Minimum cytocidal effect of different minocycline and doxycycline concentrations to human periodontal ligament fibroblasts *in vitro*

by

TANYA DE WET

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School of Dentistry

Faculty of Health Sciences

University of Pretoria

South Africa

Leader

Prof Dr SJ Botha

Centre for Stomatological Research

School of Dentistry, University of Pretoria

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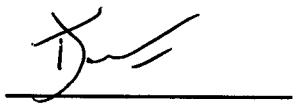
DECLARATION

I hereby declare that this dissertation, submitted by me in partial fulfilment of the requirements for the degree of MChD (Periodontics and Oral Medicine) at the University of Pretoria, is my own work and has not previously been submitted at any other university.



Tanya de Wet

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DEDICATION

To the Glory of God for giving me the ability to study.

To my darling Alfred for believing in me, your love and support helped me to continue till the very end.

To my parents, for your support, prayers and that you are always there for me.

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SUMMARY

Minimum cytocidal effect of different minocycline and doxycycline concentrations to human periodontal ligament fibroblasts *in vitro*

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Tanya de Wet

Department of Periodontics and Oral Medicine

School of Dentistry

Faculty Health Sciences

University of Pretoria

Minocycline (MC) and Doxycycline (DC) are used worldwide as locally applied adjuncts in the treatment of periodontal diseases. As a group the tetracyclines are well known for their advantageous properties. There is however possible cytotoxicity towards cells in the area of application.

This study determined the minimum cytocidal concentration of MC and DC on the growth and proliferation of Human Periodontal Ligament Fibroblasts (HPLF) *in vitro.* This was facilitated by growing cells (PDL1 and PDL2) in the presence of MC and DC in media in 96 tissue wells starting at a concentration of 1400 $\mu g.ml^{-1}$ (100%). Serial dilutions of the MC and DC at 10% increments were investigated in order to detect significant HPLF cell growth inhibition. The significant LD_{50} was further determined at one percent increments in order to arrive at a specific percentage value.

The results were read as LD_{50} values from growth concentration curves. The LD_{50} of MC on PDL1 and PDL2 after one hour exposure was 686 $\mu g.ml^{-1}$ and 896 $\mu g.ml^{-1}$

respectively while for DC it was 252 $\mu g.ml^{-1}$ and 546 $\mu g.ml^{-1}$. The LD_{50} of MC on PDL1 and PDL2 after 24 hour exposure was 196 $\mu g.ml^{-1}$ and 266 $\mu g.ml^{-1}$ respectively while for DC it was 252 $\mu g.ml^{-1}$ for both. The LD_{50} of MC on PDL1 and PDL2 after 48 hour exposure was 252 $\mu g.ml^{-1}$ and 182 $\mu g.ml^{-1}$ respectively while for DC it was 154 $\mu g.ml^{-1}$ and 168 $\mu g.ml^{-1}$.

Based upon the LD_{50} values this study found that DC is more cytotoxic than MC and linked to this, the two cell lines reacted slightly differently. The concentrations MC and DC tested in this study did however not influence growth of HPLF significantly.

CHAPTER 1: INTRODUCTION

Periodontitis is an infectious disease caused by periodontopathic bacteria in the gingival crevice. It is known that more than 500 species of bacteria are found in dental plaque and is present in periodontal pockets (Moore & Moore, 1994; Paster *et al.*, 2001). Periodontopathic bacteria such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* have the ability to penetrate and survive within host cells (Meyer *et al.*, 1997). Mechanical debridement of pockets is not always effective on its own for treating certain forms of periodontitis. Tetracyclines are broad-spectrum bacteriostatic antibiotics that are widely used as systemic and locally delivered antimicrobial adjuncts to periodontal therapy.

In certain forms of periodontitis antibiotics are administered as adjunctive therapy either systemically or locally. Antibacterial agents are directly administered to the site of infection as chemotherapeutic treatment - either by conditioning of the dentin, local irrigation or agent application in a periodontal pocket (Caton *et al.*, 2000; Kim *et al.*, 2004). The use of Tetracycline-HCl (TCH) (100mg.ml^{-1}) as irrigant for 5 minutes in conjunction with mechanical debridement resulted in significantly greater attachment gain over a six months period of healing as compared to scaling and root planing alone (Christersson *et al.*, 1993). When used for root conditioning together with mechanical debridement, research has shown that it extracts bacterial endotoxins from affected roots resulting in reattachment of the periodontal fibroblasts (Gilman & Maxey, 1985). Tetracyclines proved to be not just antibacterial but have other properties such as anti-inflammatory activity, suppression of immunity, suppression of antibody production in lymphocytes, inhibition of lipase and collagenase activity and improvement of the attachment of gingival fibroblast cells (Roberts, 2002).

After topical application of tetracyclines, it exhibit dentine substantivity whilst maintaining antimicrobial activity in the periodontal pocket. Locally controlled release delivery systems for insertion directly into the periodontal pocket are available (Demirel *et al.*, 1991). Concentrations of Tetracycline (TC) in excess of 1300 $\mu g.ml^{-1}$ in the crevicular fluid can be obtained with local delivery systems with minimal detrimental effects. Adjacent soft tissue concentrations can reach values of approximately 65 $\mu g.ml^{-1}$ with little systemic uptake (Seymour & Heasman, 1995). Goodson *et al.*, (1983) reported a maintainable level in the periodontal sulcus of 1500 $\mu g.ml^{-1}$ for 10 days utilising tetracycline hydrochloride (TCH) containing fibers.

In the literature different concentrations of different tetracyclines and their effect on human gingival epithelium and human periodontal fibroblast attachment, growth and cytotoxicity has been studied (Inoue *et al.*, 2004; Maizumi *et al.*, 2002). The minimum inhibitory concentrations (MIC) of TC to periodontopathic bacteria are known (0,031-4,0 $\mu g.ml^{-1}$) (Miyake *et al,.* 1995). Research suggests that the advantageous effects of TC, MC and DC may be less at higher concentrations (Rompen *et al.*, 1993), as the effects become cytocidal (Tsukuda & Gabler, 1993). Although the treatment of periodontal disease with different TC concentrations was researched as indicated, the precise concentration of cytotoxicity for MC and DC to Human Periodontal Ligament Fibroblast (HPLF) is however still unknown and will be determined in this study.

CHAPTER 2: LITERATURE REVIEW:

2.1 The use of tetracyclines as locally applied antibiotics in periodontics

The utilization of locally delivered antibiotics is mainly as periodontal supportive therapy together with scaling and root planing in persistent deep pockets. Local delivery of antibiotics may be most beneficial in the control of localized ongoing disease, in otherwise stable patients in order to limit systemic resistance (Mombelli, 2003). Concentrations of 500 $\mu g.ml^{-1}$ TC were established initially by local fiber application in the gingival crevice and after prolonged periods of time concentrations of at least 50 $\mu g.ml^{-1}$ TC are achievable (Goodson *et al.*, 1983). This is 10-100 times the periodontal pocket concentration normally achieved by systemic administration of TC and is capable of inhibiting 345 strains of bacteria normally isolated from periodontal pockets. The concentration of TC needed to inhibit most periodontal bacteria *in vitro* is 4-8 $\mu g.ml^{-1}$ (Walker *et al.*, 1981).

TCH, DC and MC are semi-synthetic bacteriostatic antibiotics. The latter two exhibit greater oral absorption, longer half-lives and are more extensively protein bound. TCH is a chelating agent while DC and MC have less gastrointestinal side effects. Antimicrobial activity of the TC is achieved by them being concentrated in the gingival crevicular fluid after systemic treatment (Seymour & Heasman, 1995). HPLF possess active transporters that could potentially contribute to the relatively high levels of TC in gingival crevicular fluid (Yang *et al.*, 2002). In contrast after systemic administration of TC, MC and DC the average gingival crevicular fluid concentration was however found to be 20-50% lower than the plasma concentration and in 20% of cases below levels that are considered to be antimicrobial (Sakellari *et al.*, 2000).

Researchers have suggested that inter individual variability of oral absorption may be the reason for the variability of the plasma concentrations.

The substantivity of TC to cementum and dentine is very good and provides a constant TC release into the crevicular sulcus (Baker *et al.*, 1983). *In vitro* studies have shown that pre-treatment of dentine with TC enhances HPLF attachment and colonization. Incubation of HPLF with 50 $\mu g.ml^{-1}$ of MC significantly improves cell attachment compared to untreated dentine (Rompen *et al.*, 1993). Somerman *et al.* (1988) indicated in their *in vitro* study of the effects of MC on the spreading and attachment of fibroblasts, that concentrations greater than 50 $\mu g.ml^{-1}$ MC, promoted cell attachment but concentrations higher than 100 $\mu g.ml^{-1}$ prevented cell attachment, suggesting that there should be a concentration that is optimal for cell attachment.

Rompen *et al.* (1993) studied the effect of MC on HPLF populating powdered dentine. The MC was used to condition the dentine. MC conditioning enhanced the attachment and spreading of the HPLF significantly and is dose dependant. The concentrations of MC applied were 20, 50, 100 and 200 $\mu g.ml^{-1}$ and the optimum concentration was shown to be 110 $\mu g.ml^{-1}$. Higher dosages did not prove to be more beneficial. In a follow up study the same group of researchers found significantly higher rates of HPLF proliferation and significantly higher levels of total protein and collagen synthesis on MC conditioned dentine than on untreated dentine (Rompen *et al.*, 1999).

2.2 Cytocidal effect of locally applied antibiotics

The cytocidal effect of macrolide antibiotics was determined on HPLF as alternative to tetracycline antibiotics in the treatment of periodontitis. Topical administration *in vitro* of clarithromycin or azithromycin to the gingival sulcus at the minimum inhibitory concentration (MIC_{90}) for periodontopathic bacteria had little effect on the growth and differentiation of the periodontal ligament cells (Maizumi *et al.*, 2002). The minimum inhibitory concentrations of TC to periodontopathic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* range from 0,031-4,0 $\mu g.ml^{-1}$ and that of MC range from 0.031-2,0 $\mu g.ml^{-1}$ (Miyake *et al.*, 1995).

