

Theme IV

Reduction and replacement of animal use in science and industry

103961

Hemolysis vs protein denaturation on the RBC assay to predict ocular irritation of surfactants

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Red blood cells (RBC), ubiquitous and really available, have a long scientific history of use in the study of plasma membranes lysis. The RBC test was developed to assess initial cellular reactions to irritation caused by certain chemicals. Certain classes of chemical irritants (mainly surfactants) will cause damage to cell plasma membrane and/or denaturation of various types of proteins. Such reactions can be correlated with the initial events in eye tissue irritation. Numerous studies have been performed, which finally led to feasible in vitro tests with the red blood cells reported in two different Invitox protocols. In the present study we used the Invitox protocol 37, which calculates the lysis/denaturation ratio and compares with the in vivo rabbit eye data as a prediction model. Different surfactants were studied and compared based on hemolysis and protein denaturation. Classification of the surfactants in different categories from non irritants to irritants showed contradictory results depending on the variable analysed (hemolysis, protein denaturation and/or their ratio). Thus, anionic surfactants that could be considered low irritants based only on their hemolytic activity, showed high protein denaturation. By contrast, cationic surfactants presented higher hemolytic activity but lower protein denaturation. When the hemolytic effect was related to the chain length, we fail to find a linear relationship between hemolysis and hydrophobicity. The chain length only affects irritancy in the same family. From our results the RBC assay appears a better reliable model to classify our surfactants when both parameters, hemolytic activity and protein denaturation, were considered.

KEYWORDS: eye irritation; surfactants; red blood cells; Draize test

105244

Characterization of nanovesicles for in vitro toxicity assessment: role of aggregation state and chemical composition on the cellular response

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Risk assessments using in vitro methods are essential for understanding the mechanisms of biological influences induced by nanomaterials. However, these colloidal systems should be fully characterized for an accurate toxicological evaluation. Here, we developed nanovesicles containing different cationic lysine-based amphiphiles, and examined the effects of these surface modification agents on their physicochemical and in vitro cytotoxic properties.

Hydrodynamic diameter was measured by DLS in both ultrapure water and cell culture medium, and the readings were taken immediately after nanovesicle preparation and after 24h incubation at 37°C. Zeta potential and polydispersity index were also determined, together with morphology and size analysis by TEM. Evaluation of cellular response was performed in 3T3 and HeLa cells by means of MTT, NRU and LDH endpoints.

DLS measurements showed that fresh prepared nanovesicles dispersed in water have hydrodynamic size between 90-255 nm. When dispersed in cell culture medium, size increase was slight by 0h, but significant agglomeration occurred after 24h incubation at 37°C. Zeta potential values of nanovesicles dispersed in water were highly positive, whereas almost neutral values were obtained in cell culture medium. We demonstrated the cell-specific nature of nanovesicle toxicity as well as different toxic responses when in vitro endpoint varied. The specific cellular responses depended on the amphiphile and on the agglomeration state of the nanomaterials in cell culture medium. We conclude that behavioral information of each nanomaterial type is essential for in vitro evaluation of its biological effects, since each nanocarrier shows unique physicochemical properties.

KEYWORDS: nanovesicle; in vitro nanotoxicity; cationic lysine-based amphiphiles; cell culture; hydrodynamic size



105959

The reutilization of the rabbits used to prove biological products in the pyrogens test. An alternative for the animals reduction

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The pyrogens and the endotoxins (ET) taken place by the Gram-negative bacteria they are a growing concern in the biotechnical industry. There was known from end of the XIX century with the appearance of the hypodermic needle when some injectable solutions caused increments of the corporal temperature in treated people, of unknown origin. The endotoxins are very toxic substances for the mammals cells and one of the most potent modulators of the immunologic system. The collateral effects, product of the man's contact with pyrogenic substances, still represent a threat for the patients. They are activated by the contact with attenuated pathogens micro organisms or their undone and it can induce fever, dysfunctions in the circulation and organic failure, or even a fatal outcome. The presence of new biological products, product of the advances in the nano technology and the gene and cellular therapy, it puts to the scientific community in the search of new detection methods, developing several investigation projects in assays methods in vitro that mimic the fundamental phase of the human feverish reaction. In the world a more and more strong movement is developed in the search of alternative to the use of the laboratory animals in the biological assays that replaces, reduce or refine the methods and number of the same ones in the rehearsals. The demand of necessary animals for the realization of this test is high and it has not been satisfied during this period by the suppliers. With the objective of giving answer to the one climbed productive happened in our institution, it has been studied, in the laboratory, the possibility of the reutilization of the rabbits used to test different biological products in the pyrogens rabbits test that allows to get a formulation sequence, filling and adapted container, leaving of the reliable reutilization of the rabbits in this assay applying different biological products. For these we evaluated a rabbits pyrogens test for four different biological products, following the US Pharmacopoeia. After the that the 8 animals were challenged with a Secondary International Standard of E.coli with 1 ng/ml. Any rabbit shows temperature rise after to be used in the test after receive different biological products, the challenged rabbits show temperature rise. It is possible to reduce the number of rabbits in the pyrogens test reusing the rabbits when we prove biological products.

KEYWORDS: Rabbits pyrogens test; Reductions; Biological products

106211

Development and validation of an immunochemical method for the determination of neutralizing antibodies of the component *Bothrops alternatus* in antivenom produced in Argentina

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Snake antivenom is the only effective treatment in case of envenomation. Therapy depends on the ability of the antivenom to neutralize the lethal toxins injected following snakebite accident. The key in order that it could be administered, is the quality control of the immunotherapeutic, both during the development process and in the final product, provided that they must meet two requirements: they must be effective and safe. In the present study, two technologies were applied for the evaluation of the component *Bothrops alternatus* of the bothropic antivenom formulated in Argentina. On one hand, we developed an ELISA method, to assess the neutralizing antibodies of *B.alternatus* in the homologous antivenom. First, the above mentioned methodology of agreement was validated for the parameters of validation selected, and then was compared the in vitro method developed with the alive method of reference that is realized in mice. In the studied conditions of validation, was verified a coefficient of variation of 15% demonstrating that the system is precise and that recovery range between 81% and 125% indicating the accuracy of the test. The applicability of the method candidate compared with the method of reference was studied with samples available in the laboratory. By the first time there was obtained, in the above mentioned conditions, a coefficient of correlation based on the linear regression of 0,877. Our results demonstrated that with the evaluated parameters, the technology immunochemistry developed can be used as complementary method to the in alive method.

KEYWORDS: *Bothrops alternatus*; Antivenom; ELISA; Validation; Argentina



106270

Cuban experience on Monocyte Activation Test (MAT) for biological products

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Biologicals, such as protein solutions and some vaccines, raise challenges for pyrogenicity test. Some of them can interfere with LAL or be immunogenic in rabbits. Monocyte Activation Test (MAT) has emerged as a promising alternative for pyrogen control, which has been adopted by European Pharmacopea since 2010, though it is not regulated for a particular product yet. The FDA accepted MAT as a substitute for endotoxin test in parenteral drugs on a case-by-case basis. Our group has evaluated the performance of MAT in biologicals such as 20% Human Serum Albumin, and Diphtheria-Tetanus -Pertussis (DPT) and Neisseria meningitidis vaccines. Both whole blood and cryopreserved blood were used as monocyte sources. The results were compared with LAL and Rabbit test (RPT). In the case of HSA, it was demonstrated that glucans contamination derived from cellulose filters induced a synergistic effect on the IL-6 response to LPS, which could promote harmful effects in patients. Similar to HSA, higher endotoxin equivalent units with IL-6 as readout were obtained in DPT vaccines, probably because the antigens induce more secretion of IL-6 in comparison with IL-1B. Conversely to a previous report, we found that Pertussis antigen was the only responsible for cytokines secretion in DPT vaccine. Conversely to HSA, the results obtained with LAL and MAT in DPT and Neisseria vaccines were similar. Taking into account the results obtained, MAT is a suitable assay to evaluate the pyrogenic and immunomodulating response of such biologicals, which overcomes the disadvantages of LAL and RPT.

106276

Combination of erythrocyte and cell line-based methods as alternative to animal models for the evaluation of potential antioxidant ingredients for topical formulations

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Skin possesses a variety of enzymes as well as small molecular antioxidants that can inhibit oxidative damage. Excessive Reactive Oxygen Species (ROS) generation can overwhelm the antioxidant defense capacity of the skin, resulting in oxidative stress and consequently in oxidative damaging of the main skin biomolecules. Oxidative stress by ROS can damage DNA, proteins or membrane lipids of the cells, and induce several skin disorders. To prevent this damage, antioxidants are frequently added into topical formulations. To study the effect of antioxidants, different models have been developed in vivo, based on the determination of different stress parameters such as superoxide dismutase and catalase in the skin of animals after stress exposition and the topical application of the potential antioxidant. To replace the use of animals to study the antioxidant properties of different extracts obtained from natural sources, we propose a combination of erythrocyte and cell line-based methods. As erythrocyte-based method, AAPH and TBARS assays evaluate different antioxidant mechanisms and are regarded as complementary. For the assessment of biocompatibility, HaCat and 3T3 cells were used in a cytotoxicity assay which showed good tolerability of the extracts. To further study their antioxidant capacity, the protective effect of the extract towards hydrogen peroxide-induced oxidative stress was also evaluated in these cells. We concluded that the combination of erythrocyte and cell line-based methods is a useful and promising battery of tests to evaluate antioxidant capacity of natural products *in vitro*.

