The evaluation of the effect of latex condoms using cell culture techniques
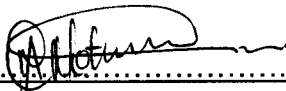
by

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A research dissertation submitted to the Department of Anatomy, School of Health Sciences, of the University of Pretoria in fulfillment of the requirements for the Degree of Masters of Science (MSc) in Anatomy

Declaration

I, Nana Arcilia Motsoane hereby declare that this research dissertation is my own work and has not been presented for any degree of another University;

Signed.........................

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Dedications

To my family and most especially to my father.

Abstract

Increased awareness of protection against infections such as Hepatitis B and Human Immune-deficiency Virus (HIV)/ Acquired Immune Deficiency Syndrome (AIDS) and other sexually transmitted diseases has led to an increase in the demand for latex gloves and condoms leading to an increase in latex allergy. Besides latex, condoms also contain several undisclosed chemicals including antioxidants, accelerators, emulsifiers, stabilizers, lubricants, and in some cases flavourings and colourants. Though extensive testing is done to evaluate the physical quality of condoms, little information is available regarding the biological safety of condoms.

In this study a modification of the direct cell culture testing method that is specified by the American Test Method F813-83 of 1998 was used to determine the cytotoxicity of the surface material of latex condoms prepared at time intervals that represents normal physiological exposure times T_2, T_4 and T_8. The L929 cells were exposed to medium containing increasing amounts of condom washings (0-66%) for 20 hours. After exposure cell number and viability was determined using the Crystal violet (CV) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-3H-tetrazolium bromide (MTT) assays respectively. Data was evaluated using a split-plot design with the appropriate Analysis of Variance (ANOVA).

The effect of the condom washings on cell morphology and CV staining, MTT metabolism and Neutral red (NR) uptake at a fixed condom washing (16%) and exposure time T_8 was evaluated microscopically. Cell membrane integrity was evaluated by Propidium iodide (PI) uptake and with PI staining after fixation and Hoechst 33324 (H33342) staining nuclear structure was evaluated with fluorescence microscopy. Apoptosis induced DNA fragmentation was evaluated by agarose gel electrophoresis. The effects of condom washings at 16% condom

washing and exposure times T_2, T_4 and T_8 was further evaluated in the HeLa cell line, a cell line in origin and type closer to that of the cervical lining. Cytotoxicity was evaluated using the CV, MTT and NR assays.

In the L929 cell line, condom types Non-lubricated condoms (NLC), Lubricated condoms (LC) and Lubricated and flavoured condoms (LFC) behaved differently over time of exposure and the concentrations of condom washings. LFC were found to induce a decrease in cell number compared to other condom types, followed by LC and NLC revealed increases in cell number. Split-plot analysis, revealed that condom type x time (CT x Time) is significantly different due to the effect observed at T_2 for LC. The MTT usually considered being more sensitive than the CV assay showed only toxicity for LFC and not for NLC and LC as with the CV assay. Exposure to LFC revealed significant decrease of 70 % decrease in cell viability at T_8.

Condom washings, LC, LFC and LFCC had no effect on cell morphology following CV staining. MTT metabolism and NR uptake was reduced and altered cell morphology was observed for L929 cells exposed to LFC and LFCC. Little PI uptake was observed for all cells exposed to condom washings. Condensed nuclei were observed for L929 cells exposed to LFC and LFCC while Hoechst staining revealed peripheral arrangement of DNA with Hoechst 33342 staining. Cell death in L929 cells were found to be mediated by apoptosis with L929 exposed to LFC showing the most damage. All effects of LFC is greater than that observed for LFCC indicating that other factors rather than the number of components present in each type of condom may account for toxicity.

Toxicity of condom washings were compared to that found in the L929 cell line using the CV and MTT assays and an additional bioassay the NR assay was included. Condom types, LC, LFC and LFCC had a significant effect on cell

viability and lysosomal membrane integrity. Differences observed between the L929 and HeLa cells were due to the increased viability observed for LC and the decrease in membrane integrity for LFC on HeLa cells. With LC and LFC no decrease in cell number and viability was observed as previously reported for the L929 cell line.

Although no decreased in cell viability is observed for LFC a decrease of 75% in lysosomal membrane integrity is observed. The increase in cell viability found for HeLa exposed to LC (although statistically not significant) cannot be explained. Changes in cell viability and membrane integrity was only observed for HeLa cells, indicating that the HeLa cell line is more sensitive to the cytotoxic effects of condom washings. Furthermore the NR assay is a more sensitive assay than the MTT assay in detecting the cytotoxic effects of LFC condom washings at low concentrations. These are assays address only the effects of short-term exposure and not possible genotoxic effects that may occur following repeated and long-term exposure as reported in other latex products.

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List of publications and presentations

Full Articles

1. Motsoane NA, Pretorius E, Bester MJ, Becker PJ. The Biological safety of condom material can be determined using an in vitro cell culture system. Analytical Cellular Pathology 2001; 23(2): 51-59.

2. Motsoane NA, Pretorius E, Bester MJ, Becker PJ. An in vitro study of biological safety of condoms and their additives. Human and Experimental Toxicology. (In press)

Scientific Letter

1. Pretorius E, Motsoane NA, Bester MJ. Evaluation of the biological safety of condoms using an in vitro cell culture method. (Scientific Letter) South African Medical Journal Jan 2000; 91(1): 50.

Abstracts

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Posters

1. Motsoane NA, Pretorius E, Bester MJ. Evaluation of the biological safety of condoms using an in vitro cell culture method. Presented on faculty day, Faculty of Health Sciences, UP 2000

2. Motsoane NA, Bester MJ, Pretorius E, Becker PJ. The evaluation of the cytotoxic Effect of Condom Material on HeLa Cells in vitro. Anatomical Society of South Africa: 32nd Annual Conference and Faculty day, 2002, Health Sciences, UP.

List of Abbreviations, Symbols and Chemical Formulae

AIDS	Acquired Immune Deficiency Syndrome
%	Percentage
µg	Micro gram
µL	Micro liters
µm	Micro meter
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CCL	Certified Cell Line
CM	Condom medium
cm^2	Centimeters squared
CO_2	Carbon dioxide
CT	Condom type
CV	Crystal Violet
ddH_2O	Double distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate buffered saline
EDTA	Ethylene diamine tetra acetic acid ($C_{10}H_{16}N_2O_8$)
EL900	Elisa plate reader model EL900
EMEM	Eagle's minimum essential medium
ET	Exposure time
EtBr	Ethidium bromide
FCS	Foetal Calf serum

FDA	Food and Drug Administration
FLIPs	Flice-like Inhibitory proteins
H 33342	Hoechst 33342
HBSS	Hanks Buffered Salt Solution
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
ICC	Immunocytochemical
ICE	Interleukin 1ß-converting enzymes
IAPs	Inhibitors of apoptosis
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KCl	Potassium chloride
KDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
LC	Lubricated condoms
LFC	Lubricated and flavoured condoms
LFCC	Lubricated, flavoured and coloured condoms
LSD	Lowest Standard Deviation
Mg^{2+}	Magnesium ion
ml	Milliliters
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-3H-tetrazolium bromide
Na_2HPO_4	Sodium Disodium hydrogen phosphate
NaCl	Sodium chloride
NADH	
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
$NaHCO_3$	Sodium hydrogen carbonate

NCTC	National Cancer Institute Tissue Culture unit
NLC	Non-lubricated condoms
nm	Nano meters
NR	Neutral Red
TNF-R	Tumor Necrosis Factor
NRL	Natural Rubber latex
$^{\circ}$C	Degrees centigrade
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution.
PS	Phosphotydalserine
p-Value	Probability value
r^2	Square Correlation
rpm	Revolutions per minute
SABS	South African Bureau of standards
STD	Sexually Transmitted Diseases
T_0	Exposure time 0 minutes
T_2	Exposure time 2 minutes
T_4	Exposure time 4 minutes
T_8	Exposure time 8 minutes
U	Units
W/v	Weight per volume
W_0	Condom washings exposed for 0 minutes
W_2	Condom washings exposed for 2 minutes
W_4	Condom washings exposed for 4 minutes
W_8	Condom washings exposed for 8 minutes
xg	Gravitational acceleration

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CHAPTER 1: INTRODUCTION

Latex products have long been recognized as a cause of latex protein allergy (1-4). The use of protective gloves and condoms is being encouraged both nationally and internationally for protection against infectious body fluids and diseases such as Hepatitis B and Human Immuno-deficiency Virus (HIV)/ Acquired Immuno Deficiency Syndrome (AIDS) and other sexually transmitted diseases (1, 3, 5). Most of the HIV/AIDS awareness campaigns and associated organizations encourage the use of condoms and gloves as a preventive measure against HIV infection (6).

Unfortunately, due to repeated use and exposure to latex products, studies have emerged, indicating that the natural rubber latex protein (NRL) causes allergy and/or sensitivity (7). The incidence of allergy to latex varies widely depending on the usage of the products. According to research, health care workers, latex industry workers, people undergoing multiple surgeries, atopic-prone individuals (those who get atopic eczema, allergic rhinitis, hay fever and asthma) and individuals experiencing repetitive exposure to latex products are the most susceptible (1-3, 5, 7-13).

Allergy and/or sensitivity is particularly noted in medical and dental personnel because of the frequent donning and taking off of the gloves (14-18). Sensitization to latex has been found to range from itching and in some cases simple dermatitis to life threatening conditions such as anaphylaxis (2, 4, 5, 8-10, 13, 16, 17, 19-22). Several reports have also highlighted death due to such sensitization (7, 9, 20, 21). Allergy to NRL is therefore a real threat, in particular to sensitized, but undiagnosed patients in health care.

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The use of latex condoms in particular, has gained much popularity due to availability and ease of use. Condoms made from natural rubber latex have been developed as an alternative to lambskin condoms due to their durability (23). Condoms are also freely available in most countries including South Africa, and here are widely distributed to the public at work, to households and schools by the Department of Health.

Individuals already sensitized to e.g. latex gloves may be particularly vulnerable when using latex condoms. Like all other allergies, with repeated exposure to latex condom surface material a threshold concentration or point is reached. Once a threshold point is reached, an allergic effect might be seen in some individuals. Should the conditions not change, more serious conditions may ensue (1, 10, 15, 24). Studies have shown that latex products contain not only one but several proteins that are potential allergens (1, 3, 25). Since the use of latex condoms has gained popularity, the prevalence of latex sensitive individuals amongst the populations has also increased (3, 7, 9-13).

Although extensive testing is done to evaluate the physical quality of condoms both nationally and internationally, the biological safety of these products needs to be assessed. Besides needing to assess the allergenic potential of NRL and other added compounds it is also necessary to determine the biological safety of all constituents. The latter can be achieved by using an *in vitro* cell model. Although cell models provide little information regarding allergenic potential, these do provide information on the toxicity of the constituents on condom material. Cell models allow rapid testing of different products under different experimental conditions.

Cytotoxicity process is a complex *in vitro* cellular process, and its expression in the cell may manifest itself in a wide spectrum of cellular events. These events range from simple cell death (apoptosis or necrosis) to functional changes such as alterations in metabolism and membrane integrity (26, 27). *In vitro* cytotoxic assays or bioassays used to measure these changes must be sensitive enough to detect the effect of very small concentrations of cytotoxic substances and must be easy to perform (27)

The purpose of the research presented in this dissertation was to develop an appropriate, simple, reproducible and cost effective method for evaluating the biological safety of commonly used latex condoms in South Africa. Literature reviews have shown several methods of evaluating the cytotoxicity of different products and toxins; only in a study by Pretorius and co-workers in 2000 a method for evaluating the cytotoxicity of latex glove materials using the L929 cell line (28) has been described. This study showed that the surface material of latex gloves caused a decrease in L929 cell viability when measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-3H-tetrazolium bromide (MTT) assay.

The test method used by Pretorius and co-workers involves a modification of a direct contact cell culture testing method (FDA test method F813-83). The original F813-83 developed by the FDA (Food and Drug Administration) is used as a standard test method for the evaluation of the cytotoxic potential of medical materials and devices (22)

The basic composition of condoms is the same as that of gloves although often other compounds such as lubricants, spermicides, colourants and flavourants are added. Therefore a similar test system can be used to determine the biological safety of condoms.

The main aims of this study will be addressed by answering the following research question.

1. Can the modified F813-83 method be used to determine the cytotoxicity measured with the (Crystal Violet) CV and MTT assay of non-lubricated (NLC), lubricated (LC) and lubricated flavoured (LFC) latex condoms on L929 cells *in vitro?* These results are presented in Chapter 3.

2. If condom washings (including an additional condom type, lubricated, flavoured and coloured condoms (LFCC)) are toxic to L929 cells *in vitro* by what cellular mechanism do these effects occur? This will be investigated using light and fluorescence microscopy as well as gel electrophoresis and the results will be presented in Chapter 4.

3. Further research included the evaluation of a cell line more closely related to the lining of the human cervix namely the HeLa cell line a cervical epithelial cancer cell line. The effects of condom washings on this cell line will be compared to the cytotoxic effects observed in the L929 cell line using the MTT, CV and the Neutral Red (NR) assays. These results and a concluding discussion of all findings are presented in Chapter 5 and 6 respectively.

CHAPTER 2: LITERATURE REVIEW

2.1 Latex

Latex is a milky natural sap extracted from trees and plants of Euphobiaceae family. In everyday usage, latex refers to rubber produced from the Brazilian rubber tree, *Hevea brasiliensis* (1, 3, 5, 9, 13, 20, 21, 28-32). The milky sap is ammoniated to preserve it at harvesting time and then transferred to factories where latex products are manufactured. Processing of natural latex result in an elastic pliable product called NRL.

Many chemicals are added to the sap before and during processing. Accelerators, antioxidants, emulsifiers and stabilizers are added later and this results in a product that has many commercial uses. Latex is used commercially in the production of various gums and rubbers with extensive industrial applications of over 40 000 products (3). Amongst other things, latex is used to manufacture surgical gloves, condoms, catheters, tyres, chewing gums, balloons, babies' teats and toys (3, 4, 15, 17, 20).

Although natural rubber latex is inexpensive, and versatile, its problem and that of its products is that they contain many potential allergens. The chemical structure of the elastic component of the latex is cis 1-4 isoprene (1, 3) and is associated with latex proteins with molecular masses ranging from 4 to 70 kDa (3). Included are molecules such as Hevein, Prohevein, Hovamine, and the enzyme phenyl transferase and rubber elongation factor that are implicated as allergens (20, 25, 32, 33). Thus the antigenicity of latex is heterogeneous and

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therefore the diagnosis of Immunoglobulin E (IgE) mediated sensitivity to latex is often difficult to confirm. (25).

2.1.1 Latex Allergy

Allergy to NRL was first reported in a German literature in 1927 (19), and it was only in 1979 when Nutter reported the first English literature where he noted a case of contact urticaria due to latex sensitivity (1, 2, 5, 9, 19, 20, 28, 29, 31, 34). Since then, there has been an exponential increase in the number of reported cases amongst health care workers, latex industry workers and amongst subjects with positive risk factors such as children with spina bifida, those undergoing multiple surgeries and atopic subjects (1, 5, 12, 13, 19- 21, 28, 29, 34-38).

A large worldwide increase of allergy to latex proteins has been observed due to increased usage of latex gloves and condoms due to diseases such as HIV/ AIDS and Hepatitis B (28) and a recommendation for universal precautions for protection from potentially infectious body fluids (3, 5, 12, 21, 39, 40). Latex products, particularly gloves and condoms, offer functionally, inexpensive and effective disposable barriers to diseases such as HIV, Hepatitis B and other sexually transmitted diseases (STD).

Latex allergy has become a health and occupational hazard and a risk to latex protein-sensitized subjects (7, 10, 21, 29, 35). Reactions to an allergen are usually of the immediate type (Type 1) and are elicited by IgE (1, 3, 21, 25, 31, 35, 36, 39-41). The predominant manifestation is contact urticaria; less frequent are rhinitis, conjunctivitis, asthma or anaphylactic shock (10, 19-21, 28, 29, 34,

37, 39). If the environment does not change, symptoms can progress to serious life-threatening reactions like asthma, anaphylaxis and death (1, 10, 20, 30, 38). With the growing use of condoms to prevent transmission of STD and for contraception, the incidence of allergy to condoms has also increased (7, 12, 13, 20, 28).

The worldwide increase in reported reactions of latex is related particularly to the increased use of poorly manufactured rubber gloves and condoms (3, 5). Sensitization can occur through direct contact with the skin, percutaneously, mucosal or it can be through parental routes (3, 12, 25) and the antigen can be transferred by direct contact or aerosol (25). Mucosal exposures are said to be more dangerous than the other exposures (3, 12). It is, however, not known exactly what quantity of latex allergen exposure is required for sensitization or to elicit a response in an allergic individual.

In cytotoxicity studies, an effect is only elicited when sufficient amounts of toxins or allergens are exposed to the subject under study. Depending on the allergen or toxins, when a certain level or amount is reached, the effects would be seen. These levels are known as the threshold exposure levels. According to Poley and Slater (2000), a threshold exposure level to latex is a complicated phenomenon because of the following:

1. Latex is a mixture of potent allergens with different stability and bioavailability characteristics that could be present in different levels in different environments, depending on the source of exposure. Therefore an aggregated threshold level of different allergens may mask biologically significant allergen thresholds.

2. An individual needs to first be sensitized to an allergen so that an immune system can develop the clonal responses for subsequent immune responses, then the repeated exposure to an allergen elicits the effector response. The threshold levels for both sensitization and eliciting responses may be different thus making it necessary to outline both the doses necessary for sensitization and those necessary for eliciting a response in a sensitized individual.

3. The route of exposure may affect the minimum exposure level necessary to sensitize and elicit reactions, since sensitized individual can react to latex allergens to which they have been exposed by different routes. It is also likely that the sensitizing and the eliciting doses of each allergen will vary by the route of exposure.

Three basic categories of adverse latex associated conditions have been described as irritant dermatitis (Type IV), allergic and immediate/Type 1 hypersensitivity (3, 5, 12, 13, 19, 28, 32, 34, 38, 42).

2.1.1.1 Irritant dermatitis

Irritant or non-allergic dermatitis causes itching or burning, redness, swelling, dry crusted skin, fissures, peeling, sores and papules. It is limited to the area of contact, and only acquired by skin contact.

2.1.1.2 Allergic contact dermatitis

In allergic contact dermatitis (a delayed hypersensitivity or chemical allergy) the onset of symptoms is usually 6 to 48 hours, and produces itching, pain, redness, blisters, dry crusted skin, papules, vesicles and peeling open sores. The reaction may become systemic, and is acquired by skin contact. The primary causative agents might probably be either the latex protein itself or the processing chemicals associated with products manufacturing.

2.1.1.3 Immediate/Type 1 hypersensitivity

The immediate or Type 1 hypersensitivity is the least common but causes the worst reactions. Its symptoms include itching, tingling, hives (contact or systemic urticaria), flushing, facial swelling, rhinitis, rhinoconjuctivitis, wheezing, nausea, abdominal cramps, tachycardia, hypertension, bronchospasm and anaphylaxis. The reaction is caused by latex proteins and is acquired by skin or mucous membrane contact with latex product.

2.1.2 Cross-reactivity to latex

Cross-reaction to latex may occur after ingestion of food, which contains similar proteins such as some fruits (banana, peaches, nectarines, plums, avocados and kiwi fruit), vegetables (tomatoes and raw potatoes), chestnuts and cereals (1, 12, 20, 25, 29, 37). Latex products contain some compounds that causes medical

conditions, these includes added chemicals such as antioxidants that cause dermatitis and natural proteins that cause systemic, potentially life threatening allergic reactions (1, 10, 20, 40).

2.1.3 Prevalence of latex allergy

Contact urticaria in relation to rubber gloves was first described in 1979 (9). More recently, IgE -mediated reactions associated with severe anaphylactic shock and cardiovascular collapse have been attributed to latex. According to Poley and Slater in 2000 and Tan *et al* (1997), the most important factor in latex sensitization is the degree of exposure (5, 25). The actual rates of prevalence of latex sensitized people are not entirely clear. Studies have shown prevalence ranging from 1% to 17% with less found amongst the general population and higher levels amongst the risk groups identified previously (5, 25, 41)

In 1995 the Center for Disease Control of America reported 600 cases of type I latex sensitivity and at least 16 of those identified patients died as a result of their sensitization (29). Between 1988 and 1992 the Food and drug Administration (FDA), received more than 1000 reports of adverse health effects from exposure to latex, including 15 deaths due to such exposure (20, 29).

Although dentists are constantly exposed to latex allergens, dental assistants as well as patient may potentially be at risk to develop latex allergies. In a study by Nkomo and co-workers in 2001, 81 dentists in the Pretoria area were questioned regarding latex sensitivity. All respondents were found to be using latex gloves at the time of questioning or had used latex gloves at some time in their practice.

Among the respondents, 31% suffered from latex allergy symptoms (defined in the questioning process as allergic reaction to latex after the use of latex gloves). Of these 31,27% reported that latex allergy was noticed when using powdered gloves, whereas 4 noticed the allergy using both powdered and powder-free gloves. Itching (19%), redness or rash (19.2%), and dry skin (14.1%) are the symptoms most frequently associated with latex allergy in dentists (43).

There was no significant relationship found to prove if length of time that dentists had used gloves influenced allergies. It can be concluded from this study that latex allergy within dental practices is a significant occupational health risk as 31% of the respondents in the study by Nkomo and co-workers suffered from latex allergies (43). This is comparable with the 38% found among Danish dentists (44) and the 38,2% latex allergy prevalence amongst hospital employees in the Pretoria area (28). Furthermore, it was noted that dentists consider latex allergy as an important occupational hazard to be endured as a daily irritation (28, 44).

2.1.4 Respiratory problems due to latex glove usage

Since the 1980s numerous respiratory allergies and asthma-like attacks in hospital employees and patients have been reported because of exposure to latex products. The problem was ascribed to inhalation of airborne natural latex allergen in the areas of heavy use of powdered gloves (14-16, 45). Baur and co-workers in 1993 suggested that up to 30% of natural latex-sensitive individuals develop respiratory problems. These authors also suggested that the aerosolized

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glove powder in areas of frequent glove usage might affect direct users, as well as those who do not use the latex products, but are in the same areas (16).

Beezhold *et al* (1992) investigated the ability of glove powder usually cornstarch to absorb the latex protein, and serve as an allergen. The study indicated that cornstarch not exposed to the latex protein contained no allergenic proteins. Cornstarch exposed to natural latex as well as cornstarch already in powdered latex gloves (and extracted from the gloves) had a significant amount of allergenic proteins bound to its particles. The results indicated that cornstarch binds allergenic proteins, and the studies supported the causal relationship between asthma reactions in individuals with natural latex allergy (17).

Pisati and co-workers in 1994 performed a bronchial provocation test by exposing allergic individuals to inhalations from the following products: extracts from powder-free surgical gloves, extracts from powdered surgical gloves and clean cornstarch powder extracts. Clean cornstarch powder caused no bronchial reaction, exposure to powder-free glove extracts induced immediate broncho-constriction in half of the subjects, while broncho-constriction was induced in all the subjects exposed to extracts of powdered gloves (36).

In 1990, Ellis provided background information and a summary of the problems associated with the use of glove powder (18), while the negative influences of latex powder has also been indicated by numerous researchers (46). Zaza and co-workers in 1994 reported on natural latex sensitivity with some reference to glove powder (47), while other researchers pleaded for powder-free gloves because they believe that the powder plays a role in the contamination of surgical wounds and in peritoneal adhesions (48, 49). Authors like Becker and

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co-workers in 1996 discussed the fact that peritoneal adhesions appear after the use of powdered latex gloves (50).

In a study by Pretorius and Bester in 2000 the authors investigated the cytotoxic potential of washings from powdered latex gloves, non-powdered latex gloves and latex-free nitrile gloves. The washings contained all surface material from the gloves. Cells in culture were exposed to these glove washings and cell number and viability was determined using the CV and MTT assays respectively. Results indicated cytotoxic effects on cell number and viability. From the results, the authors concluded that non-powdered latex gloves have the least effects on the cells, while powdered latex gloves were the most cytotoxic. This might be because more latex protein was present in the washings from the powdered latex gloves, due to the affinity of the latex proteins to the powder (28).

Although results from the study by Pretorius and Bester (2000) cannot give an indication of the type of allergic reaction, it is clear that latex proteins cause a decrease in cell viability and interferes with cell growth. This might play a role in the development of an allergic reaction.

2.1.5 Diagnosis, treatment and management

Diagnosis is based on an accurate history of documented symptoms occurring on exposure to different rubber products and on identifying individuals with latex-specific IgE and symptoms consistent with IgE mediated reactions to latex products (5, 20, 31, 32). The diagnosis is confirmed by the blood CAP RAST, skin prick and patch tests (3, 5, 12, 34). Though many methods have been

developed, no single accurate and reliable method has been developed due to the heterogeneous allergenic potential of latex proteins (3, 12, 25).

Several medications and immunotherapies can be used in the treatment latex allergy, but avoidance of latex products by sensitive individuals is the most important factor in preventing the most serious effects of latex allergy (12) since exposure may occur through airborne particles and through intravenous fluids. It has been observed that continued exposure to latex allergens is detrimental to affected individuals (20). The discreet nature of latex exposure could easily lead to failure to detect latex allergy. Many instances of latex allergy may still be undiagnosed or misclassified as cases of idiopathic anaphylaxis.

The following recommendations were made by the task force

- Medical and recreational devices should be labeled and certified as to natural rubber content.

- Manufacturers of natural rubber products and growers of natural rubber producers are encouraged to work closely with investigators to identify key antigens and adopt strategies to decrease and if possible eliminate the antigenic components of their products.

- All health care facilities and providers should be prepared and willing to offer latex- free procedures according to the level of service provided (20).

2.1.6 Allergy to latex condoms

The use of condoms to prevent sexually transmitted diseases has been encouraged worldwide (2, 5-7, 40). The recent increase in demand of both latex gloves and condoms has led to poorly manufacturing of less refined products containing higher concentrations of latex allergens. There is also considerable variation in the total protein and allergen contents of different gloves and condoms brands (5). Condoms have also been found to induce latex allergy (2-4, 7, 19, 34, 40, 42).

At present, there are few latex free condoms that prevent the transmission of sexually transmitted diseases (12, 40). Like rubber gloves, most condoms contain both natural rubber latex and chemical allergens (1, 2, 9, 10, 13, 19, 30). Contact dermatitis attributable to processing of chemicals is probably the most common problem (10, 13, 30), and anaphylactic reactions to condoms have also occurred (2, 13, 19).

Typical reactions to latex condoms include swelling; pruritus of the vulva and the vulvovaginal area during or after coitus followed by vesiculation lichenification and generalized eczema. Immediate-type hypersensitivity to latex in condoms involving local swelling, pruritus, vulvoviginitis in women (12), penile dermatitis, swelling of the penile shaft (22) rhinoconjuctivitis, cutaneous urticaria and generalized reactions has also been observed (2, 4, 30, 42).

Condoms can cause local or systemic reactions in latex allergic individuals (4, 34, 42, 51). In a study conducted by Turjanmaa and Reunala in 1989, of the 46

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patients with latex glove contact urticaria, 7 of the 29 patients with a history of condom use had experienced local swelling and/ or pruritus during intercourse (4). In another study by Fisher (1994), a woman was found to have had immediate type reaction to latex condoms and gloves. She had a history of contact urticaria due to latex gloves and rubber condoms (42). Another woman developed itchy dermatitis of the vulva and vagina with discharge in a study conducted by Bircher *et al* (1993)(30).

Likewise men also often present with sensitivity towards latex condoms. Shenot et al (1994) presented two cases of males with severe allergic reactions to latex condom catheters. In one case, a paraplegic patient developed a chronic penile dermatitis. Attempts at establishing urinary drainage using an indwelling urethral catheter were abandoned after the development of urethro-cutaneous urethro-scrotal fistulae. The patient underwent a definitive therapy with cystectomy and ileal conduit diversion because of morbidity associated with the latex urinary drainage device (34).

2.1.7 Effect of other additives to latex gloves and condoms

Allergic reactions to brand- specific condom contents such as spermicides, dry (silicon based) or wet (surgical jelly or aqueous gums) lubricating agents, perfumes, local anesthetics (intended to prolong intercourse), colourants, flavourings, or other chemical reagents should also be of great concern (7, 13, 40, 47). According to the study done by Docena *et al* (2000), allergenic and antigenic components in latex condoms were identified and were comparable with those found in other latex products such as latex gloves (7). These authors

showed that different condom brands show a wide variation in protein composition and in protein quantities. In a previous study of Motsoane and co-workers in preparation of this dissertation, it was found that cell culture medium containing latex condom washings induced cell death in the L929 mouse fibroblast cell line (52).

Occupational asthma was identified by Cullinan *et al* in two patients employed in a manufacture of condoms. These condoms were made from *Lycopodium clavatum* used as a rubber dusting agent, in this case used for precoating of condoms (53). *Lycopodium* powder, made from the spores found in ground pine or club moss, has been used as the first lubricant (54). In 1930 the surgeons realized that this causes granuloma and adhesion formation and as a result was unacceptable to use as a glove lubricant (54) but still continued to be used in condoms (53).

Many studies have been done on identifying chemical allergens associated with some brands of condoms, and identifying clinical symptoms of latex allergy (7, 40). The method developed by Pretorius and Bester in 2000 and Motsoane *et.al* in 2001 (52) in preparation of this dissertation is the only available documented protocol for determining the biological safety of latex products (28, 52). The advantage of such biological system would determine the safety of all components of a specific type of condom. This will include the effects of latex, lubricants, colourants and flavourings and the possibility of an additive, synergistic, antagonistic or coalitive effect between any components.

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2.2 Toxicity and cytotoxicity

Toxicity can be defined as the biological or chemical process where a product (pollutant, chemical, bacteria or virus) has a toxic effect on a biological system (e.g. organisms) or on the environment (e.g. water sources). In order to determine toxicity, two models exist, a chemical and a biological model. Chemical-based analysis of toxicants determines the specific constituents of a sample, but not the cytotoxic effects it could have on organisms or cells. In the biological model, toxicity tests determine the effects of a toxicant on living material, and particularly on the cells of an organism (27).

The toxic effect on living material (including cells in culture) is termed cytotoxicity. Cytotoxicity tests are important additions to chemical-specific measurement used in the chemical model, where e.g. High Pressure Liquid Chromatography (HPLC) is used to determine chemical composition of a particular sample[i]. When using the biological model, the organism or cells in culture should adhere to specific requirements. The researcher must have knowledge of the origin of organism or cells, the cells must be disease-free, acclimatize to test conditions and be handled and treated with care to limit variation in their response (27). The biological model may utilize either *in vivo* or *in vitro* methods to determine cytotoxicity. Methods used in this dissertation utilize the *in vitro* method; therefore specific attention will now be focused on procedures and definitions of the *in vitro* method.

In vitro cytotoxicity assays are also known as bioassays and may use sensitive cells in culture exposed to different toxicants. The cytotoxicity process is a complex *in vitro* cellular process, and its expression in the cell may manifest itself

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in a wide spectrum of cellular events (26, 27). Furthermore cytotoxicity may not only affect cells but also different tissues, organ systems even different species of organism (26, 55).

