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In Vitro Cytotoxicity Evaluation of Highly Absorbent Foam Dressings Based on Silver Zirconium Phosphate via IC_{50} Value

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Abstract

In this study, we, for the first time, tried to apply IC_{50} values (inhibitory concentration estimated to affect the endpoint in question by 50%) in the MTT colorimetric assay to investigate the cytotoxic effects of highly absorbent foam dressings based on silver zirconium phosphate, a newly nano-based matrix. Our results showed that silver released from dressings based on silver zirconium phosphate attributed mainly to highly cytotoxic to L929 cells cultured with MEM containing 10% fetal bovine serum. In addition, we have also compared the IC_{50} values among different dilutions of $AgNO_3$ solution, silver based dressing extracts and material reference control (ZDEC) extracts using the optimized MTT assay, along with characterizing the silver content in the dressing extracts using atomic absorption spectroscopy. Results have shown that the IC_{50} values of $AgNO_3$ solution, silver based dressing extracts and ZDEC extracts are 3.5 μg/mL, 3.8 μg/mL and 8.4%, respectively. And there exist some good agreements between qualitative and quantitative evaluation method as well. In conclusion, our study has led to the view that the IC_{50} value is a promising quantitative index for screening cytotoxicity with regard to silver based dressings.

Keywords

Cytotoxicity, Silver Zirconium Phosphate, IC_{50}

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1. Introduction

In recent decades, the silver ion is commonly used in functional dressings as topical antimicrobial agents [1]-[3]. Though historical use of silver can be looked back to hundred years ago [4], as heavy metal ions, it has been observed that silver released from dressings may possess cytotoxic effects. Such severe side effects, no doubt, have extremely restricted its clinical applications.

To date, the matrix used for silver-bearing, which is of importance to silver release has been well developed, including zeolite silver, SiO_2 silver and the zirconium phosphate silver. Among these, the zirconium phosphate silver has been widely used in functional dressings for its nano-based matrix and good performance. However, cytotoxicity is still the major side effect that should be taken into account in safety control of these dressings [5]. In this regard, optimized approaches by which to determine the cytotoxicity of these silver based dressings have generated considerable interest.

In vitro cytotoxic test is an important approach to screen the potential human health hazards in biomaterials and medical devices. To illustrate, several qualitative and quantitative methods are proposed to evaluate the cytotoxic results. Among these, the MTT assay is a sensitive, quantitative, and reproducible testing method which based on the measurement of the viability of cells via metabolic activity [6] [7]. Similar to qualitative evaluation method, for materials with potential cytotoxicity, we also need a defined value to evaluate the cytotoxic effects by calculating the quantitative results we obtained. This promoted us to develop a possible value and apply it in the quantitative evaluation of the cytotoxic results. So far, accumulating evidence has shown that IC_{50} value, an inhibitory concentration, which reduces the maximum possible viability of tested cells by 50% is a promising value in the cytotoxic assay. Interestingly, IC_{50} value is a significantly toxic value which is calculated through concentration dependent inhibition curves using a known computer program [8]. In addition, some researchers have used this value to evaluate the cytotoxicity of reference biomaterials [9] [10].

Though the IC_{50} value has been well established in cytotoxic test such as V79 colony assay [11], there is little information available on its application in cytotoxicity mediated by medical device yet. In the present study, based on characterizing precisely the silver release by atomic absorption spectroscopy (AAS), we try to compare the cytotoxicity of silver-based dressing extracts, $AgNO_3$ solution and ZDEC extracts in order to illustrate the relationship between silver release and cytotoxicity of these dressings based on silver zirconium phosphate, and then to investigate the possibility of quantitatively evaluating MTT cytotoxic results with IC_{50} value.

2. Materials and Methods

2.1. Materials

Silver-based foam dressings and corresponding control dressings only without silver was obtained from commercial sources. Silver nitrate (CAS Number, 7761-88-8), MTT stock solutions and isopropanol solutions were purchased from Sigma Company. The ZDEC polyurethanes and high-density polyethylene are purchased from the Food and Drug Safety Center, Hatano Research Institute, Japan. L929 cell lines were acquired from the American Type Culture Collection. Media and fetal calf serum used in cell cultures were purchased from Hangzhou Sijiqing Company, China.

