Tart Cherry Supplement Enhances Skeletal Muscle Glutathione Peroxidase Expression and Functional Recovery After Muscle Damage

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Abstract

Introduction

Montmorency cherry concentrate (MCC) supplementation enhances functional recovery from exercise, potentially due to antioxidant and anti-inflammatory effects. However, to date, supporting empirical evidence for these mechanistic hypotheses is reliant on indirect blood biomarkers. This study is the first to investigate functional recovery from exercise alongside molecular changes within the exercised muscle following MCC supplementation.

Methods

Ten participants completed two maximal unilateral eccentric knee extension trials following MCC or placebo supplementation for 7 days prior to and 48 hours following exercise. Knee extension maximum voluntary isometric contractions (MVC), maximal isokinetic contractions, single leg jumps, and soreness measures were assessed before, immediately, 24 and 48 h after exercise. Venous blood and vastus lateralis muscle samples were collected at each time point. Plasma concentrations of IL-6, TNF-α, C-reactive protein, creatine kinase, and phenolic acids were quantified. Intramuscular mRNA expression of SOD 1 and 3, GPX1, 3, 4 and 7, Catalase, and Nrf2 and relative intramuscular protein expression of SOD1, Catalase and GPX3 were quantified.

Results

MCC supplementation enhanced recovery of normalized MVC 1s average compared to placebo (Post- Exercise PLA: 59.5±18.0% vs MCC: 76.5±13.9%; 24 h PLA: 69.8±15.9% vs MCC: 80.5±15.3%; supplementation effect p=0.024). MCC supplementation increased plasma hydroxybenzoic, hippuric and vanillic acid concentrations (supplementation effect p =
0.028, p = 0.002, p= 0.003); SOD3, GPX3, GPX4, GPX7 (supplement effect p < 0.05) and
GPX1 (interaction effect p = 0.017) gene expression; and GPX3 protein expression
(supplementation effect p = 0.004) versus placebo. There were no significant differences
between conditions for other outcome measures.

Conclusion

MCC supplementation conserved isometric muscle strength and upregulated antioxidant gene
and protein expression in parallel with increased phenolic acid concentrations.

Keywords: Phenolic Acids, Resistance Exercise, Muscle Damage, Antioxidant,

Oxidative Stress

Abbreviations

Akt (Protein Kinase B)
ANOVA (Analysis of Variance)
BCA (Bicinchoninic Acid)
βME (Beta-mercaptoethanol)
BSA (Bovine Serum Albumin)
CAT (Catalase)
cDNA (complementary Deoxyribonucleic Acid)
CK (Creatine Kinase)
CRP (C-Reactive Protein)
ddH₂O (double-distilled water)
ECL (Enhanced Chemiluminescence)
ECMax (Maximal Eccentric Knee Flexion Contractions)
EDTA (Ethylenediaminetetraacetic Acid)
ELISA (Enzyme-linked Immunosorbent Assay)
GPX 1,3,4,7 (Glutathione Peroxidase 1,3,4,7)
High performance liquid chromatography (HPLC)
IKCon\textsuperscript{Max} (IK\textsuperscript{Max} Concentric Phase)
IKEcc\textsuperscript{Mac} (IK\textsuperscript{Max} Eccentric Phase)
IK\textsuperscript{Max} (Maximal Isokinetic Knee Extension and Flexion)
IL-6 (Interleukin-6)
Keap1 (Kelch-like ECH-associated protein 1)
LSD (Lithium Dodecyl Sulphate)
MCC (Montmorency Cherry Concentrate)
mRNA (messenger Ribonucleic Acid)
mTOR (Mammalian Target of Rapamycin)
MVC (Maximal Voluntary Isometric Contraction)
NADPH (Nicotinamide Adenine Dinucleotide Phosphate)
Nrf2 (Nuclear factor erythroid 2-related factor 2)
PARQ (Physical Activity Readiness Questionnaire)
PLA (Placebo)
PPT (Pain Pressure Threshold)
PPT\textsuperscript{VL/VM/RF/SUM} (Pain Pressure Threshold at Vastus Lateralis/Vastus Medialis/Rectus Femoris/sum of VL, VM and RF)
RIPA (Radioimmunoprecipitation Assay)
RNA (Ribonucleic Acid)
ROM (Range of Motion)
ROS (Reactive Oxygen Species)
95 rRNA (ribosomal Ribonucleic Acid)
96 RTqPCR (Real-Time Polymerase Chain Reaction)
97 SLJ (Single Leg Jump)
98 SOD 1, 2, 3 (Superoxide Dismutase 1, 2, 3)
99 SR (Sarcoplasmic Reticulum)
100 TBS (Tris-buffered Saline)
101 TBST (TBS with tween)
102 TNFα (Tumour Necrosis Factor Alpha)
103 VAS (Visual Analogue Scale)
Introduction

Intense exercise may induce muscle damage, resulting in muscle soreness and associated reductions in force generating capacity of the muscle. This damage occurs through a complex combination of mechanisms including structural damage to the contractile apparatus, as well as disruption to biochemical pathways such as those governing skeletal muscle calcium handling. This is due, in part, to high intra-muscular forces and increased reactive oxygen species (ROS) exposure, generated during exercise (1, 2).

ROS are generated during exercise (1, 2) and are understood to play an important role in maintaining homeostasis; when levels exceed the capabilities of the endogenous antioxidant defence mechanisms, cellular redox balance is altered resulting in oxidative stress (3). This in turn causes further disruption and damage to cellular processes and structures (1-4). For example, structures within the sarcoplasmic reticulum (SR) are sensitive to ROS, such that increased exposure to ROS impairs muscle calcium handling and sensitivity (5). This leads to decrements in muscle contractile force development and consequently exercise performance (2, 4). Furthermore, disruptions in skeletal muscle calcium handling, are also likely to impair recovery; for example via elevating muscle protein breakdown and reducing phosphorylation of protein kinase B (Akt) and mammalian target of rapamycin (mTOR) (6), thereby reducing protein synthesis required for repair.