2.2.1 Cytotoxicity in an organism

Cytotoxicity results in changes in the normal physiological functioning of the cell in an organism. These changes may lead to the alterations in the metabolic functioning of the entire cell or parts thereof that can eventually result in the total structural, biochemical or physiological changes within a cell or even cell death. The cytotoxic effects can be local or systematic affecting either a specific point of exposure or may affect other areas that may include the organ or systems of an organism, respectively; delayed or immediate; direct or indirect (affecting the exposed area or may affect other areas that were not in direct exposure with the compound) and reversible or irreversible (recovering from the damage or having a definite damage) (26, 55).

When different toxins are mixed, the resulting toxicity might either be the same as the sum of the toxicity of the components (additive) or may be greater than the sum of added toxicity (potentiation or synergism), less (antagonism) or it may be different or coalitive. Repeated exposure to a toxic compound may lead to a reduction of a cytotoxic effect, a phenomenon known as tolerance (26). Toxicity shows a dose-response relationship and may range from complex biochemical changes to lethality and may involve receptor interactions (56, 57).

Cytotoxicity due to a chemical is being classified by Timbrell (26) as can be divided into six types and may appear in individual cells and may affect organs of

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an organism. The six types are: direct tissue lesion; physiological, pharmacological and biochemical effects; teratogenesis; immunotoxicity; mutagenesis and carcinogenesis.

2.2.1.1 Direct tissue lesions

This type of cytotoxicity appears in organs of organisms. Direct tissue lesion results in damage to a target organ. The target organ is determined by various factors that includes function and position of the organ, its blood supply, whether it has the uptake systems, pathways of intermediary metabolism, biotransformation capabilities, repair mechanism, or it can bind to particular macromolecules or its vulnerability to damage or disruption (26).

Mechanisms and response in cellular injury may result from primary, secondary or tertiary underlying events. Primary events result in initial damage and include lipid peroxidation, ischaemia, changes in thiol status, enzyme inhibition and covalent binding to crucial macromolecules. One or more of these events may be initiated following an exposure to a cytotoxic compound (26, 27).

Secondary events result from primary and include both biochemical and structural changes such as changes in membrane structure or permeability, mitochondrial damage, lysosomal destabilization, inhibition of mitochondrial function, DNA damage, changes in Ca^{2+} concentrations, damage to the endoplasmic reticulum and activation of apoptosis pathways.

Tertiary events are the final observable manifestations of exposure to a cytotoxic substance and include fatty changes, apoptosis, blebbing and necrosis. These changes may occur together or sequential and are irreversible (26).

2.2.1.2 *Physiological, pharmacological and biochemical responses*

The toxic foreign compound can affect the homeostasis of an organism by altering basic biochemical processes. These processes are physiological, pharmacological and biochemical responses and may result from interactions of chemicals with receptors or specific enzymes leading to anoxia, inhibition of cellular respiration, respiratory failure, changes in pH, temperature, changes in blood pressure or electrode balance. These effects may be reversible if inhibition of an enzyme or binding to a receptor is involved. A well-defined dose- response observation is often observed with such effects, and the end point may be death of the animal if a process of central importance is affected. (26).

2.2.1.3 *Teratogenesis*

Teratogenesis is a specific interference with the development of the embryo or the foetus. There are four outcomes of this interference namely; death, malformation, functional deficiencies, and growth retardation. The development of a foetus or embryo involves a sequence of events, many chemicals and other agents can interfere with it leading to one or more of the above four manifestations. Timing, dosage and the type of teratogen is crucial and will determine the nature of the outcome. Teratogens are usually not toxic to the

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maternal organism but are either directly toxic to the developing embryo or it may interfere with its developmental pathways (26, 27).

2.2.1.4 Immunotoxicity

Immunotoxocity is a specific type of cytotoxicity involving either the effect of a chemical on the immune system (immunosuppression or immunotoxicity) or stimulation of the immune system that leads to the toxic response (allergy). The function of the immune system is to protect the organism against infection, foreign proteins and neoplastic cells. It is organized into primary lymphoid tissue (bone marrow and thymus) and secondary lymphoid tissues (spleen and lymph nodes) (26).

The cells of the immune system are lymphocytes are the T-cells (derived from the thymus) and B-cells depending on the site of maturity. T-cell population consists of T-helper (T_H) and T-cytotoxic (T_C) cells. The B-lymphocytes development into plasma cells that produce antibodies (immunoglobulins (Ig)). Activation of the immune system is a complex process but essentially the foreign compound usually a protein is recognized as an antigen. The following events involve either humoral or cellular systems. Molecules, haptens that are structurally too small to illicit an immune response will bind larger molecules and thereby elicit an immune response (26).

Once the antigen has been produced it can then interact with the immune system and be recognized as "non-self" by the immune system in order for a response to be initiated. The response does not necessarily depend on the nature of the

hapten and may involve more tissues than the site of exposure. This occurs in two stages, namely the sensitization and elicitation stages (26).

The sensitization stage involves exposure of a foreign compound, the antigen formation and recognition of the antigen as foreign by the immune system. The immune system responds by producing Ig or activating T-cells. Macrophages and T- and B- cells may collaborate to produce specific memory cells that synthesize Ig or lymphocytes. Five classes of Ig, namely IgA, IgD, IgE, IgG and IgM are produced depending on the type of immune response and the tissue involved. More than one Ig may be produced in response to a single antigen.

The elicitation stage follows a further exposure when the presence of an antigen starts an immune response which may be cell mediated or humoral or both. The response of an animal occurs at this stage and is variable. There are four basic types of adverse reactions that can occur following exposure to an allergen these responses, Type I-IV. Types I-III are humeral responses and Type IV is cell-mediated (26). These types of reactions are outlined by Timbrell in 2000 as the following:

2.2.1.4.1 Type I

Type I reaction is also known as anaphylaxis or immediate hypersensitivity and involves the IgE. Binding of the antigen to Ig releases mediators that induce anaphylaxis resulting in a variety of physiological effects that may be severe, causing difficulty in breathing, loss of blood pressure, anoxia, inflammation, oedema in the respiratory tract and bronchospasms that may be fatal.

2.2.1.4.2 Type II

Type II or the cytolitic reactions involve the free Ig (IgG, IgA, IgM) and antigens bound to blood cells such as leucocytes, erythrocytes and platelets that are agglutinated by the Ig and leads to haemolytic anaemia, leucocytopoenia and thrombocytopoenia. The agglutination may also stimulate the cascade of physiologically active proteins, which result in the release of enzymes such as histamine, kinins and lysosomal enzymes that may cause cell lysis.

2.2.1.4.3 Type III

The type III reaction involves both free antigens and Ig (mainly IgG), which interact to form an immune complex that can precipitate in the blood vessels. Complement activation and precipitation can give rise to thrombosis, inflammation, and damage to the vascular system. The main targets are the joints (rheumatoid arthritis), skin, kidneys (glomerulonephritis), lungs and circulatory system.

2.2.1.4.4 Type IV

Type IV reaction, also known as delayed hypersensitivity is cell- mediated and involves the target of physiologically altered cell membranes by the activated T-cells or T_K and destruction of the antigenic cells. Various mediators such as the lymphokines may be released causing aggregation of lymphocytes, macrophages and basophils.

2.2.1.5 Mutagenesis

Genetic toxicity or mutation is a hereditable change in cell genotype often caused by chemical substances. This may lead to aneuplody where a chromosome may be lost or acquired, clastogenesis where a chromosome or part thereof can be acquired, lost or rearranged, or mutagenesis where a small number of base pairs substitutions, deletions and point mutations may occur. Larger alterations in chromosome arrangement often leads to cell death while smaller changes such as point mutations are often inherited by the next generation of cells (26). Induction of point mutations by chemicals that lead to the development of cancer is known as chemical carcinogensis.

2.2.1.6 Carcinogenesis

Carcinogenesis or the process of cancer formation is a multistage process consisting of initiation, promotion and progression phases. Chemical carcinogensis is the induction of cancer by a chemical substance. The process of carcinogenesis consists of initiation, promotion and progression (26, 27, 55).

2.2.1.6.1 Initiation

The initiation stage is usually rapid and irreversible and involves mutations of the genetic material of a cell. As a result a cell undergoes a transformation in which it gains the potential to develop into a clone of preneoplastic cell.

2.2.1.6.2 Promotion

After the transformation, a promoter is needed to facilitate the necessary proliferation of a preneoplastic cell. The promotion stage is characterized by an alteration in the genetic expression and the growth of the clone from the initiated cell. Promoters may release preneoplastic cells from regulatory control and may also increase the number of tumors produced, and may decrease the latency period between exposure to the initiator and the development of tumors.

2.2.1.6.3 Progression

Progression stage is a stage in which neoplastic cells becomes malignant tumours and may involve changes in the phenotype. This stage is characterized by changes in the number and/ or organization of chromosomes. The result is an increased growth rate, invasion of healthy tissue and formation of metastases.

Several factors influence the process of chemical carcinogenesis such as predisposition and age of an organism, activation and excretion pathways, DNA repair mechanisms and the status of the organism's immune system.

2.2.2 Mechanisms of chemical cytotoxicity

The cytotoxic effect of a chemical on an organism is determined by several factors such as absorption, distribution, metabolism and excretion of a chemical compound/ toxin (26, 27).

2.2.2.1 Absorption

Absorption of a chemical may cause a local effect at the site of administration. Alternatively it is absorbed and then exerts a systemic effect. The most common entry of toxic compounds is via the skin, gastrointernal tract, and lungs; where the toxic compound penetrates through the membranes of the cells by the process of absorption (58). Cell membranes are selectively permeable furthermore absorption can be receptor mediated or may occur via active uptake pathways depending on the cell type and the pathway of absorption. Furthermore the characteristics of the chemical such as size, shape, lipid solubility and polarity will also affect absorption.

2.2.2.2 Distribution

Distribution is a process in which absorbed compounds are carried to various sites of the body. Distribution from cell to cell may occur through diffusion or blood flow to different tissues. Some compounds accumulate in a specific tissue because of their affinity towards a particular macromolecule (e.g.

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carbonmonoxide binds to Haemoglobin in red blood cells being the target site). These compounds may be directed to the liver where they may be removed (extracted/ metabolized).

2.2.2.3 Metabolism

Metabolism is the transformation of a molecule into one or more different chemical entities. This process is catalyzed by enzymes found in various tissue and cells but occurs predominantly in the liver. The metabolism of a molecule will change its physicochemical properties, usually increasing water solubility, size and molecular weight and therefore increasing excretion.

2.2.2.4 Excretion

This is the elimination stage whereby the molecules of the compound are eliminated from the body. The urine is the major excretory route, but other routes including expired air, bile, milk, saliva, and semen may also be utilized. Excretion through bile may reach saturation and lead to hepatic damage while excretion through milk may be dangerous to the exposed infant. Rapid excretion is important to reduce the likelihood of toxic effects.

2.3 Cell Death

Cell death is an essential part of normal development and maturation cycle and is a component of many response patterns of living tissues to xenobiotic (microorganisms and chemicals) and to endogenous modulations, such as inflammation and disturbed blood supply. Two types of cell deaths are identifiable necrosis and apoptosis. Necrosis is unregulated cell death while apoptosis is active and is a regulated sequence of intracellular signals thus influencing the outcome of exposure to a cytotoxic substance (27, 56, 59, 60).

Cellular components such as the plasma membrane, mitochondria, lysosomes, endoplasmic reticulum, nucleus and other cellular organelles can be considered as vulnerable sites of a cell whose destruction or malfunction threatens the functioning of the cellular unit. Necrosis is caused by external stimuli exerting pressure to the cell surface resulting in damage to the cell membrane (24, 56, 59). During this process mitochondria changes its shape and function, and then loses its ability to regulate osmotic pressure, swells, bursts and releases its contents into the surrounding areas. Characteristics of a necrotic cell are chromatin flocculation, loss of membrane integrity, cell lyses and generation of local inflammatory reaction (27, 56, 59, 60).

Apoptosis is an integral part of life and is involved in many physiological processes and plays a significant role in pathogenesis of many diseases. It is responsible for cell death in development, normal tissue turnover, atrophy induced by endocrine and other stimuli, negative selection on the immune system, and a substantial proportion of T- cell killing. . It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral

infections. It is also a major factor in the cell kinetics of tumour, growth and regression and disease such as Alzheimer's disease, Parkinson's disease and heart failure (27, 56, 59, 60).

Exposure of a cell to a chemical can also lead to apoptosis characterized by cell shrinkage, cell membrane blebbing and the formation of membrane bound apoptotic bodies. Usually the organelles structures are preserved intact into the apoptotic bodies but the nucleus undergoes a characteristic condensation of the chromatin. Other characteristics are nuclear and cytoplasmic shrinkage, DNA fragmentation, phosphatidalserine externalization and chromatin condensation. These changes in the cell surface molecules also ensure that in tissues, apoptotic cells are immediately recognized and phagocytosed by their neighbours (27, 56, 59).

Whether necrosis or apoptosis is induced following exposure of a cell to a chemical is a function of concentration and exposure time. Apoptosis is mediated by receptors and several pathways that include death receptors, and apoptosis modulating genes/ proteins, protease cascade, membrane alterations, mitochondrial damage and DNA fragmentation (59).

2.3.1 Death receptors

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Whether necrosis or apoptosis is induced following exposure of a cell to a chemical is a function of concentration and exposure time. Apoptosis is mediated by receptors and several pathways that include death receptors, and apoptosis modulating genes/ proteins, protease cascade, membrane alterations, mitochondrial damage and DNA fragmentation (60).

Apoptosis is induced by stimulation of cell surface receptors in association with caspase activation, eg. CD95 receptor ligand system is a mediator of several physiological and pathophysiological processes including homeostasis of the peripheral lymphoid compartment and CTL- mediated target cell killing. Upon cross-linking by ligand or agonist antibody, the Fas receptor initiates a signal transduction cascade that leads to caspase- dependent programmed cell death (27, 56, 59).

2.3.2 Apoptosis modulating genes/ proteins

Apoptosis is controlled by suppressors and effectors genes working through a number of routes, to regulate the process of apoptosis. These intracellular regulators are the Bcl-2 family of proteins, the FLIPs (Flice-like inhibitory proteins) the IAPs (Inhibitors of apoptosis). The suppressor genes belonging to the Bcl-2 superfamily include the Bcl-2, Bcl-X_L, bcr-abl and the v-abl while the effector genes are the Bax, Bcl-X_S, p53 and the c-myc. The regulators interfere

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with apoptotic signaling pathway at different levels. Cell destruction is mediated by the TNF-R superfamily and under physiological conditions; their corresponding ligand members of the Tumour Necrosis Family (TNF) family activate these receptors (59, 61).

2.3.3 Protease cascades

Signals that lead to the activation of a family of intracellular cysteine proteases, known as the caspases, (cysteinyl- aspatate- specific proteases) play a significant role in initiation and execution of apoptosis induced by various stimuli. Caspases are synthesized as inactive pro-enzymes that are activated following cleavage at specific aspartate cleavage (Asp residue) sites to activate enzymes containing both large (p20) (caspase -2, -4,-5, -8, -9 and -10) and small (p10) subunits (caspase- 3, -6 and -7). A linker region that may be involved in the regulation of caspase activation sometimes separates the large and small subunits (59, 61-63).

2.3.4 Membrane changes during apoptosis

Throughout the process of apoptosis the cell membrane remains intact and only in the final stages of apoptosis it buds into apoptotic vesicles containing cell material. Cells undergoing apoptosis fragment into membrane-bound apoptotic bodies that are readily phagocytosed and digested by neighboring cells or macrophages without generating an inflammatory response (24, 64, 59). Early phases of apoptosis are characterised by changes at cell surface and plasma membrane. One of these changes in a plasma membrane is the translocation of

phosphotydalserine (PS) from the inner side of the plasma membrane to the outer layer by which PS becomes exposed at the external surface of the cell.

2.3.5 Mitochondrial changes

During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Damage to the mitochondrial membrane leads to the distribution of cytochrome c in the cytosol followed by subsequent depolarization of the inner mitochondrial membrane. Cytochrome c (Apaf -2) release promotes caspase activation by binding to Apaf-1 and therefore activating Apaf- 3 (caspase 9). The Apoptotic Inducing Factor (AIF) has proteolytic activity that once in the cytoplasm induces apoptosis (59, 60).

2.3.6 DNA fragmentation

The DNA is considered the most vulnerable site for cell death. Damage to the nuclear DNA. The DNA is degraded by cleavage between nucleosomes to form internucleosomal fragments. The chromatin condenses forming membrane bound apoptotic bodies. The fragmentation of DNA marks the irreversible stage of apoptosis that commits the cell to die. This stage occurs prior to changes in plasma membrane permeability. This DNA fragmentation has been shown in many instances to result from activation of an endogeneous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease enzyme. This enzyme selectively cleaves the DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragmentation (27, 56, 59-67).

According to Zahm *et al* (2001), apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes that includes incompaction and chromatin condensation, DNA fragmentation into membrane bound apoptotic bodies, cytoplasmic membrane permeability including the release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria, changes in plasma membrane phospholipids, combination of the fluorescent Annexin V with DNA fluorochromes (e.g. propidium, Hoechst 33342 and aminoactinomycin D), cell membrane blebbing with no loss of integrity, activation of caspase cascade and alteration in membrane asymmetry (68).

The final phase, execution phase of apoptotis is marked by the activation of a proteolytic cascade by an interleukin 1ß-converting enzymes (ICE) or the ICE-like protease, thus forming a cleavage of several substrates. Several methods can be used to visualize these morphological changes. Such methods are used for the detection, quantification and characterization of apoptotic cell death. During this phase of both pathways is mediated by activation of the caspases with long domains, such as caspase- 8 or –9, either directly or indirectly activate effector caspases, such as caspases- 3 and –7 (59-62). The effector caspases then cleave (61) and inactivate intracellular factors that are critical for cell survival, such as poly (ADP-ribose) polymerase (PARP) and lamins, and cause the typical apoptotic morphology (59, 61, 62).

2.4 Measurements and tests for cellular cytotoxicity

In an organism cytotoxicity is a complex process involving several different organ and cellular systems. The rationale for testing a chemical or toxin is based on reported case studies or on epidemiological evidence. The first level of testing is usually laboratory based using a cell line as a test system. Once a compound is

shown to affect cells *in vitro* the following level of testing are primary cultures derived from the target tissue followed by animal studies. This data can be carefully extrapolated to case study and epidemiological findings.

To enable initial testing of a toxic agent an *in vitro* test system needs to be developed together with bioassay systems that can detect cellular damage. The different types of bioassay systems that can be used in conjunction with an in *vitro* cell system will be discussed. Many bioassays can be used to measure cytotoxicity and often depend on changes in mitochondria function, membrane permeability, increased or decreased protein expression and changes in DNA structure and morphology. In this study cytotoxicity is measured using the CV, MTT, NR assays and fluorescence microscopy to identify cellular changes associated with apoptosis only these bioassays will be discussed.

2.4.1 Cell division and growth

Cell death occurs as an end result of cytotoxic insult and CV stains cellular protein with a direct correlation between protein staining and cell number being reported (69). Cytotoxic effects can cause cell detachment or cell damage with the resulting leakage of protein and therefore a corresponding decrease in protein staining (26, 60).

2.4.2 Mitochondrial damage

Results of mitochondrial damage due to cytotoxic agents may lead to changes in the structure (contraction or swelling of the organ) or leakage of some enzymes into the cytoplasm. Contraction may take place with increase in ADP/ATP ratio while prolonged contraction may lead to deterioration of the inner membrane that may eventually resulting in rapture and deterioration of the membrane. This process normally happens before cell death (55, 59, 67).

MTT assay is a bioassay used to determine cell viability and/or the metabolic state of the cell by demonstrating the cell's oxidative systems. It is based on the conversion of MTT tetrazolium salt, a soluble yellow dye to a water insoluble blue formazan derivative by mitochondrial enzymes such as succinic dehydrogenase. Reduction of the tetrazolium salt, however, does not occur as a direct effect of the specific dehydrogenase but most cellular MTT reduction occurs outside the mitochondrial inner membrane and involves NADH and NADPH-dependent mechanisms (70-75).

2.4.3 Membrane integrity

Damage to the membrane structure may be due to lipid peroxidation. Some substances or compounds are metabolically activated to reactive intermediates, which are responsible for initiating toxic effects. Process of lipid peroxidation is initiated by the attack of a free radical on unsaturated lipids, and resulting chain reaction is terminated by production of lipid breakdown products: lipid alcohols, aldehydes or smaller fragments such as malondialdehyde (55).

As many enzymes and transport processes are membrane bound, this will affect the structure and function of the organelle and results in changes in membrane permeability and transport and subsequently cell death. Damage to plasma membrane may lead to changes such as blebbing often associated with apoptosis prior to permeability changes. Changes in membrane structure and permeability can be determined by immunocytochemical (ICC) detection and cellular uptake of fluorescent dyes such as Propidium Iodide (PI) (70, 77)

2.4.4 Apoptosis

Several methods of quantifying the type of cell death (apoptosis or necrosis) have been developed. Some of them rely on the apoptosis-associated changes in distribution of plasma membrane phospholipids or transport function of the membrane. Immunocytochemistry, flow cytometry and Western blotting are techniques usually used to detect these changes in protein and or phospholipid expression. Other techniques measure changes in the mitochondrial transmembrane potential that decreases during early apoptosis. Alternatively the detection of endonucleolytic DNA degradation either through the extraction of low molecular weight DNA or the separation of these smaller fragments from the larger intact DNA by agarose gel electrophoresis or by the Comet assay can be used (65, 78-80).

2.5 Aims of the study

The use of latex gloves and condoms is promoted to reduce the transmission of Hepatitis B and Human Immuno-deficiency Virus (HIV)/Acquired Immuno Deficiency Syndrome (AIDS) as well as other sexually transmitted diseases (1, 3, 5). The repeated use and exposure to latex products has resulted in an increase in the incidence of latex allergy and sensitivity.

Latex products such as condoms contain other several different proteins with allergenic potential as well as other added compounds such as lubricants, flavouring and colourants that besides being allergenic may also be toxic. Extensive testing is done to evaluate the physical quality of condoms both however the biological safety of these products needs to be accessed. *In vitro* cell culture model systems are usually used to access the toxicity of drugs, toxins and medical devices. Although these cell models provide little information regarding allergenic potential, they do provide information on the toxicity of the constituents on condom material. Cell models allow rapid testing of different products under different experimental conditions.

Bioassays are used to measure the effect of a compound *in vitro* and can be used to measure several different cellular properties such as membrane integrity, cell number and viability. Furthermore these tests must be able to detect low concentrations of the test compound and must be easy to perform. The purpose of this research presented in this dissertation was to developing an appropriate, simple, reproducible and cost effective method for evaluating the biological safety of commonly used condoms in South Africa.

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To achieve this aim a method described by Pretorious et al (2000) for the testing of the cytotoxicity of latex gloves that involves a modification of a direct contact cell culture method (FDA test method F813-83) will be used.

Specific aims of this study were:

1. To develop a method to obtain condom surface material that is representative of that to which an individual is exposed.

2. To determine the protein content of the surface material of non-lubricated (NLC), lubricated (LC) and flavoured (LFC) condoms.

3. To use the modified culture testing method (FDA test method F813-83) to test the biological safety of non-lubricated (NLC), lubricated (LC) and flavoured (LFC) condoms.

4. To measure the cytotoxic effects of condom washings derived from each condom type on cell number and viability using the CV and MTT assays respectively.

5. To determine the effect of condom type (CT), Condom washing added to media (CM) and Time on cell number and viability by using a Split-plot design using the appropriate Analysis of Variance (ANOVA).

6. To assess with microscopy the toxic effects of condom washings derived from LC, LFC and an additional possibly more toxic type LFCC (lubricated, flavoured and coloured condoms) in L929 cells following CV, MTT and Neutral Red (NR) staining.

7. To determine if cell death in L929 cell line is mediated by necrosis or apoptosis. Fluorescence microscopy using fluorescence stains Propidium Iodide (PI) and Hoechst 33342 (H33342) will be used to access plasma membrane integrity and nuclear morphology respectively, and agarose gel

electrophoresis will be used to determine the mode of cell death (either necrosis or apoptosis).

8. To further determine the cytotoxity of condom washings in a physiologically relevant cell line, the HeLa cell line that is a cervical epithelium cancer cell line.

9. To access the safety of these condoms using an additional bioassay, the NR assay used to determine lysosomal membrane integrity.

10. To determine the effect of condom type (CT), Exposure time (ET) and cell type on cell number and viability by using a Split-plot design with the appropriate Analysis of Variance (ANOVA).

CHAPTER 3: THE ESTABLISHMENT OF A BIOLOGICAL SYSTEM TO TEST THE SAFETY OF CONDOMS

3.1 Introduction

Latex products have long been recognized as a cause of latex protein allergy (1- 4). The increased usage of latex gloves, with the consequent increased occurrence of latex allergies appears to have escalated with increasing awareness of the transmission of HIV-AIDS and other infections. The use of condoms as a means to prevent the transmission of STD's (sexually transmitted diseases) and HIV-AIDS has been widely promoted (3, 7, 9-13). Although extensive testing is done to evaluate the physical quality of condoms, no information is available regarding the biological safety of condoms.

This study was undertaken to determine *in vitro* the effects of short-term exposure to physiological levels of condom surface material. A direct contact cell culture testing method (FDA test method F813-83 used to evaluate the cytotoxic potential of medical materials and devices) was used. The effects of the condom material on cell number and viability were determined using the CV and MTT assays respectively. Split-plot analysis was used to evaluate the effects of different types of condom washings derived from non-lubricated condoms (NLC), lubricated condoms (LC) and lubricated and flavoured (LFC) on cell number and viability *in vitro*.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Cell lines

Mouse fibroblast (ATCC, CCL1 NCTC clone 929 strain designated L-929) cells were obtained from Highveld Biological Company, Johannesburg, South Africa.

3.2.1.2 Media, supplements and reagents

Eagles Minimum Essential Medium (EMEM) powder, Hanks Balanced salt solution (HBSS) and Foetal Calf Serum (FCS) were from Highveld Biological Company, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters 0.22µm were from National Separations, Johannesburg, South Africa. Fixatives, acids and organic solvents, such as glutaldehyde, hydrochloric acid (HCl), acetic acid, isopropanol, and formic acid were analytical grade and were purchased from Merck, Johannesburg, South Africa.

Streptomycin sulphate, penicillin G (sodium salt), Amphotericim B and Trypsin were obtained from Life Technologies Laboratory supplied by Gibco BRL Products, Johannesburg, South Africa. Ethylene Diamine Tetra Acetate

(EDTA), Dimethyl sulphoxide (DMSO), Potassium Chloride (KCl), Potassium dihydrogen phosphate (KH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and Sodium hydrogen carbonate ($NaHCO_3$) were from Merck, Johannesburg, South Africa.

The Bio-Rad protein assay kit was from Bio-Rad Laboratories supplied by Scientific Products, Randburg, South Africa. MTT (1- (4,5- Dimethylthiazol- 2-yl) -3,5- diphenylformazan) and Crystal Violet (CV) powder from Sigma-Aldrich, Atlasville, South Africa. Bovine Serum Albumin (BSA) was from Boehringer Mannheim, Randburg South Africa.

Water was double distilled and deionized (ddH_2O) with a Continental Water System and sterilized by filtration through a Millex 0.2µm filter. Glassware was sterilized at -140^0C in a Prestige Medical Autoclave (Series 2100).

3.2.1.3 Plasticware

The 24 well and 96 well plates, $25cm^2$ and $75cm^2$ cell culture flasks, 10ml and 5ml pipettes, 15ml and 50ml centrifuge tube, micro centrifuge tubes were from NUNC^{TM} supplied by AEC- Amersham, Johannesburg, South Africa.

3.2.1.4 Condoms

Three types of latex condoms were used, namely non-lubricated condoms (NLC), lubricated condoms (LC) and lubricated and flavoured condoms (LFC). LC were obtained from the South African Health Department in Pretoria,

South Africa. NLC and LFC were obtained over the counter from a local adult shop.

3.2.2 Methods

3.2.2.1 Cultivation, maintenance and preservation of the L-929 fibroblast cell line

The L929 fibroblast cell line was maintained in EMEM supplemented with 5% FCS and 1% antibiotic solution. An antibiotic stock solution was prepared by mixing 10,000U/ml Penicillin G (sodium salt), 10,000µg/ ml Streptomycin sulphate and 25µg/ml Amphotericim B in 0.85% saline. A volume of 10 ml of the working solutions was added to a litre of the prepared medium. The antibiotic solution was kept at -10 $^{\circ}$C and thawed when needed. The media was sterilized by filtration through a 0.22µm membrane filters under aseptic conditions in a laminar flow cabinet. Aliquots of 100 ml were prepared and the medium was stored at 4°C and warmed to 37°C before use.

The cells were plated at a concentration of $5X10^4$ cells per ml in $25cm^2$ and $75cm^2$ cell culture flasks and were maintained at 37°C and 5% CO_2 in a CO_2 - water-jacketed incubator from Forma Scientific. The cell culture medium was changed every three days or when the medium had become acidic. The L929 fibroblasts cells were passaged once confluent with a 0.05% Trypsin solution. A 10X Trypsin/EDTA stock solution of 5g/l Trypsin, 2g/l EDTA.4Na was prepared by mixing 0.25g Trypsin, 0.1g EDTA and 0.425g NaCl in 50ml Hanks buffer. Hanks buffer was prepared by dissolving 9.86g/ l Hanks salt and 0.35g/l $NaHCO_3$ in ddH_2O. A working solution of 1X was prepared by diluting the stock solution with DPBS that was stored at -10 $^{\circ}$C and warmed

up at 37^0C before use. The solutions were filtered through a 0.22μm membrane filter under aseptic conditions.

The cells were passaged by firstly removing the medium from the confluent monolayer. A volume of 0.08 ml/cm^2 trypsin working solution was added and the flask was placed at 37^0C for 5-10 minutes. The medium containing the detached cells was added to 10 ml medium. The cells were collected by centrifugation in a BTL Bench centrifuge from Baird and Tatlock at 6000 x g (2000 rpm) for 5 minutes. The medium was removed and the cells were suspended in 10 ml fresh medium and the centrifugation step was repeated. The number of cells was determined by counting a 10 μl aliquot of cells using a haemocytometer from Brand supplied by Merck, Johannesburg, South Africa.

The L929 fibroblasts were either used for experiments described below or stored at −70°C. For storage the cells were suspended in cell culture freezing medium at a concentration of 5×10^6 cells per ml. The freezing medium was prepared by adding 10% DMSO and 5% FCS to supplemented EMEM. One ml of the cell suspension was transferred to the 1.5ml freezing vials and stored by slow freezing (the vials were wrapped with tissue paper and placed into a large Styrofoam box) in a −70°C freezer. The cells were stored for indefinitely with minimum loss of viability.

The vials containing the L929 fibroblasts were thawed rapidly by stirring the vial in warm water at 37^0C. The cells were suspended in supplemented EMEM to a volume of 15 ml. The cells were collected by centrifugation, the supernatant was removed and the cells were suspended in fresh medium. This step was repeated twice before the cells were suspended in a final volume of 3ml culture medium and plated in 25cm^2 cell culture flasks.

