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PATRÍCIA CORREIA DE SIQUEIRA

CITOTOXICIDADE DE CIMENTOS DE IONÔMERO DE VIDRO COM ADIÇÃO DE NANOPARTÍCULAS DE PRATA

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PATRÍCIA CORREIA DE SIQUEIRA

CITOTOXICIDADE DE CIMENTOS DE IONÔMERO DE VIDRO COM ADIÇÃO DE NANOPARTÍCULAS DE PRATA

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Goiás para obtenção do título de Mestre em Odontologia, área de concentração Clínica Odontológica.

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Citotoxidade de cimentos de ionômero de vidro com adição de nanopartículas de prata.

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“E você aprende que realmente pode suportar, que realmente é forte, e que pode ir muito mais longe depois de pensar que não se pode mais.”

(Willian Shakespeare)

RESUMO

O objetivo do presente trabalho foi avaliar e comparar a citotoxicidade de dois cimentos de ionômero de vidro (CIV) indicados para forramento, sendo um convencional (GC Gold Label 1 – GC Corporation) e um modificado por resina (Vitrebond – 3M ESPE), com e sem adição de nanopartículas de prata (NAg). As NAg foram incorporadas aos materiais durante sua manipulação em duas concentrações em massa: 0,1% e 0,2%. Espécimes com dimensões padronizadas (4 x 2 mm), com e sem NAg, foram confeccionados, e para o preparo de extratos líquidos dos cimentos, os espécimes foram imersos em 400 µL de meio de cultura e incubados em estufa a 37ºC e 5% de CO_2 por 48 horas. Os extratos obtidos foram incubados em contato com as células por 48 horas em estufa. Como controles negativo e positivo foram usados, respectivamente, meio de cultura e solução de NAg a 0,78% em massa. Dois testes para realizar a avaliação da viabilidade celular foram utilizados: o ensaio colorimétrico do MTT e o ensaio de Azul de Tripano. Os dados obtidos foram tabulados e submetidos à análise estatística com ANOVA e Tukey (α=0,05). Foi observada redução significativa na viabilidade celular em todos os grupos do Vitrebond ($p<0,001$), em comparação ao controle negativo. Não foram observadas diferenças estaticamente significantes entre os grupos desse cimento com NAg e o grupo sem NAg ($p>0,05$). Para o GC Gold Label 1, não foram observadas diferenças estaticamente significantes da viabilidade celular entre os grupos experimentais em comparação ao controle negativo ($p>0,05$). Também não houve diferença significante entre os grupos com NAg e sem NAg ($p>0,05$). No controle positivo observou-se redução significante da viabilidade celular ($p<0,001$). Pela metodologia empregada, concluiu-se que as NAg não influenciaram na citotoxicidade dos CIVs avaliados.

Palavras-chave: Cimentos de ionômeros de vidro. Citotoxicidade.Técnicas de cultura de células. Nanotecnologia. Nanopartículas metálicas.

ABSTRACT

CYTOTOXICITY OF GLASS IONOMER CEMENTS WITH ADDITION OF SILVER NANOPARTICLES

The aim of this study was to evaluate and compare the cytotoxicity of two glass ionomer cements (GIC), a conventional (GC Gold Label 1 – GC Corporation) and a resin modified (Vitrebond – 3M ESPE), both indicated for lining, with and without addition of silver nanoparticles (NAg). The NAg were incorporated at the materials in two different concentrations by weight: 0.1% and 0.2%. Specimens with standardized dimensions (4 x 2 mm) were prepared, and for preparing liquid extracts of the cements, the specimens were immersed in 400 µL of culture medium and incubated at 37°C and 5% CO_2 for 48 hours. The extracts obtained were incubated in contact with cells for 48 hours. As negative and positive controls were used respectively culture medium and solution of NAg at 0.78% by weight. To evaluate cellular viability, MTT and Trypan Blue assays were used. Data were subjected to statistical analysis with ANOVA and Tukey (α=0.05). Significant decrease in cell viability was observed in all groups of Vitrebond ($p<0.001$) compared to negative control. There were no statistically significant differences between the groups of this cement with and without NAg ($p>0.05$). For GC Gold Label 1, no statistically significant differences were observed in cell viability between any of the groups compared with the negative control ($p>0.05$). There was also no difference between the groups with and without NAg ($p>0.05$). The positive control showed significant reduction in cell viability ($p<0.001$). It is concluded that the NAg did not influence on cytotoxicity of GICs evaluated.

Keywords: Glass Ionomer Cements. Cytotoxicity. Cell Culture Techniques. Nanotechnology. Metal Nanoparticles.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância
ATP	Adenosine triphosphate
CIV	Cimento de ionômero de vidro
DMEM	Dulbecco's Modified Eagle's Medium
DO	Densidade óptica
EDTA	Ethylenediamine tetraacetic acid
GIC	Glass Ionomer Cement
IL	Illinois
ISO	International Organization for Standardization
MDPC-23	Mouse Dental Papilla Cells
MEV	Microscopia eletrônica de varredura
MI	Michigan
MN	Minnesota
MO	Missouri
MTT	Brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil-tetrazólio
NAg	Nanopartículas de prata
NY	New York
PBS	Phosphate Buffered Saline
ROS	Reactive oxygen species
rpm	Rotação por minuto
SDS	Sodium Dodecyl Sulfate
SFB	Soro fetal bovino
UNESP	Universidade Estadual Paulista
USA	United States of America
UV	Ultravioleta
VA	Virgínia

LISTA DE SÍMBOLOS

Ag	Prata
nm	Nanômetro
μg	Micrograma
%	Porcentagem
mL	Mililitro
U	Unidade
°C	Graus Celsius
g	Grama
L	Litro
CO_2	Dióxido de carbono
M	Molar
μL	Microlitro
cm^2	Centímetro quadrado
mg	Miligrama
α	Nível de significância
p	Valor de probabilidade

SUMÁRIO

1 INTRODUÇÃO

A função primária da polpa dentária é a formação de dentina pelos odontoblastos dispostos em sua periferia, que mantém seus prolongamentos no interior do tecido recém-formado, criando canais que são responsáveis pela nutrição da dentina (ARANA-CHAVEZ; MASSA, 2003; MODENA *et al.,* 2009; HEBLING *et al.,* 2010). O transporte de fluidos e nutrientes mantém a vitalidade pulpar e a resiliência necessária para neutralizar as tensões mastigatórias sobre a dentina (MODENA *et al.,* 2009). Além da formação de dentina, a polpa é responsável também pela resposta a diferentes estímulos, principalmente pelo mecanismo da inflamação (HEBLING *et al.,* 2010; KRIFKA *et al.,* 2013).

O tratamento restaurador visa o restabelecimento funcional e estético dos dentes. Para que seja alcançada plenitude funcional, os procedimentos operatórios e restauradores em polpas vitais devem respeitar seus preceitos biológicos (HEBLING *et al.,* 2010). Estudos têm demonstrado que além de bactérias e seus produtos tóxicos, os próprios materiais dentários aplicados sobre o complexo dentino-pulpar podem liberar componentes que apresentam capacidade de se difundir através dos túbulos dentinários e causar danos à polpa (HEBLING *et al*., 1999; COSTA *et al*., 2001; KRIFKA *et al.,* 2013).

A proteção do complexo dentino-pulpar tem como finalidade o restabelecimento da vitalidade pulpar, e consiste na aplicação de uma ou mais camadas de um material específico entre o material restaurador e o tecido dentário para evitar dano adicional causado pelos procedimentos operatórios, pela toxicidade do material restaurador e penetração de bactérias pela infiltração marginal (MODENA *et al.,* 2009). O complexo dentino-pulpar apresenta uma capacidade inerente de resposta defensiva frente a diferentes estímulos agressores, o qual tem como principal finalidade minimizar ou limitar os danos causados (HEBLING *et al.,* 2010).

Diferentes materiais têm sido propostos como agentes de proteção do complexo dentino-pulpar, e a compatibilidade biológica desses materiais é de suma importância para limitar ou evitar irritação ou degeneração do tecido pulpar (MODENA *et al.,* 2009).

O material forrador cavitário ideal deveria apresentar as seguintes características: apresentar boa compatibilidade biológica, não interferir nas propriedades do material restaurador (mecânicas e estéticas), apresentar propriedades mecânicas adequadas, apresentar atividade antimicrobiana (bactericida ou bacteriostática), ser isolante térmico e elétrico, apresentar bom selamento da dentina e preferencialmente, apresentar adesividade às estruturas dentárias (COSTA *et al*., 2007; HEBLING *et al.,* 2010).

Um dos materiais indicados para proteção indireta do complexo dentino-pulpar é o cimento de ionômero de vidro (CIV). Esse material foi desenvolvido na década de 70 a partir de estudos de Wilson e Kent (1972), e vem sendo aprimorado com o passar do tempo a fim de oferecer melhores propriedades e ampliar suas aplicações clínicas. As principais características que levaram ao crescimento da utilização desse material são as suas propriedades de adesividade à estrutura dentária, liberação de flúor e biocompatibilidade (MOUNT, 1995; KOVARIK *et al*., 2005).

A compatibilidade biológica dos CIVs tem sido amplamente estudada, principalmente a citotoxicidade, que pode ser definida pela capacidade desses materiais liberarem substâncias que possam causar danos ou morte celular, direta ou indiretamente, por meio da inibição de vias metabólicas (ORÉFICE *et al.,* 2006). Porém a interpretação dos resultados desses estudos é complexa, devido à variedade de formulações dos materiais incluídos na classe dos CIVs (KOVARIK *et al*., 2005).