2.2. Extracts of Dressing and Control Preparation

Silver-based dressings were aseptically cut into 5 cm × 6 cm size. And then perform the absorbent test compatible with the European reference of Test methods for primary wound dressings EN 13726-1:2002 and the absorbent capacity was calculated by the following formula: absorbent capacity = $(W_b - W_a)/W_a$, where: W_a is the original weight of the dressings; W_b is the weight after fully soaked with 0.9% NaCl solution. Then the extract was conducted with MEM culture containing 10% FCS at a ratio of 3 cm^2/mL under the condition of 72 h at 37˚C with 60 rpm horizontal vibration in accordance with ISO 10993-12:2012. Subsequently, extracts were used for silver content determination and then being diluted into six concentrations for the cytotoxicity assay. Control dressing was prepared in just the same way as above. The ZDEC polyurethanes and high-density polyethylene extracts were prepared with MEM culture containing 10% FCS at a ratio of 0.1 g/mL under the condition of 24 h at 37˚C in accordance with ISO 10993-12:2012, The ZDEC polyurethanes extracts were diluted 20%, 10%, 8%, 6%, 4% and 2% to calculate IC_{50} value.

2.3. Preparation of $AgNO_3$ Solution

In brief, $AgNO_3$ powder was weighed and dissolved by demonized water to a final concentration of 1 mg/mL. Solution is sterilized by sterile filtration using syringe filters (pore size ≤ 0.22 μm). After analyzed by AAS for the spike recovery, the primary $AgNO_3$ solution was then serial diluted two-fold with MEM culture containing 10% FCS starting from 64 μg/mL to 0.5 μg/mL, totally 8 dilutions used for the cytotoxicity assay.

2.4. Silver Characterization in Dressing Extract and $AgNO_3$ Solution

Atomic absorption spectroscopy (AAS) is a spectroanalytical method for quantitative determination of silvers in silver-based dressing extracts and $AgNO_3$ solutions. Briefly, 1 mL extract of dressings were digested by 5 mL HNO_3 and 2 mL H_2O_2 and dissolve all of the silver present. Then, solutions were diluted with demonized water at 1:10 ratio and were determined by AAS (Thermo Electron Corporation, iCE 3500) under the instruction. Sensitivity of AAS is 50 ppb. The silver content of dressing extract was subsequently determined and expressed in μg/mL.

2.5. Cytotoxicity Assay

Briefly, 100 μl cell suspension at 1×10^5 cells/mL was prepared and seeded in the designated 96-well plate. After 24 h incubation to form a half-confluent monolayer, aspirate culture medium from the cells, then add 100 μl of treatment medium with the appropriate concentration, including 8 different concentrations of the test extract and $AgNO_3$ solutions, 100% the positive and negative control extract, vehicle control and blank. In another test, the ZDEC polyurethanes extracts were diluted 20%, 10%, 8%, 6%, 4% and 2% to calculate IC_{50} value. After incubating cells for 48 h, carefully observe and record changes in the morphology of the cells due to cytotoxic effects and then remove the culture medium from the plates, and add 50 μl of the MTT solution to each indicate well and the plates are further incubated for 2 h in the incubator at 37˚C. Then the MTT solution is decanted and 100 μl of isopropanol is added into each well. Vibrate the plate for 30 seconds and subsequently transfer it to a microplate reader equipped with a 570 nm filter to read the absorbance.

2.6. Statistical Analysis

The results were reported in mean ± SD. The significance of differences between different groups and controls was assessed by the Student's t-test using origin8 software. $P < 0.05$ was regarded as significant.

3. Results

3.1. Absorbent Capacity of Highly Absorbent Foam Dressings Based on Silver Zirconium Phosphate

To testify the absorbent capacity of dressings based on silver zirconium phosphate, we used the indicated method compatible with the European reference of Test methods for primary wound dressings EN 13726-1:2002 to calculate the absorptive efficiency. Results have revealed that the average absorbent capacity of dressings is 11.3 and the highest absorbency is 0.93 mL/cm^2, which demonstrated that these dressings had high absorbent performance (**Table 1**). Also, these results suggest that in the extraction process, the samples should be presoaked with 28 mL of MEM containing 10% FCS to obtain a ratio of 3 cm^2/mL in the subsequent extract procedure.

3.2. Silver Content in the Dressing Extract and $AgNO_3$ Solution

Silver contents of silver-based dressings are of importance to the following cytotoxic study. Therefore, we now used AAS to analyze the silver contents in silver-based dressing extracts and $AgNO_3$ solution, respectively. Results have revealed that the total silver content in the MEM containing 10% FCS extract was 0.625 mg and the $AgNO_3$ spike recovery was 112%, as demonstrated in **Figure 1**.