The minimum cytocidal (LD_{50}) concentration of MC applied to human gingival epithelium at a cell density of $1x10^6$ $cells.ml^{-1}$ was 21.234 ± 3.012 $\mu g.ml^{-1}$ and for TC 28.522 ±1.106 $\mu g.ml^{-1}$ after an exposure period of 48h, while the maximum non cytocidal concentration for MC was 0.148 $\mu g.ml^{-1}$ and for TC 0.481 $\mu g.ml^{-1}$ (Inoue *et al.*, 2004). Tsukuda & Gabler (1993) investigated the influence of different DC concentrations administered to PDLF. Dosages higher than 50 $\mu g.ml^{-1}$ DC reduced the number of adherent cells significantly. Cytotoxicity was determined by lactic dehydrogenase assay and this increased significantly at dosages higher than 50 $\mu g.ml^{-1}$ DC. This suggested that DC in higher dosages may be cytotoxic to PDLF and affect the spreading and attachment of these cells.

MC has a greater cytotoxicity compared to DC and TC when applied to human epithelioid S-G cells (Babich & Tipton, 2001) and HPLF (Omori *et al.*, 1999). It is postulated that the cytotoxicity of an antimicrobial agent is determined by its

lipophilicity resulting in easier cellular penetration. As MC is the most lipophilic it is the most cytotoxic. Babich & Tipton (2001) reported irreversible damage of S-G cells when exposed for one hour to 400 $\mu g.ml^{-1}$ and higher concentrations of MC but the cells were able to recover after one hour exposures to 25-200 $\mu g.ml^{-1}$ MC.

According to Chang *et al.* (2001) it is necessary to evaluate the concentration of the drug applied, exposure time and the surface area exposed when investigating the cytotoxicity of a drug. The methods they used to evaluate cell toxicity were protein synthesis assay, mitochondrial activity and propidium iodide fluorescence cytotoxicity assay.

Although cytotoxicity of TC against human cells has been indicated the relative toxicity of tetracyclines and fluorquinolones in terms of cytocidal effects (LD50) proved to be in rank order Demeclocycline>MC>TC>TCH, where Demeclocycline, MC and TC were 6 times more cytocidal than TCH (Omori *et al.*, 1999).

CHAPTER 3: AIM:

As the relative cytotoxicity of MC and DC is not known, this study was designed to determine the minimum cytocidal concentration of MC and DC on the growth and proliferation of HPLF *in vitro* by growing cells in the presence of MC and DC in media in 96 tissue wells starting at a concentration of 1400 $\mu g.ml^{-1}$ (100%). Serial dilutions of the MC and DC at 10% increments were investigated in order to detect significant HPLF cell growth inhibition. The significant LD_{50} will be further determined at one percent increments to arrive at a specific percentage value.

CHAPTER 4: MATERIALS AND METHODS:

4.1 Tetracyclines tested

Two different tetracyclines were used in this study namely MC and DC. The concentrations in the media are given in table 4.1 starting at a concentration of 1400 $\mu g.ml^{-1}$.

Table 4.1 Concentration of MC and DC in EMEMS for cultivation of HPLF with 1400 $\mu g.ml^{-1}$ taken as 100%

Media	Percentage %	Concentration MC/DC ($\mu g.ml^{-1}$)
A	100	1400
B	90	1260
C	80	1120
D	70	980
E	60	840
F	50	700
G	40	560
H	30	420
I	20	280
J	10	140
Control	0	0

After the initial tests were done the range of the tetracycline concentrations was narrowed in order to determine the MIC and/or LD_{50} of tetracycline. During this phase the concentration of the tetracycline in the media was varied with 1% increments (Table 4.2) after the MIC of the specific cell line for the specific tetracycline concentration with 10% increments was determined. In all media

preparations, care was taken to ensure that all solutions were freshly made and used immediately.

Table 4.2 Concentrations of MC and DC in EMEMS for cultivation of HPLF at 1% increments

Media	%	**MC/DC PDL1 and PDL2 (µg.ml^{-1})**	%	MC PDL2 t=1 (µg.ml^{-1})	%	**MC PDL1 t=1 (µg.ml^{-1})**	%	DC PDL2 t=1 (µg.ml^{-1})
A	**19**	**266**	69	966	**49**	**686**	39	546
B	**18**	**252**	68	952	**48**	**672**	38	532
C	**17**	**238**	67	938	**47**	**658**	37	578
D	**16**	**224**	66	924	**46**	**644**	36	504
E	**15**	**210**	65	910	**45**	**630**	35	490
F	**14**	**196**	64	890	**44**	**616**	34	476
G	**13**	**182**	63	882	**43**	**602**	33	462
H	**12**	**168**	62	868	**42**	**588**	34	448
I	**11**	**154**	61	854	**41**	**574**	31	434
J	**10**	**140**	60	840	**40**	**540**	30	420
Control	**0**	**0**	0		**0**		0	

4.2. Cell cultures

Two different lines of human periodontal ligament fibroblasts (HPLF) were grown in EMEMS (Highveld Biological, PO Box 1456, Lyndhurst, 2106, RSA). Standardized, calibrated cell suspensions at a concentration of 2-4 X 10^4 cells.ml^{-1} media (Wilken *et al.*, 2001) were inoculated into a series of 96 well tissue culture plates (200µl per well) (AEC-Amersham, PO Box 1596, Kelvin, 2034, RSA). After 24 hours incubation at 37°C in 5% CO_2 and 95% air in a humidified atmosphere, the media was removed, cells were washed and the prepared media with known concentrations (see Table 4.1 & 4.2) of the two different Tetracycline's were added to the cells. Three wells per cell

line were used for each concentration of tetracycline. Cytotoxicity was determined after 1, 24 and 48 hours using the MTT [3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide] technique (Mosmann, 1983). Control wells of the different fibroblasts were incubated with EMEM only. All experiments were done in triplicate.

4.3. Cytotoxicity screening

A standard MTT assay (Mosmann, 1983) was used in this study. In this test MTT was reduced by mitochondrial dehydrogenases in living cells; this reaction produces formazan crystals, which were quantified by photometry after extraction. In this study 20*µl* MTT (98%) (Sigma-Aldrich, 17 Pomona St, Aviation Park, Unit 4, Kempton Park, 1619, RSA) was added to the wells and the cells were incubated for 4 hours at 37°C. The incubation media was carefully removed and 100*µl* of Dimethyl Sulfoxide (98.6%) (Sigma-Aldrich, 17 Pomona St, Aviation Park, Unit 4, Kempton Park, 1619, RSA) was added to the wells. Well plates were carefully shaken and the absorbance read at 560nm. The results were normalised considering the control well as 100% - non-cytotoxic (Mosmann, 1983).

4.4 Statistical analysis:

After normalization of all the cytotoxicity readings and calculation of average values, results were statistically compared with ANOVA using Statistix 8 software (Analytical Software, PO Box 12183, Tallahasee, FL 32317-2185, USA).

CHAPTER 5: RESULTS:

5.1 Growth percentages of two different human periodontal ligament fibroblast (HPLF) cell lines after exposure to test agents:

The average growth percentages of two different cell lines (PDL1 and PDL2) after exposure to different concentrations MC and DC for 1h, 24h and 48h are shown in Table 5.1. In general an initial increase in growth was observed with MC but not with DC.

Table 5.1 Average growth percentages of HPLF after exposure to 10% incremental dilutions of MC and DC starting at a concentration of 1400 µg.ml^{-1} (100%) determined by standard MTT assay of each well after 1, 24 and 48 hours.

Concentration (µg/ml)	0	140	280	420	560	700	840	980	1120	1260	1400
MC PDL1 t=1	100	104.94	108.95	109.40	109.59	93.96	82.20	68.38	69.00	68.82	71.28
MC PDL2 t=1	100	130.15	129.69	131.12	137.50	117.09	105.21	82.81	92.62	88.00	82.98
MC PDL1 t=24	100	107.4	85.24	55.45	52.18	50.46	53.48	50.38	58.94	51.90	47.12
MC PDL2 t=24	100	108.53	96.70	60.96	58.00	54.06	56.15	56.33	61.24	62.42	57.32
MC PDL1 t=48	100	131.32	60.70	62.48	57.17	65.99	65.32	65.64	77.91	73.65	95.26
MC PDL2 t=48	100	138.06	66.38	65.04	63.68	69.56	68.16	71.00	89.67	91.45	113.26
DC PDL1 t=1	100	97.35	84.11	94.23	89.49	84.11	79.11	80.05	74.57	68.06	69.03
DC PDL2 t=1	100	100.33	95.36	105.42	99.45	87.67	91.56	84.17	75.46	78.92	77.91
DC PDL1 t=24	100	119.63	56.91	60.03	61.43	58.15	61.26	62.84	67.29	75.48	80.27
DC PDL2 t=24	100	84.75	48.64	58.22	54.69	55.32	54.56	57.15	60.90	59.20	59.58
DC PDL1 t=48	100	74.13	55.57	67.52	67.29	64.06	70.74	71.60	85.07	88.09	93.62
DC PDL2 t=48	100	61.09	52.24	59.87	61.52	55.24	59.90	60.31	68.75	72.82	62.70

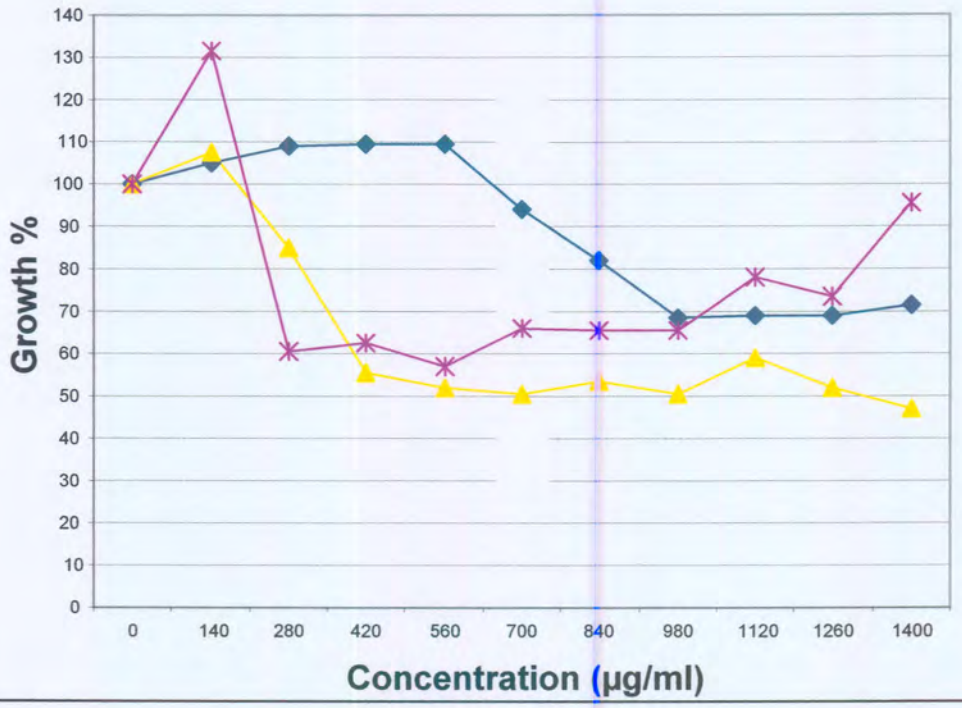

Fig 5.1: The average growth percentage of PDL1 after exposure to 10% increments of MC concentrations. Standard MTT assays were done after incubation of the HPLF for t=1h (blue), 24h (yellow) and 48h(purple) in the different MC concentrations.