Na literatura, é descrito que independentemente do material selecionado para aplicação sobre o assoalho de cavidades profundas, a polpa apresenta capacidade de reparação, desde que a contaminação por micro-organismos seja prevenida (COX *et al.,* 1987). Após a restauração do dente, ainda pode haver sob as restaurações micro-organismos que permaneceram no substrato dentinário após a remoção do tecido cariado e preparo cavitário ou em caso de microinfiltração (WEERHEIJIM *et al.,* 1999; CASTILHO *et al.,* 2012). Esses micro-organismos remanescentes podem se proliferar e induzir o desenvolvimento de lesões de cáries secundárias, reduzindo a longevidade da restauração e causando danos pulpares, (DAUGELA *et al.,* 2008).

O CIV apresenta propriedade antimicrobiana devido à liberação de flúor, que atua na redução da desmineralização, estimula a remineralização, interfere na

formação de biofilme e inibe o crescimento e o metabolismo bacteriano (KOVARIK *et al.,* 2005; DAUGELA *et al.,* 2008; XIE *et al.,* 2011). Porém estudos têm buscado melhorar ainda mais a atividade antimicrobiana desses materiais, adicionando agentes antimicrobianos, a fim de diminuir a ocorrência de lesões de cáries secundárias (TAKAHASHI *et al.,* 2006; TURKUN *et al.,* 2008; SAKU *et al.,* 2010; XIE *et al.,* 2011; CASTILHO *et al.,* 2012).

A prata tem sido estudada e utilizada em diversas áreas, por sua atividade antimicrobiana (MORONES *et al.,* 2005; WEIR *et al.,* 2008; MONTEIRO *et al.,* 2009; BURGERS *et al.,* 2009; KUREK *et al.,* 2011), devido ao seu amplo espectro de atividade antimicrobiana, atuando sobre bactérias Gram-positivas e negativas, fungos, protozoários e alguns vírus (MONTEIRO *et al.,* 2009; ALLAKER, 2010).

A nanotecnologia permite uma exploração eficiente das propriedades antimicrobianas de prata, usando-a sob a forma de nanopartículas (MONTEIRO *et al.,* 2009; PARK *et al.,* 2011). Estas são usadas em diferentes aplicações tais como conservantes em cosméticos, indústria têxtil, sistemas de purificação de água, revestimentos em cateteres e curativos (PARK *et al.,* 2011). Nanopartículas são caracterizadas como partículas insolúveis menores que 100 nm (MONTEIRO *et al.,* 2009; ALLAKER *et al.,* 2010; KUREK *et al.,* 2011). As nanopartículas de prata (NAg) apresentam vantagem pelo seu pequeno tamanho, que confere a elas maior área de superfície e consequentemente menor concentração de partículas necessárias para sua eficácia (MORONES *et al.,* 2005; LI *et al.,* 2013).

Recentemente a nanotecnologia tem ganhado espaço também na Odontologia. Pesquisas têm buscado a possibilidade de incorporar NAg a materiais odontológicos, a fim de melhorar suas propriedades antimicrobianas (AHN *et al.,* 2009; CHENG *et al.,* 2011; DURNER *et al.,* 2011; ZHANG *et al.,* 2012; MAGALHÃES *et al.,* 2012; LI *et al.,* 2013). Magalhães *et al.* (2012) observaram que a incorporação de NAg em um CIV modificado por resina (Vitrebond – 3M ESPE) aumentou seu efeito antimicrobiano, através do teste te difusão em ágar.

O mecanismo de ação das NAg ainda não é totalmente elucidado (ALLAKER, 2010; CHALOUPKA *et al.,* 2010), mas provavelmente está relacionado à liberação de íons prata, formação de radicais livres (ROS) e interação direta das partículas com as membranas dos micro-organismos (MORONES *et al.,* 2005; ALLAKER, 2010; CHALOUPKA *et al.,* 2010; KUREK *et al.,* 2011;).

Porém, um fator importante a ser considerado com relação à incorporação de NAg em materiais odontológicos é o seu potencial efeito tóxico sobre as células da polpa e do tecido periodontal, que poderão estar em contato direto ou indireto com esse material. Poucos trabalhos têm avaliado a influência da adição das nanopartículas de prata na citotoxicidade dos materiais dentários (ZHANG *et al.,* 2012; LI *et al.,* 2013). Alguns estudos têm relatado potencial citotóxico e genotóxico das NAg (FOLDBJERG *et al.,* 2009; PARK *et al.,* 2011; SHAVANDI *et al.,* 2011; LIM *et al.,* 2012; MARTÍNEZ-GUTIERREZ *et al.,* 2012; NYMARK *et al.,* 2012).

Frente à necessidade de assegurar uma boa resposta biológica do tecido pulpar em relação aos materiais utilizados para proteção do complexo dentino-pulpar, é justificável a realização de estudos sobre a citotoxicidade de materiais indicados para forramento de cavidades em associação com nanopartículas de prata, a fim de fornecer subsídios científicos para sua aplicação clínica de maneira segura, evitando danos adicionais à polpa.

2 OBJETIVOS

OBJETIVO GERAL

Avaliar e comparar a citotoxicidade de um CIV convencional e de um CIV modificado por resina, com e sem adição de nanopartículas de prata, em cultura de células de linhagem odontoblástica murina MDPC-23, usando os ensaios de MTT e de Azul de Tripano.

OBJETIVOS ESPECÍFICOS

- Avaliar e comparar a viabilidade de células de linhagem odontoblástica MDPC-23 após exposição a extratos dos cimentos Vitrebond e GC Gold Label 1;
- Comparar a citotoxicidade dos extratos dos cimentos Vitrebond e GC Gold Label 1 adicionados com nanopartículas de prata a 0,1% e 0,2%;
- Avaliar a influência da adição de nanopartículas de prata (NAg) na citotoxicidade desses cimentos.

3 MATERIAL E MÉTODOS

Para a avaliação da citotoxicidade dos materiais, foi realizado um estudo laboratorial *in vitro*, utilizando técnica de cultura de células. Foram utilizados dois ensaios para avaliação da viabilidade celular. As etapas dos experimentos são descritas a seguir.

3.1 CULTURA DE CÉLULAS

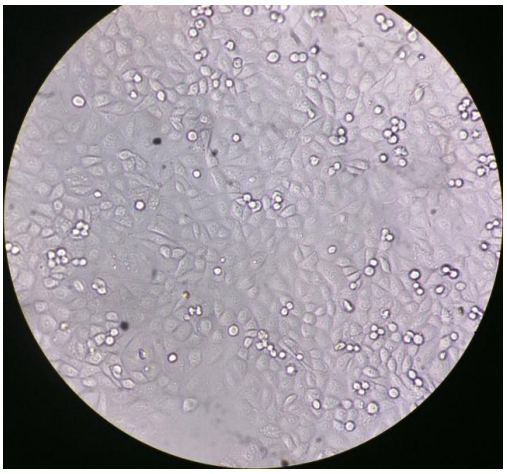
No presente estudo foram utilizadas células de linhagem odontoblástica MDPC-23 (Mouse Dental Papilla Cells), originadas de fetos de camundongo (HANKS *et al.,* 1998). As células foram cultivadas em garrafas de poliestireno para cultura de células (Figura 1) contendo meio de cultura DMEM (Dulbecco's Modified Eagle's Medium - Sigma Chemical Co., St. Louis, MO, USA), acrescido de 100 μg/mL de estreptomicina, 100 U/mL de penicilina e suplementado com 10% de soro fetal bovino (SFB - Gibco, NY, USA).

O crescimento exponencial das células foi realizado em garrafas mantidas em estufa a 37°C com atmosfera saturada em 5,0% de CO_2. Ao atingirem confluência (Figura 2), o meio de cultura foi descartado e as células lavadas com 5,0 mL de DMEM sem SFB. A seguir, aplicou-se 4,0 mL de solução de tripsina 2,500 g/L + EDTA (Ethylenediamine tetraacetic acid) 250 mg/L, e as células permaneceram por 4 minutos na estufa. Após esse período, foram aplicados 4,0 mL de meio de cultura suplementado (DMEM + SFB) e as células centrifugadas em tubo Falcon a 1500 rpm durante 10 minutos. O sobrenadante foi descartado e as células ressuspendidas em 2,0 mL de meio de cultura suplementado. A contagem de células desta suspensão foi realizada em microscópio óptico utilizando corante azul de tripano e câmara de Neubauer. Posteriormente, as células foram congeladas em nitrogênio líquido.

Os procedimentos de passagens celulares (subcultivo) foram realizados até a obtenção da quantidade necessária de células para a realização dos experimentos. Para o presente estudo foram utilizadas células que apresentavam viabilidade superior a 90,0%.

Figura 1 – Cultivo das linhagens celulares MDPC-23 em garrafas de poliestireno e meio de cultura.

Figura 2 – Células MDPC-23 em confluência. Imagem obtida em microscópio invertido (Leica Microsystems) com aumento de 1000x.



3.2 MATERIAIS

3.2.1 Cimentos de ionômero de vidro

Para a realização deste estudo foram utilizados dois cimentos de ionômero de vidro indicados para forramento de cavidades: GC Gold Label 1 (CIV convencional) e Vitrebond (CIV modificado por resina). A descrição de cada cimento está apresentada na tabela 1.

Tabela 1 - Descrição dos cimentos de ionômero de vidro utilizados no estudo.