3.3. Cytotoxicity of the Dressing Extract and $AgNO_3$ Solution

The cytotoxicity of dressing extracts and $AgNO_3$ solution is determined by the MTT method and the cytotoxic

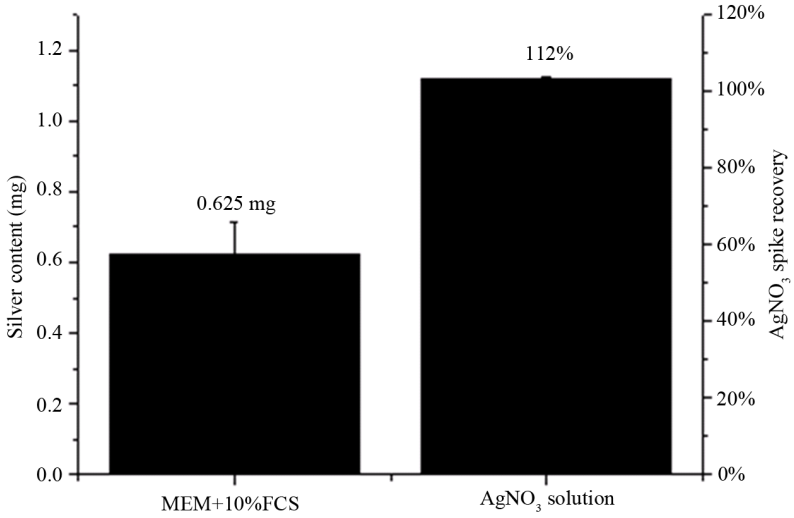

Figure 1. Silver contents were determined by AAS in MEM supplemented with 10% FCS after extracted for 72 h under the condition of 37˚C. Simultaneously, another 1.0 mg $AgNO_3$ solution was analyzed by AAS to validate the $AgNO_3$ spike recovery.

Table 1. Absorbent capacity of high absorbent foam dressings.

Sample	1	2	3	4	5	6	7	8	9	10
W_a (g)	2.450	2.451	2.45	2.401	2.452	2.451	2.453	2.453	2.451	2.454
W_b (g)	30.116	30.105	30.115	30.111	30.104	30.112	30.105	30.101	30.1	30.116
W_b-W_a	27.666	27.654	27.665	27.71	27.652	27.661	27.652	27.648	27.649	27.662
Absorbent capacity	11.292	11.282	11.291	11.541	11.277	11.285	11.272	11.271	11.280	11.272
Average								11.306		

potential was expressed as the IC_{50} value, an inhibitory concentration, which reduces the maximum possible viability of tested cells by 50%. As showed in **Figure 2**, the IC_{50} values of $AgNO_3$ solution, silver-based-dressing extracts and SPU-ZDEC extracts are 3.5 μg/mL, 3.8 μg/mL and 8.4%, respectively. These results have shown a proper cytotoxic potential of extracts of dressings based on silver zirconium phosphate. **Figure 3** has demonstrated the cell morphology of different groups under a microscope. And also in **Table 2**, qualitative results also illustrated that there were good correlations between quantitative and qualitative evaluation methods of $AgNO_3$ solution, silver-based dressing extracts and SPU-ZDEC extracts.

4. Discussion

During recent years, varieties of silver-based dressings are available on the burn care domain partly because of its broad antibacterial effect [12]. Meanwhile, accumulated evidences suggest that silver ions released from these dressings may give rise to cytotoxicity [4]. To date, silver release and its cytotoxicity for silver-based dressings contain multiple factors, involving at least silver existential state, silver contents, silver release rate and mode and it is clear that highly absorbent capacity of silver-based dressings may possess obvious silver release and perform cytotoxic effect on monolayer cells [13]. In this investigation, we have determined the absorptive capacity of silver-based dressings. As showed in **Table 1**, the dressing we evaluated has highly absorbent capacity, which has the absorbent capacity of 11.3 and the highest absorbency of the tested sample is 0.93 mL/cm^2. Surely, this provided the feasibility for the following experiments.

