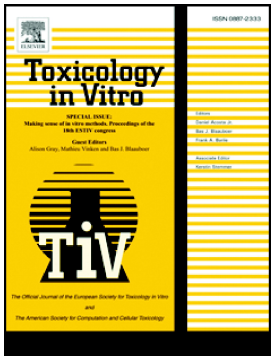

The variable toxicity of silver ions in cell culture media

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The variable toxicity of silver ions in cell culture media

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Abstract

The elevated interest in silver ions (Ag^+) as a broad spectrum antimicrobial for use on medical devices has increased the number and importance of *in vitro* biocompatibility testing, however little consideration is given to the culture environment in which the assessments are performed. The current investigation assessed the viability of mouse fibroblasts (L929) exposed to different concentrations of Ag^+ in both Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium Eagle, alpha modification (αMEM). We identified a significant increase in the EC_{50} of L929 cells exposed to Ag^+ in αMEM compared to DMEM, which was matched by a corresponding decrease in Ag^+ availability in αMEM at concentrations ≤400 µM, as detected by inductively coupled plasma mass spectrometry (ICP-MS). The reduced availability was not observed for Ag^+ >400 µM, the concentration above which caused *in vitro* cytotoxicity in L929 cells in αMEM; while linear quantification of Ag^+ was observed in DMEM. Equilibration of the chloride and glucose components between media did not affect cytotoxicity on primary test cells; mesenchymal stromal cells (MSCs). Overall, our results present evidence of the importance of culture conditions on the *in vitro* evaluation of silver, with DMEM providing a reliable basal media in which to conduct assessments.

Keywords

Silver, EC_{50}, culture media, L929 fibroblasts, mesenchymal stromal cells

Introduction

The original cell culture medium developed by Harry Eagle in the 1950s, was made up of components designed to enhance *in vitro* fibroblast viability and growth (Eagle, 1955). Subsequent adaptations of Eagle's basal medium have led to the plethora of formulations that are currently available, with changes and selection based primarily on the improvement of similar positive cell characteristics such as enhanced proliferation of specialised cells (Contreras et al., 2010; Sotiropoulou et al., 2006). For example, the development of minimal essential medium (MEM) altered the protein content for the culture of human cells (Eagle, 1959), while Dulbecco's modified Eagle's medium (DMEM) allowed improved culture of mouse embryonic cells (Dulbecco, 1964; Morton, 1970) and minimal essential medium Eagle, alpha modification (αMEM) for the culture of mouse-hamster hybrid cells (Stanners et al., 1971). In all cases, however, new media formulations were designed to promote cell performance with little consideration given to conditions required for cytotoxicity assays.

The search for alternative antimicrobials in the face of growing resistance to current treatments has led to the rise in the use of silver for its broad spectrum properties. With the incorporation of silver into medical devices comes the requirement for thorough cytotoxicity testing; a process that is vital from the early stages of development through to the finalised product. While the International Standards Organisation (ISO) has published guidelines on the cytotoxicity testing of medical devices (ISO10993-5), the majority of data is published using laboratory-specific methodology using a range of techniques and reagents. An example is the varied selection of culture media, with literature reporting the effects of silver on mesenchymal stromal cells (MSCs) using Roswell Park Memorial Institute (RPMI), DMEM, DMEM/F-12 and αMEM basal media during testing (Greulich et al., 2012; Jaiswal et al., 1997; Loza et al., 2014; Pauksch et al., 2014; Samberg et al., 2012; Zhao et al., 2015).

This is complicated further during MSC culture, with some laboratories using αMEM for proliferation, switching to DMEM during differentiation (Both et al., 2007; Jaiswal et al., 1997; Nöth et al., 2002; Sekiya et al., 2002). This selection is often justified on the basis of beneficial cellular characteristics and differentiation efficiency, however, the danger is that this is performed without consideration of the effects on test article behaviour during analysis.

Reports on the biocompatibility of silver have presented both positive and negative consequences of exposure, however, as previously noted, the use of different media types can make comparison of biocompatibility difficult (Contreras et al., 2010; Cortizo et al., 2004; Luo et al., 2010). Therefore, for research to progress to effective clinical therapies, it is important that the *in vitro* data is consistent, and conclusions drawn between laboratories not compromised as a consequence of media selection. With regards to silver, it is antimicrobial in its ionic form (Ag^+), however, it rapidly forms silver chloride (AgCl) in biological systems. AgCl has lower bioavailability and does not possess antimicrobial properties (Schierholz et al., 1998). It is also less cytotoxic to mammalian cells, as demonstrated through improved viability following the formation of AgCl in chloride-controlled test media (Behra et al., 2013; Zhang et al., 2013). However, large increases in chloride (NaCl ≥20 g/L) were shown to result in the generation of water soluble AgCl anions that retained a bactericidal property (Gupta et al., 1998). While this was observed in bacterial culture media, the comparatively low chloride content of culture media means that this is an unlikely scenario, the result being the production of insoluble chloride precipitates, restricted in size by a 'protein corona' (200-350 nm) (Loza et al., 2014). Despite this, the generation of AgCl has been shown to increase cytotoxicity to monolayer cultures. For example, viability was reduced in MEM (containing Cl^- = 124.5 mM) through the sedimentation and subsequent engulfment of AgCl precipitates, an effect that was less pronounced in media of reduced chloride (≤87.3 mM) (Kaiser et al., 2017).

The influence on chloride content of media is not the only reported influence on media choice for biocompatibility studies. An increased tolerance of HepG2 (human hepatocytes) to silver nanoparticles (AgNP) was observed following prolonged expansion (>1 month) in low-glucose DMEM (although no data were presented for a high-glucose equivalent) (Zuberek et al., 2015). The improvement was determined to be the elevation of antioxidant protein activation, a result of a general elevation in reactive oxygen species (ROS) production as a consequence of the switch to oxidative phosphorylation that has been observed elsewhere (Sarre et al., 2012). They explain that the increased oxidative stress and the subsequent priming of the ROS pathways permitted the increased tolerance to AgNP.

These examples of the influence of culture conditions on experimental outcome therefore prompted us to investigate the effect of two commonly used media, αMEM and DMEM, on the cytotoxicity of silver to L929 mouse fibroblasts, an immortalised cell line recommended for use by ISO 10993-5 (Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity) and MSCs, validated for the use in acute cytotoxicity testing (Scanu et al., 2011). The biocompatibility data were used to correlate the findings of analytical studies that determined the silver content of media following incubation.

