicity between treatment times in each product; different letters indicate statistically significant difference.

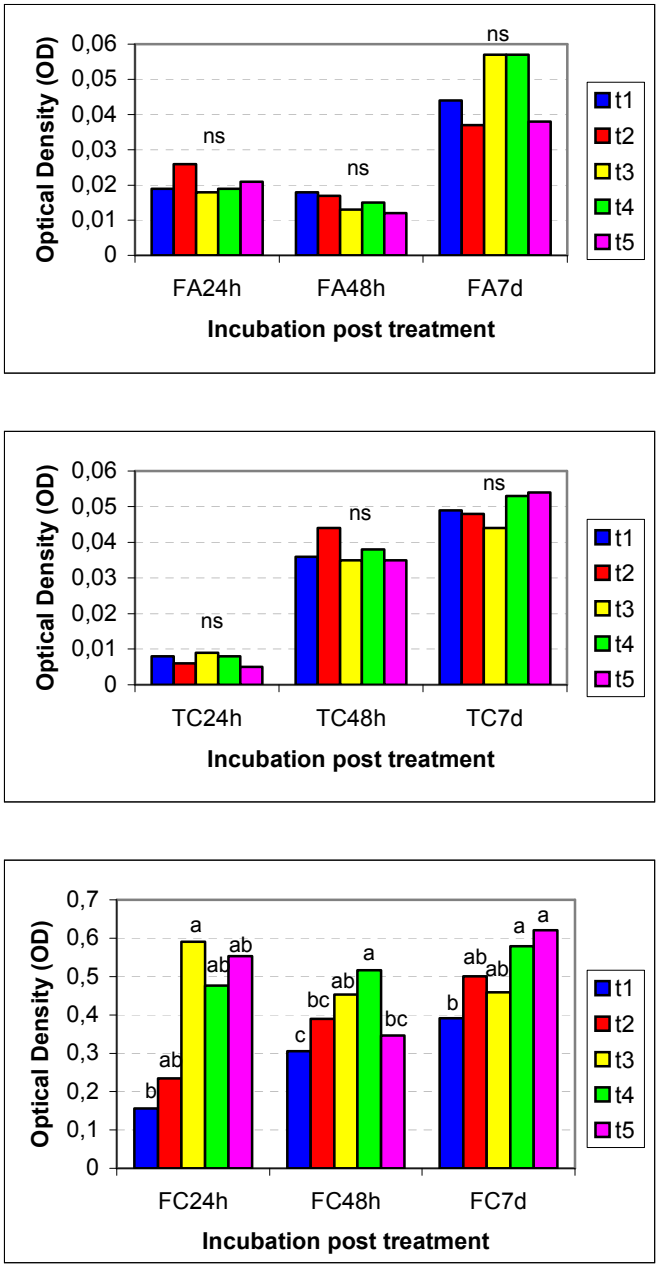


Figure 4 – Cell viability of HeLa cell line in different times of treatment (1, 2, 3, 4 and 5 minutes) for each test product: FA (formaldehyde), TC (tricresol formalin) and FC (formocresol) and incubated for 24 hours, 48 hours and 7 days post treatment. *p*FA24h=0.191; *p*FA48h=0.125; *p*FA7d=0.406; *p*TC24h=0.144; *p*TC48h=0.823; *p*TC7d=0.174; *p*FC24h=0.043; *p*FC48h=0.0042; *p*FC7d=0.033 (Tukey Test). ns: statistically non significant difference. Letters (a, b and c) correspond to statistical significance analysis of cytotoxic effect of tested products (FA, TC, FC) in each incubation time (24h, 48h, 7d). Same letter indicates no statistically significant difference of toxicity between treatment times in each product; different letters indicate statistically significant difference.

