Free Rad. Res., Vol. 32, pp. 157–170
Reprints available directly from the publisher
Photocopying permitted by license only

© 2000 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of The Gordon and Breach Publishing Group.
Printed in Malaysia.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

Studies of Free Radical-Mediated Cryoinjury in the Unicellular Green Alga *Euglena gracilis* Using a Non-Destructive Hydroxyl Radical Assay: A Novel Approach for Developing Protistan Cryopreservation Strategies

ROLAND A. FLECK[a,†], ERICA E. BENSON[b,*], DAVID H. BREMNER[b] and JOHN G. DAY[a]

[a]*Culture Collection of Algae and Protozoa, NERC Institute of Freshwater Ecology, Windermere Laboratory, Far Sawrey, Ambleside, Cumbria, LA22 0LP, UK;* [b]*Plant Conservation Biotechnology Group, Division of Molecular and Life Sciences, School of Science and Engineering, University of Abertay Dundee, Bell Street, Dundee, DD1 1HG, Scotland, UK*

Accepted by Prof. B. Halliwell

(Received 30 April 1999; In revised form 28 June 1999)

The development of cryoconservation methods for the long-term storage of algal cultures is important for the *ex situ* preservation of biological diversity and the maintenance of genetic stability within this group of important organisms. However, as many unicellular algae are recalcitrant to cryogenic storage, this study aims to evaluate the role of oxidative stress in cryoinjury. A non-invasive, non-destructive assay method previously applied to animal cells has been developed to evaluate free radical mediated oxidative stress in *Euglena gracilis* exposed to different cryopreservation treatments. The procedure employs dimethyl sulphoxide as a probe for the hydroxyl radical. Adopting this approach it was possible to identify those components of the cryopreservation protocol which were the most damaging. These were identified as preparative centrifugation and sub-zero freezing treatments. Post-storage survival in *E. gracilis* was significantly ($P < 0.05$) enhanced when the chelating agent desferrioxamine was included in the recovery medium whilst methane production was significantly ($P < 0.004$) reduced, suggesting that the additive was capable of ameliorating oxidative stress. The potential of using novel, exogenous antioxidant treatments developed for medical applications and applying them to enhance cryopreservation tolerance in recalcitrant unicellular algae is discussed.

Keywords: Cryoprotection, freezing injury, cryopreservation, algae, *Euglena gracilis*, conservation, oxidative stress, free radicals, hydroxyl radical

INTRODUCTION

Cryogenic storage, in liquid nitrogen (LN), at −196°C, provides one of the most important means of conserving viable cells[1] and the Culture Collection of Algae and Protozoa (CCAP) presently holds a cryopreserved collection of

* Corresponding author. Tel.: 01382 308674. Fax: 01382 308663. E-mail: e.e.benson@abertay-dundee.ac.uk.
† Present address: Department of Soil, Crop and Atmospheric Sciences, Cornell University, Ithaca, NY 14853, USA.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

unicellular freshwater algae.[1] However, many protistan species cannot be cryopreserved as they are susceptible to freezing injury and cryoprotectant toxicity.[2] As the long-term maintenance of algae in an actively growing state risks genetic drift and contamination,[1,3] there is an urgent need to improve cryopreservation methodology. This is important as microalgae are increasingly used in the pharmaceutical, aquaculture and biotechnology industries[4,5] and for educational and ecological purposes. The cryogenic storage of these organisms will ensure their safe maintenance, as reference cultures. To date, storage methodology for freshwater algae has been developed empirically and cryoinjury has been evaluated using microscopy and the assessment of post-storage viability and regrowth.[6] These approaches are useful but they cannot be used to elucidate the biochemical basis of cryoinjury. Cryopreservation involves many different steps and in order to develop improved protocols it is essential to determine which parts of the preservation procedure cause damage and take preventative measures to limit injury.

Free radical damage occurs in tissues exposed to low and ultra low storage temperatures and radical injury is exacerbated when sub-optimal storage protocols are applied.[7–10] Thus, oxidative stress occurs in mammalian transplant organs exposed to low temperature storage and free radical-mediated loss of organ function can be ameliorated by applying antioxidants and free radical scavenging agents.[9,10] Free radical damage is also implicated in plant cryopreservation and seed storage recalcitrance[11–13] and antioxidant treatments have been used to enhance the longevity of seeds stored at sub-zero temperatures.[14] Cryopreserved plant cells and tissues undergo free radical mediated oxidative stress as evidenced by singlet oxygen formation, lipid peroxidation and free radical generation.[11,15–18] Furthermore, environmentaly induced acclimation responses of higher plants to cold stress have been associated with the enhancement of antioxidant enzyme activities.[19]

The aim of this study is to explore the possibility that free radical damage is a component of cryoinjury in microalgae. The role of oxidative stress in algal freeze-recalcitrance has not been previously investigated and as antioxidant treatments have been found to enhance cryotolerance in other organisms it is important to assess their potential for improving cryopreservation methodology in microalgae. These are a group of organisms, which have to date, proved difficult to cryopreserve.

Euglena gracilis is a member of the Euglenaphyta which has previously been chosen as the test organism[20] and is considered to be cryopreservation recalcitrant by the CCAP. The first objective of this investigation was to evaluate the potential of using dimethyl sulphoxide (DMSO) as a probe[21] for hydroxyl radical formation and cryoinjury in *E. gracilis*. Cohen and Cederbaum[21] applied this assay to study $^{\bullet}OH$ generation in microsomal systems and it is based on the fact that when $^{\bullet}OH$ radicals react with DMSO, methyl radicals ($^{\bullet}CH_3$) are formed, these subsequently abstract $H^{\bullet}$ and form methane gas which can be monitored directly. A second objective was to profile free radical production throughout a cryopreservation protocol by using the non-destructive monitoring of methane in algal headspace samples in combination with gas chromatography. Using this approach it was possible to determine which components of the cryopreservation protocol were the most damaging to *E. gracilis* and develop strategies which enhance cryotolerance in the alga. A final objective of the study was to explore the possibility of using the iron chelating agent desferrioxamine,[22,23] to reduce hydroxyl radical-mediated cryoinjury in cryopreserved *E. gracilis*. This pharmaceutical compound is a potent iron cation chelating agent and it is routinely used to prevent the clinical and pathological generation of $^{\bullet}OH$ radicals in human medicine.[22,23] The possible role of iron-mediated free radical damage has recently been examined in cryopreserved blood cells[24] and desferrioxamine together with free radical scavenging

RIGHTS LINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

treatments[25,26] has been found to be beneficial in reducing free radical-mediated low temperature injury in mammalian transplant organs and tissues.[25,26] Similar studies of cryopreserved rice cells also showed that recovery was enhanced in the absence of metal cations and in the presence of desferrioxamine.[27]

MATERIALS AND METHODS

Culture Protocols

Euglena gracilis Klebs CCAP 1224/5Z was used throughout the study and routinely cultured[28] in *E. gracilis* medium (designated as EG) and Jaworski's medium (designated as JM) mixed in 1:1 proportions.[28] Cultures were maintained at 15°C under a 12:12 h light:dark regime. Illumination was provided by cool white fluorescent lamps with a photon flux density of $50\,\mu mol \cdot m^{-2} \cdot s^{-1}$ at the surface of the culture vessel. During recovery, cultures were maintained using initial incubation temperatures of either 22°C or 15°C, followed by maintenance at 15°C.