Due to this involvement of oxidative damage, there has been an abundance of research investigating the use of exogenous antioxidant supplements as a means of reducing exercise induced muscle damage and the associated recovery time (7, 8). Numerous studies have shown that polyphenol supplementation reduces blood markers of oxidative damage and
inflammation (9-13). Montmorency cherries contain high concentrations of polyphenols (14), and there is evidence that their consumption in supplement form may attenuate oxidative stress, inflammation and muscle soreness; aiding muscular recovery from multiple exercise modalities (9-11, 13, 15, 16). However, to-date, research in this area has relied on proxy markers of intra-muscular oxidative stress and inflammation within blood plasma or serum, rather than analysis of the exercised muscle tissue itself. This approach is unlikely to comprehensively elucidate the effects of intensive exercise and supplementation strategies to support recovery, since these proxy measures have been shown to respond differently in recovery from intensive exercise to direct muscle measures (17).

The mechanisms by which supplementation may enhance recovery are unclear, and the limited evidence available is equivocal. Initial theories proposed radical scavenging as the primary mechanism, due to the ability of phenolic compounds to donate electrons via hydrogen atom transfer from a hydroxyl unit. However, the low concentrations of polyphenols and phenolic metabolites present in the plasma suggest they are unlikely to act as direct antioxidants \textit{in vivo} (18). We and others have hypothesised that it is more likely that antioxidant effects \textit{in vivo} arise from nuclear translocation and activation of Nuclear Factor Erythroid 2–related Factor-2 (Nrf2) signalling following exposure to phenolic metabolites (7, 19, 20).

This study is the first to investigate the potential biological mechanisms within human skeletal muscle that underpin improvements in exercise recovery in response to Montmorency cherry concentrate (MCC) supplementation. We quantify antioxidant mRNA and protein expression within exercised muscle tissue. We also quantify the phenolic metabolites of MCC in plasma throughout the MCC loading and exercise recovery phases. It
was hypothesised that supplementation would amplify gene and protein expression of endogenous antioxidant enzymes thus enhancing functional recovery.

Methods

This study employed a double-blind cross-over design in which participants completed two trials separated by a two-week washout period in line with previous literature (10, 15, 21, 22). The study received ethical approval from the Sport and Health Sciences ethics committee at the University of Exeter and Human Research Ethics committee at the University of Queensland and all testing conformed to the guidance set out by the Declaration of Helsinki (see additional information for details).

Participants

Ten recreationally active male participants (age = 23.4±5.4 years, weight = 78.0±21.9 kg, height = 178.4±6.9 m), asymptomatic of illness and injury, completed the study. Twelve participants were recruited and consented, but two withdrew prior to completion of the study. One participant was unable to participate following consent and familiarisation, due to commitment to another study which required dietary manipulation, and one participant completed one arm of the study but was un-responsive to all subsequent contact attempts. Participants completed a physical activity readiness questionnaire (PARQ) and medical and exclusion screening questionnaire, before providing written informed consent. PARQ was also used to exclude sedentary individuals. Exclusion criteria comprised individuals aged below 18 or above 40 years, females, individuals allergic to fruit, and highly trained individuals. Females were excluded to avoid any confounding influence of low-grade
inflammation caused by menstrual cycle symptoms (23), and highly trained individuals were
excluded due to their familiarity with high intensity exercise, which may have dampened the
effects of the exercise damage protocol (24). Individuals who are highly trained will have
chronic training adaptations, such as increased antioxidant and buffering capacity, as well as
increased monocarboxylate transporters, which can improve the speed of post-exercise
recovery (25). Furthermore, trained individuals’ familiarity with eccentric exercise may be
protective against muscle damage from subsequent eccentric exercise during the damage
protocol (26). Trained individuals were excluded by verbal questioning prior to giving
informed consent, and their responses were subsequently confirmed by visual assessment of
their activity diary. Trained individuals were defined as completing more than 3 h.wk-1 of
deliberate planned exercise outside of normal physical activity as defined by Caspersen et al.
(27).

Sample size was calculated as 10 participants to provide 80% power to detect a 5% difference
between trials, based on the expected difference between MCC and placebo (PLA) (effect
size of 1). Calculations were based on the maximum voluntary isometric contraction (MVC)
force recovery data from Bowtell et al. (10) and the anticipation of a curvilinear relationship
between dosage and functional effects on performance markers. 12 participants were initially
recruited to account for dropouts.

Supplementation Period

Trials were counterbalanced for trial order and leg dominance, with participants and
investigators blinded to treatment to avoid potential bias. A researcher, who had no further
involvement with data collection, prepared the supplement packs for the participants. The
participants were randomised to supplement code ‘A’ or ‘B’ using a sealed envelope system. The MCC and PLA supplements were then provided to investigators by a member of the research team not involved in data collection, in opaque bags coded ‘A’ or ‘B.’

During each trial participants ingested two 30 mL daily doses (morning and evening) of either MCC or PLA, for ten days. MCC supplement was a commercially available product (CherryActive®, ActivEdge, U.K) produced from US Montmorency cherries. PLA was a commercially available fruit concentrate (Morello Cherry Cordial, Blossom Cottage, Gloucester, U.K) with additional carbohydrate added to ensure it was isoenergetic. Analysis of the phenolic content of the MCC by high performance liquid chromatography (HPLC) (28) was conducted by Atlas Bioscience, Inc. (Tucson, AZ). Total content was 20.167 mg·mL⁻¹ polyphenolics, 7.211 mg·mL⁻¹ total anthocyanins, with pelargonidin (3.319 mg·mL⁻¹) and delphinidin (1.299 mg·mL⁻¹) the most prevalent anthocyanins (see Table A, SDC, for details on polyphenol composition of MCC supplement). This supplementation protocol has previously been shown to enhance recovery of MVC knee extensors following exercise induced muscle damage (10). Participants were also asked to maintain any normal exercise habits throughout the trial, but to refrain from high volume and high intensity exercise, such as resistance, interval or unaccustomed endurance type exercise, for 48 hours prior to experimental visits. Participants were also asked to maintain their normal diet, but to avoid any increase in consumption of foods with high polyphenol concentrations during the supplementation and wash out periods, in addition to alcohol and caffeine 48 hours before the test. To this end, participants were provided with diet and exercise diaries, to record all food and beverages consumed in the final 6 days of the supplementation period, and exercise completed over the final five days of each supplementation period. Avoidance of an increase in consumption of polyphenol rich foods, rather than avoidance in toto was advised in order
to maintain ecological validity. Participants were also asked to refrain from eating or drinking anything apart from water for 10 h prior to the laboratory visits during both supplementation periods, and all lab tests were conducted at the same time of day for each participant. On days where experimental testing occurred, morning doses of MCC were consumed by participant prior to arrival at the laboratory.