For each experiment, cells were plated at a cell concentration of 2×10^4 cells per ml in 24 flat well plates with the culture area of $1.9 cm^2$/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

3.2.2.2. The protein content of the condom surface washings

The protein content of the surface material of each type of condom NLC, LC, and LFC was determined using the Bio-Rad protein kit, which is based on the Bradford assay (81-84).

A 0-0.25 µg/µl protein standard curve protein standard was prepared from a 0.1mg/ ml Bovine Serum Albumin (BSA) solution prepared in ddH_2O. The dye working solution was prepared by adding 100ml of the dye solution to 400ml ddH_2O (1:4) that was stored at 10°C.

A 0-50µl volume of protein standard was added to a series of test tubes and the volume was adjusted to a final volume of 200µl with ddH_2O. To each tube 300µl of the Bio-Rad dye working solution was added. The samples were mixed and the absorbency was measured at 595 nm using a Perkin-Elmir Lambda 2 Spectrophotometer. A standard curve was prepared from an average of three experiments with each data point assayed in triplicate.

One gram of each type of condom was cut into thin strips and incubated separately in 5 ml ddH_2O for 20 hours at 37^0C. The protein content of 20µl aliquots was determined using the same method as was used to prepare the standard curve.

3.2.2.3 The effect of condom washings on the L929 fibroblast cell line in vitro

The condom washings were prepared as follows: 1g of each type of condoms NLC, LC and LFC was cut into thin strips, suspended and incubated in 20ml EMEM for 0, 2, 4 and 8 minutes at room temperature. The medium containing condom washings was removed and sterilized by filtration and stored at 10°C. The same packet of condoms from the same manufacturer was used and for each experiment one condom was used. This medium containing the condom washings is referred to throughout this study as T_0, T_2, T_4 and T_8.

For each experiment, L929 fibroblasts were plated at a cell concentration of 2×10^4 cells per ml in 24 well cell culture plates with a culture area of $1.9cm^2$/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment. Cells were exposed to different dilutions (0-66%) of this medium for 24 or 48 hours. Following exposure, cell viability and number were determined by the MTT and Crystal Violet assays respectively. All experiments were done in quadruples and each point in each experiment was an average of three assays.

3.2.2.3.1 The Crystal Violet assay

To determine the number of cells adherent to the surface of the tissue culture surface following exposure to the condom washings, a 100µl volume of 11% glutaldehyde was added to the medium. The plates were shaken for 30 minutes, washed with water and dried overnight at room temperature. A 300µl volume of a 0.1% (weight/volume (w/v)) Crystal Violet (CV) dye solution

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prepared in 200mM of formic acid pH 3.5 was added to each well. The plates were shaken well for 30 minutes, washed and dried. The bound dye was dissolved by shaking for 10 minutes in 300μl of a 10% acetic acid solution. The dye solution was transferred to a 96 well plate and the absorbency at 595nm was measured using a spectrophotometer (EL900) plate reader.

3.2.2.3.2 The MTT assay

Cell viability was measured using the MTT assay. A 0.1 mg/ml 3-(4,5-Dimethylthiazol-2 -yl) -2,5-diphenyltetrazolium bromide (MTT) solution was prepared in DPBS. A 50μl volume of the MTT solution was added to each well and the cell culture plates were maintained for 20 hours at 37°C and 5% CO_2. The medium was then removed and 200μl of isopropanol: HCl solution (24:1(1M HCl)) was added to each well to dissolve the water insoluble formazan product. After shaking the plates for 20 minutes the solution was transferred into a 96-flat well plate and absorbency at 545nm was measured using an EL900 plate reader.

3.2.3 Statistical analysis

The Crystal Violet assay and MTT Assay were analysed separately with an appropriate Analysis of Variance (ANOVA) for the split-split plot study design that was used to conduct the experiment (85). Testing was performed at a 0.05 level of significance and pair wise comparisons were done using Fisher's test.

For Figure 3.2 a-c and Figure 3.4a-c the percentage cell number or viability respectively is calculated as the absorbance measurement obtained for cells exposed to a certain percentage of medium containing condom washings/

control cells not exposed to condom washings (T_0) X100. Only plots of exposure times T_0, T_4 and T_8 are included.

Absorbance readings were used for the statistical evaluation of each condom type at exposure times T_0, T_2, T_4 and T_8 and the mean plots are of results showing significant differences and are presented in Figure 3.3a, 3.5a and 3.6a. For easier evaluation of the effect of each condom type on cell number and viability the same statistical data is plotted as percentage of T_0 and is presented in Figure 3.3b, 3.5b and 3.6b.

3.3 Results

3.3.1 Protein content of condom washings

The protein content of the surface material of the condoms, NLC, LC and LFC was determined prepared by washing condom fragments in ddH_2O for 20 hours. The protein content was determined from the standard curve (linearity: $r^2 = 0.9903$) shown in Figure 3.1a.
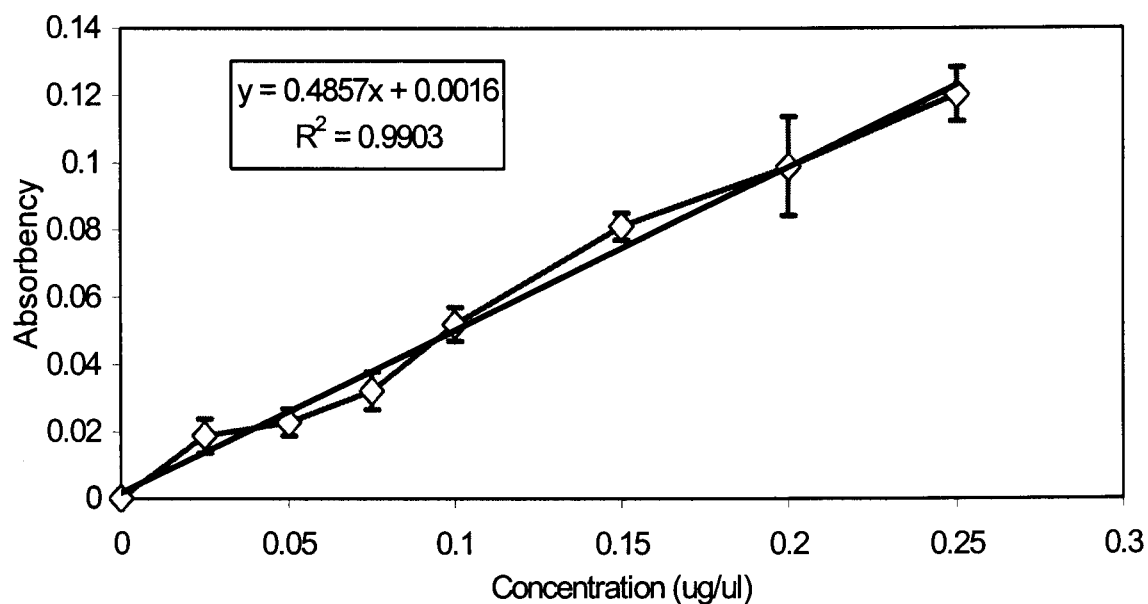
Figure 3.1a: BSA standard curve for the determination of the protein content of NLC, LC and LFC.
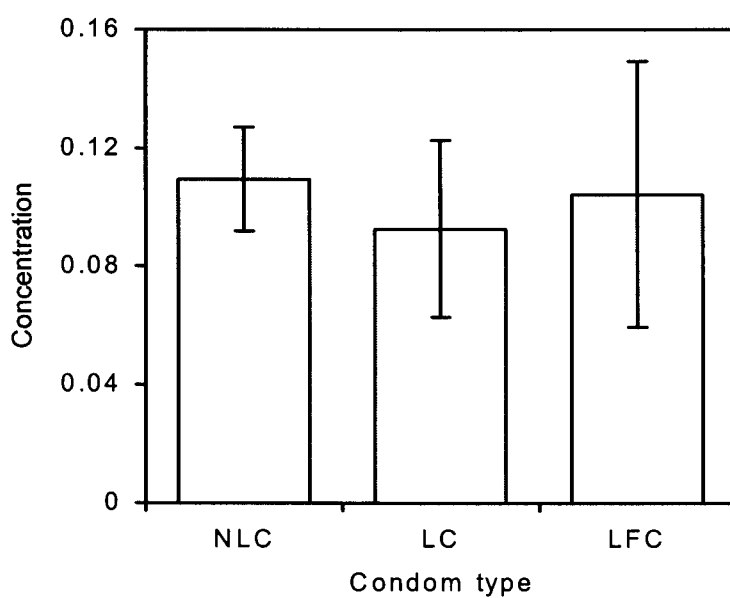
Figure 3.1b: The protein concentration (mg/ml) of condoms, NLC, LC and LFC determined from BSA standard curve (Figure 3.1a).

The protein content of the condom types, NLC, LC, and LFC were similar and the mean protein content of 0.092, 0.0923 and 0.104 mg/ml were observed for LC, NLC and LFC respectively (Figure 3.1b).

3.3.2 Exposure of L929 fibroblasts to condom washings

3.3.2.1 The effect of condom washings on L929 fibroblast number measured using the CV assay

Condom washings were prepared by washing the same mass of condom material for 0, 2, 4, and 8 minutes. The washings were used to prepare mediums T_0, T_2 T_4 and T_8. L929 cells were exposed to serial dilutions of each of these mediums from 0- 66% condom washing (T_{0-8}). Cell number was determined following 48 hours exposure. Figures 3.2a-c shows the effect of different concentrations of condoms prepared at T_0-T_8 on cell number. The effect of different exposure times on cell number was determined using the CV assay (Figure 3.2a-c). Figure 3.2a-c displays the average percentage of cell number plotted against the percentage of the condom medium of the control, T_0.
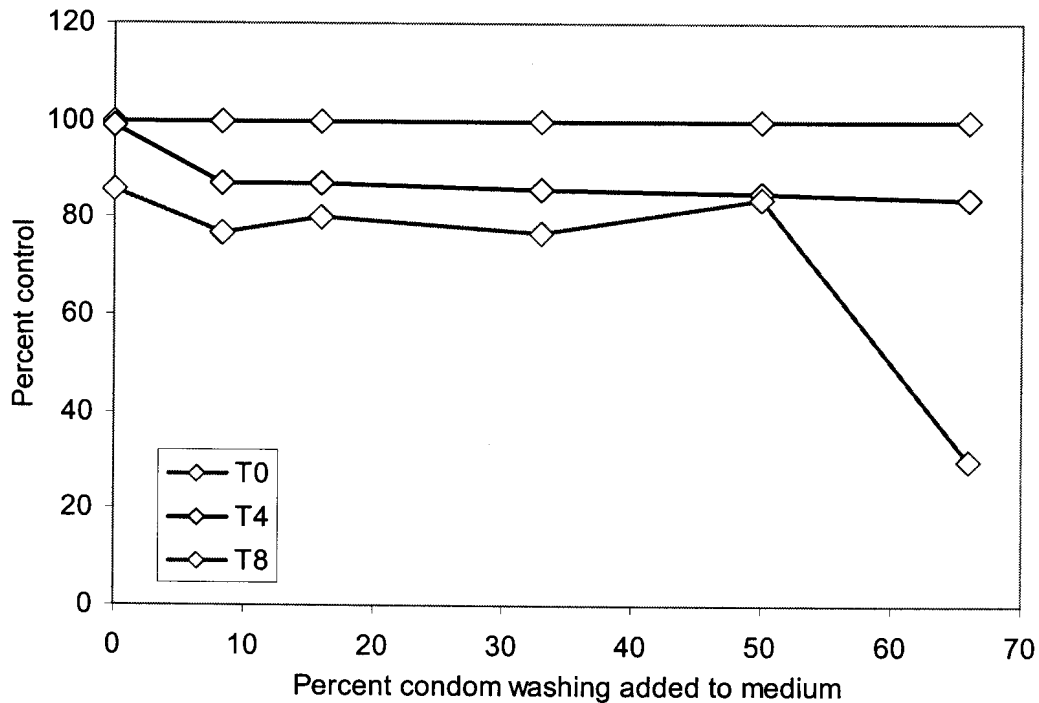
Figure 3.2a: Percentage cell number determined by the Crystal Violet assay following exposure of cells to condom washings T0, T4 and T8 from NLC.

A decrease in cell number was observed for all condom types. For NLC (Figure 3.2a) a 10-20% decrease in cell survival was observed for exposure times T_4 and T_8 where 8,3% to 50% of the medium was replaced with condom washings. A dramatic decrease in cell survival was seen at T_8 for NLC for higher concentrations where 66% of the medium was replaced with condom washings (Figure 3.2a).

The effect of LC condom washings on cell number was greater than observed for NLC. For exposure times T_4 and T_8 a decrease in cell number is observed (Figure 3.2b). Cell numbers decreased to 60% and 22% for T_4 and T_8 respectively compared to 80% and 25% for NLC.
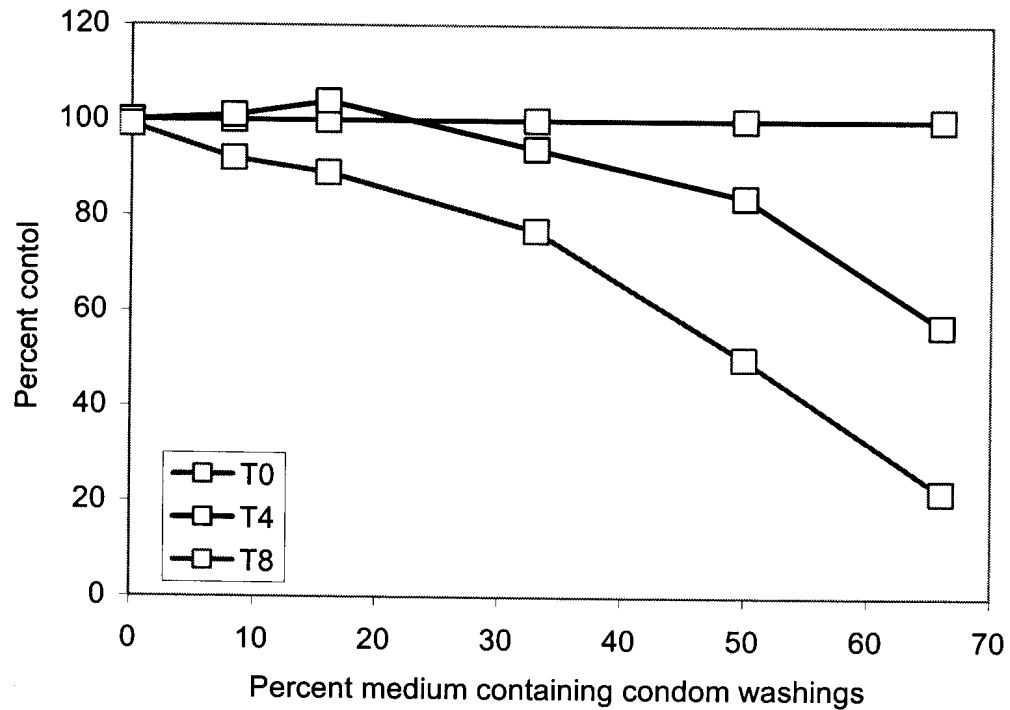
Figure 3.2b: Cell number determined by the Crystal Violet assay following exposure of cells to washings T_0, T_4 and T_8 from lubricated condoms (LC).

The effect of condom washing on cell number was the greatest for LFC where a 10% decrease in mean cell number was observed where 0 to 33% of the medium was replaced with condom washings. A further decrease of 50-70% was observed at higher volumes of condom washings for both exposure times (Figure 3.2c). At exposure time T_4 and T_8 and condom washing replacements of 66%, only 25% and 28% of the cells survived.
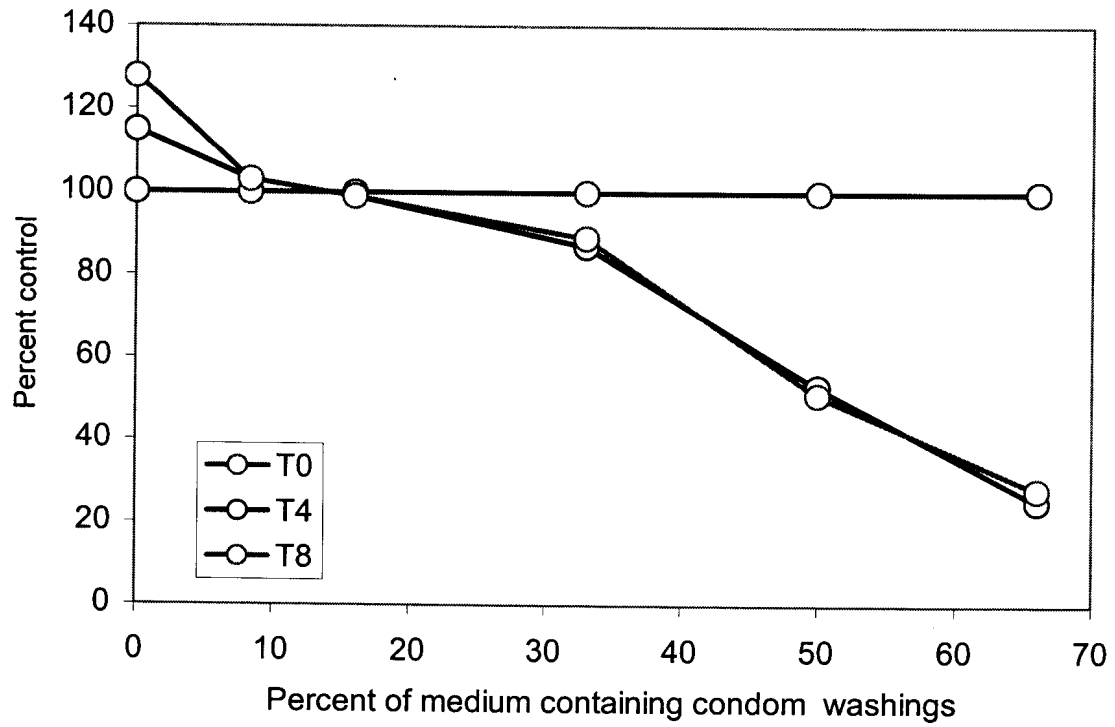
Figure 3.2c: Cell number determined by the Crystal Violet assay following exposure of cells to washings, T_0, T_4 and T_8 derived from FLC.

Table 3.1: Analysis of variance table for cell number

Source of variance	Df	p-values
Condom Replicate	2	0.2561
Condom type (CT)	2	0.0102
Condom replicate X condom type	4	na
Condom washing volume added to media (CM)	5	0.0735
CT X CM	10	0.9249
Condom replicate X CT X CM	30	na
Time	3	0.0001
CT X Time (Figure 3.4)	6	0.0001
CM X Time	15	0.4468
CT X CM X Time	30	0.9885
Condom replicate X CT X CM X Time	108	

The effect of each condom type (CT) on cell number is significant (p=0.0102). Evaluation of CT X CM (p=0.9249) and CT X time (0.0001) indicates that time and not amount of medium added contributes to this significant difference. From Figure 3.3a and b this effect is mainly due the increase in cell number observed at T_2 for NLC.
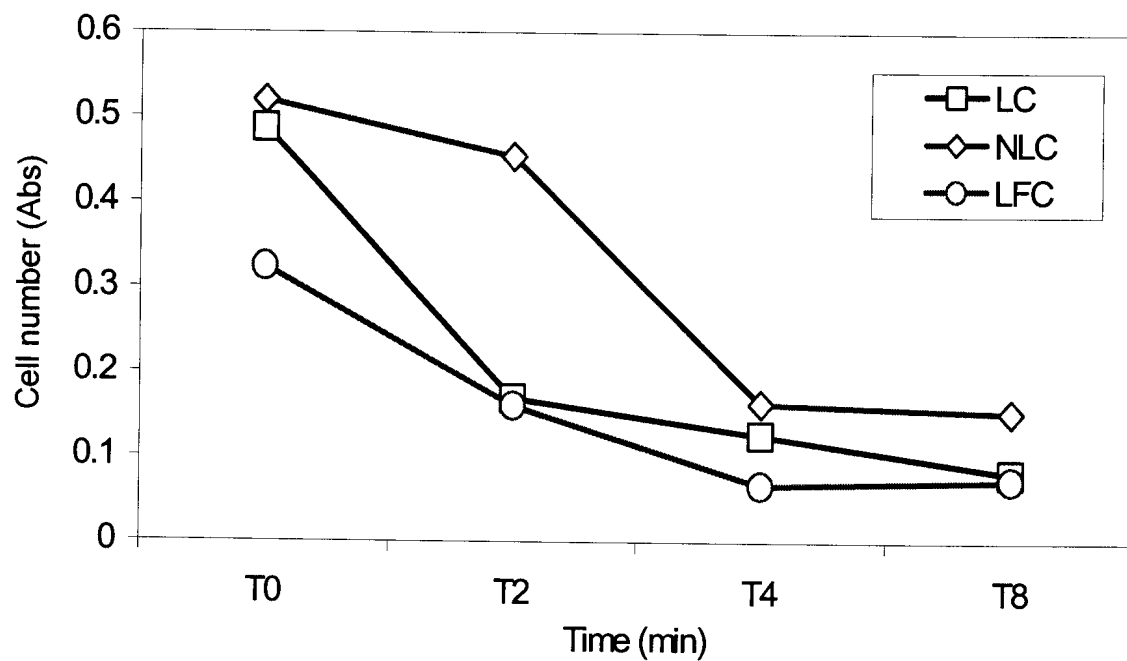
Figure 3.3a: Means of cell number (CV assay) of CT X time for LC, NLC and LFC.
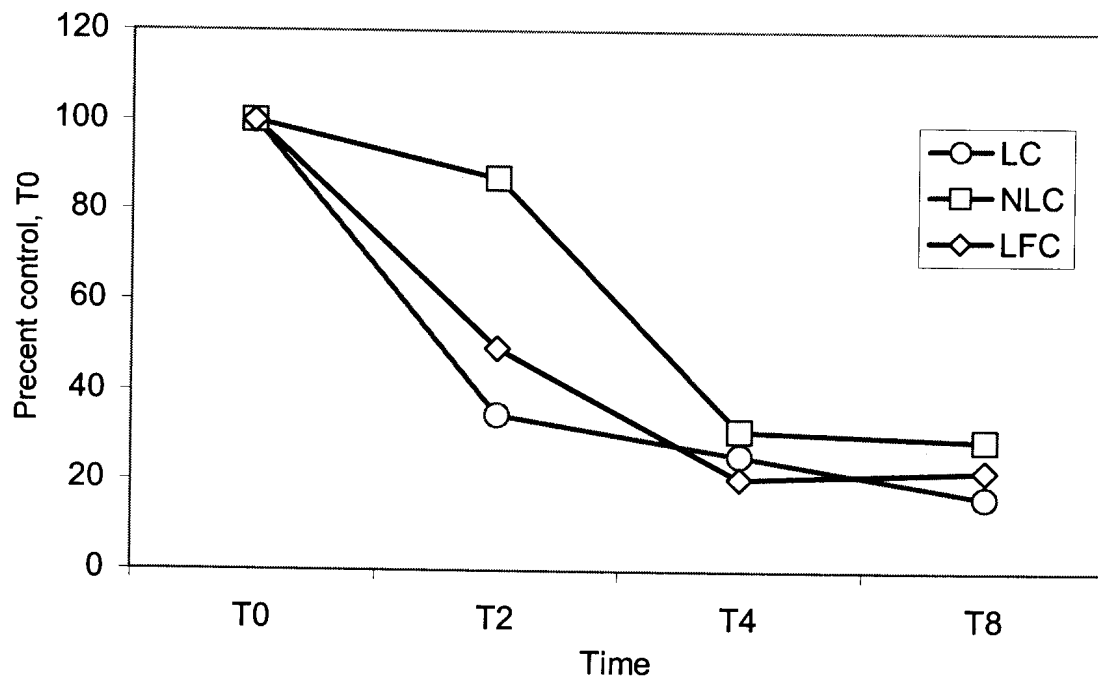
Figure 3.3b: Means of cell number as percentage of control, T0 of CT X time for LC, NLC and LFC

By expressing the data as a percentage of the T_0 as shown in Figure 3.4b relative differences are easier to evaluate and the effect of T_2 for NLC is 90% compared to 50 and 35% for LFC and LC respectively. All other sources of variance such as CT X CM X time (0.4468) and condom replicate X CT X CM X time (0.9885) are not significant.

3.3.2.2 *The effect of condom washings on L929 fibroblasts cell viability measured using the MTT assay.*

Cell viability of the cells using MTT assay was determined following 20 hours exposure to medium containing different volumes of condom washings. The decrease in cell viability is expressed as the percentage of plot T_0. Results of different exposure times T_0, T_4 and T_8 are shown in Figure 3.4a-c. These figures represent the average percentage cell viability plotted against percentage condom washings for each condom type. Different volumes of condom washings appear to have only slight effect on cell viability for NLC (Figure 3.4a) and LC (Figure 3.4b).
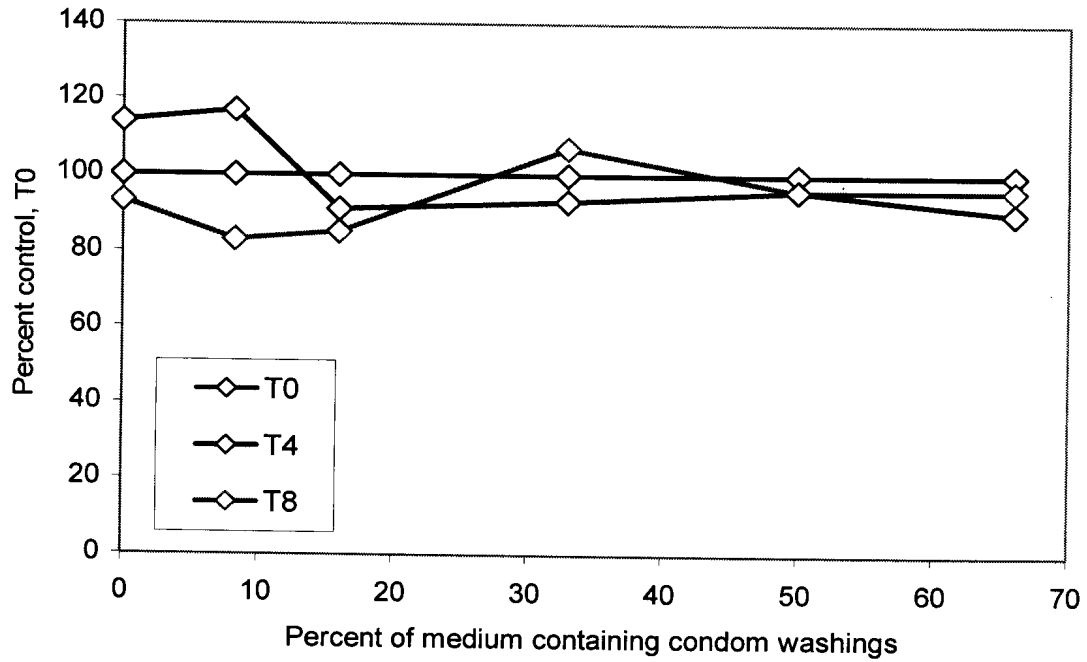
Figure 3.4a: Cell viability determined by the MTT assay following exposure of cells to condom washings derived from NLC.
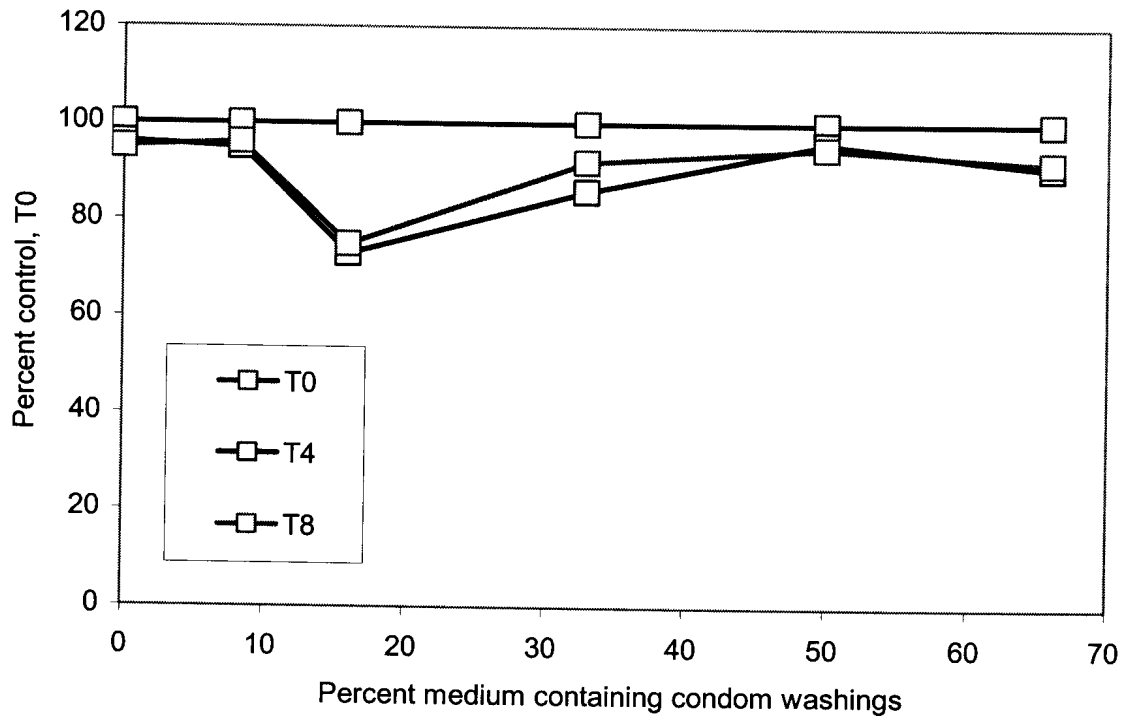
Figure 3.3b: Cell viability determined by the MTT assay following exposure of cells to washings derived from LC.
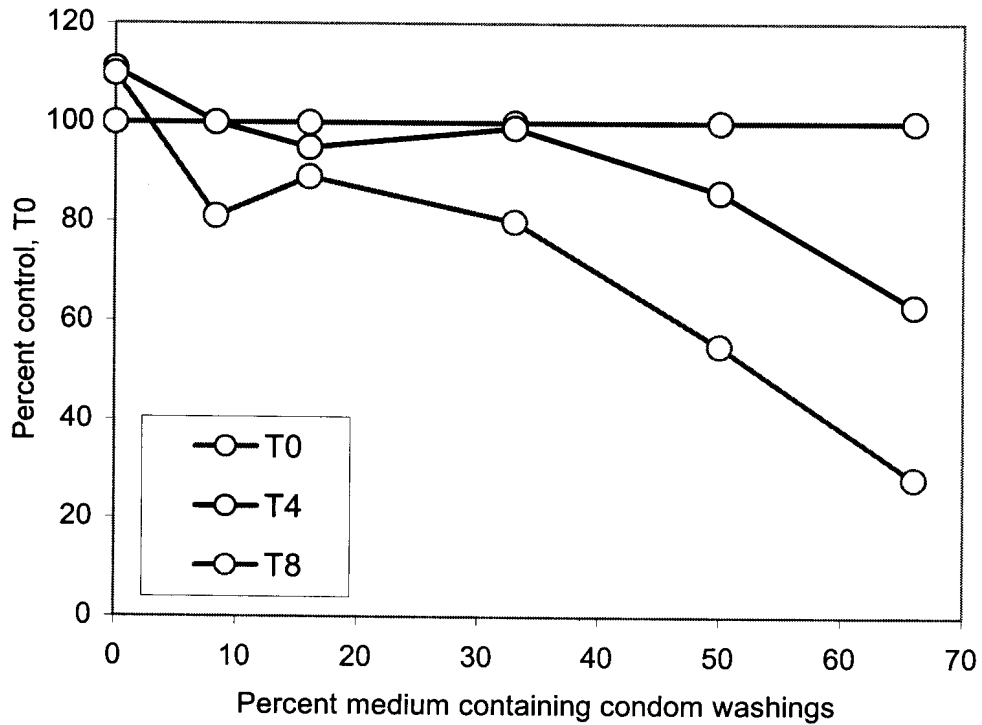
Figure 3.4c: Cell viability determined by the MTT assay following exposure of cells to washings derived from FLC.

