RESEARCH ARTICLE

Blueberry fruit polyphenolics suppress oxidative stress-induced skeletal muscle cell damage *in vitro*

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Skeletal muscle damage can result from disease and unaccustomed or excessive exercise. Muscle dysfunction occurs *via* an increased level of reactive oxygen species and hence there is potential in antioxidants as amelioration strategies. We explored the putative benefit of fruit polyphenolic extracts in reducing the susceptibility of skeletal muscle cells to oxidative stress. Muscle myotubes were simultaneously challenged with fruit extracts (1–50 μg/mL) and calcium ionophore (A23187), hydrogen peroxide, or 2,4-dinitrophenol and damage monitored by release of cytosolic enzymes. A blueberry fruit extract displayed a potent and significant dose-dependant protective capacity. Evaluation of the protective capacity of anthocyanin sub-extracts of blueberry fruit and pure individual glycosides, with identification of extract polyphenolic components using MS, suggested that malvidin galactoside and/or glucoside were the active compounds. These *in vitro* data support the concept that blueberry fruits or derived foods rich in malvidin glycosides may be beneficial in alleviating muscle damage caused by oxidative stress. More research on the benefits of blueberry fruit consumption in human intervention studies is warranted.

Keywords:
Anthocyanins / Blueberry fruits / Fruit polyphenolics / Oxidative stress / Skeletal muscle cells

1 Introduction

During excessive, strenuous, or unaccustomed exercise, muscles are exposed to very high levels of mechanical and metabolic insults. Due to increased energy demands for exercise, there is a marked increase in oxygen uptake and cellular metabolism in various organs, especially in skeletal muscle [1]. Reactive oxygen species (ROS) are produced as a consequence of the increased metabolism and have potential to mediate tissue destruction with long term consequences culminating in decreased physical performance, muscular fatigue, and/or muscle damage. The mechanism by which muscle is damaged is reported to be *via* several key pathways including an energy crisis and loss of calcium homeostasis [2, 3]. However, while the exact process remains to be elucidated, a central role for intracellular and extracellular ROS is apparent [4]. A key source of ROS liberation is several enzyme complex sites of the mitochondrial respiratory chain [5, 6].

Fruits and vegetables are known to be a good source of a range of polyphenolics compounds and secondary plant compounds, which contain multiple hydroxyl groups directly attached to benzene rings. These polyphenolics compounds (including non-flavonoids (phenolic acids and stilbenes) and flavonoids (flavanols, flavanones, flavonols, flavones, isoflavones, and anthocyanidins)) exhibit strong

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Abbreviations: CK, creatine kinase; **DCF,** dichlorofluorescein; **DCFDA,** carboxy-2′,7′-dichlorohydrofluorescein diacetate; **DNP,** dinitrophenol; **D-PBS,** Dulbecco's PBS; **FCS,** fetal calf serum; **LDH,** lactate dehydrogenase; **NADP$^+$,** nicotinamide adenosine dinucleotide phosphate; **PDA,** photodiode array; **ROS,** reactive oxygen species

antioxidant properties. Many of the biological effects of polyphenolics have been attributed to their antioxidant properties, which include the ability to scavenge ROS, to inhibit lipid peroxidation, and to chelate metal ions. The vast majority of this evidence is derived from *in vitro* studies and there is much debate as to whether these actions translate into the *in vivo* situation because of metabolism and bioavailability constraints predominantly occurring in the gastrointestinal tract. Until more is understood, however, the dietary supplementation with antioxidants from fruits and their components may still provide a potential strategy to enhance health [7–9]. *In vitro* studies have demonstrated that antioxidants can inhibit the biological changes occurring in muscle during fatigue and furthermore slow the decline in force output. Hence, dietary supplementation with antioxidants is also an attractive choice for athletes or health conscious individuals engaged in intense or excessive training programmes [10]. It is also possible that novel innovation will address barriers to polyphenolic bioavailability in the near future.

A variety of model systems (*in situ* and *in vitro*) have been developed to enable the controlled monitoring of responses to stress in muscle that cannot be easily evaluated in human trials. These methods provide an easy and cost effective method of screening for possible antioxidants for protection against muscle cell oxidative stress. Furthermore they are useful in the determination of underlying mechanisms of action. A useful and simple system in this regard is the use of cell culture and skeletal muscle myotubes (differentiated from muscle myoblast cell lines) exposed to calcium ionophores. Calcium ionophores have been shown to induce a stress *via* processes known to occur in exercising muscle *in vivo*, *i.e.* loss of intracellular calcium homeostasis, loss of energy supplies, activation of apoptosis pathways, and/or an increased activity of oxidizing free radical-mediated reactions [4].

The New Zealand Institute for Plant and Food Research Ltd., has an interest in evaluating fruits (and compounds) for antioxidant health benefits. Our aim in this study therefore was to evaluate whether polyphenolic compounds, extracted from fruit (*e.g.* blueberry), provide a defense from oxidative damage in skeletal muscle cells *in vitro*. For this purpose we used differentiated skeletal muscle myotubes challenged with an oxidative stress relevant to excessive exercise.

2 Materials and methods

2.1 Experimental approach

An undifferentiated skeletal muscle cell line (myoblasts) that can be differentiated into muscle cells (myotubes) was utilized in this study. Myotubes were exposed to chemical agents (*e.g.* the calcium ionophore A23187) of relevance to oxidative stress processes known to occur *in vivo* during muscle exercise. During oxidative stress challenge, myotubes were co-incubated with fruit polyphenolic extracts and determinations of enzyme release from the cells served as an indicator of cell damage and/or protection.