Nome comercial (Fabricante)	Composição	Proporção pó/líquido em peso (g)
Vitrebond (3M ESPE Dental Products, St. Paul, MN, USA) Lote nº 1303800842	Pó: - vidro de fluoraluminosilicato (90-100%) - fotoiniciador (canforoquinona) Líquido: - ácido polialcenóico (35-45%) - HEMA (hidroxietilmetacrilato) (20-30%) - água (30-40%) - fotoiniciador (canforoquinona)	1.4:1
GC Gold Label 1 (GC Corporation, Tokyo, Japan) Lote nº 1111221	Pó: - vidro de fluoraluminosilicato (95%) - ácido poliacrílico (5%) Líquido: - ácido poliacrílico (30-40%) - água destilada (50-55%)	1.8:1

3.2.2 Síntese das nanopartículas de prata

As nanopartículas de prata (NAg) foram sintetizadas pelo grupo de pesquisa do Instituto de Física da Universidade Federal de Goiás, utilizando o método de síntese descrito por Solomon *et al.* (2007). O método se baseia na redução do nitrato de prata (0,001 M) (Sigma-Aldrich, St. Louis, MO, EUA) com o boridreto de sódio (0,002 M) (Sigma-Aldrich, St. Louis, MO, EUA) em baixas temperaturas controladas em agitador magnético (TE 080, Techal, São José dos Campos, SP, Brasil). Esse método pode ser descrito pela reação:

$$AgNO_3 + NaBH_4 \rightarrow Ag + ½\ H_2 + 1/2\ B_2H_6 + NaNO_3$$

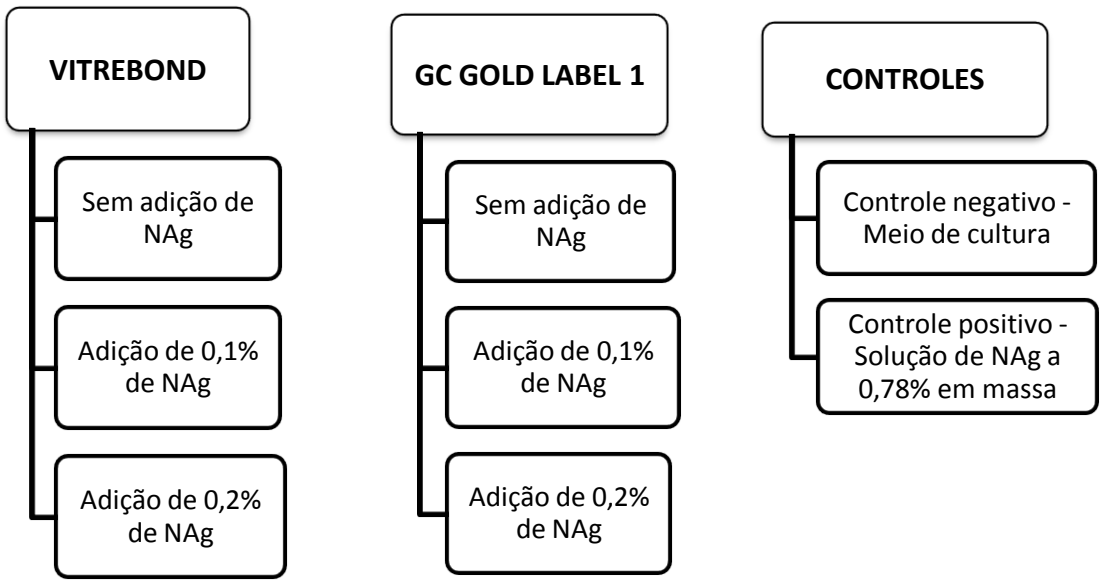
A solução obtida de NAg em água foi dividida em frascos de 45 mL e em cada frasco foi adicionado de 3,0 mL de uma solução 0,75 M de cloreto de sódio (Sigma-Aldrich, St. Louis, MO, EUA) para desestabilizar a solução e estimular a precipitação das NAg (SOLOMON *et al.*, 2007). Essa solução foi concentrada até a obtenção de uma solução aquosa com concentração de 0,78% de prata em massa. As soluções finais foram colocadas em agitador ultrassônico (USC-2800, Unique, Indaiatuba, SP, Brasil) por 3 ciclos de 10 minutos para completa dispersão do metal na solução.

3.3 CONFECÇÃO DOS ESPÉCIMES E DIVISÃO DOS GRUPOS

Os cimentos foram manipulados de acordo com as especificações de cada fabricante, adicionando prata ou não de acordo com o grupo ao qual pertenceu (Figura 3). A solução de NAg foi incorporada aos materiais durante sua manipulação, de maneira a se obter espécimes com duas concentrações em massa de prata: 0,1% e 0,2%. As porções de pó dos cimentos foram pesadas em balança analítica e as proporções foram feitas com relação ao peso do pó do cimento.

Cada cimento foi dividido em três grupos: um grupo sem NAg, um grupo com adição de 0,1% de NAg em massa e um grupo com adição de 0,2% de NAg em massa. Foram confeccionados três espécimes para cada grupo. Como controle negativo foi utilizado somente meio de cultura e como controle positivo foi utilizada a solução de NAg a 0,78%.

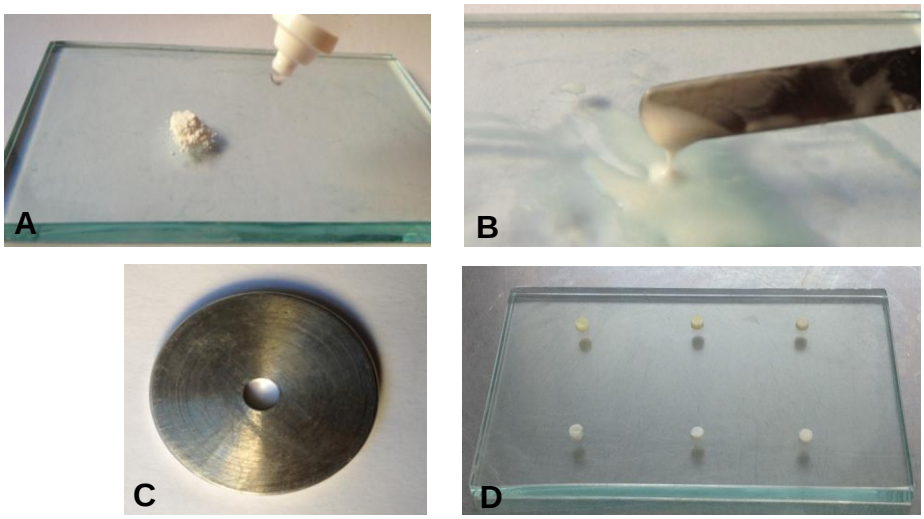
Figura 3 – Diagrama apresentando a divisão dos grupos experimentais e controles.



Para a confecção dos espécimes (Figura 4), os materiais foram manipulados e inseridos em uma matriz de aço inoxidável com geometria interna circular de 2,0 mm de espessura e 4,0 mm de diâmetro, confinada entre duas tiras de poliéster (COSTA *et* al., 2003; ARANHA *et al*., 2006). O CIV convencional (GC Gold Label 1) permaneceu na matriz por 10 minutos afim de garantir a reação de presa do material. O CIV modificado por resina (Vitrebond) foi fotopolimerizado

através da tira de poliéster com LED (LED radii-cal, SDI, Austrália) com densidade de potência de 600 mW/cm² e faixa de comprimento de onda entre 440-480nm, por 40 segundos de cada lado. A manipulação dos materiais e confecção dos espécimes foram realizadas em fluxo laminar. Após a reação de presa dos cimentos, os espécimes foram removidos da matriz e expostos a radiação ultra-violeta (UV) por 40 minutos de cada lado a fim de prevenir contaminação durante o experimento.

Figura 4 - Confecção dos espécimes. (A) Proporção dos materiais; (B) Manipulação dos cimentos de acordo com as especificações do fabricante; (C) Matriz de aço inoxidável; (D) Espécimes após a remoção da matriz e exposição à UV.

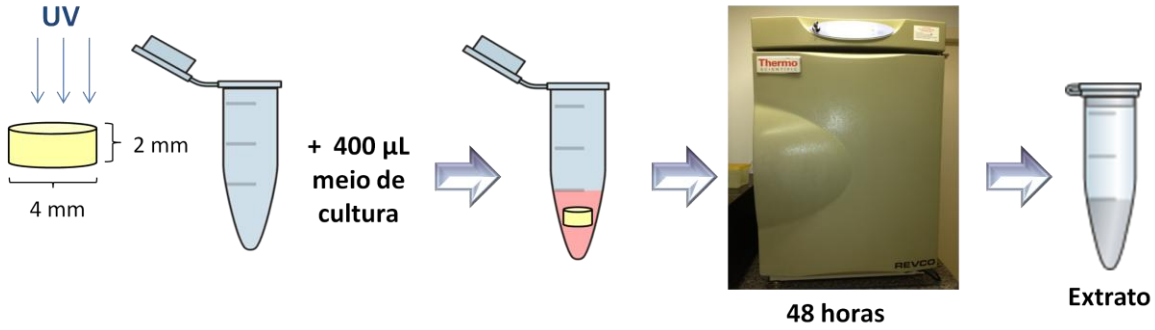


3.4. PREPARO DOS EXTRATOS

Para simular o contato indireto das células com os cimentos, foi utilizada a técnica de preparo de extratos líquidos dos materiais, preconizada pela ISO 10993-5. Para a obtenção dos extratos dos cimentos, os espécimes foram inseridos separadamente em microtubos (Eppendorf Ltda., São Paulo, SP, Brasil) e em cada microtubo foi adicionado 400 µL de meio de cultura suplementado, mantendo uma relação de área de superfície por volume de 1,25 cm^2/mL, de acordo com as especificações da norma ISO 10.993-12 (Figura 5). Os espécimes permaneceram incubados no meio de cultura por 48 horas, em estufa a 37ºC e 5,0% de CO_2.