For LFC at percent medium containing condom washings greater than 40%, a rapid decrease in cell viability to 63% at T_4 and 28% at T_8 were observed

(Figure 3. 4c).

Table 3.2: Analysis of variance table for cell viability

Source of variance	Df	p-values
Condom Replicate	2	0.9858
Condom type (CT)	2	0.1673
Condom replicate X condom type	4	na
Condom washing volume added to media (CM)	5	0.0001
CT X CM	10	0.2640
Condom replicate X CT X CM	30	na
Time	3	0.0001
CT X Time (Figure 3.7)	6	0.0001
CM X Time (Figure 3.8)	15	0.0001
CT X CM X Time	30	0.0015
Condom replicate X CT X CM X Time	108	

Condom washing volume added to the medium (CM) and time shows significant differences of p=0.0001. This difference for CM is due to the interaction between CM X time and CT X CM and time. The same effect is also observed for time (CM X time and CT X CM and time). Mean plots for CT X time is presented in Figure 3.5a and the corrected data for T_0 in Figure 3.5b. From Figure 3.5b the effect of condom type LFC at T_8 at 60% compared to 80 and 85% for LC and NLC respectively causes this significant difference.
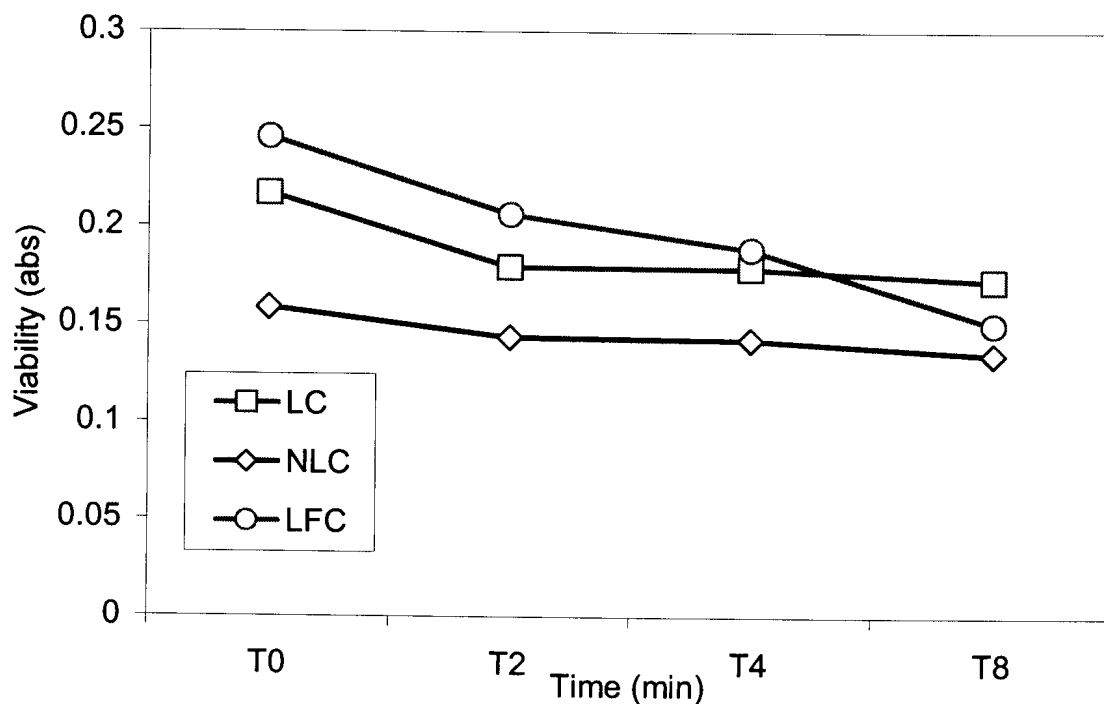
Figure 3.5a: Means of cell viability for CT X Time for LC, NLC and LFC.
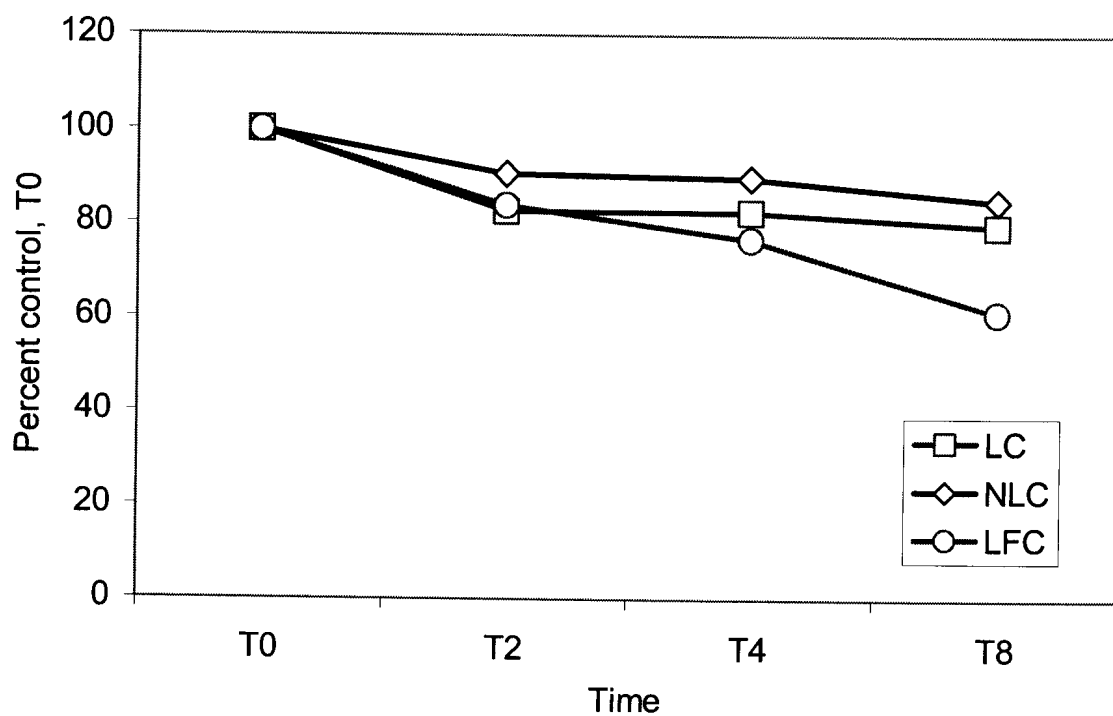
Figure 3.5b: Means of cell viability as percentage of control, T_0 of CT X Time for LC, NLC and LFC

The mean plot for CM X time is presented in Figure 3.6a and the corrected CM X time is presented in Figure 3.6b. Differences are significant with $p=0.0001$ the greatest effect being the difference between 0 and all other dilutions 8 through to 66 (Figure 3.6b).
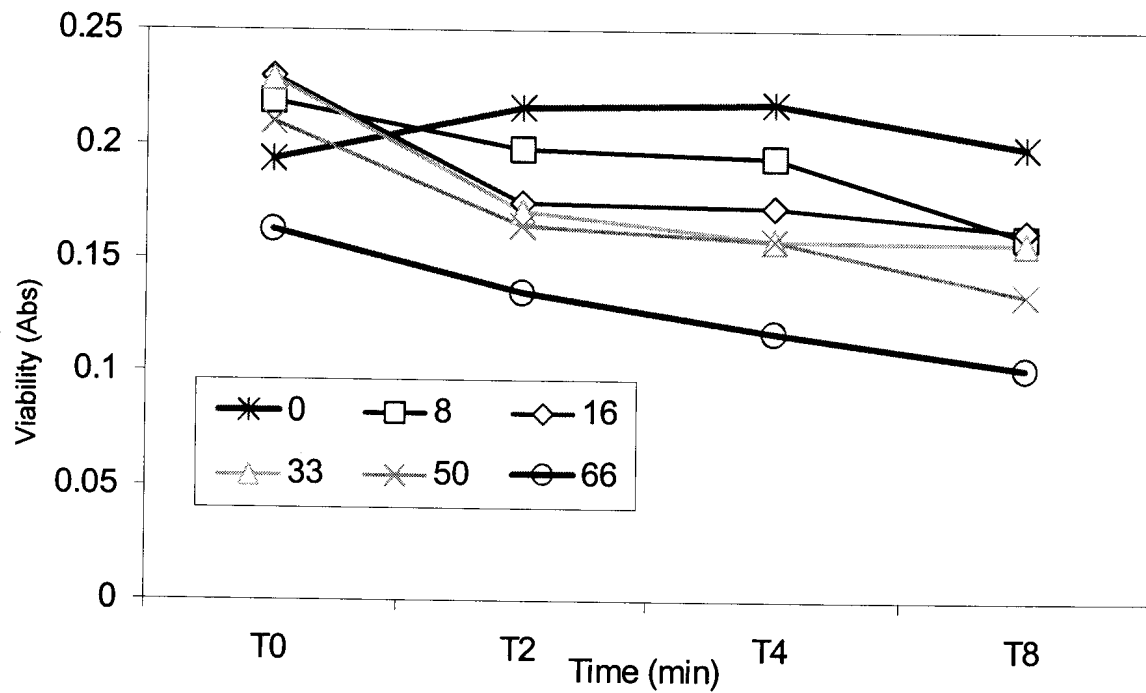
Figure 3.6a: Means of cell viability of condom washings volume (CM) X time (MTT assay).
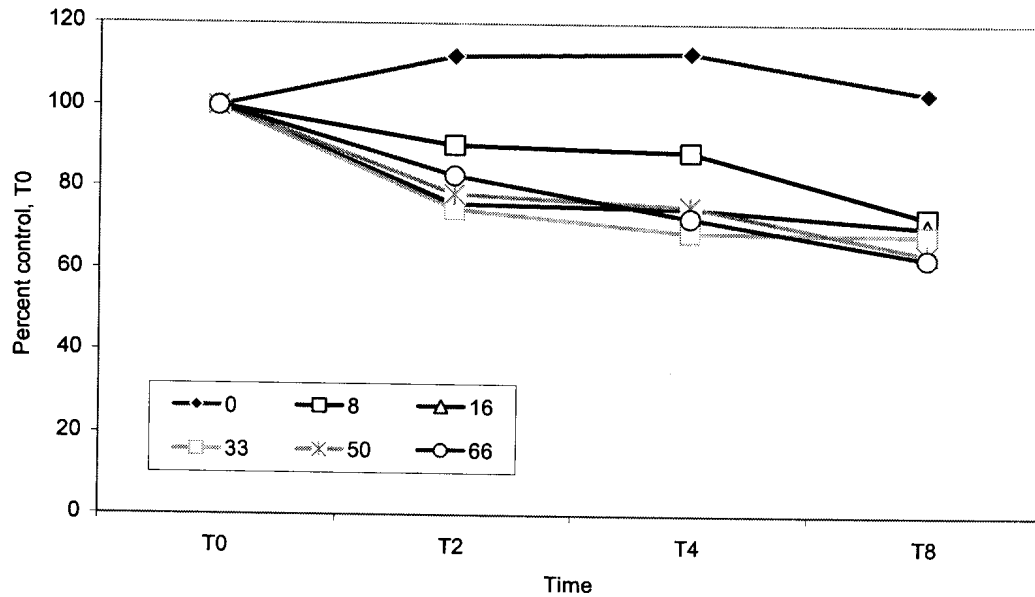
Figure 3.6b: Means of cell viability expressed as percentage of the control, T_0 for condom washing volume (CM) X time (MTT assay).

3.4 Discussion

Continued HIV/ AIDS awareness campaigns encourage the use of (latex) condoms to prevent sexually transmitted diseases with more emphasis on prevention of HIV infections. This led to escalation in reported allergic reactions to NRL (1, 3, 5, 11). The question that may arise is whether latex condoms are safe to use or not.

Three types of condoms were evaluated namely, NLC, LC and LFC. The NLC were without lubrication but covered with talcum powder and resembled the powder used in glove powder. The LC and LFC were dimethyl silicone. Although each type of condoms may contain different types of material the protein content derived from the surface was determined. The protein content of each condom type was determined using the Bio-Rad assay.

From the standard curve the protein content of condom washings prepared from each condom type was determined after washing 1 g of material in 5 ml H_2O for 20 hours. The protein content of the condom washings was 0.092, 0.0923 and 0.104 mg/ml for LC, NLC and LFC respectively. No significant difference in the protein content of the washings prepared each condom type was observed. The actual amount of condom material added to the medium is less being prepared from a 2-8 minute washing rather than the 20-hour washing used for the protein determination.

Cells *in vitro* were exposed to medium containing LC, NLC and LFC surface material. The FDA direct culture testing method traditionally used to assess the cytotoxic potential for established medical materials and devices was used. Since there is no specific documented cell culture testing method available for testing latex condoms, it was hypothesized that this test method with minor changes may be suitable for use to test for the cytotoxic effects of latex condoms on cells in culture.

To test the cytotoxic effects of the condom washings, condom washings were prepared by washing condom material with cell culture medium for 2, 4 and 8 minutes. This time period of 2-8 minutes was chosen to represent normal physiological exposure and to determine if a dose response effect occurs. Furthermore medium was also prepared containing different amounts of each of the condom washing medium (0-66%). The L929 cell line was exposed to the different mediums for 20 hours before cell number and viability was determined using the MTT and CV assays respectively.

Crystal violet stains cellular protein with a direct correlation between protein staining and cell number being reported. Cytotoxic effects can cause cell detachment or cell damage with the resulting leakage of protein and therefore

a corresponding decrease in protein staining (70, 71). A decrease in cell number was observed for all condom types. For NLC a 10-20% decrease in cell number was observed for exposure times T_4 and T_8 where 8,3% to 50% of the medium was replaced with condom washings. At higher concentrations for the longer exposure time T_8 a dramatic decrease in cell survival was seen at T_8 to 20% when 66% of the medium was replaced with condom washings.

The effect of LC condom washings on the effect on cell number was greater than observed for NLC. For both T_4 and T_8 a decrease in cell number is observed to 60% and 22% for T_4 and T_8 respectively when 66% of the medium was replaced with condom washings. The effect of condom washing on cell number was the greatest for LFC where a 10% decrease in mean cell number was at 0 to 33% condom medium and a further decrease of 28% was observed at higher volumes of condom washings for both exposure times. At exposure time T_4 and T_8 and condom washing replacements of 66%, only 25% and 28% of the cells survived. This indicates that all condom washings after 24 hours caused a decrease in cell number with LFC being the most cytotoxic and LC the least.

Split-plot analysis determines if a single parameter such as condom type (CT), condom washings (CM) and time has a significant effect of cell number. Further evaluation then considers the effect of several parameters in combination such as CT X CM and CT X CM and Time. Split-plot analysis determines whether the parameter considered can show significant differences between groups. Further evaluation of data by plotting the average means against for example time can be used to identify the factor or data that causes the significant difference (85).

These differences become easier to identify if data is expressed as percentage of the control and is plotted as such. From the plots of percentage control, T_0 vs. time all condom types cause a decrease in cell number.

Differences between each condom type (CT x Time) is significantly different due to the effect observed at T_2 for LC.

The cytotoxic effects measured with the Crystal violet assay are determined by exposure time and concentration of condom washings. The longer the time of exposure and the higher the concentration of the condom washings, the lower survival was observed. Different condom types behaved differently over time of exposure and the concentrations of condom washings. LFC were found to induce lesser survival compared to other condom types, followed by LC and NLC revealed better survival rate.

Another bioassay used to evaluate the cytotoxic effects of many agents is the MTT assay. The dye MTT is metabolised by the mitochondria enzyme succinate dehydrogenase to an insoluble formazan compound (70-75). The coloured product is solubilised and the absorbency is measured. This assay was also used to measure the effect of the different types condom washings on cell viability.

Cell viability of the cells using MTT assay was determined following 20 hours exposure to medium containing different volumes of condom washings. The decrease in cell viability is expressed as the percentage of the control (exposure time T_0). Different volumes of condom washings appear to have only slight effect on cell viability for NLC and LC. For LFC at percent medium containing condom washings greater than 40%, a rapid decrease of cell survival to 63% at T_4 and 28% at T_8 were observed. The MTT usually considered being more sensitive than the CV assay showed only toxicity for as LFC and not for NLC and LC as with the CV assay.

The effect of condom type (CT) on cell viability was not significant (p=0.1673) whereas the effect of condom washing volume added to media (CM) and time

is significant with p=0.0001 was significant different for both. A significant difference was observed for CT X time (p=0.0001) caused by the effect of LFC at T_8.

Though MTT assay could not reveal the difference between different condom types, a definite distinction was observed when using CV assay. It remains clear that LFC was observed to be the most toxic type of condom with NLC being the least toxic condom type among the three types of condoms used. There is no absolute reason why LFC were very toxic, but speculations can be made on the fact that not only does LFC contain latex proteins like all the other condom types, but they also contain lubricants and flavourings as compared to other condoms.

Though reports have been made on the latex glove powder as being the major route of transportation of latex allergens in latex gloves, NLC, which are also covered with talcum powder, were found to induce very little cytotoxicity as compared to other condom types. As a result their cytotoxicity in this study was found to be insignificant.

Furthermore the assays used to determine cytotoxicity measure different parameters such as the amount of protein compared to the ability of a cell to metabolise a substrate. Therefore each test should be carefully evaluated. Evaluation of condoms by the South African Bureau of Standards (SABS) involves the testing of the physical properties such as strength and durability of condoms. However little is known regarding the biological safety of condoms. In this study a biological system has been developed to determine the toxic effects of condom material and additives. It has been shown that some condoms are more toxic than others and therefore may have a potential health risk.

CHAPTER 4: THE EVALUATION OF THE EFFECTS OF CONDOM WASHINGS ON THE L929 CELL MORPHOLOGY AND STRUCTURE

4.1 Introduction

Microscopic evaluation of cells can provide important information regarding the effects of a toxic compound on cellular morphology and structure. Furthermore it can verify some of the findings of quantification bioassays such as the MTT, CV and NR assays. The use of fluorescent dyes such as Propidium Iodide and Hoechst 33342 can also provide information regarding membrane integrity and changes in nuclear structure respectively (78-80). The cell membrane of a cell is the first level of insult when a cell *in vitro* is exposed to a toxic compound.

Changes in membrane integrity can be determined by studying the uptake of a fluorescent dye such as Propidium Iodide (PI). PI cannot cross an intact cell membrane, however with damage the cell membrane become more permeable, the dye can move across the cell membrane and once in the nucleus binds DNA with a resulting increase in fluorescence (76, 77, 87-90). In contrast Hoechst 33342 (H 33342) is a fluorescent dye that can cross the cell membrane and intercalate with the DNA helix with an increase in fluorescence (88, 89). Both dyes are used for the detection of cellular apoptosis and necrosis in cells exposed to a toxic compound *in vitro*.

Necrosis is characterised by loss of membrane integrity, chromatin flocculation, and cell lyses, whereas in apoptosis death is a programmed

process of cell death associated with an intact cell membrane that shows blebbing and DNA fragmentation (66, 86). Differences between apoptosis and necrosis can be determined by microscopic evaluation of nuclear DNA using fluorescent dyes or by agarose gel electrophoresis used to separate the fragmented DNA from intact genomic DNA.

The purpose of this study is to microscopically evaluate cellular and structure following exposure of L929 cells to washings of condom types, lubricated (LC), lubricated and flavoured (LFC) and lubricated, flavoured and coloured (LFCC) condom types. The first two condom types are the same as used in the study presented in Chapter 3 where LFC was shown to be toxic *in vitro*. Condom type LFCC is an additional type that is included in this study. The effects of the condom washings were determined at a specific concentration of condom washing and at a specific exposure time.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Condoms

LC and LFC are the same type of condoms as used in Chapter 3. An additional condom type lubricated, flavoured and coloured condom (LFCC) was used and was obtained over the counter from a South African convenience store shop.

4.2.1.2 Solvents and reagents

Acetic acid, formalin, ethanol, methanol, Tris, Boric acid and Sucrose were all from Merck, Johannesburg, South Africa. Neutral red powder was from Sigma-Aldrich, Johannesburg, South Africa. The fluorescence stains Propidium Iodide (PI) and Hoechst 33342, Ethidium bromide and Bromophenol blue were obtained from Sigma/ Aldrich, Johannesburg, South Africa. QIAamp DNA Isolation kit was from Qiagen, Southern Cross Biotechnology, Johannesburg, South Africa. Agarose D1 LE gel and all electrophoresis equipment were from Whitehead Scientific, Cape Town, South Africa.

4.2.2 Methods

4.2.2.1 Exposure of the L929 cells to condoms washings

The L929 cells were exposed to 16% of the medium containing condom washings derived from lubricated (LC), lubricated and flavoured (LFC) and lubricated, flavoured and coloured (LFCC) for 3 hours at an exposure time of T_8.

4.2.2.2 MTT, CV and NR assay

4.2.2.2.1 MTT Assay

Following exposure of L929 cells to condom washings MTT was added to each well as described in Chapter 3 Section 3.2.2.3.2 and the plates were incubated for further 1 hour at 37°C and 5% CO_2. The cells were fixed as described for CV assay in Chapter 3 Section 3.2.2.3.1.

4.2.2.2.2 CV Assay

Following exposure of L929 cells to condom washings; the attached cells were fixed and stained as described for CV assay Chapter 3 section 3.2.2.3.1.

4.2.2.2.3 NR Assay

Following a 3 hour incubation with condom washings, a 100µl volume of a 0.15% Neutral Red (NR) solution in DPBS was added to each well. Plates were maintained at 37°C and 5% CO_2 for a further 120 minutes. The medium was discarded and the cells were fixed for 10 minutes with 200µl of a 1% acetic acid and 1% formaldehyde solution. The 24 well plates were then dried overnight at room temperature.

4.2.2.2.4 Microscopic evaluation of L929 cell structure following the metabolism, staining and uptake of MTT, CV and NR

Cellular structure and staining was evaluated with a Nikon ACT-1 digital camera DXM 1200 fitted to the Zeiss Axiovert 200 inverted microscope using a 20XLD lens with the final magnification of 200X and the photos were processed using Irfan view software.

4.2.2.3 Fluorescence staining with Propidium Iodide (PI) and Hoechst 33342

4.2.2.3.1 Preparation of cells for fluorescence staining

L929 were maintained as described in Chapter 3 Section 3.2.2.1. Cells were plated at a concentration of 1×10^5 cells/ml in 24 welled plates. Different condom washings were exposed to the cells at 8 minutes (T_8). Following the incubation of cells with condom washings, one set of cells was fixed with 10% ethanol solution for 5 minutes and these cells were used for the evaluation of the cellular structure. The unfixed cells were used with PI staining and Hoechst 33342 for the evaluation of the membrane integrity and nuclear changes in cells following exposure to different latex condom washings.

4.2.2.3.2 Propidium Iodide (PI) staining

A 1mg/ml PI in H_2O was prepared and the solution was kept in the dark. Following a 3 hour incubation of the cells with condom washings LC, LFC and LFCC the medium was discarded, and cells were rinsed with DPBS. A 200µl volume of DPBS and 5µl of the PI solution were added to each cell well and incubated in a dark room at room temperature. Following an incubation period of five minutes, the DPBS/PI solution was discarded and the wells were rinsed with fresh DPBS to remove excess PI that was not taken up by cells. A little DPBS (50µl) was added to protect the cells from drying off before viewing.

The plates were viewed under fluorescence light using a Zeiss Axiovert 200 inverted microscope. The samples were viewed under the green filter and an orange-red emission filter. The magnification was the same as for section 4.2.2.2.4. The pictures were taken with a Nikon digital camera DXM 1200 fitted to the microscope and the photos were processed using NIKON ACT-1 software.

4.2.2.3.3 Hoechst 33342 staining

A solution of 1mg/ml Hoechst 33342 was prepared in H_2O. Following an incubation of 3 hours of L929 cells with condom washings, LC, LFC and LFCC, the medium was discarded, and cells were rinsed with DPBS. A volume of 200µl of DPBS/ Hoechst 33342 at a final concentration of 2.5 µg/ml was added to each well and incubated in a dark room at room temperature. Following an incubation period of five minutes, the solution was discarded and the wells were rinsed with fresh DPBS to remove excess staining. A volume of 50µl DPBS was added to protect the cells from drying off before viewing.

The samples were viewed under the UV light and a blue/ white emission filter and photos were taken as described for PI.

4.2.2.4 Agarose gel electrophoresis of L929 cells exposed to condom washings, LC, LFC and LFCC.

L929 cells were plated at a concentration of 6×10^4 cells/ ml in $75cm^2$ cell culture flasks. The cells were exposed to condom washings LC, LFC and LFCC derived from T_8 for an hour. The cells were collected by scraping the monolayer, washed and the pellet containing the cells was used for DNA isolation with the QIAamp DNA Mini Kit (90).

To the pellet of cells a 20µl volume of the QIAGEN Protease solution and 200µl Buffer AL (the composition of the buffers and solutions was not provided by the manufacturers) was pipetted into the bottom of 1.5ml micro-centrifuge tube. The sample was mixed by pulse-vortexing for 15 seconds. The sample was incubated at 56°C for 10 minutes in a water-bath. Following incubation the sample was centrifuged at 6 000 x g (8 000rpm) for 30 seconds in a Hereus Biofuge 15 Centrifuge to remove droplets from the inside of the lid. The tube was opened and 200µl ethanol was added to the sample, the sample was mixed again by pulse-vortexing for 15 seconds and the centrifugation step was repeated.

The lysed cell sample was carefully applied with a pipette to the QIAamp spin column ensuring that the rim remained dry. The cap was closed, and the tube with the spin column was centrifuge at 6 000 x g for 1 minute. The spin column was placed into a clean 2 ml collection tube and the tube containing the filtrate was discarded. The QIAamp spin columns were opened carefully and 500µl of Buffer AW1 were added without wetting the rim. The cap was closed and the sample was centrifuged at 6000 x g (8 000 rpm) for 1 minute.

The spin column was removed, placed into a clean 2ml collection tube, and the collection tube containing the filtrate was discarded. The spin column was carefully opened and 500µl Buffer AW2 was added without wetting the rim. The cap was closed and the sample was centrifuged at full speed (20 000 x g; 14 000rpm) for 3 minutes. The spin column was removed from the collection tube, dried with a paper towel to reduce possible buffer carryover. The collection tube was replaced with a new tube and centrifuged at 8000 xg for 1 minute.

The spin column was placed in a clean 1.5ml micro-centrifuge tube and the collection tube containing the filtrate was discarded. The column was carefully opened and 200µl AE Buffer was added. The spin column was incubated at room temperature for 1 minute, then centrifuged at 6000 xg for 1 minute to extract the DNA. The tube containing the DNA sample was closed, labelled and stored at 4°C.

A 0.8% agarose gel was prepared in a TBE buffer (0.089 M Tris, 0.079M Boric acid, 0.002M EDTA at pH 8.3) containing 0.01% ethidium bromide (EtBr). Electrophoresis was carried out using a Submarine Gel Electrophoresis System coupled to an Amersham Pharmacia biotech 301 DC power supply at 65 V for 150 min. The gel was then visualized by ultraviolet (UV) radiation and photographed using UVIdoc Gel Documentation System manufactured by UVItec Limited, St John's Innovation Centre, Cambridge, UK and supplied by Whitehead Scientific, Cape Town South Africa. The DNA samples were diluted 1:1 with loading buffer (60% sucrose in 1x TBE buffer containing 0.001% bromophenol blue (BPB)) and loaded onto the gel.

4.3 Results

4.3.1 Microscopic evaluation of L929 cell structure following the metabolism, staining and uptake of MTT, CV and NR

The cell line L929 was exposed to washings derived from condom type LC, LFC and LFCC prepared following an exposure time of 8 minutes. To each wells a 16% of the condom washing solution was added. The cells were exposed for 3 hours. For the CV staining the cells were fixed and stained with CV. For the L929 control the cells presented with typical characteristics of sub confluent fibroblast cultures where the cells have multipolar and bipolar shapes. Condom washings LC, LFC and LFCC have little effect on cell morphology (Figure 4.1) or the degree of staining (Figure 4.1 a-d).

