Comparative Evaluation of Biocompatibility Assays used in Polymer Biomaterials Assessment

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Most of the biocompatibility assays - metabolic, DNA quantitation and membrane permeability - are commonly used to evaluate biomaterials with medical and dental applications. Comparing the results and efficacy among these various protocols may lead to a faster and more accurate choice for a specific assay according to the evaluated biomaterial and its physical/chemical features. The aim of the present work was to perform a reliability comparison between metabolic, membrane integrity and DNA content assays, with respect to biocompatibility of biomaterials with biomedical use. Using chitosan films as polymer support we have observed a good correlation (0.7<R2<1) between cell proliferation evaluated by MTT assay and the DNA quantitation for both tumor and normal phenotype cell lines. For all investigated cell lines grown on chitosan films, less epithelial phenotype MCF10A, there was a good correlation for the two metabolic assays, MTT and CellTiterBlue. Same good correlation was noticed for SytoxGreen (membrane integrity assay) and CyQuant (DNA quantitation). Correlation study performed for metabolic/membrane integrity/DNA quantitation assays demonstrated the usefulness of a permanent adaptation of the cytotoxicity assay for biomaterial evaluation according to physical/chemical and mechanical sample features. For the polymer films used in the present study (chitosan), metabolic assays and DNA quantitation assays showed well correlated results for both tumor and normal phenotype cells. Both assay types may be used, but the final choice will respect potential interferences of the reagents in the assays with biomaterial compounds.

Keywords: biocompatibility, biomaterials, metabolic/membrane integrity/DNA quantitation assays, chitosan film, normal phenotype cells

Polymers are widely used in dental applications. Prosthetic and restorative materials are currently applied in oral and maxillofacial surgery, periodontology or orthodontic treatments and impression materials [1-3]. In most clinical and experimental applications, biomaterials are getting in contact with a cellular element of a tissue or organ and thus, assays to evaluate biocompatibility should be carefully selected in order to provide reliable and reproducible results [4, 5]. The most used biocompatibility assays are falling in one of the three categories: metabolic, DNA quantitation and membrane permeability evaluation. Metabolic assays may speculate tetrazolium salts reduction (MTT, MTS, XTT or WST-1 assays), resazurin-resorufin reduction assays or ATP/LDH quantitation assays. Membrane integrity-based assays include but they are not limited to: trypan-blue exclusion assay, propidium iodide inclusion assay, neutral red assay, LDH leakage assay, SytoxGreen assay [6-8]. DNA quantitation assays include CyQuant and PicoGreen, in which cyanine stain shows reduced fluorescence in free state and important fluorescence while binding nucleic acids [9]. Most of the biocompatibility assays are based on viability/proliferation evaluation by metabolic, membrane integrity or DNA content means. Comparing the results and efficacy among these various protocols may lead to a faster and more accurate choice for a specific assay according to the evaluated biomaterial and its physical/chemical features. Results comparison for the mentioned assays is purely visual and thus a comparative analysis is useful to help the selection of the most appropriate one.

The aim of the present work was to perform a reliability comparison between metabolic, membrane integrity and DNA content assays, with respect to biocompatibility of biomaterials with biomedical use. Comparisons were performed between variations of viable cell number for metabolic assays and membrane integrity and DNA content for DNA-quantitation assays. While the comparisons among the up mentioned assays is purely visual, a correlation between their results was performed for 4 selected cell lines on chitosan polymer films, as shown in material and methods.

Experimental part

To evaluate cell viability in contact with polymer films, chitosan was used as biomaterial, as being used in numerous biomedical devices and composite biomaterials. Chitosan films were generated in black 96 well flat clear bottom plates, from a sterile 1% acetic acid solution. Plates were placed in a cell culture hood for 5 h to allow the films to dry, then in a standard cell culture incubator, at 37°C, 5%CO_2 and 95% humidity, overnight. Prior to cell placement in the chitosan coated plates, wells were washed twice by sterile phosphate buffered saline (PBS).

Cell lines

The experimental design included four cell lines, normal dermal human fibroblasts (NDHF), human osteosarcoma cells (HOS), normal human osteoblasts (HOB), normal epithelial mammary cells (MCF10A). All cell lines were

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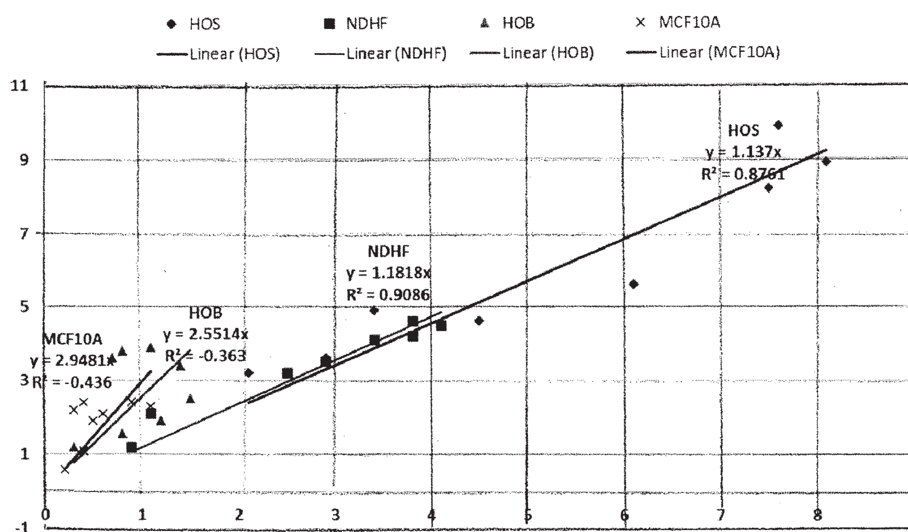