Passado esse período, os espécimes foram removidos dos microtubos com auxílio de pinças, sendo então obtidos os extratos dos cimentos.

Figura 5 - Esquema do preparo dos extratos.



3.5 ENSAIOS DE VIABILIDADE CELULAR

As células foram semeadas na densidade de 3 x 10^4 células/cm^2 em placas de 96 poços contendo 200 µL de meio de cultura e mantidas em estufa por 24 horas para permitir a aderência das células na placa. Após esse período, o meio de cultura foi descartado da placa e a seguir foi realizado o tratamento das células. Para o controle positivo e para os grupos experimentais foram realizadas diluições de 10% dos materiais em meio de cultura. Nos grupos experimentais foram adicionados em cada poço 180 µL de meio de cultura e 20 µL dos extratos dos cimentos. No controle positivo, foram aplicados 180 µL de meio de cultura e 20 µL da solução de NAg a 0,78%. Para o controle negativo, foi utilizado somente meio de cultura suplementado (200 µL). A seguir, as células tratadas foram incubadas por 48 horas em estufa. Os experimentos foram realizados em triplicata, tendo em cada experimento 3 poços representativos para cada grupo, totalizando 9 poços por grupo.

3.5.1 Ensaio do MTT (Metiltetrazólio)

Para avaliar o metabolismo celular foi utilizado ensaio colorimétrico do MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil-tetrazólio) (MOSMANN,1983). Para o teste do MTT, ao final do período de incubação das células com os materiais, o meio de cultura dos poços foi descartado e em cada poço foi aplicado 40 µL de

PBS (phosphate buffered saline) e 10 µL de MTT na concentração de 5,0 mg/mL. Após 3 horas de incubação com o MTT, foram acrescentados 50 µL de SDS (sodium dodecyl sulfate) para paralisação da reação do MTT. A quantificação da densidade óptica (DO) foi medida em espectrofotômetro (Awareness Technology INE, Palm City, FL, USA). A porcentagem de viabilidade celular foi determinada a partir da seguinte fórmula:

$$\% \text{ Viabilidade} = \left(\frac{\text{Absorbância do tratamento}}{\text{Absorbância do controle negativo}} \right) \times 100$$

3.5.2 Ensaio do Azul de Tripano

Para avaliação da integridade celular em nível de membrana, foi realizado o ensaio do Azul de Tripano. Após o período de incubação das células com os materiais, o conteúdo dos poços foi aspirado e inserido em microtubos identificados. As células que permaneceram aderidas à placa foram lavadas com DMEM e posteriormente foram aplicados 150 µL de tripsina + EDTA em cada poço. A seguir, a placa foi incubada em estufa por 5 minutos, e após esse período, foi aplicado 150 µL de meio de cultura suplementado. O conteúdo de cada poço foi aspirado e inserido nos respectivos microtubos, e os mesmos centrifugados a 1500 rpm por 10 minutos. O sobrenadante foi descartado e as células ressuspendidas em 100 µL de meio de cultura. Uma alíquota de 10 µL da suspensão de células foi retirada e misturada com 10 µL de corante azul de tripano. Após homogeneização, foi realizada a contagem de células vivas e mortas em contador eletrônico (Luna, Logos Biosystems, Annandale, VA, USA) e os valores de viabilidade foram calculados em porcentagem pela seguinte fórmula:

$$\% \text{ Viabilidade} = \left(\frac{\text{Nº de células vivas}}{\text{Nº total de células}} \right) \times 100$$

3.6 ANÁLISE DOS DADOS

A análise estatística foi realizada pelo software *GraphPad Prism* versão 4.0. Os dados foram inicialmente analisados quanto à normalidade de distribuição, pelo teste *Kolmogorov–Smirnov*. Como a distribuição dos dados em cada grupo foi normal, foi utilizado o teste paramétrico ANOVA *one-way* e complementação com teste *Tukey* para as comparações múltiplas. O nível de significância adotado foi $\alpha=0,05$.

4 ARTIGO

CYTOTOXICITY OF GLASS IONOMER CEMENTS WITH ADDITION OF SILVER NANOPARTICLES

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Abstract

The aim of this study was to evaluate and compare the cytotoxicity of two glass ionomer cements (GIC), a conventional and a resin modified, with and without addition of silver nanoparticles (NAg). The NAg were incorporated to the materials in two different concentrations by weight: 0.1% and 0.2%. Specimens with standardized dimensions were prepared, and to prepare liquid extracts of the GICs, the specimens were immersed in 400 µL of culture medium and incubated at 37°C and 5% CO_2 for 48 h. The extracts obtained were incubated in contact with cells for 48 h. As negative and positive controls, culture medium and solution of NAg at 0.78% by weight were used respectively. To evaluate cellular viability, MTT and Trypan Blue assays were used. Data were subjected to statistical analysis with ANOVA and Tukey (α=0.05). In both tests, significant decrease in cell viability was observed in all groups of Vitrebond ($p<0.001$). There were no statistically significant differences between the groups of this cement with and without NAg ($p>0.05$). For GC Gold Label 1, no statistically significant differences were observed in cell viability between any groups compared with the negative control ($p>0.05$). It is concluded that NAg did not influence the cytotoxicity of the GICs evaluated.

Keywords: Glass Ionomer Cements. Cytotoxicity. Cell Culture Techniques. Nanotechnology. Metal Nanoparticles.

Introduction

Restorative treatment aims to reestablish the teeth function and esthetic. To obtain complete functional, operative and restorative procedures in vital pulps should respect their biological principals [1]. Studies have shown that in addition to bacteria and their toxic products, the dental materials applied on the dentin-pulp complex can also release components that are able to diffuse through the dentinal tubules and cause damage to the pulp [2-4].

The protection of dentin-pulp complex aims the re-establishment of pulp vitality, since the pulp has an inherent ability of defensive response against aggressors stimuli [1], and consists on the application of one or more layers of specific material between the restorative material and dental tissue to avoid additional damage to the pulp tissue caused by operative procedures, toxicity of restorative materials and bacteria penetration due to microleakage [4]. Different materials have been proposed as protective agents of the dentin-pulp complex and their biological compatibility is very important to avoid or limit pulp tissue irritation or degeneration [4]. One of these materials indicated for indirect protection of the dentin-pulp complex is the glass ionomer cement (GIC). The use of this material has grown due to the properties of adhesion to tooth structure, fluoride release and biocompatibility [6,7].

According to the literature, regardless of the material selected for application on the bottom of deep cavities, the pulp is capable to repair, since micro-organisms contamination is prevented [8]. After restoring a tooth, bacteria may still be present under the restoration when the tissue affected by caries is not fully removed or if there is microleakage [9,10]. This may cause an increase of bacterial

colonies under the restoration inducing secondary caries, reducing its longevity and leading to pulp damage [11].

GIC has antimicrobial property due to the release of fluoride, which reduces demineralization, stimulates remineralization, interferes in biofilm formation and inhibits bacterial growth and metabolism [7,11,12]. However, studies have been done to improve the antimicrobial activity of these materials, adding antimicrobial agents to reduce the occurrence of secondary caries [10,12-15].

Silver has been studied and used in several areas [16-20] due to its broad spectrum of antimicrobial activity against Gram positive and negative bacteria, fungi, protozoa and some viruses [18,21]. Nanotechnology allows an efficient exploitation of the silver antimicrobial properties, using it in nanoparticles form. These particles have different applications such as cosmetics, textile industry, water purification systems, catheters coatings and dressings [22]. Silver nanoparticles (NAg) are clusters of silver atoms that are insoluble and smaller than 100 nm in size [18,20,21]. Their size is an important characteristic because smaller particles give rise to higher specific surface areas, and therefore reduce the particle concentration necessary for efficacy [16,23].

Recently, studies have investigated the possibility of incorporating silver nanoparticles to dental materials in order to improve its antimicrobial properties [23-28]. However, it is important to consider the potential toxic effect of NAg incorporated in dental materials on pulp and periodontal cells, which may be in direct or indirect contact with this material. Only two studies have evaluated the influence of incorporated NAg on the cytotoxicity of dental materials [23,28], and some studies have reported cytotoxic and genotoxic effect of NAg [22,29-33].

The aim of this study was to evaluate and compare the cytotoxicity of a conventional GIC (GC Gold Label 1 – GC Corporation) and a resin modified GIC (Vitrebond – 3M ESPE), with and without addition of NAg, in an odontoblast cell line.

Materials and Methods

For this study two GICs indicated for cavity lining were used: GC Gold Label 1 (conventional GIC) and Vitrebond (resin modified GIC). The description of each cement is shown in Table 1.

Silver nanoparticles synthesis

The NAg were prepared in the Physics Institute of Federal University of Goias (UFG), by reduction of silver nitrate (0.001 M) (Sigma-Aldrich, St. Louis, USA) with sodium borohydride (0.002 M) (Sigma-Aldrich, St. Louis, USA) in controlled low temperature and magnetic stirrer (TE 080, Techal, São José dos Campos, Brazil). This method can be described by the reaction [34]:

$$AgNO_3 + NaBH_4 \rightarrow Ag + ½\ H_2 + 1/2\ B_2H_6 + NaNO_3$$

The solution obtained was added with 3.0 mL of a 0.75 M sodium chloride solution (Sigma-Aldrich, St. Louis, USA) to destabilize the solution and stimulate the precipitation of NAg [34]. Then, this solution was concentrated to obtain an aqueous solution with a concentration of 0.78% silver by weight. The final solutions were

shaken in an ultrasonic apparatus (USC-2800, Unique, Indaiatuba, Brazil) for 3 cycles of 10 minutes for complete dispersion of the metal in the solution.

