Acute aerobic exercise-conditioned serum reduces colon cancer cell proliferation through IL-6-induced regulation of DNA damage *in vitro*

Short-title: Exercise reduces colon cancer proliferation through DNA repair

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Novelty and Impact

This study demonstrates that the acute systemic responses to aerobic exercise inhibit colon cancer cell proliferation *in vitro*, which may be driven by IL-6-induced regulation of DNA damage and repair. This novel mechanism of action may at least partly underlie epidemiological associations linking regular physical activity with reduced colon cancer risk.

Abbreviations

95% CI	95% confidence interval
BSA	Bovine serum albumin
BMI	Body mass index
DMEM	Dulbecco's Modified Eagle's Medium
DSB	Double-strand break
EV	Extracellular vesicle
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IQR	Interquartile range
PBS	Phosphate-buffered saline
RPE	Rating of perceived exertion
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
STR	Short-tandem repeat
TNF-α	Tumour necrosis factor-alpha

ABSTRACT

Epidemiological evidence shows that regular physical activity is associated with reduced risk of primary and recurrent colon cancer. However, the underlying mechanisms of action are poorly understood. We evaluated the effects of stimulating a human colon cancer cell line (LoVo) with human serum collected before and after an acute exercise bout versus non-exercise control serum on cancer cell proliferation. We also measured exercise-induced changes in serum cytokines and intracellular protein expression to explore potential biological mechanisms. Blood samples were collected from 16 men with lifestyle risk factors for colon cancer (age ≥50 years; body mass index ≥25 kg/m^2; physically inactive) before and immediately after an acute bout of moderate-intensity aerobic interval exercise (6 x 5 min intervals at 60% heart rate reserve) and a non-exercise control condition. Stimulating LoVo cells with serum obtained immediately after exercise reduced cancer cell proliferation compared with control (-5.7%; p=0.002). This was accompanied by a decrease in LoVo cell γ-H2AX expression (-24.6%; p=0.029), indicating a reduction in DNA damage. Acute exercise also increased serum IL-6 (24.6%, p=0.002). Furthermore, stimulating LoVo cells with recombinant IL-6 reduced γ-H2AX expression (β=-22.7%; p<0.001) and cell proliferation (β=-5.3%; p<0.001) in a linear dose-dependent manner, mimicking the effect of exercise. These findings suggest that the systemic responses to acute aerobic exercise inhibit colon cancer cell proliferation *in vitro*, and this may be driven by IL-6-induced regulation of DNA damage and repair. This mechanism of action may partly underlie epidemiological associations linking regular physical activity with reduced colon cancer risk.

Key Words: Acute exercise; physical activity; colon cancer; exercise-oncology; cancer therapy

INTRODUCTION

There is strong epidemiological evidence that regular physical activity protects against colon cancer.[1–3] Current estimates suggest that achieving the highest versus lowest level of physical activity reduces the relative risk of developing colon cancer by 12-28%.[1] Physical activity after a colon cancer diagnosis is also associated with a decreased risk of cancer-specific mortality[2] and recurrence.[4]

The biological mechanisms underlying how physical activity reduces colon cancer risk have mainly been attributed to decreased adiposity and associated reductions in circulating insulin and pro-inflammatory cytokines.[5,6] However, the epidemiological evidence, including both observational and Mendelian randomisation studies, demonstrates that physical activity is inversely related to colon cancer risk independent of adiposity.[7–10] Murine studies also show that physical activity alone, without dietary modification or changes in body weight, reduces tumour progression.[11,12] This indicates that physical activity might reduce colon cancer risk - at least partly - through biological mechanisms other than body fat-associated pathways.

Exercise is a subcomponent of physical activity encompassing planned, structured activities purposefully carried out to improve physical fitness.[13] During a bout of exercise, skeletal muscle and other secretory organs release bioactive molecules (proteins, nucleic acids, metabolites) into the systemic circulation.[14] These molecules can elicit biological effects on distant cells via endocrine-like signalling and are thought to mediate some of the multisystemic benefits of exercise.[14]

Early preclinical evidence shows that the systemic responses to exercise can regulate cancer cell proliferation *in vitro*.[15,16] Indeed, our recent meta-analysis demonstrated that stimulating a range of cancer cell lines with human serum obtained immediately after exercise reduces cell proliferation by ≈9%.[17] The transient modulation of humoral factors during acute exercise may represent a novel mechanism underlying the association between regular physical activity and reduced colon cancer risk. However, only one study included in the meta-analysis used a cell line model of colorectal cancer, comprising an uncontrolled, pre-post design (n=10 colorectal cancer survivors).[18] The inclusion of a non-exercise control experiment is needed to account for biological variation and regression to the mean effects.[19] Previously we have also highlighted concerns about the overall quality of evidence,[15,17] warranting the conduct of well-controlled studies to strengthen the evidence-base.