Experimental Design

Familiarisation

Participants’ baseline measures of height and weight were assessed, before familiarisation with all experimental procedures, and measurement of MVC. Performance of the warm-up during familiarisation was force limited to 100 N for all participants. Familiarisation for all tests occurred for both dominant and non-dominant legs on the same day. A maximum of one set of the damage protocol was performed sub-maximally. Chair and dynamometer arm settings for the Biodex were determined during the familiarisation visit and recorded for use in all subsequent tests. Leg dominance was determined by defining the non-dominant leg as the stabilising leg during single leg movements such as kicking.

Damage Protocol and Functional Testing

Overnight fasted participants returned to the laboratory on day 8 of supplementation at which point resting venous blood samples were collected from an antecubital vein. Serum samples were collected in tubes containing clot activator and gel for serum separation and kept at room temperature for 30 minutes, and plasma samples were collected in tubes containing
lithium heparin. Tubes were centrifuged at 4500 rpm for 15 minutes at 4°C to fractionate samples and remove the cellular components. Serum and plasma were distributed into microcentrifuge tubes before storage at -80°C until analysis. *Vastus lateralis* muscle biopsy samples were taken using the suction-modified percutaneous Bergstrom needle technique (29). The leg from which the biopsy was taken was sterilised using iodine and anaesthetised locally with 2% lidocaine. An incision of approximately 0.8 cm was then made, before a biopsy needle was used to collect a sample of muscle (~150 mg). The incision was then closed with butterfly stitches and covered with a waterproof dressing. Eight biopsies were taken from each participant, with four per experimental trial, prior to and after exercise, at 24 and 48 hours. All biopsies were taken from the same leg during the course of each trial, each time from a new incision.

Muscle soreness was assessed with participants seated and knee extensors in a stretched position, with a knee angle of 90° via the use of a (200 mm) visual analogue scale (VAS) and pain pressure threshold (PPT) using a handheld algometer (FDX 50, Wagner, Greenwich, CT 06836-1217 USA) (10, 16). Algometer measures were taken by application of increasing pressure, with a handheld algometer, to the participant determined point of being ‘uncomfortable but not painful,’ at the vastus lateralis, vastus medialis and rectus femoris. One measure was taken at each site by the same investigator before, post, and 24 and 48 hours after completion of the damage protocol as in Bowtell *et al.* (10). For VAS analysis participants were instructed to mark their level of soreness on a line from 0 to 10, with 0 being no pain, and 10 being extreme pain. VAS of this length have previously been shown to have good reliability for measuring acute pain and detecting changes in pain intensity (30).
The exercise protocol (Figure 1) consisted of a warm-up, muscle function measures (single-leg maximal isokinetic knee extension and flexion repetitions (IK\textsuperscript{Max}); MVC, single leg jumps (SLJs)) and a muscle damage protocol, using a Biodex Isokinetic Dynamometer (Biodex System 3 Medical Systems 830-200, Shirley, N.Y. 11967 USA). Functional measures and muscle damaging exercise via eccentric contractions of knee extension exercise were selected in line with previous research to allow for direct comparisons (10, 31).

Before beginning experimental measures (IK\textsuperscript{Max}, MVC and SLJ), participants completed the warm-up protocol using the leg from which the pre-exercise biopsy had been taken. The warm-up consisted of 5 sets of 5, single-leg sub-maximal isokinetic knee extension and flexion repetitions with a force limit set at 50\% of familiarisation MVC for that leg, separated by 1 minute of rest. Following the warm-up protocol, participants completed, 3 sets of 3 IK\textsuperscript{Max} repetitions and 3 MVCs, separated by 1 minute of rest, and 3 SLJs performed consecutively with a rest period of at least 10 seconds. Jumps were performed on a mat (Jump Mat Pro, SL Electronics Ltd., Cookstown, UK) with hands on hips and a single leg take-off to isolate performance as much as possible to the limb of interest. A two-footed landing was used to account for discrepancies in participants ability to balance upon landing, especially with fatigue following the exercise protocol. Jump height was recorded in mm.

Warm up and IK\textsuperscript{Max} repetitions were performed over a range of motion (ROM) of 80\° from full flexion at the knee, at 60\°·s\textsuperscript{-1} for both the concentric and eccentric phases. MVC repetitions were performed at a knee angle of 90\°, with the Biodex arm stationary, as this has previously been shown to be the angle at which the maximum amount of force can be produced, due to optimal overlap of sarcomeres (32).
The damaging exercise protocol was performed 5 minutes after completion of SLJs, and consisted of 10 sets of 30 maximal eccentric knee flexion contractions (EC\textsuperscript{Max}), with each set separated by a period of 1-minute. Repetitions were performed over the same ROM of 80° as that used for the warm-up and IK\textsuperscript{Max} repetitions; with a passive (no contraction) concentric phase at 180°·s\textsuperscript{-1}, and a maximal eccentric phase, at 60°·s\textsuperscript{-1}. There were no significant differences in joint angles between legs (p = 0.528). Performance tests were then repeated following the damage protocol. Throughout the experimental measures and damage protocol, participants were given verbal encouragement. A further muscle biopsy and blood sample were taken immediately following completion of the post-damage protocol performance tests, in addition to further measures of muscle soreness.

Twenty-four and forty-eight hours later, participants returned to the laboratory following an overnight fast. During both visits, resting venous blood samples, a further assessment of muscle soreness, and a further muscle biopsy were taken, before repetition of the warm-up, functional performance measures (Figure 1). All biopsies were taken from the same leg during the course of a trial. Following a two-week supplement wash-out period, this protocol was repeated with the functional measures and damage protocol performed using the contralateral leg, in order to minimise any repeated bout effects. All visits for each participant were performed at the same time of day.