KEYWORDS: antioxidant; topical formulations; natural products; oxidative stress



106341

Differential profile of Cyclosporine nephrotoxicity: PKC, PKA and p38 MAPK signaling involvement

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Nephrotoxicity is one of the main effects caused by Cyclosporine, a nephrotoxic immunosuppressant, which may vary among nephron segments. The present study aims to evaluate the involvement of nephrotoxicity signaling pathways and the effects caused by cyclosporine using BGM (monkey), MDCK (dog), and LLC-PK1 (pig) cell lines. The cell lines were incubated in 8 concentrations of drug for 1, 6, and 24 hours, and the cytotoxicity was evaluated through MTT and Neutral Red assays. For cell signaling studies, the specific blocking of the PKC, PKA, and p38 MAPK pathways was performed using, respectively, Calphostin C, H89, and PD169316. While in BGM cells, the blocking of PKC and p38 MAPK pathways has intensified the toxic effects of cyclosporine, in LLC-PK1 cells, cyclosporine effects were only intensified when the p38 MAPK pathway was blocked. For MDCK cells, the toxicity of cyclosporine was intensified only when the three pathways were simultaneously blocked. Therefore, the involvement of the PKC, PKA, and p38 MAPK signaling pathways in the nephrotoxicity of cyclosporine can vary depending on the region of the organ tested as it did with the cell strains assayed. Also, the present study showed the importance of the drugs screening at a molecular level using more than one cell strain as a model.

KEYWORDS: *nephrotoxicity; cyclosporine; BGM; LLC-PK1; MDCK*

106404

In vivo acute toxicological studies of mangiferin

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Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-D-glucoside) is a polyphenol of natural origin of the xanthone derivatives and C-glycosylxanthones from *Mangifera indica* L. This compound has well documented anti-oxidant, anti-diabetic, anti-HIV, and anti-cancer, properties as well as an immunomodulatory, antiallergic and anti-inflammatory effects. The pharmacological properties of mangiferin have been reported but only a few studies have investigated its toxicity. In view of the ethnomedical, preclinical, and clinical uses of this compound, and also because of the need of assessing its possible toxicological effect in man, an in vivo toxicology analysis of the mangiferin is reported in this work. Acute toxicity was evaluated in mice and rats of both genders by oral, dermal, and intraperitoneal (i.p.) administration using the method of the acute toxic classes, using 3 animals per sex in each step, and taking into account the principle of the 3 Rs. No toxicity effects were observed after oral and dermal exposition at 2000 mg/kg doses of mangiferin on Sprague Dawley rats or Balb C mice. Some transient toxicity signs like dyspnoea, abdominal position, piloerection, and reduced locomotion activity were observed after mangiferin oral and i.p. exposure. The i.p. route induced mice deaths at the highest dose. No animal death was observed when doses of 200 mg/kg were administered by i.p. route. The DL50 of mangiferin was over 2000 mg/kg of body weight in rats and mice through oral and dermal routes according to the globally harmonized system. However, the DL50 in mice in which mangiferin was administered by i.p. route ranged from 200 to 2000 mg/kg body weight. Following oral and dermal application, mangiferin had minimal toxicity.



106425

Protective effects and repair effects of the green and black tea of *Camellia sinensis* on the genetic material of *Allium cepa* bioassay

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The great world consumption of *Camellia sinensis* tea and its many benefits to human health have stimulated the implementation of this research. The aim of this study was to evaluate the protective and repair effects of green and black teas on genetic material using the *Allium cepa* bioassay as a model, observing the cell cycle, before and after exposure of roots to the alkylating agent mitomycin C (MMC - SIGMA), and the chromosomal changes frequency. The protocol consisted of two treatments: (a) immersion in green and black tea and subsequent exposure to aqueous solution of MMC (protective treatment), and (b) immersion in MMC and recovery in green and black tea (reparative treatment). Mineral water was used as a negative control (NC) in all tests. Each treatment lasted 48 hours. Mitotic index, phase index, and frequency of cell changes were analyzed in 3,000 cells per bulb, and five bulbs were used for each condition. The results were analyzed by analysis of variance at 5% probability. While the analysis of cells treated with green tea indicated only its protective effect against changes caused by MMC, no repair effects were observed under the conditions employed in this study. However, treatment of cells with black tea showed both protective and repair effects when compared to NC. The changes more frequently observed were C-metaphase, chromosomes scattered and anaphase bridges. The *Allium cepa* bioassay was effective for achieving the aim of this research.

KEYWORDS: *Antiproliferative potential; antimutagenicity; cells changes; cell cycle*

106430

Antiproliferative potential of the white tea of *Camellia sinensis* in *Allium cepa* bioassay

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White tea is an aqueous extract, obtained by the infusion of leaves and buds of *Camellia sinensis*. It has several chemicals with medicinal properties. The objective of this study was to evaluate the antiproliferative potential of white tea of *Camellia sinensis* in *Allium cepa* bioassay. The protocol consisted of two treatments: immersion in white tea and subsequent exposure to an aqueous solution of mitomycin C (MMC SIGMA) and immersion in MMC and recovery in white tea. The mineral water was used as negative control (NC) in all experiments. Each treatment with MMC and with the tea lasted 48 hours. Mitotic index, phase index and frequency of cell changes were analyzed in 3000 cells per bulb. Five bulbs were used for each treatment and 5 bulbs for NC. The results were analyzed using analysis of variance, at 5% probability level. Mitotic index was reduced when the roots were exposed to white tea for 48 hours. There was no significant difference in frequency of changes in cells when roots were treated with white tea and later with MMC, compared to the results of the CN. When roots were treated with MMC and subsequent recovery in tea, there was significant reduction in mitotic index and in frequency of changes in cells. Under the conditions used in the present study, the white tea showed potential antiproliferative, preventing abnormal cells of multiply and transmit such changes to daughter cells when roots were pretreated with MMC, and subsequently with the white tea of *C. sinensis*.

KEYWORDS: *Allium cepa; genetic material; cell cycle*



106494

Pros and cons of allometric parity for biological models under the light of new experimental necessities

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Animal models used in experimental research relate human to animal, in all its idiosyncrasies, and should always respect the inter-species particularities, ethics in research, rules and stages of research, with clear criteria and objectives. Fundamentally, these models must allow the extrapolation of results to other species. However, it is difficult to find a model that covers all aspects of the relevant translational existing between the model used and the reality. The choice of experimental models enfances the difficulty to standardize techniques and extrapolation/ comparison of results. Differences in morphology, body mass, energy metabolism and eating, reproductive and locomotion habits make such choices critical points of concern. The interspecies allometric extrapolation is based on mathematical calculations that allow the pairing of different taxonomic groups, of different sizes and body masses. To compare aerobic microorganisms, plants, invertebrates, fishes, amphibians, reptiles, birds and mammals, it is necessary to verify the metabolic rate as a function of temperature and body mass. This suggests a simple metabolic rate for organisms in function of body size and temperature. Allometric parity methods are available to perform the interrelation of biological models used in research; even so, their proper use will depend on the researcher talent to join the research aims to the actual/experimental model. Here, we present a compilation of data from several authors suggesting resources (e.g. allometric indexes) to enable extrapolation of interspecific variables, as well as their positive and negative points.

106498

Evaluation of *in vitro* cytotoxicity by different methodologies in samples submitted to extraction procedure

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In vitro cytotoxicity tests using cell culture methods have been used as an alternative to laboratory animals as an effort to achieve the three R's described by Russel and Bursch (1959). Among the used methods, the agar-diffusion assay requires that the test sample pieces are placed on a semi-solid medium. However, large, heavy and difficult to fragment samples must be submitted to an extraction procedure in culture media. The United States Pharmacopeia recommends that these extracts are immersed in filter paper and placed on agar or placed directly in contact with the cell monolayer. The objective of this study was to observe whether differences can be observed in the cytotoxic response on samples extracts when tested by both agar diffusion method or placed in direct contact with the cell monolayer. The cell line used was NCTC clone 929 and samples (adhesive labels, bone grafts, metal materials, rubber stoppers) were tested in three ways: (1) placed directly on agar, (2) extract placed directly on the cells, and (3) extract immersed in filter paper placed on agar. The results showed that most of the samples tested by the agar diffusion method have greater cytotoxic responses than their extracts tested directly onto cells. However, no cytotoxic response was observed when extracts were immersed in filter paper and then placed onto agar. These results suggest that when the sample requires extraction, the best method of evaluation is to place extracts in direct contact with the cells.