Table 5.2: Results of statistical analysis of the average growth of PDL1 after exposure to 10% increments of MC for 1h, 24h and 48h (P<0.05 is statistically significant).

Concentration (µg/ml)	0	140	280	420	560	700	840	980	1120	1260	1400
MC PDL1 t=1	100	104.94	108.95	109.40	109.59	93.96	82.20	68.38	69.00	68.82	71.28
P value		0.17	<0.05	<0.05	<0.05	0.12	<0.05	<0.05	<0.05	<0.05	<0.05
MC PDL1 t=24	100	107.40	85.24	55.45	52.18	50.46	53.48	50.39	58.94	51.90	47.13
P value		0.127	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
MC PDL1 t=48	100	131.32	60.70	62.48	57.17	66.00	65.32	65.64	77.91	73.66	95.26
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

This study determined the cytocidal effect of MC and DC and it indicated that MC significantly decreased the ($P \leq 0.05$) growth of PDL1 after one hour exposure at a concentration of 840 $\mu g.ml^{-1}$. After 24 and 48 hours of exposure MC a significant decrease of growth of PDL1 started from a concentration of 280 $\mu g.ml^{-1}$.

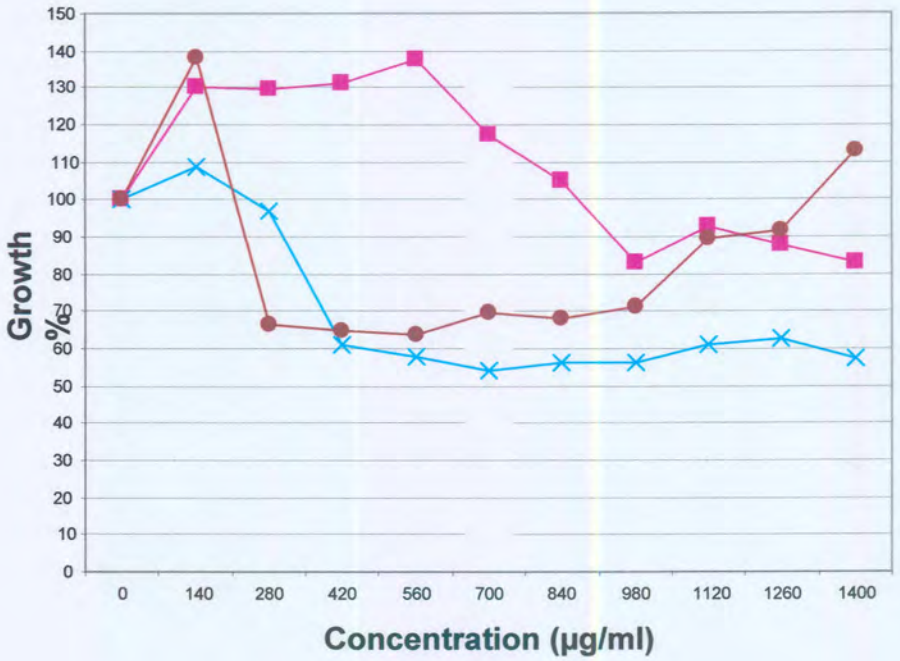

Fig 5.2: Average growth percentages of PDL2 after exposure to 10% increments of MC. Standard MTT assays were done after incubation of the HPLF for 1h (pink), 24h (blue) and 48h (plum) in the different MC concentrations.

Table 5.3: Results of statistical analysis of the average growth of PDL2 after exposure to 10% increments of MC for 1h, 24h and 48h (P<0.05 is statistically significant).

Concentration (µg/ml)	0	140	280	420	560	700	840	980	1120	1260	1400
MC PDL2 t=1	100	130.15	129.69	131.12	137.50	117.09	105.21	82.81	92.62	88.00	82.98
P value		<0.05	<0.05	<0.05	<0.05	0.19	0.42	<0.05	0.27	<0.05	<0.05
MC PDL2 t=24	100	108.53	96.71	60.96	58.00	54.06	56.15	56.33	61.24	62.41	57.32
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
MC PDL2 t=48	100	138.05	66.38	65.05	63.68	69.56	68.16	71,00	89.67	91.45	113.26
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

After one hour exposure to MC the growth of PDL2 showed an initial increase but was significantly decreased from a concentration of 980 µg.ml^{-1}. After 24 and 48 hours of exposure a significant decrease of growth started from a concentration of 280µg.ml^{-1} MC.

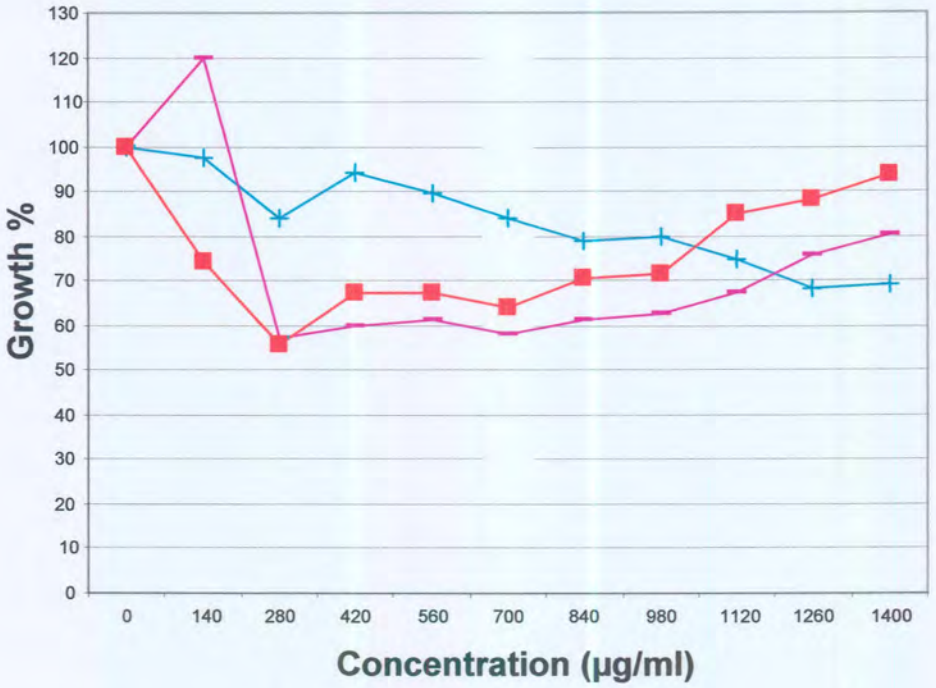

Fig 5.3 Average growth percentages of PDL1 after exposure to 10% increments of DC concentrations. Standard MTT assays were done after incubation of HPLF for t=1h (blue), 24h (pink) and 48h (red) in the different DC concentrations.

Table 5.4 Results of statistical analysis of the average growth of PDL1 after exposure to 10% increments of DC for 1h, 24h and 48h (P<0.05 is statistically significant).

Concentration (µg/ml)	0	140	280	420	560	700	840	980	1120	1260	1400
DC PDL1 t=1	100	97.35	84.11	94.23	89.49	84.11	79.11	80.05	74.57	68.06	69.03
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DC PDL1 t=24	100	119.63	56.91	60.03	61.43	58.15	61.26	62.84	67.29	75.48	80.28
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DC PDL1 t=48	100	74.13	55.57	67.52	67.29	64.06	70.74	71.60	85.07	88.10	93.62
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.28

After one hour exposure to DC the growth of PDL1 showed a decrease from a concentration of 140 $\mu g.ml^{-1}$. After exposure times of 24 and 48 hours the growth decreased significantly at concentrations of 280 and 140 $\mu g.ml^{-1}$ respectively.

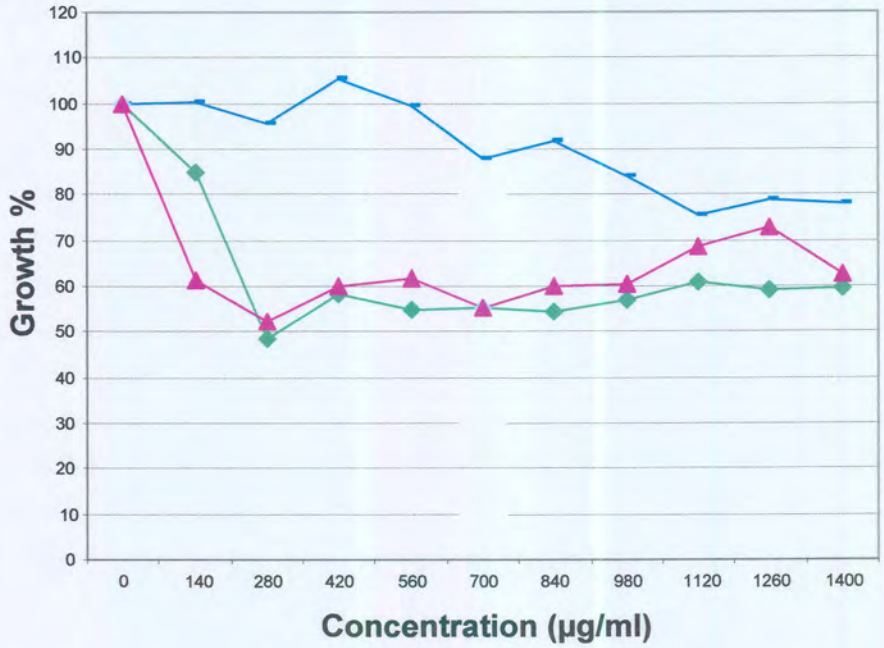

Fig 5.4: Average growth percentages of PDL2 after exposure to 10% increments of DC concentrations. Standard MTT assays were done after incubation of HPLF for 1h (blue), 24h (green) and 48h (purple) in the different DC concentrations.