**Force Recordings**

Force produced during knee extension exercise was measured using a Biodex isokinetic dynamometer. Torque was displayed in Newton metres (Nm). Force data were recorded and analysed using a custom written script in Spike2 ver.6 software (CED, Cambridge, UK).
Work done during the damaging exercise protocol was determined via calculation of the area under the force time curve:

\[ Work = \text{force} \times \text{time trace} \]

Force data from MVCs were analysed to calculate both peak force output and the highest average value over a 1 second period, occurring within the plateau of each contraction. The reported MVC value for each respective time point was represented by the highest value achieved across the 3 MVC contractions for each measurement time point to ensure the maximal possible peak values was recorded.

Force data for maximal isokinetic contractions were assessed by measurement of peak force during each set for the three individual concentric and eccentric contractions respectively completed during each set at each measurement time point. Three sets of contractions were completed at each time point, from which the highest values of peak concentric and eccentric force were taken.

**Sample Analyses**

**Blood Sample Analysis**

Serum samples were analysed for activity of Interleukin-6 (IL-6); C-reactive protein (CRP), and Tumour Necrosis Factor Alpha (TNF- α) via ELISA (IL-6: HS600C; TNF- α: HSTA00E; CRP: DCRP00, R&D Systems Quantikine High Sensitivity ELISA, R&D Systems,
Minneapolis, United States), according to the manufacturer’s instructions to assess muscle
damage and systemic inflammation. Creatine Kinase (CK) analysis was performed by the
Royal Devon and Exeter NHS Trust on the 702 module of the Cobas 8000 automated
platform according to the manufacturers recommended protocol (Roche Diagnostics, Basel,
Switzerland).

Measurement of Plasma Phenolic Metabolites Profile by High-Resolution Accurate-
Mass (HRAM) Mass Spectrometry

Phenolic metabolite analysis were performed at the Bioanalytical Facility, University of East
Anglia. Plasma concentrations of protocatechuic acid, 4-hydroxybenzoic acid, hippuric acid,
vanillic acid, ferulic acid and isoferulic acid were quantified using an Orbitrap Velos Linear
Trap Quadrupole (LTQ) high-resolution accurate-mass (HRAM) mass spectrometry system
coupled with an Accela autosampler and ultra high-pressure liquid chromatography pump
(Thermo Scientific, Cheshire, UK). The Orbitrap system was operated in Fourier transform
MS mode at the resolution of 30,000 in negative electrospray ionisation (ESI) mode.

To prepare the samples for analysis, 200 µL of plasma/calibration stands/quality controls and
20 µL of internal standard containing ferulic acid-[2H3] (100 nmol.L-1) and hippuric acid
[13C6] (200 µmol.L-1) (Toronto Research Chemicals, Ontario, Canada). in 0.1% formic acid
(Merck, Germany), were pipetted into a microcentrifuge tube and mixed. To this, 1 mL of
methanol was added slowly with gentle mixing, the mixture was then incubated at room
temperature for 15 minutes, followed by centrifugation at 14000 rpm for 7 minutes. The
supernatant was transferred to borosilicate glass tube and placed in an evaporator to dry
under a constant stream of nitrogen at a temperature of 60°C. To the dried supernatant 200
µL of methanol (Merck, Germany) with 0.1% formic acid was added into each tube and vortex mixed for 30 seconds, followed by 2.5 mL of ethylacetate (Merck, Germany) and vigorously mixed for 10 minutes. After centrifugation at 4000 rpm for 10 minutes, 2 mL of the ethylacetate in the upper layer was transferred to a fresh set of borosilicate glass tubes and again evaporated to dryness as described above. The dried residue was resuspended in 250 µL of LCMS grade deionised water with 1% acetic acid (Merck, Germany), then vortex mixed followed by centrifugation at 4000 rpm for 10 mins. The final mixture was transferred into polypropylene autosampler vials, 50µL was injected into the liquid chromatography high-resolution mass spectrometry system for analysis.

Chromatographic separation was achieved using a ModusCore C18 reverse phase column (2.1 m x 50 mm, 2.7 µm) (Chromatography direct, Runcorn, UK) maintained at a temperature of 40°C. Mobile phases A consisted of 1% acetic acid in LCMS grade deionised water with LCMS grade methanol as mobile phase B. The binary gradient program was: 0 min 1% B, 0–1 min 1% B with a linear increase to 45% B at 10 min, 10-10.5 min 95% B and held to 12 min, returned to 1% B at 12.5 min to re-equilibrate with a cycle time of 15 mins. Mobile phase flow rate was 0.5 mL per min throughout the run.

The mass scan range used to quantify each phenolic metabolite was determined by direct infusion of pure standards into the ion source via a T-connector. European Pharmacopoeia (EP) reference standards used to prepare the calibration standards were obtained via Merck (Germany). The quantitation mass range (Da) for protocatechuic acid (152.99800-153.00300), 4-hydroxybenzoic acid (137.005-137.010), hippuric acid (178.026-178.032), vanillic acid (167.012-167.017), ferulic acid and isoferulic acid (193.023-193.030). Xcalibur software version 2.1 (Thermo Scientific, Cheshire, UK) were used for system control, data
acquisition, baseline integration and peak quantification (see Table B, SDC, for summary of assay performance).

**Muscle Sample Analysis**

Muscle samples were analysed for protein concentrations of SOD1, Catalase, GPX3, GPX4 and GPX7, as well as gene expression of SOD1, SOD2, SOD3, GPX1, GPX3, GPX4, GPX7, Catalase, and Nrf2.

**Gene Expression (Real-Time Quantitative Polymerase Chain Reaction (RTqPCR))**

Superoxide dismutase (SOD) 1, SOD2, SOD3, Glutathione Peroxidase (GPX) 1, GPX3, GPX4, GPX7, Catalase (CAT), and Nrf2 mRNA expression were quantified using TaqMan® Array 96-Well Fast Plates. RNA was extracted from muscle samples by immersion in 500 µL of TRIzol reagent (Sigma-Aldrich Company Ltd., Dorset, United Kingdom), and bead homogenisation (Speedmill Plus, Analytik Jena AG, Jena, Germany) for 30 seconds and 1 minute sequentially. RNA was extracted according to the TRIsol manufacturer’s instructions and was resuspended in 30 µL nuclease free water (Severn Biotech, Limited, Kidderminster, United Kingdom). Samples were heated for 5 minutes at 60°C to ensure complete solubilisation.