The MTT assay is used to measure cell viability that is achieved by incubating the cells with MTT. The MTT is metabolised by the mitochondrial succinate dehydrogenase to form a water insoluble formazan product that is observed on the surface of the cells as thin crystals (Figure 4.2a and b). For the control and L929 cells exposed to LC, cell morphology is retained and crystal formation is observed on the surface of the cells. In L929 cells exposed to

LFC and LFCC the MTT is metabolised and is retained in the cells with very little crystal formation. Furthermore for L929 exposed to LFCC showed cells with different cell morphology. Both typical fibroblasts and rounded cells were observed. For cells exposed to LFC the rounded cells were predominant type found in culture.

a) **L929/CONTROL**
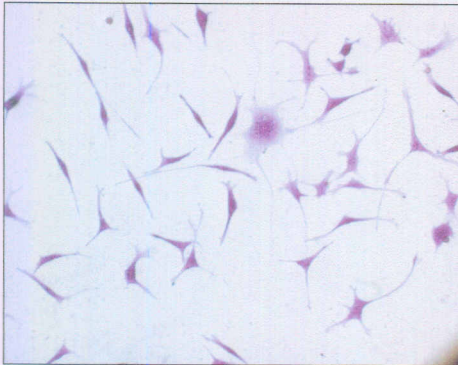
b) **L929/LC**
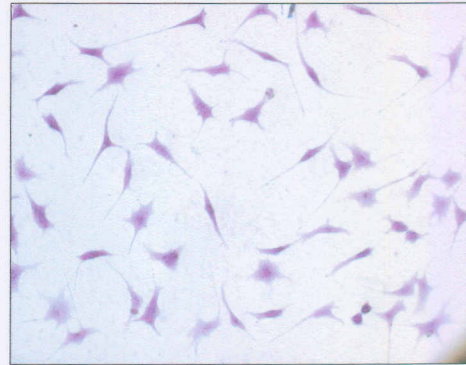
c) **L929/LFC**
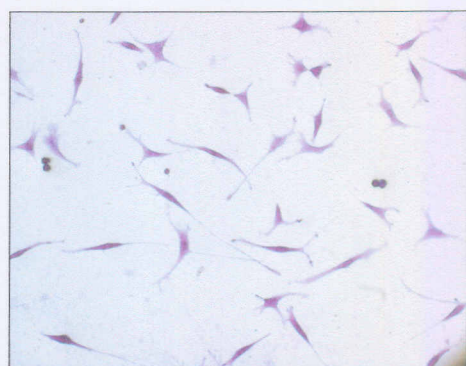
d) **L929/LFCC**

Figure 4.1: Microscopic evaluation of condom washings on cell number using the CV assay in the L929 (a-d) cell line. Photographs 4.1 (a-d) show normal fibroblast morphology and staining.

a) L929/CONTROL
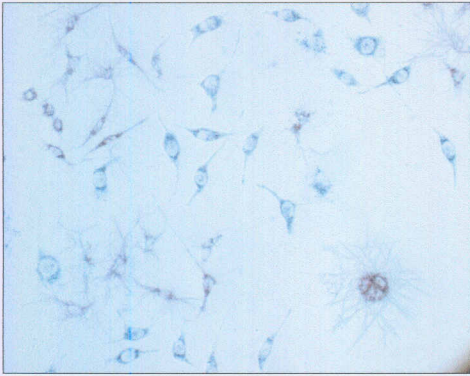
b) L929/LC
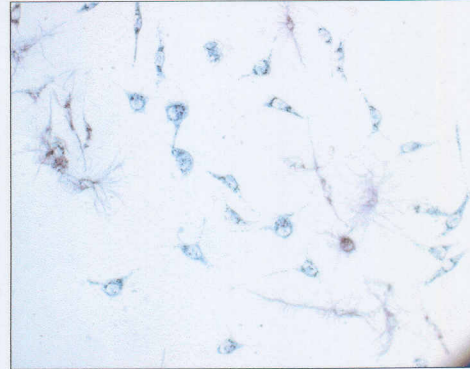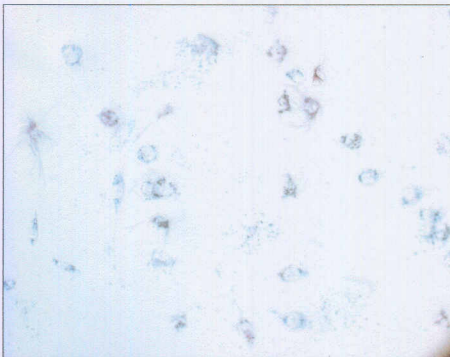
c) L929/LFC

d) L929/LFCC
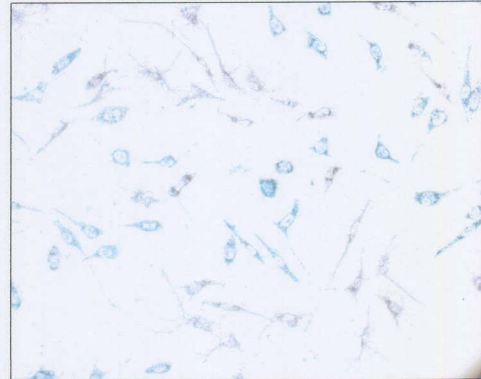
Figure 4.2. Microscopic evaluation of condom washings on cell viability measured using the MTT assay in the L929 (a-d) cell line. Formazan crystal formation can be observed on the surface of the L929/Control and L929/LC. Altered morphology and reduced staining L929/LFC and L929/LFCC.

Viable cells take up NR and the dye accumulates in the lysosomes. Damage to lysosome structure causes decreased NR uptake. NR also stains known as Nuclear Fast Red also stains the nuclei of cells as can be seen in Figure 4.3a. For L929 cells exposed to LC, LFC and LFCC reduced staining is observed. In L929/LFC fewer cells are present and most remaining cells are rounded with irregular staining.

4.3.2 Fluorescence staining of L929 cells exposed to condom washings, LC, LFC and LFCC with Propidium Iodide (PI) and Hoechst 33342

PI is a fluorescent stain that can be used to measure cell membrane permeability and it excludes the dye from viable and apoptotic cells. Therefore PI will cross the cell membrane of cells where cell membrane is damaged and bind nucleic acids. In Figure 4.4a no PI staining for L929/Control indicating no cell membrane damage or cell death has occurred. For LC, LFC and LFCC some cells are stained with most staining observed for LFC (Figure 4.4c) however it is not possible to determine the percentage of the total cell population that shows positive fluorescence.

Fixation prior to PI staining will result in the staining of all nucleic acid. From the pattern of staining can it be concluded whether apoptosis or necrosis has occurred. A hallmark of apoptosis is the degradation and the compaction of the chromatin. In Figure 4.5a staining of DNA and RNA is observed for

a) **L929/CONTROL**
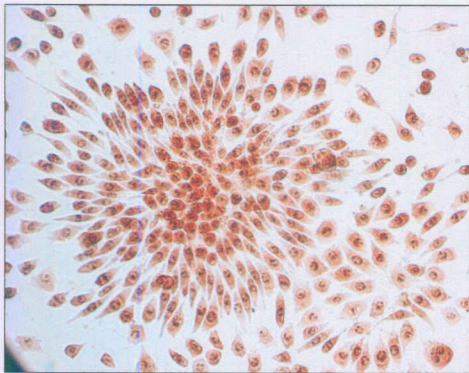
b) **L929/LC**
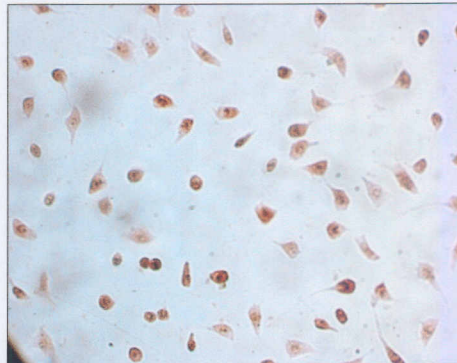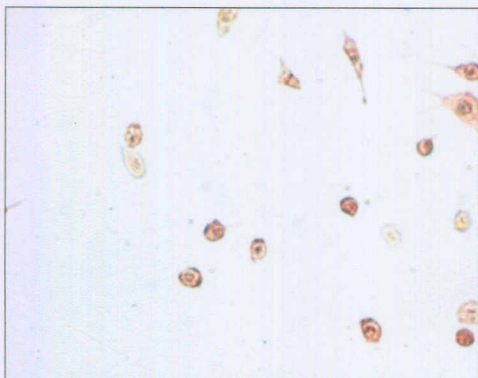
c) **L929/LFC**

d) **L929/LFCC**

Figure 4.3: Microscopic evaluation of condom washings on lysosomal membrane integrity measured using the NR assay in the L929 (a-d) cell line. Reduced staining is observed for L929/LC, L929LFC and with L929/LFCC. L929/LFC is also associated with altered cell morphology.
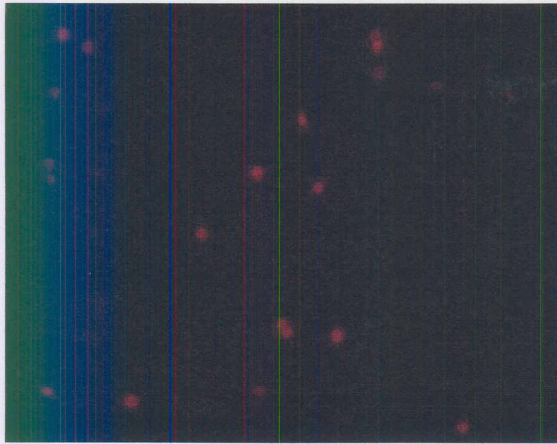
a) <u>L929/CONTROL</u>   b) <u>L929/LC</u>
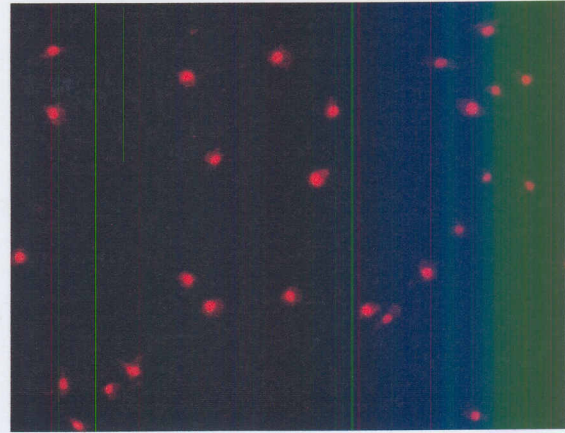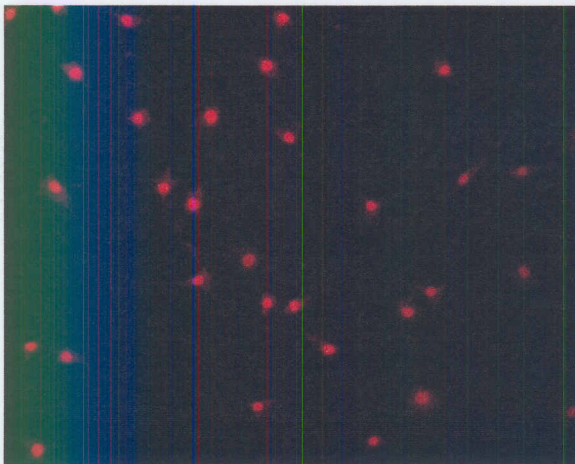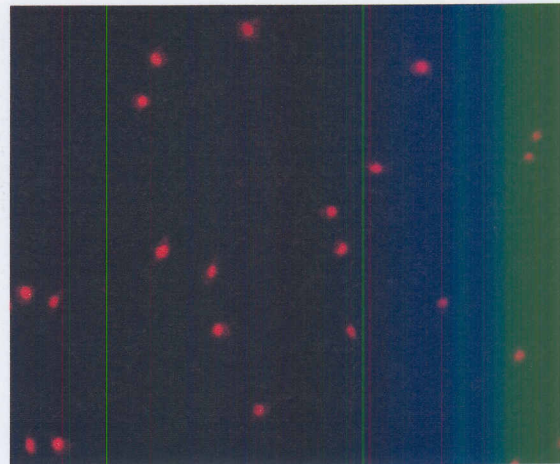
c) <u>L929/LFC</u>   d) <u>L929/LFCC</u>

Figure 4.4: Microscopic evaluation of cell membrane integrity with Propidium Iodide staining without fixation in the L929 (a-d) cell line.

a) L929/CONTROL
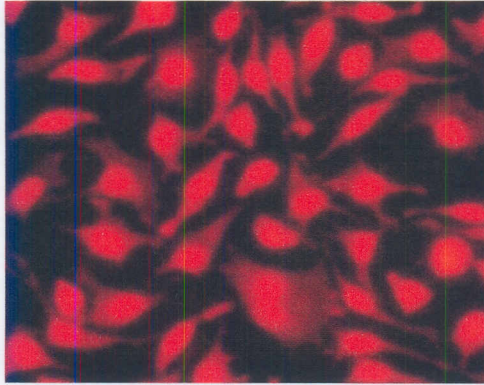
b) L929/LC
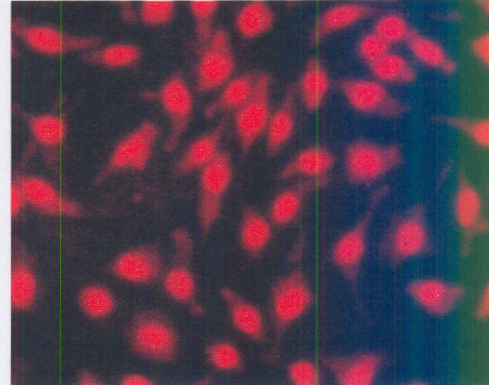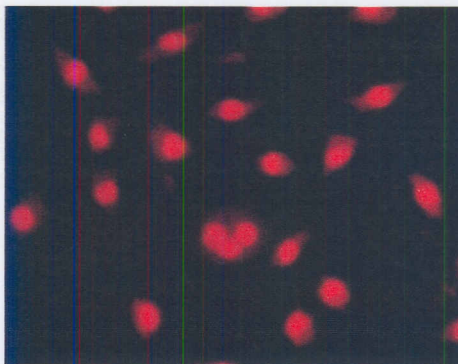
c) L929/LFC
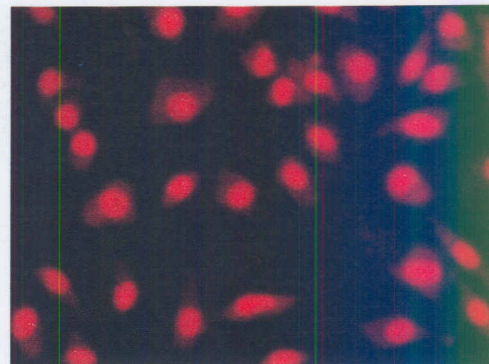
d) L929/LFCC

Figure 4.5: Microscopic evaluation of nuclear structure after fixation and staining with Propidium Iodide in the L929 (a-d) cell line.

L929/Control. For L929/LFC and LFCC fewer cells are present and the cell that are PI positive show condensation of the nuclei with little staining in the cytoplasm where RNA is usually found.

Hoechst 33342 intercalate in A-T regions of DNA with a resulting increase in fluorescence. Normal nuclear morphology is observed for L929/Control and L929/LC in Figure 4.6a and b. Both L929/LFC and LFCC show nuclear condensation. At a higher magnification for L929/LFC the DNA shows peripheral chromatin condensation that resembles apoptosis.

4.3.3 Agarose gel electrophoresis of L929 cells exposed to condom washings, LC, LFC and LFCC.

For all cells including the control not exposed to condom washings DNA laddering associated with apoptosis was observed (Figure 4.7). For all cells the same amount of DNA was loaded as can determined from the amount of DNA close to the position where the samples were loaded. The degree of laddering was in the following order, LC, Control, LFCC and LFC. The greatest degree of DNA laddering occurred in LFC exposed cells.
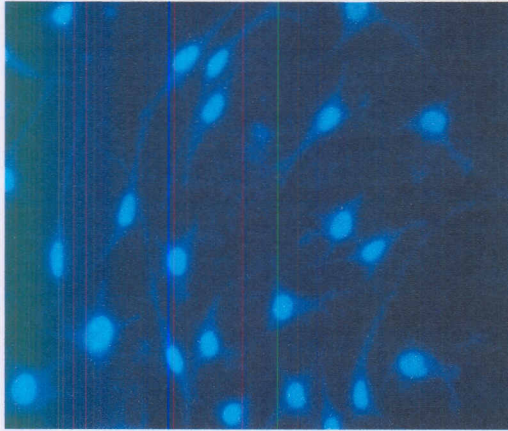
a) L929/CONTROL
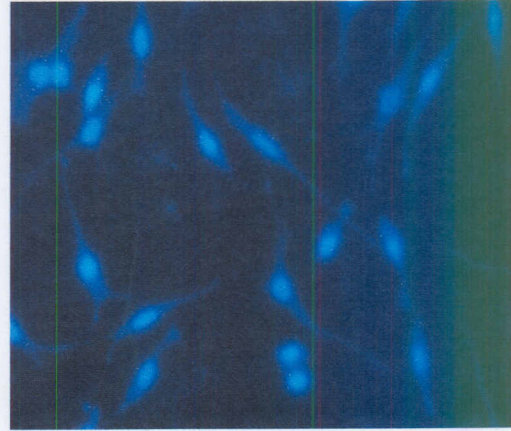
b) L929/LC
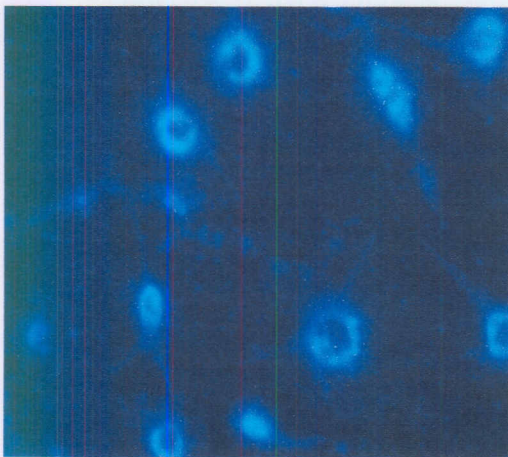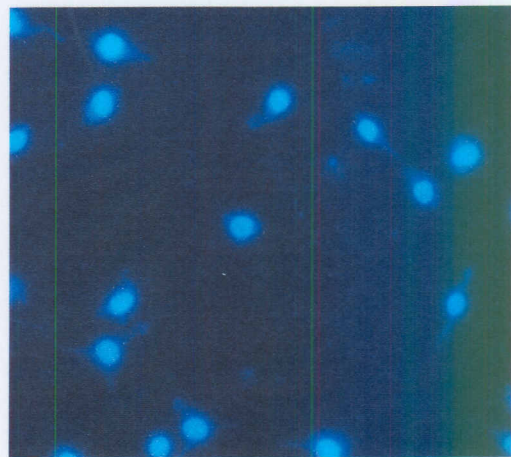
c) L929/LFC

d) L929/LFCC

Figure 4.6: Microscopic evaluation of nuclear staining with H33342 in the L929 (a-d) cell line.
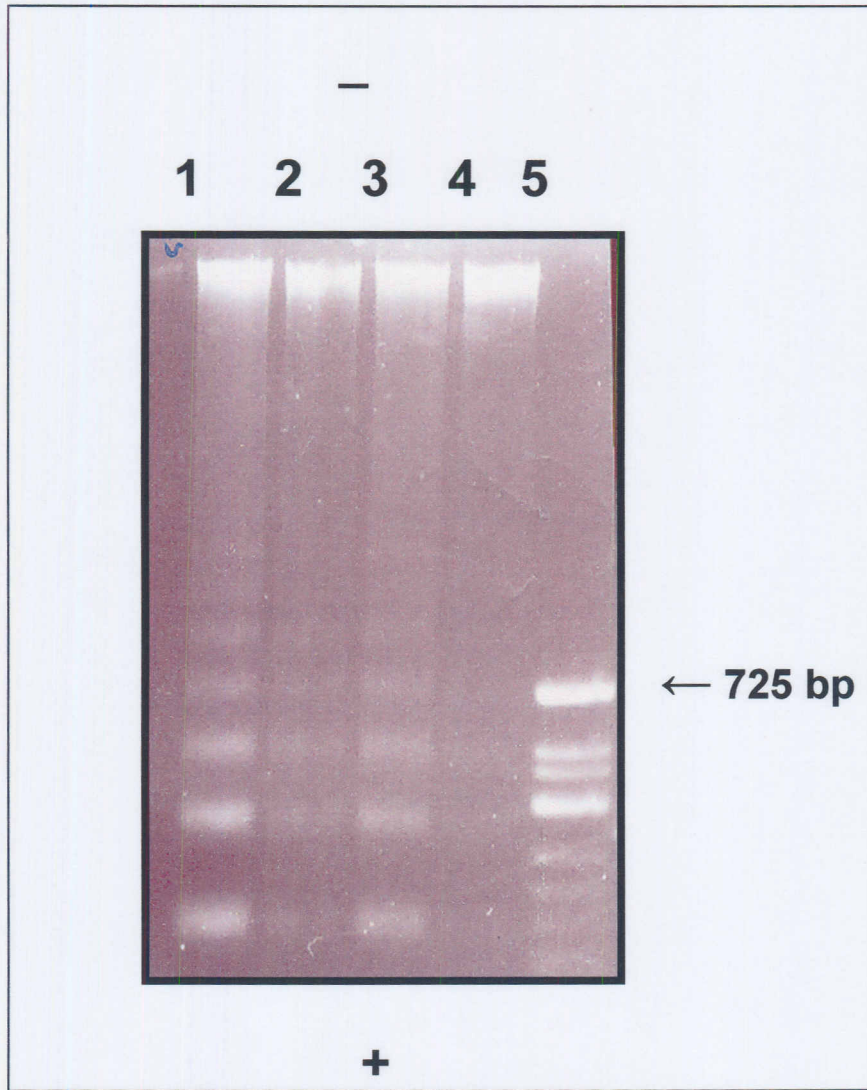
Figure 4.7: Agarose gel electrophoresis of DNA isolated from L929 cells following exposure to LC, LFC and LFCC. Typical DNA laddering patterns associated with apoptosis is observed in Lane 1: LFC, Lane 2: LFCC, Lane 3: Control (no exposure to condom washings, Lane 4: LC and Lane 5 Molecular Size Markers (725 -   bp).

4.4 Discussion

Previously in Chapter 3 it was shown that washings containing the surface material of condom type LFC were the most cytotoxic compared to other condom types (NLC and LC). Since little effect was observed for NLC an additional condom type was included in this following study. LFCC contain lubricants (dimethyl silicone), flavourings and an additional component colourants that may show increased toxicity.

In Chapter 3 the L929 cell line was exposed to increasing concentrations of condom washings for To, T_2, T_4 and T_8. In this study a single concentration of 16% and an exposure time of 8 minutes (T_8) was used.

CV staining can be used to study cell morphology and can also be used to quantify cell number. The amount of staining is either a function of the number of cells present in a cell culture or the extent of cellular damage that has occurred following exposure to a toxin. Cell damage may result in the detachment of the cell membrane and to some extent leakage of the cell contents into the surrounding (55).

No differences in staining was observed for L929 cells exposed to LC, LFC and LFCC and these results verify the findings of Chapter 3 where at 16% condom concentration at T8 no decrease in cell number was observed.

The MTT assay is used to measure cell viability after the formazan formed via the enzymatic catalysis of MTT is solubilized (70-74). Microscopic evaluation of the distribution of the formazan and cell morphology provides additional

information about the effects of condom washings LC, LFC and LFCC on cell structure. The greatest effect is observed for L929 cells exposed to LFC.

Although the MTT seems to be metabolised to form an intracellular blue product, few formazan crystals are observed on the surface of the cells when compared to L929/Control and L929/LC. These results indicate that mitochondrial function is compromised. Furthermore cells do not present with the typical cell morphology associated with fibroblasts *in vitro*.

The NR assay is a cytotoxic test that is used to measure the immediate toxic effects of test substances on the cell membrane, resulting in the leaking of intracellular contents. The assay is based on the ability of viable cells to incorporate and bind neutral red dye. When cells are exposed to a cytotoxic agent, they may lead to irreversible changes to the lysososmal membrane thus resulting in a decreased uptake and binding of NR (71, 75, 91).

For NR and MTT similar cell morphology is observed for L929/LFC. Furthermore reduced staining is observed for LFC and LFCC indicating that the NR assay where accumulated NR is solubilized with acetic acid solution is a sensitive assay for the measurement of cellular toxicity if cells exposed to condom washings.

Cell death occurs either by necrosis or apoptosis (61, 62, 66). Necrosis is unregulated cell death while apoptosis is active and involves regulated sequence of intracellular signals. Necrosis is characterised by changes in mitochondrial shape and function, and the cell loses its ability to regulate osmotic pressure. It then swells, bursts and releases its contents into the surrounding areas (27, 56, 61-63, 66).

Exposure of a cell to a chemical can also lead to apoptosis. Apoptosis is characterised by cell shrinkage, cell membrane blebbing and the formation of membrane bound apoptotic bodies. Usually the organelles structures are preserved intact into the apoptotic bodies but the nucleus undergoes a characteristic condensation of the chromatin. Microscopically, characteristics like nuclear and cytoplasm shrinkage, DNA fragmentation, apoptotic bodies, raptured cytoplasm and membrane blebbing can be seen. Whether necrosis or apoptosis is induced following exposure of a cell to a chemical is a function of concentration and exposure time (27, 56, 61- 63, 66).

No membrane damage is observed for L929/Control when evaluated using PI uptake while for L929 cells exposed to condom washings LC, LFC and LFCC some cells do show PI staining. Cultures were 50% confluent and therefore this represents only a small proportion of cells. Apoptosis in cell culture is associated with shrinkage and detachment of cells from adjacent cells and the cell surface (as has been seen for MTT and NR staining of L929/LFC).

For PI staining only chromatin condensation is observed for LFC and LFCC when compared to control cells. No apoptotic bodies or the arrangement of DNA along the cell membrane. Vapour-fixation of cells *in vitro* that has been described in other literature may be a better method if the fine cellular structure needs to be retained (76, 77).

Staining of the L929 cells exposed to condom washings with H33342 revealed distribution of the chromatin along the cell membrane for L929/LFC. Fragmentation of nuclear DNA into lengths of characteristic size creating a DNA "ladder" is a characteristic feature of apoptosis (27, 56, 60-67). Agarose gel electrophoresis of isolated DNA revealed that all cells including the control

cells underwent apoptosis. Many experimental factors can induce apoptosis in cells *in vitro* not exposed to a toxin such as trypsin, hypoxia and pH. Furthermore the amount of laddering observed for LC was less than that seen for L929/Control.

These results although appearing unusual corroborates the findings of Chapter 3 where LC caused an increase in cell number at T_2. The effect of LFC is greater than that observed for LFCC indicating that other factors rather than the number of components present in each type of condom may account for toxicity.

In conclusion condom type LFC is the most toxic for L929 cells *in vitro* and toxicity is mediated through the process of apoptosis. Further studies should include the effect of these condom washings on other cell types that may be more representative of the tissue exposed to condom material. In addition the NR should be evaluated as an additional bioassay for the evaluation of condom cytotoxicity.

CHAPTER 5: FURTHER EVALUATION OF THE CYTOTOXIC EFFECTS OF CONDOMS ON L929 AND HELA CELLS

5.1 Introduction

The use of latex condoms to prevent sexually transmitted diseases with more emphasis on prevention of HIV infections are widely encouraged (3, 7, 9-13). Natural latex proteins (NRL) have, however, been identified as source of latex allergy. In a previous study by Pretorius and Bester in 2000 the authors indicated that, at cellular level the latex protein causes a decrease in cell numbers as well as viability of cells in culture (11). In the previous chapter, a cell culture testing method was developed to evaluate the cytotoxicity of latex condoms on cells in culture, by modifying the FDA test method.

In this study, the developed method will be applied to compare the cytotoxic effects of latex condom washings on L-929 mouse fibroblast cells (suggested as a sensitive cell line by the FDA for direct cytotoxicity testing) and HeLa cells (a permanent human cervical carcinoma cell line). In this study, besides lubricated (LC) and lubricated flavoured (LFC) condoms, lubricated flavoured and coloured condoms (LFCC) were also evaluated. In addition to the MTT and CV assays used to measure cell number and viability the Neutral Red (NR) assay that measured lysosomal membrane integrity was also used to evaluate the cytotoxic effects of the different types of condoms evaluated.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Cells Lines

Two permanent cell lines, namely mouse fibroblast (ATTC, CCL1 NCTC clone 929 strain designated L-929) and HeLa cells (cervical cancer cell line) were obtained from Highveld Biological Company, Johannesburg, South Africa.

5.2.1.2 Condoms

Three different types of latex condoms were tested, lubricated condoms (LC), lubricated and flavored condoms (LFC), and lubricated, flavored and colored condoms (LFCC). LC were obtained from the South African Health Department in Pretoria, South Africa. LFC were obtained from Remed Pharmacia, Belgium and LFCC were obtained over the counter from a South African convenience store shop.

5.2.2 Methods

5.2.2.1 Cultivation, maintenance and preservation of the L-929 and HeLa cell line

Both L929 cells and HeLa cells were cultivated, maintained and preserved as described for L-929 fibroblast cell line in Section 3.2.2.1. For each experiment, cells were plated at a cell concentration of 3×10^4 cells per ml in 24 flat well plates with the culture area of $1.9cm^2$/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

5.2.2.2. The protein content of condom surface washings

Protein concentrations in different latex condoms were quantitatively analyzed by using the Bio Rad system based on the Bradford assay with BSA as standard as described in Section 3.2.2.2

5.2.2.3 The effect of condom washings on the L929 fibroblast cell line in vitro

5.2.2.3.1.The MTT assay

The cell viability of the L929 and the HeLa cells following a 24-hour exposure to the various condom washings was determined using the MTT assay as described in Section 3.2.2.3.1.

5.2.2.3.2 The combined Neutral Red and Crystal Violet Assay

This assay is a combined method to determine the lysosome membrane integrity (70) and the number of cells in culture. A 100µl volume of a 0.15% Neutral Red (NR) solution in DPBS was added to each well. Plates were maintained at 37°C and 5% CO_2 for a further 90 and 120 minutes for the HeLa and L929 cells respectively. The medium was discarded and the cells were fixed for 10 minutes with 200µl of a 1% acetic acid and 1% formaldehyde solution. This solution was discarded and the dye was solubilized with a 1% (w/v) acetic acid and 50% ethanol solution. The absorbency of the solubilized dye was measured at 570 nm. The 24 well plates were then rinsed with DPBS and dried overnight at room temperature.

A 300µl volume of a 0.1% CV solution in 200mM formic acid at pH 3, 5 was added to the dried wells. The plates were incubated at room temperature for 30

minutes rinsed with DPBS to remove excess dye and then dried overnight at room temperature. The bound dye was dissolved by shaking for 10 minutes in $300\mu l$ of a 10% acetic acid solution. The absorbency of the solubilized dye was measured at 570 nm.

5.2.3 Statistical analysis

MTT, NR and CV were conducted separately and analyzed with an ANOVA for the split-split plot study design. Exposure time zero was omitted as it had no effect on any of the three outcomes viability, lysosome membrane integrity and cell number. Condom washings, W_2, W_4, and W_8 observations were expressed in terms of washings W_0. The testing was performed at the 0.05 level of significance and pair wise comparisons were done using Fisher's Least Significant Differences (LSD) test. The ANOVA tables for viability, lysosomal integrity and survival (Table 1) show the degrees of freedom and p-value where W_0 was omitted from the analyses. Only LSD (T) that is significant is included.