Methods

Silver ion preparation, media incubation and ICP-MS analysis

Stock solutions of 35 mM Ag^+ were prepared in distilled water from silver sulphate (Ag_2SO_4, CAS: 10294-26-5, Alfa Aesar, Cat: 41443) before spiking in to 2 mL αMEM +10 % FBS, DMEM +10 % FBS or deionised water (dH_2O, 15 MΩ) to give an Ag^+ of 25, 250, 400 & 600 µM. Samples were incubated at 37 °C, 5 % CO_2 for 0, 1, 3 & 24 hours. Additional time points of 20, 40, 60 & 80 minutes were performed in αMEM for Ag^+ ≥250 µM.

Following incubation, the solutions were filtered (0.2 µm, Phenomenex), diluted in 1 % nitric acid and analysed using Agilent Technologies 8800 ICP-MS triple quad. Samples were measured against a matrix (cell culture media) matched Ag^+ standard curve using a 500 ppb rhodium internal standard (Agilent Technologies). Data were analysed by two-way ANOVA, Tukey's correction for multiple comparison.

Cell culture

Mouse fibroblast L929 and primary MSCs (isolated from human bone marrow aspirate and bone samples for use as a model primary cell) were expanded in DMEM (high glucose) supplemented with 10 % Foetal Bovine Serum (FBS), 100 Units/mL Penicillin and 100 µg/mL Streptomycin. Both were passaged once 80-90 % confluency was achieved. All assays were performed using a 96-well plate format, with cells seeded at $3.125x10^4$ cells/cm^2 24 hours prior to testing, incubated at 37 °C, 5 % CO_2.

In vitro viability

Viability was determined using ISO10993-5 as a guideline (*Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity (ISO10993-5)*, 2009). Output measure was through optical density reading (440/600 nm) of the change in colorimetric proliferation reagent, WST-1 (Roche Diagnostics), with data converted to viability as a percentage of the untreated control. L929 cells were cultured with an Ag^+ dose response (range 8.75-100 µM (DMEM) or 50-700 µM (αMEM)) added in triplicate. After 24 hours viability was determined as above. Data were analysed by one-way ANOVA, Sidak's correction for multiple comparison and EC_{50} values (the concentration that provides a 50 % response) calculated.

NaCl supplementation

The difference in sodium chloride content between the two basal media (DMEM, 6.4 g/L; αMEM, 6.8 g/L) was equilibrated through the addition of NaCl (prepared in DMEM) to

DMEM+10 % FBS (to be referred to as DMEM+NaCl). MSCs were seeded (as above), in their respective test medium (DMEM, DMEM+NaCl, αMEM), after which an Ag^+ dose response was added. Viability was assessed as before.

Glucose supplementation

To provide a high glucose αMEM, equivalent to that of DMEM (4.5 g/L), D-(+)-Glucose (Sigma) was added to the basal αMEM to provide DMEM+10 % FBS and αMEM+10 % FBS preparations available in 'Low' (1 g/L) and 'High' (4.5 g/L) formulations. Replicating published work, MSCs were expanded in the four different media types before the addition of an Ag^+ dose response in their respective medium and viability assessed as before (Zuberek et al., 2015).

Statistics

Two-way ANOVA and EC_{50} calculations performed using GraphPad Prism version 7.00, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

αMEM reduces Ag^+ availability at low concentrations

Quantification of Ag in dH_2O vehicle controls using ICP-MS matched target molar concentrations across all incubation periods, from 0 hours to 24 hours (Figure 1). Although equivalent to each other (P>0.05), the Ag concentration in both αMEM and DMEM culture media was significantly below that of the dH_2O control at 0 hours when Ag^+ ≥250 µM (P<0.0001, Figure 1a). At all subsequent time points, the measured Ag from αMEM (at 250 & 400 µM) remained significantly below that of dH_2O control (P<0.0001) and DMEM

(*P*<0.0001). However, this effect was not observed at 600 µM, where Ag levels in αMEM were elevated above that of DMEM, at 3-24 hours, equating to approximately 89% of the dH_2O control (Figure 1b-d).

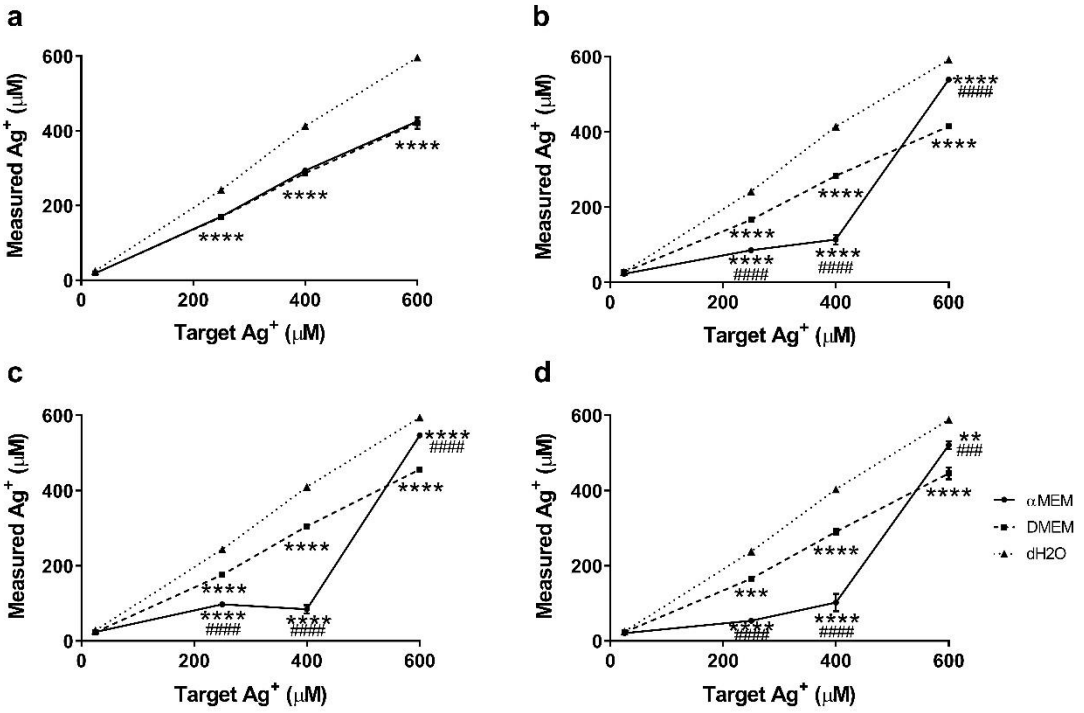

Figure 1: Quantification of Ag^+ from cell culture media and deionised water.

Measured Ag^+ from αMEM, DMEM and dH_2O determined at a) 0, b) 1, c) 3 and d) 24 hours, using ICP-MS and following incubation at 37°C, 5 % CO_2. Data presented as target Ag^+ against mean Ag^+ concentration ±SEM (n=3). Significance against dH_2O represented by ** *P*<0.001, *** *P*<0.005, **** *P*<0.0001, significance between DMEM and αMEM represented by ### = *P*<0.005, #### = *P*<0.0001.