Cryopreservation Regimes

Euglena gracilis is difficult to cryopreserve and the following regime comprises a promising protocol which the CCAP has developed empirically and which does offer survival potential. Thus, cells were harvested for cryopreservation by centrifugation (1 min at 1000 rpm) and cryoprotected for 15 min using 10% (v/v) methanol applied at 0°C. To avoid excessive osmotic shock cryoprotectant solutions were always added to the cell suspensions. Vials containing 0.5 ml of *E. gracilis* cells mixed with the cryoprotectant were cryopreserved using a controlled two-step cooling procedure for which the temperature gradient was regulated by a Planer Kryo 10 Programmable Freezer (Planer, UK). Cells were cooled at a rate of $-0.5°C\,min^{-1}$ to a terminal transfer temperature of either −10°C or −60°C and held at this intermediate temperature for 30 min. The −60°C samples were then directly plunged into LN. Vials were thawed using a two-step protocol in which they were first warmed slowly at ambient air temperature for 1 min, followed by rapid warming in a pre-heated 40°C water bath. All vials were agitated until the last ice crystals had melted.[3] The thawed material was aseptically transferred to appropriate fresh, sterile, medium and biochemical and viability assays were performed as outlined below. All cryopreservation experiments were repeated in triplicate and data are presented as means, and errors are expressed as standard errors of the mean.

Viability Testing Using Flow Cytometry

Viability was assessed[29] using the vital stain, fluorescein diacetate (FDA), and viable cells in a population were quantified using Flow Cytometry. Treated and untreated cells were analysed in a FACStar Plus flow cytometer (Becton Dickinson, UK) set to trigger on forward scatter. Three parameters were measured: forward light scatter (FSC), 90° side-scatter (SSC) and fluorescence at 530 ± 30 nm. The laser power at 488 nm was set to 20 mW and all data were recorded on a log scale. The photomultiplier voltages were set at 400 V for SSC and 450 V for the fluorescence detector. The cytometer was aligned with 0.5 μm diameter Fluoresbrite YG fluorescent latex beads (Polysciences, USA). The nozzle diameter was 70 μm. For each sample, 5000 events were recorded. The FACStar Plus computing package was used to determine the proportion of cells that were fluorescently labelled with FDA and to display the data as single-dot plots or histograms. For each data set, unstained sub-samples were removed and used as negative controls to determine the baseline fluorescence of non-labelled cells.[30] Cut off points were then determined as being areas where no background fluorescence was detected. In subsequent stained samples, it

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

was possible to measure the proportion of the population labelled with vital stain.

Photosynthetic Activity and Chlorophyll Measurements

Oxygen evolution rates were measured using a Rank oxygen electrode according to Whitelam and Codd.[31] Illumination was constant and non-limiting for oxygen evolution steps ($440\,\mu mol \cdot m^2 \cdot s^{-1}$). Chlorophyll levels were determined as described by MacKinney[32] and all experiments were performed in triplicate.

Cell Preparation and Experimental Design for •OH Radical Assessments

Aliquots of *E. gracilis* derived from a single culture, 24 h prior to cooling, were pre-cultured, under standard conditions, in identical volumes of EG : JM medium. Immediately before chilling, non-centrifuged and untreated controls cells (2 ml) were transferred to sterile recovery tubes. The remaining *E. gracilis* cultures were dispensed (2 ml) into sterile centrifuge tubes, concentrated by centrifugation (1 min at 1000 rpm) and resuspended in 0.25 ml EG : JM medium. Cells were treated using the standard cryopreservation protocol described above. During post-thaw manipulations, cryoprotectants, if present, were removed by centrifugation and all cells were resuspended in 2 ml of EG : JM recovery medium supplemented with 1% (v/v) DMSO, and, as appropriate, $10\,mg \cdot l^{-1}$ desferrioxamine. For logistical purposes sufficient cell samples were prepared to permit the removal of triplicate samples at all stages of the cryopreservation protocol for later analysis. All GC analyses were performed on cell cultures of identical volume and cell density. Samples corresponding to the following treatment steps were collected and treated with 1% (v/v) DMSO and designated as: (a) untreated control cells which were not centrifuged at any stage during preparation; (b) cells which were centrifuged to concentrate them prior to cryopreservation; (c) concentrated cells which had been exposed to cryoprotectant [10% (v/v) methanol] for 15 min; (d) concentrated, cryoprotected cells which had been cooled at $-0.5°C\,min^{-1}$ to $-10°C$; (e) concentrated, cryoprotected cells which had been cooled at $-0.5°C\,min^{-1}$ to $-60°C$ and (f) concentrated, cryoprotected cells which had been cooled at $-0.5°C\,min^{-1}$ to $-60°C$, and held for 30 min prior to being plunged into LN (at $-196°C$).

Monitoring •OH Activity Using a DMSO Probe and Gas Chromatography. Preparation of Cell Samples for Head Space Sampling

Identical glass vials with volumes of either 4 or 15 ml (Suplico, USA) were used throughout. The volume of each individual glass vial was accurately determined thus ensuring that it was possible to calibrate the head space sampling procedures accurately. Vials were sealed with air tight silicon–teflon septa (Suplico) and sterilised with their silicon–teflon lids in place by autoclaving (10 Bar). These enclosures were inert and they did not produce contaminating hydrocarbon volatiles. Immediately post sterilisation, the vials were opened and allowed to vent for 2 h in a sterile laminar flow bench. This ensured that any volatiles which were retained in vials as a result of autoclaving were removed. Control vials were routinely assessed for background methane which was found to be negligible. For head space sampling, cell suspensions (2 ml) were placed in pre-prepared sterile glass vials (4 or 15 ml) and sealed with air tight silicon–teflon septa. The exact time was recorded at the point of sealing the vials, permitting calculation of the time interval between sealing the vial and injecting the head space samples into the gas chromatograph, and the rate of methane production per hour was thus accurately determined. Methane evolved by the DMSO/algal system was captured in the vial head space which was sampled for gas chromatography at post-treatment recovery intervals of 4, 24, 48, 72 and 120 h.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

Head Space Sampling and Gas Chromatography

All procedures are based on the previously reported methods[16,29] as adapted from a method developed by Cohen and Cederbaum.[21] Head space samples (1 ml) were removed via the septa using gas-tight calibrated syringes and injected into a Perkin-Elmer 8310 Gas Chromatograph (Perkin-Elmer, USA), fitted with a 2 m Poropak Q column and a hydrogen/air flame ionisation detector. Methane was clearly separated from the injection peak by using an oven temperature of 35°C and an isothermal time of 2 min. The injector and detector temperatures were 200°C and 225°C respectively. A nitrogen carrier gas at a flow rate of $10\,\mathrm{ml \cdot min^{-1}}$, was employed throughout. The gas chromatograph was pre-calibrated using a standard methane/N_2 mixture. Identified peaks and volatile concentrations (ppm) were stored in the computerised data handling facility. At all stages, appropriate controls were incorporated into the analyses to determine the presence of any background contaminating volatiles and the presence of non-DMSO (in the absence of the algal cells) derived methane which may be evolved from the DMSO, media, vessels, septa, cryoprotectants or the laboratory atmosphere. Contamination of methane from all these controls was either not detectable or negligible. However, throughout all the experiments, control vials (9 vials) were also included in the experimental design and the methane concentrations in the lab atmosphere recorded immediately after the vials were sealed (3 vials) and at the start (3 vials) and end (3 vials) of the experimental gas chromatographic analysis. These data sets were employed as background methane levels and where necessary subtracted from the cell suspension/ DMSO sample data.