Specimens preparation

The cements were manipulated according to manufacturer's specifications, adding or not silver, according to the group it belongs. The NAg solution was incorporated into the material during its manipulation, so as to obtain specimens with two concentrations of silver by weight: 0.1% and 0.2%. The portions of powder were weighed on analytical balance and proportions were made considering to the cement powder weight.

Each cement was divided into three groups: one group without NAg, one group with addition of NAg 0.1% by weight and one group with addition of NAg 0.2% by weight. Three specimens for each group were prepared. After manipulation, the cements were placed in a circle shaped stainless steel mold (2.0 mm thick, 4.0 mm diameter) and confined between two opposing polyester strips (3M ESPE, St. Paul, MN, USA). The conventional GIC (GC Gold Label 1) remained in the mold for 10 minutes in order to ensure this setting reaction. The resin modified GIC (Vitrebond) was cured using a light-curing unit based on light emitting diodes (LED) (Emitter, Schuster, Santa Maria, Brazil) with continuous polymerization technique (600 mW/cm^2) for 40 s in each side.

The materials manipulation and specimens preparation were performed in laminar flow. After the setting reaction of the cements, the specimens were removed from the mold and exposed to ultra-violet (UV) radiation for 40 minutes on each side to prevent contamination during the tests.

Extracts preparation

To simulate indirect contact of the cells with the cements, liquid extracts of the cements were prepared as recommended by ISO 10993-5 [11]. To obtain the extracts, specimens were placed separately in microtubes (Eppendorf Ltda., São Paulo, SP, Brazil) and 400 µL of culture medium DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% of fetal bovine serum (FBS) were added, maintaining a 1.25 cm^2/mL ratio of surface area/volume, according to ISO 10993-12 [12]. The medium, with the specimens immersed, were maintained for 48 hours in humidified incubator at 37 °C and 5% CO_2. After this period, the specimens were removed from the microtubes and the extracts were used for toxicity testing.

Cell viability assays

The cytotoxicity tests were performed in cultured odontoblast cell line MDPC-23 [40]. Cells were seeded at a density of 3 x 10^4 cells/cm^2 in a 96-well plate containing 200 µL of supplemented culture medium in each well. The plates were maintained in a humidified incubator at 37 °C and 5.0% CO_2 for 24 hours to allow cell adherence. After this period, the culture medium was discarded and 180 µL of supplemented culture medium and 20 µL of the extracts of the cements were added to each well, allowing a 10% dilution of the original extracts.

As negative control, only the supplemented culture medium was used (DMEM + 10% fetal bovine serum) and as positive control, solution of 0.78% NAg by weight was used. In negative control was applied 200 µL of culture medium. In positive control, 20 µL of the NAg solution and 180 µL of culture medium were applied. The treated cells were incubated for 48 hours. The experiments were

performed in triplicate, with each experiment representing 3 wells, totaling 9 wells for each material.

To assess cellular metabolism, methyltetrazolium (MTT) colorimetric assay was used. After the incubation of the cells with the extracts, the culture medium of the wells was discarded and 40 µL of PBS (Phosphate Buffered Saline) and 10 µL of MTT (5.0 mg/mL) were added. After 3 hours of incubation with MTT, 50 µL of SDS (Sodium Dodecyl Sulfate) were added to interrupt the reaction of MTT. The quantification of optical density was obtained in spectrophotometer (INE Awareness Technology, Palm City, FL, USA). These values were used to calculate the cell viability percentage.

For assessment of cell integrity at membrane level, the Trypan Blue assay was performed. After the cells incubation with the extracts, the culture medium was discarded, washed with the DMEM, and 150 µL of Trypsin + EDTA (Ethylenediamine tetraacetic acid) were applied in each well. The plate was incubated for 5 minutes, and then 150 µL of supplemented culture medium were applied. The contents of each well were aspirated, inserted into microtubes identified and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 100 µL of culture medium. An aliquot of 10 µL of cell suspension was removed and mixed with 10 µL of Trypan Blue. After homogenization, the live / dead cells count was performed with an electronic counter (Luna, Logos Biosystems, Annandale, VA, USA) and the percentage of cell viability calculated.

Statistical analysis

Results obtained for the tests were described by mean and standard deviation. Due to the normal distribution of the variables (Kolmogorov-Smirnov),

ANOVA was performed to verify the effect of the different variables and Tukey test for multiple comparisons. Statistical analysis was carried out in software GraphPad Prism version 4.0 with a significance of 5% (α=0.05).

Results

The results with the percentage of cell viability obtained by the MTT and Trypan Blue assays are shown in Table 2. The negative control presented means of cell viability of 100.6% by MTT and 93.2% by Trypan Blue.

Statistically significant decrease in cell viability was observed in Vitrebond without NAg group compared to negative control ($p<0.001$) in both testes, showing means of 61.4% by MTT and 71.3% by Trypan Blue. This significant decrease in viability occurred also in groups of this cement with both NAg concentrations ($p<0.001$). No statistically significant differences between groups with different NAg concentrations ($p>0.05$) were observed, as well as between the groups with and without NAg ($p>0.05$).

There were no statistically significant differences in cell viability in both tests between any of the GC Gold Label 1 groups (without NAg, with 0.1% and 0.2% of NAg) and negative control ($p>0.05$). Similarly, there was no significant difference between the groups with and without NAg ($p>0.05$).

In positive control (NAg solution 0.78%), statistically significant decrease in cell viability compared to the negative control was observed ($p<0.001$), with means of 68.1% by MTT and 65.4% by Trypan Blue. However, no significant difference between the positive control and the groups of Vitrebond (with and without NAg) were observed ($p>0.05$).

Figures 1 and 2 show the percentage cell viability distribution in each group by MTT and Trypan Blue assays respectively.

Discussion

The cytotoxicity of materials used on the bottom of deep cavities is very important considering the possibility of indirect contact between components of these materials and pulp cells [1]. Residual components of dental materials can diffuse through the dentinal tubules and reach the odontoblasts layer [38]. Therefore, cultures of odontoblasts are widely used to evaluate the cytotoxic effects of various dental materials [39]. Immortalized odontoblast cell line MDPC -23 (Mouse Dental Papilla Cells) was used in this study, which exhibit phenotypic markers of odontoblasts and synthesize proteins of dentinal matrix [40].

In this study, two viability assays were used. The MTT assay described by Mossman [37] reflects cell viability based on the dehydrogenase enzyme activity, located in the mitochondria of cells and is related to cell metabolism. The Trypan Blue assay was also used, and it is based on the fact that viable cells are not permeable to the dye, reflecting cell viability according to integrity of the cell membrane [41]. Results of both assays led to the same conclusions when comparing the groups studied.

The conventional GIC assessed in this study (GC Gold Label 1) showed percentages of cell viability similar to negative control ($p>0.05$). Under the employed conditions, this material was not cytotoxic to MDPC-23 cells. Despite there are no other studies evaluating the cytotoxicity of this specific cement, it is reported in literature that, in general, conventional GICs have good biocompatibility, particularly when compared to the resin modified GICs [7,42-44]. The GICs powder contains

glass particles (SiO_2, Al_2O_3, CaF_2, Na_3AlF_6, AlF_3, $AlPO_4$) and during the setting reaction, metal ions (Al^{3+}, Na^+ e Ca^{2+}) are dissolved from the powder to liquid. These metal ions are considerably non-toxic or non-irritating for living cells and tissues, as well as silica (SiO_2), considered as base substance of glass powder [7,44].

The resin modified GIC (Vitrebond) evaluated in this study showed toxic effect on the MDPC-23 cells, with significant reduction of cell viability even without addition NAg. This result is consistent with other studies in the literature [39,42-47] which report that Vitrebond shows high cytotoxicity, causing morphological changes and cell death.

The incorporation of resin components (monomers and photoinitiators) in conventional formulation of GICs is associated with their increased cytotoxic effects [4,39,46,48]. The residual HEMA released can easily diffuse through the dentinal tubules due to its hydrophilic character and low molecular weight, and reach pulp cells [3,39]. Recent studies have also shown strong correlation between fluoride release and cytotoxic effects of GICs [47,49]. The results showed that the most cytotoxic GICs released more fluoride, whereas the materials proved less cytotoxic released minor amounts of fluoride. In both studies, Vitrebond was the GIC that released the largest amount of fluoride ions compared to other GICs, which may be a determinant factor in the cytotoxicity of this material [47,49].

In order to improve the antimicrobial properties of dental materials, studies have been done to investigate antimicrobial agents that may be added without adversely affecting other material properties [23-28]. NAg application in Medicine and Dentistry has been encouraged by the broad-spectrum antimicrobial effect in low concentrations, and the ability to not cause resistant bacterial strains to develop [16,26,28]. The antimicrobial mechanism of NAg is still not fully elucidated [21,51],

but is probably related to the release of silver ions, formation of reactive oxygen species (ROS) and direct interaction of the particles with the membrane of microorganisms [16,20,21,51]. However, cytotoxic effects of NAg are observed not only in microbial cells, but also in human cells. Studies associate these cytotoxic effects with the induction of ROS formation by the cells, leading to cell death, in a dose-dependent and time-dependent manner [22,29,52].