<u>5.3 Results</u>

The protein content of the surface material of the condoms, LC, LFC and LFCC was determined prepared by washing condom fragments in H_2O for 20 hours. The protein content was determined from the standard curve (linearity: $r^2 = 0.9874$) shown in Figure 5.1a. The protein content of the condom types, LC, LFC, and LFCC was 0.048, 0.079 and 0.078 mg/ml (Figure 5.1b).
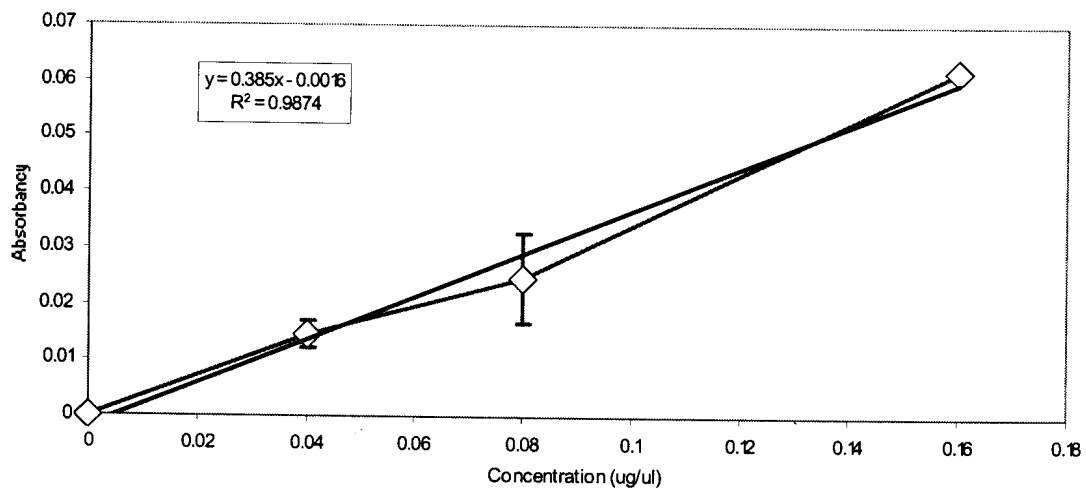
Figure 5.1a: BSA standard curve for the determination of the protein content of the LC, FLC, LFCC
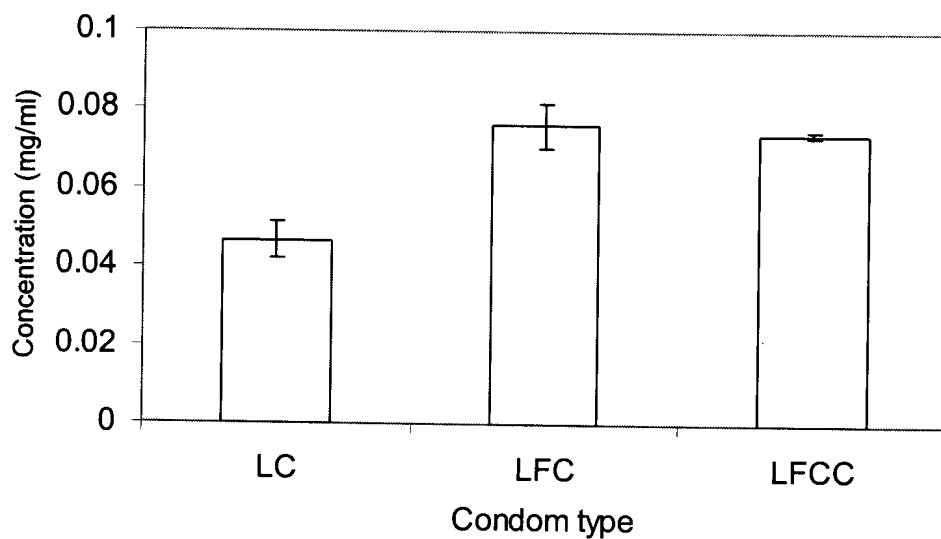
Figure 5.1b: The protein content of condoms, LC, LFC and LFCC determined from the BSA standard curve Figure 4.1b.

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5.3.1 Exposure of the L929 fibroblast and the HeLa cell line to condom washings

The cell lines L929 and HeLa were exposed to condom washings derived from LC, LFC and LFCC at 16% of the culture medium. Following 3 hours exposure cell viability and number as well as lysosome integrity was determined.

5.3.1.1 The effect of condom washings on the cell viability

Cell viability was determined following 24 hours exposure to the condom washings derived from LC, LFC and LFCC (Figure 5.2). For L929 cells exposed to condom washings, LC, LFC and LFCC no changes in cell viability was observed. For HeLa cells an increase in cell viability was observed for LC to 130% for T_2, T_4 and T_8 while no effect for LFC and LFCC was observed.
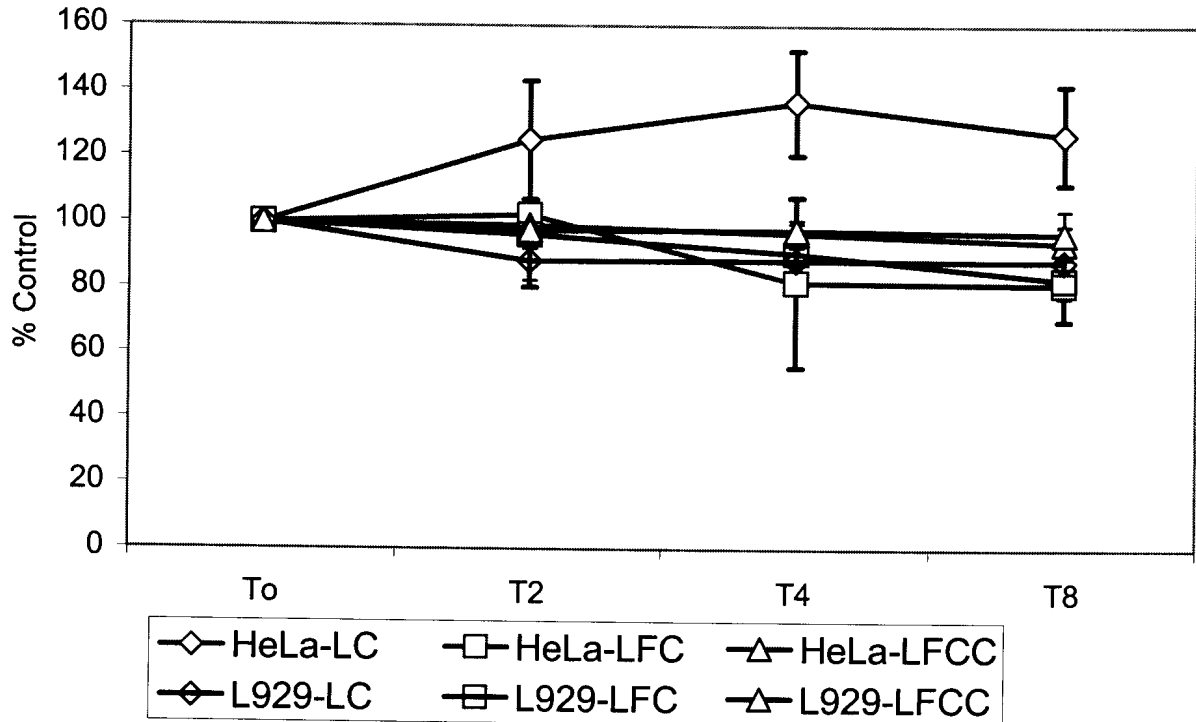
Figure 5.2: The effect of condom washings T_0, T_2, T_4 and T_8 of cell viability in L929 and HeLa cells.

The data was analyzed using a Split-plot design and the data obtained for CT, Cell type, Exposure time (ET), CT X Cell type, and CT x ET was determined and are presented in Table 5.1.

Table 5.1: Summarized ANOVA table for cell viability

Source of variance	Degrees of freedom	p-value
Condom replicate	3	na
Condom type (CT)	2	0.0015
Cell type	1	0.0034
CT X Cell type	2	0.0006
Exposure time (ET)	2	0.0916
CT X ET	4	0.1519
Cell type X ET	2	0.9461
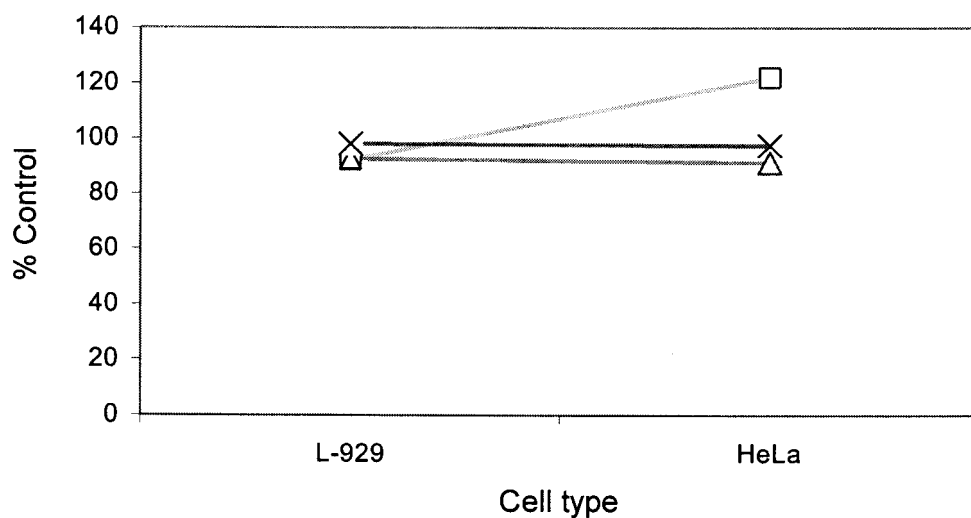
Figure 5.2a: Mean viability for condom type LC (□), LFC (▲) and LFCC (x) by cell type interaction

Parameters CT and cell type showed significance with p=0.0015 and 0.0034 respectively. Comparisons of CT X cell type showed significant differences (p=0.006). From the mean viability plot this difference is due to the increase in viability observed for HeLa cells exposed to LC (Figure 5.2). No significant differences were observed for EC (p=0.0916), CT X ET (p=0.1519) and Cell type X EC (p=0.9461).

5.3.1.2 The effect of condom washings on the cell number

Cell number was determined following 24 hours exposure to the condom washings derived from LC, LFC and LFCC (Figure 5.3).
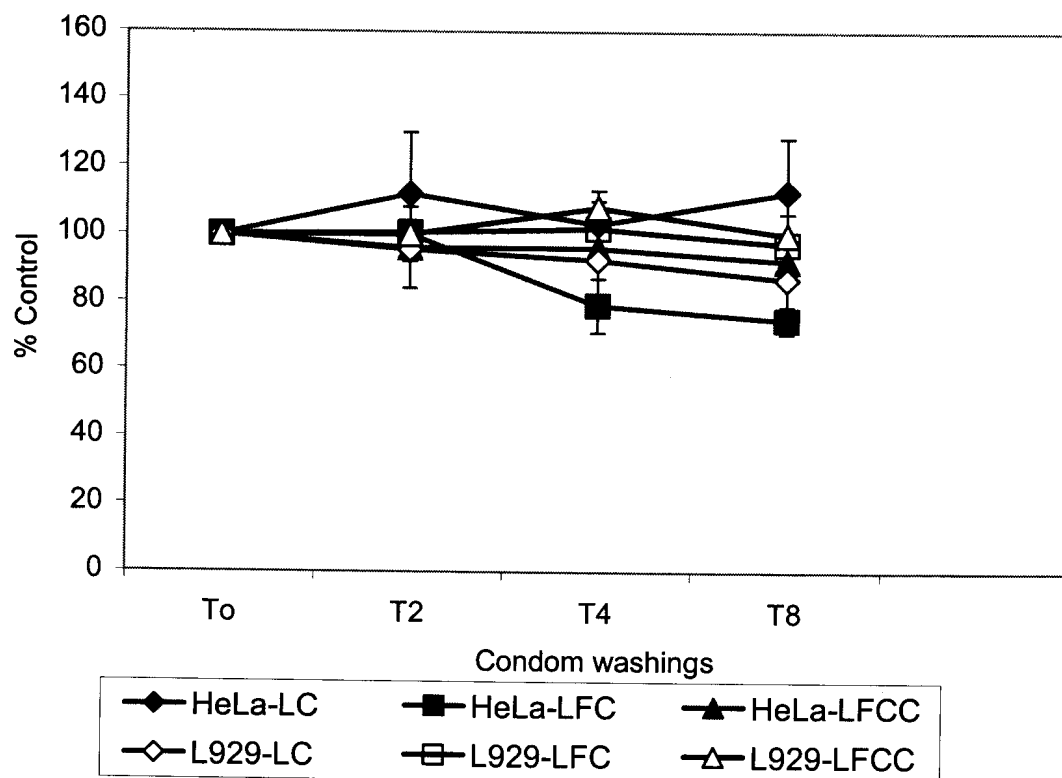
Figure 5.3: The effect of condom washings T_0, T_2, T_4 and T_8 of cell number in L929 and HeLa cells

For L929 cells and HeLa cells exposed to condom washings, LC, LFC and LFCC no changes in cell number was observed.

No statistical differences (Table 5.2) were found for CT (p=0.2483) and cell type (p=0.5869). Further evaluation of CT X Cell type however was significant and from the mean cell number plot this is due to a slight increase in cell number observed for HeLa exposed to LC (Figure 5.3a).

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Table 5.2: Summarized ANOVA table for cell number

Source of Variation	Degrees of freedom	p-value
Replicates	3	n/a
Condom type (CT)	2	0.2483
Cell type	1	0.5869
CT X Cell type	2	0.0116
Exposure time (EC)	2	0.0383
CT X ET	4	0.0805
Cell type X EC	2	0.0521
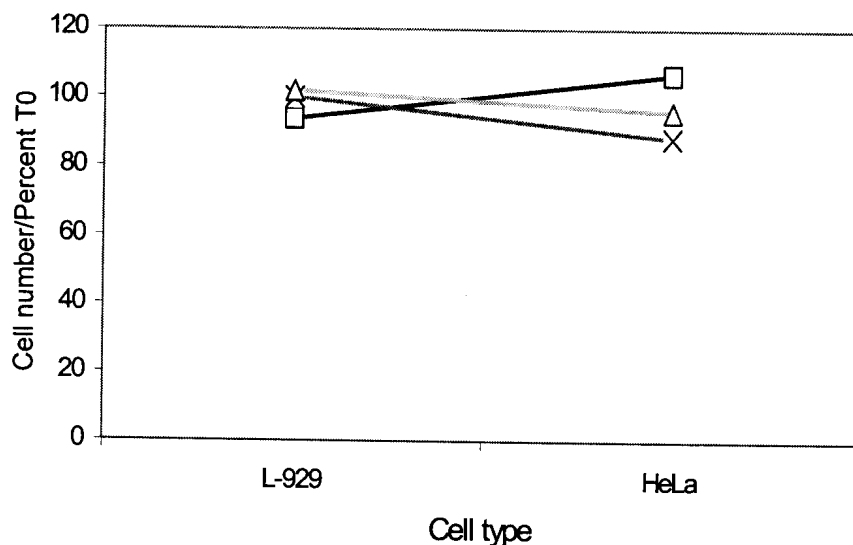
Figure 5.3a: Mean viability for condom type LC (□), LFC (▲) and LFCC (x) by cell type interaction Table 4.2b: Mean viability for condom type by exposure time.

The effect of exposure time (ET) is statistically different with p=0.0383 and this effect is possibly due to the interaction between Cell type X ET (p= 0.0521). As p is above the level of significance this data will not be evaluated.

5.3.1.3 The effect of condom washings on the lysosome membrane integrity

The effect of condom washings on lysosome membrane integrity was determined following 24 hours exposure to the condom washings derived from LC, LFC and LFCC (Figure 5.3).

No changes in lysosomal membrane integrity were observed for L929 cells exposed to LC, LFC and LFCC. Likewise no changes in lysosome membrane integrity were observed for LC and LFCC. In contrast, for LFC an exponential decrease in lysosome membrane integrity from 100% to 20% at T_8 was observed (Figure 5.4a).
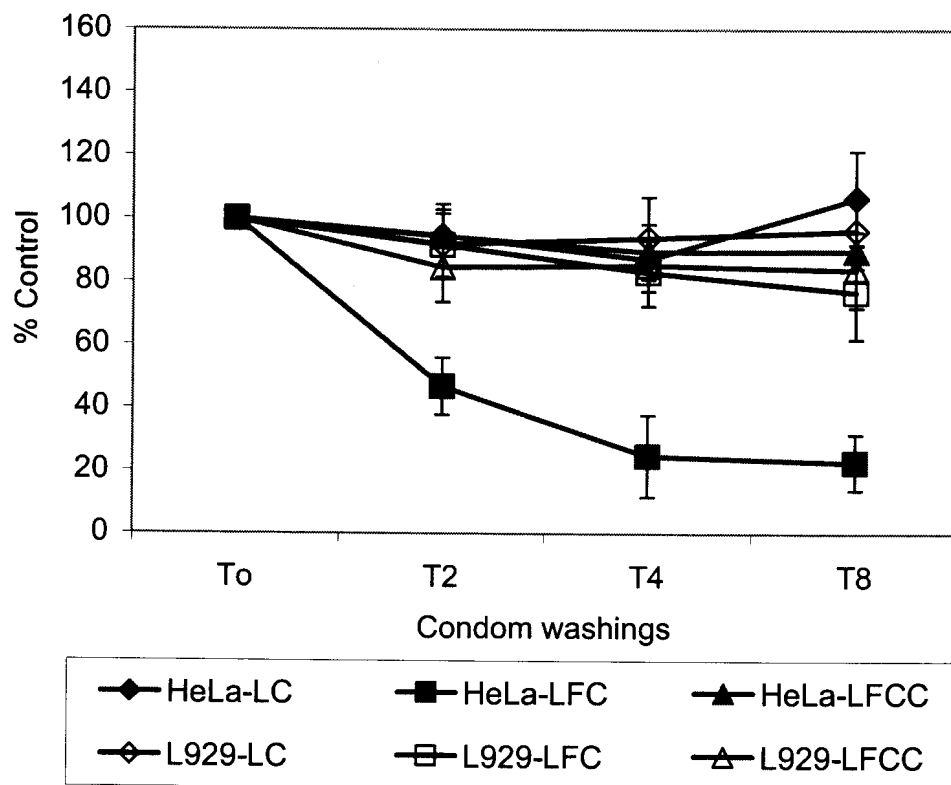
Figure 5.4a: The effect of condom washings T0, T2, T4 and T8 on lysosome membrane integrity in L929 and HeLa cells.

Table 5.3: Summarized ANOVA table for lysosome membrane Integrity

Source of Variation	Degrees of freedom	p-value
Condom replicate	3	na
Condom type (CT)	2	0.0003
Cell type	1	0.0252
CT X Cell type	2	0.0030
Exposure time (ET)	2	0.0653
CTX ET	4	0.0116
Cell type X ET	2	0.4100

Split-plot analysis of data reveals a significant difference between Condom type (CT) (p=0.0003) and Cell type (0.0252). The interaction between CT X Cell type was significant with p=0.0030. From the mean plot (Figure 4.4b) it can be seen that this effect is due to the effect of LFC on HeLa lysosome membrane integrity.
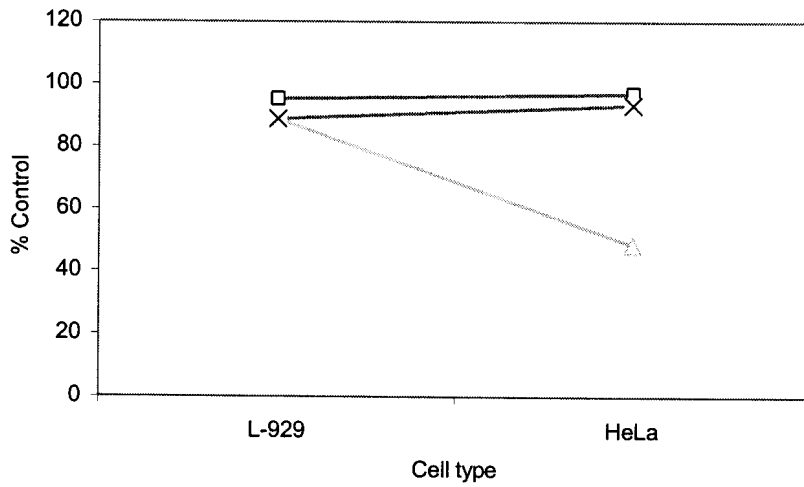
Figure 5.4b: Mean lysosome membrane integrity for condom type LC (□), LFC (▲) and LFCC (x) by cell type interaction
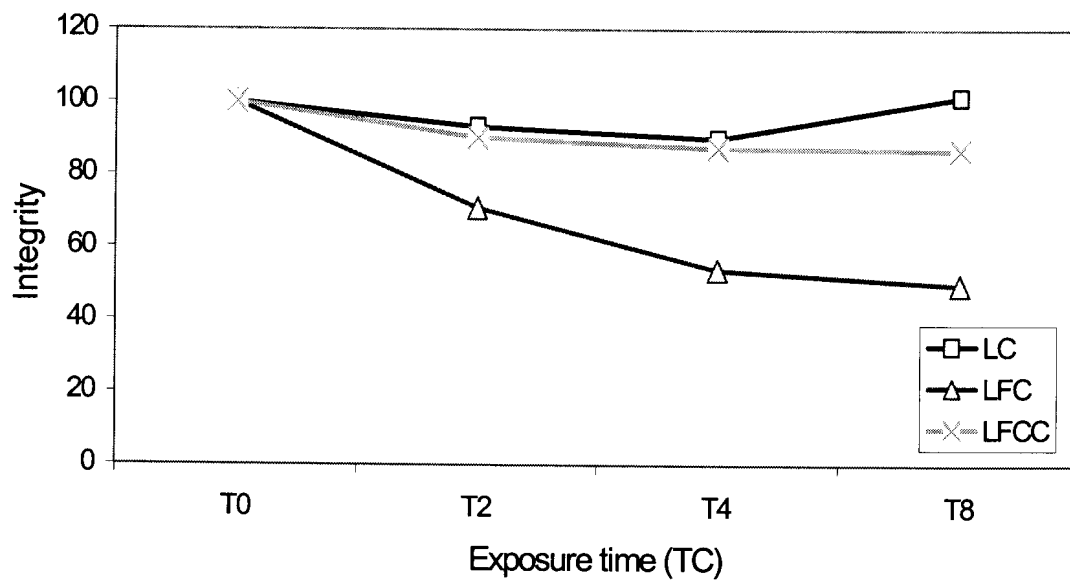
Figure 5.4c: Mean lysosome integrity for condom types, LFC and LFCC by exposure time (EC) interaction

The effect of exposure time is marginal with p=0.0653 while the interaction between CT and EC is significant with p=0.0116. This difference is due to the different amounts of LFC condom material present in mediums T_2 T_4 and T_8 (Figure 5.4c).

5.4 Discussion

Condoms contain latex, non-spermicidal lubricants (such as dimethylsiliconium) and other non-specified compounds such as colourants and flavourings. The manufacturers provide very little information regarding the type and amount of each compound added. The FDA method traditionally used to determine the safety of medical devices (22), was modified by Pretorius and Bester (11) and Motsoane *et al* (52) and was used to determine the biological safety of condoms. These authors exposed L929 cells to medium containing 0-66% condom washings from non-lubricated, lubricated (same as LC) and lubricated and flavoured (same as LFC) condoms prepared using the same method as described in this study. In this previous study at W_4 and W_8 both LC and LFC showed a decrease in cell number and viability.

The objective of this study was to determine the cytotoxic effects of condoms in a cell line that was in origin and type closer to that of the cervical lining. Assays that involve the uptake and/or the metabolism of a dye are more sensitive and effective in measuring early changes in cell metabolism and function. The MTT assay measures the ability of mitochondrial succinate dehydrogenase to metabolize MTT to a formazan product (70-74).

NR is a weak cationic dye that readily moves across cell membranes by non-ionic diffusion accumulating intracellularly in the lysosome where it binds with anionic sites in the lysosomal matrix (70, 91). Change in the lysosomal matrix result in reduced NR uptake. Cytotoxicity is a complex process where its effect may occur at different sites within the cell and therefore both the MTT and NR assay has been included to evaluate the cytotoxity of condom washings.

Three different types of condoms were evaluated lubricated condoms (LC), lubricated and flavored condoms (LFC), and lubricated, flavored and colored condoms (LFCC). The effect of condom washings on cell number, viability and lysosome membrane integrity was determined using the CV, MTT and NR assays. Condom types, LC, LFC and LFCC had a significant effect on cell viability and lysosomal membrane integrity.

Differences observed between the L929 and HeLa cells were due to the increased viability observed for LC and the decrease in membrane integrity for LFC on HeLa cells. With LC and LFC no decrease in cell number and viability was observed as previously reported for the L929 cell line (52). This is due to the dilutions of the condom washings used. In this study the cells were exposed to medium containing 20% condom washings compared to 66% used previously. Although no decreased in cell viability is observed for LFC a decrease of 75% in lysosomal membrane integrity is observed. The increase in cell viability found for HeLa exposed to LC (although statistically not significant) cannot be explained. Changes in cell viability and membrane integrity was only observed for HeLa cells, indicating that the HeLa cell line is more sensitive for the cytotoxic effects of condom washings. Furthermore the NR assay is a more sensitive assay than

the MTT assay in detecting the cytotoxic effects of LFC condom washings at low concentrations.

Testing material with unspecified composition is difficult and care must be taken not to use a single cell line or assay, as it may not detect other cytotoxic effects. Therefore testing should include a range of tests that can measure different aspects of cytotoxicity. For this reason, cell number and viability as well as membrane integrity was determined using CV, MTT and NR assays respectively. It has been shown (Chapter 4) that condom washings mediate their effect through the process of apoptosis in the L929 cell line. Further research must include the evaluation of apoptosis in HeLa cells using techniques such as Western blotting and flow cytometry to measure the levels of apoptosis related proteins such as caspase 3 and Annexin V.

CHAPTER 6: CONCLUDING DISCUSSION

Latex products including latex condoms and gloves have now been identified as sources of latex protein allergy (1-4) with a high prevalence amongst health care workers, latex industry workers, people undergoing multiple surgeries and atopic subjects (1-3, 5, 7-13). An increase in the prevalence of reported cases has been found to be associated an increase in awareness of protection against infections such as Hepatitis B and HIV/ AIDS and other sexually transmitted diseases (1-3, 5, 9, 12, 13, 19- 21, 28, 29, 31, 34-38). This has led to an increase in the demand for latex gloves and condoms and the use of poorly manufactured products has further added to this problem (3, 5).

In preparation of latex condoms, several chemicals including antioxidants, accelerators, emulsifiers, stabilizers, lubricants, and in some cases flavourings and colourants are normally added. Unfortunately the chemical composition of these chemicals is normally undisclosed by manufacturers and furthermore their toxicity potentials are not known. Though extensive testing is done to evaluate the physical quality of condoms, little information is available regarding the biological safety of condoms.

Four different types of condoms were used in the entire study. Latex condoms without lubricants (NLC) lubricated condoms (LC), lubricated and flavoured condoms (LFC) and lubricated, flavoured and coloured condoms (LFCC). In the selection of condoms only the most frequently used brands of condoms in South Africa were selected. The condoms were either bought over the counter in the local adult shop (NLC and LFC) or grocery shop (LFCC) while some were freely available from the Health Department in Pretoria (LC). These condoms contain latex, non-spermicidal lubricants (such as dimethylsilicone) and other non-specified compounds such as colourants and flavourings. The manufacturers provide very little information regarding the type and amount of each compound added.

Testing of such mixtures can present with different types of toxic effects such as additive, synergism or antagonism. Repeated exposure to a toxic compound may lead to a reduction of a tolerance (26). Toxicity shows a dose-response relationship and may range from complex biochemical changes to lethality and may involve receptor interactions (56, 57).

Besides the type of toxins to be evaluated the test system used for the assessment of toxicity should be carefully selected. In this study a modification of the direct cell culture testing method that is specified by the American Test Method F813-83 of 1998 was used. The FDA direct cell culture testing method is useful for assessing cytotoxic potential as part of quality control for established medical materials and devices. Instead of directly adding the condom material to the L929 cell line culture, fragments of the condoms were washed in the cell culture medium for specific time intervals. The toxicity of the surface material to which there is direct contact by the person using it was evaluated. The condom washings were prepared by washing condom material with cell culture medium for 2, 4 and 8 minutes. This time period of 2-8 minutes was chosen to represent normal physiological exposure and to determine if a dose response effect occurs.

The cytotoxicity process is a complex *in vitro* cellular process, and its expression in the cell may manifest itself in a wide spectrum of cellular events. These events range from cell death either apoptosis or necrosis or/and changes in cell function (26, 27). *In vitro* cytotoxic assays or bioassays used to measure these changes must be sensitive enough to detect the effect of very small concentrations of cytotoxic substances and must be easy to perform (27). The selection of the bioassay used to measure toxicity is critical and therefore the use of several different bioassays that measure different aspects of cell function is recommended.

Cytotoxity of NLC, LC and LFC were assessed in the L929 cell line using the Crystal Violet (CV) and MTT assays that measure cell number and viability respectively. CV is based on the binding of the blue dye non specifically o cellular protein. Toxicity can cause cells to die and detach or to stop dividing. CV staining has been widely used in the literatures as a cytotoxicity assay that measures cell number or survival. It was used in this dissertation to determine the number of cells attached to the surface of the cell culture dish following exposure of the L929 cell line to condom washings.

MTT (3-(4,5- Dimethylthiazol-2 -yl) -2,5-diphenyltetrazolium bromide) is a yellowish water-soluble substance that is reduced by viable cells to purple water insoluble formazan crystals by succinate tetrazolium dehydrogenase activity in mitochondria of active, viable cells. Decrease in succinate enzyme activity is an initial marker of a physiological instability in a cell. Following exposure of the L929 cells to condom washings cell viability was measured using the MTT assay. The formazan crystals formed by viable cells was dissolved using an organic solvent and measurement spectrometrically of the coloured product indicates cell viability.

The L929 cells were exposed to different dilutions (0-66%) of the condom washings. The L929 cell line was exposed to the different mediums for 20 and 48 hours before cell number and viability were determined using the MTT and CV assays respectively. The decrease in cell number and viability is expressed as the percentage of the control (exposure time T_0). Different condom types behaved differently over time of exposure and the concentrations of condom washings. LFC were found to induce a decrease in cell number compared to other condom types, followed by LC and NLC revealed increases in cell number. Split-plot analysis used to determine the effect of parameter condom type (CT), condom washings (CM), time (T), CT X CM and CT X CM and T has a significant effect of cell number.

Differences between each condom type (CT x Time) are significantly different due to the effect observed at T_2 for LC. Cell viability of the cells using MTT assay was

determined following 20 hours exposure to medium containing different volumes of condom washings. Different volumes of condom washings appear to have only slight effect on cell viability for NLC and LC. For LFC at percent medium containing condom washings greater than 40%, a rapid decrease of cell survival to 63% at T_4 and 28% at T_8 were observed. Split-plot analysis revealed significant differences for CT X time due to the effect of LFC at T8. The MTT usually considered being more sensitive than the CV assay showed only toxicity for as LFC and not for NLC and LC as with the CV assay.

Though MTT assay could not reveal the difference between different condom types, a definite distinction was observed when using CV assay. It remains clear that LFC was observed to be the most toxic type of condom with NLC being the least toxic condom type among the three types of condoms used. LFC may be more toxic due the presence of lubricants and flavourants.

Microscopic evaluation of cells can provide important information regarding the effects of a toxic compound on cellular morphology and structure. Furthermore it can verify some of the findings of quantification bioassays such as the CV and MTT assays. The effects of condom washings LC, LFC and LFCC on cell morphology at T8 and concentration of 16% were determined following the CV and MTT staining uptake. In this study NLC was excluded as it was found that it had no effect on the L929 cells. A possibly more toxic condom type LFCC was included. In addition NR bioassay was included. This assay has been used to evaluate the cytotoxicities of various kinds of products, such as cosmetics, pharmaceuticals, industrial chemicals and household products. NR is a weak cationic dye that penetrates the cell membranes by no-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix (70, 75, 91). When cells are exposed to a cytotoxic agent, they may lead to irreversible changes to the lysososmal membrane thus resulting in a decreased uptake and binding of NR.

For all staining methods cell morphology and the distribution of staining was evaluated. No differences in staining was observed for L929 cells exposed to LC, LFC and LFCC and these results verify the findings that at 16% condom concentration at T8 no decrease in cell number was observed. For MTT assay control cells and cell exposed to LC presented with normal cell morphology and formazan crystal formation on the cell surface. Cells exposed to LFC and LFCC did not show typical fibroblast morphology but were rounded indicating cellular stress or cell death.

As for MTT similar cell morphology is observed for LFC after NR staining. Furthermore reduced staining is observed for LFC and LFCC indicating that the NR assay where accumulated NR is solubilized with acetic acid solution is a sensitive assay for the measurement of cellular toxicity if cells are exposed to condom washings.