To ensure accurate sampling, head space samples were withdrawn using a gas-tight syringe, after the head space had been thoroughly mixed by flushing the syringe out several times (5 times) without withdrawing the needle. Two, 1 ml injections were taken from each vial. Immediately after head space sampling, the vial samples were returned to a laminar flow bench and aerated for 20 min before resealing.

The rate of volatile production per hour was calculated (after data was corrected for background methane levels) and data expressed as parts per million (ppm) methane (as designated by the calibration gases) per 10^6 viable cells (measured using flow cytometry) and presented as means with standard errors of mean, where $n = 6$.

RESULTS

In this study, the effects of each individual treatment step of the cryopreservation protocol on the photosynthetic capacity of cells was examined (Figure 1). Exposure to chilling at 0°C reduced oxygen production by 45% ± 1%,

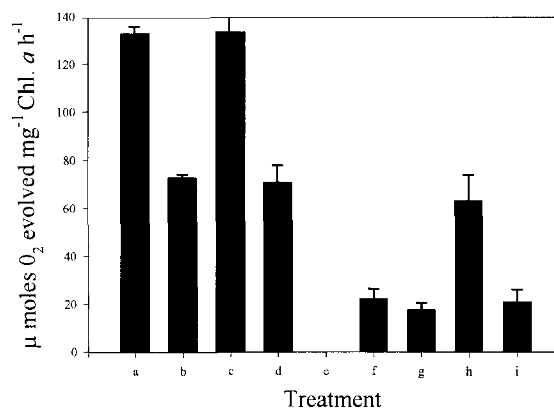

FIGURE 1 Photosynthetic activity of *E. gracilis* following exposure to different stages of a two-step, controlled rate cooling protocol. Treatments: (a) untreated control cells; (b) cells cooled to 0°C and held for 15 min; (c) cells removed from cryoprotectant (10% (v/v) methanol) after a 15 min exposure at 0°C; (d) cells exposed to cryoprotectant for 15 min at 0°C; (e) cells plunged directly into LN without cryoprotectant being present; (f) cells cooled from 0°C at $-0.5°\mathrm{C\,min^{-1}}$ to −60°C and held for 30 min, time zero (g) cells plunged into LN from −60°C, time zero and thawed; (h) cells cooled from 0°C at $-0.5°\mathrm{C\,min^{-1}}$ to −60°C and held for 30 min, 24 h. (i) cells plunged into LN from −60°C, 24 h. Cells were thawed using a simple two-step protocol involving, re-warming for 1 min in air followed by immersion in a 40°C water bath until all the ice had melted. $n = 3$, errors are expressed as standard errors of mean.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

however, this did not prove to be lethal (Figure 1; treatment b). Exposure to the cryoprotectant at 0°C reduced the photosynthetic oxygen evolving capacity by 46% ± 5% (Figure 1; treatment d), on removal of the cryoprotectant, the cells were subsequently able to recover their full photosynthetic capacity (Figure 1; treatment c). The duration of the recovery period influenced photosynthetic capacity after exposure to −60°C, with photosynthetic oxygen evolution increasing from 17% ± 3% to 48% ± 8% after 24 and 48 h of recovery respectively, (Figure 1 treatments f and h). Little change in photosynthetic capacity was observed between 24 and 48 h post-thaw recovery for cells which had been exposed to LN (Figure 1; treatments g and h). The photosynthetic oxygen evolving capacity of frozen/thawed cultures was considerably reduced as compared to the untreated control (Figure 1). Cells plunged directly into LN, without cryoprotectant, were not able to resume photosynthetic function (Figure 1; treatment e).

Treated cells recovered in the presence of DMSO evolved methane whereas, controls (Figure 2) and treatment cells recovered in DMSO-free medium either did not produce methane or did so only in trace amounts (Figures 2 and 3). When desferrioxamine was added to cells treated with DMSO, methane evolution was inhibited within 48 h of the treatment (Figure 3).

Time courses of methane evolution for each treatment were compiled to identify which components of the cryopreservation protocol produced the greatest level of oxidative stress (Figure 2). Untreated control cells only produced methane at the end of the time course, however, centrifuged cells produced high levels of methane during the early stages of post treatment (Figure 2). Exposure to cryoprotectant treatments enhanced methane production during the first 48 h of post treatment, but this effect diminished after the initial response. Cooling cells to −10°C and freezing them to −60°C stimulated methane production, whilst those cells which had been chilled evolved less methane after 48 h, the cells which had been frozen to −60°C continually evolved high levels of methane throughout the time course. In comparison, cells exposed to LN temperatures of −196°C produced lower levels of methane with a progressive increase over the time course (Figures 2 and 3).

Viability in *E. gracilis* cells was monitored 8 days after the cryopreservation treatments with a view to correlating recovery with free radical damage as evidenced by methane evolution (Figure 4). From these viability assessments it was determined that the cells were not lethally injured by the centrifugation step, cryoprotectant exposure, or controlled cooling to −10°C at $-0.5°C\,min^{-1}$ (Figure 4). In addition, recovery in EG : JM, EG : JM supplemented with 1% (v/v) DMSO and EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine did not affect post-treatment viability levels; viability was $> 99\% \pm 0.1\%$ for all treatments and recovery procedures (Figure 4). The most damaging treatments were those in which the cells were exposed to freezing temperatures at −60°C and −196°C in LN and these were $> 50\%$ and $> 40\%$ of control levels, respectively (Figure 4).