In this study, cytotoxic effect of NAg solution (positive control) on odontoblast cell line MDPC-23 was observed, with significant decrease in cell viability after 48 hours of exposure. This cytotoxicity was also reported by other authors [22,29,30,32,33,52].

The cytotoxicity and genotoxicity of NAg were evaluated by Asharani *et al.* [52]. NAg reduced the ATP (Adenosine triphosphate) content of the cells causing damage to mitochondria and production ROS in a dose-dependent manner. The authors also observed dose-dependent DNA damage. Analysis by Sanning Electron Microscopy (SEM) indicated the presence of NAg inside the mitochondria and nucleus, implicating their direct involvement in the mitochondrial toxicity and DNA damage. Based on these findings, the authors described a possible mechanism of cytotoxicity of NAg: they lead to disruption of the mitochondrial respiratory chain, leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage [52].

Besides dose and exposure time, the particle size can also influence the cytotoxic effect of NAg, whereas smaller particles have a higher toxic effect [22]. Park *et al*. [22] compared the cytotoxicity of particles with different diameters and observed that smaller particles (20 nm) were more cytotoxic compared to larger particles (80

nm to 110 nm). In the present study, the method used in the NAg synthesis produces 12 ± 2 nm particles [34], and may explain the cytotoxicity found with them.

Despite the NAg showed cytotoxic effects to the cells in this study, their incorporation did not influence the cytotoxicity of the GICs. Both Vitrebond as GC Gold Label 1 groups with NAg (0.1% and 0.2%) showed no statistically significant difference in the cell viability compared to their groups without NAg ($p>0.05$).

Recent studies evaluated the influence of NAg in the cytotoxicity of dental materials, and similar results to the present study were found, evaluating the cytotoxicity of adhesives and primers incorporated with NAg. The incorporation of NAg in dental adhesives at concentrations of 0.05% and 0.1% by weight did not change the material cytotoxicity [23,28].

Similarly, Zhang *et al.* [28] reported that addition of NAg in primers at a concentration of 0.05% by weight did not affect the material cytotoxicity in cultured fibroblasts. These results can be explained by the low concentration of NAg incorporated into materials, which do not affect the cytotoxicity. Despite these *in vitro* results are satisfactory, further studies should be conducted, including *in vivo* studies, in order to confirm the real biological safety in using NAg in dental materials.

Conclusion

According to the results of this study, it can be concluded that: the resin modified GIC (Vitrebond) showed cytotoxic effect on odontoblast line cell MDPC-23, while the conventional GIC (GC Gold Label 1) showed no cytotoxicity on cells; Silver nanoparticles did not influence the cytotoxicity of the evaluated GICs.

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Tables

Table 1 - Description of the glass ionomer cements.

Material (Manufacturer)	Composition	Powder: liquid ratio (wt)
Vitrebond (3M ESPE Dental Products, St. Paul, MN, USA) Batch nº 1303800842	Powder: - fluoralumino-silicate glass (90-100%) - photoinitiator Liquid: - polyalkenoic acid (35-45%) - HEMA (hydroxyetyl-methacrylate) (20-30%) - water (30-40%) - photoinitiator	1.4:1
GC Gold Label 1 (GC Corporation, Tokyo, Japan) Batch nº 1111221	Powder: - fluoralumino-silicate glass (95%) - polyacrylic acid (5%) Liquid: - polyacrylic acid (30-40%) - distilled water (50-55%)	1.8:1

Table 2 - Percentage of cell viability (Mean and Standard Deviation) obtained by MTT and Trypan Blue assays.

Materials	MTT	Trypan Blue
Culture medium (negative control)	100.6 (±0.10) ^{A}	93.2 (±5.23) ^{A}
NAg 0.78% (positive control)	68.1 (±4.16) ^{B}	65.4 (±11.47) ^{B}
Vitrebond	61.4 (±2.57) ^{B}	71.3 (±7.81) ^{B}
Vitrebond + 0.1% NAg	61.5 (±2.99) ^{B}	70.7 (±8.02) ^{B}
Vitrebond + 0.2% NAg	60.3 (±2.41) ^{B}	74.3 (±4.08) ^{B}
GC Gold Label 1	88.2 (±7.56) ^{A}	90.7 (±5.90) ^{A}
GC Gold Label 1 + 0.1% NAg	89.4 (±3.94) ^{A}	85.5 (±7.95) ^{A}
GC Gold Label 1 + 0.2% NAg	95.6 (±8.78) ^{A}	84.4 (±9.25) ^{A}

*Different letters indicate statistically significant difference (Tukey Test; $p<0.05$)

Figures

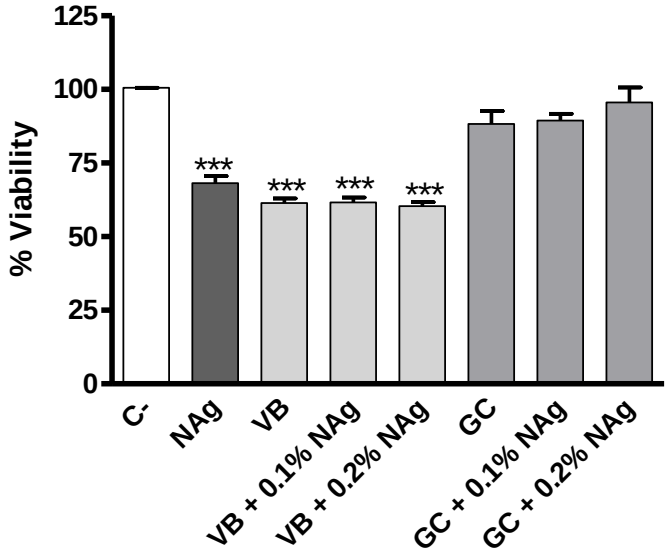

Figure 1 - Distribution of percentages of cell viability to the tested materials by MTT assay (ANOVA and Tukey.). C-: negative control; VB: Vitrebond; GC: GC Gold Label 1. *** p<0.001 compared to negative control.

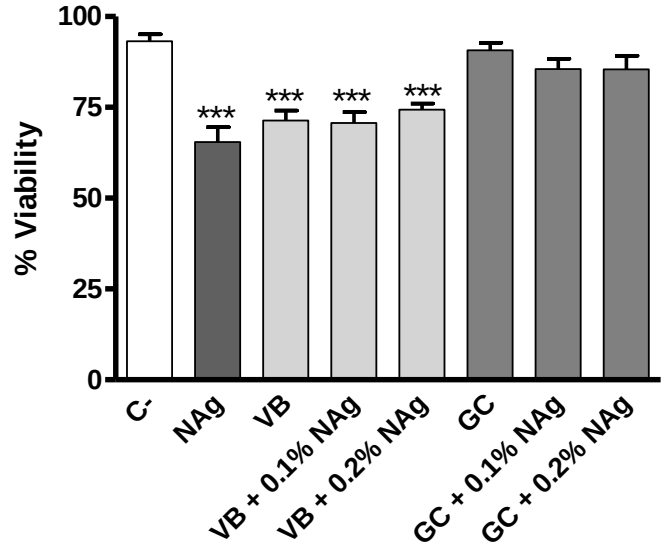

Figure 2 - Distribution of percentages of cell viability to the tested materials by Trypan Blue assay (ANOVA and Tukey.). C-: negative control; VB: Vitrebond; GC: GC Gold Label 1. *** p<0.001 compared to negative control.

5 CONSIDERAÇÕES FINAIS

Com base na metodologia empregada no presente estudo, é possível concluir que:

- O CIV modificado por resina (Vitrebond) apresentou efeito citotóxico sobre as células de linhagem odontoblástica MDPC-23, enquanto que o CIV convencional (GC Gold Label 1) não apresentou citotoxicidade sobre as células;
- As nanopartículas de prata não influenciaram na citotoxicidade dos CIVs avaliados.

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ANEXO

ANEXO A – Normas da revista a qual o artigo será submetido.

Writing Papers for Biomaterials

Introduction

Biomaterials is the leading journal that deals with biomaterials science and the related subjects of biocompatibility, medical devices, drug and gene delivery and tissue engineering. We are receiving manuscripts at 250 per month and publish over 7,000 pages per year. The high quality of the journal is beyond doubt. The maintenance, and hopefully even further improvement, of this quality is the concern and responsibility of authors, the editorial team, the referees and the publisher. The review process is central to the production of high quality published papers. The procedure, in general terms, is as follows. Manuscripts are received in the editorial office, via an online web submission and review system, and are read by the Editor-in-Chief. At this point the manuscript may be rejected because it does not match the scope of the journal. Only a few percent of manuscripts come into this category. The manuscript may also be rejected at this stage because, in the Editor's opinion, the quality of the paper is not sufficient to justify publication or because there would be very limited interest by the readership of the journal in the paper. This decision is never an easy one and the Editor takes into account the added value of the paper in comparison to other papers being published in ***Biomaterials*** in that specific area. Thus, a manuscript dealing with a slightly different way of sintering hydroxyapatite, or delivering a well-known drug in a slightly different manner, might be difficult to accept in view of the large number of papers on those subjects published recently. Approximately 35% of manuscripts are rejected on this basis, and the author is advised accordingly, usually within a couple of weeks and with a personalised letter of explanation.