2.2 Cell culture

The rat skeletal muscle cell line L6 (American Type Culture Collection, Manassas, VA, USA) was utilized in this study. The undifferentiated myoblast cells were cultured in DMEM (Sigma Aldrich, Auckland, New Zealand) containing 2 mM glutamine, 50 IU penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum (FCS). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Myoblasts were subcultured by washing in Dulbecco's PBS (D-PBS) and exposure (<5 min) to Tryple™ (Invitrogen, Auckland, New Zealand). Myoblasts were subcultured weekly when 70–80% confluent.

To induce differentiation from myoblasts into muscle myotubes, the medium was replaced with DMEM containing 2% FCS prior to confluency as described previously [11, 12]. Differentiation over 3–4 days was noted by myotube formation (light microscopy) and evaluated by the analysis of cellular activity and expression (Western blot analysis) of the muscle specific enzyme creatine kinase (CK).

2.3 Polyphenolic extracts

Polyphenolic extracts of blueberry fruits (*Vaccinium corymbosum* cv. Reka) and two other fruit extracts (not named – fruit extract 1; fruit extract 2) were evaluated in this study along with purified polyphenolic compounds purchased from commercial sources for comparison. Total polyphenolic extracts of the fruits were generated (total extract) along with anthocyanin (antho-extracts 1, 2, and 3) subextracts.

Approximately 800 g of frozen fruit was homogenized in a Waring blender with 2.4 L of acetone and the solid residue recovered by filtration through GF/A filter paper (Whatman). After the solid residue was homogenized again with acetone/water (70:30) and filtered, both acetone extracts were combined and concentrated to about 400 mL by rotary evaporation at 40°C. Lipids were removed by partitioning the aqueous extract twice with 500 mL heptane and the residual heptane in the aqueous layer removed by rotary evaporation. The polyphenolics were isolated from this crude extract with Amberlite XAD-7 (Sigma, Sydney, Australia). Portions of extract (100 mL) were treated with 40 g washed XAD-7 and the polyphenolics allowed to absorb by standing for 60 min. The XAD-7 was then recovered by filtration and washed with 1% formic acid. Finally the blueberry polyphenolics were eluted from the XAD-7 with methanol and dried by rotary evaporation to yield a friable dark red powder – this extract we have termed the total

polyphenolics extract. Further separation of this extract was carried out by LH20 chromatography. The blueberry total polyphenolics extract (1000 mg) was dissolved in 10 mL methanol/water (50:50) and applied to a Sephadex LH-20 (2.5 × 32 cm) column equilibrated with methanol/water (50/50). Once the extract was loaded, the column was eluted successively with 2 × 1 L of methanol/water (50:50), 2 × 1 L ethanol, and finally 2 × 1 L acetone/water (70:30). The volume of the collected fractions was 1 L. This provided 6 fractions which were characterized by HPLC. Fractions 1, 2, and 5 were used in this study as they were mainly composed of anthocyanins (antho-extracts 1, 2, and 3). Figure 1 specifically displays the anthocyanin profile (HPLC) of the blueberry extracts as well as identifying individual compounds by LC-MS. Table 1 displays the anthocyanin and polyphenolics content of the three fruit extracts and fractions (total polyphenolics extract, antho-extracts 1, 2, and 3) used in our study. Figure 2 also displays the anthocyanin profiles (HPLC) of the total polyphenolics extracts of the two unnamed fruit extracts (fruit extract 1; fruit extract 2).

For comparison with activities associated with the blueberry fruit total polyphenolic and anthocyanin sub-extracts,

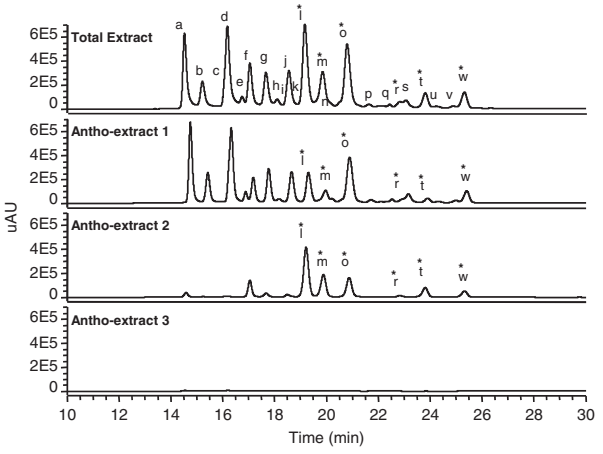

Figure 1. HPLC chromatograms of the total polyphenolics and anthocyanin sub-extracts of blueberry fruit. The total polyphenolics and anthocyanin sub-extracts of the blueberry fruit were analyzed to identify bioactives. HPLC chromatograms of the anthocyanin content of the extracts (total polyphenolics, antho-extract 1, antho-extract 2, antho-extract 3) are shown with the individual compounds identified by LC-MS: a – delphinidin galactoside, b – delphinidin glucoside, c – cyanidin galactoside, d – delphinidin pentoside, e – cyanidin glucoside, f – petunidin galactoside, g – petunidin glucoside, h – delphinidin +190[a], i – peonidin galactoside, j – petunidin pentoside, k – peonidin glucoside, l – malvidin galactoside, m – malvidin glucoside, n – peonidin pentoside, o – malvidin pentoside, p – petunidin acetyl hexoside, q – cyanidin acetyl hexoside, r – malvidin+190[a], s – petunidin acetyl hexoside, t – malvidin acetyl hexoside, u – petunidin acetyl hexoside, v – peonidin acetyl hexoside, w – malvidin acetyl hexoside. * – Highlights the polyphenolic compounds common to all the extracts. [a] – Specifies the named anthocyanin with an additional unrecognized molecule of mass 190.