Subsequently, RNA concentration and purity of samples was analysed by spectrophotometry (NanoDrop Lite Spectrophotometer, ThermoFisher Scientific, Waltham, Massachusetts, United States), before cDNA transcription of RNA was performed with Primerdesign.
Precision nanoScript 2 Reverse Transcription kit, according to manufacturer’s instructions (Primerdesign, Southampton, United Kingdom).

Following this, 5 µL of TaqMan fast advanced master mix (Applied Biosystems, Waltham, Massachusetts, United States) and 5 µL of each sample containing 2.5ng cDNA were added to custom TaqMan Gene Expression Array 96 well fast plates (Applied Biosystems, Waltham, Massachusetts, United States). Plates were then sealed and vortexed briefly to ensure contents were mixed, before centrifuging for 1 minute at 1200 g at 4°C (Sorvall ST 16 Centrifuge Series, ThermoFisher Scientific, Waltham, Massachusetts, United States). Plates were then loaded into the RTqPCR instrument for analysis (QuantStudio 6 Flex Real-Time PCR System, ThermoFisher Scientific, Waltham, Massachusetts, United States). Samples underwent 1 cycle for enzyme activation at 95°C for 20 seconds; and then underwent 40 cycles of sequential denaturing at 95°C for 1 minute, and annealing/extending at 60°C for 20 seconds. The internal control used was 18s rRNA (data available in SDC Table C).

RTqPCR fold change was calculated using the Pfaffl formula (33) for quantification relative to the pre-exercise placebo condition. These values were \( \log_{10}(x) \) transformed prior to analysis in order to linearise data. Primer efficiency was assumed to be 2.

**Protein Content Analysis**

**Protein Extraction**

25 mg muscle was placed in microcentrifuge tubes with 400 µL of radioimmunoprecipitation assay (RIPA) buffer (1 Pierce A32961 Protease and Phosphatase Inhibitor EDTA-free mini
tablet, ThermoFisher Scientific, Waltham, Massachusetts, United States, dissolved in 10 mL
Pierce 89900 RIPA buffer, ThermoFisher Scientific, Waltham, Massachusetts, United
States), before bead homogenisation (Speedmill Plus, Analytik Jena AG, Jena, Germany) for
30 seconds and 1 minute sequentially. Homogenised muscle and RIPA buffer samples were
then aspirated and placed into clean microcentrifuge tubes and vortexed thoroughly
(FB15012 TopMix Vortex Mixer, ThermoFisher Scientific, Waltham, Massachusetts, United
States), before incubation on ice for 30 minutes, with occasional vortexing. Samples were
then centrifuged for 10 minutes at 8000 g at 4°C (Sorvall ST 16 Centrifuge Series,
ThermoFisher Scientific, Waltham, Massachusetts, United States). The supernatant was
retained, and the pellet was discarded.

Protein concentrations were determined by Bicinchoninic acid (BCA) assay (Pierce 23225
BCA Protein Assay Kit, ThermoFisher Scientific, Waltham, Massachusetts, United States)
according to the manufacturer’s instructions. Protein lysate dilutions were then calculated
according to values obtained from BCA assay protein content analysis. Samples were diluted
in ddH2O and a 4x 1:0.11, lithium dodecyl sulphate (LSD): β-mercaptoethanol (βME)
solution (PCG3009 TruPAGE LDS Sample Buffer, Sigma-Aldrich, St. Louis, Missouri,
United States; 2-Mercaptoethanol, ThermoFisher Scientific, Waltham, Massachusetts, United
States).

Western Blotting

Gels were loaded with pre-stained protein molecular weight ladder (Pierce 26612 Prestained
Protein MW Marker, ThermoFisher Scientific, Waltham, Massachusetts, United States), a
pre-prepared protein standard of pooled positive control sample, produced by combining
samples of a dropout participant, and participant lysate samples for analysis. All gel
electrophoresis was run at 120 V constant (Mini-PROTEAN Tetra Cell System Tank and
PowerPac Basic Power Supply, Bio-Rad, California, United States) until the dye reached the
bottom of gels. Membranes were then incubated in a blocking solution of either 5% milk
crass powder (Marvel Dried Skimmed Milk, Premier Foods plc, Hertfordshire, United Kingdom)
or 5% BSA (BP9702-100 BSA, Fisher BioReagents, Waltham, Massachusetts, United
States), for 1 hour at room temperature before overnight incubation of at least 12 hours in a
primary antibody dilution at 4°C (see Table D, SDC, for details on individual protocols by
protein target).

Membranes were incubated in the appropriate secondary antibody dilution at room
temperature for 1 hour. The membrane was washed with tris-buffered saline (S5886 Sodium
Chloride, Sigma-Aldrich Company Ltd., Dorset, United Kingdom and Tris-Base BP152-1,
Fisher BioReagents, Waltham, Massachusetts, United States) (TBS) and Tween 20 (Tween
BP337-100, Fisher BioReagents, Waltham, Massachusetts, United States) (TBS-T) solution
at least 3 times after every cycle of antibody incubation. Protein detection was conducted on
the membrane using enhanced chemiluminescence (ECL) detection reagent (RPN2232
Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Bio-Sciences,
Pittsburgh, United States), and were then imaged for chemiluminescence (ChemiDoc XRS+
System, Bio-Rad, California, United States).

Following imaging, membranes were stained for analysis of protein load using Coomassie
blue staining solution (0.25 g C/8540/46 Coomassie Blue R250 powder (Fisher BioReagents,
Waltham, Massachusetts, United States) mixed in a solution of 10 mL Glacial acetic acid
(A/0360/PB15 Glacial acetic acid, Fisher BioReagents, Waltham, Massachusetts, United
States) and 90 mL Methanol:H₂O (1:1 v/v)) (M/4056/15 Methanol, Fisher BioReagents, Waltham, Massachusetts, United States). Membranes were de-stained using TBS-T before colourmetric imaging using automatically determined exposure (ChemiDoc XRS+ System, Bio-Rad, California, United States).

Densitometry Analysis

Blots were analysed for optical density using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Band intensity was normalised to total protein load (Coomassie blue, intensity of entire lane) and normalised across gels using the positive control sample.