There were significant differences in viability levels between cells recovered in EG : JM medium and EG : JM medium supplemented with 1% (v/v) DMSO and EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine. A one way ANOVA was performed on this data (which had been log_e transformed) and a significant increase in the level of post-thaw viability was detected when cells were recovered with desferrioxamine present ($F_{2,6} = 5.26$, $P < 0.05$). A two way balanced ANOVA incorporating treatment (exposure to −10°C, −60°C and LN) and recovery media (EG : JM, EG : JM supplemented with 1% (v/v) DMSO and EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine) indicated that treatment and recovery procedures had a significant effect on post-exposure viability, but that these effects were not synergistic. Thus, treatments and the recovery procedures were both significantly different

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

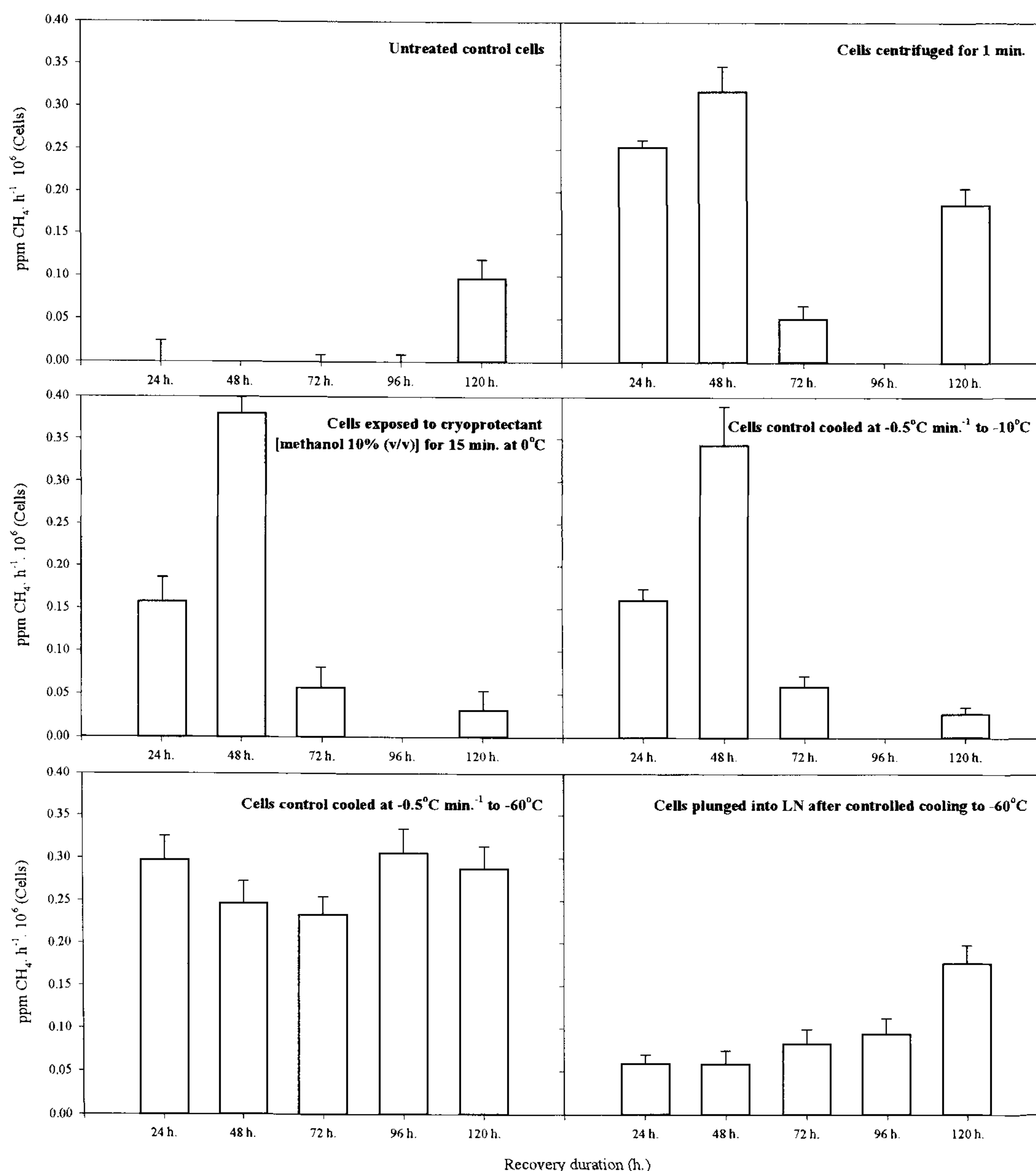

FIGURE 2 Time course of CH_4 production by *E. gracilis* cells using DMSO as a probe for hydroxyl radical activity at different stages of a cryopreservation protocol. Cells were plunged into LN after cooling to −60°C and frozen material was thawed using a simple, two-step procedure involving re-warming for 1 min in air followed by immersion in a 40°C water bath until all the ice had melted. $n = 6$, errors are expressed as standard errors of mean.

(with values of $F_{2,12} = 8.07$, $P < 0.01$, and $F_{1,2} = 6.07$, $P < 0.05$ respectively). Viability, as determined by FDA staining, for cells exposed to −60°C and LN and recovered in EG : JM were 51.1% ± 1.3%, 48.0% ± 1.6% respectively. Cells exposed to −60°C and LN (−196°C) recovered in EG : JM supplemented with 1% (v/v) DMSO and 10 mg · l^{-1} desferrioxamine had viabilities of 57.3% ± 2.4% and 55.4% ± 4% respectively (Figure 4).