For DMEM, reproducible quantification of Ag was observed at 25 µM during the 24 hour test period (Figure 2), with the mean (±SEM) measured at 92 % of the target value (23.00 µM ±1.07 µM). Across the concentration range tested (25 – 600 µM), mean quantification was linear (R^2=0.998), however, a greater spread of data was observed with increasing concentration as a consequence of time point (25 µM, range: 18.10-30.70 µM, SD±3.69; 250 µM, 161.8-179.6 µM ±4.86; 400 µM, 247.4-312.9 µM ±11.90; 600 µM, 405.7-463.9 µM ±24.26), while also equating to approximately 72% of the target.

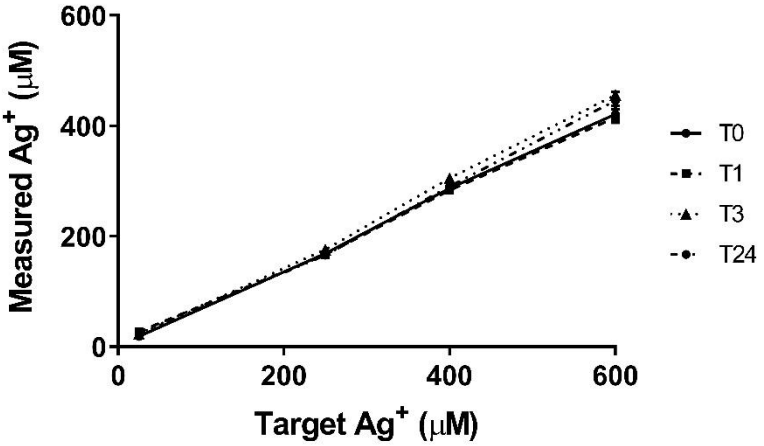

Figure 2: Quantification of Ag^+ in DMEM during 24 hour time period.

Measured Ag^+ from DMEM following incubation at 37°C, 5 % CO_2 over 24 hours in culture Data presented as mean ±SEM (n=3).

The observed decrease in measurable Ag concentrations when using αMEM was investigated at earlier time points following initial Ag addition to media. For 250 & 400 µM Ag^+, a reduction from 0 minutes was detected at 20 and 40 minutes, respectively (Figure 3). Little variation was noted during the 80 minute timeframe for samples at 600 µM.

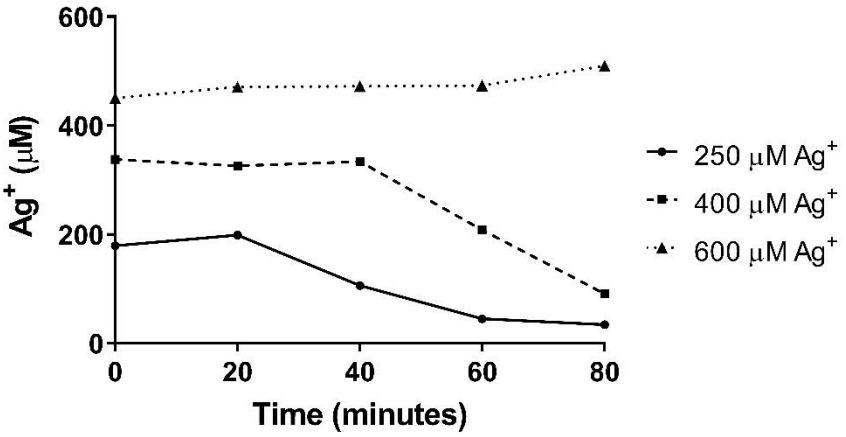

Figure 3: Quantification of Ag^+ from αMEM at short time points.

Measurement of available Ag^+ concentrations at 0, 20, 40, 60 and 80 minutes in αMEM spiked with 250 µM, 400 µM and 600 µM Ag^+ (n=1).

Reduced cell viability to Ag^+ when cultured in DMEM compared to αMEM

The viability of L929 fibroblasts was determined across the Ag^+ dose response through measurement of WST-1 conversion by metabolically active cells to a soluble formazan product. L929 cells exhibited large differences in susceptibility to Ag^+. Viability in DMEM was reduced at Ag^+ ≥10 µM, while cytotoxicity was only observed in αMEM at Ag^+ >400 µM. The EC_{50} in each media was determined as DMEM: 16.78 µM and αMEM: 451.9 µM, (P<0.0001, Figure 4).

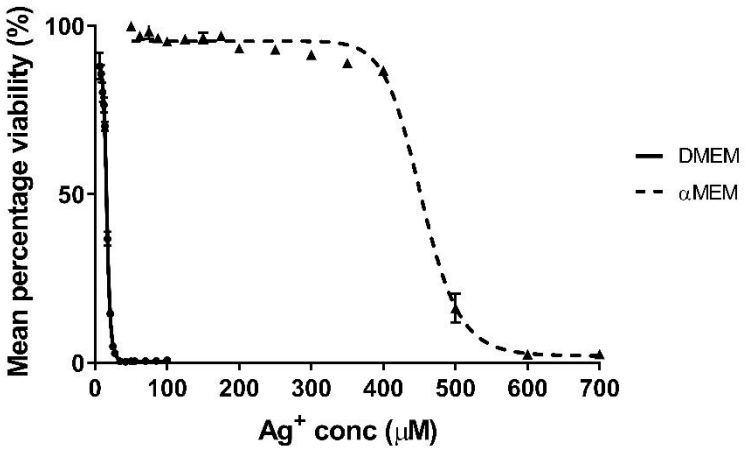

Figure 4: Cell viability of L929 cultured in αMEM and DMEM following 24 hour exposure to Ag^+.

Viability of L929 cultures as a percentage of untreated control. Dose response to silver determined for both cultures in αMEM and DMEM. Results are presented as mean percentage viability ±SEM (n=3).

Viability is unaffected by adjusting media NaCl and glucose content

The NaCl content of DMEM was adjusted to match that of αMEM to determine the effect of differences in chloride content on MSC viability following exposure to Ag^+. In unadjusted αMEM and DMEM basal media, MSC viability replicated that of the earlier L929 data whereby a large reduction was observed in DMEM compared to αMEM that provided the expected elevated Ag^+ required for cytotoxicity (Figure 5). The addition of NaCl to DMEM basal media did not affect the viability of MSCs from those cultured in unaltered DMEM (Figure 5).