Table 1. Polyphenolic content of fruit extracts

Extract	Anthocyanins (mg/g)[a)]	Polyphenolics (mg/g)[b)]
Blueberry fruit extracts		
Total extract	177	723
Antho-extract 1	387	1484
Antho-extract 2	128	491
Antho-extract 3	0	4
Fruit extract 1		
Total extract	215	656
Antho-extract 1	415	1328
Antho-extract 2	20	334
Antho-extract 3	12	3
Fruit extract 2		
Total extract	151	903
Antho-extract 1	167	920
Antho-extract 2	29	586
Antho-extract 3	0	1286

a) Concentrations reported as mg/g of powered extract (dry weight) as cyanidin 3-glucoside equivalents.
b) Concentrations reported as mg/g of powered extract (dry weight) as epicatechin equivalents.

the following purified polyphenolic compounds were evaluated: malvidin-chloride, malvidin-3-galactoside and malvidin-3-glucoside. Malvidin-chloride and malvidin glycosides were purchased from ChromaDex (Santa Ana, CA, USA) and were of high purity (secondary HPLC grade).

2.4 Determination of ROS generation using carboxy-2′,7′-dichlorohydrofluorescein diacetate (DCFDA)

For the determination of ROS, the agent DCFDA was used. DCFDA when loaded into cells in culture is cleaved into the product dichlorofluorescein (DCF), which produces an increase in fluorescence when oxidized. Muscle myoblasts were plated at 5000 cells/well in 96-well plates and were loaded with DCF by incubation with DCFDA (10 μM) for 60 min at room temperature. Following a wash in D-PBS (2 ×) cells were equilibrated at room temperature for 10 min prior to exposure to A23187 (10 μM) and fluorescence intensity determination over 30 min (excitation and emission wavelengths of 485 and 520 nm, respectively) using a fluorescence platereader (BMG FluorStar Optima, Alphatech Systems, Auckland, New Zealand). Data were expressed as relative fluorescence intensity units compared to the control analysis done in the absence of A23187.

2.5 Evaluation of damage and protection by fruit extracts

Myoblasts were plated at an initial density of 100 000 cells/well in 6-well plates. After 4–5 days of culture in DMEM

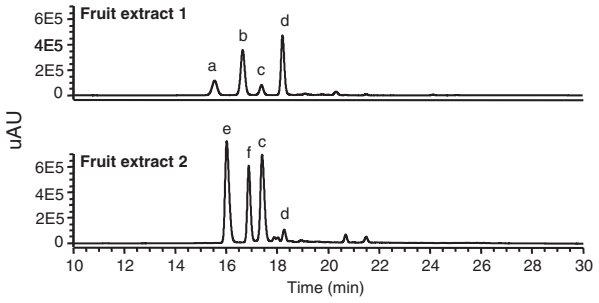

Figure 2. HPLC chromatograms of fruit extracts 1 and 2. The total polyphenolics extract of unnamed fruits 1 and 2 were analyzed to identify components. HPLC chromatograms of the anthocyanin content of the extracts are shown with the individual compounds identified by LC-MS: a – delphinidin glucoside, b – delphinidin rutinoside, c – cyanidin glucoside, d – cyanidin rutinoside, e – cyanidin sophoroside, f – cyanidin glucosyl rutinoside.

medium containing 2% FCS the myotubes were washed in D-PBS and DMEM medium (2%, 6 mL) was added. The cells were then simultaneously challenged with oxidative stress agents and the fruit polyphenolic extracts (30 min). Oxidative stress inducing agents used were the calcium ionophore A23187 (10 μM in ethanol), hydrogen peroxide (H_2O_2, 0.1–1.0 mM), or 2,4-dinitrophenol (DNP, 0.1–0.5 mM). Fruit extracts were prepared from a powdered stock in DMSO at 100 mg/mL and were applied simultaneously at the doses specified with the oxidative challenge. Controls consisted of myotubes not exposed to the oxidative stress and not treated with fruit extracts. Samples (100 μL) of the media bathing the myotubes were taken at the times indicated. Media were analyzed for lactate dehydrogenase (LDH) and CK activity. Simultaneous wells of myotubes were prepared for analysis of total cytosolic enzyme activity content. These myotubes were harvested using a dispersion agent (Tryple™), washed in D-PBS, and the total amount of enzyme activity determined from cells permeabilized by incubation with the detergent Triton-X100 (1%, 10 min). Release of LDH or CK activity from the cells was corrected for the change in well volume and expressed as a percentage of the total enzyme activity released.

2.6 Cytosolic enzyme assays

2.6.1 CK

CK activity was used as a measure of myoblast differentiation as well as a marker of cellular damage/integrity when released from cells (post stress/oxidative challenge). CK activity was determined using the method described by the German Society for Clinical Chemistry [13]. In brief, an imidazole-acetate buffer (pH 6.7) was prepared and supplemented with adenosine diphosphate (2 mM), magnesium acetate (10 mM), glucose (20 mM), nicotinamide adenine dinucleotide phosphate ($NADP^+$, 2 mM), N-acetylcysteine (20 mM), adenosine monophosphate (5 mM), diadenosine pentaphosphate (10 μM), hexokinase (2500 U/L), and glucose-6-phosphate dehydrogenase (1500 U/L). Activity of CK was determined by following the conversion of $NADP^+$ to NADPH at 340 nm in a UV spectrophotometer (UV-1601, Shimadzu Scientific Instruments) and after the addition of creatine phosphate (30 mM) and sample (80 μL).