Table 5.5 Results of statistical analysis of the average growth of PDL2 after exposure to 10% increments of DC concentrations for 1h, 24h and 48h (P<0.05 is statistically significant).

Concentration (µg/ml)	0	140	280	420	560	700	840	980	1120	1260	1400
DC PDL2 t=1	100	100.33	95.36	105.42	99.45	87.67	91.56	84.17	75.46	78.92	77.91
P value		0.93	<0.05	<0.05	0.83	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DC PDL2 t=24	100	84.75	48.64	58.22	54.69	55.32	54.56	57.15	60.90	59.20	59.58
P value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DC PDL2 t=48	100	61.09	52.24	59.87	61.52	55.24	59.90	60.31	68.75	72.82	62.70
P value	0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

The PDL2 cell line demonstrates significant suppression of growth from 700 $\mu g.ml^{-1}$ DC after exposure time of one hour. After 24 and 48 hours cell survival significantly started decreasing from the 140 $\mu g.ml^{-1}$ concentration.

Tabel 5.6 Average growth percentages of HPLF after exposure to 1% incremental dilutions of Minocycline and Doxycycline starting at different concentrations determined by standard MTT assay of each well after 1, 24 and 48 hours.

Concentration (µg/ml)	0	560	574	588	602	616	630	644	658	672	686
MC PDL1 t=1	100	118.35	139.77	113.85	131.04	112.86	84.64	93.77	57.13	56.26	49.22

Concentration (µg/ml)	0	840	854	868	882	896	910	924	938	952	966
MC PDL2 t=1	100	80.37	77.18	61.57	51.50	40.23	52.77	41.05	38.45	29.54	28.02

Concentration (µg/ml)	0	140	154	168	182	196	210	224	238	252	266
MC PDL1 t=1	100										
MC PDL2 t=1	100										
MC PDL1 t=24	100	70.38	65.60	59.00	59.02	38.13	47.91	42.57	56.98	47.70	28.05
MC PDL2 t=24	100	74.75	70.71	70.95	64.56	69.24	59.71	71.99	47.90	55.57	35.58
MC PDL1 t=48	100	81.64	59.77	61.36	62.85	67.10	59.28	53.84	51.95	50.05	28.23
MC PDL2 t=48	100	79.67	55.63	24.70	20.92	19.93	16.98	16.33	19.77	22.05	17.38
DC PDL1 t=1	100	98.91	76.98	67.86	65.27	64.38	75.30	53.75	59.01	37.72	29.93
DC PDL2 t=1	100										
DC PDL1 t=24	100	67.32	49.43	62.15	61.09	60.18	57.69	58.64	51.67	30.70	22.26
DC PDL2 t=24	100	90.82	88.46	82.19	66.13	54.23	48.63	56.01	56.327	39.21	24.49
DC PDL1 t=48	100	55.06	42.67	46.12	45.88	35.77	29.32	31.07	32.28	25.87	30.36
DC PDL2 t=48	100	72.02	67.38	41.04	44.38	43.31	36.75	39.33	42.89	33.73	32.93

Concentration (µg/ml)	0	420	434	448	462	476	490	504	518	532	546
DC PDL2 t=1	100	118.61	94.73	76.42	76.84	71.69	70.70	89.63	71.67	75.56	43.59

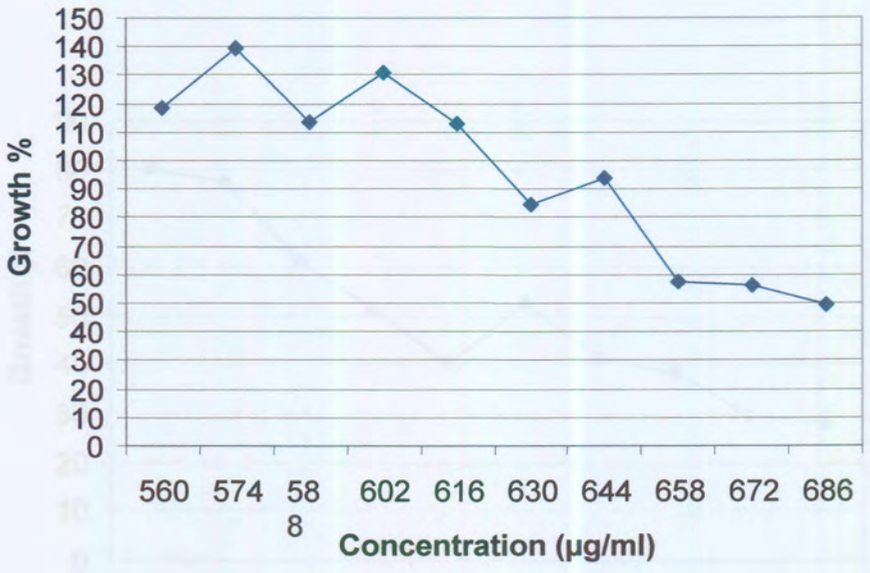

Fig 5.5 The MC concentration-growth curve of PDL1 after t=1h.

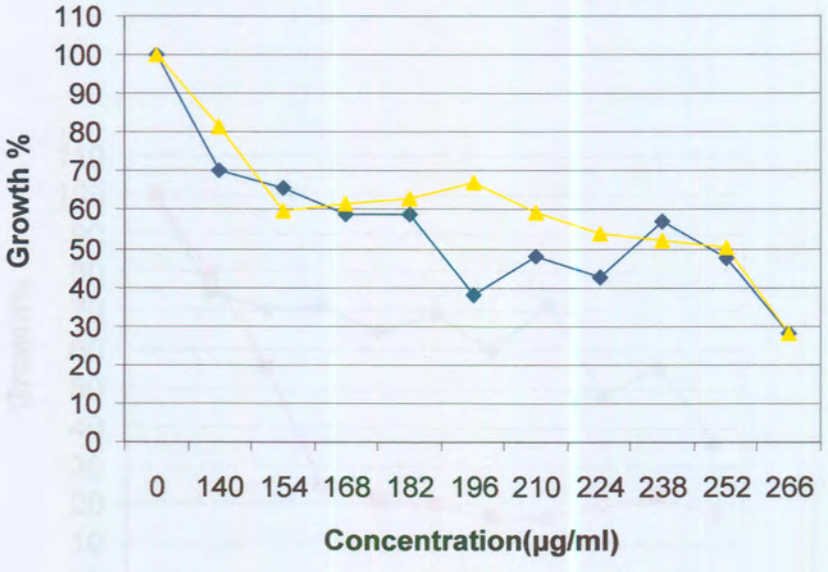

Fig 5.6 The MC concentration-growth curve of PDL1 after 24h (blue) and 48h (yellow).

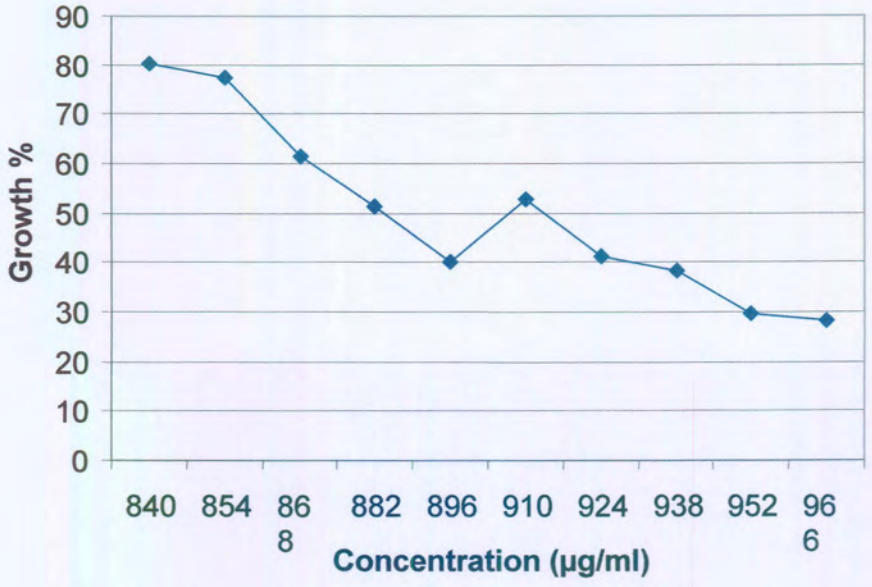

Fig 5.7 The MC concentration-growth curve of PDL2 after 1h.

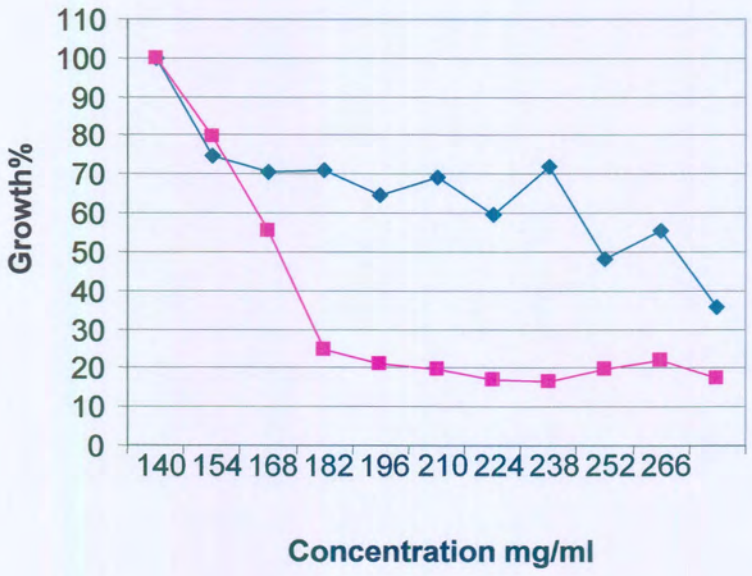

Fig 5.8 The MC concentration-growth curve of PDL2 after 24h (blue) and 48h (pink).