KEYWORDS: *cytotoxicity tests; alternative methods; cell culture; Agar difusion and tests on extracts*



106502

REDUCTION OF ANIMALS USED TO DETERMINE MEDIAN LETHAL DOSE IN MURINE MODEL OF *Acinetobacter baumannii*

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Acinetobacter baumannii is a Gram negative opportunist bacteria that causes pneumonie, urinary tract infections, and sepsis in immunocompromised patients. This pathogen is frequently associated with nosocomial outbreaks worldwide and has become particularly problematic in Brazil because of its prevalence and patterns of resistance to several antibiotics. In this study the 50% lethal dose (LD50) by intraperitoneal infection was determined in four groups of Balb/C mice, age 6-8 weeks, with *A. baumannii* using the Reed-Muench method. Although this method recommends the use of 10 animals per group, in our study we have used only four animals per group. As such, each group received the following doses: 1x10⁷, 5x10⁶, 1x10⁶e 5x10⁵ CFU. LD50 was estimated by the number of survived mice two days after infection. After 24 hours of intraperitoneal infection, all animals that received 1x10⁷ and 5 x10⁶ CFU did not survive, in contrast with the animals that received lower doses. As a result, the LD50 of *A. baumannii* established in this study was 1x10⁶ CFU. The reduction of the number of animals used allowed an improvement in the LD50 parameter, and also it is in accordance with the three R's principle (Reduction, Refinement and Replacement) that recommends a reduction in the number of animals in experiments, since it provides significant results. Thus, we suggest the use of this protocol to test others multi-resistant bacteria in order to achieve the LD50 values. In this way, we believe that vaccines can be developed faster. Also, this protocol would reduce the number of animals used in this model of experiment.

KEYWORDS: *dose letal; Acinetobacter baumannii; modelo murino*

106662

The use of alternative methods for rabies diagnosis in Brazil

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The use of laboratory animals is common practice but is also a source of increasing public concern. Some types of animal use can be replaced using in vitro methods, such as the use of a cell culture (CC) instead of the mouse inoculation test (MIT) for rabies diagnosis. The objective of this work was to describe methods for rabies diagnosis in Brazil in comparison to other countries, using a web forum. Between December 2011 and March 2012, 486 people working with rabies diagnosis in different countries were invited by e-mail to participate in the forum, to describe the methods they used and their reasons for using them. Thirty-five English-speaking and 12 Portuguese-speaking respondents answered the questions; 11 Portuguese-speaking respondents worked in Brazil. Six Portuguese-speaking and seven English-speaking respondents used the MIT. The high cost for introducing the CC was mentioned as a limitation by one English-speaking respondent and by five Portuguese-speaking respondents. The Brazilian Federal Act 9605/1998 states that animal experimentation is a crime when alternative methods exist. The Brazilian Health Ministry guidelines recognize that, once properly implemented in the laboratory, the CC is more economic and efficient than the MIT. The results of this study suggest the proportion of laboratories using mice to perform rabies diagnosis in Brazil is high and may conflict with Brazilian law and the Health Ministry recommendations.

KEYWORDS: *Animal welfare; Laboratory animals; Replacement*



107044

Three-dimensional pellet culture of human chondroblasts and adipose mesenchymal stromal cells: an efficient method for differentiation assays and drug testing

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The three-dimensional pellet system is an easily handled scaffold-free method that can best mimic physiological cell interactions. Here we describe a pellet culture system of human chondroblasts or adipose mesenchymal stromal cells, to establish models of chondrogenesis and adipogenesis, respectively. Inductions towards these lineages were done with specific inductive medium for up to 3 weeks. After that, pellets were fixed in 4% paraformaldehyde and photographed with a digital camera. The perimeter of pellets were determined by Axion Vision software using the bar size (micrometers) of each image as a reference for measurements. Chondrogenic differentiation was evaluated by Safranin-O staining performed in paraffin sections to detect sulphated glycosaminoglycans and by specific staining of frozen sections with anti-collagen type II. Adipogenic differentiation was evaluated by specific staining of frozen sections with Nile Red O to detect lipid droplets and anti-collagen type I. We observed an increase in the pellet perimeter as a consequence of differentiation assays. Histological analyses showed that chondrogenic induction was responsible for an increase in extracellular matrix synthesis, mostly characterized by a higher intensity of safranin-O staining. It was possible to detect high collagen type II content without chondrogenic stimulus. From pellets of adipose mesenchymal stromal cells we observed positivity for lipid droplets even under control medium. Collagen type I was strongly detected in adipogenic induced pellets. An efficient model for cartilage and adipose tissue generation in vitro can lead to alternative models for drug testing in orthopedics and aesthetic medicine. Research Ethics Committee: 145/09 and 146/09.

KEYWORDS: 3D pellet culture; chondrogenesis; adipogenesis; drug testing

107141

Correlation of Toxin Binding Inhibition test (ToBI) and the method of seroneutralization in mice: a promising alternative to the in vivo potency test

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The National Institute for Quality Control in Health (INCQS) is the National Control Laboratory that performs the potency test of Tetanus Antitoxin Serum (TAS), used in Brazil. Potency of TAS is determined through the inoculation of mice with a mixture of serum and tetanus toxin. This test uses more than 1,500 animals per year in INCQS. On the other hand the Toxin Binding Inhibition Test (ToBI) is an alternative for the replacement of the traditional in vivo test. However, in order to be validated, ToBI must present a good correlation with the in vivo test. The present study aimed to evaluate this correlation through the results obtained with 12 batches from one national manufacturer. Each batch was tested separately, and the average of five valid results was compared to the mean of in vivo results, performed in triplicate. Our results showed a good correlation between ToBI and the in vivo test for TAS from the manufacturer evaluated ($r=0,94$). In addition, tests performed within the same batch presented a low coefficient of variation ($CV<20\%$). These findings indicates that ToBI can be useful and appropriate for the replacement of animals used for batch release of TAS in National Control Laboratory, saving more than one thousand animals per year and optimizing quality control of immunobiologicals.

KEYWORDS: Potency; Tetanus Antitoxin Serum; neutralization test; ToBI



107143

Cytotoxic Evaluation of 4-nerolidylcatechol (4-NRC) and hydroxypropyl beta cyclodextrins (HP-B-CD) complex

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The 4-nerolidylcatechol (4-NRC), obtained from a mixed biosynthesis, is the predominant constituent of leaves and roots of *Piper umbellata* (Piperaceae) known as pariparoba, capeba and malvisco. Studies have proven that the secondary metabolite has antioxidant and anti-inflammatory actions as well as chemoprotective effect with expressive action as sunscreen, suggesting the use of this extract in topical formulations, with the aim of combating the damaging effects caused by free radicals, thus preventing skin aging. However, 4-NRC is lipophilic and as such it is difficult to solubilize in aqueous solutions as culture media. To overcome this problem, a complex has been prepared with 4-NRC and hydroxypropyl beta cyclodextrin (HP-B-CD). The aim of this work was to assay the cytotoxic profile of this complex in vitro, using cell lines to reduce animal experiments. All samples (4-NRC; HP-B-CD-4NRC; HP-B-CD and methanol) were submitted to cytotoxicity assay. Fibroblasts Balb/c 3T3 were used to test the samples in different concentrations (0.16-0.0125 µg/mL) for 24 hours at 37°C, 97% humidity, and 5% CO₂. The viable cells were measured by MTS/PMS method and formazan product was quantified at 490 nm. It is relevant to note that, under the evaluated conditions, the solvent and cyclodextrin itself are no cytotoxic. The results revealed that the complex showed a different cytotoxicity profile from the 4-NRC one, regarding the difference of solubility in the culture medium, indicating that the characteristic cytotoxicity is related to the active substance. This could be used to justify lower doses as the complex allows the 4-NRC to be more effective.

KEYWORDS: 4-nerolidylcatechol; hydroxypropyl beta cyclodextrin; balb 3T3; cytotoxic

107184

Comparison between cytotoxicity and phototoxicity of Bergamot oil in human keratinocytes and Balb/c 3T3

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Cytotoxicity is the quality of a chemical substance being toxic to cells. Phototoxicity is defined as a toxic response from a substance applied to the body which is enhanced after subsequent exposure to light and recommended by OECD TG-432 in Balb cell culture and neutral red dye. However, it's intended to validate this test with human keratinocytes and MTS dye. Bergamot, Citrus bergamia, mainly cultivated for its essential oil has, as major component, Bergapten responsible for its photosensitive activity. Bergamot oil (Givaldan) select as positive control for any essential oil was diluted at eight different concentrations over fibroblast Balb 3T3 and human keratinocytes culture and submitted to cytotoxicity test with MTS and NR to verify its cytotoxic potential, comparing the differences between dyes. The phototoxicity was performed in a chamber specially built and qualified in accordance with OECD TG-432 and ©ECVAM DB-ALM: INVITTOX. Validation and qualification of the method and chamber was made with sodium lauryl sulfate, as recommended by OECD TG-432. The chamber was built in stainless steel frame, 3 mm thick, and painted in flat black paint. It consists of two UVA lamps and a dark area for negative control. Bergamot oil has shown to be phototoxic in Balb cells, even at the same concentrations that shown to be non-cytotoxic. How expected bergamot oil showed similar behavior with human keratinocytes, without relevant differences. There were no significant differences between MTS and NR in Balb/c 3T3 cells.