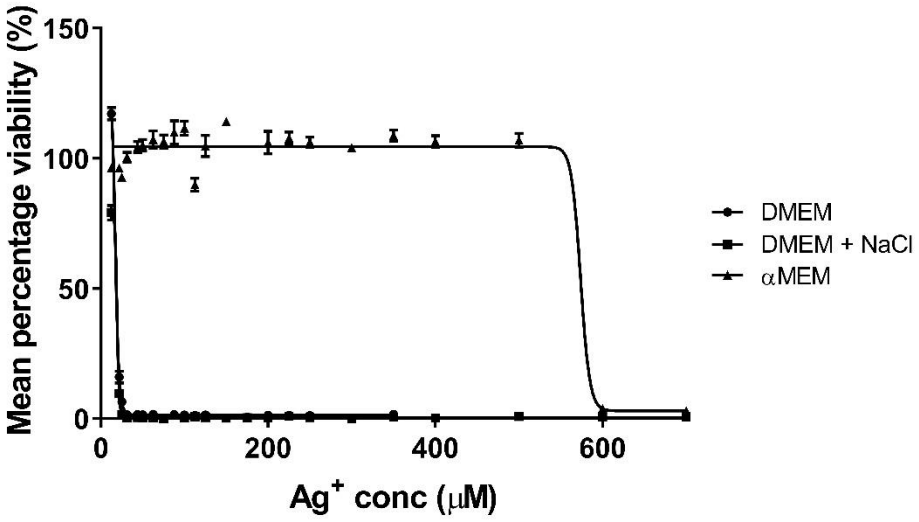

Figure 5: MSC viability cultured in DMEM, DMEM+NaCl and αMEM following 24 hour exposure to Ag^+.

Viability of MSC cultures as a percentage of untreated control. Dose response to silver determined for cultures in basal DMEM, DMEM+NaCl and αMEM. Results are presented as mean percentage viability ±SEM and represent triplicate measurements from one MSC donor.

Next, we determined the viability of MSCs to Ag^+ following expansion in medium of differing glucose concentrations. Short (maximum 2 passages, 11 days) and long-term (5 passages, 32 days) expansion periods in low and high glucose DMEM and high glucose αMEM were successfully examined (low glucose αMEM extended culture was not possible due to MSC senescence resulting in inadequate cell numbers). We determined that while MSC viability in Ag^+ was unaffected by expansion time in low glucose DMEM (Figure 6a) (short term expansion in αMEM provided for information, Figure 6c), the culture period was influential in MSC viability when using high glucose media (DMEM and αMEM). High glucose DMEM

expanded MSCs provided an EC_{50} of 26.89 µM (short term) and 16.16 µM (long term) (Figure 6c), while equivalent EC_{50} values for high glucose αMEM were 482.6 µM (short) and 418.1 µM (long) (Figure 6d).

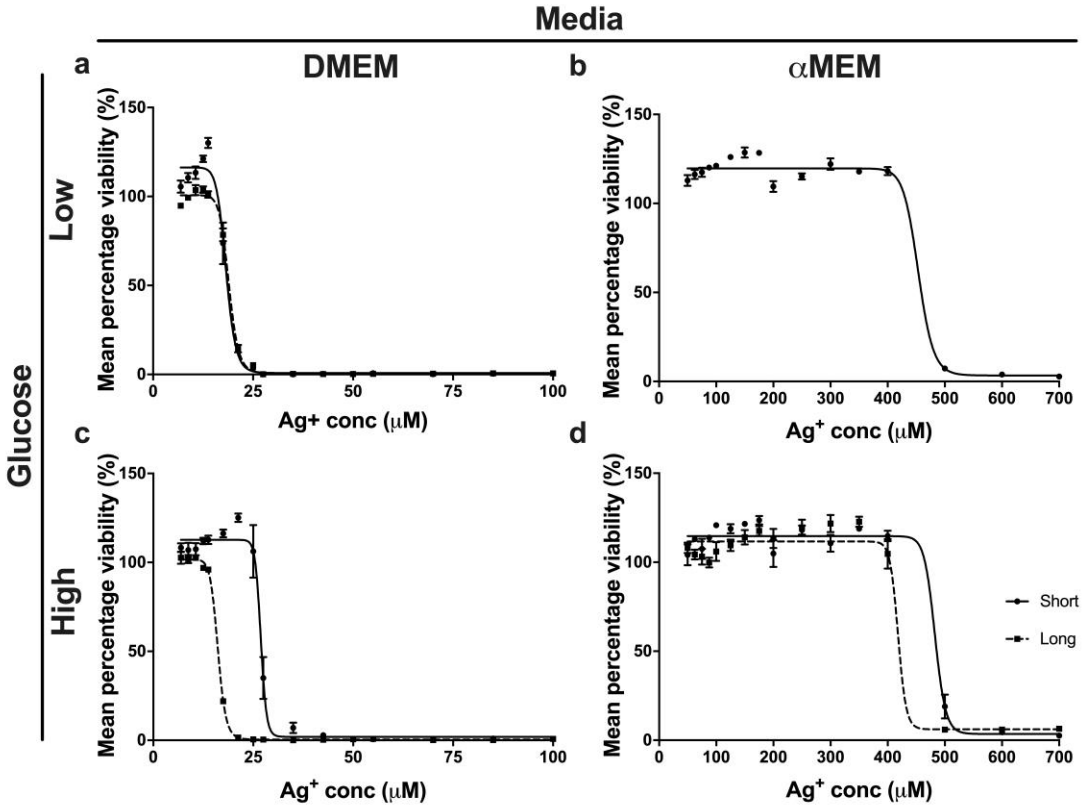

Figure 6: MSC viability following short/long term expansion in low/high glucose DMEM or αMEM following 24 hour exposure to Ag^+.

Viability of MSC cultures as a percentage of untreated control determined via WST-1 conversion. Dose response to silver determined for cultures expanded in low and high glucose DMEM (a, c) and αMEM (b, d). Results are presented as mean percentage viability ±SEM and represent triplicate measurements from two donors for short-term expansion and one MSC donor for long-term.

Discussion

Interest in the use of silver for medical applications has risen due to the increase in resistance to many conventional antibiotics. The broad spectrum antimicrobial properties of this element make it an appealing tool for various therapies, and is already widely applied to a plethora of surface dwelling wound management devices. As a consequence, a greater body of research is being published on the cytotoxic effects of silver exposure, reporting both detrimental and positive biological outcomes (Pauksch et al., 2014; Samberg et al., 2012; Zhao et al., 2015).

The biological properties of silver are influenced by the environment in which it is applied, with the container and solution both potentially affecting its bioavailability. Silver ions may adhere to different substrates, interact with protein or form inactive silver chlorides (Kaiser et al., 2017; Struempler, 1973; West and West, 1966; Williams et al., 1985). In the work presented here, the serum content of culture media was standardised, thereby allowing the individual properties of both DMEM and αMEM to be highlighted and investigated.