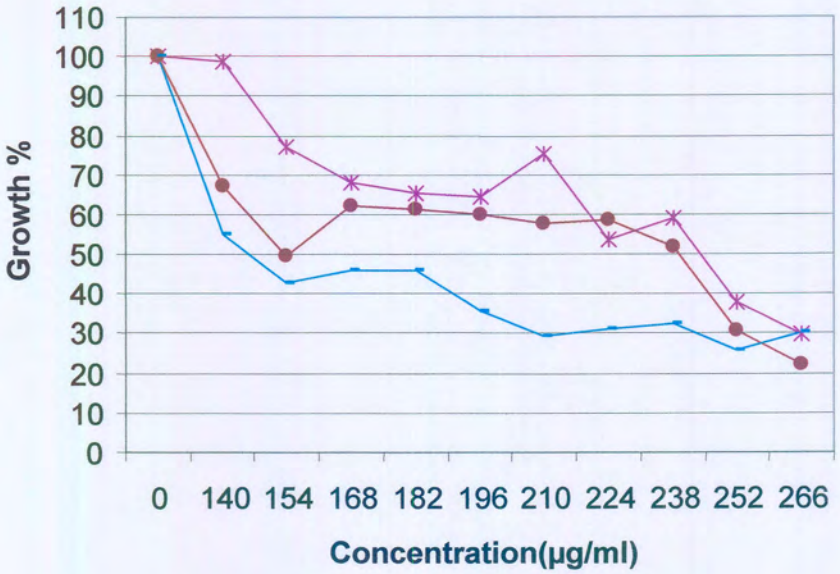

Fig 5.9 The DC concentration-growth curves PDL1 after 1h (purple), 24h (plum), 48h (blue).

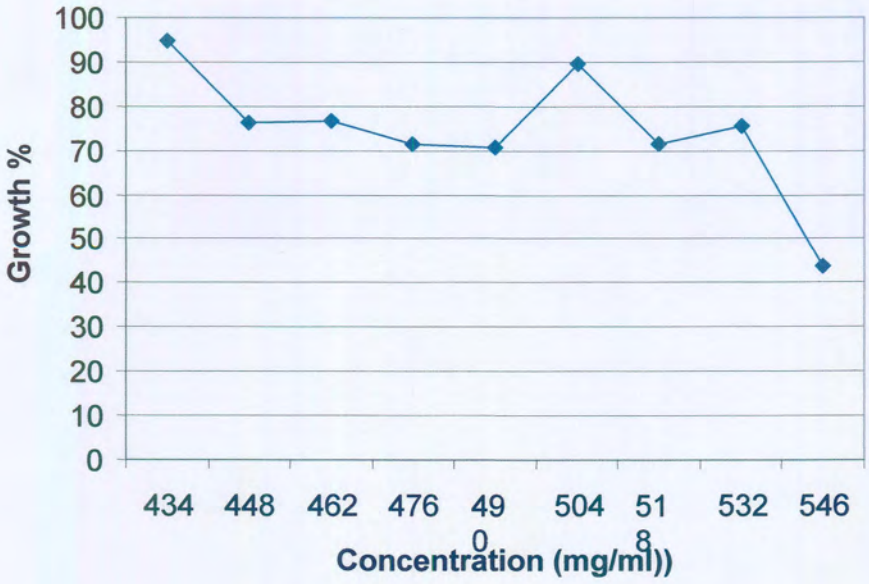

Fig 5.10 The DC concentration-growth curve of PDL2 1h.

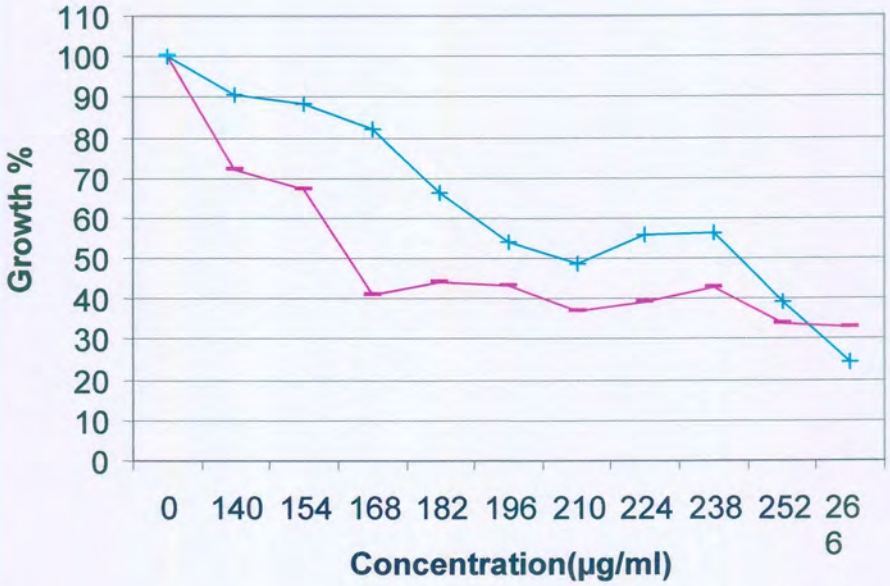

Fig 5.11 The DC concentration-growth curve of PDL2 after 24h (blue) and 48h (purple).

In order to determine the LD_{50} values of MC and DC the growth-concentration curves (Fig 5.5 to Fig 5.11) were studied to extrapolate percentage values of cytotoxicity. After 1h exposure of both cell lines to MC and DC high concentrations suppressed 50% of the cell growth (LD_{50}) in comparison to the control. After exposures of 24 and 48 hour periods, concentrations of 196 $\mu g.ml^{-1}$ and 252 $\mu g.ml^{-1}$ MC resulted in 50% reduction of cell growth for PDL1 while 266 $\mu g.ml^{-1}$ and 168 $\mu g.ml^{-1}$ for PDL2. DC is lethal for 50 % (LD_{50}) of cells at a concentration of 252 $\mu g.ml^{-1}$ for PDL1 and PDL2 after exposure periods of 24h while a concentration of 154 $\mu g.ml^{-1}$ for PDL1 and 168 $\mu g.ml^{-1}$ for PDL2 after 48 hour exposure.

Table 5.7 Summary of LD_{50} values as read from growth-concentration curves

	LD_{50} ($\mu g.ml^{-1}$)		LD_{50} ($\mu g.ml^{-1}$)
MC PDL1 t=1h	686	**MC PDL2 t=1h**	896
MC PDL1 t=24h	196	**MC PDL2 t=24h**	266
MC PDL1 t=48h	252	**MC PDL2 t=48h**	168
DC PDL1 t=1h	252	**DC PDL2 t=1h**	546
DC PDL1 t=24h	252	**DC PDL2 t=24h**	252
DC PDL1 t=48h	154	**DC PDL2 t=48h**	168

CHAPTER 6: DISCUSSION

The advantages of tetracyclines are multiple, for example immune suppression, anti-collagenase activity and improvement of fibroblast spreading and attachment (Roberts, 2002). Disadvantages of applying antibiotics locally or systemically include development of resistance and cytotoxicity towards the cells in the application area. Results of this study were based on standard MTT assay, determining the cytotoxicity quantified as the MIC or LD_{50} of MC and DC towards HPLF. Results showed that DC is more cytotoxic than MC. There is a difference in cytotoxicity between the two cell lines PDL1 and PDL2, as PDL2 is more resistant than PDL1. This difference in cell survival can possibly be explained by genetic differences between individual cell lines. The cell survival was affected in a concentration dependant manner, decreasing as the concentration of the test agent increased.

In this study HPLF appear to have a high level of resilience towards the direct application of DC and MC. Previous studies described that the effect of MC, DC and TC application appears to be specific to the cell type (Guerin *et al.*, 1992). It was indicated that human gingival epitheloid SG cells have a midpoint cytotoxicity of MC at a concentration of 204 $\mu g.ml^{-1}$ on day one, 84 $\mu g.ml^{-1}$ on day 2 and 59 $\mu g.ml^{-1}$ on day 3. The same group of researchers found that after a 24 hour exposure period the normalized ratio (NR_{50}) for MC was 226 $\mu g.ml^{-1}$ and DC 364 $\mu g.ml^{-1}$. Therefore it is clear that much higher concentrations of MC and DC can be applied to HPLF in comparison to human gingival epitheloid cells as the LD_{50} values after 24h for MC was 196-266 $\mu g.ml^{-1}$ and DC 252 $\mu g.ml^{-1}$. The epitheliod cell cytotoxicity to MC was measured by neutral red assay and it was shown that MC is

more cytotoxic than DC (Babich & Tipton, 2002) while this research found the opposite towards HPLF. In the same study epitheliodS-G cells had the ability to recover after one hour exposure to 200 $\mu g.ml^{-1}$ but after exposure to 400 $\mu g.ml^{-1}$ irreversible cell damage was caused. These results are higher than what Inoue *et al.* (2004) found when applying different concentrations of MC to a human gingival epithelial cell density of $1x\ 10^6$ resulted in a LD_{50} value of 21.234 ± 3.012 $\mu g.ml^{-1}$ after 48 hour time period but much lower than the results of this study.

The LD_{50} values in this study are much higher than the optimal concentration MC, 110 $\mu g.ml^{-1}$ for dentine conditioning which resulted in optimal human periodontal ligament cell attachment and spreading (Rompen *et al.*, 1999). This study determined that MC has a LD_{50} of 686 – 896 $\mu g.ml^{-1}$ after one hour while after longer exposures the LD_{50} started at concentrations of 196-266 $\mu g.ml^{-1}$. MC has a greater substantivity than TC (Baker *et al.*, 1983) and that is possibly the reason for the lower optimal value for the conditioning of dentine than the LD_{50} in this study. The intracellular concentration and duration of exposure to the specific concentration of MC and TC determine their cytocidal effects. MC and TC resulted in the highest percentage of apoptotic cells (LD_{50}) after 48h exposure to the respective agents (Inoue *et al.*, 2004) while in this study DC suppresses the growth of HPLF more than MC after 48h exposure.

The LD_{50} of DC in this study was affected by both concentration and by the exposure period to the HPLF. The cytotoxicity is much lower in the study by Tsukuda & Gabler (1993) who reported a significant cytotoxicity of DC at 50 $\mu g.ml^{-1}$ to periodontally derived fibroblasts after a 3h exposure time in comparison to 252-546 $\mu g.ml^{-1}$ as found in this study. The method of this study was however different as it determined the specific cytotoxicity by means of MTT assays in determining the

LD_{50} whilst the aforementioned study stopped at the first significant value of growth suppression.

By applying commercially available DC gel to the periodontal pocket a concentration of 46,73 $\mu g.ml^{-1}$ can be maintained in the periodontal pocket for at least 10 days (Kim *et al.*, 2004). Therefore the use of this product will not be toxic to the human periodontal ligament fibroblast as the LD_{50} value in this study after a 48 hour application was found to be 154-168 $\mu g.ml^{-1}$. Periodontal pathogens co-aggregate in a biofilm, resulting in a susceptibility to antibiotics 50 times lower than when the pathogens are in a sessile state (Brown & Gilbet, 1993). It should therefore be possible to apply 50 times the MIC of MC and DC to periodontopathic bacteria without influencing the growth of HPLF significantly.