Statistical Analysis

All data (with the exception of total work during the muscle damage protocol for which a paired t-test was used) were analysed by 2-way repeated measures analysis of variance (ANOVA). Where data was missing for one time point in a participant trial arm, z-scores were calculated for the missing data point. In cases where there were multiple data points missing from a participant trial arm, that participant was excluded for the corresponding analysis. MVC data are presented for each time point normalised to pre-exercise MVC to control for differences in pre-exercise MVC between legs and between participants. For transparency, absolute MVC data are also presented along with results of those statistical analyses. Fold change from RTqPCR analysis was linearised by log₁₀(x) transformation prior to analysis. In cases where a significant interaction effect was detected, post-hoc pairwise tests were conducted with Bonferroni corrections. Throughout analyses, values that did not
meet the assumption of sphericity as measured by Mauchly’s test, were Greenhouse-Geisser corrected. Where data were not normally distributed and could not be normalised by standard approaches to data transformation, a non-parametric Friedman’s test was conducted for comparison with the results of the 2-way repeated measures ANOVA. All statistical analyses were performed using IBM SPSS Statistics (Version 26). For ease of reading, main effects (supplement, time and interaction) and post-hoc differences are only reported in text, tables and figures where statistical significance was achieved.

Results

Knee extension MVC 1s average decreased to 59.5±18.0% and 76.5±13.9% of pre-exercise for PLA and MCC conditions respectively, following completion of the intensive exercise protocol (Figure 2A). There was no significant difference between conditions in work performed throughout the damage protocol (44722.4 ± 14535.7 J, PLA vs 46812.0 ± 12341.6 J, MCC). A visual inspection was conducted on food and activity diaries to ensure participants replicated dietary intake and activity. There was no effect of trial order on the MVC measures (MVC 1s Average: p = 0.467, MVC peak: p = 0.394).

MCC supplementation significantly enhanced force recovery of normalised MVC 1s average (supplementation effect, p = 0.024, interaction effect, p = 0.043) (Figure 2A). Post-hoc testing revealed a significantly higher force recovery for MCC immediately post-exercise (p = 0.033), but no significant differences at any other time point.

Recovery of normalised peak MVC force was enhanced in the MCC versus PLA condition (supplementation effect, p = 0.032, interaction effect, p = 0.049). Post-hoc testing revealed no
significant differences in normalised peak MVC between conditions pre-exercise, immediately, 24- and 48-hours post-exercise, though post-exercise was close to significance (p = 0.054) (Figure 2B). Non-normalised peak and 1s average MVC force were not significantly higher in MCC than PLA condition, however there were significant interaction effects for both 1s Average and Peak MVC force (Table 1).

There was no significant difference in recovery of normalised IK^\text{Max} (combined eccentric and concentric phases), or during separated concentric (IK\text{Con}^\text{Max}) (Figure 2C), or eccentric contraction phases (IKE\text{ce}^\text{Max}) (Figure 2D).

There was no significant difference between supplement conditions in jump height (Figure 2E) or soreness measures (VAS and PPT\text{VL}, PPT\text{VM}, PPT\text{RF}, PPT\text{SUM}) between conditions (Table 1). Soreness, as measured by VAS and PPT\text{RF}, was significantly elevated after the damaging exercise (time effect, p < 0.05).

Plasma hydroxybenzoic acid, hippuric acid and vanillic acid concentrations were significantly higher following MCC supplementation compared with placebo supplementation (p < 0.05, supplementation effect) (Figure 3A, B, C). There was no significant difference between conditions for concentrations of protocatechuic acid, ferulic acid or isoferulic acid (SDC Figure A).

MCC significantly increased mRNA expression of SOD3, GPX3, GPX4 (supplementation effect p < 0.05), GPX7 (supplementation effect p = 0.001, interaction effect p = 0.014) and GPX1 (interaction effect p = 0.017) (SOD3 1.1, GPX3 2.9, GPX4 6.0, GPX7 2.8 and GPX1 1.4 fold increase; 2.8 mean fold increase in antioxidant enzyme gene expression with MCC
supplementation) (Figure 4). Post-hoc testing revealed significantly greater expression of GPX7 for the MCC condition post-exercise (p = 0.024) and at 24-hours (p = 0.009) (Figure 4H), but no significant differences between conditions at any time points for GPX1 (Figure 4E). There was no significant difference between conditions in mRNA expression of SOD1, SOD2, CAT and Nrf2 (Figure 4).

There was a significant increase in protein expression of GPX3 following MCC supplementation (3.0-fold increase, supplementation effect p = 0.004) (Figure 5C). There was no significant difference in SOD1 and CAT protein expression between MCC and PLA (Figure 5A, B). GPX4 and GPX7 proteins were undetectable and are not presented.

There were significant increases in serum concentrations of IL-6, TNF-α, and CK (time effect p < 0.05), but not CRP (p = 0.130), following the damage protocol (Figure 6). No significant differences were found between supplementation conditions for serum concentrations of IL-6, TNF-α, CRP and CK (Figure 6). IL-6 data were not normally distributed and could not be normalised by standard approaches to data transformation. However, a non-parametric Friedman’s test produced results that were consistent with the results of our 2-way RM ANOVA analysis of these data (time effect p < 0.001, supplement effect p = 0.145).

**Discussion**

This study presents the first evidence demonstrating a significant upregulation of antioxidant gene and protein expression in human skeletal muscle following 7 days pre-load and three days post-load supplementation with MCC containing a complex blend of polyphenols.
Crucially, these effects occurred in parallel with significantly improved functional recovery after intensive exercise, as observed previously (9, 10, 13, 15, 16). Furthermore, enhancements in antioxidant expression profile and functional recovery were accompanied by a corresponding augmentation in plasma concentrations of phenolic acids. These novel findings shed new light on the mechanisms that underpin functional changes following natural polyphenol blend supplementation such as MCC.