Fig. 1. Correlations between cell proliferation evaluation by MTT and CyQuant assays for 4 cell lines (HOS – human osteosarcoma; NDHF – normal dermal human fibroblast; HOB – human osteoblast; MCF10A – epithelial phenotype)

proliferated and splitted using specific culture media for each of them.

NDHF cells were proliferated in Fibroblast growth media with supplements (Lonza). HOS were proliferated in MEM (Minimal Essential Medium) supplemented by 10% FCS, 2% Glutamax and 1% Penicillin-Streptomycin. HOB were proliferated in specific osteoblast growth medium with supplements (Lonza).

MCF10A cells were proliferated according to Brugge recommendations [10,11], in DMEM/F12 supplemented by 5% horse serum, 20ng/mL EGF, 0.5mg/mL hydrocortizone hemisuccinate, 100ng/mL cholera toxin, 10ug/mL Insulin, 1% Penicillin-Streptomycin.

Cell culture methods

All cells from the selected cell lines were grown in their specific cell media prior to placement in the 96 well plates for the current experiment. For the current experiment, cells were placed in 200 uL RPMI1640 supplemented by 10% fetal calf serum (FCS), 1% Glutamax and 1% Penicillin-Streptomycin, precoated by chitosan films.

Cells were detached by TrypLE trypsin (2mL for 3 min in a 75cm^2 cell culture flask). Trypsin was inactivated by 10 mL cell culture media. Cells were gently mixed to detach them from clumps and counted by an automated cell counter (Countess Invitrogen) prior to plate dispensing. Cells from each cell line were distributed at a concentration of 3x10^4 cells/well in a 96 well plate.

Cell viability/proliferation assays

The assays applied to the selected cell lines were MTT, CellTiterBlue (resazurin-resorufin) for metabolic kits, CyQuant for DNA quantitation kit, and Sytox Green for membrane permeability evaluation. All kits were used according to manufacturer recommendations.

Statistical methods

Correlation level between the values regarding cell proliferation (for MTT and CellTiterBlue assays) and DNA quantitation for CyQuant assay was performed by the less square method in SPSS 16.0 for Windows from IBM SPSS Data Collection.

Results and discussions

Cell viability assessed by MTT were compared to DNA quantitation results of CyQuant assay (fig.1). On chitosan films there is a good correlation ($0.7 < R^2 < 1$) between cell proliferation evaluated by MTT assay and the DNA quantitation for both HOS (R^2 = 0.876) and NDHF (R^2 = 0.908) cell lines. Even if, most of the times, tumor cell lines are more reliable for biocompatibility assays, being widely used is specific polymer/metallic biomaterial evaluation, our results show a good correlation for NDHF too. This particular behaviour may be due to fibroblast growth profile regarding surface adhesion and specific geometry of membrane expansions – different from tumor cells. Their proliferation rate is slower than tumor cells but constant and resistant to moderate starvation and *p*H variations. Thus, NDHF cell proliferation is well correlated with DNA detectable amounts during polymer films biocompatibility evaluation. This cell line may be recommended for biocompatibility assays, either for fast, cheap but less reliable proliferation assays as MTT. The other two cell lines used in the current work showed a poor correlation for the MTT and CyQuant assays. Correlation scores were of R^2 = -0.363 for HOB and 0.436 for MCF10A cell lines respectively.

In the next step, correlations were performed between MTT and CellTiterBlue metabolic assays (fig. 2). For all investigated cell lines grown on chitosan films, less epithelial phenotype MCF10A, there was a good correlation for the two mentioned metabolic assays ($0.75 < R^2 < 1$). For HOS cells, correlation was less strong than for HOB and NDHF, with recorded values of R^2 = 0.753 for HOS, R^2 = 0.83 for HOB and R^2 = 0.82 for NDHF. Malignant phenotype cells (as HOS) and normal phenotype (as HOB and NDHF but not the epithelial MCF10A) showed good correlation for the metabolic assays regarding cell proliferation/viability for biocompatibility evaluation. MCF10A showed a R^2 of 0.56 but this cell line is well known as demanding in cell culture, being easily influenced by environmental factors as temperature, *p*H and growth factor concentration. For biomaterials cytotoxicity evaluation, both metabolic assays are recommended by a high confidence degree but the selection will consider the potential interferences of the reagents on the cells used.