Recent observations indicated that the silver release from the corresponding dressings mainly depended on the test fluid we used [14]-[16] and in particular when the dressings were presoaked with fetal bovine serum, the amount of silver released into culture medium was significantly enhanced [13]. In this work, we also analyzed

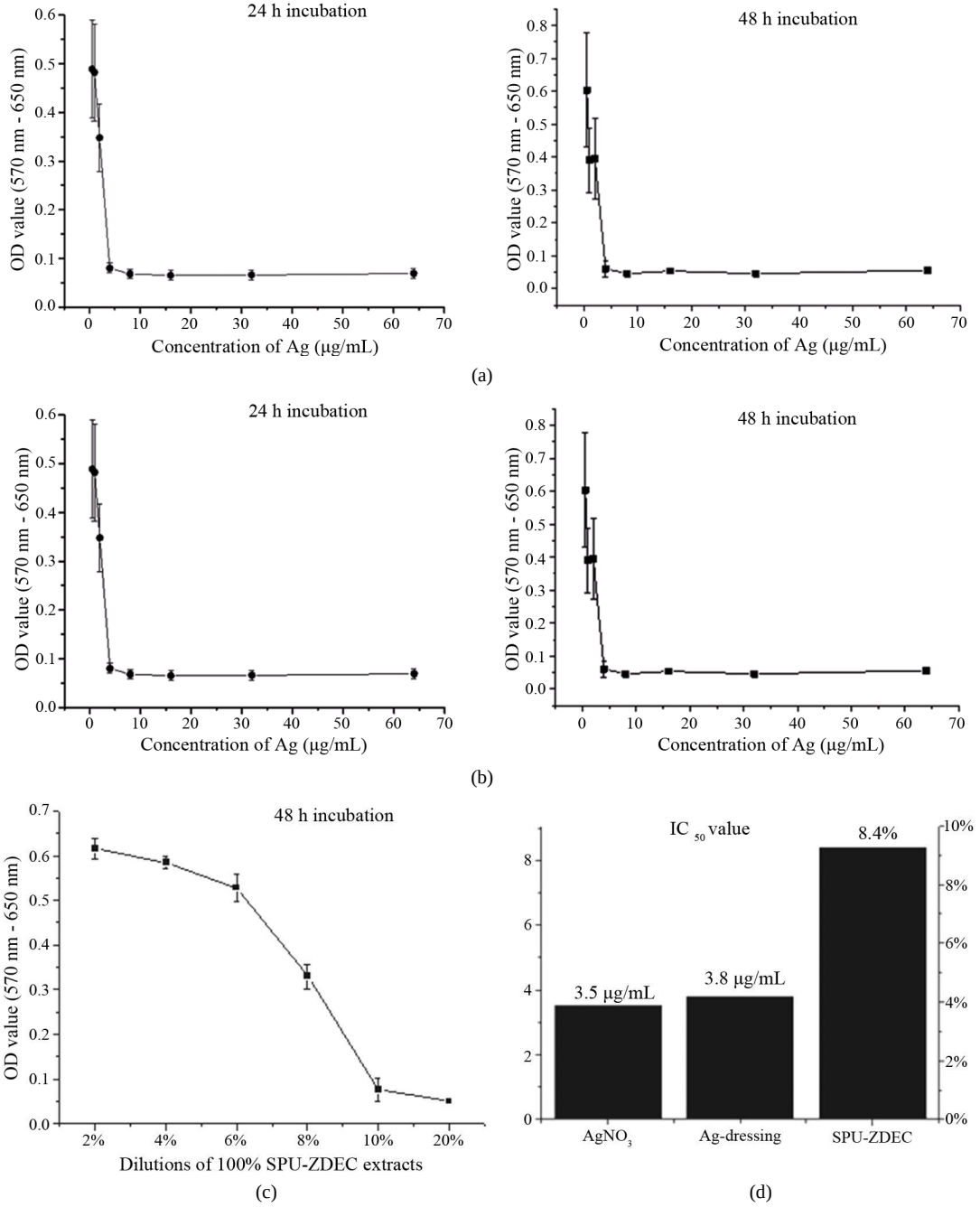

Figure 2. After silver contents were determined by AAS, toxic effects of $AgNO_3$ solution,silver based dressing extracts and SPU-ZDEC extracts on L929 cells were measured by MTT assay at indicated concentrations after 24 h and 48 h incubation. (a) The OD value variation of different doses of $AgNO_3$ solution ranging from 0.5 μg/mL to 64 μg/mL; (b) The OD value variation of different dilutions of silver from the silver based dressing extracts ranging from 0.1 μg/mL to 13 μg/mL; (c) The OD value variation of indicated dilutions of SPU-ZDEC extracts from 20% to 2% dilution of 100% extracts; (d) The IC_{50} value of $AgNO_3$ solution, silver based dressing extracts and SPU-ZDEC extracts calculated by indicatingprogram, respectively.

and compared silver release in different vehicles including MEM supplemented with 10% fetal bovine serum, human tissue simulation liquid, physiological saline and 5% glucose. Our results indicated that there are significant differences of silver release in different media, which is at least in part, in agreement with these conclusions (data not shown).