Fluorescence probes are used increasingly in cell biology studies for the visualisation of specific organelles of the cells and to distinguish between life and dead cells. Fluorescence stains can be used for viability testing and have proved to be reliable, rapid and economic test methods (76, 77, 86-90). In this dissertation, propidium iodide staining (PI) and Hoechst 33342 (H 33342) staining were used. Changes in membrane integrity can be determined by studying the uptake of a PI that cannot cross an intact cell membrane, however with damage the cell membrane become more permeable, the dye can move across the cell membrane and once in the nucleus binds DNA with a resulting increase in fluorescence (76, 77, 86-90). For L929 cells exposed to LC, LFC and LFCC membrane damage is observed when compared to the control cells. Cultures were 50% confluent and therefore this represents only a small proportion of cells.

Cell death can occur by necrosis or apoptosis (56, 59, 60). The latter is unregulated cell death characterised by changes in mitochondrial shape and function, and the cell

loses its ability to regulate osmotic pressure. It then swells, bursts and releases its contents into the surrounding areas. Low PI staining of unfixed cells indicates that little membrane damage had occurred and therefore cell death is not mediated by this process.

Apoptosis is characterised by cell shrinkage, cell membrane blebbing and the formation of membrane bound apoptotic bodies. Usually the organelle structures are preserved intact in apoptotic bodies but the nucleus undergoes a characteristic chromatin condensation. Both H 33342 and PI can be used to evaluate nuclear structure following exposure of L929 cells to LC, LFC and LFCC washings. For PI staining only chromatin condensation is observed for LFC and LFCC when compared to control cells. No apoptotic bodies or the arrangement of DNA along the cell membrane was observed with PI staining. Vapour-fixation of cells *in vitro* that has been described and may be a better method if the fine cellular structure needs to be retained (76, 77).

Staining of the L929 cells exposed to condom washings with H33342 revealed distribution of the chromatin along the cell membrane for LFC. Apoptosis is associated with the fragmentation of nuclear DNA into lengths of characteristic size creating a DNA "ladder" that is a characteristic feature of apoptosis. Agarose gel electrophoresis of isolated DNA revealed that all cells exposed to condom washings and the control cells underwent apoptosis. Many experimental factors such as hypoxia and pH can induce apoptosis. The amount of DNA laddering was assessed and occurred in the following ordered LC, control, LFCC and LFC. The findings that cell exposed to washings from LC showed less DNA laddering than the control cells could be due to the mitogenic effect of LC on cell number at T_2. The effect of LFC is greater than that observed for LFCC indicating that other factors rather than the number of components present in each type of condom may account for toxicity. From these findings it condom type LFC is the most toxic and all toxic effects are mediated by apoptosis.

Further research investigated the toxic effects of these condoms in a cell line in origin and type closer to that of the cervical lining. Toxicity of condom washings were compared to that found in the L929 cell line using the CV and MTT assays and an additional bioassay the NR assay was included. Condom types, LC, LFC and LFCC had a significant effect on cell viability and lysosomal membrane integrity. Differences observed between the L929 and HeLa cells were due to the increased viability observed for LC and the decrease in membrane integrity for LFC on HeLa cells. With LC and LFC no decrease in cell number and viability was observed as previously reported for the L929 cell line(52). This is due to the dilutions of the condom washings used. In this study the cells were exposed to medium containing 16% condom washings compared to 66% used previously (52). Although no decreased in cell viability is observed for LFC a decrease of 75% in lysosomal membrane integrity is observed. The increase in cell viability found for HeLa exposed to LC (although statistically not significant) cannot be explained. Changes in cell viability and membrane integrity was only observed for HeLa cells, indicating that the HeLa cell line is more sensitive to the cytotoxic effects of condom washings. Furthermore the NR assay is a more sensitive assay than the MTT assay in detecting the cytotoxic effects of LFC condom washings at low concentrations.

Testing material with unspecified composition is difficult and care must be taken not to use a single cell line or assay, as it may not detect other cytotoxic effects. Therefore testing should include a range of tests that can measure different aspects of cytotoxicy. Besides the MTT, CV and NR assay other tests such as the propidium iodide assay for cell membrane integrity (91, 92) and microscopic evaluation of nuclear apoptosis with a fluorescent dye such as Sytox-green (76) should included. These are assays address only the effects of short-term exposure and not possible genotoxic effects that may occur following repeated and long-term exposure.

The different types of condoms differed in their ability to induced cytotoxic effects on L929 and HeLa cells *in vitro*. LFC condoms that contained flavourings were more toxic that LFCC that contained both flavouring and colouring. These types of

additives are not the only factors that may contribute to cytotoxity. The Medical Devices Agency (MBA) has determined the presence of dithiocarbamate vulcanization accelerators in latex gloves. MBA reported that the genotoxicity of these agents was due to the type used as well as the residue concentration (93). As condoms used in this study were from different manufacturers little information is provided regarding dithiocarbamate usage and amount of residue present in each product. Therefore genotoxic testing should be included in the evaluation of condom material. Besides a single chemical that may contribute to genotoxicity synergistic effects should also be considered.

From this study it can be clearly seen that condoms contain various non-specified constituents. Further research needs to be directed towards the identification of each specific compound using techniques such as High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS) to identify compounds such as lubricants, flavourings and colourants added during the manufacturing process. Each compound must be tested individually *in vitro* to determine its specific toxic effects as well as possible additive and synergistic between different constituents. Following these assessments, recommendations should be made to condom manufactures regarding the biological safety of compounds added to condoms.

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Appendix: Publications

1. Pretorius E, Motsoane NA, Bester MJ. Evaluation of the biological safety of condoms using an in vitro cell culture method. (scientific letter) South African Medical Journal Jan 2000; 91(1): 50.

2. Motsoane NA, Pretorius E, Bester MJ, Becker PJ. The Biological safety of condom material can be determined using an in vitro cell culture system. Analytical Cellular Pathology 2001; 23(2): 51-59.

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improved response seen in this group. Reported adverse effects ranged from 2.7% to 9.4%, with the weekly iron/vitamin group reporting the least adverse effects and the daily iron group the most.

We conclude that a weekly iron and combined iron/vitamin supplement, when given to a population of rural Malawian preschool children, has haematological effects similar to a standard daily iron supplement. In this way it confirms the findings of previous research studies, including a study conducted on pregnant women at Ekwendeni.[8] Further benefit has been shown in that the weekly administration of an iron/vitamin supplement resulted in fewer adverse effects and improved compliance. It should be noted that the study did not include children with severe anaemia (Hb < 7.0 g/dl), and therefore the use of weekly iron for children with severe anaemia remains questionable and should not be recommended at this time. The other limitation of this study was the fact that causes of anaemia other than iron deficiency were not specifically examined. This would include malaria infection and HIV, both of which are thought to be prevalent among the population studied. We can report, however, that the situation of weekly iron supplementation has now been tested successfully in a primary health care setting in rural sub-Saharan Africa, and can be recommended for children with mild to moderate anaemia.

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50 EVALUATION OF THE BIOLOGICAL SAFETY OF CONDOMS USING AN *IN VITRO* CELL CULTURE METHOD

To the Editor: Latex products have long been recognised as a cause of latex protein allergy.[1-3] The increased usage of latex gloves, with the consequent increased occurrence of latex allergies, appears to have escalated with increasing awareness

of the transmission of HIV-AIDS and other infections. The use of condoms as a means to prevent the transmission of sexually transmitted diseases and HIV-AIDS has been widely promoted. Although extensive testing is done to evaluate the physical quality of condoms, no information is available regarding the biological safety of condoms. This study was undertaken to determine the effects of short-term exposure to physiological levels of condom surface material on cell viability (MTT assay) and cell growth (crystal violet assay). A direct contact cell culture testing method (FDA test method F813-83[4] used to evaluate the cytotoxic potential of medical materials and devices) was used. The cell line ATCC, L-929 was grown in media exposed to different concentrations of condom material. Three types of latex condoms were tested, namely: without spermicide, with spermicide, and flavoured condoms with spermicide. Cell studies revealed no significant decrease in cell viability or growth for condoms with and without spermicide. However, a significant decrease in cell viability was observed for flavoured condoms with spermicide and results indicate an effect on cell growth. The modified FDA test method F813-83 was found to be a sensitive test system for the evaluation of the biological safety of condoms.

The penis, and in particular the cervical and vaginal cells of the female reproductive organs, are exposed to the latex proteins and additives such as colourants and flavourings of condoms. Our results indicate that condoms without spermicide and those with spermicide do not adversely affect cells in culture. Flavoured condoms, however, affect cell numbers and cell viability adversely. We therefore believe that the additives together with the latex proteins might affect cells of both the male and female reproductive organs. Although we want to stress that we support the use of condoms, we would like to warn the general public and health care fraternity against using flavoured and coloured condoms because these added chemicals may have a toxic effect on cells of the vagina, cervix and penis. Furthermore, these results demonstrate that the biological safety of all commercially available condoms should be assessed to limit a possible health risk.

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The biological safety of condom material can be determined using an *in vitro* cell culture system

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Latex products have long been recognized as a cause of latex protein allergy. The increased usage of latex gloves, with the consequent increased occurrence of latex allergies appears to have escalated with increasing awareness of the transmission of HIV–AIDS and other infections. The use of condoms as a means to prevent the transmission of STD's (sexually transmitted diseases) and HIV–AIDS has been widely promoted. Although extensive testing is done to evaluate the physical quality of condoms, no information is available regarding the biological safety of condoms. This study was undertaken to determine the effects of short-term exposure to physiological levels of condom surface material on cell viability (MTT assay) and cell growth (crystal violet assay). A direct contact cell culture testing method (FDA test method F813-83 used to evaluate the cytotoxic potential of medical materials and devices) was used. The modified test method was found to be a sensitive test system for the evaluation of the biological safety of condoms. This study reveals the importance of evaluating the biological safety of all condoms that are commercially available, because of the potential health risk that may be associated with prolonged use of certain types of condoms.

1. Introduction

Latex products have long been recognized as a cause of latex protein allergy [2,9,11,13]. The increased us-

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age of latex gloves, with the consequent increased occurrence of latex allergies appears to have escalated with increasing awareness of the transmission of HIV/AIDS and other infections. The use of condoms as a means to prevent the transmission of hepatitis B and sexually transmitted diseases such as HIV/AIDS, has been widely promoted [2,11,12]. Latex is a natural sap extracted from the Brazilian rubber tree, *Hevea brasiliensis* [2,10,12]. It is used to produce natural rubber and is one of the important vegetable materials with extensive applications of over 40 000 products in the USA [2,9,10] including industrial and surgical products, chewing gums, balloons and toys [7,10,13]. The worldwide increase in reported reactions of latex is related particularly to the increased use of poorly manufactured rubber gloves and condoms used as a barrier against diseases [11,12]. Allergy to natural latex has become a major health problem and is frequent in certain occupational settings, especially among health care workers, among individuals producing articles containing natural latex and also in patients with a history of repeated operations [2,9–12].

Latex products contain two types of compounds that cause allergies, namely: added chemicals such as antioxidants that cause dermatitis, and natural proteins that cause systemic, potentially life threatening allergic reaction [5]. Reactions to an allergen are usually of immediate type and are elicited by an IgE response [7,10]. The predominant manifestation is contact urticaria, and the less frequent are rhinitis, conjunctivitis, asthma or anaphylactic shock [6,9,10,12,13].

The manufacturing process is similar for condoms and surgical gloves and includes the dipping of the mold in the liquid latex containing additives such as antioxidants, flavors and dyes. However, differences may exist in the stringency of the manufacturing process and the quantities of latex in different condoms may vary [4,13].

Like rubber gloves, most condoms contain both natural latex and chemical allergens. Contact dermatitis

attributable to processing chemicals probably the most common problem, but anaphylactic reactions to condoms have also occurred [4]. In men, the symptoms include itching and edema of the distal shaft of the penis. Eczematous can occur and spread to the scrotum, inguinal area, and inner thighs as well as to more distal locations including the face and neck. Women may experience symptoms that range from mild vulval pruiritus or vaginal burning to redness and edema of the vulva and a diffuse eczema or dermatitis of the adjacent skin [4].

Although extensive testing is done to evaluate the physical quality of condoms [13], no information is available regarding the biological safety of condoms. This study was undertaken to determine the effects of short-term exposure to physiological levels of condom surface material on cell viability and cell growth using cell culture experiments.

2. Materials and methods

2.1. Direct cell culture testing method

A modification of the direct cell culture testing method that is specified by the American Test Method, F813-83 of 1998 was followed [1]. This method of cell culture testing is useful for assessing cytotoxic potential as part of quality control for established medical materials and devices. It assumes that assessment of cytotoxicity potential provides a method for predicting the potential for cytotoxic or necrotic reactions to medical materials and devices during clinical applications to humans [1].

2.2. Cell cultures

Fibroblast cells (ATCC, CCL1 NCTC clone 929 strain designated L-929) were grown in minimum essential medium (MEM) with L-glutamine, 5% fetal bovine serum, 5% L-glutamine and 5% penicillin and streptomycin. Cells, medium and additives were from Highveld Biological Company, Johannesburg, South Africa. Cultures were maintained at 37°C and 5% CO_2 and passaged by trypsinisation using 0.25% trypsin in Hanks buffered saline solution. Cells were plated at a cell concentration of 2×10^4 cells per ml in 24 well plates and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

2.3. Medium containing condom washings

Three different types of latex condoms were tested, namely condoms without spermicides, with spermicides and flavored condoms with spermicides.

The different mediums were prepared as follows: 1 g of each type of condoms was cut into thin strips and placed into 20 ml medium. Following incubation times of 0, 4 and 8 minutes at 37°C, the medium was removed. Cells were exposed to different dilutions of (0–66%) this medium. Following 20 or 48-hours exposures, cell viability and cell numbers (referred to in this paper as cell survival) were determined by the MTT [3] and Crystal Violet assay [8], respectively.

2.4. MTT assay

A 50 μl volume of 0.1 mg/ml MTT stock solution was added to each well. The cultures were maintained for a further 20 hours at 37°C and 5% CO_2. The medium was removed. 200 μl of isopropanol : HCl solution (24 : 1 (1 M HCl)) was added to each cell well and the plates were shaken for 20 minutes. The solution was transferred into a 96-welled plate and absorbency at 545 nm was measured using a spectrophotometer (EL900) plate reader. All assays were done in triplicate.

2.5. Crystal Violet assay

A 100 μl volume of 11% glutaraldehyde was added to the medium, the plates were shaken for 30 minutes and then washed with water and dried over night at room temperature. A 300 μl volume of 0.1% methylene blue dye solution prepared in 200 mM of formic acid pH 3.5. The plates were shaken well, washed and dried. The bound dye was dissolved with shaking for 10 minutes in 300 μl of 10% acetic acid. Absorbency at 595 nm was determined as described for the MTT assay. All assays were done in triplicate.

2.6. Statistical analysis

The Crystal Violet assay and MTT Assay were analysed separately with an appropriate ANOVA for the split–split plot study design that was used to conduct the experiments. Testing was performed at the 0.05 level of significance and pairwise comparisons were done using Fisher's LSD test.

3. Results

The effect of different exposure times on cell survival was determined using the Crystal Violet assay (Fig. 1(a)–(c)). Figure 1(a)–(c) displays the average percentage cell survival plotted against percentage condom medium of the control (exposure time T_0). A decrease in cell survival was observed for all condom types. For non-lubricated condoms (Fig. 1(a)) a 10–20% decrease in cell survival was observed for exposure times T_4 and T_8 where 8.3% to 50% of the medium was replaced with condom washings. A dramatic decrease in cell survival was seen at T_8 for non-lubricated condoms where 66% of the medium was replaced with condom washings.

Lubricated condoms (Fig. 1(b)) at exposure time T_8 where 66% of the medium was replaced with condom washings had a survival rate of only 22%. For flavored condoms a 10% decrease in mean cell survival was observed where 0–33% of medium was replaced with condom washings and a further decrease of 28% was observed at higher volumes of condom washings for both exposure times (Fig. 1(c)). At exposure time T_4 and T_8 and condom washing replacements of 66%, only 25% and 28% of the cells survived.

Cell viability of the cells using MTT assay was determined following 20 hours exposure to medium containing different volumes of condom washings. The decrease in cell survival is expressed as the percentage of the control (exposure time T_0). Results of different exposure times T_0, T_4 and T_8 are shown in Fig. 2(a)–(c). These figures represent the average percentage cell viability plotted against percentage condom washings for each condom type. Different volumes of condom washings appear to have only slight effect on cell viability for non-lubricated (Fig. 2(a)) and lubricated condoms (Fig. 2(b)). Where medium was replaced with condom washing volumes above 40%, a rapid decrease of cell survival to 63% at T_4 and 28% at T_8 were observed (Fig. 2(c)).

Condom types are significantly different ($p = 0.0102$) and in particular non-lubricated condoms have significantly higher survival than lubricated and flavored condoms. However, there is also a significant interaction between type of condom and exposure time which is due to a much higher survival than expected for non-lubricated condoms at T_2 (Fig. 3). The significant differences between condom types are more likely as a result of this interaction. Furthermore, times are also significantly different ($p < 0.001$) and in partic-

(a)

Fig. 1. Cell survival determined from Crystal Violet assay following exposure of cells to washings derived from: (a) non-lubricated condoms, (b) lubricated condoms, and (c) flavored condoms.

(b)

(c)

Fig. 1. (Continued).

(a)

(b)
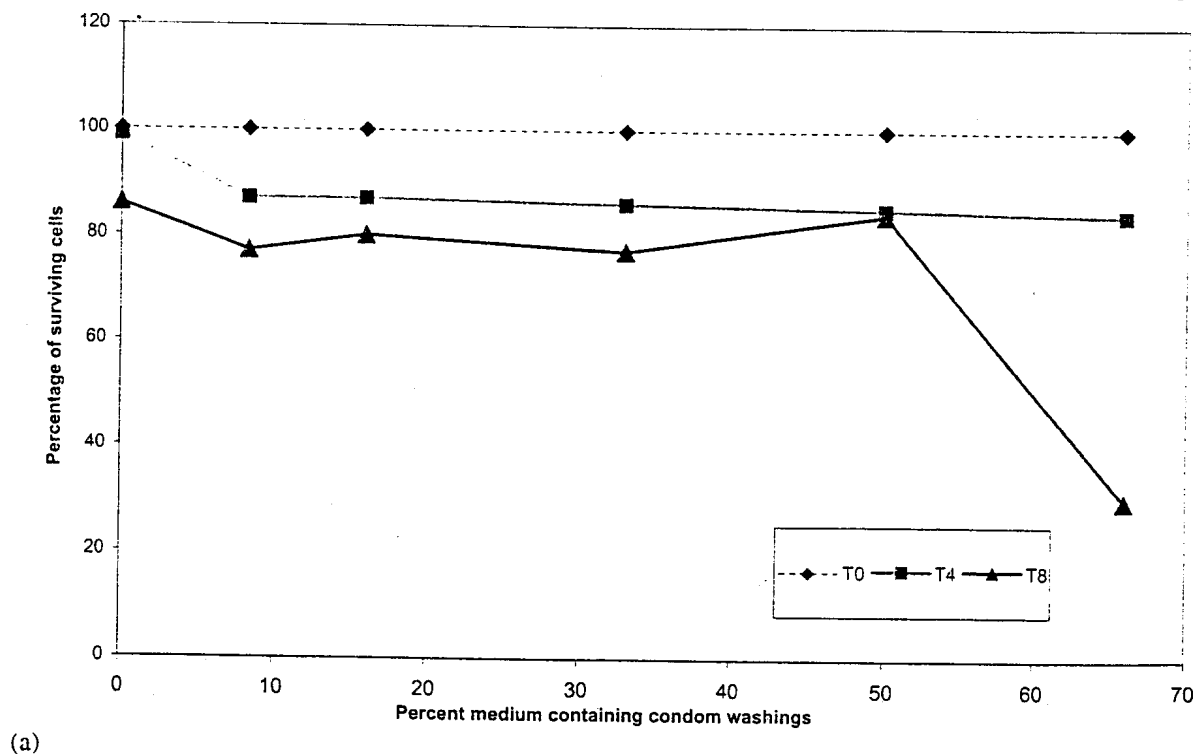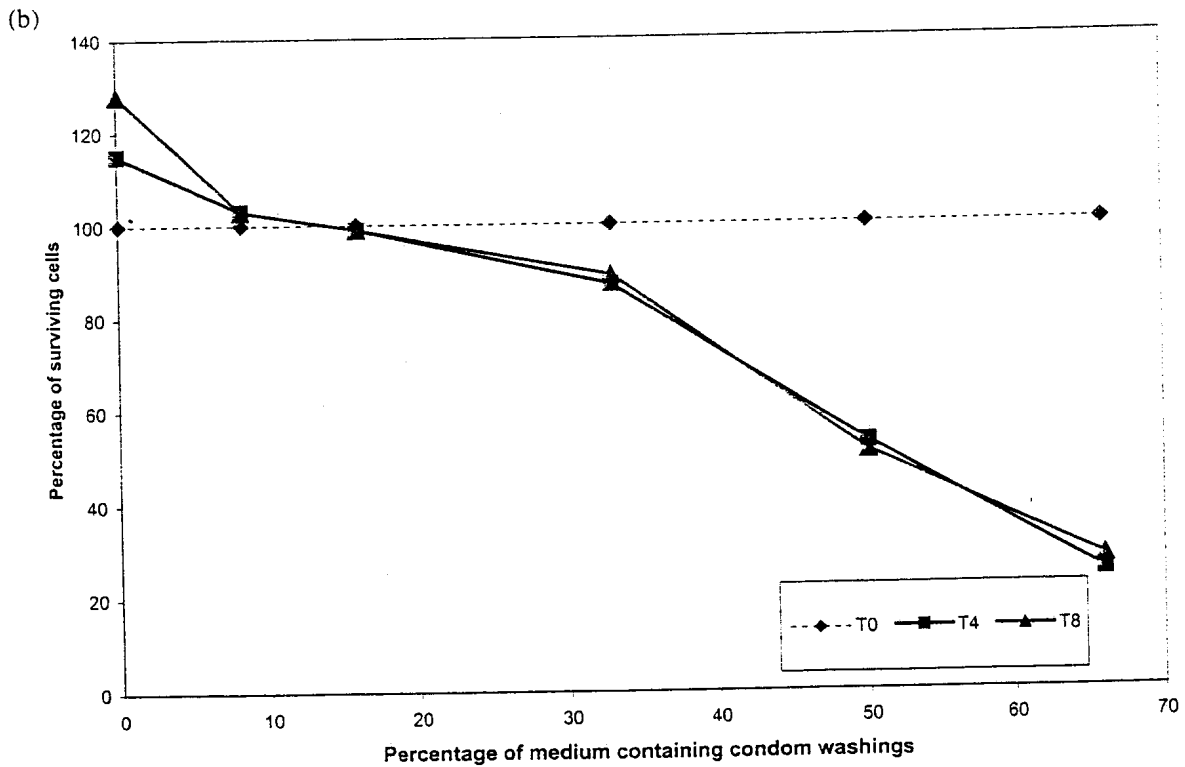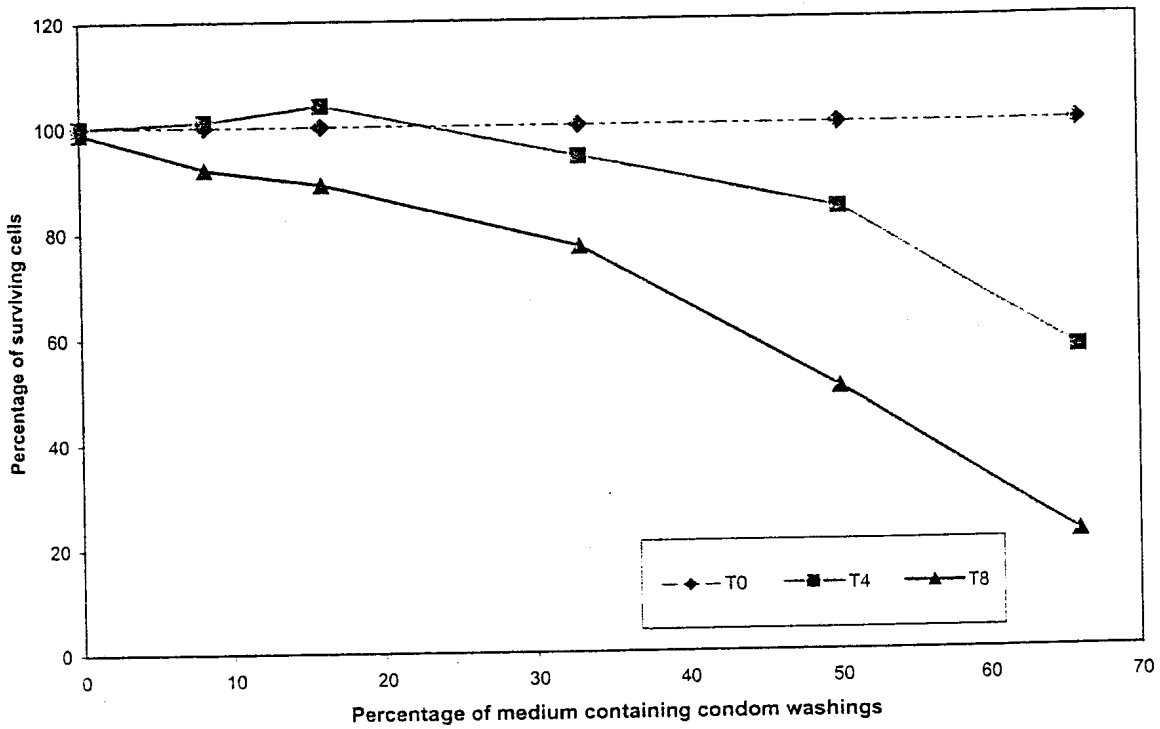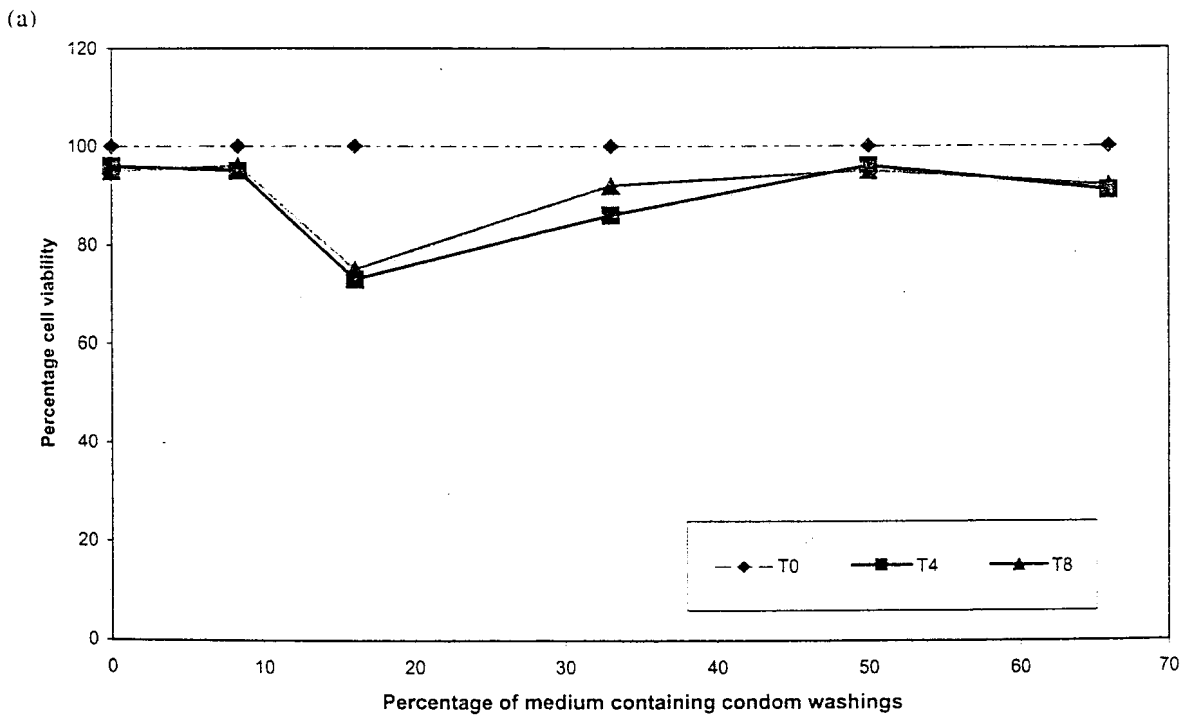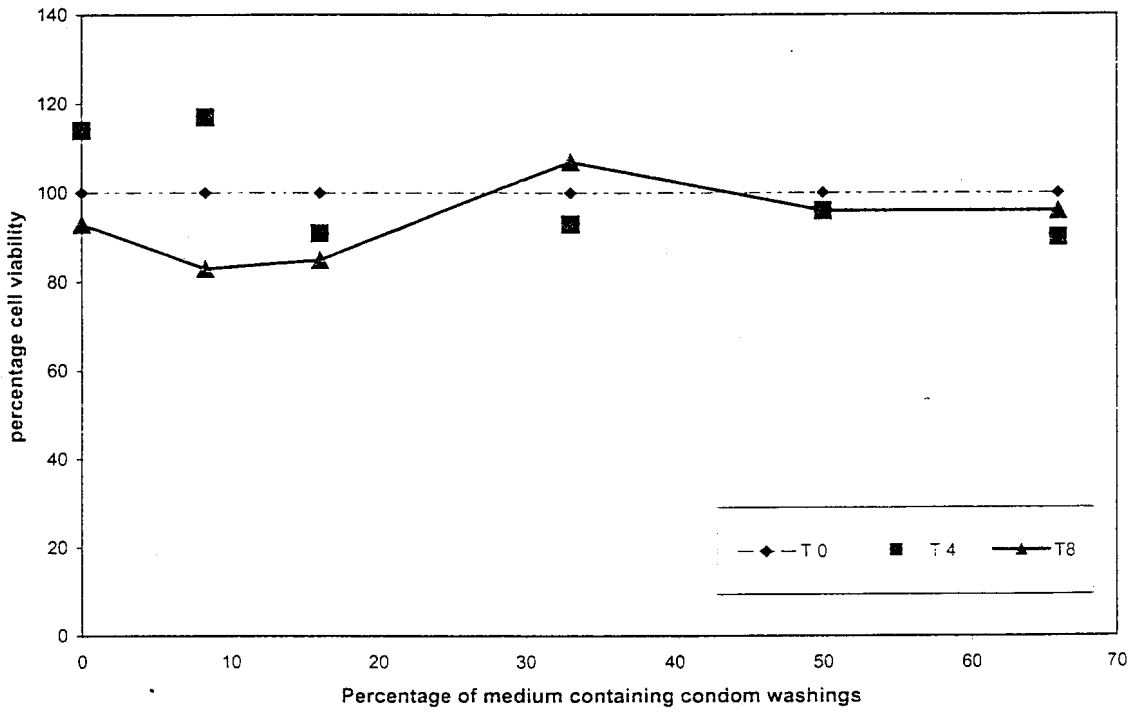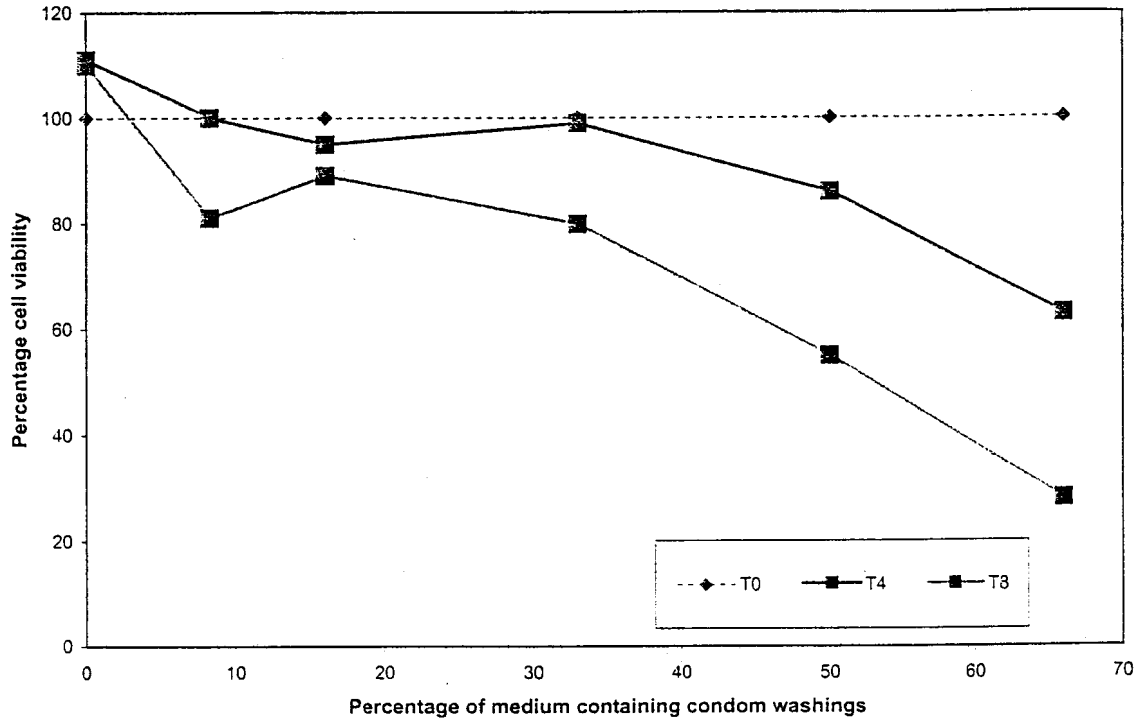
Fig. 2. Cell viability (MTT assay) following exposure of cells to washings derived from: (a) non-lubricated condoms. (b) lubricated condoms. and (c) flavored condoms.
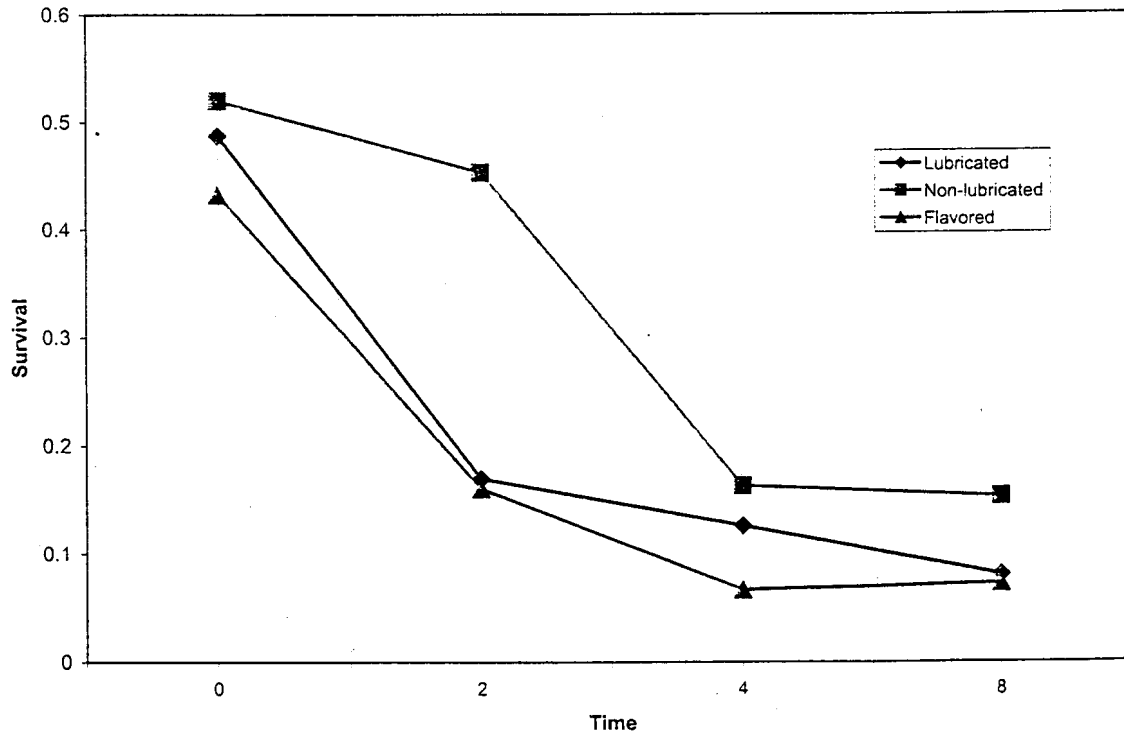
(c)