The entry of DC into periodontal ligament-derived fibroblasts is influenced by the composition of the growth media. It is well known that tetracyclines are chelating agents. By removing any Ca^{2+} or Mg^{2+} from the media the intracellular concentration of DC can be doubled. Thus the uptake of DC can be influenced by adding serum to the incubation media (Tsukuda & Gabler, 1993). The results of this study could have been influenced by the chelation of DC. Variables, such as type of growth media and *in vitro* cell density may influence results, making comparison of different research designs difficult. In this study, however LD_{50} values were determined.

In vivo use of locally applied antibiotics is challenged by gingival crevicular fluid flow. After application of fluoresceine gel in the gingival sulcus Oosterwaal reported that 50% of the gel was washed out after 12.5 minutes (Oosterwaal *et al.*, 1990).

The concentration of tetracycline (100 mg.ml^{-1}) decreased logarithmically after local application, by means of a impregnated fiber from 1500 ± 270 µg.ml^{-1} to 19 ± 5 µg.ml^{-1} in one week (Goodson *et al.*, 1983). The clinically maintainable concentration of locally applied TC is lower than the LD_{50} values of this study; therefore implying that it may be used without fear of being cytotoxic towards HPLF.

CHAPTER 7: CONCLUSION

In this study the results indicated that the cytotoxicity of MC and DC is concentration dependant as well as dependant on the exposure time to HPLF. The determination of the specific cytotoxicity of MC and DC to HPLF may maximize the clinical application of the adjunctive benefits of these antimicrobial agents and reduce the detrimental effects. The two cell lines showed a difference in LD_{50} values possibly because of the genetic difference of the cell lines.

It is important to study the cytotoxicity of drugs utilised clinically on a regular basis. The toxicity of the MC and DC has been speculated upon in the literature. In this study it was determined that the local application of commercially available MC and DC products will not be cytocidal towards HPLF but only suppressed growth in such a way that this was eventually interpreted as LD_{50}. DC is more cytotoxic than MC after local application to HLPF *in vitro*. MC and DC at concentrations tested in this study will not influence the growth of HPLF significantly.

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ADDENDUM A: DATA

Mono cyclin	1	2	3	4	5	6	7	8	9	10	11	12
A	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
B	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
C	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
D												
E												
F	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	
G	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	
H	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	

Plate no →
1. Immediately
2. 24 hours
3. 48 hours

Mino
~~Doog~~

Bio-Tek ELx800

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Temp:

Lot: Plate 1 : Inne
Operator:
Plate ID:

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B	0.096 SMP2	0.078 SMP10	0.097 SMP18	0.091 SMP26	0.082 SMP34	0.089 SMP42	0.096 SMP50	0.094 SMP58	0.093 SMP66	0.110 SMP74	0.103 SMP82	0.041 SMP90
C	0.071 SMP3	0.074 SMP11	0.085 SMP19	0.077 SMP27	0.099 SMP35	0.106 SMP43	0.086 SMP51	0.083 SMP59	0.090 SMP67	0.093 SMP75	0.108 SMP83	0.042 SMP91
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E	0.055 SMP5	0.042 SMP13	0.044 SMP21	0.043 SMP29	0.043 SMP37	0.042 SMP45	0.043 SMP53	0.043 SMP61	0.042 SMP69	0.042 SMP77	0.044 SMP85	0.042 SMP93
F	0.073 SMP6	0.080 SMP14	0.079 SMP22	0.073 SMP30	0.088 SMP38	0.079 SMP46	0.081 SMP54	0.087 SMP62	0.087 SMP70	0.091 SMP78	0.100 SMP86	0.042 SMP94
G	0.080 SMP7	0.071 SMP15	0.090 SMP23	0.090 SMP31	0.080 SMP39	0.076 SMP47	0.085 SMP55	0.090 SMP63	0.088 SMP71	0.098 SMP79	0.096 SMP87	0.042 SMP95
H	0.079 SMP8	0.094 SMP16	0.071 SMP24	0.078 SMP32	0.086 SMP40	0.068 SMP48	0.068 SMP56	0.087 SMP64	0.083 SMP72	0.076 SMP80	0.101 SMP88	0.043 SMP96

Bio-Tek ELx800

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Lot: 2 : 24h.
Operator:
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COMMENTS

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B	0.099 SMP2	0.095 SMP10	0.087 SMP18	0.146 SMP26	0.102 SMP34	0.096 SMP42	0.102 SMP50	0.123 SMP58	0.116 SMP66	0.108 SMP74	0.101 SMP82	0.038 SMP90
C	0.101 SMP3	0.100 SMP11	0.087 SMP19	0.091 SMP27	0.090 SMP35	0.111 SMP43	0.101 SMP51	0.089 SMP59	0.103 SMP67	0.136 SMP75	0.137 SMP83	0.042 SMP91
D	0.049 SMP4	0.041 SMP12	0.044 SMP20	0.043 SMP28	0.042 SMP36	0.042 SMP44	0.045 SMP52	0.042 SMP60	0.048 SMP68	0.043 SMP76	0.041 SMP84	0.043 SMP92
E	0.044 SMP5	0.042 SMP13	0.040 SMP21	0.045 SMP29	0.042 SMP37	0.042 SMP45	0.035 SMP53	0.041 SMP61	0.040 SMP69	0.040 SMP77	0.044 SMP85	0.042 SMP93
F	0.109 SMP6	0.111 SMP14	0.097 SMP22	0.109 SMP30	0.077 SMP38	0.092 SMP46	0.102 SMP54	0.115 SMP62	0.188 SMP70	0.131 SMP78	0.112 SMP86	0.058 SMP94
G	0.122 SMP7	0.100 SMP15	0.095 SMP23	0.105 SMP31	0.098 SMP39	0.092 SMP47	0.106 SMP55	0.121 SMP63	0.106 SMP71	0.107 SMP79	0.166 SMP87	0.043 SMP95
H	0.087 SMP8	0.112 SMP16	0.085 SMP24	0.094 SMP32	0.104 SMP40	0.086 SMP48	0.121 SMP56	0.067 SMP64	0.102 SMP72	0.119 SMP80	0.123 SMP88	0.041 SMP96

Bio-Tek ELx800

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MMENTS

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B	0.125 SMP2	0.091 SMP10	0.121 SMP18	0.127 SMP26	0.099 SMP34	0.093 SMP42	0.088 SMP50	0.121 SMP58	0.131 SMP66	0.116 SMP74	0.188 SMP82	0.042 SMP90
C	0.108 SMP3	0.094 SMP11	0.104 SMP19	0,079 SMP27	0.090 SMP35	0.074 SMP43	0.078 SMP51	0.086 SMP59	0.109 SMP67	0.128 SMP75	0.190 SMP83	0.042 SMP91
D	0.040 SMP4	0.040 SMP12	0.041 SMP20	0.041 SMP28	0.041 SMP36	0.041 SMP44	0.041 SMP52	0.041 SMP60	0.041 SMP68	0.041 SMP76	0.042 SMP84	0.044 SMP92
E	0.042 SMP5	0.042 SMP13	0.042 SMP21	0.042 SMP29	0.042 SMP37	0.042 SMP45	0.042 SMP53	0.042 SMP61	0.042 SMP69	0.041 SMP77	0.041 SMP85	0.042 SMP93
F	0.085 SMP6	0.103 SMP14	0.081 SMP22	0.119 SMP30	0.094 SMP38	0.115 SMP46	0.078 SMP54	0.109 SMP62	0.105 SMP70	0.123 SMP78	0.113 SMP86	0.042 SMP94
G	0.087 SMP7	0.081 SMP15	0.065 SMP23	0.117 SMP31	0.068 SMP39	0.102 SMP47	0.099 SMP55	0.099 SMP63	0.112 SMP71	0.095 SMP79	0.167 SMP87	0.042 SMP95
H	0.090 SMP8	0.116 SMP16	0.070 SMP24	0.096 SMP32	0.061 SMP40	0.104 SMP48	0.106 SMP56	0.092 SMP64	0.087 SMP72	0.081 SMP80	0.118 SMP88	0.042 SMP96

Doxy cyclin	1	2	3	4	5	6	7	8	9	10	11	12
A	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
B	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
C	100% PDL1	90% PDL 1	80% PDL 1	70% PDL 1	60% PDL 1	50% PDL 1	40% PDL 1	30% PDL 1	20% PDL 1	10% PDL 1	Control PDL1	
D												
E												
F	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	
G	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	
H	100% PDL2	90% PDL 2	80% PDL 2	70% PDL 2	60% PDL 2	50% PDL 2	40% PDL 2	30% PDL 2	20% PDL 2	10% PDL 2	Control PDL2	

Plate no 4. Immediately
5. 24 hours
6. 48 hours

Oxy

Bio-Tek ELx800

say: Quick Read Date: 24/05/05 Lot: Plate 4 : Imm

Time: 01:00:02PM Operator:

velength: 490 Temp: Plate ID:

MMENTS

	1	2	3	4	5	6	7	8	9	10	11	12
A												
OD	0.073 SMP1	0.083 SMP9	0.089 SMP17	0.093 SMP25	0.086 SMP33	0.089 SMP41	0.092 SMP49	0.090 SMP57	0.119 SMP65	0.078 SMP73	0.085 SMP81	0.041 SMP89
B												
OD	0.084 SMP2	0.080 SMP10	0.074 SMP18	0.099 SMP26	0.083 SMP34	0.081 SMP42	0.088 SMP50	0.076 SMP58	0.088 SMP66	0.077 SMP74	0.097 SMP82	0.041 SMP90
C												
OD	0.086 SMP3	0.093 SMP11	0.055 SMP19	0.092 SMP27	0.085 SMP35	0.080 SMP43	0.087 SMP51	0.077 SMP59	0.083 SMP67	0.082 SMP75	0.068 SMP83	0.042 SMP91
D												
OD	0.043 SMP4	0.041 SMP12	0.042 SMP20	0.043 SMP28	0.041 SMP36	0.041 SMP44	0.042 SMP52	0.041 SMP60	0.041 SMP68	0.041 SMP76	0.042 SMP84	0.041 SMP92
E												
OD	0.042 SMP5	0.042 SMP13	0.042 SMP21	0.042 SMP29	0.042 SMP37	0.042 SMP45	0.042 SMP53	0.041 SMP61	0.043 SMP69	0.043 SMP77	0.042 SMP85	0.042 SMP93
F												
OD	0.109 SMP6	0.078 SMP14	0.078 SMP22	0.053 SMP30	0.072 SMP38	0.086 SMP46	0.081 SMP54	0.104 SMP62	0.084 SMP70	0.086 SMP78	0.084 SMP86	0.043 SMP94
G												
OD	0.073 SMP7	0.072 SMP15	0.070 SMP23	0.090 SMP31	0.085 SMP39	0.076 SMP47	0.084 SMP55	0.097 SMP63	0.078 SMP71	0.077 SMP79	0.085 SMP87	0.044 SMP95
H												
OD	0.082 SMP8	0.081 SMP16	0.078 SMP24	0.090 SMP32	0.100 SMP40	0.088 SMP48	0.074 SMP56	0.077 SMP64	0.079 SMP72	0.075 SMP80	0.095 SMP88	0.044 SMP96