Silver quantified by ICP-MS from αMEM was reduced compared to expected concentrations following incubation and subsequent filtration, however, at high Ag^+ (600 µM), the measured concentration of Ag was greater in αMEM than in DMEM and near to the water control. Although the species of Ag was not identified, the expectation following the initial reductions at ≤400 µM, was for a continuation of this trend. Any observed increase was expected to be minimal following exhaustion of the components of αMEM media that were responsible for the initial decline, with the concentration maintained below that of DMEM. However, it appears that high Ag^+ bypasses the same chemical complexing that occurs at lower concentrations, allowing the ions to remain in solution following filtration. The biological effect of these data was demonstrated in the cytotoxicity profile of Ag^+ on L929, where the EC_{50} in αMEM (451.9 µM) was beyond the concentration at which a reduced Ag was quantified from ICP-MS (400 µM); while that of the linearly quantified Ag in DMEM was 27-fold lower. While there is some correlation with the metabolic activity of untreated L929s in

different media and their subsequent susceptibility to silver, the correlation does not fit all instances of MSC exposure.

Silver quantification at short time points demonstrated the time- and concentration-dependent nature in which silver can be filtered from αMEM, we speculate this to be the result of particle formation. However, while previous research has identified that the rate of particulate formation is dependent on concentration, an inverse relationship was found where suspected Ag complexes were formed more rapidly at lower concentrations and removed via filtration, with no formation occurring at 600 µM (Zhang et al., 2013). The explanation provided by Zhang *et al* for their findings was that increased Ag caused a more rapid formation of primary colloids, however, our data suggests that this reasoning does not apply to the complexed milieu of cell culture media.

The composition of culture media therefore defines the biocompatibility of Ag^+, with reports discussed earlier suggesting that chloride and glucose could both affect the biological outcomes, but through different mechanisms. The data tested the hypothesis that the difference in chloride content between media could be the defining factor in the reduction of viability observed with DMEM. Although the chloride content of DMEM (119.3 mM) and αMEM (126.2 mM) media is derived from several sources e.g. potassium chloride and calcium chloride; NaCl is the primary contributing factor (Cl^-, DMEM = 109 mM; αMEM = 116 mM) (Lee et al., 2011). However, the elevation of this salt within DMEM was found to have no effect on the viability between the basal media, suggesting that the difference in viability is likely a result of multiple media components. Formation of further insoluble particles may also arise from the sulphur content of the media, resulting in the generation of silver sulphide (Ag_2S). The impact of this element was not investigated as part of this work, however the total sulphur content of DMEM and αMEM is equal (0.8 mM) (Lee et al., 2011). While identical formation of Ag_2S cannot be assumed from this parity, the likelihood of its impact on cytotoxicity is believed to be small.

The availability of glucose during expansion was investigated with regards to MSC viability during silver exposure. While a reduction in the EC_{50} was observed following expansion in high glucose media, there was no effect of improving viability following expansion in low glucose medium. This is in contrast to the data published by Zuberek and co-workers (previously discussed), but is in line with the increased susceptibility of aged cells as a consequence of elevated oxidative stress and reduced antioxidant enzyme activity (Stolzing et al., 2008; Stolzing and Scutt, 2006; Zuberek et al., 2015), in addition to the use of oxidative phosphorylation during active proliferation in glucose rich medium. The difference observed here, is therefore likely due to the choice of primary over an immortalised cell.

While accuracy and clinical translation should be considered during *in vitro* modelling, the normal functioning of this system (i.e. cell proliferation) should not be compromised, nevertheless, there should be an awareness of the potential effects that the model can exert. The viability findings presented here, provide evidence for the first time of the impact that culture media can have on the cytotoxicity of test materials, specifically silver and links this effect to the availability of this element during culture. Therefore an emphasis should also be placed on determining the behaviour of a test material during *in vitro* assessments of efficacy and biocompatibility.

Acknowledgements

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Highlights

- Antimicrobial concentrations of silver ions (Ag^+) may cause cytotoxicity
- Ag^+ cytotoxicity is significantly modified by culture media (αMEM versus DMEM).
- Bioavailability of Ag^+ is reduced in αMEM at concentrations ≤400 μM
- Ag^+ quantification in DMEM is linear, providing a predictive media for testing