If the Editor-in-Chief believes that the manuscript is of sufficient quality and interest to be peer reviewed, he will select appropriate referees from his database. The manuscript and abstract are sent by e-mail to referees who are invited to accept or decline the invitation within 10 days. If the referee agrees to conduct the review he is

requested to complete his assessment and provide his report, in the EES web system, within three weeks. If a referee cannot review the paper, for any reason, the manuscript is sent to an alternate reviewer. This continues in cascade until the required number of reports is received. When the referee reports have been received, the Editor-in-Chief reads them and re-reads the manuscript. At this point he will either reject the paper, accept it without revision or request that the author revises the manuscript. A further 40% are rejected at this stage. Very few are accepted without revision. The authors normally receive copies of the referee's reports, although on occasion the Editor-in-Chief consolidates the referee's comments into his own report on the manuscript. If a revision is required, the authors are usually requested to complete this within a short, defined period of time, usually between 1 and 3 weeks. This time limit is specified to avoid the publication of work that becomes out of date. If revised manuscripts are received after the deadline, the editorial office may decide to have the paper re-refereed. It should be noted that only rarely will the Editor-in-Chief require that significant additional experimental work is required. If referees suggest that more work needs to be done in order to make the work publishable, the Editor-in-Chief will usually reject the paper, with a recommendation to the author.

It will be seen from the above summary that some 75-80% of manuscripts are rejected and 95% of those eventually accepted have to be revised. These are not exceptional figures for a high quality scientific journal. It is unlikely that the rejection rate will be lowered since it is the intention to increase the quality of the journal, so that the acceptance criteria will be gradually raised. There are, however, many ways in which the overall quality of submitted manuscripts can be improved. This is important for several reasons. Too many manuscripts are received with obvious errors and poor quality presentation, which makes the editorial and review process more time consuming and difficult. It is not an easy task persuading referees to review papers and it is clear that they usually respond positively to well presented manuscripts but negatively (i.e. by refusing to do the review, or being late with the report) with poorly presented scripts. Obviously the shorter the editorial process the quicker will be the publication of the paper.

This present paper has been produced to give advice to authors on the presentation and submission of manuscripts to ***Biomaterials*** from the editorial perspective. It is

not concerned with the logistics of submission, although a few aspects of this will be touched upon. It will cover manuscript content, style and length and will deal specifically about each part of a manuscript, from title to references.

Manuscript Content

Types of Manuscripts

Papers published in regular issues of ***Biomaterials*** are normally original research papers. Some Review papers are published but these are specially commissioned by the Editor-in-Chief. Leading Opinion Papers, which provide evidence-based scientific opinions on topical and important issues in biomaterials science, are also commissioned by the Editor-in-Chief. In both these cases, we do not accept unsolicited papers although the Editor would be happy to receive proposals for manuscripts in either category.

Scope

Because of the changing role of biomaterials in many areas of medical technology, the scope of the Journal is constantly evolving. The journal is relevant to all applications of biomaterials including implantable medical devices, tissue engineering and drug delivery systems. Indeed the journal is now divided into 9 sections, Biomaterials & Tissue Engineering, Biomaterials & Drug Delivery, Biomaterials & Medical Devices, Biomimetic & Natural Materials, Biocompatibility, The Materials Science of Biomaterials, Modelling of Biomaterial Performance, Biomaterials and Gene Transfer and Biomaterials for Biotechnology. Authors are requested on submission to specify the most appropriate section although the Editor-in-Chief may override the selection. It is very important that authors remain within the limits set out by these instructions. Thus, whilst we accept and indeed encourage manuscripts on drug delivery systems, the work must address materials science issues of these systems and not solely the pharmacology. Similarly, papers dealing with implantable devices must relate to the materials of those devices and not solely to clinical performance or biomechanics.

Papers dealing with the synthesis and characterisation of new materials that might have potential as biomaterials cannot be accepted unless they are able to demonstrate some relevant biological performance data. Any manuscript that does not mention the materials actually used cannot be accepted and detailed information

about the materials is normally required. It should also be noted that we rarely publish papers that only describe techniques, without any substantive new biomaterials science content.

Intellectual Property

Quite often, questions about proprietary names, trademarks or materials of an undisclosed specification, arise and great care has to be taken. It is acceptable for a material or a device to be described by a trade name as long as there is also a description of that material or device. However, we normally prefer that trade name not to be used in the title or in the list of key words. It is not acceptable for a paper to discuss a material that cannot be specified for confidentiality reasons. It should also be said that all authors have the responsibility of ensuring that they consider the intellectual property implications of manuscript submission. Authors should be aware that the act of transmitting a manuscript to an editor, with the implicit assumption that the manuscript will be sent to referees, has already undertaken an act of disclosure which some legal jurisdictions may argue prevents a patent filing related to any aspect of the subject matter of the manuscript. Although in many jurisdictions of publication online is considered to be the date of disclosure we urge authors to take great care with the transmission of unprotected intellectual property. Also in the context of commercial aspects of biomaterials related products, we try to be very careful over the language used by authors to describe products, as they can be very misleading, often being written for ‘marketing’ purposes; papers that overtly promote a product, or denigrate competing products, are not acceptable.

Testing Results

A number of manuscripts have been received recently that report on tests carried out on biomaterials to determine biological safety, usually by compliance with the international standard ISO 10993. These manuscripts are normally rejected since this type of testing is done for regulatory purposes and is not scientifically based.

Splitting of Work

We have noticed recently that a number of authors are splitting pieces of work into very small packages and trying to publish these as series of papers. Whilst it is quite possible for a sequence of papers from one research group to be published, each paper has to be of sufficient significance to publish in its own right. It is unacceptable to submit a series of articles on the same subject matter, with duplication of much of

the introduction, the methods, the discussion and references, and only small differences in the experimental work and results. Such submissions are usually returned with a request to consolidate them into one manuscript. It is also noticeable that a number of authors are submitting papers to ***Biomaterials*** that bear much similarity to papers submitted elsewhere, perhaps with sufficient differences to avoid any claim of publishing the same work twice, but only just. The editorial office is monitoring this situation and authors are asked to avoid this practice.

Incremental Work

There is one further feature about the manuscript content that should be emphasised. Quite often a manuscript is received that is technically and scientifically sound and fits the overall scope of the journal, but adds very little to our body of knowledge on the subject. Typically this happens when the data obtained and the conclusions drawn show only a minor incremental advance. When considering the publication times of journals and the overall level of interest generated in each paper, it is often difficult to justify the inclusion of such papers. Equally we do not usually accept papers that only provide data that supports or confirms existing knowledge.

Manuscript Style, Length and Structure

The guide to authors gives some sound advice about the structure and style of a manuscript. Authors should note that the following sequence is normally required: title, authors, affiliations, abstract, keywords, introduction, materials and methods, results, discussion, conclusions, acknowledgements, appendix (where necessary), figure captions and tables. Review papers may have a different format within the main text. Failure to follow these instructions leads to delays.

Language

Somewhere in the region of 75% of papers submitted are from authors who do not have English as their first language. The editorial team are sympathetic to these authors and try to help when there are difficulties, but it is in the best interests of authors to produce manuscripts with high quality English in their first submission. The referees chosen for ***Biomaterials*** all understand the situation, and we in fact use many referees who do not have English as their first language, but it is inevitable that their view of a paper will be adversely affected if it is very difficult to read. In many cases we have to ask authors to have their manuscript checked and re-written by an

English speaking person. Better use of spell-check and grammar-check software would also be helpful. It would be very beneficial if this could be done with the original submission rather than during the revision stage.

Length

There is no prescribed length of papers however the current average is 10 printed pages and we are seeking to reduce this to 8. The guide to authors urges them to write as concisely as possible. There are good reasons for this. Papers that are concise are more easily read by referees and by ultimate users of the journal. It also means that more papers can be published in each issue, thereby reducing publication times. It is important, of course, that the manuscript is sufficiently robust and substantive to convey accurately the significance of the work, but this can be achieved with careful attention to style of the text.

Title

The title is obviously the major factor that determines who will find and read the paper and great care should be taken with it. The title should be sufficiently informative so that the reader can immediately assess its likely relevance, but without being excessively long. The title does not have to convey the results or the conclusion, nor indeed does it have to specify the techniques. It is best to avoid sentences as titles; the best titles have between six and twelve words, with no verbs. As noted earlier, trade marks or proprietary names should be avoided.

Authorship

This is extremely important. In order to avoid later recriminations or even lawsuits, it is essential that all people who have played a significant role in the work and preparation of the manuscript are included in the list of authors and that, equally, there should be no authors who listed purely out of courtesy or local politics. Papers may be published by a single author or by a group of up to ten or twelve authors. Lead authors should be aware that papers lose some credibility if there are far more named authors than could have possibly been involved with the work in any significant way. It is important that authors follow the declaration of consent to submission as given in the guidelines.

Each paper should have a corresponding author. It does not matter where in the list of authors the corresponding author is placed, and it is recognised that different laboratories and institutions have different policies on this. However, the

corresponding authors should, as the name implies, be the person who corresponds with the editorial office and who will be the lead correspondent with any reader who wishes to communicate with the authors once the paper has been published. Far too often the editorial office receives requests for information about a manuscript from authors who are not the corresponding author. The editorial office communicates only with the corresponding author. . The corresponding author must provide a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from biomaterials@online.be.

We would also like to standardise the way in which the author's names are quoted, but this is difficult because of cultural differences. We request the use of Christian name (given name), middle initial (if any) followed by surname (family name).

The affiliations of all authors should be unambiguously stated.