2.6.2 LDH

The cellular release of LDH following challenge and/or fruit extract exposure was used as a measure of cellular damage/integrity. The enzyme activity was determined as described previously [14] by monitoring NAD absorbance (340 nm). The assay medium consisted of D-PBS (pH 7.4) with added pyruvate (0.35 mM) and NADH (8 mM) with absorbance being monitored using a UV spectrophotometer.

2.7 Quantification and identification of fruit extract total polyphenolics and anthocyanins

Quantification of fruit extract polyphenolics and anthocyanin content was performed by RP HPLC with detection at 530 nm for anthocyanins and 280 nm for polyphenolics. The total polyphenolics were calculated by summing the areas of all the peaks observed (3–35 min) and expressing data as epicatechin equivalents. Anthocyanin content was expressed as cyanidin-3-glucoside equivalents.

MS was employed to identify the flavonoid components of the different fruit polyphenolic extracts. Anthocyanins were identified by LC-MS using a LTQ linear ion trap fitted with an electronic service interface (ThermoQuest, Finnigan, San Jose, CA, USA) trap fitted with an ESI interface (ThermoQuest, Finnigan) coupled to an Ettan™multidimensional liquid chromatograph (GE Healthcare Bio-Sciences, Auckland, New Zealand) and Surveyor™ photodiode array (PDA) detector (ThermoQuest, Finnigan). Compound separation for blueberry extracts was achieved using a LiChroCart Superpher® 100 RP-18 endcapped column (Merck KgaA, Darmstadt, Germany), 250 × 2 mm maintained at 35°C, and for the other extracts, a Synergi 4 m Hydro-RP 80Å column (Phenomenex, Torrence, CA, USA), 250 × 2 mm also maintained at 35°C was used. Solvents were (A) 5:3:92 ACN:formic acid:water v/v/v and (B) ACN + 0.1% formic acid and the flow rate was 200 μL/min. The initial mobile phase, 100% A was ramped linearly to 83% A at 17 min, 80% A at 20 min, 70% A at 26 min, 50% A at 28.5 min, 5% A at 32 min and held for 3 min before resetting to the original conditions. Sample injection volume was 10 μL. Detection was enabled by a Surveyor™ PDA detector which was capable of absorbance scanning at 530 nm. MS data were acquired in the positive ion mode using a data-dependent LC-MS^3 method with dynamic exclusion enabled

and a repeat count of 2. This method isolates and fragments the most intense parent ion twice to give MS^2 data, then isolates and fragments the most intense daughter ion twice (MS^3 data). Each ion interrogated is then added to an exclusion list for a period of 1 min to enable mass spectral fragmentation data to be obtained from less intense ions. The ESI voltage, capillary temperature, sheath gas pressure, and auxiliary gas were set at 27 V, 300°C, 45 psi, and 10 psi, respectively. Data provided proof of anthocyanin structure by allowing us to remove the glycosylated side chains in a controlled, stepwise manner to provide molecular weight information about the aglycone core and glycosyl substituents.

Polyphenolics compounds were identified by LC-MS using a LCQ Deca 3D ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan) and coupled to a Surveyor HPLC and PDA detector. Compound separation was achieved using a Prodigy 5 μm ODS(3) 100Å, 150 × 2 mm column maintained at 35°C. Gradient elution was performed using water containing 0.1% formic acid (solvent A) and ACN containing 0.1% formic acid (solvent B). The initial mobile phase, 95% A 5% B, was held for 5 min, then programmed using a linear solvent gradient to 90% A 10% B over 5 min, then 83% A 17% B over 15 min, 77% A 23% B over 5 min, 70% A 30% B over 10 min, 3% A 97% B over 8 min and held for 5 min before resetting to the original conditions. The flow rate was 200 μL/min, the injection volume 10 μL, and the PDA detector scanned from 200–600 nm. The ESI MS data were acquired in the negative mode using a data-dependent LC-MS3 method. Proof of structure for polyphenolic compounds identified was confirmed by comparing the fragmentation pattern to that of standard compounds where available.

2.8 Statistical analysis

Results are expressed as means ± SEM for triplicate observations from at least three separate experiments in each case. All statistical significance analysis was undertaken by comparison of two groups and was assessed by use of the Student's *t*-test. A probability value (p) of less than 0.05 was considered significant. Statistical significance is displayed in each figure using the symbol *.

3 Results

3.1 Development of myotubes in culture and oxidative stress-induced cell damage model

We have used differentiated L6 skeletal muscle myotubes as a model to study stress-mediated damage and protection by fruit compounds. We characterized the differentiation of L6 muscle cells by monitoring the activity and expression of the muscle specific enzyme CK and myotube formation. Progressive myotube formation was observed (but not quantified) by light microscopy and appeared to mirror the development of CK enzyme activity in the cells. Cultured undifferentiated myoblasts contained a low level of CK activity, which rose steadily from day 1 following the change to 2% FCS. The CK activity in developing myotubes reached a maximum on day 3 (30.4 ± 5.1 mIU/mg), to plateau on day 4 (31.1 ± 5.1 mIU/mg). The enzyme activity on days 3 and 4 were increased 123 ± 0.1% and 126 ± 0.1%, respectively, ($p < 0.05$) compared with the activities at day zero. The profile of the CK enzyme activity of the developing myotubes was also confirmed by the expression of the protein observed by Western blot analysis (data not shown). We interpret these data to indicate that development of differentiation reaches a plateau at days 3–4 – this time point was therefore considered to be the most appropriate time for evaluation of cell damage.