RIGHTSLINK

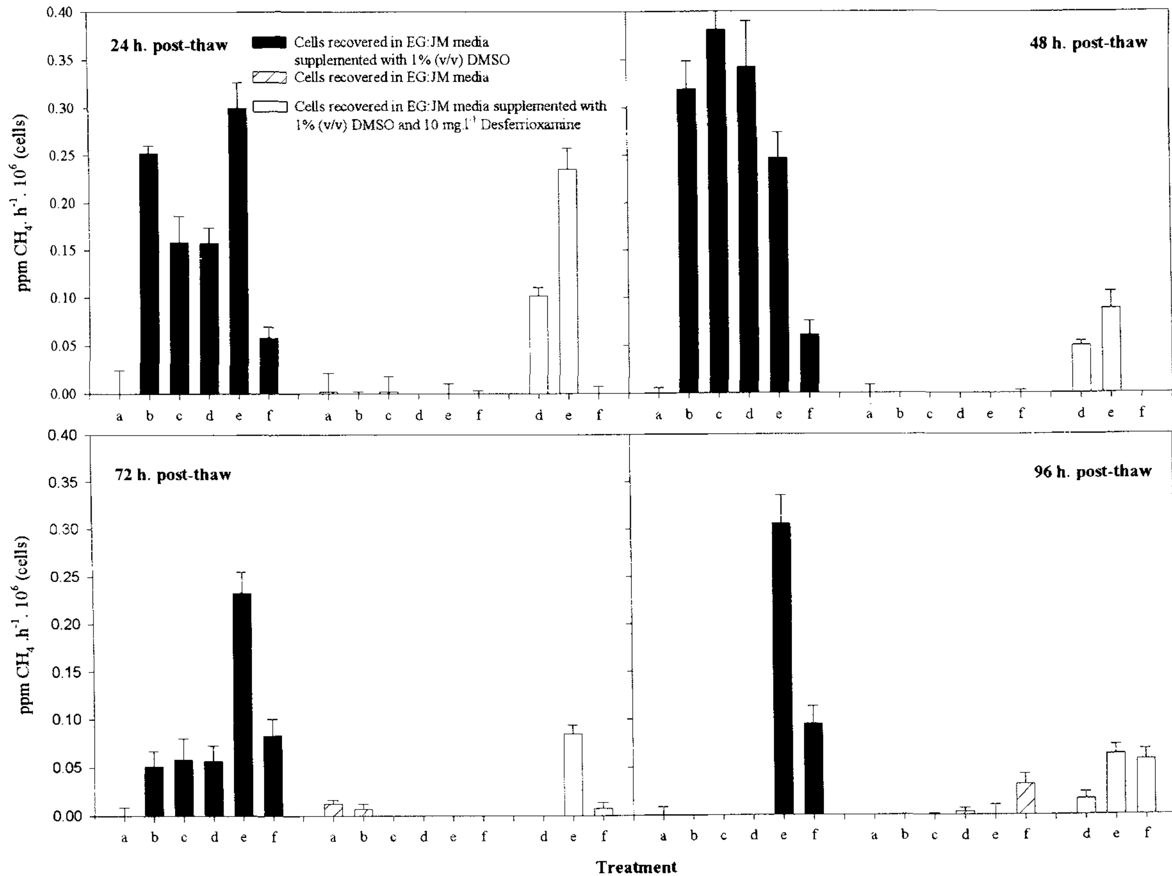

FIGURE 3 Time course of CH_4 production by *E. gracilis* following different cryopreservation protocol treatments. Methane from the headspace of cultures recovered in EG : JM medium supplemented with 1% (v/v) DMSO, assessed by gas chromatography at 24 h intervals over a 5 day recovery period. Treatments: (a) untreated controls; (b) cells centrifuged for 1 min; (c), cells exposed to cryoprotectant at 0°C {10% (v/v) methanol} for 15 min; (d) cells control cooled to −10°C; (e) cells control cooled to −60°C and held for 30 min; (f) cells plunged into LN from −60°C two-step. Frozen material was thawed using a simple, two-step protocol involving, re-warming for 1 min in air followed by immersion in a 40°C water bath until all the ice had melted. $n = 6$, errors are expressed as standard errors of mean.

DISCUSSION

To date, *E. gracilis* has proved difficult to cryopreserve using standard cryopreservation protocols.[20] The first objective of the study was to explore the possibility (using DMSO as a probe for the hydroxyl radical[16,29]) that oxidative stress pre-disposes the alga to cryopreservation-related injury. A second objective was to pinpoint the most damaging components of the cryopreservation protocol and the third objective was to improve post-cryopreservation recovery by the application of desferrioxamine. This potent chelating agent is known to reduce low temperature damage in mammalian tissue and its mode of action involves the removal of Fe^{3+} thereby, inhibiting Fenton chemistry and the production of $^{\bullet}OH$ radicals.[22,23]

It is first important to consider that DMSO is a cryoprotectant and that the assay system may also be protective. However, it is unlikely that DMSO confers colligative cryoprotection to *E. gracilis* at the levels applied in the present assay. Concentrations of 10% (v/v) and greater are

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

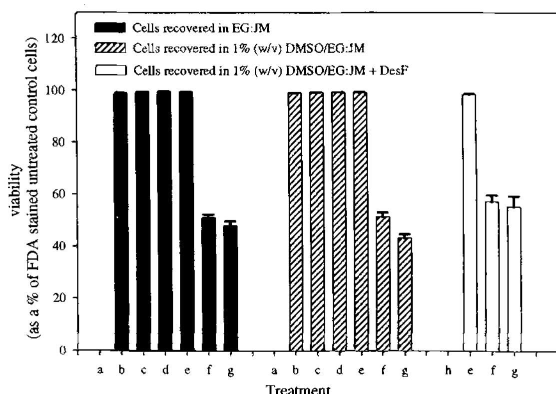

FIGURE 4 The effect of different cryopreservation treatments and post-treatment recovery media on cell viability in *E. gracilis*. Treatment effects (control culture medium {EG : JM}; culture medium with DMSO; and culture medium with DMSO and desferrioxamine {DesF}) were assessed by FDA staining 8 days after thawing and designated as: (a) unstained, untreated control; (b) untreated control; (c) cells centrifuged for 1 min; (d) cells exposed to cryoprotectant at 0°C {10% (v/v) methanol} for 15 min; (e) cells control cooled to −10°C; (f) cells control cooled to −60°C and held for 30 min; (g) cells plunged into LN from −60°C two-step thaw 1 min in air followed by immersion in a 40°C water bath; (h) unstained cells after controlled cooling to −10°C. Frozen material was thawed using a simple, two-step protocol involving, re-warming for 1 min in air followed by immersion in a 40°C water bath until all the ice had melted. $n = 3$, errors are expressed as standard errors of mean.

the usual rates of application required for DMSO to act as a cryoprotectant. It is however, very likely that DMSO will afford some protection to *E. gracilis* as it is a free radical scavenger.

Free radical production is known to be associated with metabolic uncoupling and our first level of investigation was to establish if *E. gracilis* cells undergo photosynthetic inhibition during exposure to cryogenic treatments. Damage to chloroplasts by low temperatures and freezing has been reported elsewhere[33–38] suggesting the inactivation of photophosphorylation in freeze-damaged thylakoid membranes. This study concurs with previous findings as photosynthesis was impaired in *E. gracilis* cells exposed to low and sub-zero temperatures (Figure 1). Cryoprotection moderated the freeze-induced inhibition of photosynthesis and cryoprotected cells which had been frozen at −60°C were, on recovery, able to increase their photosynthetic capacity from 17% ± 3% at 24 h after thawing to 48% ± 8% at 48 h after thawing. This suggests that injury was non-lethal, however, the population of cells recovered from the −60°C treatment will comprise damaged and surviving cells and lethally damaged cells. Flow cytometry studies support this premise as approximately 50% of the cell population survived exposure to −60°C. It is possible that surviving, photosynthetically active cells may manifest impaired or uncoupled metabolism which could lead to •OH production. Non-cryoprotected *E. gracilis* cells exposed to LN were not able to resume photosynthetic metabolism and, in this case, their injuries were considered lethal. Cryoprotected, *E. gracilis* cells exposed to LN recovered some photosynthetic activity (Figure 1) and the level of inhibition suggested that metabolic uncoupling may have taken place. As considerable structural damage has also been observed[38–42] in higher plants, *E. gracilis* and other algae exposed to low temperatures and cryopreservation treatments these findings suggest the algae may be predisposed to free radical-related stress during cryogenic treatment.

