Title: Assessment of strategies to increase chondrocyte viability in cryopreserved human osteochondral allografts: evaluation of the glycosylated hydroquinone, arbutin

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Running title: Cartilage cryoprotection by arbutin

Abstract

Objective: Allogeneic cartilage is used to repair damaged areas of articular cartilage, requiring the presence of living chondrocytes. So far, no preservation method can effectively meet that purpose. Identification of more effective cryoprotective agents (CPAs) can contribute to this goal. The aim of this study was to determine whether the glycosylated hydroquinone, arbutin, alone or in combination with low concentrations of other CPAs, has cryoprotective properties towards human articular cartilage.

Material and Methods: Human tibial plateaus were procured from multi-organ donors, with the approval of the Ethics Committee of the University Hospital of Coimbra. The tibial plateaus were treated with or without arbutin (50 or 100 mM), alone or in combination with various concentrations of dimethyl sulfoxide (DMSO) and glycerol, for 0.5 to 1.5h/37ºC, then frozen at -20ºC and 24h later transferred to a biofreezer at -80ºC. Two to three months later, thawing was achieved by immersion in cell culture medium at 37ºC/1h. Chondrocyte viability was assessed before and after freeze/thawing using a colorimetric assay based on the cell's metabolic activity and fluorescent dyes to evaluate cell membrane integrity.

Results: Before freezing, chondrocyte metabolic activity was identical in all the conditions tested. After freeze/thawing, the highest activity, corresponding to 34.2 ± 2.1% of that in the fresh control, was achieved in tibial plateaus incubated in 50 mM arbutin for 1h whereas in those left untreated it was 11.1 ± 4.7. Addition of DMSO and glycerol to arbutin did not increase chondrocyte viability any further. Fluorescence microscopy confirmed these results and showed that living chondrocytes were mainly restricted to the superficial cartilage layers.

Conclusion: Arbutin seems to be an effective cryoprotective agent for osteochondral allografts with potential benefits over DMSO and glycerol.

Keywords: arbutin; chondrocyte viability; cryopreservation; human; tibial plateau.

Introduction

Damage to articular cartilage because of trauma, degenerative and tumour diseases, or infection represents a major clinical problem. Of the existing treatment alternatives, the transplantation of osteochondral grafts is increasingly employed as means of biological resurfacing for articular cartilage defects. Osteochondral grafts, consisting of an articular cartilage layer and the underlying subchondral bone, can either be collected from the patient (autografts)[1] or from multi-organ donors (allografts)[2,3] which, unlike autografts, allow the treatment of large cartilage defects.[4,5]

Cryopreserved allografts enable size-matching of the allograft and the recipient, screening of infectious diseases of the donor and quarantine of the allografts, which are mandatory for safe transplantation, and transportation of tissues to distant locations.[3] However, the clinical outcome of cryopreserved versus fresh osteochondral allografts is poor and formation of fibrocartilage, chondromalacia and loss of the cartilage surfaces occur frequently sometime after implantation, leading to a variable graft failure rate of 10 to 50%.[3] The efficacy of the transplanted osteochondral grafts seems to be proportional to their content of living chondrocytes.[6-8] Since these cells are essential to maintain cartilage matrix integrity[9], any injury that kills even a small number, can have severe consequences on cartilage integrity and durability.[8] Cryoprotective agents (CPAs), such as glycerol or dimethyl sulfoxide (DMSO), effectively protect isolated chondrocytes from the damaging effects of freezing.[10] However, freezing of intact articular cartilage, even in the presence of the same CPAs, seriously compromises chondrocyte viability.[7,8,11-14]

In most studies, a maximum of 20-30% of the cartilage chondrocytes were reported to survive freezing when CPAs, such as DMSO or glycerol, were employed[11,12,14], whereas, in their absence, none or a very small number of viable chondrocytes was retrieved.[14,15] The CPAs currently available are cytotoxic, depending on the duration and temperature of exposure and on the concentrations used. To avoid toxicity, most cryopreservation protocols use low temperatures of exposure to the CPAs.[11,12] However, Carsi and co-workers[15] found that the diffusion of either DMSO or glycerol through human articular cartilage decreased at lower temperatures and with increasing concentrations. Thus, identification of less toxic CPAs should allow longer exposure times at higher temperatures, probably eliciting a more effective cryoprotection. Possible strategies based on this principle are the combination of low concentrations of various CPAs and the identification of new more effective and less toxic CPAs. In this regard, compounds extracted from organisms naturally resistant to drought and cold are being evaluated for cryoprotective properties. One such compound is arbutin (4-hydroxyphenyl-β-D-glucopyranoside, Fig.1), a glycosylated hydroquinone that has been shown to have a membrane stabilizing effect and to protect isolated cell membranes and liposomes from the damaging effects of both freeze-thawing and drying.[16,17]