Mandatory Author Declaration

An Author Declaration is a mandatory and integral part of a submission. This Declaration covers a number of logistic and ethical issues. A template for the covering letter is found on the ***Biomaterials*** website. Authors may save the template, obtain the required signatures and then upload it as a part of their submission. All authors need to physically sign the form. It cannot be emailed, faxed or sent by post.

Keywords

Keywords have become very important with respect to literature searches and many search engines operate through the listing of these words. It is in the author's interest to think carefully about the words that will attract interested readers to their paper. A list of preferred key words has been compiled by the Editor-in-Chief and may be found in the Guide for Authors. There is little point in using very generic terms such as biomaterial, implant, drug, tissue engineering and prosthesis as key words. Equally there is no point in using obscure names, and it is best to avoid the author's own abbreviations. As noted earlier, trade names should be avoided.

Abstract

Next to the title, the abstract will be the second most important point of entry to the paper since most search facilities will print the abstract as part of the service, and far more people will read the abstract than the full papers. The abstract should be concise and informative. It is not the place to expand on techniques or discuss philosophy, and the conclusions that it expresses have to be an accurate reflection

on what was found. Abstracts should be not used to exaggerate the significance of the work and they should not contain subjective opinions on this importance or speculate how a material might be used. Very commonly submitted abstracts will include a phrase such as ‘material X is very biocompatible and shows promise for use in orthopaedic implants’. This is rarely a sensible approach to writing an abstract. We do not require the abstract to be split into sections (e.g. background, experiments, results, conclusions) as demanded by some other journals. The instructions specify a length of 100 – 200 words. Most good abstracts are around 150 words in length, as a single paragraph.

Introduction

The Introduction, as the name implies, should introduce the background to the work that has been carried out, effectively providing the scientific rationale. It should contain sufficient citations to the key literature to support this rationale and should lead to a clearly stated hypothesis or set of objectives. Authors should assume that the readership of the journal is well-informed and there is no need for any generic educational background. For example, in a paper on wound healing it is not necessary to take the first page to explain the ideal characteristics of wound dressing materials, or in a paper on drug eluting stents it is not necessary to describe all of the competing technologies that address in-stent restenosis. The introduction should rarely be more than two manuscript pages long. It should not pre-empt the Results, Discussion or Conclusions.

Materials and Methods

This section should specify exactly what was done experimentally, with sufficient detail for the reader to be able to repeat the experiments if he wishes. It is acceptable to refer to other publications if the methods have been used elsewhere, for example the MTT test is used very widely and it is unnecessary to repeat the details unless there has been a departure from standard practice. It is not, however, acceptable to refer to the author’s own work if it has been published in relatively inaccessible places, including PhD theses and non-English language journals. All of the experimental work discussed in the paper should be described in this section. Materials used in the work should be described in appropriate detail, including sources of commercial supply or synthetic routes, and all major equipment should be specified with the manufacturers name, reference number and location. Animal

experiments should be described in good detail but with sensitivity. Where institutional or regulatory rules apply to the conduct of the experiments they should be quoted. If any of the experimental work has been performed by a laboratory or organisation that is not represented in the list of authors, it should be explained here.

Results

Ideally the Results Section should be separate from the Discussion, but there is some flexibility here. The section should, obviously, be factual and it is best to avoid any philosophy or speculation. Authors should consider very carefully how to present their data. It should not be presented in multiple formats (i.e. the same data should not appear in figures and tables). If the data is displayed very effectively in either a table or figure, it should not be necessary to explain results in great detail in the text, but rather to use the text as a medium for emphasising the most significant data. It is occasionally acceptable not to provide actual evidence of the data, but this should not be done when the data is critical to interpretation, for example discussing crystallinity without showing XRD graphics.

Discussion

This section should summarise the nature of the observations and attempt to place this data into the context of the existing body of literature and, where appropriate, to express opinions about the significance of the work as far as biomaterials science is concerned. It should not be repetitive of the Introduction. It is entirely valid to suggest the potential implications of the work but without too much speculation. It is particularly important not to extend the discussion into areas that are not supported by the facts that are in evidence. Experiments that address the mutagenicity potential of implantable metals should not lead to discussions about the generic biocompatibility of these materials, for example.

It is also important that new data is not brought into evidence in the discussion. Several recent manuscripts have set out the experimental methods and results in the correct sections, but then the authors described quite briefly some additional experiments in the discussion and used those results to support their conclusions. This is not acceptable. Equally authors cannot cite their as-yet unpublished work to support the discussion.

Conclusions

Many authors end the Discussion section with a paragraph on the conclusions. This is not the best way to draw the manuscript to an end, and we require that conclusions be separated into a distinct section. This should not be too long, nor should it be repetitive of the discussion, and especially should not bring new ideas into the paper. The conclusions have to be based on the facts in evidence and should be limited to reasonable speculation about the significance of the work. The editorial team are particularly vigilant over the use of unjustified, exaggerated language in the Conclusions section.

Acknowledgements

It is perfectly acceptable for authors to acknowledge any person, institution or organisation that have made a significant contribution to the work, including any funding agency or other sponsor of the work, or individuals who contributed to the work but who are not named as authors. It is always sensible to show a draft manuscript to such individuals to ensure they are comfortable about this citation. The Editor-in-Chief currently does not require statements to be made about the funding or sponsorship, nor is any declaration concerning conflict of interest required. Authors are encouraged, however, to consider using this Acknowledgements section to make any personal comments they wish about such issues.

References

Instructions for the preparation of the list of references are given in the guidelines to authors; the designated form is modified Vancouver. These instructions should be followed exactly; failure to do this is one of the most common faults with manuscripts and causes frustration all round. Note that this system requires the names of all authors. Only where there are more than six authors can the abbreviation et al be used, after the name of the sixth author. There is no formal guidance on the number of references quoted, but in practice the best papers have between 20 and 30. It is better to avoid too many citations to the author's own work, and it is good to have a balance between the older seminal papers that lay the groundwork for that particular area and recent quality papers that have contributed serious input into the subject. Documents that have limited circulation, obscure journals or books, especially those out of date, and electronic sources (e.g.web-sites) should also be avoided wherever possible. It is always helpful to the reputation of the journal to include citations to previous papers published in ***Biomaterials***.

Figures and Tables

As noted earlier, experimental data should be represented in figures or tables wherever possible. Advice is not given here about the preparation of figures, detail being given in the guide to authors. Authors should note, however, that since figures and tables take up a considerable amount of space, they should be limited in number. Many authors used flow charts to represent experimental strategy or line drawings or photographs of equipment, most of which are unnecessary. Sometimes multiple figures are used with very little data on each, and which could be consolidated.

Colour is reproduced in high resolution online. However, consistent with Elsevier's global policy on colour that became effective in June 2008, colour will not be used in the print version, apart from exceptional circumstances, for example with papers commissioned by the Editor-in-Chief where colour is essential. The reason for this is that the vast majority of readers access the journal solely on-line and the high cost of colour reproduction in printed versions that are only rarely accessed cannot be justified. We suggest that authors may wish to alter their own methods of producing illustrations to take this into account. It is rare that colour actually enhances graphs and charts, and authors should resist the submission of such illustrations where the sole means of distinguishing lines or columns is by colour. We also suggest that PowerPoint illustrations are avoided as they are not usually consistent with the serious scientific information that is contained within the figure. It is recognised that some figures that appear within papers submitted to Biomaterials are rendered un-readable in black and white, for example those that display multiple colour stains or fluorescent images. In such cases, in order to avoid the frustration of readers, the Appendix to a paper which will indicate that some Supplementary Information associated with the article can be found on the on-line version, quoting the doi, will also explicitly state that the differential colours of such illustrations can be seen in the online version. This indicator will be included at the discretion of the Editor-in-Chief.

This will specify, for example:

Appendix: Figures with essential colour discrimination.

Certain figures in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:……..

Figure and table captions should be constructed with care. There should be sufficient information for the reader to understand the subject matter, but it is not necessary to write an extensive text to explain all the detail. All figures must be numbered by the author as the system does not automatically generate them.

Supplementary Data

If an author wishes to include supplementary information for the online version of the paper, including video-clip or raw data, he may do so. Supplementary material is made available via links in the online article but not published in print. Further technical details for uploading supplementary data may be found at http://authors.elsevier.com/ArtworkInstructions.html?dc=AI43 .

The Editorial Decision

After the completion of the review process, the Editor-in-Chief will normally be in a position to advise the authors of the first editorial decision. There are three possibilities, acceptance without revision, revision and rejection. Exceptionally, the Editor-in-Chief may advise the author that there is a delay in coming to this decision because of a serious conflict in the recommendations of the referees, in which case a further report may be requested for arbitration. If the author is advised that the paper is rejected and cannot be published, the decision is final and not available for negotiation. This does not mean that the author is prevented from submitting further papers to the journal on a similar subject, but the authors are strongly advised to take into account any critical comments of the referees and any further papers will be considered as new submissions and submitted to the review process from the beginning.

If the author is requested to revise the paper, it is important for all of the points raised by the referees and / or editor to be addressed. This does not mean that the referee has to make all of the changes suggested, but it is expected that the author will make most of these changes (the Editor-in-Chief will often remove referees recommendations that he does not consider to be sensible) and will provide reasons why he is unable to make the remainder. The preferred format for the re-submission of a revised paper is a covering letter explaining the responses to the referees together with a clear copy of the revised version and a copy which tracks the

changes that have been made. It is essential that the author follows the detailed instructions when submitting a revised paper.

In this respect, it is essential that the authors are vigilant with the version of the paper that they are working with. It is not unusual to have authors submit a ‘revised’ paper, but only send the original version in error. This provides serious problems for the office.