Bio-Tek ELx800

say: Quick Read
/elength: 490

Date: 25/05/05
Time: 10:36:27AM
Temp:

Lot: S 24h
Operator:
Plate ID:

MENTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.158 SMP1	0.116 SMP9	0.161 SMP17	0.200 SMP25	0.201 SMP33	0.144 SMP41	0.135 SMP49	0.140 SMP57	0.108 SMP65	0.118 SMP73	0.109 SMP81	0.042 SMP89
B	0.130 SMP2	0.127 SMP10	0.147 SMP18	0.168 SMP26	0.169 SMP34	0.128 SMP42	0.123 SMP50	0.119 SMP58	0.139 SMP66	0.086 SMP74	0.080 SMP82	0.042 SMP90
C	0.127 SMP3	0.178 SMP11	0.140 SMP19	0.134 SMP27	0.146 SMP35	0.096 SMP43	0.157 SMP51	0.136 SMP59	0.102 SMP67	0.128 SMP75	0.099 SMP83	0.042 SMP91
D	0.042 SMP4	0.042 SMP12	0.041 SMP20	0.043 SMP28	0.042 SMP36	0.042 SMP44	0.042 SMP52	0.042 SMP60	0.035 SMP68	0.042 SMP76	0.042 SMP84	0.036 SMP92
E	0.043 SMP5	0.042 SMP13	0.042 SMP21	0.042 SMP29	0.043 SMP37	0.041 SMP45	0.043 SMP53	0.041 SMP61	0.042 SMP69	0.041 SMP77	0.042 SMP85	0.041 SMP93
F	0.195 SMP6	0.113 SMP14	0.127 SMP22	0.153 SMP30	0.127 SMP38	0.157 SMP46	0.142 SMP54	0.141 SMP62	0.123 SMP70	0.092 SMP78	0.114 SMP86	0.041 SMP94
G	0.185 SMP7	0.159 SMP15	0.152 SMP23	0.185 SMP31	0.157 SMP39	0.124 SMP47	0.119 SMP55	0.146 SMP63	0.099 SMP71	0.122 SMP79	0.089 SMP87	0.042 SMP95
H	0.206 SMP8	0.215 SMP16	0.164 SMP24	0.126 SMP32	0.144 SMP40	0.155 SMP48	0.141 SMP56	0.121 SMP64	0.101 SMP72	0.089 SMP80	0.106 SMP88	0.043 SMP96

Bio-Tek ELx800

say: Quick Read
velength: 490

Date: 26/05/05
Time: 10:39:49AM
Temp:

Lot: 6 ; 48h
Operator:
Plate ID:

MMENTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.135 SMP1	0.164 SMP9	0.093 SMP17	0.132 SMP25	0.119 SMP33	0.098 SMP41	0.092 SMP49	0.107 SMP57	0.103 SMP65	0.096 SMP73	0.155 SMP81	0.041 SMP89
B	0.122 SMP2	0.140 SMP10	0.104 SMP18	0.102 SMP26	0.109 SMP34	0.138 SMP42	0.124 SMP50	0.112 SMP58	0.085 SMP66	0.096 SMP74	0.180 SMP82	0.042 SMP90
C	0.100 SMP3	0.096 SMP11	0.097 SMP19	0.138 SMP27	0.085 SMP35	0.094 SMP43	0.109 SMP51	0.090 SMP59	0.094 SMP67	0.103 SMP75	0.182 SMP83	0.041 SMP91
D	0.042 SMP4	0.042 SMP12	0.042 SMP20	0.042 SMP28	0.041 SMP36	0.041 SMP44	0.041 SMP52	0.041 SMP60	0.042 SMP68	0.041 SMP76	0.041 SMP84	0.042 SMP92
E	0.042 SMP5	0.043 SMP13	0.041 SMP21	0.041 SMP29	0.041 SMP37	0.041 SMP45	0.042 SMP53	0.041 SMP61	0.041 SMP69	0.042 SMP77	0.041 SMP85	0.041 SMP93
F	0.124 SMP6	0.137 SMP14	0.139 SMP22	0.080 SMP30	0.121 SMP38	0.125 SMP46	0.114 SMP54	0.096 SMP62	0.095 SMP70	0.098 SMP78	0.082 SMP86	0.041 SMP94
G	0.135 SMP7	0.105 SMP15	0.129 SMP23	0.128 SMP31	0.100 SMP39	0.131 SMP47	0.116 SMP55	0.112 SMP63	0.128 SMP71	0.103 SMP79	0.101 SMP87	0.045 SMP95
H	0.141 SMP8	0.117 SMP16	0.116 SMP24	0.118 SMP32	0.118 SMP40	0.126 SMP48	0.109 SMP56	0.096 SMP64	0.086 SMP72	0.125 SMP80	0.082 SMP88	0.042 SMP96

MC	PDL 1	1h	40 – 50
MC	PDL 2	1h	60 – 70
MC	PDL 1	24h	10 – 20
MC	PDL 2	24h	10 – 20
MC	PDL 1	48h	10 – 20
MC	PDL 2	48h	10 – 20

DC	PDL 1	1h	0 – 10
DC	PDL 2	1h	30 – 40
DC	PDL 1	24h	10 – 20
DC	PDL 2	24h	10 – 20
DC	PDL 1	48h	0 – 10
DC	PDL 2	48h	0 – 10

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Bio-Tek ELx800

MC

ay: Quick Read
elength:490

Date:13/09/05
Time:02:49:16PM
Temp:

Lot:
Operator:
Plate ID: 1h

IMENTS

1	2	3	4	5	6	7	8	9	10	11	12
0.222 SMP1	0.261 SMP9	0.314 SMP17	0.464 SMP25	0.425 SMP33	0.575 SMP41	0.471 SMP49	0.567 SMP57	0.689 SMP65	0.584 SMP73	0.579 SMP81	0.041 SMP89
0.283 SMP2	0.272 SMP10	0.258 SMP18	0.491 SMP26	0.491 SMP34	0.630 SMP42	0.764 SMP50	0.587 SMP58	0.665 SMP66	0.579 SMP74	0.517 SMP82	0.039 SMP90
0.185 SMP3	0.241 SMP11	0.228 SMP19	0.360 SMP27	0.290 SMP35	0.398 SMP43	0.556 SMP51	0.441 SMP59	0.582 SMP67	0.482 SMP75	0.339 SMP83	0.040 SMP91
0.044 SMP4	0.043 SMP12	0.045 SMP20	0.046 SMP28	0.047 SMP36	0.043 SMP44	0.043 SMP52	0.045 SMP60	0.045 SMP68	0.048 SMP76	0.044 SMP84	0.046 SMP92
0.045 SMP5	0.047 SMP13	0.048 SMP21	0.046 SMP29	0.048 SMP37	0.046 SMP45	0.047 SMP53	0.049 SMP61	0.044 SMP69	0.048 SMP77	0.048 SMP85	0.046 SMP93
0.111 SMP6	0.118 SMP14	0.134 SMP22	0.160 SMP30	0.178 SMP38	0.150 SMP46	0.173 SMP54	0.167 SMP62	0.340 SMP70	0.289 SMP78	0.361 SMP86	0.043 SMP94
0.108 SMP7	0.128 SMP15	0.175 SMP23	0.187 SMP31	0.228 SMP39	0.145 SMP47	0.206 SMP55	0.281 SMP63	0.191 SMP71	0.247 SMP79	0.393 SMP87	0.038 SMP95
0.115 SMP8	0.104 SMP16	0.150 SMP24	0.139 SMP32	0.227 SMP40	0.188 SMP48	0.241 SMP56	0.298 SMP64	0.395 SMP72	0.437 SMP80	0.445 SMP88	0.046 SMP96

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Bio-Tek ELx800

say: Quick Read
velength: 490

Date: 14/09/05
Time: 12:21:37PM
Temp:

Lot:
Operator: MC
Plate ID: 24h

MMENTS

	1 100%	2	3	4	5	6	7	8	9	10 10%	11 Control	12
A OD	0.210 SMP1	0.378 SMP9	0.466 SMP17	0.343 SMP25	0.445 SMP33	0.294 SMP41	0.452 SMP49	0.498 SMP57	0.481 SMP65	0.579 SMP73	0.741 SMP81	0.041 SMP89
B OD	0.203 SMP2	0.307 SMP10	0.403 SMP18	0.310 SMP26	0.335 SMP34	0.256 SMP42	0.406 SMP50	0.415 SMP58	0.462 SMP66	0.501 SMP74	0.743 SMP82	0.037 SMP90
C OD	0.206 SMP3	0.367 SMP11	0.389 SMP19	0.287 SMP27	0.279 SMP35	0.291 SMP43	0.444 SMP51	0.390 SMP59	0.504 SMP67	0.474 SMP75	0.723 SMP83	0.041 SMP91
D OD	0.046 SMP4	0.046 SMP12	0.046 SMP20	0.046 SMP28	0.048 SMP36	0.045 SMP44	0.046 SMP52	0.054 SMP60	0.046 SMP68	0.046 SMP76	0.044 SMP84	0.045 SMP92
E OD	0.045 SMP5	0.050 SMP13	0.046 SMP21	0.046 SMP29	0.049 SMP37	0.046 SMP45	0.048 SMP53	0.050 SMP61	0.048 SMP69	0.046 SMP77	0.046 SMP85	0.045 SMP93
F OD	0.205 SMP6	0.375 SMP14	0.305 SMP22	0.486 SMP30	0.377 SMP38	0.443 SMP46	0.433 SMP54	0.487 SMP62	0.535 SMP70	0.632 SMP78	0.661 SMP86	0.047 SMP94
G OD	0.246 SMP7	0.366 SMP15	0.340 SMP23	0.489 SMP31	0.370 SMP39	0.421 SMP47	0.363 SMP55	0.399 SMP63	0.484 SMP71	0.471 SMP79	0.640 SMP87	0.046 SMP95
H OD	0.214 SMP8	0.303 SMP16	0.255 SMP24	0.379 SMP32	0.369 SMP40	0.430 SMP48	0.410 SMP56	0.441 SMP64	0.319 SMP72	0.316 SMP80	0.574 SMP88	0.046 SMP96