The purpose of this study was to determine whether arbutin has cryoprotective properties towards human articular cartilage. For this and based on the strategies identified above, we evaluated the ability of arbutin, alone and in combination with low concentrations of DMSO and glycerol, to increase the recovery of viable chondrocytes

from frozen/thawed whole human tibial plateaus, which represent a good model of the whole bone.

Materials and methods

REAGENTS

Calcein AM (Cal) was purchased from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

OSTEOCHONDRAL SAMPLES

As a model of the whole bone, the right and left lateral and medial tibial plateaus, composed of the intact articular cartilage and an underlying bony layer of 0.5-1.5 cm thick, were harvested from 18 multi-organ donors (10 males and 8 females) and processed within 24h *post-mortem*, according to the standards of the Tissue Bank of the University Hospital of Coimbra (HUC), Portugal, and the Portuguese Transplantation Organization. Donor age ranged from 17 to 53 years old. All the tibial plateaus used appeared macroscopically normal without signs of cartilage damage. All the procedures had the approval of the Ethics Committee of HUC.

CRYOPROTECTION TREATMENTS AND FREEZE-THAWING CONDITIONS

Immediately upon harvesting, the tibial plateaus were incubated at 37ºC for 0.5 to 1.5h in Ham F-12 nutrient mixture in the presence or absence of 50 or 100 mM arbutin, alone or in combination with DMSO and glycerol in the concentrations indicated in the figures and legends. Then, the tibial plateaus were removed from the cryoprotective solutions, placed in sterile plastic bags, frozen at -20ºC/24h, transferred to a biofreezer at -80ºC and kept in those conditions for 2 to 3 months. Thawing was achieved by immersion in Ham F-12 nutrient mixture at 37ºC/1h.

EVALUATION OF CHONDROCYTE VIABILITY *IN SITU*

Chondrocyte viability *in situ* was assessed before freezing and after thawing. For this, two cartilage cylinders were cut with a 5 mm diameter trephine from each tibial plateau immediately before freezing and twelve identical cylinders were obtained immediately after thawing. Two methods were used to evaluate chondrocyte viability: 1) an adaptation[18] of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay[19] which is a quantitative colorimetric method based on the reduction of the MTT salt by the mitochondrial enzymes of viable cells; and 2) a cell membrane integrity assay using a fluorescent dye combination consisting of Calcein AM (Cal) and Propidium Iodide (PI). Cal is a cell membrane-permeable ester that upon hydrolysis by intracellular esterases, emits green fluorescence and becomes cell membrane-impermeable, being retained within cells bearing an intact plasma membrane. PI is cell membrane-impermeable and stains the nuclei of cells with damaged membranes with strong red fluorescence.

The two cylinders collected from each tibial plateau before freezing and ten of those collected after thawing were weighted and incubated in Ham F-12 Nutrient Mixture containing 1 mg/ml MTT, for 1h at 37ºC. The dark blue crystals of formazan produced by reduction of the MTT salt were dissolved in acidified isopropanol and quantified in an automatic plate reader (SLT, Austria) set at a test wavelength of 570 nm and a reference wavelength of 620 nm.

The remaining two cylinders collected after thawing were incubated at 37ºC for 30 min in PBS containing 5 µM Cal and 5 µg/ml PI. Cryostat sections 30 µm thick were obtained from each cylinder and viewed under a fluorescence microscope.

DATA ANALYSIS

Where applicable, statistical analysis was performed using one-way ANOVA with Dunnett's post test. Results were considered significant for $P<0.05$.

Results

The MTT reduction assay preformed in fresh cartilage immediately before freezing (Fig. 2A), showed that arbutin did not affect the ability of chondrocytes to reduce the MTT salt, as compared to chondrocytes in cartilage incubated in culture medium alone (Control). These results showed that arbutin was not toxic, nor increased the metabolic activity of chondrocytes *in situ*, under the conditions tested.