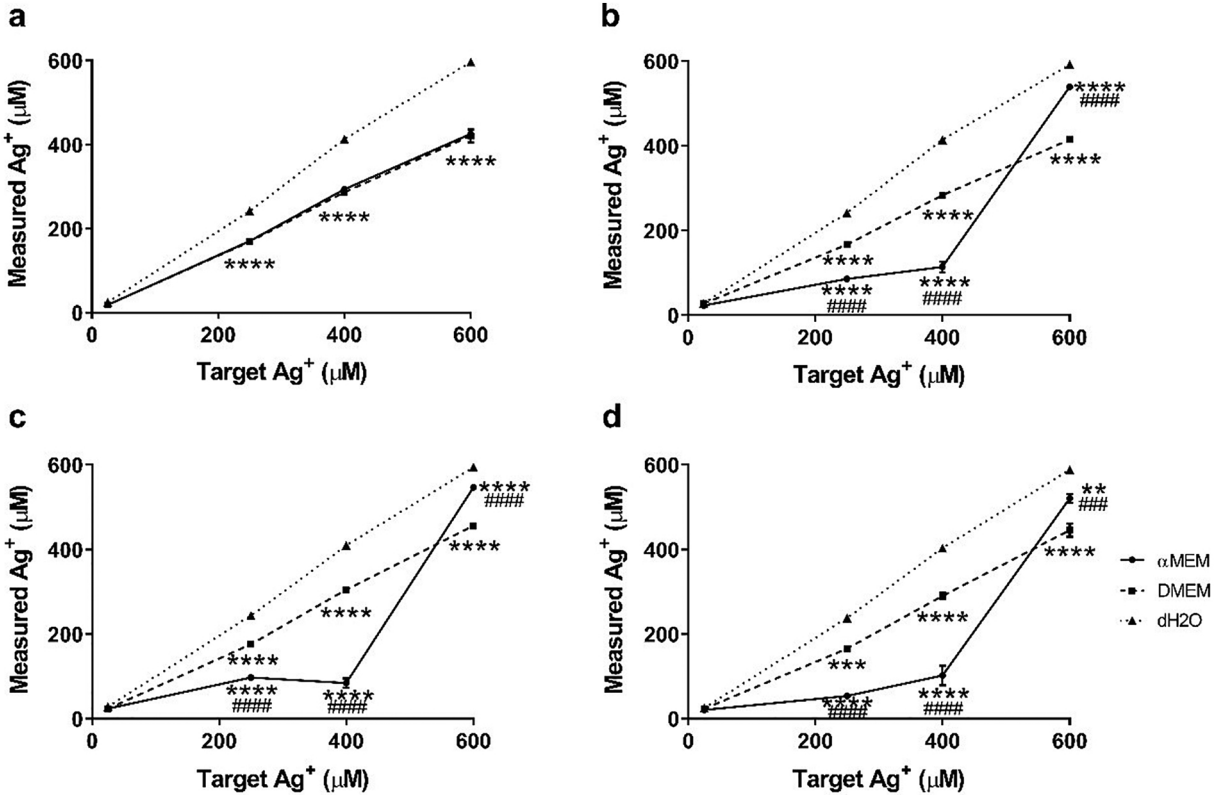

Figure 1

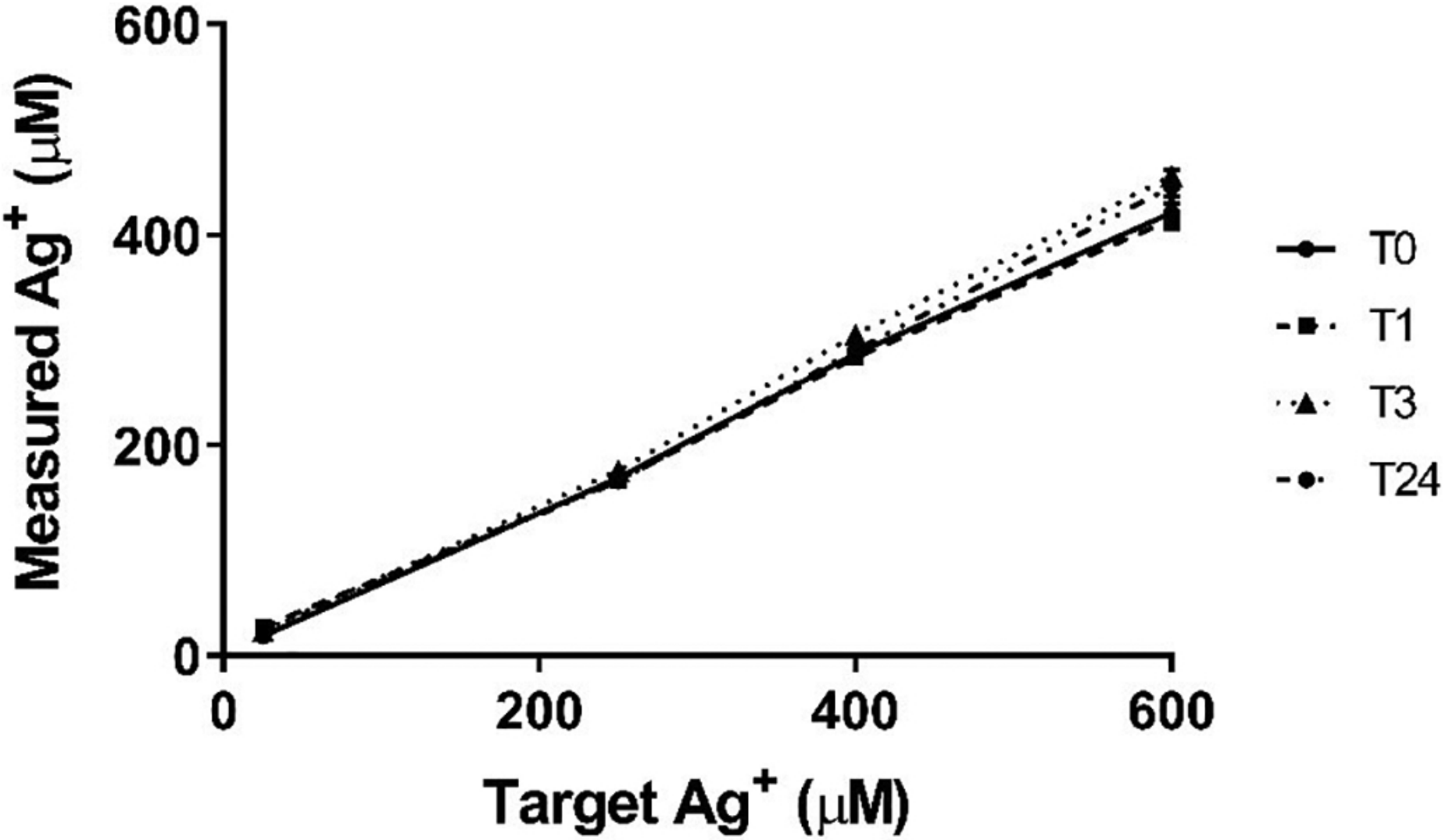
600
400
200
0
Measured Ag+ (μM)
0
200
400
600
Target Ag+ (μM)
T0
T1
T3
T24

Figure 2

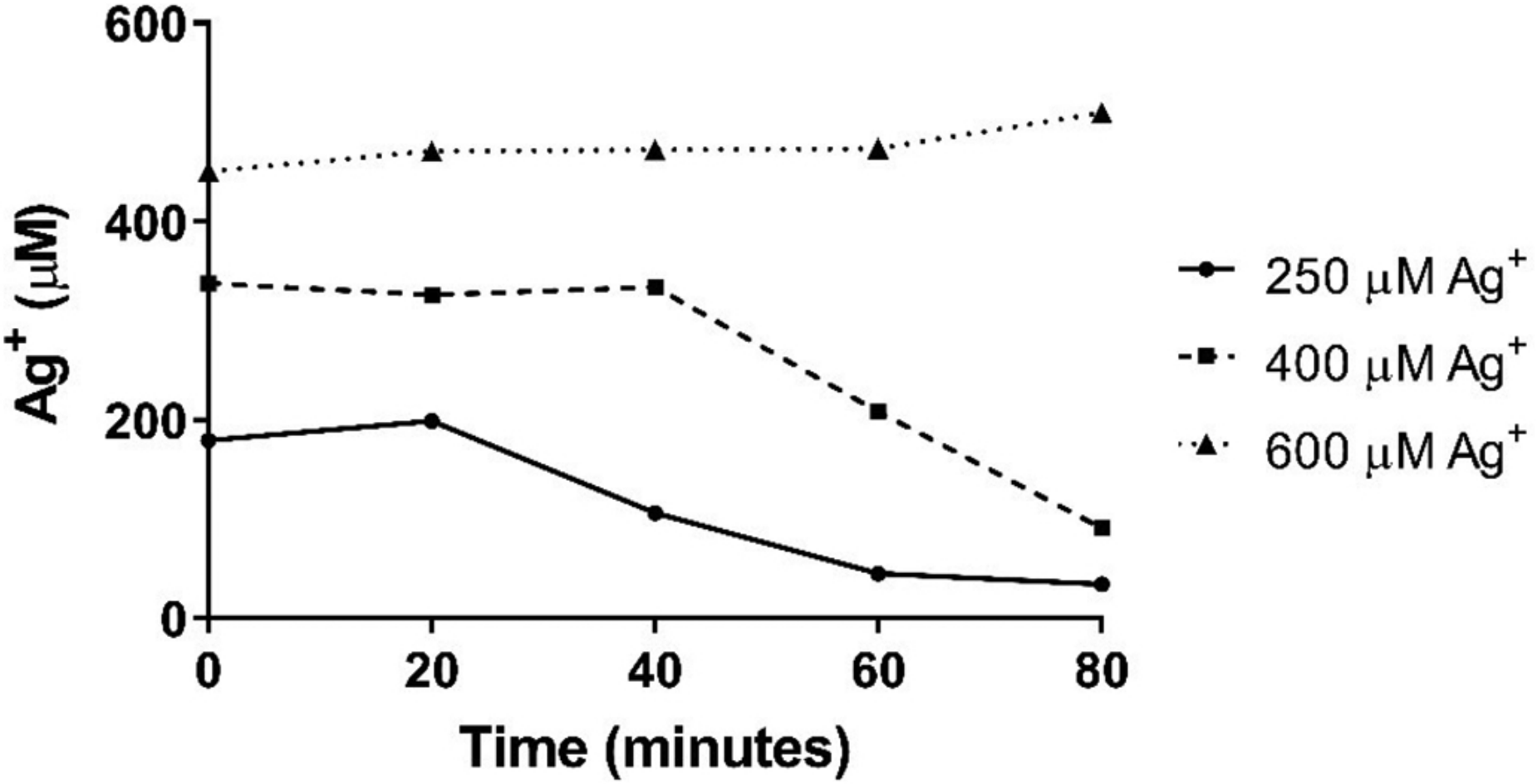
600
400
200
0
Ag+ (μM)
0
20
40
60
80
Time (minutes)
250 μM Ag+
400 μM Ag+
600 μM Ag+