Development of a Non-Destructive Assay to Monitor Cryoinjury

The application of a non-invasive, non-destructive assay procedure was a priority approach as this allows the continuous and cumulative monitoring of stress throughout the entire cryopreservation protocol. The use of DMSO as a non-destructive "marker probe" for the •OH free radical was considered to be the most suitable method of investigating free radical stress in *E. gracilis*. Furthermore, as DMSO is a cryoprotectant[11] as well as a free radical scavenger, its general use in the study of cryoinjury in cryopreserved higher plant cells has a dual advantage.[11] Unfortunately, this is not the case for algal cells, as DMSO is toxic to algae at the levels required

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

for cryoprotection (10% and above). Its use, at low concentrations ($\leq 5\%$), as a non-toxic free radical probe in algal systems is however possible. It has been previously adapted for examining $^{\bullet}OH$ radical production and oxidative stress in higher plants exposed to cryopreservation treatments.[16,29]

DMSO was introduced into the post-treatment recovery media and the success of the assay is dependent upon the use of stringent controls. Preliminary gas chromatography investigations established that only low levels of methane could be detected in head space samples taken from media controls, DMSO controls, empty vials and cryoprotectant controls [methanol 10% (v/v)]. These low levels of methane were identical in each set of vials and were attributed to background methane present in the laboratory atmosphere. Furthermore, the pre-preparation of vials (venting of vials following autoclaving and prior to use) before head space analysis ensured that trace background methane was removed (Figure 2). In general, methane levels were negligible compared to those of test treatments, however, throughout the study, all test treatments were corrected for background levels of the gas if it was detected to be present. When desferrioxamine was applied to cells in the presence of DMSO (Figure 3) there was a reduction in methane production. Control cells recovered in EG : JM media did not display a large increase in methane evolution with respect to treatment (Figure 2) and this was attributed to the absence of the hydroxyl radical trap, DMSO. Cells recovered in media supplemented with 1% (v/v) DMSO produced a significant increase ($P < 0.004$) in methane where cultures had previously experienced stresses (Figure 2). The correlation between post stress, increased methane evolution, in the presence of DMSO, indicates that it is most likely acting as a putative free radical scavenger during recovery (Figure 2). Further confirmation that methane production in the presence of DMSO was due to oxidative stress was indicated by the ability of the iron chelator desferrioxamine to reduce/inhibit methane production (Figure 3).

Assay validation required us to determine any potential effects of DMSO on algal recovery responses. DMSO would not have the capacity to act as a cryoprotectant at the concentrations used to detect $^{\bullet}OH$ in this system. Importantly, in the case of EG : JM supplemented with 1% (v/v) DMSO did not affect cellular viability levels, viability was $> 99\% \pm 0.1\%$ of the untreated control for DMSO exposure control and non-lethal treatments (Figure 4).

It is important to note that other probes[43,44] can be used to detect $^{\bullet}OH$ radicals *in vivo*. In this study, the DMSO/methane evolving system does offer a number of major advantages, since as it is non-destructive the sequence of different treatments can be examined in terms of cumulative stress parameters.

Elevated levels of methane (Figures 2 and 3) were detected during the first 24 h post treatment for cultures which had been exposed to controlled cooling to $-10°C$, or $-60°C$, and were recovered with desferrioxamine present (Figure 3). Initial increases in methane are likely to be due to the experimental design, in which all cultures were exposed to cryopreservation protocol treatment steps in the absence of DMSO and desferrioxamine and were only subsequently recovered in medium supplemented with these chemicals. Thus, no hydroxyl radical scavengers (DMSO) or iron chelator was present during first-stage treatments to limit, or influence free radical production. During the later stages of the time course hydroxyl radicals generated from initial damaging events would presumably have been scavenged by the DMSO, leading to enhanced methane evolution. Interestingly, following subsequent incubation in the presence of desferrioxamine, methane evolution diminished considerably (Figure 3). Therefore, the introduction of desferrioxamine into the recovery medium was, able to limit hydroxyl radical production and as a consequence the compound may be expected to reduce injury and enhance viability.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

Profiling Stress Responses in Cryopreserved Cells

A profile of *E. gracilis* stress responses to the sequential and different components of the cryopreservation protocol are shown in Figure 2. Viability levels of *E. gracilis* used for monitoring changes in methane were initially determined by vital staining {using fluorescein diacetate (FDA) and flow cytometry} as shown in Figure 4. By considering the combined results of Figures 2 and 4 it is possible to ascertain the effects of the cryopreservation treatments on cell viability and oxidative stress. Thus, untreated control cells had 100% viability levels and methane was only detected after 120 h. The increase in methane at this point of the time course can be attributed to normal culture ageing which should also be taken into account for the later stages of the time course for all treatments. Interestingly, centrifugation, although non-lethal, promotes methane evolution. This method is routinely used to concentrate cultures for cryopreservation[20] and it is now recommended as a result of this study, to substitute less stressful preparative procedures (e.g. passive filtration, sedimentation). At present it is not known why centrifugation is especially stressful, it is possible that sheer forces may damage the flagellum of the organism. The cumulative, stressful effects of non-lethal injury may, in the longer term influence the overall survival responses of cells after cryopreservation.

Exposure to the cryoprotectant, methanol, and/or controlled cooling to $-10°C$ at $-0.5°C\,min^{-1}$ produced an initial (24 h) increase in post-stress methane evolution, which peaked after 48 h (Figure 3). The cells were able to moderate this response after 72 h (Figure 3). Methane evolution by cells subjected to centrifugation increased more rapidly during the first 24 h compared to cultures which had been exposed to the additional stress of cryoprotectant exposure and/or cooling to $-10°C$. This delay in methane evolution by the cells exposed to cryoprotectant at 0°C and/or controlled cooling to $-10°C$ may be due to the fact that the metabolic rate of *E. gracilis* was initially lower (following exposure to low temperatures) and this may have influenced the initial rate of radical production (Figure 2). Temperatures applied during chilling and freezing could influence the initial rate of methane formation. For example, $^{\bullet}CH_3$ abstraction of $^{\bullet}H$ may proceed at a slower rate than reactions involving $^{\bullet}OH$, which occur so rapidly that temperature affects are unlikely. However, the time course of the study largely examines damage on transfer to ambient temperatures (following thawing) and investigates long-term, post-treatment damage (i.e. 24 h and greater).

At 48 h, rates of methane evolution were higher in cells exposed to cryoprotective/cooling treatments. During the subsequent 24 h, rates of methane decreased (Figure 2) in cultures which had been exposed to non-lethal stresses (specifically, cells centrifuged for 1 min; cells exposed to cryoprotectant; cells control cooled to $-10°C$). After 96 h incubation under standard conditions, methane levels in these cultures returned to untreated control levels and the limited methane production observed attributed to cell ageing. From these results it appears that whilst cultures may experience non-lethal stress due to preparative manipulations prior to cryopreservation, these stresses, in isolation, do not affect cellular viability and the cultures are capable of recovery (Figures 2 and 4).

To ensure survival after cryogenic storage it is essential (in the case of controlled rate freezing protocols) for cells to reach an optimal, sub-zero, intermediate transfer temperature which will allow the movement of unfrozen intra-cellular water to move to the extra-cellular frozen environment.[6] This reduces the intra-cellular water available for ice nucleation, preventing damage once the cells are plunged into LN.[6] Many freeze-recalcitrant plant cells are not even capable of surviving the intermediate transfer temperature. In this system the effects of a $-60°C$ transfer temperature on cryoprotected *E. gracilis* were investigated. The profile of methane production

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

in cultures which had been control cooled to −60°C indicates a considerable degree of stress in these cultures (Figure 2). Flow cytometry investigations showed that >40% of cells may experience lethal injury during a freeze/thaw cycle to −60°C. The final stage of a cryopreservation protocol is to transfer the cell from −60°C and plunge them directly into LN. Significantly lower levels of methane evolution were detected in cultures which had been exposed to cryogenic storage. The difference in methane evolution between cultures exposed to LN and −60°C may be a result of gross lethal injury in cells exposed to LN. These cells are not metabolically active and this may limit radical production, with only the non-lethally stressed (but damaged) cells capable of producing •OH. Thus, in cultures exposed to −60°C a proportion of intact, but ultimately non-viable cells may be present which are a source of radicals.