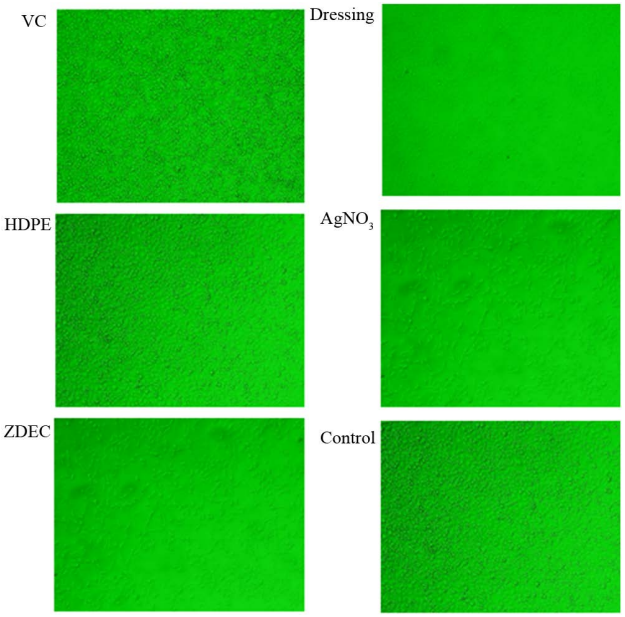

Figure 3. Comparison of cellular sensitivity among indicated concentrations of silver based dressing extracts, $AgNO_3$ solution, ZDEC extracts, HDPE extracts, dressing control extracts and vehicle controls. L929 cells were tested according to the optimized protocol of ISO 10993-5:2009 with the corresponding test materials using MEM containing 10% FCS and the cellmorphology was taken at48h after incubation (×100).

Table 2. Cytotoxicity of dressing extracts, $AgNO_3$ solution and SPU-ZDEC extracts by qualitative evaluation.

Groups	Grades	Reactivity	Conditions of all cultures
Vehicle control	0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
HDPE control	1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
Silver based dressing control extracts	1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
$AgNO_3$ solution (3.5 μg/mL)	3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50% growth inhibition observable.
Silver based dressing extracts (3.8 μg/mL)	3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50% growth inhibition observable.
SPU-ZDEC extracts (100%)	4	Severe	Nearly complete or complete destruction of the cell layers.

Due to the general applicability and their widespread use in evaluating a large range of devices and materials, *in vitro* cytotoxic tests using MTT assay has been widely adopted since it was developed [17] [18]. In this study, we firstly analyze the silver content in the dressing extracts with AAS (see **Figure 1**) and then try to take an optimized protocol to investigate the cytotoxic potentials of $AgNO_3$ solution, silver-based dressing extracts and SPU-ZDEC extracts by MTT assay and evaluate the results using IC_{50} value, a qualitative index used to evaluate toxic effect of compounds. As showed in **Figure 2**, the IC_{50} values are 3.5 μg/mL, 3.8 μg/mL and 8.4%, respectively. Interestingly, the results of $AgNO_3$ solution and silver-based dressings show good correlation depending on the silver concentration. Of note, we also compare the results between quantitative and qualitative evaluation methods, as presented in **Figure 3** and **Table 2**, which have shown that these two methods also possessed a good agreement with respect to silver release.

5. Conclusion

In conclusion, our results demonstrate that there are good correlations between silver release and cytotoxicity of highly absorbent foam dressings based on silver zirconium phosphate, which also indicates good agreements between quantitative evaluation and qualitative morphological evaluation. Importantly, we confirmed that it is possible to apply the IC_{50} value in quantitative evaluation of optimized MTT method and it is also a promising method for screening cytotoxicity of silver based dressings. Further studies will focus on elucidating the precise molecular mechanism of silver based dressings mediated cytotoxicity in order to fulfill their safely clinical applications in future.