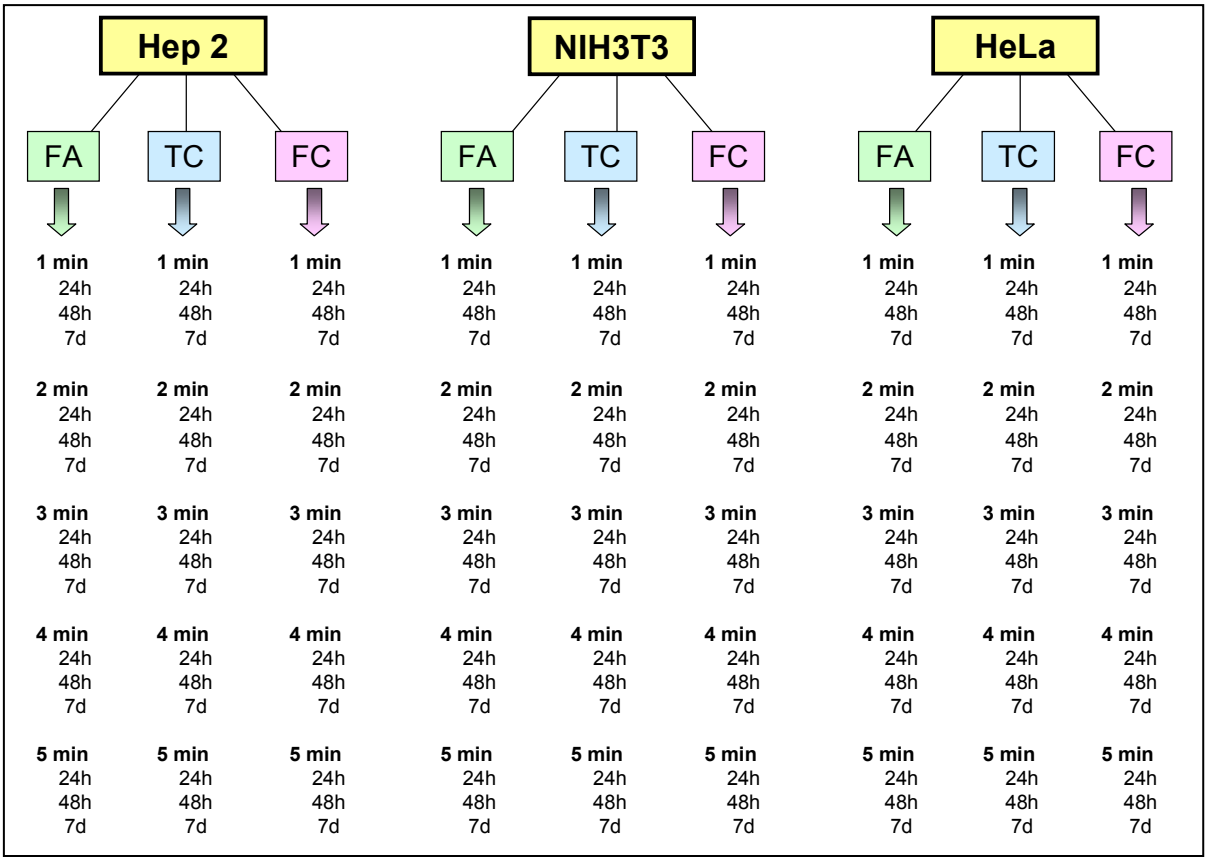
CONSIDERAÇÕES FINAIS

A proposta deste estudo foi avaliar a toxicidade de produtos usados na endodontia para desinfecção do canal radicular: formocresol, tricresol formalina e formaldeído, em nível celular, através de testes de citotoxicidade em linhagens celulares estabelecidas. A partir dos resultados obtidos, é possível concluir que:

- O formocresol, o tricresol formalina e o formaldeído apresentaram citotoxicidade quando testados na linhagem celular Hep2 (células epiteliais de carcinoma de laringe humano)
- O formocresol, o tricresol formalina e o formaldeído apresentaram citotoxicidade quando testados na linhagem celular NIH3T3 (fibroblastos de camundongos)
- O formocresol, o tricresol formalina e o formaldeído apresentaram citotoxicidade quando testados na linhagem celular HeLa (células originárias de câncer cervical humano)
- O formocresol, o tricresol formalina e o formaldeído foram citotóxicos nos diferentes tempos de exposição testados (1, 2, 3, 4, e 5 minutos), nas três linhagens celulares (Hep2, NIH3T3, HeLa)
- O efeito citotóxico do formocresol, do tricresol formalina e do formaldeído permaneceu após 24 horas, 48 horas e 7 dias de incubação pós-tratamento, nas três linhagens celulares testadas (Hep2, NIH3T3, HeLa)

- O formocresol foi o produto que apresentou menor citotoxicidade, em todas as condições testadas, sendo a diferença de toxicidade estatisticamente significante quando comparada com o tricresol formalina e o formaldeído.