Fig. 2. (Continued).

Fig. 3. LSH (T) means of survival by type versus time (Crystal Violet assay).

Table 1

Analysis of variance table for cell survival (Crystal Violet assay)

Source	Degrees of freedom	p-value
Condom replicate	2	0.2561
Condom type	2	0.0102
Condom replicate v. condom type	4	
Condom washing volume added to media	5	0.0735
Condom type v. condom washing volume added to media	10	0.9249
Condom replicate v. condom type v. condom washing volume added to media	30	
Time	3	0.0001
Condom type v. time	6	0.0001
Condom washing volume added to media v. time	15	0.4468
Condom type v. condom washing volume added to media v. time	30	0.9885
Condom replicate v. condom type v. condom washing volume added to media v. time	108	

Table 2

Analysis of variance table for cell viability (MTT assay)

Source	Degrees of freedom	p-value
Condom replicate	2	0.9858
Condom type	2	0.1673
Condom replicate v. condom type	4	
Condom washing volume added to media	5	0.0001
Condom type v. condom washing volume added to media	10	0.2640
Condom replicate v. condom type v. condom washing volume added to media	30	
Time	3	0.0001
Condom type v. time	6	0.0001
Condom washing volume added to media v. time	15	0.0001
Condom type v. condom washing volume added to media v. time	30	0.0015
Condom replicate v. condom type v. condom washing volume added to media v. time	108	

ular T_0 higher survival than T_2 which in turn is higher than T_4 and T_8 which are not different from each other. The increase of survival at T_2 relative to T_4 and T_8 can also be attributed to the interaction between time and condom type resulting from the higher than expected survival for non-lubricated condoms at T_2.

Exposure times are significantly different ($p <$ 0.0001) and in particular T_0 has significantly higher survival than T_2 and T_4 which in turn have higher survival than T_3. Had there not been a significant interaction between time and percentage of condom washings added to medium. the better survival will have been even more marked (Fig. 4). The influence of the interaction between type and time is such that without interaction T_8 will have had even lower survival (Fig. 5).

The interactions between condom washing volumes and time and between condom type and time had a conservative influence on the main effect time. In the absence of these interactions the above differences will have been even more marked.

4. Discussion

The original FDA direct cell culture testing method is used to assess the cytotoxic potential for established medical materials and devices [1]. Since there is no specific documented cell culture testing method available for testing latex condoms. it was hypothesized that this test method may be suitable for use. Our re-

sults indicate that the modified direct cell culture testing method is an effective, simple and reproducible method to determine the biological safety of condoms.

The cytotoxic effects of condom washings can be measured either by using cell survival (using the Crystal Violet assay) [8] or cell viability tests (using the MTT assay) [3]. However, we believe that measurement of cell viability may be the more sensitive method as it measures succinate dehydrogenase activity, an enzyme which is a component of the mitochondria complex II electron transport chain and is highly sensitive to changes in cell homeostasis [3].

For medium derived from *non-lubricated condoms* cell survival did not significantly decrease at exposure times of T_4. However, at T_8, where 66% of the medium was replaced with condom washings of media a decrease in cell survival was observed suggesting that exposure times may have an important role in cytotoxic effects. Cell viability was not significantly affected for all time intervals and percentage condom washings present in the media.

Cell survival for both time intervals, T_4 and T_8 decreased to 60% and 20% for *lubricated condoms* respectively, however, cell viability was not significantly affected for all time intervals and percentage condom washings added to media. These results indicate that condoms without spermicides (non-lubricated) do not decrease cell survival or cell viability, but condoms with spermicides (lubricated) decrease cell survival but do not affect cell viability of the remaining cells.

Flavoured condoms coated with spermicides on the other hand show a significant decrease in cell survival as well as cell viability. This is probably not necessarily due to spermicides, but due to the added chemicals used for flavouring.

Even though these tests were performed using the mouse fibroblast cell culture, the method is used by the FDA as a direct cell culture method. We therefore believe that our results reflect the cytotoxic effects of the added chemicals and latex proteins. Further research endeavors will focus on using human epithelial cells to confirm these results.

Promotion of condom use for contraception and the spread of HIV and STD's is essential, however, we believe that the type of condom used is also important as little is known of the toxic and even carcinogenic effects of certain types of condoms. Furthermore, these results demonstrate that the biological safety of all commercially available condoms should be assessed to limit a possible health risk associated with prolonged use of certain types of condoms.

5. Conclusion

The modified, FDA test method F813-83 was found to be a sensitive test system for the evaluation of the biological safety of condoms. This study reveals the importance of evaluating the biological safety of all condoms that are commercially available, because of the potential health risk that may be associated with prolonged use of certain types of condoms.

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An *in vitro* study of biological safety of condoms and their additives

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The use of condoms to prevent sexually transmitted diseases, especially HIV, is widely encouraged. Condoms contain latex, nonspermicidal lubricants (such as dimethylsiliconium) and other nonspecified compounds, such as colorants and flavorings. Latex causes allergy reaction in susceptible individuals but little is known regarding the cytotoxic effects of other additives. The objective of this study was to develop a sensitive *in vitro* system to determine the toxic effects of condom material. The modified L929 FDA method and a more specific cell type, such as the cervical epithelial tumor cell line HeLa, was used. Lubricated (LC), lubricated and flavored (LFC), and lubricated, flavored and colored condoms (LFCC) were evaluated. Washings containing condom surface material were prepared by washing condom fragments in medium for different time intervals. Changes in cell number, viability and lysosome integrity in the L929 and HeLa cell lines was determined using the Crystal Violet, MTT and Neutral Red assays, respectively. The condom type affected cell viability and lysosome integrity, with LC inducing an increase in cell viability and LFC a decrease in lysosome integrity. The HeLa cell line in combination with the MTT and NR assay was the most sensitive *in vitro* system to determine the toxic effects of condom material. *Human & Experimental Toxicology* (2003) 22, 659–664

Key words: cell survival; cell viability; cytotoxicity; latex condoms; lysosome membrane integrity

Introduction

During the past 15 years there has been a worldwide increase in the use of latex products due to increased awareness of human immunodeficiency virus (HIV) and hepatitis B viruses,[1–3] and as a universal precaution for protection from potentially infectious body fluids. Since then, there has been an increase in the number of reported cases among health care workers,[4] latex industry workers and among subjects with positive risk factors, such as children with spina bifida, those undergoing multiple surgeries and atopic subjects.[1,5–8]

Latex proteins cause allergies ranging from contact dermatitis to more severe reactions and even anaphylaxis has been mentioned in some publications.[4–12] In 1995, the Center for Disease Control of America reported 600 cases of type I latex sensitivity and at least 16 of identified patients died as a result of sensitization.[9] Between 1988 and 1992 the Federal Drug Association (FDA) received more than 1000 reports of adverse health effects from exposure to latex, including eight deaths.[1,4–12]

With the increased awareness of HIV and other sexually transmitted diseases, there has been an increase in the promotion of use of latex condoms. Not only do these products contain latex,[13,14] but often other compounds such as lubricants, colorants and flavorings. Little is known regarding the cytotoxic effects of condoms on the cells lining the vagina and cervix. Pretorius and Bester[15] and Motsoane *et al.*[16] developed a method to evaluate the cytotoxicity of latex to cells in culture, by modifying the FDA F813-83 test method.[17] The original FDA test method was used to determine the cytotoxic effect of medical materials and devices as part of quality control.[17] The modified direct cell culture testing method, which makes use of the mouse L-929 fibroblast cell line, has shown to be effective in determining the effects of short-term exposure to physiological concentrations of latex condom and glove surface materials.[15,16] Furthermore, the method proved to be very sensitive and reproducible for the evaluation of the biological safety of latex products. In the study of Motsoane *et al.*,[16] the cytotoxicity of three different types of

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latex condoms was assessed. Latex condoms with spermicides and flavorings were found to have the most negative effect on cell survival and viability while latex condoms with spermicides only and those with no spermicides and no flavorings had the least effect on cells.[16] This study revealed the need for further cytotoxicity testing to include more types of condom and testing methods to determine the type of interaction that occurs between the latex proteins/additives and cellular structures.

In this study, the cytotoxicity of lubricated (LC), lubricated and flavored (LFC), and lubricated, flavored and colored (LFCC) latex condoms was assessed on mouse fibroblast cells (L929) that was used previously[15,16] as well as on HeLa cells a permanent, human cervical carcinoma cell line. The effects on cell viability, lysosome membrane integrity and cell number were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),[18-20] Neutral Red (NR),[18,21,22] and Crystal Violet (CV) assays,[18,23,24] respectively.

Materials and methods

Cell cultures

Two permanent cell lines, namely mouse fibroblasts (ATTC, CCL1 NCTC clone 929 strain designated L929) and HeLa cells were obtained from Highveld Biological Co., Johannesburg, South Africa. Both L929 cells and HeLa cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 5% fetal calf serum (FCS), and 1% antibiotic solution (10 000 U/mL penicillin, 10 000 μg/mL streptomycin and 25 μg/mL amphotericin in 0.85% saline). Cultures were maintained at 37°C and 5% CO_2 and passaged by using trypsin/EDTA solution (0.5 g/L trypsin, 0.2 g/L EDTA and 8.8 g/L NaCl in Hanks buffer). Cells were plated at a concentration of 3×10^4 cells/mL in 2 cm^2 wells and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

Medium containing condom washings

The following three different types of latex condoms were tested: LC, LFC, and LFCC. LC were obtained from the South African Health Department. LFC and LFCC were obtained over the counter from a South African adult shop. Condom washings were prepared as follows: 1 g of each type of condom was cut into thin strips, suspended and incubated in 20 mL EMEM for 0, 2, 4, and 8 min at room temperature. The same packet of condoms from the same manufacturer was used and for each experiment one condom was used. The medium containing the condom washings, W_2, W_4, W_8, was removed.

Each cell line was exposed to a 1:5 dilution of condom washings with medium for 3 hours at 37°C and 5% CO_2. Each assay was in quadruple and each data point is an average of six experiments.

MTT assay

Fifty microliters of 0.1 mg/mL MTT (Sigma Chemical, Johannesburg, South Africa) stock solution in DPBS (Dulbecco's phosphate buffered saline) was added to the medium in each well. After incubation for a further 90 min at 37°C and 5% CO_2, the medium was removed and 0.2 mL of isopropanol:1 M HCl solution (24:1) was added to each well[20] to solubilize the formazan product. The plates were shaken for 10 min and absorbancy was measured at 545 nm using a spectrophotometer (EL900) plate reader.

Combined Neutral Red and Crystal Violet assay

This assay is a combined method to determine lysosome membrane integrity[18] and the number of cells in culture. 0.1 mL of a 0.15% NR (Sigma Chemical) solution in DPBS was added to each well. Plates were maintained at 37°C and 5% CO_2 for a further 90 and 120 min for the HeLa and L929 cells, respectively. The medium was discarded and the cells were fixed for 10 min with 0.2 mL of a 1% acetic acid and 1% formaldehyde solution. This solution was discarded and the dye was solubilized with a 1% acetic acid and 50% ethanol solution. The absorbancy of the solubilized dye was measured at 570 nm. The plates were then rinsed with DPBS and dried overnight at room temperature.

0.3 mL of a 0.1% CV (Sigma Chemical) solution in 200 mM formic acid at pH 3.5 was added to the dried wells. The plates were incubated at room temperature for 30 min rinsed with DPBS to remove excess dye and then dried overnight at room temperature. The bound dye was dissolved by shaking for 10 min in 0.3 mL of 10% acetic acid solution. The absorbancy of the solubilized dye was measured at 570 nm.

Statistical analysis

MTT, NR and CV were conducted separately and analyzed with an ANOVA for the split-split plot study design.[25] Exposure time zero was omitted as it had no effect on any of the three outcomes viability, lysosome membrane integrity and cell number. Condom washings, W_2, W_4, and W_8 observations were expressed in terms of washings W_0. The testing was performed at the 0.05 level of significance and pair wise comparisons were done using Fisher's test. The ANOVA tables for viability, lysosomal integrity and survival (Table 1) show the degrees of freedom

Table 1 Summarized ANOVA table of P values for cell viability, number and lysosomal membrane integrity

Source of variation	df	Viability	Cell number	Integrity
		MTT assay	CV assay	NR assay
Condom type[a]	2	0.0015	0.2483	0.0003
Cell type[b]	1	0.0034	0.5869	0.0252
Condom type × cell type[b]	2	0.0006	0.0116	0.003
Condom washings ($W_{2\text{-}8}$)[c]	2	0.0916	0.0383	0.0653
Condom type × condom washings ($W_{2\text{-}8}$)	4	0.1519	0.0805	0.0116
Cell type × condom washings ($W_{2\text{-}8}$)[a]	2	0.9461	0.0521	0.410

[a,b,c] Calculated using the appropriate residual terms.

and P value where W_0 was omitted from the analyses.

Results

The HeLa and the L929 cell lines were exposed for 3 hours to condom washings prepared by washing each of LC, LFC and LFCC material for 2, 4, and 8 min in cell culture medium. Cell viability, number and lysosomal membrane integrity were measured using the MTT, CV and NR assays respectively. For both L929 and HeLa cells no decrease in cell number was observed using the CV assay after 3 hours exposure to condom washings, $W_{2\text{-}8}$ (Figure 1). Only for HeLa exposed to W_2, W_4, and W_8 was an increase in cell viability using the MTT assay, was observed from 100% to 130% from $W_{2\text{-}8}$ (Figure 2). No changes in cell viability were observed in L929 exposed to all condom washings and HeLa exposed to LFC and LFCC. For HeLa cells exposed to condom washings $W_{2\text{-}8}$ a decrease in lysosomal membrane integrity using the NR assay, from 100% to 20% was observed with LFC (Figure 3) from W_0 to W_8. No changes in lysosomal membrane integrity were
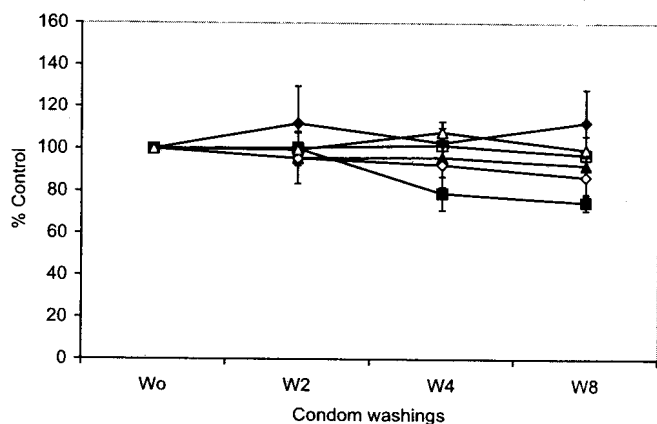

Figure 1 Comparative cytotoxicity of LC (◆), LFC (■) and LFCC (▲) condom washings on L929 (open symbols) and HeLa (closed symbols) cells measured with the CV assay. Mean $(n = 4) \pm$ standard deviation (SD).

observed in L929 exposed to any condom washings and HeLa exposed to LC and LFCC.

Because several parameters, such as the type of condom, cell type and condom washings $W_{2\text{-}8}$ were evaluated, a split-split plot study design was used to evaluate the interaction between condom type × cell type, condom type × condom washings ($W_{2\text{-}8}$) and cell type × condom washings ($W_{2\text{-}8}$).[25] The ANOVA tables for viability, cell number and survival are summarized in Table 1 giving the degrees of freedom (df), the P values where W_0 was omitted from the analysis.

In vitro, condom types had a significant effect on cell viability ($P = 0.0015$) and lysosomal membrane integrity ($P = 0.0003$). Differences between cell lines HeLa and L929 were significant when cell viability ($P = 0.0034$) and lysosomal membrane integrity ($P = 0.0252$) was used to determine the cytotoxic effects of condom washings. For the interaction between each condom type and cell type significant differences were observed in cell viability ($P = 0.0006$) and lysosomal membrane integrity ($P = 0.003$). Although there was no significant differences in cell number when only condom type and cell type are evaluated, the interaction between the two groups is significant with a $P = 0.0116$. These differences were due to the increased cell viability and number observed for LC and the decrease in lysosomal membrane integrity for LFC on HeLa cells when compared to the L929 cells (Figure 4).

The statistical analysis on interactions with washings ($W_{2\text{-}8}$) indicated that, in general, the responses of different length condom washings differed statistically only in the analysis of cell number ($P = 0.0383$) though nearly statistically significant for cell viability and integrity (Table 1). The interaction of different condom types with washings were stronger for cell integrity, $P = 0.0116$ than cell number, $P = 0.0521$. The main determinant was likely the dose-dependent decrease in lysosomal membrane integrity by LFC determined by the NR assay in HeLa cells (Figure 5).
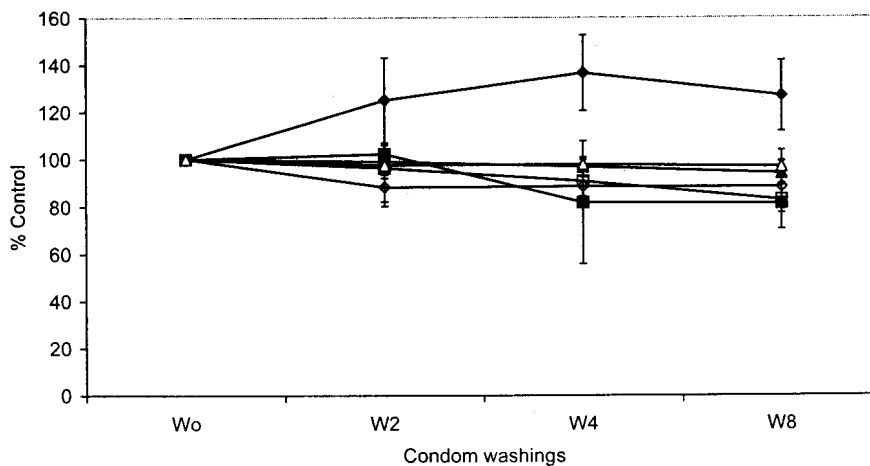
Figure 2 Comparative cytotoxicity of LC (♦), LFC (■) and LFCC (▲) condom washings on L929 (open symbols) and HeLa (closed symbols) cells measured with the MTT assay. Mean $(n = 4) \pm$ standard deviation (SD).
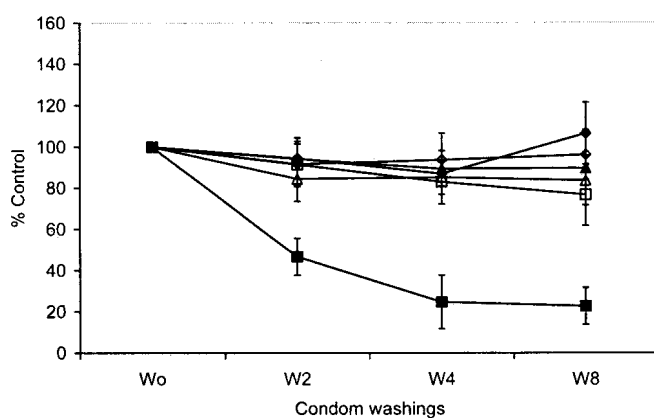

Figure 3 Comparative cytotoxicity of LC (♦), LFC (■) and LFCC (▲) condom washings on L929 (open symbols) and HeLa (closed symbols) cells measured with the NR assay. Mean $(n = 4) \pm$ standard deviation (SD).
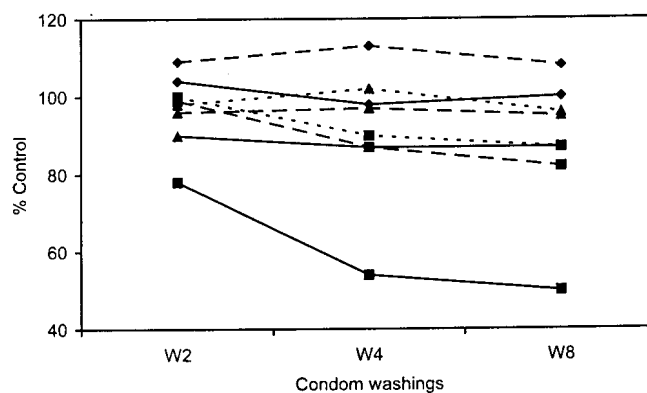

Figure 5 Comparison of means for cell viability (– – –), number (---) and lysosomal integrity (—) × condom washings (W_{2-8}) of LC (♦), LFC (■) and LFCC (▲).
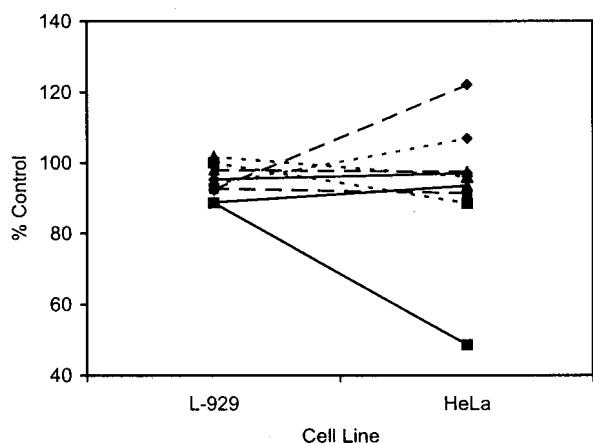

Figure 4 Comparison of means for cell viability (– – –), number (---) and lysosomal integrity (—) × cell types L929 and HeLa exposed to condom washings LC (♦), LFC (■) and LFCC (▲).

Discussion

Condoms contain latex, nonspermicidal lubricants (such as dimethylsiliconium) and other nonspecified compounds such as colorants and flavorings. The manufacturers provide very little information regarding the type and amount of each compound added. The FDA method traditionally used to determine the safety of medical devices,[17] was modified by Pretorius and Bester[15] and Motsoane *et al.*[16] and was used to determine the biological safety of condoms. These authors exposed L929 cells to medium containing 0–66% condom washings from nonlubricated, lubricated (same as LC) and lubricated and flavoured (same as LFC) condoms prepared using the same method as described in this study. In this previous study at W_4 and W_8 both LC and LFC showed a decrease in cell number and viability.

The objective of this study was to determine the cytotoxic effects of condoms in a cell line that was in origin and type closer to that of the cervical lining. Assays that involve the uptake and/or the metabolism of a dye are more sensitive and effective in measuring early changes in cell metabolism and function. The MTT assay measures the ability of mitochondrial succinate dehydrogenase to metabolize MTT to a formazan product. NR is a weak cationic dye that readily moves across cell membranes by nonionic diffusion accumulating intracellularly in the lysosome where it binds with anionic sites in the lysosomal matrix. Change in the lysosomal matrix result in reduced NR uptake. Cytotoxicity is a complex process where its effect may occur at different sites within the cell and therefore both the MTT and NR assay has been included to evaluate the cytotoxity of condom washings.

Condom type had a significant effect on cell viability and lysosomal membrane integrity. Differences observed between the L929 and HeLa cells were due to the increased viability observed for LC and the decrease in membrane integrity for LFC on HeLa cells. With LC and LFC no decrease in cell number and viability was observed as previously reported for the L929 cell line.[16] This is due to different dilutions of the condom washings used. In this study the cells were exposed to medium containing 20% condom washings compared to 66% used previously.[16] Although no decreased in cell viability is observed for LFC a decrease of 75% in lysosomal membrane integrity is observed. The increase in cell viability found for HeLa exposed to LC (although statistically not significant) cannot be explained at present. Changes in cell viability and membrane integrity were only observed for HeLa cells, indicating that the HeLa cell line is more sensitive for the toxic effects of condom washings.

Bogdanffy and Valatine[26] have reported that exposure of rodent upper gastrointestinal tract to vinyl acetate leads to mitogenic proliferation while exposure of nasal tissue leads to mitogenic proliferation and cytotoxicity. Both pathways may lead to a greater risk for DNA mutations due to an overextend DNA replication/repair system during reparative proliferation associated with cytotoxicity and rapid mitogenic cellular proliferation. Similarly condom washings have a mitogenic and cytotoxic effect on HeLa as observed for LC and LFC condom washings respectively. Not only do various cell types respond differently to the same toxic insult, but also different chemicals may induce different pathways that may lead to cellular transformation.

Testing material with unspecified composition is difficult and care must be taken not to use a single cell line or assay, as it may not detect other cytotoxic effects. Therefore testing should include a range of tests that can measure different aspects of cytotoxicy. Besides the MTT, CV, and NR assay other tests such as the propidium iodide assay for cell membrane integrity[27] and microscopic evaluation of nuclear apoptosis with a fluorescent dye such as Sytox-green[28] could also be included. These assays address only the effects of short-term exposure and not possible genotoxic effects that may occur following repeated and long-term exposure.

The different types of condoms differed in their ability to induced cytotoxic effects on L929 and HeLa cells *in vitro*. LFC condoms that contained flavorings were more toxic than LFCC that contained both flavorings and colorings. These types of additives are not the only factors that may contribute to cytotoxity. The Medical Devices Agency (MBA)[29] has determined the presence of dithiocarbamate vulcanization accelerators in latex gloves. MBA reported that the genotoxicity of these agents was due to the type used as well as the residue concentration. As condoms used in this study were from different manufacturers little information is provided regarding dithiocarbamate usage and amount of residue present in each product. Therefore, genotoxic testing might also be justified in the evaluation of condom material. Besides a single chemical (e.g., dithiocarbamate) that may contribute to genotoxicity synergistic effects should also be considered.

Though extensive testing is done to evaluate the physical quality of condoms, little information is available regarding the biological safety of condoms. The reproductive organs of both males and females and in particular the cervical and vaginal cells of the female, are exposed to the latex proteins and additives. Our results indicate that the MTT and NR assay in HeLa cell line is a more sensitive system than the previously used L929 cell line with the MTT assay to test the cytotoxicity of condom material. Certain constituents of condom material are toxic to cells *in vitro* and therefore a potential health risk may exist. Therefore, condoms should be subjected to the same constringency of testing as for latex gloves. Testing should include assessment of genotoxicity and determination of the type and amount of additives and vulcanization accelerator residue present. The use of condoms is still strongly advocated in individuals that are not allergic to latex. However, other compounds added must be better specified, tested and possible additive or synergistic effects should be investigated as these may affect the health of the user.

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