KEYWORDS: phototoxicity, cytotoxicity, Balb/c 3T3, human keratinocytes, bergamot essential oil



107189

Two techniques validation to obtain suitable Langerhans cells for an immunocompetent three-dimensional human skin model

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An immunocompetent three-dimensional human skin model presenting in vitro more complex structures like melanocytes and dendritic cells represents a new generation for human skin substitute, allowing the studies of primary sensitization processes and skin sensitization, being able to overcome the need for animal models at least in the early stages of allergenicity tests of cosmetics and drugs. The setting of skin models are typically based on a de-epidermized dermis or collagen scaffold, supplanted with fibroblasts and keratinocytes. This study presents the validation of two different techniques to isolate and cryopreserve Langerhans cells (LC) to permit its subsequent introduction into the immunocompetent three-dimensional human skin model. After the epidermis mechanical separation and cell suspension by trypsinization, there were tested two different purification methods in parallel: (a)collecting the supernatant of the cultured keratinocytes before the first change of medium and (b) mechanical purification system based on cell separation according to density - Ficoll. Both systems were evaluated for their efficiency in terms of purification and yield by phenotype using FITC conjugated with HDL-DR marker in flow cytometer. The first system was simple to apply, had high cell yield but low LC coefficient. The second system, although having higher complexity and requiring longer period to settle, brings higher degree of LC recovery. LC cryopreservation from both systems was performed using a rich fetal bovine serum cold-freezing medium. After thawing, the cell viability was checked and verified that the freezing method is suitable for this cell type.

KEYWORDS: *Langerhans Cells; Ficoll; cryopreservation; three-dimensional human skin model*

107193

Alternative methods to animal testing in the biological potency of rhEPO: Current situation

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rhEPO acts on the erythroid progenitor cells from bone marrow. It is indicated in the treatment of anemia of various etiologies (Costa et al, 2010). Safety and efficacy testing should be performed by the producer and the regulator. One of these tests is the biological potency. Currently, two methods are recommended by the European Pharmacopoeia (EP), one using polycythemic mice and the other normocythaemic mice (EP, 2008). With the strong influence of the 3R's the scientific community has been studying new methods in order to replace the use of laboratory animals or at least to minimize their suffering. In this context, the European Centre for the Validation of Alternative Methods (ECVAM) recommends, since 2002, that the only method for biological potency of rhEPO in mice normocythaemic should be used, because the polycythemic assay induces a lot of stress and to Suffering to the animals (ECVAM, 2002). Some authors recommend alternatives to animal use, using different cell lines for proliferation /differentiation tests, e.g. Erickson-Miller et al (2000), Wu et al (1997), Fukumoto et al (1989), Gassmann et al (1995), Wei et al (2007), Seong et al (2005), Sakatoku & Inoue (1997), Hammerling & Sjodin (1998). Others, like Mykleubust et al (2000), Erickson-Miller et al (2000) suggested testing in the field of molecular biology, which evaluates the increased expression of membrane receptors for rhEPO in erythroid precursor cells. Some other authors studies the increase of iron uptake e.g Goldwasser et al (1975), Gilg et al, 1996. There are even authors such as Barth et al (2007), Sepehrizadeh et al (2008) and Bietlot & Girard (1997) who advocate the use of physico-chemical (reversed phase liquid chromatography , real time PCR and high-performance capillary electrophoresis) as substitutes for in vivo testing. As we can see, several efforts are being made by the scientific community in the search for alternatives to the in vivo test for evaluation of biological potency of rhEPO. However, there is not a validated method yet for this purpose.

KEYWORDS: *Alternative Methods; Human Recombinant Erythropoietin; Potency Test*



107200

EVALUATION of *Drimys brasiliensis* Miers ESSENTIAL OIL BY CYTOTOXICITY ALTERNATIVE METHOD

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Drimys brasiliensis Miers is a native Brazilian species popularly known as casca-de-anta. Aromatic and antimicrobial properties of *D. brasiliensis* essential oil confers a potential use in cosmetics. As such it is important to verify its safety. The use of alternative methods for new ingredients toxicity determination has been recommended by the 7th Amendment of the European Cosmetics Directive 2003/15/EC. In this scenario the goal of this work was to evaluate the in vitro toxicity of *D. brasiliensis* essential oil by the MTS cytotoxicity test. The essential oil was obtained by hydrodistillation of the aerial parts of the plant. It was dispersed in the culture medium composed of DMEM supplemented with 10% Donor Bovine Serum (v/v), and added to the surfactant Polysorbate 20, which was based on the Hydrophile-Lipophile Balance (HLB) theory. The dispersion test was conducted at 37 °C and the results observed after 24 and 48 hours; clear dispersions have ensured the full contact between samples and cells. For the cytotoxicity test, 10,000 murine fibroblasts cells BALB/c 3T3 per well on 96-well plates were used, followed by incubation at 37 °C on 5% CO₂. After the incubation period, the MTS/PMS solution was added and the amount of violet formazan formed spectrophotometrically measured at 490 nm. The 10% inhibitory concentration (IC₁₀) for 24 and 48 hours were, respectively, 0.125 mg.ml⁻¹ and 0.133 mg.ml⁻¹. Thus, concentrations up to the IC₁₀ values of *D. brasiliensis* essential oil can be safely used as a natural substance in cosmetic formulations.

KEYWORDS: *Drimys brasiliensis* Miers; essential oil; Cytotoxicity; IC₁₀; alternative method; BALB/c 3T3

107202

EVALUATION OF CRYOPROTECTANTS IN Balb/c 3T3 CELLS

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Cryopreservation consists on the storage of isolated cells under ultra low-temperature to avoid any biochemical activity. This preservation process allows the recovery of the cells to the same living state before freezing. Slow cooling freezes mainly the outside water of the cells, leading to their dehydration by the osmolarity rise of the unfrozen solution surrounding them. The lack of a suitable cryoprotectant implies in cell viability reduction after thawing due to the effect of the extra and intracellular water. The freezing medium outlined for BALB/c 3T3 cells (ATCC number: CCL-163) contains DMSO as a cryoprotectant agent, although some laboratories use Donor Bovine Serum (DBS) instead. The aim of this study was to compare the performance of DMSO and a mixture of DMSO with DBS, as cryoprotectants. Standard freezing medium (SFM) consisted of 5% of DMSO and 95% of Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100UI.ml⁻¹), streptomycin (100 mg.ml⁻¹), amphotericin (0.025 mg.ml⁻¹), L-glutamine (4 mM), and DBS 10% (v/v); Laboratory freezing medium (LFM) consisted of DMSO 10% (v/v), DBS 30% (v/v) and DMEM 60% (v/v). BALB/c 3T3 cells (2 x 10⁴) in 0.3 mL of each of the freezing formulations were frozen at the rate of 1 °C/min. After 30 days, the cells were thawed to evaluate the viability towards the cryoprotectant solutions by the Trypan Blue exclusion test. The average of viability for SFM and LFM were, respectively, 81.76 and 77.84%, with no statistical differences (Tukey HSD test) between the two freezing solutions in a short term evaluation. Studies should now proceed after long-term freezing, allowing the evaluation of the effects of DBS as a non-toxic and efficient cryoprotectant.

KEYWORDS: Alternative methods; science education; animal ethics



107247

Establishment of mixed cell cultures from the visual system of the crab *Ucides cordatus*

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The Three Rs (replacement, refinement, and reduction) represent a practical method for implementation of the principles that mandate responsible and human care and use of experimental animals. Replacement includes the substitution of vertebrates with animals that are phylogenetically lower. Additionally, cell cultures derived from tissues of the nervous system have proven to be powerful tools for elucidating cellular and molecular mechanisms of biological function. In this context, our objective was to establish and characterize purified cell cultures obtained from the visual system of the crab *Ucides cordatus*.

The animals were crioanesthetized and had their optic stalk dissected, trypsinized and dissociated, and the suspension was plated on coverslips previously treated (or not) with collagen, poly-L-Lysine and poly-L-ornithine. The cells were maintained in L-15 medium supplemented with 10% FBS, during 7 days at 28°C. The culture was divided into 2 groups: a group in which the culture medium was changed every 48 hours; a group that had no medium exchange. The cells were then fixed with 4% paraformaldehyde and were phenotypically characterized by immunocytochemistry with anti-GFAP and anti-NeuN antibodies; their nuclei were labeled with DAPI. Our results showed an increased number of cells when the culture was maintained with no replacement of the medium and on ornithine. Specific markers revealed a larger number of glial cells when compared with neurons.

The protocol followed by us provided for the first time, the maintenance of neural crustacean cells *in vitro*, helping us abolish the use of traditional animal tests in developmental neurotoxicity studies.