Figure 3

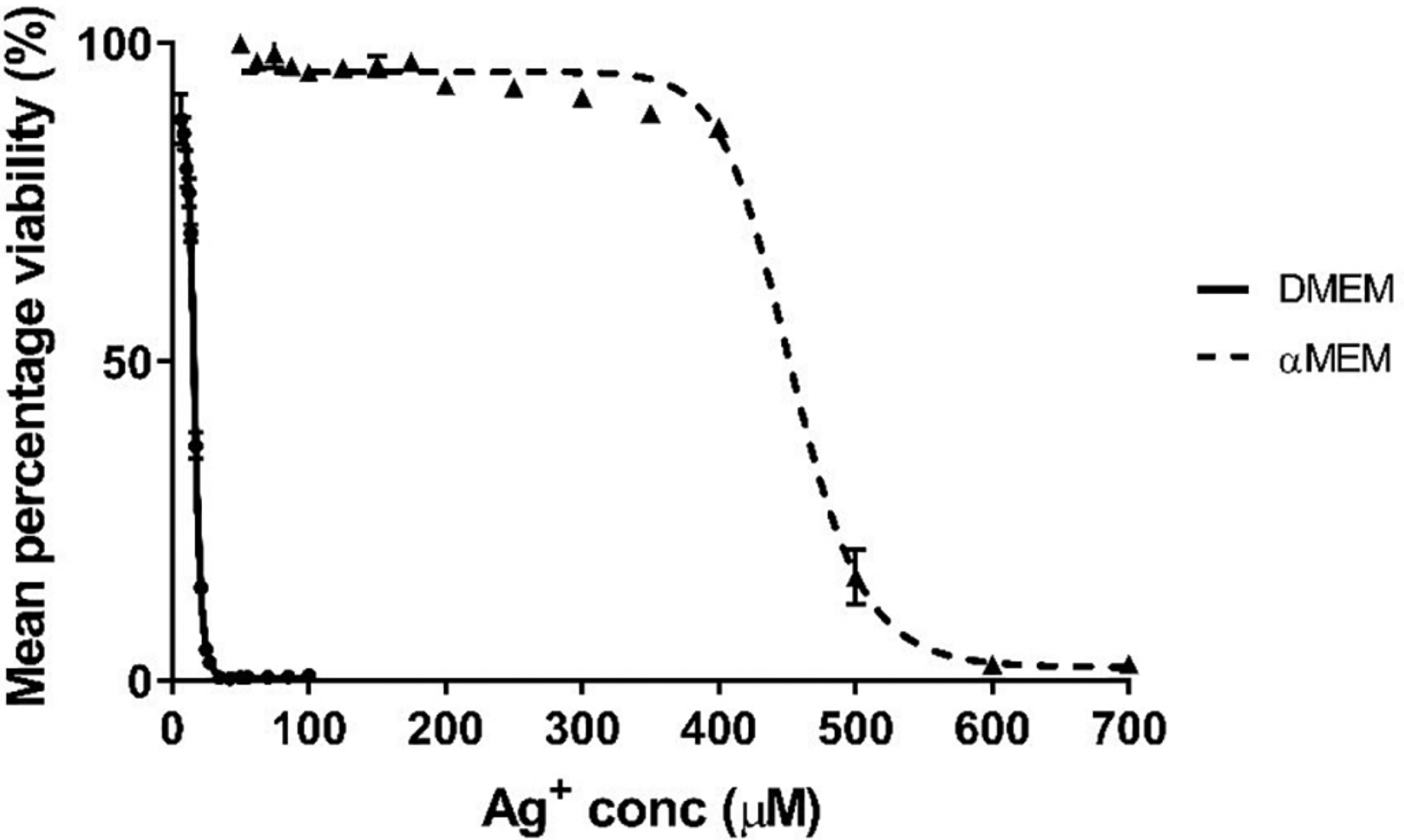
Mean percentage viability (%)
100
50
0
0
100
200
300
400
500
600
700
Ag+ conc (μM)
DMEM
αMEM