After freeze-thawing, the metabolic activity of chondrocytes in tibial plateaus pre-incubated with 50 or 100 mM arbutin for 0.5 or 1h, was compared to that in the fresh and post-thawed controls. As shown in Fig. 2B, chondrocyte metabolic activity relative to the fresh Control, which corresponds to the condition of maximal viability, was significantly reduced in all conditions tested. Nevertheless, it was significantly higher in tibial plateaus exposed to arbutin, in any of the conditions tested, than in the frozen-thawed control, which represents the lowest viability, corresponding to 11.1 ± 4.7% of the metabolic activity of the fresh control. The highest activity, corresponding to 34.2 ± 2.1% of the fresh control, was achieved in tibial plateaus incubated in 50 mM arbutin for 1h, but a shorter incubation period (30 min) elicited almost the same activity (32.0 ± 5.4%). Increasing arbutin concentration to 100 mM resulted in a slightly lower activity of 28.5 ± 2.8% relative to the fresh control, although the difference was not statistically significant.

Attempting to further enhance *in situ* chondrocyte cryoprotection without significantly increasing direct toxicity, we tested various combinations of low

concentrations of DMSO and glycerol with arbutin and extended the incubation period to 1.5h, hypothesising that diffusion of the CPAs could be improved. The results showed that before freezing, the differences between conditions were not statistically significant, although mean values were slightly variable (Fig. 3A). After freeze-thawing, MTT reduction relative to that obtained in the fresh control, was significantly higher with any of the combinations tested than in the post-thawed control (Fig. 3B), but slightly lower than the highest activity obtained with arbutin alone (Fig. 2B).

Membrane integrity stains showed that the apparent number and distribution of viable chondrocytes was similar in the three conditions using arbutin (Figs. 4B, C, D), whereas in the frozen-thawed control very few or no viable cells were observed (Fig. 4A). When compared to cartilage sections pre-treated with 10% DMSO (Fig. 4E) or 10% glycerol (Fig. 4F) for 30 min at 37ºC, used as positive standards, the apparent number of viable chondrocytes was higher in those treated with arbutin, either alone (Fig. 4B) or in combination with those CPAs (Figs. 4C, D). These results confirmed those of the MTT reduction assay and showed that, even in the condition eliciting the highest activity (50 mM arbutin/1h), viable chondrocytes were mainly restricted to the superficial cartilage layers (Fig. 4B). Cell density in each cartilage section was estimated by observing the cells stained with PI (Figs. 4a, b, c, d, e, f) and by viewing each section under light transmission. No apparent differences were observed between the different conditions tested in tibial plateaus obtained from the same donor, although some variability, unrelated to age or gender, was observed among different donors.

Discussion

The results presented show that arbutin increased the metabolic activity in frozen-thawed tibial plateaus to a maximum of 34% of the fresh control. Some studies indicate, however, that metabolic activity assays overestimate cell viability due to activation by cold exposure, whereas membrane integrity stains seem to reflect cell viability more accurately.[11,20] This is also probably the case in our study since fluorescence microscopy showed only very few viable chondrocytes in the frozen/thawed control, whereas metabolic activity in the same samples represented 11% of the fresh control.

Our previous study, using the same osteochondral model and experimental conditions (temperature of incubation with the CPAs and duration and temperatures of freeze-thawing) similar to those described in the current study, showed that treatment with 10% DMSO or 10% glycerol increased the metabolic activity in the frozen-thawed tibial plateaus to approximately 15% of the fresh control.[14] Thus, comparatively, the cryoprotective effect of arbutin was approximately twice that elicited by those CPAs. It can be argued that arbutin may simply have increased the metabolic activity of the cells that survived freeze-thawing, but since before freezing, arbutin, either alone or in combination, did not affect the metabolic activity of chondrocytes (Fig. 2), that hypothesis is unlikely.

On the other hand, if the metabolic activities were corrected by subtracting the result of the frozen-thawed control from that of each experimental condition, viability in tibial

plateus treated with arbutin 50 mM/1h would be approximately 23%, whereas in those treated with 10% DMSO or 10% glycerol it would not exceed 4%.

In either case, the ability of arbutin to preserve the metabolic activity and likely the viability of chondrocytes is significantly higher than that of DMSO or glycerol tested under similar conditions. Moreover, if compared to the results reported for DMSO and glycerol in studies using small osteochondral cylinders, the metabolic activity obtained with arbutin is within the same range.[6,12,21] Since the surface area through which the cryoprotective agents can diffuse through the articular cartilage is considerably smaller and the volume much larger in the whole tibial plateaus than in the small osteochondral cylinders generally used, the results obtained with arbutin in our study seem especially relevant.