107250

In vitro assays evaluating the anticoagulation and antiplatelet effects of two diterpenes isolated from the algae *Dictyota menstrualis*

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The cardiovascular diseases, in which platelet aggregation and blood coagulation are involved, represent a major cause of disability and death worldwide. The current antithrombotic therapy has an unsatisfactory performance and may produce side effects. Therefore, alternative therapies are under hard investigation, and natural products have been routinely studied. Marine organisms produce several and complex substances with different ecological functions and also pharmacological properties. In this work, we evaluated the effects of two diterpenes (dichotomane and pachy/isopachydictyol A) isolated from the Brazilian marine brown algae *Dictyota menstrualis* upon platelet aggregation and blood coagulation, using *in vitro* assays only. The tests carried out in this work were realized on digital devices (aggregometer, coagulometer and microplate reader) using human blood derivatives. The dichotomane inhibited platelet aggregation on platelet-rich plasma (PRP) induced by collagen (IC50 1,1 mM) and ADP (IC50 0,32 mM). Meanwhile, the pachy/isopachydictyol A did not inhibit platelet aggregation upon PRP, but inhibited upon washed platelets induced by collagen (IC50 0,12 mM) and thrombin (IC50 0,22 mM). Moreover, both diterpenes were able to delay the clotting time or inhibit the coagulation measured through Plasma Recalcification, Fibrin Clot, Prothrombin Time and activated Partial Thromboplastin Time, despite showing different intensities. As seen, the diterpenes evaluated here showed anticoagulant and/or antiplatelet properties, thus revealing biotechnological potential on the antithrombotic therapy. This work also shows the importance of bioprospecting studies of the Brazilian marine biodiversity in the search for novel products that could be used in drug developmen.

Support: CAPES, CNPq, FAPERJ, IFS. Propii-UFF and Reuni

KEYWORDS: *anticoagulation; antiplatelet; Dictyota menstrualis; in vitro assays; marine bioprospecting*



107251

Evaluation of 1,2,3 triazoles effects on platelet aggregation and coagulation through in vitro assays

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In some Latin American countries the incidence of cardiovascular diseases varies between 135-151/100.000 inhabitants. These diseases, such as stroke and thrombosis-related ischemia, are a major cause of death worldwide. These events may occur due to an imbalance in the haemostatic system, in which blood coagulation and platelet aggregation participate. The regular treatment is the administration of heparinoid drugs, which have some disadvantages, as decrease in efficacy and development of side effects (bleeding or thrombocytopenia). For this reason, there is a huge effort on seeking novel compounds that could replace or complement such therapies. The objective of this work was to evaluate, using only in vitro assays, the antithrombotic potential of a series of 1,2,3-triazole derivatives. The coagulation effects were evaluated by the Prothrombin Time (PT) and the activated Partial Thromboplastin Time (aPTT) tests, realized on a digital coagulometer (Amelung KC4A) using human plasma. The platelet aggregation was monitored on an aggregometer (Chrono log 490 2D) using human platelet-rich-plasma. According to these assays, some of the synthetic compounds evaluated have the ability to inhibit blood coagulation (by prolonging controls PT and aPTT) and have potential effects inhibiting platelet aggregation induced by collagen, ADP and ristocetin. Our results point out promising aspects that may indicate the use of triazoles as molecular models for antithrombotic drugs. This work also shows that in vitro assays should be carried out as a trial, before the use of animal models, in the early studies on drug development.

Support: CAPES, CNPq, FAPERJ, IFS and Propii-UFF.

KEYWORDS: anticoagulation; antiplatelet; in vitro assays; triazoles

107264

In vitro seroneutralization test to evaluate the potency of clostridial vaccines

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Approximately 150 million doses of clostridial vaccines are produced in Brazil each year. The standard technique to evaluate clostridial vaccine potency is the seroneutralization assay (SN) in mice. Despite the known sensitivity and reliability, this method is expensive, time-consuming and cause pain and suffering to a large number of animals. The objective of this work was standardized in vitro SN test to evaluate the potency of clostridial vaccines by comparison with the animal bioassay. Clostridium perfringens epsilon toxin (CPE), C. septicum alpha toxin (CSA), and C. novyi type B alpha toxin (CNA) were standardized to the test level of L+/50 in MDCK cells, L+/25 and L+/200 in VERO cells, respectively. For each vaccine it was evaluated in a pool of serum from rabbits immunized with the commercially clostridial vaccines. Results of in vivo and in vitro SN were analyzed by Student's T test for comparison of mean of treatments and the parametrical correlation using Pearson's correlation. The analysis of the vaccine potency test demonstrates that results of SN and bioassay are statistically the same ($p \leq 0.05$). The correlation of the two methods were, 99.73% ($p < 0.05$), 98.24% ($p < 0.05$), and 98.38% ($p < 0.01$) for CPE, CSA and CNA respectively. Cells culture is a viable alternative in the evaluation of clostridial vaccine potency. In vitro SN presents a series of advantages over the animal bioassay, smaller variation in the individual responses, and decrease in the amount of standard reagents and drastic reduction in the number of animal used.

KEYWORDS: seroneutralization; cell; vaccine; potency



108616

Maximum Reclaiming for Zoological Specimens in Search, Training and Disclosure

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The use of teaching resources to exemplify academic questions is, undoubtedly, a great help to the teacher in the classroom at universities. With the use of some specific techniques you can develop scientific, teaching and dissemination collections from materials that would be discarded. Preserved objects could be used in class or be exposed, providing the public with a more detailed view of this material. Thus, in the present study we use a technique for the production of scientific and educational material aimed at maximizing the use of the zoological resources of vertebrates. Through reflections on the best use of the material set in the wet, in the laboratory of taxidermy at the National Museum of Rio de Janeiro, we discussed the maximum use of a fixed *Rinella jimi* in 10% formalin and subsequently kept in alcohol 70%. Due to packaging errors, such copy was in an advanced state of putrefaction. Predicting the disposal of this material, which no longer belonged to a research collection, we experimented his expository taxidermy and preparation of skeletal material by Dermestes. The results of such preparations allowed the recovery of material that would be discarded and the reintegration of the parts in this exemplary scientific collection and use of other parties in teaching and dissemination.

KEYWORDS: *Preparing zoological; Taxidermy expository; Didactic material; scientific collection*

108617

New Feature for Preparing Educational Material in Entomology

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The use of teaching resources to exemplify academic inquiries is, undoubtedly, a great help to the teacher in the classroom. In the teaching of entomology, the scientific study of insects, it is a big challenge to exemplify the great diversity of shapes and characters of these animals. Most of the educational materials in entomology resources are fragile like, for example, entomological boxes and materials preserved in the wet. With the use of such techniques is also possible to develop educational collections, where the material preserved that way it can be used in the classroom, providing students with a more detailed view of the object of study, enabling their manipulation, allowing a close relationship between student and object of study. The great challenge for this educational resource is the durability of entomological parts that must withstand excessive handling. In this paper we describe a new technique for the production of teaching resources aiming at teaching with durable entomological materials. The technique consists of preserving in dry the parts of the insects. They are fixed in a mixture of 70% alcohol and camphor, the material is then oven-dried and packed in glass jars sealed with cork cap. Insects are pinned on the lid of the bottle, in order to demonstrate their anatomical structures. At the bottom of the bottle wax is used to promote greater adhesion of the condom material, naphthalene. This technique has been shown to be efficient and the indirect manipulation of insect corroborates for durability of this didactic material.

KEYWORDS: *Entomological material; didactic material; Scientific collection; Didactic collection*



108620

New Technique for Production Educational Materials in Internal Anatomy of Vertebrates: Emphasis on Durability, Resistance and Economy of Exemplary Used

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The use of teaching resources to exemplify academic questions is, undoubtedly, a great help to the teacher in the classroom at universities. With the use of fusion of some specific techniques you can develop teaching and broadcast collections from materials intended to this function, where the objects preserved can be used indefinitely. Thus, the present work we developed a technique from other pre-existing ones, for the production of teaching and diffusion materials in internal anatomy, which aims at better utilization of the resource zoology of vertebrates. According this technique the material is fixed in 10% formalin, then follows a chain of dehydration, and then bathed in xylene, infiltrated with paraffin and packaged in blocks of crystal resin. Thereby, we manage to create durability and resistance, allowing a few copies to be used in the preparation of such collections. Through reflections on the best preparation material for teaching through didactics collections we conclude that it must resist intensive handling, allowing students a more detailed view of the copies, and has a considerable durability. So it is enough to use this technique just once by each educational institution since the parts suffer very low action of time and from handling, allowing many animals to be spared from such a fate.

KEYWORDS: *Zoological material; Didactic collection; Resistance; Durability; Animals spared*

108638

The use of the 3Rs in nutrition experiments: replace, reduce and refinement do not affect either results or quality

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The use of animals in scientific research has been subject of intense debate for many years by academic society. At least, nowadays the majority researchers have been conducting animals studies reasoned in ethical principles and the 3Rs replacement, reduction and refinement. The 3Rs have been changing the conduct in bioassays, assuming that scientific use of animals were generally compatible with their welfare. Our group has been considering the 3Rs as basis in all works. Regarding to general refinement, we have been changing techniques in way to minimize the suffering of animals used in the experiments (e. g. analgesia and anesthesia have been replacing decapitation). Concerning reduction, since 2010 we have used 66.57% less animals in experiments involving nutrition and related diseases. The number of animal to be used, disregarding the 3Rs, were estimated based on the mean of those used in a standard bioassay by our researcher group (n= 6-8 per group). It was used approximately 25 animals/ experiment (n= 4-5), saving 21.5% of them, in 2010. This percentage rose to 30.71% in 2011. Lastly, before April (2012), 27 animals/ experiment (n= 4-5) were used, which trend to decrease until December. Finally, regarding to replacement, we have looking for replace gradually the in vivo assays to the in vitro ones. In 2011, we started use human cells based analysis, and we do believe that the use of the 3Rs is essential to reduce the number and suffering of animals in research and it do not affect the quality of data.