For SytoxGreen (membrane integrity assay) and CyQuant (DNA quantitation) assays, correlation between viable cell count and DNA amount variations was performed (fig. 3). All investigated cell lines grown on chitosan films showed a good correlation for the two investigated assays. For HOS cells R^2 = 0.941 while for NDHF R^2 = 0.949. Despite different phenotype, these two cell lines can be successfully used for biocompatibility assays (mainly for polymer biomaterials). HOB cell line (normal osteoblasts) showed also a remarkable correlation for the results of the two investigated assays (R^2 = 0.922). For the epithelial MCF10A cell line the correlation was

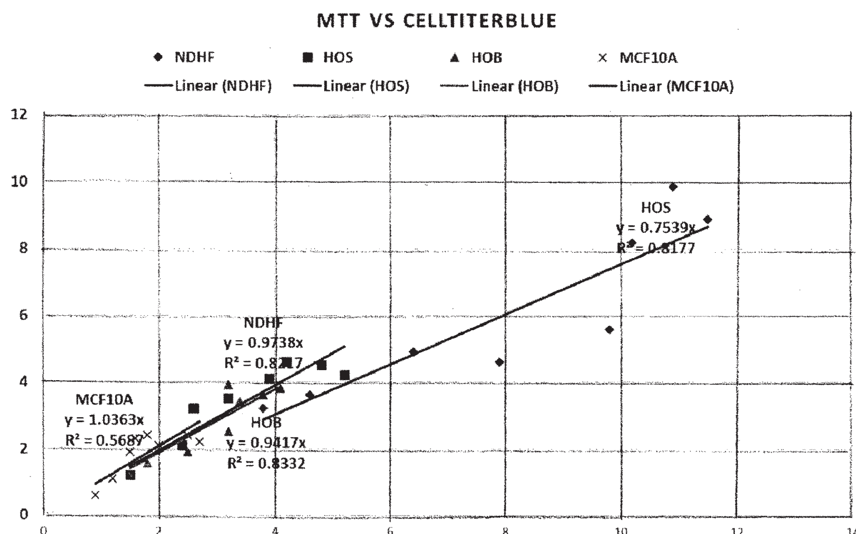

Fig. 2. Correlations between cell proliferation evaluation by MTT and CellTiterBlue for 4 cell lines (HOS – human osteosarcoma; NDHF – normal dermal human fibroblast; HOB – human osteoblast; MCF10A – epithelial phenotype)

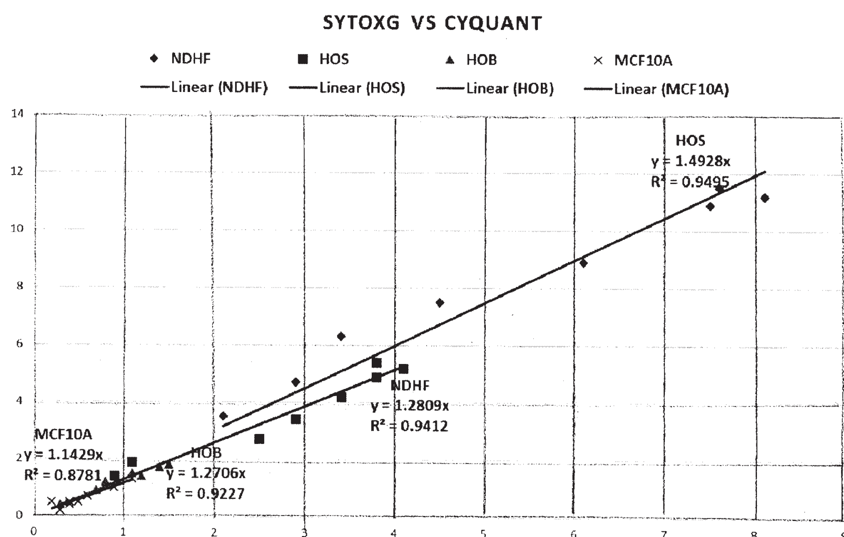

Fig. 3. Correlations between cell proliferation evaluation by SytoxGreen and CyQuant assays for 4 cell lines (HOS – human osteosarcoma; NDHF – normal dermal human fibroblast; HOB – human osteoblast; MCF10A – epithelial phenotype)

slightly reduced but the R^2 value was higher than 0.8 (R^2 = 0.878). The assay to be selected will definitely have to consider the cost and availability, mainly for screening purposes.

Biomaterial cytotoxicity depict the events developed at molecular level in cells in direct/indirect contact with the samples to be evaluated [12]. In respect to the chemical/mechanical signaling incoming from the biomaterial, cells may show extended viability and proliferation or, by contrary, cell death induction (e.g. necrosis, apoptosis) [13,14]. Membrane permeability assay (SytoxGreen) may be used either as end-point assay or in time-lapse assays, being cheap and quite reliable [15].

Conclusions

Correlation study performed for metabolic/membrane integrity/DNA quantitation assays demonstrated the usefulness of a permanent adaptation of the cytotoxicity assay for biomaterial evaluation according to physical/chemical and mechanical sample features. For the polymer films used in the present study (chitosan), metabolic assays and DNA quantitation assays showed well correlated results for both tumor and normal phenotype cells. Both assay types may be used, but the final choice will respect potential interferences of the reagents in the assays with biomaterial compounds.

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