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Conflict of Interest Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

[1] Wright, J.B., Lam, K. and Burrell, R.E. (1998) Wound Management in an Era of Increasing Bacterial Antibiotic Resistance: A Role for Topical Silver Treatment. *American Journal of Infection Control*, **26**, 572-577. http://dx.doi.org/10.1053/ic.1998.v26.a93527

[2] Elliott, C. (2010) The Effects of Silver Dressings on Chronic and Burns Wound Healing. *British Journal of Nursing*, **19**, S32-S36. http://dx.doi.org/10.12968/bjon.2010.19.Sup5.77707

[3] Lansdown, A.B.G., Williams, A., Chandler, S., *et al*. (2005) Silver Absorption and Antibacterial Efficacy of Silver Dressings. *Journal of Wound Care*, **14**, 205-210. http://dx.doi.org/10.12968/jowc.2005.14.4.26762

[4] Klasen, H.J. (2000) Historical Review of the Use of Silver in the Treatment of Burns. Part I. Early Uses. *Burns*, **26**, 117-130. http://dx.doi.org/10.1016/S0305-4179(99)00108-4

[5] Poon, V.K. and Burd, A. (2004) *In Vitro* Cytotoxicity of Silver: Implication for Clinical Wound Care. *Burns*, **30**, 140-147. http://dx.doi.org/10.1016/j.burns.2003.09.030

[6] Scudiero, D.A., Shoemaker, R.H., Paull, K.D., *et al*. (1988) Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. *Cancer Research*, **48**, 4827-4833.

[7] Gerlier, D. and Thomasset, N. (1986) Use of MTT Colorimetric Assay to Measure Cell Activation. *Journal of Immunological Methods*, **94**, 57-63. http://dx.doi.org/10.1016/0022-1759(86)90215-2

[8] Tallarida, R.J. and Murray, R.B. (1981) Manual of Pharmacologic Calculations with Computer Programs. Springer-Verlag, New York, 69-146. http://dx.doi.org/10.1007/978-1-4684-0101-1

[9] Tsuchiya, T. (1994) Studies on the Standardization of Cytotoxicity Tests and New Standard Reference Materials Useful for Evaluating the Safety of Biomaterials. *Journal of Biomaterials Applications*, **9**, 138.

[10] Ikarashi, Y., Toyoda, K., Ohsawa, N., *et al*. (1992) Comparative Studies by Cell Culture and *in Vivo* Implantation Test on the Toxicity of Natural Rubber Latex Materials. *Journal of Biomedical Materials Research*, **26**, 339-356. http://dx.doi.org/10.1002/jbm.820260306

[11] Tsuchiya, T., Ikarashi, Y., Hats, H., Toyoda, K., Takahashi, M., Uchima, T., *et al*. (1993) Comparative Studies of the Toxicity of Standard Reference Materials in Various Cytotoxicity Tests and *in Vivo* Implantation Tests. *Journal of Applied Biomaterials*, **4**, 153-156. http://dx.doi.org/10.1002/jab.770040206

[12] Burrell, R.E. (2003) A Scientific Perspective on the Use of Topical Silver Preparations. *Ostomy Wound Management*, **49**, 19-24.

[13] Burd, A., Kwok, C.H., Hung, S.C., *et al*. (2007) A Comparative Study of the Cytotoxicity of Silver-Based Dressings in Monolayer Cell, Tissue Explant, and Animal Models. *Wound Repair and Regeneration*, **15**, 94-104. http://dx.doi.org/10.1111/j.1524-475X.2006.00190.x

[14] Schierholz, J.M., Wachol-Drewek, Z., Lucas, L.J. and Pulverer, G. (1998) Activity of Silver Ions in Different Media. *Zentralblatt für Bakteriologie*, **287**, 411-420. http://dx.doi.org/10.1016/S0934-8840(98)80178-3

[15] Walker, M., Cochrane, C.A., Bowler, P.G., Parsons, D. and Bradshaw, P. (2006) Silver Deposition and Tissue Staining Associated with Wound Dressings Containing Silver. *Ostomy Wound Management*, **52**, 42-50.

[16] Rigo, C., Roman, M., Munivrana, I., Vindigni, V., Azzena, B., Barbante, C. and Cairns, W.R.L. (2012) Characterization and Evaluation of Silver Release from Four Different Dressings Used in Burns Care. *Burns*, **38**, 1131-1142. http://dx.doi.org/10.1016/j.burns.2012.06.013

[17] Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**, 55-63. http://dx.doi.org/10.1016/0022-1759(83)90303-4

[18] Landegren, U. (1984) Measurement of Cell Numbers by Means of the Endogenous Enzyme Hexosaminidase. Applications to Detection of Lymphokines and Cell Surface Antigens. *Journal of Immunological Methods*, **67**, 379-388. http://dx.doi.org/10.1016/0022-1759(84)90477-0