108642

Cytotoxicity & detachment evaluation of papain in fibroblasts murines

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Papain is a proteolytic enzyme extracted from the latex of green papaya leaves and fruits. It has been used in various industrial fields and medical, used mainly as debriding agent for scars and wound healing agent. In view of their use acuity assessment of cytotoxicity of papain is necessary. This study show the in vitro action of papain on murine fibroblast cells (CCL-92) and were tested using several papain different concentrations (from 0,01 to 0,2 % (w/v)) for 24, 48 h of contact at 37°C, 97% humidity and 5% CO₂ in cell culture flasks. In attempt to observe a reverse mechanism, the cells were also maintained for 7 days after 24 and 48 hours of contact. The viable cells were measured by MTS method, where the active component is a tetrazolium compound and the living cells reduce it to a colored formazan product that is quantified at 490 nm. The different inhibitory concentrations estimated to affect endpoint in question by 50% were IC₅₀ - toxic potential. Basal cytotoxicity can be used in combination with other information for many purposes in the process of safety or risk evaluation, e.g. to predict starting doses for in vivo acute oral LD₅₀ values in rodents. Moreover, was detected simultaneously an effect detachment, highlighting the need of performed a bioassay when dealing with proteolytic enzymes, avoiding false-positive results, thereby obtaining reproducibility in new studies of bioenzymes based products.

109243

Interaction of Gold Nanoparticle with Epithelial Cells an in vitro Lung Tissue-Equivalent Model

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The properties of their interaction with human body are not fully understood. Nanoparticles are also present in environmental pollution and in industrial environment of units that deal with production of nanostructured goods. Understanding interaction of lung cells with nanoparticles is relevant for nanotoxicology, since lung is the major interface with air-born particles. The use of in vitro models is mandatory for monitoring the response of human cells and tissues to nanostructured materials. Lung epithelial cells have been used in several research fronts, such as release of inflammatory mediators, chemokines and growth factors. Most studies have been made in conventional monolayer systems, which do not represent in vivo conditions. The in vitro cultivation in three dimensions (3D) is an important tool for studies of cell behavior. It is required to establish a model that mimics the microenvironment of lungs, expected to achieve: (1) definition structural and functional interactions between epithelial and mesenchymal cells, (2) determination of cellular response to nanoparticles, (3) definition internalization and intercellular transport of nanoparticles, (4) definition of their pathogen activity at the tissue level. We established spheroids containing A549 epithelial and MRC-5 mesenchymal cells. Spheroid diameter was stabilized at 0.2 to 0.3 mm diameter, and epithelial cells constituted a fully differentiated external layer with mucus-producing cells. Gold nanoparticles added to 3D cultures adhered to villousities of epithelial cells, they were internalized and accumulated in the vicinity of mucous granules. In this distal part of the secretory pathway, they are expected to be released, in association with mucus, and the properties of their interaction with other cells may be considerably modified. We propose that 3D lung cells spheroids will facilitate studies of cell-to-cell interactions that occur in vivo, concerning the traffic and the final destiny of nanoparticles at the body interface with environment. [1] This research was supported by CNPq, FAPERJ and INMETRO (Brazil).



109273

Cell assays for stents evaluation: Safety margin and proliferative efficacy of the Ruthenium-NO donors coated stainless steel

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Neointima is observed in 20-25% of PTCA following for stents implantation and can be prevented by nitric oxide (NO) eluting stent. The aim of the present study was the evaluation of the NO donors trans-[Ru(NO)Cl(cyclam)]²⁺ and trans-[Ru(NH₃)₄(inaH)(NO)]³⁺ embedded in xerogel polymers coating stents on the cytotoxicity effects and proliferative efficacy on rabbit arterial smooth muscle cells (RASM). The cellular viability (MTT, Neutral Red assays), proliferation (3H-thymidine assay), migration (transwell analyses), nitric oxide (NO) release (Sievers® NOA280i), apoptosis index (Tunel+Hoechst, Caspase activity) and protein expression (Western Blot) responses were determined on cells exposed to stent samples (1mm²) and test agents. RASM cells cytotoxicity was absent at all concentrations tested for trans-[Ru(NO)Cl(cyclam)]²⁺ whereas it was present only at 0.8 mM for trans-[Ru(NH₃)₄(inaH)(NO)]³⁺. No change on RASM proliferation was observed with trans-[Ru(NO)Cl(cyclam)]²⁺, but 0.8 mM trans-[Ru(NH₃)₄(inaH)(NO)]³⁺ induced 75% of inhibition. Migration response on transwell analyses showed 50% reduction. NO released after 24 hours exposition to trans-[Ru(NH₃)₄(inaH)(NO)]³⁺ was similar to basal levels (6±0.4nmol/mg protein) whereas trans-[Ru(NO)Cl(cyclam)]²⁺ induced 20% reduction compared to basal levels. Only at 0.8 mM apoptosis response was present and they were correlated with 100% increase of caspase activity from 0.26 to 0.8 mM of both NO donors. SM-MHC, alpha-actin and Akt/p-Akt protein expression were correlated to contractile vs. synthetic phenotype. In conclusion, NO donors on solution or in the stainless steel coating displayed low cytotoxicity profile and inhibitory proliferative/migratory action. The different biological response showed by the compounds was related to the different specific rates of NO release from each compound.

KEYWORDS: nitric oxide (NO) donors; stents; cell proliferation cell migration

109290

Development of a 3-D Atherosclerotic Vessel Model

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Cardiovascular diseases (CVD) represent a major global concern, demanding the development of more effective drugs with a lower cost. The process of development and validation of new drugs face ethical problems and comparison between animal models. Pre-clinical studies using human cells and tissues can minimize such issues. Thus, it is clear the need of creating an in vitro model of "vessel-equivalent" which can generate reliable results, reduce testing costs and eliminate ethical problems. Methodologically, a co-culture model was developed using endothelial (HUVEC) and vascular smooth muscle cells (VSMC) obtained from human umbilical cord vein by enzymatic digestion according to Jaffe et. al. Phenotypes were confirmed by flow cytometry and immunofluorescence. In order to assemble, two cell populations were grown in a 1:1 ratio in 96-well plate coated with agarose gel. The formation of spheres was verified by scanning electron microscopy (SEM). Once characterized, the spheres were treated with cholesterol-water soluble and then stained with Oil-Red-O and DAPI to visualize in a confocal microscopy. Our results showed that primary cultures of HUVEC and VSMC of co-culture were able to generate an equivalent-tissue blood vessel, with endothelial cells on the surface and smooth muscle cells at the core of the spheres. SEM revealed endothelial cells after 15 days co-culture completely covering the stromal layer. When treated with cholesterol-water soluble, we observed neutral lipids accumulation in the core of the sphere. Our "vessel-equivalent" represents a promissory tool to study cell-to-cell interactions as well as a model to test new drugs eliminating ethical problems.

KEYWORDS: Atherosclerosis; HUVEC; VSMC; Co-culture; cholesterol



109329

In vitro toxicity analysis of gold nanoparticles

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The gold nanoparticles are one of the most versatile and widely researched materials for novel biomedical applications. Some studies have reported the gold nanoparticles toxicity in vitro and in vivo, but the results remain controversial in the literature. One of the main routes of human nanoparticles exposure is the lung since the exposure may occur through the contact with the air containing such nanoparticles. Rodent inhalation models are still performed to predict the toxicity and pathogenicity of nanomaterials. The aim of this study is to assess the toxicity of gold nanoparticles performing the in vitro model. Mycoplasma-free MRC5 cell culture was obtained from the Rio de Janeiro Cell Bank. The cells were treated or not (control) for 24, 48 and 72 h at different concentrations (1.43 nM, 0.143 nM, 0.0143 nM, 0.00143 nM) of gold nanoparticles with 13nm (± 2). Evaluation of two toxicity endpoints allowed the determination of cell viability: CVDE and NR. Morphological changes in the cytoskeleton were observed through the labeling with red phalloidin. No toxicity was observed at any concentration or time of exposure assessed. Also, no morphological alterations in treated cells when compared to control were reported. Membrane integrity and proliferation of MRC-5 cells seems to be unaffected by gold nanoparticles under our experimental conditions and cell culture shown to be a suitable method for evaluating nanotoxicidade.

109732

Obtention Of 3D Spheroidal Culture From Mc3t3-E1 Murine Pre-Osteoblastic Cells For Biocompatibility Studies

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Several studies on the biocompatibility of medical/odontological materials intended for bone therapy employ monolayer bidimensional cell cultures of MC3T3 pre-osteoblasts. However, alternative models of cell culture are constantly being investigated, in order to achieve closer proximity with in vivo animal models and, in the near future, contribute to the reduction on animal testing. Among such models, 3D spheroidal cell culture could allow to evaluate simultaneously material-cell, cell-cell and cell-environment interactions. This work presents the trial of protocols employing different initial cell densities in order to determine the ideal procedure to obtain pre-osteoblast spheroidal cultures for material cytocompatibility tests. Either 0.5, 1, 2, 3, 4, 5, 7.5, 9 and 11×10^4 cells ($n=15$ per group) were seeded on 24-well plates coated with agar, and incubated in agitation from 1 to 7 days at $37^\circ\text{C}/5\%\text{CO}_2$. Cell aggregate morphology was observed and photographed with an inverted microscope, and qualitatively evaluated by a score methodology, considering the regularity of shape, size, repeatability, handling, stability and presence of multiple spheroids on the same well. It was observed that, while the lower initial cell densities presented faster spheroid formation, most groups were already aggregated by day 3. Higher cell numbers formed more stable spheroids. Handling and observation of spheroids was considered easier starting from 3×10^4 cells. Cell densities above 7.5×10^4 formed irregularly shaped and multiple spheroids. It is concluded that the initial density of 3×10^4 cells provides the ideal protocol, as it was among the best scores on all parameters considered, being also responsive to XTT assay.