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Bio-Tek ELx800

ssay: Quick Read
avelength: 490

Date: 15/09/05
Time: 12:42:45PM
Temp:

Lot:
Operator: MC
Plate ID: 48h

OMMENTS

	1 100 %	2	3	4	5	6	7	8	9	10 10 %	11 Control	12
A	0.235 SMP1	0.464 SMP9	0.354 SMP17	0.347 SMP25	0.501 SMP33	0.421 SMP41	0.529 SMP49	0.569 SMP57	0.509 SMP65	0.588 SMP73	0.736 SMP81	0.042 SMP89
B	0.231 SMP2	0.338 SMP10	0.430 SMP18	0.522 SMP26	0.361 SMP34	0.549 SMP42	0.423 SMP50	0.376 SMP58	0.471 SMP66	0.571 SMP74	0.654 SMP82	0.044 SMP90
C	0.122 SMP3	0.248 SMP11	0.294 SMP19	0.242 SMP27	0.382 SMP35	0.421 SMP43	0.364 SMP51	0.345 SMP59	0.267 SMP67	0.544 SMP75	0.700 SMP83	0.051 SMP91
D	0.045 SMP4	0.045 SMP12	0.045 SMP20	0.046 SMP28	0.047 SMP36	0.046 SMP44	0.047 SMP52	0.047 SMP60	0.046 SMP68	0.045 SMP76	0.047 SMP84	0.048 SMP92
E	0.047 SMP5	0.053 SMP13	0.046 SMP21	0.047 SMP29	0.050 SMP37	0.047 SMP45	0.047 SMP53	0.057 SMP61	0.045 SMP69	0.048 SMP77	0.049 SMP85	0.047 SMP93
F	0.111 SMP6	0.128 SMP14	0.124 SMP22	0.099 SMP30	0.097 SMP38	0.149 SMP46	0.136 SMP54	0.150 SMP62	0.318 SMP70	0.469 SMP78	0.507 SMP86	0.045 SMP94
G	0.102 SMP7	0.181 SMP15	0.135 SMP23	0.102 SMP31	0.121 SMP39	0.102 SMP47	0.116 SMP55	0.182 SMP63	0.373 SMP71	0.400 SMP79	0.672 SMP87	0.046 SMP95
H	0.096 SMP8	0.089 SMP16	0.094 SMP24	0.091 SMP32	0.088 SMP40	0.097 SMP48	0.119 SMP56	0.111 SMP64	0.310 SMP72	0.554 SMP80	0.637 SMP88	0.046 SMP96

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Bio-Tek ELx800

Assay: Quick Read
Wavelength: 490

Date: 13/09/05
Time: 02:50:53PM
Temp:

Lot: DC
Operator: 1h
Plate ID:

COMMENTS

	1 100%	2	3	4	5	6	7	8	9	10 control	11 10%	12
A	0.234 SMP1	0.280 SMP9	0.442 SMP17	0.444 SMP25	0.643 SMP33	0.484 SMP41	0.496 SMP49	0.499 SMP57	0.600 SMP65	0.763 SMP73	0.759 SMP81	0.050 SMP89
B	0.234 SMP2	0.286 SMP10	0.476 SMP18	0.466 SMP26	0.643 SMP34	0.503 SMP42	0.514 SMP50	0.612 SMP58	0.625 SMP66	0.803 SMP74	0.823 SMP82	0.050 SMP90
C	0.223 SMP3	0.304 SMP11	0.445 SMP19	0.335 SMP27	0.458 SMP35	0.499 SMP43	0.497 SMP51	0.461 SMP59	0.554 SMP67	0.744 SMP75	0.705 SMP83	0.068 SMP91
D	0.056 SMP4	0.081 SMP12	0.091 SMP20	0.089 SMP28	0.059 SMP36	0.079 SMP44	0.065 SMP52	0.080 SMP60	0.049 SMP68	0.066 SMP76	0.074 SMP84	0.085 SMP92
E	0.041 SMP5	0.053 SMP13	0.067 SMP21	0.105 SMP29	0.059 SMP37	0.078 SMP45	0.080 SMP53	0.063 SMP61	0.068 SMP69	0.062 SMP77	0.052 SMP85	0.057 SMP93
F	0.198 SMP6	0.341 SMP14	0.369 SMP22	0.450 SMP30	0.376 SMP38	0.352 SMP46	0.372 SMP54	0.395 SMP62	0.353 SMP70	0.449 SMP78	0.415 SMP86	0.046 SMP94
G	0.220 SMP7	0.360 SMP15	0.247 SMP23	0.331 SMP31	0.283 SMP39	0.372 SMP47	0.384 SMP55	0.384 SMP63	0.478 SMP71	0.627 SMP79	0.459 SMP87	0.071 SMP95
H	0.176 SMP8	0.334 SMP16	0.360 SMP24	0.440 SMP32	0.298 SMP40	0.245 SMP48	0.285 SMP56	0.251 SMP64	0.473 SMP72	0.553 SMP80	0.498 SMP88	0.050 SMP96

100% 10% control

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Bio-Tek ELx800

DC

Assay: Quick Read
Wavelength: 490

Date: 14/09/05
Time: 12:19:52PM
Temp:

Lot:
Operator:
Plate ID: 24h

COMMENTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.169 SMP1	0.267 SMP9	0.440 SMP17	0.545 SMP25	0.464 SMP33	0.602 SMP41	0.455 SMP49	0.470 SMP57	0.390 SMP65	0.543 SMP73	0.951 SMP81	0.041 SMP89
B	0.191 SMP2	0.318 SMP10	0.399 SMP18	0.468 SMP26	0.466 SMP34	0.489 SMP42	0.500 SMP50	0.586 SMP58	0.468 SMP66	0.577 SMP74	0.827 SMP82	0.044 SMP90
C	0.206 SMP3	0.300 SMP11	0.481 SMP19	0.493 SMP27	0.540 SMP35	0.462 SMP43	0.596 SMP51	0.704 SMP59	0.403 SMP67	0.597 SMP75	0.795 SMP83	0.056 SMP91
D	0.047 SMP4	0.049 SMP12	0.054 SMP20	0.057 SMP28	0.064 SMP36	0.053 SMP44	0.049 SMP52	0.057 SMP60	0.062 SMP68	0.061 SMP76	0.058 SMP84	0.045 SMP92
E	0.046 SMP5	0.053 SMP13	0.050 SMP21	0.060 SMP29	0.055 SMP37	0.047 SMP45	0.056 SMP53	0.053 SMP61	0.046 SMP69	0.060 SMP77	0.046 SMP85	0.056 SMP93
F	0.136 SMP6	0.277 SMP14	0.263 SMP22	0.368 SMP30	0.235 SMP38	0.332 SMP46	0.360 SMP54	0.521 SMP62	0.553 SMP70	0.560 SMP78	0.670 SMP86	0.039 SMP94
G	0.147 SMP7	0.228 SMP15	0.356 SMP23	0.330 SMP31	0.329 SMP39	0.294 SMP47	0.446 SMP55	0.443 SMP63	0.485 SMP71	0.553 SMP79	0.485 SMP87	0.039 SMP95
H	0.132 SMP8	0.169 SMP16	0.325 SMP24	0.260 SMP32	0.248 SMP40	0.303 SMP48	0.304 SMP56	0.447 SMP64	0.478 SMP72	0.432 SMP80	0.577 SMP88	0.042 SMP96

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Bio-Tek ELx800

DC

Assay: Quick Read
Wavelength: 490

Date: 15/09/05
Time: 12:44:56PM
Temp:

Lot:
Operator:
Plate ID: 48h

COMMENTS

	1 100 %	2	3	4	5	6	7	8	9	10 10%	11 Control	12
A	0.205 SMP1	0.211 SMP9	0.250 SMP17	0.296 SMP25	0.183 SMP33	0.231 SMP41	0.312 SMP49	0.365 SMP57	0.379 SMP65	0.493 SMP73	0.815 SMP81	0.047 SMP89
B	0.265 SMP2	0.210 SMP10	0.263 SMP18	0.240 SMP26	0.288 SMP34	0.266 SMP42	0.423 SMP50	0.429 SMP58	0.391 SMP66	0.459 SMP74	0.880 SMP82	0.052 SMP90
C	0.283 SMP3	0.220 SMP11	0.287 SMP19	0.234 SMP27	0.259 SMP35	0.385 SMP43	0.405 SMP51	0.354 SMP59	0.293 SMP67	0.415 SMP75	0.790 SMP83	0.052 SMP91
D	0.046 SMP4	0.047 SMP12	0.045 SMP20	0.045 SMP28	0.045 SMP36	0.045 SMP44	0.045 SMP52	0.047 SMP60	0.047 SMP68	0.045 SMP76	0.045 SMP84	0.045 SMP92
E	0.045 SMP5	0.044 SMP13	0.046 SMP21	0.046 SMP29	0.047 SMP37	0.044 SMP45	0.045 SMP53	0.048 SMP61	0.044 SMP69	0.045 SMP77	0.051 SMP85	0.046 SMP93
F	0.181 SMP6	0.191 SMP14	0.202 SMP22	0.256 SMP30	0.236 SMP38	0.270 SMP46	0.294 SMP54	0.268 SMP62	0.421 SMP70	0.443 SMP78	0.594 SMP86	0.044 SMP94
G	0.206 SMP7	0.204 SMP15	0.272 SMP23	0.235 SMP31	0.235 SMP39	0.278 SMP47	0.264 SMP55	0.239 SMP63	0.372 SMP71	0.367 SMP79	0.506 SMP87	0.044 SMP95
H	0.202 SMP8	0.210 SMP16	0.226 SMP24	0.208 SMP32	0.176 SMP40	0.216 SMP48	0.230 SMP56	0.225 SMP64	0.422 SMP72	0.504 SMP80	0.731 SMP88	0.043 SMP96