We evaluated three stress mediating agents as tools to induce muscle cell damage as measured by the release of the cytosolic enzymes LDH and CK. Figure 3 shows the effect of exposure of differentiated myotubes to the calcium ionophore A23187 (10 μM), which has been shown to replicate the processes of exercise-induced muscle cell damage *via* a number of possible pathways including the generation of reactive oxidizing species [4, 12]. LDH enzyme activity was detected first, and it demonstrated the greatest accumulative release with a 16.2% increase above that of CK at 4 h. Control (not treated with A23187) cells showed a low level of LDH release that was slowly progressive and reached a maximum at 4 h of 7.4 ± 0.4%. Following A23187 exposure, however, a significant increase in LDH release was detected which occurred from 30 min (8.1 ± 0.3%, $p = < 0.05$ *versus* control) and progressively increased to the peak detected at the 4 h evaluated (22.9 ± 5.2%, $p < 0.05$ *versus* control). In contrast CK release was undetectable until the 2-h time point and had reached a plateau with a 5.6 ± 0.5% release at 2 h ($p < 0.05$ *versus* control) compared to 6.7 ± 0.9% release at 4 h ($p < 0.05$ *versus* control). Control cells showed no significantly detectable CK release over the times evaluated. We interpret these data to suggest that the release of the cytosolic enzyme LDH is the most appropriate tool as a marker of damage to myotubes as compared with CK. Exposure to H_2O_2 (0.1–1.0 mM) induced an immediate release of LDH that did not markedly accumulate any further with time (at 0.5 mM the 30 min release was 19.8 ± 4.2% compared to 26.7 ± 5.5% at 2 h). In a similar fashion to the A23187 data, DNP induced LDH release that was progressive with a 7.2 ± 1.2% release at 30 min, and a 16.0 ± 1.6% release at 2 h ($p = < 0.05$, 0.5 mM DNP). We interpret these data to suggest that exposure to A23187 and/or DNP are the most appropriate tools for mediating myotube damage that is of relevance to excessive exercise. For simplicity we have opted to use A23187 as our routine stress (oxidative) challenge in our evaluation

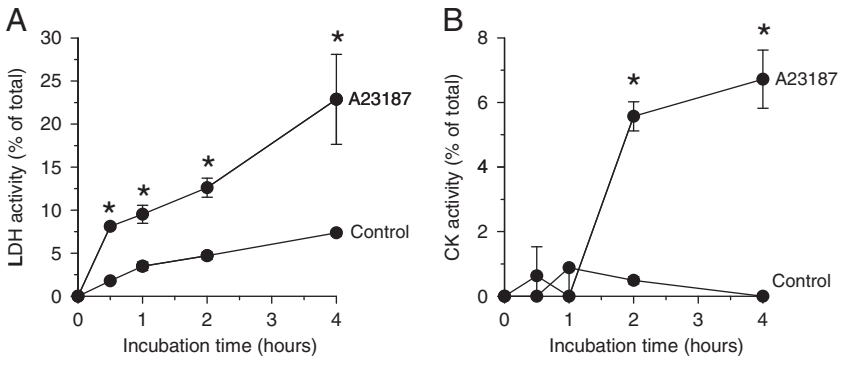

Figure 3. Time course of release of cytosolic enzymes from differentiated muscle cells following challenge with A23187. Differentiated muscle cells were challenged with the calcium ionophore A23187 (10 μM) and the release of the cytosolic enzymes LDH (A) and CK (B) released monitored. Data are displayed as the activity of the enzymes released into the bathing culture medium over the times indicated and expressed as a percentage of the total activity released following complete cell rupture (detergent exposure). Values are means ± SEM from eight observations *per* data point. $^{*}p < 0.05$ compared with untreated cells.

of the protective capacity of fruit polyphenolics extracts in this study. Cells were challenged and exposed simultaneously with A23187 (10 μM) and the fruit polyphenolics extracts for 30 min. Furthermore, as protective mechanisms of fruit extracts would most likely be mediated by an inherent antioxidant activity we clarified whether A23187 indeed mediated an oxidative stress in the muscle cells by determining ROS generation intracellularly. Using the ROS detection agent DCFDA, which when loaded into cells is cleaved into the oxidation sensitive product DCF, we demonstrated that this was indeed the case. A23187 (10 μM) doubled (57% increase) the levels of ROS over the control (as measured by relative fluorescence) in the muscle cells monitored over 30 min. The relative fluorescence of A23187 exposed cells at 30 min was increased by 13.6% from baseline compared with 7.7% for control non-treated cells (data not shown).

3.2 Effect of fruit extracts on stress-induced damage to myotubes

Figure 4 shows the effect of three different polyphenolics fruit extracts on A23187 mediated (10 μM, 30 min exposure) LDH release from myotubes with trolox as a well-characterized standard antioxidant for comparative purposes. Trolox (10–100 μM), added simultaneously with the A23187 challenge, significantly and dose-dependently prevented the oxidative stress-induced damage to the myotubes (as measured by block of LDH release). At the highest concentration of Trolox tested, a 92% inhibition of damage was observed clarifying further that A23187 mediated damage was predominantly *via* an oxidative stress process. Three fruit total polyphenolic extracts were evaluated. The extract derived from blueberry fruit displayed the most protective potential of the fruits. At the highest concentration evaluated (50 μg/mL) the blueberry extract resulted in a 54% protection ($p < 0.05$) while the other two fruit extracts (unnamed) at these concentrations demonstrated no protection against the calcium ionophore (Fig. 4).