KEYWORDS: *osteoblast; tridimensional culture; biocompatibility*



109733

Evaluation of solvents and controls for in vitro human cell-based neurotoxicological studies

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Neurotoxicological studies of chemicals are important to risk assessment and new drug evaluation. Ethical, economical and methodological issues encourage the development of alternative methods, such as computational modeling and in vitro tests to, in the future, replace or reduce animal testing. Multiparametric human cell line based tests, evaluating simultaneously cytotoxicity, neurite outgrowth, membrane potential, and enzyme activity, are potential endpoints to in vitro neurotoxicology. However, adequate controls and solvents should be established for such novel tests. Aims: Analyze the effects of two solvents, Methanol and Dimethyl Sulfoxide (DMSO), and Eserine, a classical cholinesterase inhibitor used in palliative treatment for Alzheimer's disease, on human neuroblastoma cell line (SH-SY5Y). Methodology: Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) high-glucose, supplemented with 10% of fetal bovine serum (SFB). 2x10⁴ cells per well were seeded in 96-well plates with 24 hours adherence. 24h of incubation at different concentrations of DMSO, methanol and eserine for a dose-response curve evaluating mitochondrial activity (XTT) and DNA quantification (CVDE). Results and conclusion: methanol and DMSO did not cause significant effects ($p \leq 0,01$) at concentrations less than 4% and 0.8% for XTT, and 1.2% and 8% for CVDE, respectively. Eserine was not toxic at concentrations less than 40 ug/ml for XTT and CDVE. These data enable the test with other chemicals poorly soluble in aqueous environment and use of eserine as a control to test simultaneously cytotoxicity and cholinesterase inhibition. Further assessments on reference substances should be performed for the development of this new method.

KEYWORDS: Eserine; Cholinesterase inhibitor; Neuroblastoma; Cytotoxicity; SH-SY5Y neuroblastoma

In vitro Assessment of Shampoos Eye Stinging Potential Using Transient Receptor Potential Vanilloid Type 1 (TRPV1)

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Unmyelinated c-fibers are implicated in nociception and pain signal transduction. Transient receptor potential vanilloid type 1 (TRPV1) is described as a key integrator of nociceptive signal in sensory neurons. TRPV1 is a calcium permeable channel activated by heat, acidic conditions and capsaicin₂ present in c-fibers.

In order to evaluate ability of 10 shampoos formulas to activate TRPV1, we used a neuronal-like sensory cell model SH-SY5Y surexpressing TRPV1. These formulas (children or adults shampoos) had been selected considering their clinical eye comfort classification. Formulas were evaluated at a 0.1% final concentration in culture medium. TRPV1 activation was assessed by measurement of cellular calcium influx using fura₂-AM, a fluorescent ratiometric indicator after contact between cells and formulas. TRPV1 specific activation was confirmed by using TRPV1 specific antagonist capsazepine.

Some formulas were able to induce calcium influx by a TRPV1 specific manner. Capsazepine reveals that some calcium influx is independent of TRPV1. Considering calcium influx concentration, formulas were classified in 3 discomfort categories: No - Slight - Moderate. This classification seems to correlate with clinical one.

These results confirmed that TRPV1 target seems to be a relevant biological target to understand and study eye stinging. This kind of assay offer the ability to evaluate eye stinging potential in vitro of shampoo formulas before commercialization and to understand what type of raw material play an important role in eye pain.



2/

A Mapping Based on Physico-chemical Features: Lessons Learned

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The marketing ban on animal tests for systemic toxicity testing of cosmetic products in Europe is foreseen in 2013. Several research programs involving public and private sectors have been initiated in this area with the aim of fulfilling regulatory constraints and complying with the transformative shift in toxicology. Typically, L'Oréal has been promoting such efforts with emphasis on developing its capacity for predicting the toxicity of the so-called “real-life” chemicals. Many efforts have been made in order to handle the complexity, diversity and even specificity of cosmetic ingredients in terms of chemical reactivity and classes or solubility profiles.

The study presented herein describes exploration of the chemical space considered for development of a predictive model for systemic toxicity using the ToxCast™ high throughput screening data, where 309 compounds of the Phase I program were used as a starting point for the elaboration of the training set. In order to enrich that selection list and eventually expand the applicability domain of the proposed model, a comparative study of the US EPA and L'Oréal chemical spaces (673 and 708 compounds, respectively) has been completed using a panel of 19 calculated structural and physico-chemical properties.

While the simple comparison of the two inventories by strict structural similarity (CAS) yielded a poor overlap, using the physico-chemical properties showed a high degree of overlap—indicating that the knowledge and models derived from the Phase I and Phase II ToxCast effort could be extended to a broader chemical space. This result will help to define the rationale for selecting additional compounds from the US EPA library in order to enrich the training set of the proposed model. However, future work is required to identify physico-chemical properties that would best describe systemic toxicity and extend the exercise to categories like mixtures and polymers, which are not considered in this study but commonly used in consumer products. This abstract does not necessarily reflect U.S. EPA policy.

3/

Cutaneous Absorption on Reconstructed Human Epidermis (RhE): Guidance to the Choice of Solvent for Topical Application

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Risk assessment from topical exposure to chemical in human requires reliable models and test procedures. For such a study, ex vivo human skin samples are the tools recommended by regulators. However, its use is time consuming and requires numerous replicates due to the variability of the donors. According to their similarities to native human tissue in terms of morphology, lipid composition and biochemical markers, Reconstructed Human Epidermis (RhE) have been identified as useful tools for the in vitro testing of phototoxicity, corrosivity and irritancy. These last years, some papers claim that RhE are appropriate alternatives to human skin for the assessment of skin permeation and penetration in vitro.

For topical application, the question to ask is how to select the vehicle for an optimized cutaneous absorption. To answer this question, two chemicals, Caffeine and Butylated hydroxytoluene (BHT), were used. Kinetic experiments with infinite dose were performed, allowing to measure the permeability coefficient and flux at the steady state.



4/

Alternative Approaches for Skin Sensitization Evaluation: Statistical and Integrated Approach for the Combination of Non animal Methods

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L'Oréal is developing approaches for safety evaluation (skin sensitization) of ingredients by combining multiple in vitro and in silico methods in an integrated strategy. Contact sensitizers are reactive molecules (haptens) that have the ability to modify skin proteins to form an antigen which will be recognized by specific T cells activated during the sensitization process. In addition to the haptentation mechanism, contact sensitizers induce several phenotypic and functional changes of dendritic cells (DC) either directly or indirectly through intercellular signaling pathways implicating keratinocytes, fibroblasts and other skin cells. This rather complex and still not fully unraveled maturation process of DC induced by contact sensitizers, allow them, to migrate to the lymphnode, present antigen and prime efficiently hapten-specific T cells. Due to the complexity of the skin sensitization process, it is now agreed that alternative hazard identification and risk assessment need to be addressed by combining a battery of complementary methods covering the different known AOPs (Adverse Outcome Pathways). The aim of this study was to combine in silico and in vitro tools, from chemical reactivity assay to DC-based assay, into an integrated testing strategy for the evaluation of skin sensitization. For this purpose, we used a full data set on 165 chemicals composed of different variables, representing the results from in silico predictions (Derek, TIMES, Toxtree), from DPRA, MUSST, Nrf-2 and PGE-2 assays as well as numerous physicochemical experimental or calculated parameters. Many prediction models are described in the statistic literature. In order to avoid any bias brought by the use of one single statistical method, we chose the Stacking model allowing us to combine five statistical methods (Boosting, Bayesian, SVM, Sparse PLS-DA and Scoring). These models correspond to various families of prediction models which tackle the problem from different angles through complementary statistical approaches (linear, non-linear, probabilistic...). The outcome of the final stacking model is a probability for a chemical to be a sensitizer. By setting the thresholds to classify a chemical as a sensitizer at > 80 % of probability, and as a non sensitizer at < 20 %, the performances of the optimal model are: 85 % concordance, 90% sensitivity and 75 % specificity (obtained on a validation data set). This kind of alternative prediction will ultimately contribute to the risk assessment decision making in a Weight of Evidence approach.

5/

Human Reconstructed Tissues for the toxicology of the XXIth century

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SkinEthic 1992-2012, twenty years of industrial tissue engineering experience. Alain Alonso will present the goals and the achievements of modeling human tissues for in vitro research and testing. He will illustrate the ethical, technical and scientific advantages of 3D reconstructed human tissue through several examples of industry uses.