Although not quantitatively expressed, evidence from membrane integrity stains also indicates that treatment with arbutin, either alone or in combination with traditional CPAs, increases the recovery of viable chondrocytes. Nonetheless, viable chondrocytes were still restricted to the superficial cartilage layers, even when cryoprotective treatment was extended to 1.5h. This suggests that none of the compounds used reached the entire cartilage depth, as also found in other studies with DMSO and glycerol.[7,14]

Additionally, the conditions used in our study for the cryoprotection treatments are easily applicable in tissue banks and as such, the results obtained can be expected to more closely represent the actual chondrocyte viability achievable in the clinical context. Worthwhile noticing is the fact that human articular cartilage seems to be

intrinsically more difficult to cryopreserve than cartilage from other species,[21] which is agreement with studies showing that the structural features and organization of human articular cartilage differ considerably from those of other animal species commonly used in orthopaedic research.[22]

Taken together, our results identify arbutin as a potential new CPA for use with large human osteochondral allografts, showing that it is more effective than DMSO or glycerol. Nonetheless, improving other conditions, namely freezing at a lower temperature, eventually by a step-cooling method, and promoting a more complete diffusion of arbutin through the cartilage, seems mandatory to significantly increase chondrocyte viability any further. One such possibility is the application of forces to the cartilage surface that can drive the cryoprotective agent through it, as occurs *in vivo* where joint motion drives the movement of nutrients from the synovial fluid into the articular cartilage.[23] Developing mechanical devices that can apply the required forces and combining them with highly effective CPAs, such as arbutin, may be the key to achieve a more complete protection of chondrocytes in the entire depth of the articular cartilage and thus, to ensure the clinical success of cryopreserved osteochondral allografts.

Acknowledgments

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Figure legends

Figure 1. Chemical structure of arbutin (4-hydroxyphenyl-β-D-glucopyranoside).

Figure 2. Chondrocyte viability, measured by the MTT reduction assay, in tibial plateaus treated with arbutin. A) chondrocyte viability, expressed in percentage of the Control which was taken as 100%, in fresh tibial plateaus incubated for the periods indicated in the absence (Control) or presence of the indicated concentrations of arbutin. B) chondrocyte viability, expressed in percentage relative to fresh cartilage incubated in culture medium alone (Fresh Control), in freeze/thawed tibial plateaus previously incubated for the periods indicated in the absence (Post thawed Control) or presence of the indicated concentrations of arbutin.. Each bar represents the mean ± SEM of six independent experiments. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared to the post thawed control; $^{***}P<0.001$ compared to the fresh control.

Figure 3. Chondrocyte viability, measured by the MTT reduction assay, in tibial plateaus treated with combinations of arbutin, DMSO and glycerol. A) chondrocyte viability, expressed in percentage of the Control which was taken as 100%, in fresh tibial plateaus incubated for 1.5h in the absence (Control) or presence of the indicated concentrations of arbutin combined with 5% (2.5% DMSO + 2.5% glycerol)or 10% (5% DMSO + 5% glycerol) CPAs. B) chondrocyte viability, expressed in percentage relative to fresh cartilage incubated in culture medium alone (Fresh Control), in freeze/thawed tibial plateaus previously incubated for the periods indicated in the

absence (Post thawed Control) or presence of the indicated concentrations of arbutin combined with 5% (2.5% DMSO + 2.5% glycerol) or 10% (5% DMSO + 5% glycerol) CPAs. Each bar represents the mean ± SEM of, at least, six independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to the post thawed control; $^{***}P<0.001$ compared to the fresh control.

Figure 4. Chondrocyte viability and cell density evaluated as a function of cell membrane integrity by fluorescence microscopy of cartilage sections stained with Calcein AM (A, B, C, D, E, F) and Propidium Iodide (a, b, c, d, e, f). Representative fluorescence microscopy photographs of full depth cartilage sections obtained from frozen-thawed tibial plateaus left untreated (A, a) or pre-treated at 37ºC with 50 mM arbutin/1h (B, b), 50 mM arbutin plus 5% CPAs (2.5% DMSO + 2.5% glycerol)/1.5h (C, c), 100 mM arbutin plus 10% CPAs (5% DMSO + 5% glycerol)/1.5h (D, d), 10% DMSO/30 min (E, e) or 10% Glycerol/30 min (F, f). Chondrocytes with intact cell membranes are stained in green and those with damaged cell membranes are stained in red (100x). The images shown are representative of at least four independent experiments for each condition.

Figure 1

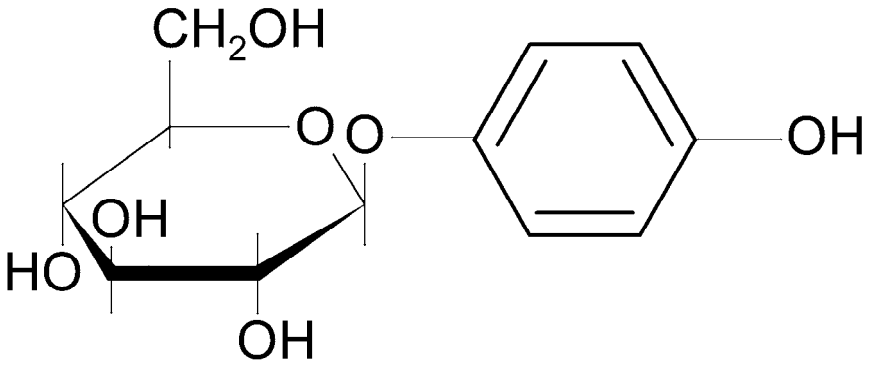
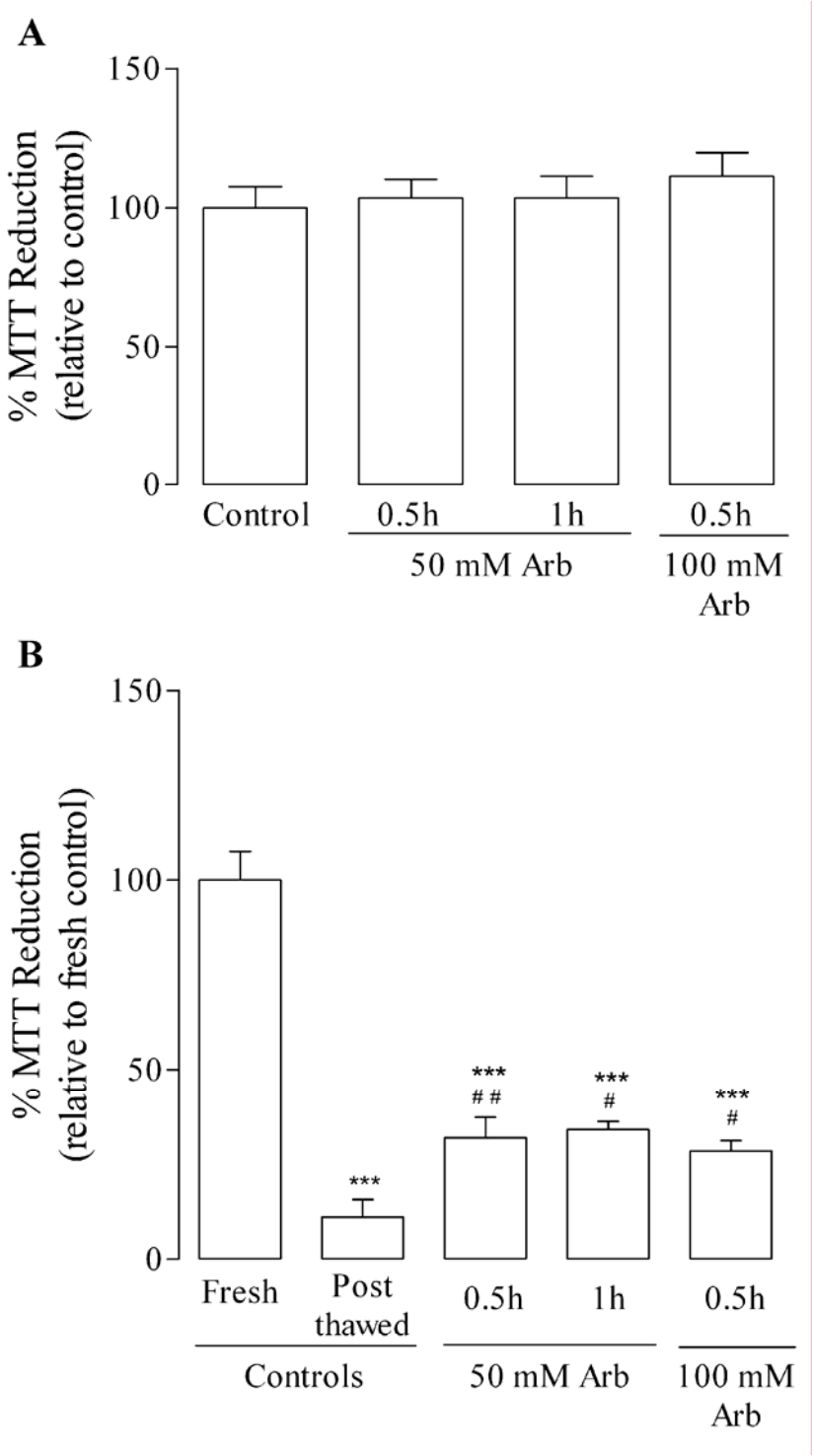


Figure 2


A
150
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% MTT Reduction
(relative to control)
Control
0.5h
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50 mM Arb
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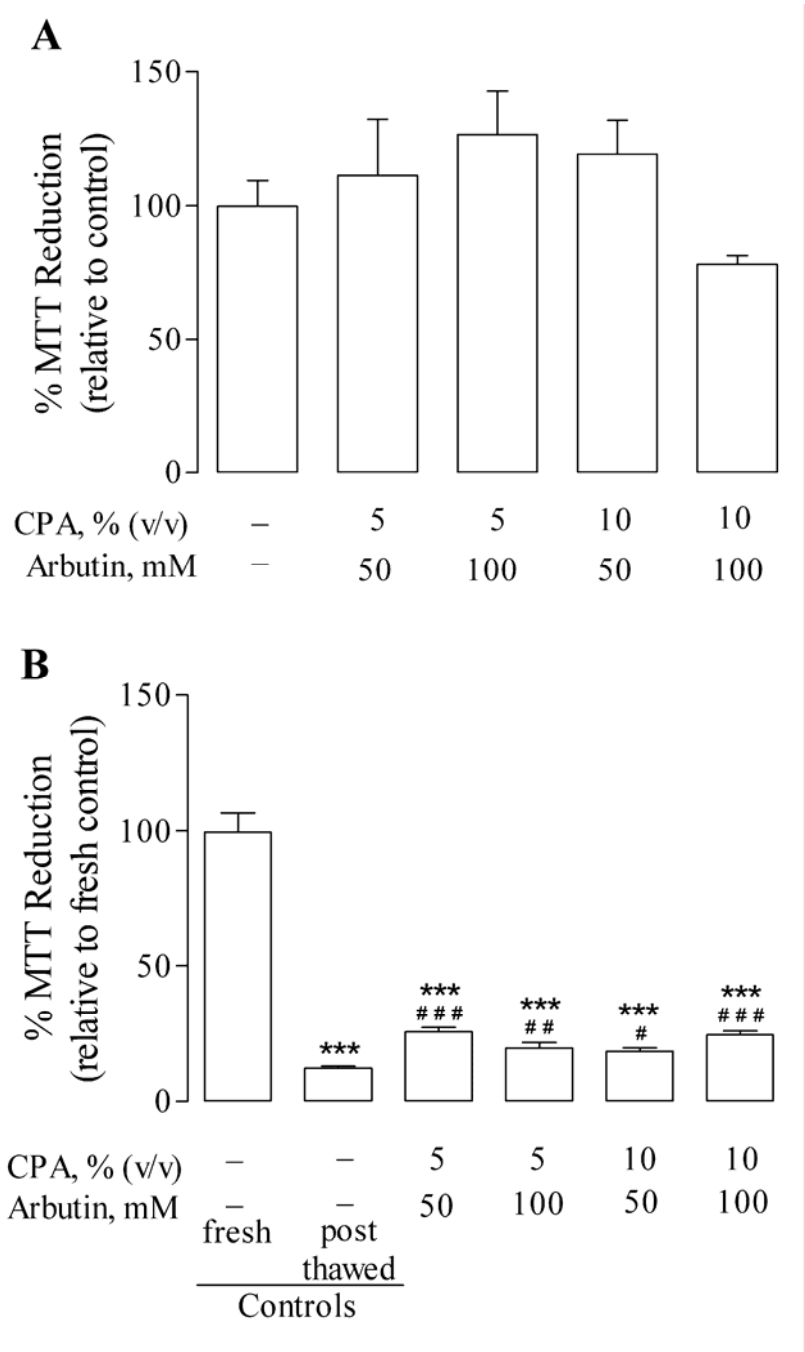
Arb

Figure 3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CPA, % (v/v) – – 5 5 10 10
Arbutin, mM – – 50 100 50 100
fresh
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Controls

Figure 4