Plasma concentrations of 4-hydroxybenzoic acid, hippuric acid and vanillic acid were significantly elevated following MCC supplementation, which demonstrates that supplementation increased circulating exogenous antioxidant concentrations. However, direct ROS scavenging by these compounds is not thought to be the primary mechanism of reduced oxidative damage observed following polyphenol supplementation (18). Indeed, the greatest mean value of plasma phenolic acids measured in the current study did not surpass 40 \mu\text{mol}\cdot\text{L}^{-1} (Vanillic Acid), which is 4-10 fold lower than the values observed for endogenous extracellular antioxidants such as plasma urate, for which concentrations range between 150-450 \mu\text{mol}\cdot\text{L}^{-1} (7). Although not sufficient to elicit direct antioxidant effects, the increase in plasma concentrations of phenolic compounds appears to have been sufficient to elicit a significant upregulation of endogenous antioxidant gene and protein expression in skeletal muscle in the MCC condition. The mechanism hypothesised to underpin these changes is an upregulation of endogenous antioxidant production via the Nrf2 antioxidant response element pathway following exposure to the aforementioned elevation in phenolic metabolites (19, 20). Nrf2 is widely accepted as the ‘master regulator’ of antioxidant defence, and upregulation induces an expression profile protective against oxidative stressors through augmented expression of endogenous antioxidants and cytoprotective genes, (34).
Under normal homeostatic conditions, Nrf2 is repressed through binding to Keap1 within the cytoplasm, where it is ubiquitinated and subsequently proteolysed (35). The proposed activation of Nrf2 following supplementation of MCC is hypothesised to occur via exposure of Keap1 to phenolic metabolites. Indeed, plasma levels of phenolic acids were augmented following MCC supplementation in the present study, supporting previous research which has observed bioavailability of phenolic compounds following consumption of other polyphenol rich supplements (36, 37). These phenolic compounds are then hypothesised to undergo conversion to quinones, semi-quinones and superoxide radicals via dismutation of phenoxy radicals and redox complexes produced during radical scavenging (38, 39). The literature suggests that cellular exposure to these compounds then causes oxidative modification of Keap1 cystine residues via alkylation (40). Consequently, Nrf2 dissociates from Keap1, enabling nucleic accumulation of Nrf2 and the observed upregulation of endogenous antioxidant gene and protein expression (34, 38, 40).

Previous *in vitro* and rodent model research has shown evidence that polyphenol exposure can induce Nrf2 gene expression and translocation, as well as augment activity of antioxidant enzymes including GPX and SOD (19, 41), however, to the authors’ knowledge, this study presents the first *in vivo* evidence of up-regulation of antioxidant enzyme gene and protein expression in human skeletal muscle following polyphenol supplementation. These novel data provide strong evidence that increased expression of endogenous antioxidant and cytoprotective genes following exposure to phenolic acids, confers protection against oxidative stressors, such as intensive exercise and the resulting inflammatory response; thereby contributing to observed improvements in functional recovery (20, 34) (Figure 7).
This study is the first to demonstrate an increase in both GPX3 mRNA and protein expression following MCC supplementation. One of the primary postulated mechanisms for reductions in force generating capacity after intensive exercise is altered myofibrillar Ca\(^{2+}\) sensitivity (1, 42). Oxidised Troponin I cysteine residues have been shown *in vitro* to bind with glutathione (43). This glutathionylation has a protective effect on Troponin I molecules against oxidative stressors, and increases the Ca2+ sensitivity of the contractile apparatus (41). These mechanisms have in turn been suggested to beneficially affect exercise performance (43). GPX enzymes catalyse the reduction of H\(_2\)O\(_2\) and organic hydroperoxides by glutathione and thus their induction (MCC-induced induction of GPX3 in this instance) may contribute to the MCC-induced attenuation of the MVC force reduction identified immediately post-exercise (Figure 2A); this requires considerable further study.

We describe an ergogenic effect of MCC on recovery of maximal isometric force production in accordance with previous research (9, 10, 13, 15, 16), demonstrating that the aforementioned biological changes occur in parallel with significant functional effects. Importantly, the intensive bout of eccentric exercise induced significant muscle damage as indicated by the significant impairment of all functional measures; measures of perceived soreness; pain pressure threshold at the rectus femoris, and all blood inflammatory markers. Therefore, an experimental paradigm was created in which the favourable effects of MCC supplementation were detectable. Whilst there were no significant differences between supplementation groups for soreness, some of these measures may have been affected by the use of lidocaine local anaesthetic, which, whilst required for the *vastus lateralis* biopsies, may have influenced participants’ ability to detect pressure pain. The measure most likely to have been affected in the current study was the PPT measure at the vastus lateralis, as this location was at the closest proximity to the biopsy site. Indeed, there were no significant
effects detected for supplement or time point at the vastus lateralis or vastus medialis.

However, as there was a significant time effect at the rectus femoris, suggesting that the
damage protocol did elicit significant muscle soreness, but pain sensitivity was reduced in the
areas closest to the biopsy site. It must also be noted, that a similar phenomenon has been
observed previously, where functional enhancements in recovery are not necessarily reflected
in soreness or PPT measures (10, 11, 15).

The lack of difference between conditions in measures of circulating inflammatory cytokines
may be due to the methodological limitations associated with the ability of proxy measures
from blood to detect subtle changes occurring at the intramuscular level. Indeed, previous
research of MCC supplementation that has found significant functional effects have
demonstrated equivocal results for blood measures of inflammatory cytokines (9-11, 13).
This further highlights the importance of conducting analysis on the exercised tissue, a major
limitation of previous research in the area. Unfortunately, due to limited muscle tissue
availability, we were unable to characterise inflammatory processes within muscle in the
present study. A further limitation in the current study is the lack of quantified dietary intake,
without which we cannot be certain of the extent to which background diet, including
polyphenol intake, was replicated between trial arms. This may have influenced the observed
functional and molecular responses to MCC supplementation. However, visual inspection of
dietary logs suggested that intake was faithfully replicated between study arms. Notably, such
logs, whether quantified or not are prone to participant reporting errors (44).

In conclusion, this study showed for the first time that supplementation with US
Montmorency cherry concentrate, a polyphenol rich fruit concentrate, significantly increased
expression of antioxidant genes and proteins in human skeletal muscle, in parallel with a
significant increase in plasma concentrations of phenolic acids. This study also confirmed previous findings that MCC supplementation improved functional muscle recovery from exercise induced muscle damage. This study provides new and compelling evidence to support an upregulation of the antioxidant response element pathway, perhaps due to increased nuclear translocation of Nrf2 following exposure to elevated phenolic metabolites, as the primary mechanism underpinning enhanced functional recovery following polyphenol supplementation.
Additional Information

Data availability statement

Data are available within the figures and tables of this manuscript and are also included in the statistical summary document.

Competing interests

The authors have no competing interests/conflicts of interest to disclose.