3.3 Identification of protective blueberry fruit compounds

We set out to identify the active component(s) responsible for the marked protection against oxidative stress in the total polyphenolics extract of blueberry fruit. Figure 5 displays the effect of three sub-extracts derived from the original total polyphenolics extract. Comparing the protective effect of exposure to antho-extracts 1, 2, and 3 to that of the original total polyphenolics extract demonstrated that only antho-extract 2 retained activity. At 5 μg/mL the antho-extract 2 mediated a significant ($p < 0.05$) block of LDH release from the cells that was comparable to that of the total polyphenolics extract (21.8 and 18.1% inhibition, respectively). The protective effect of the antho-extract 2 was only evident at the 5 μg/mL concentration.

HPLC and MS (LC-MS) analysis (Fig. 1) highlighted extract polyphenolic components potentially responsible for the observed protective capacity. HPLC chromatograms indicate that a range of anthocyanin compounds typical of blueberry were present in the total blueberry polyphenolics extract and extracts antho-1 and 2 were rich in anthocyanins (Fig. 1). Antho-extract 3 contained no anthocyanins (Fig. 1). The absolute levels of polyphenolics in the fruit extracts confirmed these findings (Table 1). Additionally, as a useful comparison, Table 1 shows the polyphenolics concentrations of the extracts including those of the unnamed fruits, which were ineffective at protecting against calcium ionophore-induced damage. Furthermore, Fig. 2 displays the anthocyanin profiles (HPLC chromatographs) of the unnamed fruit total polyphenolics extracts. The extracts from the different fruits contained a range of concentrations of polyphenolics and a range of different anthocyanins typical of those particular fruits. Some anthocyanins were found in more than one fruit. We further identified the major polyphenolic components in the blueberry extracts. The principal polyphenolic components of the blueberry extracts were chlorogenic acid and rutinoside glycosides and were predominantly found in antho-extract 1. Quercetin glycosides were also identified in the total polyphenolics extract but not in the antho-1 or antho-2 extracts. Neither chlorogenic acid nor the rutinoside glycosides is likely to be

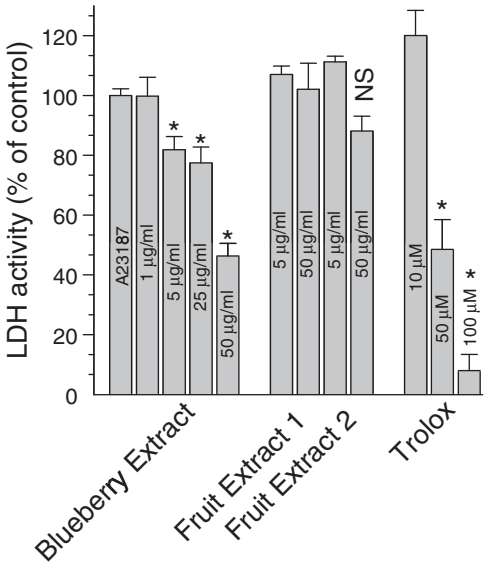

Figure 4. Prevention of LDH release from muscle myotubes by exposure to a blueberry fruit polyphenolics extract. Muscle myotubes were simultaneously exposed to the calcium ionophore A23187 (10 μM) and three different fruit total polyphenolic extracts (1–50 μg/mL) and cytosolic LDH activity determined. The effect of trolox (10–100 μM) was also evaluated. Enzyme activity expressed as a percentage of the activity released by A23187 alone (100%). Values shown are means ± SEM from at least four observations per data point. $^{*}p<0.05$ compared with A23187 alone.

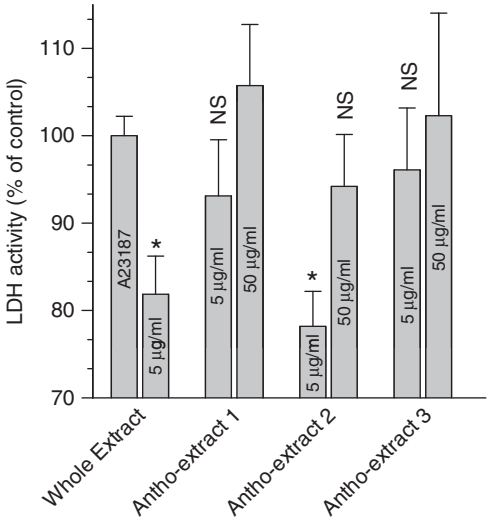

Figure 5. Effect of anthocyanin polyphenolic extracts of blueberry fruit on oxidative stress challenged myotubes. The protective effect of the total polyphenolics and anthocyanin subextracts of the blueberry fruit was determined by monitoring LDH release from muscle myotubes after challenge with A23187 (10 μM). Data are means ± SEM from at least four observations. Enzyme activity expressed as a percentage of the activity released by A23187 alone (100%). $^{*}p<0.05$ compared with A23187 alone.

the responsible actives as only the total extract and antho-extract 2 displayed a protective action on skeletal myoblasts. LC-MS confirmed that the anthocyanins that were present in all the extracts were predominantly malvidin glycosides (highlighted in Fig. 1). Antho-extract 3 contained no anthocyanins. Given that only the total polyphenolics and antho-extract 2 demonstrated activity (compared with the antho-extracts 1 and 3 – no activity), the comparison of the individual anthocyanin components and levels present in each of these extracts should highlight the most likely actives. Our analysis suggests that malvidin glycosides and particularly malvidin galactoside, and/or malvidin glucoside (Fig. 1) are components that could be providing the protection from oxidative stress. While these components are present in the inactive antho-extract 1, the concentrations of malvidin galactoside and glucoside are higher in antho-extract 2 (Fig. 1). Malvidin glycosides were not detected in the ineffective fruit extracts from the unnamed fruits (Fig. 2). Quantitation of the malvidin glycosides was performed by integration of the relevant peaks in the LC-MS HPLC-PDA profiles at 530 nm and using MS and MS^n to confirm compound identification. Quantitation of the biologically active antho-extract 2 indicated that malvidin galactoside was the major malvidin glycoside present (46% of the total malvidin glycosides). Malvidin glucoside accounted for 20% of the total malvidin glycosides with malvidin pentoside accounting for 19% and malvidin acetyl hexoside 15%.