Figure 4

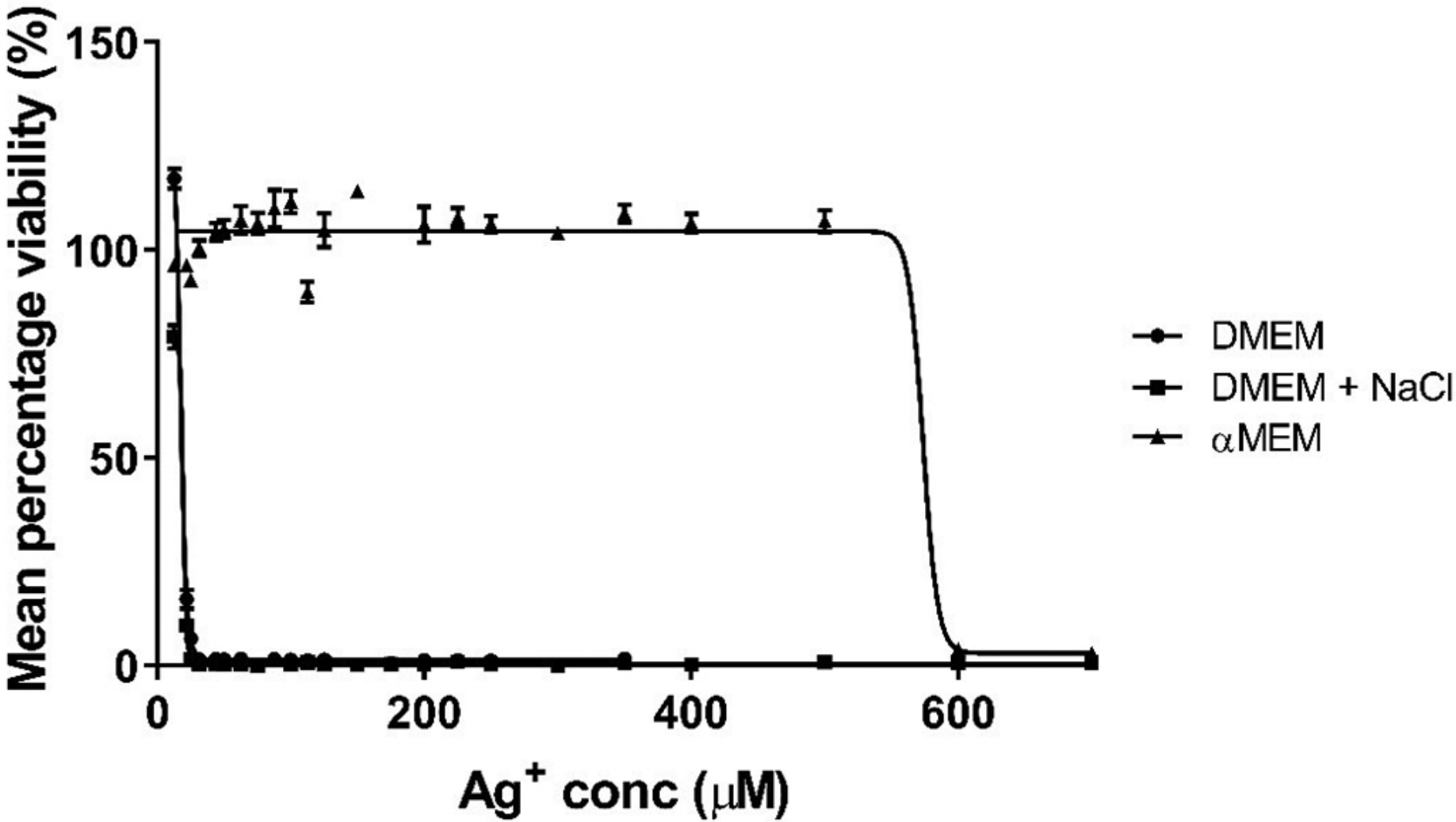
Mean percentage viability (%)
150
100
50
0
0
200
400
600
Ag+ conc (μM)
DMEM
DMEM + NaCl
αMEM

Figure 5

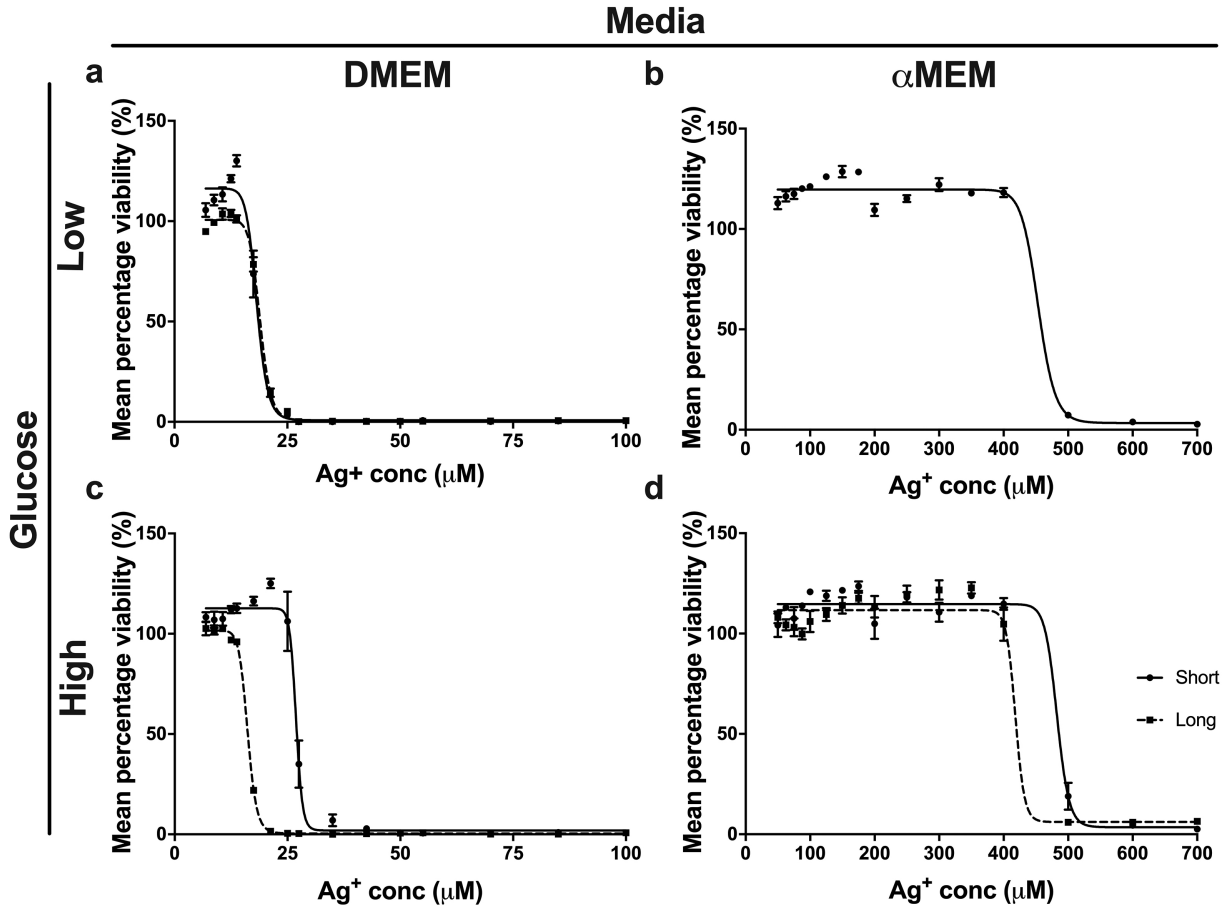
Media
DMEM
αMEM
Glucose
Low
High
a
b
c
d
Mean percentage viability (%)
Ag+ conc (μM)
Ag⁺ conc (μM)
Short
Long

Figure 6