Author Contributions

Conception or design of the work (J.T.W., M.F.O., V.G.K., J.L.B.). Acquisition, analysis or interpretation of data for the work (J.T.W., M.F.O., V.G.K., S.R.J., J.D, J.C.Y.T., J.L.B.). Drafting the work or revising it critically for important intellectual content (J.T.W., M.F.O., V.G.K., J.C.Y.T., J.L.B.)

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship and are listed.

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Ethics

The individual responsible for research governance at the University of Exeter Sport and Health Sciences Ethics Committee was Dr Melvyn Hillsdon.

The individual responsible for research governance at the University of Queensland’s Human Research Ethics Committees A & B was Chris Rose’ Meyer.

Institutional Ethics Committee Approval Numbers were as follows:

University of Exeter: 180314/B/05
University of Queensland: 2018000928

The results of the current study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the current study do not constitute endorsement by ACSM.
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**Figure Captions**

**Figure 1:** Experimental protocol. Montmorency cherry concentrate (MCC), placebo (PLA), warm up (WU), maximal voluntary isometric contraction (MVC), maximal isokinetic contraction (IKMax), single leg jump (SLJ), maximal eccentric contraction (ECMax), Ex (exercise).
Figure 2: Comparison of A: maximal voluntary contraction 1s average force B: maximal voluntary contraction peak force C: peak concentric phase maximal isokinetic contractions D: peak eccentric phase maximal isokinetic contractions E: maximal single leg jump measured pre-exercise, immediately, 24 hours and 48 hours post exercise for Montmorency Cherry Concentrate (MCC) and Placebo (PLA). *denotes a significantly higher value at that time point for MCC condition vs the placebo condition (Bonferroni Corrected p<0.05) or a significantly greater main effect of supplementation for MCC condition vs the placebo condition (p<0.05). † denotes a significantly greater interaction effect for MCC condition vs the placebo condition (p<0.05). *denotes a significant time effect (p<0.01##, p<0.001###) N = 10.

Figure 3: Comparison of plasma phenolic acid concentration measured pre-exercise, immediately and 24 hours post exercise for Montmorency Cherry Concentrate (MCC) and Placebo (PLA). Metabolites displayed are A: Hydroxybenzoic Acid B: Hippuric Acid C: Vanillic Acid. Values are displayed as means±SD. * and ** denote a significantly higher value at that time point for MCC condition vs the placebo condition (Bonferroni Corrected *p<0.05) or a significantly higher main effect of supplementation for MCC condition vs the placebo condition (*p<0.05/**p<0.01). ## denotes a significant time effect (p<0.01). N = 10

Figure 4: Comparison of antioxidant enzyme gene expression measured pre-exercise, immediately and 24 hours post exercise for Montmorency Cherry Concentrate (MCC) and Placebo (PLA) expressed as A: log10(x) fold change relative to the pre-exercise condition for all measured antioxidant genes, and B-J: fold change relative to PLA pre-exercise. B: Superoxide Dismutase 1 (SOD1) C: SOD2 D: SOD3 (N=8) E: Glutathione Peroxidase 1
(GPX1) **F:** GPX3  **G:** GPX4  **H:** GPX7 (N=8)  **I:** Catalase (CAT)  **J:** Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2). All data are displayed as mean±SD. Data are presented in a manner which allows the reader to visually appreciate both the overall pattern (A – this figure is for visualisation purposes only and does not represent the statistical analysis employed) and actual magnitude (fold-change B-J) of mRNA expression changes. For statistical analysis RTqPCR fold change was calculated using the Pfaffl formula relative to the pre-exercise placebo condition. These values were log10(\(x\)) transformed prior to analysis * and ** denote a significantly higher value at that time point for MCC condition vs the placebo condition by 2-way repeated measures analysis of variance (ANOVA) (Bonferroni Corrected *p<0.05/**p<0.01) or a significantly higher main effect of supplementation for MCC condition vs the placebo condition (p<0.05). ‡ denotes a significant interaction effect for MCC condition vs the placebo condition (p<0.05). N = 9 (unless stated otherwise where values were excluded) (1 participant excluded due to insufficient sample).

**Figure 5:** Comparison of western blot protein expression for **A:** Superoxide Dismutase 1 (SOD1) (N=10) and **B:** Catalase (CAT) (N=10), and **C:** Glutathione Peroxidase 3 (GPX3) (N=9) measured pre-exercise, immediately, 24 hours and 48 hours post exercise for Montmorency Cherry Concentrate (MCC) and Placebo (PLA). All values are presented as mean±SD. * denotes a significantly higher main effect of supplementation for MCC or a significant difference at that time point (p < 0.05). **D:** Example blots are displayed for PLA and MCC conditions pre-exercise (Pre), immediately (Post), 24, and 48 hours post exercise. Coomassie stain for protein load is shown for GPX3, stains for SOD1 and CAT are available in supplementary material.
Figure 6: Inflammatory and proxy muscle damage markers in serum A: Interleukin 6 (IL-6)(N=9), B: Tumour Necrosis Factor Alpha (TNF-α)(N=10), C: Creatine Kinase (CK)(N=10), D: C-Reactive Protein (CRP)(N=7) measured pre-exercise, immediately, 24 hours and 48 hours post exercise for Montmorency Cherry Concentrate (MCC) and Placebo (PLA). All values are presented as mean±SD. *denotes a significant time effect (p<0.05*, p<0.01**, p<0.001***).

Figure 7: Hypothesised mechanism underlying increased expression of glutathione peroxidase (GPX) following Montmorency cherry concentrate (MCC) supplementation. Mechanisms demonstrated by the current study are demarcated from hypothesised mechanisms. Kelch-like ECH-associated protein 1 (Keap1), Small musculoaponeurotic fibrosarcoma (sMAF). Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), Created with BioRender.com

Supplemental Digital Content
Supplemental Digital Content. Docx
Figure 1

A $p$ Supplement = 0.024
$p$ Interaction = 0.043
$p$ Time < 0.001

B $p$ Supplement = 0.032
$p$ Interaction = 0.049
$p$ Time < 0.001

Figure 2

$\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{p}}}}}}}}}}$ Time = 0.001

$\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{p}}}}}}}}}}$ Time = 0.004

$\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{\bar{p}}}}}}}}}}$ Time = 0.009
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7