To confirm the conclusions from HPLC and MS analysis regarding the active(s), we further evaluated the protective ability of ''standard'' highly pure commercially sourced compounds. Figure 6 shows the evaluation of the standard pure malvidin aglycone and glycosides. Malvidin aglycone demonstrated no protective effect on challenged myotubes while malvidin galactoside and glucoside at 5 ug/mL (the concentration of the antho-extract 2 which demonstrated activity) mediated significant ($p<0.05$) protective capacity that was comparable to that of the whole polyphenolic extract (20.5 and 20.6% inhibition compared to 18.1% inhibition, respectively). These data confirm that the blueberry fruit compounds responsible for protection against oxidative stress in muscle cell culture is malvidin galactoside and/or glucoside.

4 Discussion

Rat and mouse myoblast cell lines (L6 and C2C12, respectively) can be induced into fully differentiated skeletal muscle myotubes [11] and therefore act as a model system for both the evaluation of muscle damage and as a means to study the regulatory processes involved in myogenesis [12]. We report here the use of differentiated (induced by low serum) L6 myoblast cells, exposed to oxidative stress mediating agents, as a means to evaluating the potential protective benefit of fruit polyphenolics. Differentiation into skeletal myotubes was induced by low FCS and as reported

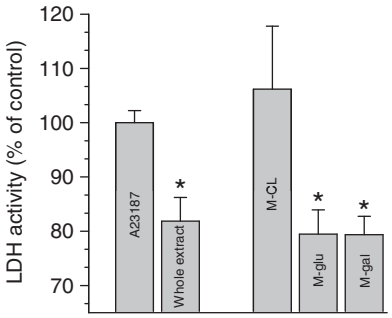

Figure 6. Evaluation of the protective effect of purified standard polyphenolics compounds on oxidative stress challenged myotubes. The protective action of standard polyphenolic compounds (malvidin aglycone (malvidin chloride, M-CL), malvidin galactoside (M-gal), and malvidin glucoside (M-glu), all at 5 μg/mL) were evaluated on muscle myotubes challenged with A23187 (10 μM) by monitoring LDH release. Data shown are means ± SEM from at least four observations per data point. Enzyme activity is expressed as a percentage of the activity released by A23187 alone (100%). $^{*}p < 0.05$ compared with A23187 alone.

by others [11, 12], myotube formation and CK expression/activity was increased over a number of days with peak and plateau CK activity observed at days 3–5.

Due to increased energy demands during exercise, cellular metabolism is increased in skeletal muscle which leads to the generation of ROS and free radicals [1], oxidative stress, and the potential for muscle damage [4]. Maglara *et al.* [11] advocated the exposure of muscle myotubes to calcium ionophores (*e.g.* A23187) and the mitochondrial uncoupler DNP as a means to induce oxidative stress. In our study we compared the effect of exposure to A23187, DNP, with that of H_2O_2 and showed that A23187 and/or DNP were the most appropriate tools for monitoring muscle damage. By comparing in our study the release of LDH and CK from muscle myotubes as indicators of cell damage and/or plasma membrane permeability [15], we demonstrate that both enzymes are released from myotubes after damage and that the release of LDH was immediate, while CK release was delayed (Fig. 3). The release of CK from muscle cells during *in vivo* animal or human exercise trials or from isolated muscle and skeletal cell preparations does not necessarily reflect changes in cell membrane permeability. Even though CK is a smaller enzyme, it is reluctantly released from damaged muscle [16] and the release process is independent of size and not due to non-specific leaks in the plasma membrane [11]. A suggested possibility is that the damaged muscle cell maintains viability by actively extruding certain cytoplasmic contents, which are different (potentially because of different locations and the intracellular binding of different proteins) from those released following detergent exposure [17]. Data from this study support the conclusions that the mechanisms of cytosolic enzyme release following muscle damage are not well understood and for whatever reasons, the release of LDH was prior to that of CK. For the purposes of our evaluation therefore the release of LDH was a more useful marker of damage in this study.