Using Desferrioxamine to Enhance Post-Cryopreservation Survival

By reducing or eliminating the events which promote oxidative stress in injured cryopreserved cells it may be possible to significantly increase post-thaw viability levels in the longer term. This possibility was explored in *E. gracilis*, using the additive desferrioxamine. The basis of this approach is related to the role of metal cations in free radical generation.[45] Desferrioxamine is a powerful Fe^{3+} chelator and is therefore able to prevent the production of the hydroxyl radicals, by limiting the availability of Fe^{3+}. Where cells were recovered in EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine, a significantly lower level ($P < 0.004$) of methane was evolved 24–120 h after thawing. Introduction of desferrioxamine into recovery media was, therefore, able to limit methane evolution and presumably •OH production. Desferrioxamine may thus be expected to reduce cryoinjury. That this is the case has been previously reported in studies performed on low-temperature stored mammalian tissues[7,26] and cryopreserved higher plant systems.[27]

Recovery in EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine did not affect post-treatment viability levels, viability was >99% ± 0.1% of the untreated control for treatments without a lethally injurious treatment step. Viability after exposure to −60°C and LN were >50% and >40% respectively in all cases. Significantly ($F_{2,6} = 5.26$, $P < 0.05$) higher viability levels were obtained where cultures exposed to LN were recovered in EG : JM supplemented with 1% (v/v) DMSO and $10\,mg \cdot l^{-1}$ desferrioxamine. This indicated that the application of the chelating agent may offer considerable scope for enhancing post-thaw viability levels in cryopreserved algae and may assist in cryopreserving a wider spectrum of presently freeze-recalcitrant algae in the future. Although the increase in post-thaw viability was relatively small it is worth noting that viability was assessed 8 days following thawing, during which time cultures would have undergone a number of cell divisions. It is important to note that *E. gracilis* has an absolute requirement for iron for growth,[46] with iron depletion causing arrest of cell division. If cellular division was arrested in cultures, recovered in the presence of desferrioxamine, then the difference in post-thaw viability between cultures with and without desferrioxamine present may be greater than the data suggests. In this study and work by Benson *et al.*,[27] desferrioxamine was employed to suppress oxidative stress at critical points of cryoinjury and during the initial stages of recovery. Desferrioxamine should be removed immediately following these periods to minimise iron limitation effects on re-growth.

SUMMARY

Methane production by *E. gracilis* cells exposed to DMSO has been used as a putative marker of the hydroxyl radical. This system has been used to identify which components of a cryopreservation

RIGHTS LINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

protocol are the most damaging to the unicellular alga. Desferrioxamine has been found to reduce methane production by cryopreserved *E. gracilis*, and enhance post-storage survival. These findings concur with the previously reported use of the pharmaceutical compound in low-temperature stored mammalian tissues and cryo-preserved higher plant systems. Future studies of freeze-recalcitrant algae will explore the role of antioxidant protection in cryoinjury and tolerance with a view to developing robust cryoconservation methods for a wider range of algal protists which are currently cryopreservation-recalcitrant.

Acknowledgements

The authors acknowledge the Esmée Fairbairn Charitable Trust for the provision of a Studentship Award to RAF. The authors wish to thank the technical staff at the Institute of Freshwater Ecology and at the University of Abertay Dundee.

References

[1] J.G. Day (1998) Cryoconservation of microalgae and cyanobacteria. *Cryo-Letters*, (Suppl.), **1**, 7–14.
[2] R.A. Fleck (1998) Mechanisms of cell damage and recovery in cryopreserved freshwater protists. Ph.D. Thesis, University of Abertay Dundee.
[3] J.G. Day, M.M. Watanabe, G.J. Morris, R.A. Fleck and M.R. McLellan (1997) Long-term viability of preserved eukaryotic algae. *Journal of Applied Phycology*, **9**, 121–127.
[4] J.G. Day and M.F. Turner (1992) Algal culture collections and biotechnology. In: *Proceedings of The Symposium on Culture Collection of Algae*, Tsukuba, February 15, 1991. (Ed. M.M. Watanabe), National Institute For Environmental Studies Environment Agency, Japan, pp. 11–27.
[5] F.B. Metting Jr. (1996) Biodiversity and application of microalgae. *Journal of Industrial Microbiology and Biotechnology*, **17**, 477–489.
[6] J.G. Day and M.R. McLellan (1995) Methods in molecular biology, Vol. 38. *Cryopreservation and Freeze-Drying Protocols*. Humana Press Inc., Totowa. New Jersey, USA.
[7] B.J. Fuller and C.J. Green (1986) Oxidative stress in organs stored at low temperatures for transplantation. In: *Free Radicals Cell Damage and Disease* (Ed. C. Rice-Evans), Richelieu Press, London, pp. 223–240.
[8] L.A. Cotterill, J.D. Gower, B.J. Fuller and C.J. Green (1989) Oxidative stress during hyperthermic storage of rabbit kidneys: Possible mechanisms by which calcium mediates free radical damage. *Cryo-Letters*, **10**, 119–126.
[9] G.S.W. Whitely, B.J. Fuller and K.E.F. Hobbs (1992) Deterioration of cold-stored tissue specimens due to lipid peroxidation: Modulation by antioxidants at high subzero temperatures. *Cryobiology*, **29**, 668–673.
[10] M.A. Pickford, B. Sandin, J.D. Gower and C.J. Green (1990) The effect of allopurinol and nifedipine on oxidative damage and functional outcome of rat lung isografts after a 48 hour cold storage. *Cryo-Letters*, **11**, 265–276.
[11] E.E. Benson (1990) *Free Radical Damage in Stored Plant Germplasm*. Rome, International Board for Plant Genetic Resources Publishers.
[12] G.A.F. Hendry, W.E. Finch-Savage, P.C. Thorpe, N.M. Atherton, S.M. Buckland, K.A. Nilsson and W.E. Seel (1992) Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytologist*, **122**, 273–279.
[13] W.J. Magill, N. Deighton, H.W. Pritchard, B.A. Goodman and E.E. Benson (1994) Physiological and biochemical studies of seed storage parameters in *Carica papaya*. *Proceedings of the Royal Society of Edinburgh*, **102B**, 439–442.
[14] R.N. Basu and M. Dasgupta (1978) Control of seed deterioration by free radical controlling agents. *Indian Journal of Experimental Botany*, **26**, 1070–1073.
[15] R.J. Gorecki and G.E. Harman (1987) Effects of antioxidants on viability and vigour of ageing pea seeds. *Seed Science and Technology*, **15**, 109–117.
[16] E.E. Benson and L.A. Withers (1987) Gas chromatographic analysis of volatile hydrocarbon production by cryopreserved plant cultures: A non-destructive method for assessing stability. *Cryo-Letters*, **8**, 35–36.
[17] E.E. Benson and A.A. Noronha-Dutra (1988) Chemiluminescence in cryopreserved plant tissue cultures: The possible role of singlet oxygen in cryoinjury. *Cryo-Letters*, **9**, 120–131.
[18] E.E. Benson, P.T. Lynch and J. Jones (1992) The detection of lipid peroxidation products in cryoprotected and frozen rice cells: consequences for post-thaw survival. *Plant Science*, **85**, 107–114.
[19] M.C. Anderson, T.K. Prasad and C.R. Stewart (1995) Changes in izosyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiology*, **109**, 1247–1257.
[20] J.G. Day, M.M. Watanabe and M.F. Turner (1998) *Ex situ* conservation of protistan and cyanobacterial biodiversity: CCAP-NIES collaboration 1991–1997. *Phycological Research*, **46** (Suppl.), 77–83.
[21] G. Cohen and A.I. Cederbaum (1979) Chemical evidence for the production of hydroxyl radicals during microsomal electron transfer. *Science*, **204**(6), 66–68.
[22] H. Keberle (1967) The biochemistry of desferrioxamine and its relation to iron metabolism. *Annals of the New York Academy of Science*, **119**, 758–768.
[23] B. Halliwell (1989) Protection against tissue damage *in vivo* by desferrioxamine: What is the mechanism of action? *Free Radical Biology and Medicine*, **7**, 645–651.
[24] P. De Loecker, B.J. Fuller, V.A. Koptelove, V.I. Grischenko and W. De Loecker (1997) Cryopreservation of isolated rat hepatocytes: Effects of iron-mediated oxidative stress on metabolic activity. *Cryobiology*, **34**, 150–157.
[25] J.F. McAnulty and X.Q. Huang (1996) The effect of hypothermic preservation with trolox and ascorbate on lipid peroxidation in dog kidneys. *Cryobiology*, **33**, 217–225.