Christian Pellevoisin, will detail the contribution of 3D human skin models to the development of alternative methods to animal testing and their usefulness to be compliant with regulatory changes in Europe thanks to their integration in OECD guidelines. A live demonstration of the models and of their use will give the opportunity to everyone to see how these tools are both powerful and easy to use.



6/

Methods Development Through Recognition in 3Rs : L'Oréal Commitment

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Dissemination of advances on alternative methods represents a step to promote alternatives to animal testing in line with the EU Cosmetics Directive. L'Oréal has, based on these principles, developed test methods to screen and test potential effects on chemicals.

We have focused initially on approaches for skin irritation. A peer review on various aspects of alternative techniques was performed at all stages of the R&D with a focus on in vitro methods improvement of chemicals selection (screening) as well as quality testing. To ensure quality and objectivity, experts from international committees oversee the content of EpiSkin and SkinEthic RHE protocols. Details of the approach will be presented for both skin corrosion and irritation with a set of 50 reference chemicals. Using computational approaches, an automated workflow algorithm was developed to predict a molecule's potential for skin irritancy based on in vivo Draize data. The practical approaches developed by L'Oréal in the areas of eye irritation (SkinEthic HCE defined with 90 chemicals), skin sensitization (MUSST assay optimized with 50 chemicals), phototoxicity and genotoxicity will be also described. For chronic and systemic toxicity testing, a realistic approach relied on the combination of data generated for multiple endpoints. Preliminary studies indicated that the method had good sensitivity and specificity (91% and 78%) while defining a LD50 threshold at 2000 mg/kg.

Combination of in silico, read across and in vitro strategies assure realistic scientific approaches suitable for the safety assessment process within industry.

Cytotoxicity and Adhesion Studies of Osteoblast to Multifunctional Titanium Implant Surfaces

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Titanium and its alloys have been widely used in biomedical applications, namely in dental and orthopedic implants, however failures still continue to occur many of the times due to an increased corrosion and/or wear degradation mechanisms at the interface implant bone. Surface modification techniques has been widely used on titanium surfaces, the biomaterial commonly used in dental implants, in order to induce the osseointegration process. The main aim of this work was to develop multifunctional titanium surfaces that stimulate the osseointegration process and with superior corrosion and wear performance (tribocorrosion). Prior to in vitro tests, surfaces were fully characterized at structural, topographical and tribocorrosion point of view. The cytotoxicity effect of titanium surfaces was evaluated following the ISO 10993-5. In vitro tests using human osteoblasts derived from bone explants were also used to study the adhesion mechanisms of the cells on titanium surfaces. Results demonstrate that the anodic treatment produced a bioactive protective oxide layer rich in calcium and phosphorus on Ti surface that are non-toxic. The oxide layers with thickness in the micrometre range present both titanium phases (rutile and anatase) distributed along the surface. The oxide layers produced reveal higher corrosion and wear performance and superior cells adhesion comparing with untreated surfaces. This work is expected to decisively contribute to the fundamental knowledge on the effect of surface properties on the biological interaction with materials. It is expected that understanding the mechanisms of osteoblast adhesion, are fundamental to design novel multifunctional surfaces for dental implants enhancing osseointegration process and patient healthcare and comfort.

KEYWORDS: *Titanium; Functionalization; Osteoblasts; cytotoxicity*



106919

Cytotoxic activity of Chitosan Films containing herbal substances

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In Brazil, standards for the study of the toxicity of herbal products have been published in the form of Directive 90/2004 (ANVISA, 2004), but the majority of the tests described are *in vivo*. Various formulations such as ointments and wound dressings have been developed for the treatment of severe skin wounds or ulcers including bedsores and burn wounds. Chitosan membranes can be used as wound dressing. These preparations have attracted much attention because of their potentially beneficial biological properties, like non-toxicity, biocompatibility, high hydrophilicity, fiber/membrane forming ability, hemostasis, antimicrobial activity, stimulation of healing and the chemical and mechanical resistance. Additionally chitosan promotes the activation of inflammatory cells in granular tissues and consequently accelerating cleaning the wound and the properties of re-epithelialization. These and other properties can be potentiated with the incorporation of herbal extracts. The aim of this work is to evaluate the cytotoxic effect of chitosan films containing herbal extracts, one hydrophilic and the other one hydrophobic, on balb/c fibroblasts cells as previous test to verify the biocompatibility of these films. No cytotoxicity was observed by the indirect method. In Brazil it is still not possible to use *in vitro* methods to evaluate the toxicity of plants used for medicinal purposes, since the legislation requires *in vivo* tests. With more-focused publications in the use of *in vitro* methods to aid in toxicity assessments, Brazilian researchers hope to change this current situation.

KEYWORDS: *herbal extract; in vitro; toxicity; chitosan*

106968

Evaluation of the mutagenicity of butanolic and acetate extract of *Campomanesia lineatifolia*

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The use of medicinal plants is an old practice in Brazil, mainly among the most lacking populations. *Campomanesia lineatifolia* Ruiz and Pav. (Myrtaceae) is one of those plants. A native edible species found in the Amazon Rainforest, commonly known as gabirola. *Campomanesia* species are frequently used in traditional medicine for dysentery, stomach problems, diarrhea, cystitis, urethritis and hepatic disorders. In this study, the Ames test (*in vitro*) was used to evaluate the mutagenic potential of the butanolic and acetate extract of the leaves of *Campomanesia lineatifolia*. Three concentrations of butanolic and one of acetate extracts were tested, using DMSO as solvent. The assays were performed according to the plate direct incorporation procedure, with the TA98 and TA100 strains of *Salmonella typhimurium*, with and without metabolic activation. After statistical analysis, indications of mutagenicity of both extracts were verified in the TA98 and TA100 strains, with some of the tested concentrations being statistically different from the values of the negative control. However, none of the strains the mutagenicity ratio was larger or equal than 2, for any of the evaluated concentrations, not allowing the characterization of the vegetable extract as mutagenic. Furthermore, the assays with metabolic activation any significant alteration was verified in the revertants frequency regarding the negative control.



109544

Establishment of Tissue-Equivalent Model: 3d Structure from Prostate Cells Present *In Vitro* Characteristic Similar *In Vivo*

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Prostate cancer is the most common cancer and the second most lethal men in Western society, but is uncommon among Asians and occurs more frequently in individuals of black race. In Brazil, data from 2008 projected an estimated 49,530 new cases of prostate cancer. Knowing that the interactions between the epithelial and stromal compartments are important for the development, growth and differentiation of normal prostate as well as in pathological prostate cancer, understanding these interactions *in situ* are important for studying the action of substances that can potentially to act on the malignant cell. The goal of this study is the *in vitro* establishment of the 3-D structures from epithelial and stromal cells of the prostate, in order to understand biological responses that recapitulate the human *in vivo* tissue, in a facile and robust system to be used by researchers with diverse scientific interests. To obtain the 3-D structures cell lineage (PC-3 and DU-145) and primary cells were cultured in the proportion of 1:1 (1,5 × 10⁴ cells) in 1% agarose gel coating tissue culture plate 96-well, containing DMEM culture medium with 10% fetal bovine serum. We analyzed the extracellular matrix components distribution by immunofluorescence and cell morphology by transmission and scanning electron microscopy (EM). Immunofluorescence and EM analysis revealed epithelial cells in the inner and stromal cells organized in cortex zone of 3-D structures. The 3-D structures of prostate cells can represent an important tool for the investigation of various biological processes related to prostate cancer.

109231

3-D *in vitro* Model of human gastric cells in simulated microgravity of a NASA Bioreactor: a new model for Studying Epithelial Cells and Helicobacter pylori Interaction

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The WHO concluded, in 1994, that *Helicobacter pylori* (Hp) is a definite or class I carcinogen in humans. The lack of readily available experimental systems has limited knowledge pertaining to the development of Hp-induced much gastrointestinal disease in humans [1]. Our aim was establish a 3-D model of human gastric epithelial and stromal cells for studying molecular aspects between epithelial cell and Hp. These cells were isolate from endoscopy gastric biopsies that were enzymatically dispersed. To obtain the 3-D structures (gastrospheres) cells were cultured in the proportion of 1:1 (1,5 × 10⁴ cells) without carrier beads in tissue culture plate 96-well, containing DMEM culture medium with 10% fetal bovine serum. After 48 h, gastrospheres were transfer upon NASA rotary cell culture system and cultivated by days 7, 14, 21, and 28. In this work we analyzed the extracellular matrix components distribution by immunofluorescence and cell/Hp interaction assays mainly by electron microscopy (EM). Immunofluorescence and EM analysis of 3-D gastrospheres revealed that the epithelial cells were rounded, organized in cortex zone and expressed cytokeratin, and stromal cells were sprayed localized inner spheroid with vimentin positive. EM revealed groups of cohesive gastric cells surrounded by stromal structures and reticulin fibers. Interaction assays showed that Hp established infection of epithelial cells in both culture models. The presence of phagosome in the gastrospheres infected by Hp was visualized. Considering our results, these models can be used as *in vitro* equivalent tissue models for a better understanding of studies of cell-to-cell interactions that occur *in vivo*. [1] S. Wen and S. F. Moss, *Cancer Lett.* (2010) 282(1): 18. [2] This research was supported by CNPq (Brazil), FAPERJ and INMETRO).