ANEXOS


Hep 2
NIH3T3
HeLa
FA
TC
FC
1 min
24h
48h
7d
2 min
24h
48h
7d
3 min
24h
48h
7d
4 min
24h
48h
7d
5 min
24h
48h
7d

Anexo 2: Especificações da linhagem celular HeLa utilizada (Fonte: ATCC)

HeLa **(human, Black, cervix, carcinoma, epitheloid)**

IZSBS BS TCL20
Morphology: epithelial-like
Species: human, Black female 31 years old; Tissue: cervix; Tumor: carcinoma, epitheloid
Properties: antitumour testing; transformation; tumorigenicity; cytotoxicity; cell biology; bacterial invasiveness; virology; Susceptible to: adenovirus 3, measles, poliovirus 1, echovirus, vaccinia, arbovirus, respiratory syncytial virus, reovirus 3, rhinovirus, Coxsackie

Available in the following LABORATORY:

- Istituto Zooprofilattico Sperimentale (IZSBS, Brescia)
  continuous culture, grown as monolayer; MEM (EBSS) + 10% FBS; 37C, 5% CO2
  Further information
  Freezing medium: Culture medium + 10% Glycerol; mycoplasma negative, culture
  Karyology: aneuploid
  tumorigenic in nude mice

Availability in cell line catalogues: ATCC CCL 2; ECACC 85060701; DSMZ ACC 57; ICLC HTL95023;

Anexo 3: Especificações da linhagem celular Hep2 utilizada (Fonte: ATCC)

Hep 2 **(human, Caucasian, larynx, carcinoma, epidermoid)**

IZSBS BS TCL 23
Morphology: epithelial-like
Species: human, Caucasian male 56 years old; Tissue: larynx; Tumor: carcinoma, epidermoid; Validated by isoenzymes: confirmed as human with NP, G6PD, PEPB, AST, LD
Depositor: obtained from Centro Virus Respiratori, Roma (I)
Properties: virology; expressing xenobiotic metabolising enzymes; Susceptible to: adenovirus 3, poliovirus 1, herpes simplex, vesic. stomatitis (Indiana), respiratory syncytial virus

Available in the following LABORATORY:

- Istituto Zooprofilattico Sperimentale (IZSBS, Brescia)
  continuous culture, grown as monolayer; MEM (EBSS) + 10% FBS; 37C, 5% CO2 Passages: 363
  Further information
  Freezing medium: Culture medium + 10% DMSO; mycoplasma negative, culture
  Karyology: 2n = 46 in 47 cells
  tumorigenic

Availability in cell line catalogues: ATCC CCL 23; ECACC 86030501;

Bibliographic references:

- Cancer Res 1955;15:598
- J Clin Microbiol 1990;28:1049 - PMID: 2161864
- J Gen Virol 1983;64:825 - PMID: 6834007
- Virology 1969;38:42 - PMID: 4306525

Anexo 4: Especificações da linhagem celular NIH3T3 utilizada (Fonte: ATCC)

NIH 3T3 **(mouse, NIH Swiss, embryo)**

ECACC 93061524
Morphology: Fibroblast
Mouse Swiss NIH embryo contact inhibited
Depositor: Obtained from ATCC, USA
No restrictions. Patent: None Specified By Depositor
Properties: Applications: DNA transfection studies

Available in the following LABORATORY:

- CAMR Centre for Applied Microbiology & Research (ECACC, Salisbury, Wiltshire)
  DMEM + 2mM Glutamine + 10% Calf Serum (CS). Split confluent cultures 1:2 to 1:6 i.e. seeding at 2-5x10,000 cells/cm2 using 0.25% trypsin/EDTA; 5% CO2; 37C. Do not allow culture to become fully confluent; the use of fetal calf serum is not recommended. Passages: 136
  Hazard: CY
  Established from a NIH Swiss mouse embryo. These cells are highly contact inhibited and are sensitive to sarcoma virus focus formation and leukaemia virus propagation.

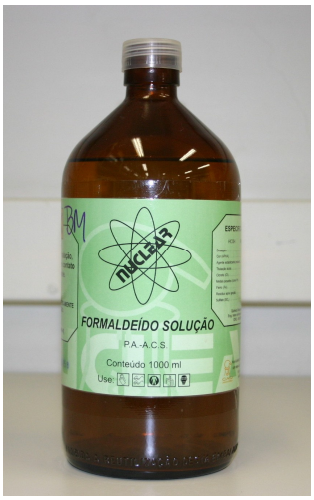
Availability in cell line catalogues: ATCC CRL 1658; DSMZ ACC 59;

Bibliographic references:

J Virol 1969;4:549; Cell 1979;16:63; Cell 1979;16:347

Anexo 5: Produtos utilizados nos testes de viabilidade celular

Formaldeído (FA)



Formocresol (FC)

Tricresol formalina (TC)