RIGHTSLINK

[26] C.J. Green, G. Healing, S. Simpkin, B.J. Fuller and J. Lunec (1986) Reduced susceptibility to lipid peroxidation in cold ischemic rabbit kidneys after addition of desferrioxamine, mannitol or uric acid to the flush solution. *Cryobiology*, **23**, 358–365.

[27] E.E. Benson, P.T. Lynch and J. Jones (1994) The effects of the chelating agent desferrioxamine on the post-thaw recovery of rice cells: A novel approach to cryopreservation. *Plant Science*, **10**, 249–258.

[28] J. Tompkins, M.M. De Ville, J.G. Day and M.F. Turner (1995) *Culture Collection of Algae and Protozoa: Catalogue of Strains*. Titus Wilson & Son Ltd. Kendal, UK.

[29] K. Harding and E.E. Benson (1995) Biochemical and molecular methods for assessing damage, recovery and stability in cryopreserved plant germplasm. In: *Genetic Preservation of Plant Cells In Vitro* (Ed. B.W.W. Grout) Springer-Verlag, Germany, pp. 113–167.

[30] R.W. Pickup, G. Rhodes, R.J. Cobban and K.J. Clarke (1996) The postponement of non-culturability in *Aeromonas salmonicida*. *Journal of Fish Diseases*, **19**, 65–74.

[31] G. Whitelam and G.A. Codd (1983) Photoinhibition of photosynthesis and *in vivo* chlorophyll fluorescence in the green alga *Ankistrodesmus braunii*. *Plant Cell Physiology*, **25**, 465–471.

[32] G. MacKinney (1941) Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry*, **140**, 315–322.

[33] R.R. Wise (1995) Chilling enhanced photooxidation: The production, action and study of reactive oxygen species produced during chilling in the light. *Photosynthesis Research*, **45**, 79–97.

[34] T. Ginsburger-Vogel, S. Arbault and R. Perez (1992) Ultrastructural study of the effect of freezing-thawing on the gametophyte of the brown alga *Undaria pinnatifida*. *Aquaculture*, **106**, 171–181.

[35] G.J. Morris, G.E. Coulson and E.A. Leeson (1985) Changes in the shape of mitochondria following osmotic stress to the unicellular green alga *Chlamydomonas reinhardii*. *Journal of Cell Science*, **76**, 145–154.

[36] U. Heber, J.M. Schmitt, G.H. Krause, R.J. Klossen and K.A. Santarius (1981) Freezing damage to thylakoid membranes *in vitro* and *in vivo*. In: *Effects of Low Temperatures on Biological Membranes* (Eds. G.J. Morris and A.J. Clarke), Academic Press, London, pp. 263–284.

[37] K.A. Santarius (1996) Freezing of isolated thylakoid membranes in complex media. X. Interactions among various low molecular weight cryoprotectants. *Cryobiology*, **33**, 118–126.

[38] R.A. Fleck, J.G. Day, K.J. Rana and E.E. Benson (1997) Visualisation of cryoinjury and freeze events in the coenocytic alga *Vaucheria sessilis* using cryomicroscopy. *Cryo-Letters*, **18**, 343–354.

[39] R.A. Fleck, J.G. Day and E.E. Benson (1996) An investigation of viability loss and cell damage during the application of cryopreservation to the unicellular microalge: *Euglena gracilis* and *Haematococcus pluvialis*. *Cryobiology*, **33**, 645–646.

[40] A. Sakai and K. Otsuka (1967) Survival of plant tissue at super-low temperature V. An electron microscope study of ice in cortical cells cooled rapidly. *Plant Physiology*, **42**, 1680–1694.

[41] R.A. Fleck, J.G. Day, E.E. Benson and R.W. Pickup (1997) Factors affecting post-thaw viability in the microalga *Euglena gracilis*, *Cryobiology*, **35**, 340–341.

[42] R.A. Fleck, J.G. Day, K.J. Clarke and E.E. Benson (1999) Elucidation of the metabolic and structural basis for the cryoperservation recalcitrance of *Vaucheria sessilis*, Xanthophyceae. *Cryo-Letters* (in press).

[43] M. Grootveld and B. Halliwell (1986) Aromatic hydroxylations as a potential measure of hydroxyl radical formation *in vivo*: Identification of hydroxylated derivatives of salicylate in human body fluids. *Biochemical Journal*, **237**, 499–504.

[44] H. Kaur and B. Halliwell (1995) Detection of hydroxyl radicals by aromatic hydroxylation. *Methods in Enzymology*, **233**, 67–82.

[45] V. Nivière and M. Fontecave (1995) Nitric oxide: Chemistry and biology. In: *Analysis of Free Radicals in Biological Systems* (Eds. A.E. Favier, J. Cadet, B. Kalyanaraman, M. Fontecave and J.L. Pierre), Birkhäuser Verlag, Germany, pp. 11–20.

[46] T. Ishikawa, T. Takeda, S. Shigeoka, O. Hirayama and T. Mitsunaga (1993). Requirement for iron and its effect on ascorbate peroxidase in *Euglena gracilis*. *Plant Science*, **93**, 25–29.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK