

•Hyaluronic Acid as a Cryopreservant of Mesenchymal Stem Cells

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INTRODUCTION: Human mesenchymal stem cells (MSCs) hold great therapeutic potential for a number of diseases including osteoarthritis. Optimal cryopreservation of these cells for such applications will be fundamental for the desired cellular efficacy. Cryopreservation methods to date have required inclusion of high levels of toxic DMSO (10%). The naturally-occurring glycosaminoglycan, hyaluronan or hyaluronic acid (HA) is found predominantly in the extracellular matrix of adult cartilage, where it plays a role in a plethora of processes including cell aggregation, proliferation, migration, inflammation and tissue stability (Bastow et al 2008). A number of HA variants exist, all with varying properties, molecular weights and viscosities. Although HA has previously been reported as a useful additive in cryostorage medium for some cell types, there are no previous reports of its application as a cryoprotectant for human MSCs. Here the cryoprotective properties of HA were investigated, focusing on the effect of various HAs on preserving cell viability, proliferation and chondrogenic differentiation of preserved MSCs.

METHODS: Cell culture: Human and ovine MSCs were isolated from bone marrow aspirates. Cells were expanded in α MEM/10% fetal calf serum (FCS) + 5ng/ml FGF-2. HA variants: A, B, C, D, E, F, G, H & I (see Table 1 for description). Cryopreservation: Cells were trypsinised and cryopreserved at 1×10^6 cells/ml in combinations of dimethylsulphoxide (DMSO, 1-10%), Trehalose (30-200mM), HA (0.5-20mg/ml) and FCS or human serum albumin (HSA). Cell-cryovials were placed in controlled-rate containers and cryopreserved at $-1^\circ\text{C}/\text{minute}$ to -80°C , prior to storage in liquid nitrogen. Cell viability assay: The Guava-VIACOUNT assay was used to assess cell viability. Cells were resurrected at 37°C and washed in medium, prior to combination with Guava Viacount solution and analysis on the Guava cytometer. Cell proliferation assay: Cell proliferation was measured using Picogreen (Molecular Probes) reagent. Chondrogenic differentiation: Cells were pellet cultured in the presence of standard chondrogenic differentiation factors and assessed for glycosaminoglycan content using histological Safranin O staining and the quantitative DMMB assay as previously published (Mort JS & Roughley PJ 2007).

HA	Source	HA Concentration (mg/ml)	MW (Da)	Property
A	Avian	10	620,000 - 1,170,000	Single Chain
B	Synthetic	20	10^{11}	Cross Linked
C	Synthetic	20	Unknown	Cross Linked
D	Synthetic	20	Unknown	Single Chain
E	Synthetic	12	Unknown	Cross Linked
F	Avian	8	6,000,000	Cross Linked
G	Bacterial	10	2,400,000 - 3,600,000	Cross Linked
H	Bacterial	10	500,000 - 730,000	Single Chain
I	Avian	10	600,000	Single Chain

Table 1: Descriptive outline of HA compositions used in this study including the source, molecular weight and physical properties of the HA used.

RESULTS SECTION: Cell viability following cryopreservation (up to 19 days) of both ovine and human MSC was enhanced ~10% in the presence of 5mg/ml high molecular weight HA-A (Figure 1). Species differences were observed between ovine and human MSC with respect to cryopreservation requirements. Unlike human MSC, ovine MSC were more robust, able to withstand cryopreservation (7-days) in low levels of DMSO (1% DMSO), whilst HA-A alone (10mg/ml) in absence of DMSO failed to maintain cell viability in both species (data not shown). Furthermore, in the presence of 5mg/ml HA-A, ovine cell viability remained above 70% when DMSO was replaced completely with 30mM trehalose. Human MSC viability however, was significantly compromised (<50%) when DMSO was replaced with trehalose, even at higher concentrations (results not shown). Addition of HSA in place of FCS in the cryopreservation medium of both human and ovine MSC had little effect on cell viability (data not shown). With respect to human MSC, cryopreservation in the presence of 5mg/ml HA enabled

lower concentrations of DMSO to be applied in short term cryostorage (Figure. 2). A dose-dependant effect on HA concentration was also observed, with a concentration of 4-5mg/ml HA being optimal (data not shown). It was evident that different HA variants elicited different levels of cryoprotection on human MSC (in the presence of 10% DMSO), with variant F resulting in the least favorable (data not shown). Following cryopreservation in different HA variants, cell proliferation was also influenced, whereby HA-A & B enhanced proliferation above control level and HA-I reduced. The effect on subsequent chondrogenic differentiation is currently under investigation.

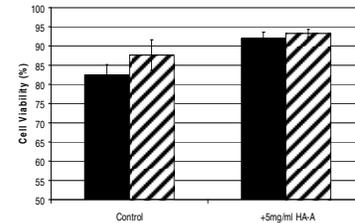


Fig 1: Cryopreservation in HA enhances MSC viability. Human (solid) & Ovine (lined) MSC were cryopreserved (19 days) in FCS/10% DMSO (control) and in the presence of 5mg/ml HA-A.

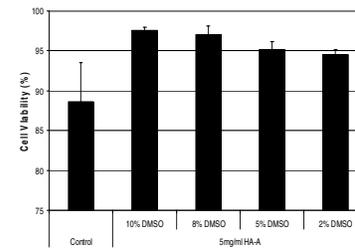


Fig 2: Cryopreservation in HA permits reduction of DMSO. Human MSC were cryopreserved (7 days) in FCS/10% DMSO (control) and in the presence of 5mg/ml HA-A with 10-2% DMSO.

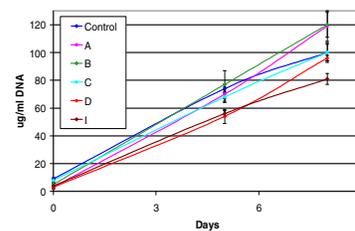


Fig 3: HA property influences proliferation following cryopreservation. Human MSC cryopreserved (21 days) in FCS/10% DMSO in absence (control) and presence of 5mg/ml HA-A, B, C, D & I were assessed for proliferation.

DISCUSSION: Cryopreservation of both ovine and human MSCs in the presence of various forms of HA enhances cell viability. Significant species differences were observed however, and should be considered when performing in vivo pre-clinical studies in view of a human clinical trial. Most interestingly, the presence of 5mg/ml HA enabled lower concentrations of DMSO to be applied in human MSC cryostorage, thereby enhancing direct use of a MSC product in the clinic. Following cryopreservation in variations of HA, differences were observed with respect to human MSC viability & proliferation, however these observations were independent of HA source and / or cross-linking property. HA is therefore a suitable cryopreservant for both ovine and human MSCs. Selection of the optimal HA-type and concentration are important factors to consider, whilst modifications to the HA structure or properties might enhance performance further with respect to cryopreservation property & subsequent proliferation and differentiation.

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