We show that polyphenolic extracts derived from blueberry fruit protect skeletal muscle myotubes from oxidative stress-mediated cell damage. Two other (unnamed) fruit extracts were ineffective despite also possessing a high polyphenolics and anthocyanin content (Fig. 4 and Table 1). These results suggest that for protection against calcium ionophore-mediated damage in muscle cells *in vitro* the absolute level of antioxidant anthocyanins and/or polyphenolics is of little relevance and that protection must be the consequence of specific polyphenolic compound(s) present in the blueberry fruit and not present in the other ineffective fruit extracts. There are many publications on the potential therapeutic health promoting action of polyphenolic anthocyanins (flavonoids), especially derived from berry fruits for human pathologies such as heart disease, cancer, stroke, neurodegeneration, diabetes, and vision [18, 19]. Many of these biological effects have been proposed to relate to the classical antioxidant properties of many of these compounds [20]; however, recent reports highlight direct effects of these compounds that are not necessarily related to antioxidant activity. Actions on important cellular signaling mechanisms from altering enzyme activities, to receptor function and transport processes are reported [21]. With regard to potential protective effects and benefits to muscle this study adds to a range of publications in cell studies and human/animal feeding studies where supplements containing antioxidants and various fruits and polyphenolic compounds were consumed. While polyphenolic anthocyanins have been reported to decrease proliferation in smooth muscle cells and inhibit the expression of pro-angiogenic and pro-atherosclerotic factors [22, 23], a number of reports highlight the ability of anthocyanins to mediate artery (vaso) relaxation by direct effects on vascular smooth muscle. Delphinidin-3-rutinoside a major anthocyanin component of blackcurrant has been reported to stimulate nitric oxide production, which mediates relaxation in ciliary smooth muscle [24]. However, red wine polyphenols such as the stillbene derivative trans-resveratrol appear to modulate artery relaxation *via* a process involving nitric oxide for intrapulmonary arteries and systemic arteries while pulmonary artery relaxation is promoted by mechanisms independent of nitric oxide [25]. Furthermore, with regard to exercise-induced oxidative muscle damage, Rossi *et al.* [26] showed that soybean ingestion affords an improvement in antioxidant status, which potentially is reflected by a decrease in exercise-induced oxidative damage. Similar conclusions were drawn from a study in athletes following supplementation with a red orange extract [27] and Connolly *et al.* demonstrated a decrease in symptoms of exercise-induced muscle damage in students fed a cherry juice blend [10].

In our study blueberry fruits were suggested as good candidates to combat muscle oxidative damage although

further investigations especially at an *in vivo* level are needed. Blueberries have one of the highest antioxidant capacities of all fruit and vegetables [28], which may explain the association of blueberry supplementation with the prevention of the decline of aged-related brain function, [29] and the improvement of memory through new neuron growth [30, 31]. With regard to muscle physiology a wild blueberry rich diet has been reported to affect the contractile machinery of rat aortic smooth muscle, which may be of benefit in controlling vascular tone [32]. Furthermore, Kalea *et al.* demonstrated that wild blueberry consumption results in structural changes in rat aorta glycosaminoglycans, which might affect vascular smooth muscle signal transduction pathways and function [33]. Moreover, Shaughnessy *et al.* recently demonstrated that diets containing blueberry extract slowed the development of hypertension in a rat model of human essential hypertension [34]. Blueberry supplementation in humans has been suggested to be beneficial in an antioxidant manner for athletic training [8].

In our studies not only was a blueberry polyphenolics extract found to be protective against skeletal muscle oxidative stress but we further evaluated anthocyanin sub-extracts for bioactivity. The antho-extract 2 retained protective activity and only at the lower dose of 5 μg/mL. Indeed higher concentrations (50 μg/mL) of all of the extracts other than the total polyphenolics extract (*i.e.* antho-extracts 1, 2, and 3) showed a trend for decreased protective activity (at 50 μg/mL) compared to the total polyphenolics extract. It remains unclear why only a lower concentration of the active compound(s) in antho-extract 2 afforded protection and high doses of all the extracts displayed decreased protective activity. However, we speculate that these effects may be due to the presence of low concentrations of unknown cytotoxic components in the extracts that counteract any protective effect at higher doses. Utilizing HPLC, LC-MS, and commercially sourced pure standard compounds, we identified and confirmed malvidin glycosides as the likely active components of blueberry. Area under the curve analysis indicated that the predominant malvidin glycoside was malvidin galactoside. While malvidin glycosides are abundant compounds in red grape varieties and blueberries, little data have been reported on the health promoting activities of this anthocyanin, although malvidin aglycone has growth-inhibitory properties. Malvidin inhibits the growth of tumor cells *in vitro*, is a potent inhibitor of cyclic adenosine monophosphate phosphodiesterases, and appears to modify other cellular signaling events that remain to be identified but are relevant to cell growth arrest [35]. To our knowledge, our findings are the first to specifically suggest that malvidin glycosides from blueberry fruits are beneficial in protecting skeletal muscle from oxidative stress-induced damage.

The mechanism(s) of action of the fruit extracts is of interest but it is not within the scope of this study and hence has not been addressed here. There is much debate about the true efficacy of polyphenolics because of transformation during absorption, microbial degradation, and a poor knowledge of the bioavailability of the actives at tissues. However, in rats and humans anthocyanin glycosides are rapidly and effectively absorbed from the stomach and small intestine, appear in plasma as glycosides, and are furthermore excreted in urine and bile as intact glucosides as well as methylated and glucuronidated forms [36–38]. With these aspects in mind it is difficult to extrapolate the *in vitro* data generated in this study to the *in vivo* situation. Much further research using human intervention studies is warranted to fully understand the implication of the findings reported here with our *in vitro* evaluations. Bioavailability concerns also make it difficult to evaluate if the doses used in this and many other published *in vitro* studies are appropriate. Because of the wide availability of these compounds in natural food beverages and products the daily intake of anthocyanins can be as much as 215 mg/day while concentrations appearing in plasma and urine are extremely variable but probably very low. We observed a dose-dependent effect in our *in vitro* studies and used a concentration range that is similar to those published in other manuscripts using *in vitro* techniques.

In summary, data presented in this report further advocate the use of differentiated muscle myotubes and oxidative stress mediators as a model system of skeletal muscle damage of relevance to exercise. Although it is difficult to deduce the biological significance of the data presented here from *in vitro* studies, one may speculate that consumption of blueberry fruit polyphenolics and particularly malvidin glycosides may be beneficial in alleviating the damaging consequences of oxidative stress in muscle tissue. Our data further endorse that more research in the action of blueberry fruit polyphenolics and muscle function is warranted. Detailed research, especially utilizing human intervention trials may provide the robust evidence required to support the use of blueberry fruit polyphenolics in functional foods and/or sports supplements.

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