An improved understanding of the precise signalling molecules and pathways driving the growth-inhibitory effects of exercise could help inform guidelines for the optimal exercise dose needed for cancer prevention. It could also increase the likelihood of physical activity being integrated into standard cancer preventive care as a therapeutic intervention. Therefore, this study assessed the effects of stimulating a colon cancer cell line with human serum collected before and after an acute exercise bout versus non-exercise control serum on colon cancer cell proliferation. We also quantified exercise-induced changes in serum cytokines and intracellular protein expression to explore potential molecular mechanisms of action.

METHODS

This trial was prospectively registered at ClinicalTrials.gov (ID: NCT04057274). Minor deviations from the original protocol are documented and justified in Table S1.

Participants

We recruited males who had lifestyle risk factors for colon cancer between August 2019 and February 2020. Inclusion criteria were: age ≥50 years, body mass index (BMI) ≥25 kg/m^2 or waist circumference ≥94 cm, and not engaged in ≥30 min of moderate to vigorous-intensity physical activity on ≥3 days·wk^{-1} for the last three months. Main exclusion criteria were: signs/symptoms of cardiovascular, metabolic or renal disease, hypertension (≥160/≥90 mmHg), previous stroke, taking beta-adrenergic blocking agents, previous treatment for malignancy, respiratory disease with peak respiratory flow <300 l/min, or any musculoskeletal, neurological, or rheumatoid condition that could be exasperated due to exercise.

Study design

This study used a two-site, prospective, randomised, controlled, crossover design. Participants completed an acute bout of moderate-intensity aerobic interval exercise and a non-exercise control experiment in a randomised, counterbalanced order, separated by two to seven days (median: six days). The randomisation sequence was stratified by site and generated in block sizes of four by an independent researcher using online randomisation software. The order was concealed from the research team using opaque, sealed envelopes until eligibility was confirmed. Before visiting the laboratory, participants were instructed to eat the same meal 2-5 hours prior, not to engage in moderate- to vigorous-intensity physical activity or consume alcohol for ≥24 hours, avoid caffeine intake for ≥12 hours, and to arrive fully hydrated.

Acute exercise bout

The moderate-intensity aerobic interval exercise was performed on a cycle ergometer (Lode Excalibur sport, Groningen, Netherlands) under the supervision of research staff in the exercise science facilities at Northumbria University and York St John University (both UK). Following a 10 min warm-up that involved pedalling against a light resistance (60 W), participants completed 6 x 5 min intervals at 60% heart rate reserve, separated by 2.5 min of active recovery (60 W). Heart rate reserve was determined as the difference between an individual's resting and estimated maximum heart rate (220 minus age). We employed an interval exercise protocol rather than continuous exercise to ensure all participants were able to complete a total of 30 min of moderate-intensity exercise without reaching the limit of tolerance. A pedal cadence of 60 rev·min^{-1} was maintained throughout. Heart rate was monitored continuously, rating of perceived exertion (RPE) was collected in the final minute of each 5 min interval using the 6-20 Borg Scale,[20] and blood pressure was measured once during each active recovery period. The load was reduced by ≈10% if heart rate reserve increased to >65% or RPE was >14.

Non-exercise control

The control experiment intended to control for the natural deviation of serum analytes, and thus serum-stimulated cell proliferation, in the absence of exercise. The experiment involved 60 min of quiet, seated rest, conducted at the same time of day as the acute exercise bout (±1 hour) to control for diurnal variation.

Serum collection

Blood samples were drawn before the warm-up and immediately after completing the acute exercise bout, and at the same time-points before and after the control experiment. Each ≈20ml blood sample was drawn from an antecubital vein and collected in 10 ml Vacutainer serum tubes (BD, New Jersey, USA). Samples were allowed to clot at room temperature for 60 min, centrifuged at 1,000 x g for 20 min, apportioned into 0.5-1 ml aliquots, and cryopreserved at -80°C for later analysis.

Cell line

A human colon cancer cell line (LoVo, RRID:CVCL_0399) was purchased from Sigma-Aldrich (Dorset, UK) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4500 mg/L glucose, 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. We chose to use the LoVo cell line because it harbours *APC* and *KRAS* mutations but is wildtype for *TP53*,[21] which are genetic features associated with the early stages

of cancer development.[22] LoVo cells also have the genomic instability phenotype microsatellite instability.[21] Thus, the LoVo cell line serves as a useful model to study the potential role of exercise in modulating early colorectal carcinogenesis through the oncogene-induced DNA damage model for cancer development.[23] Cells were thawed and passaged 4-8 times at ≈70% confluence before being used for experiments, and were maintained at 37°C in a humidified atmosphere of 5% CO_2. The cell line was authenticated using short-tandem repeat (STR) profiling within the previous year (NorthGene, Newcastle upon Tyne, UK). All experiments were performed with mycoplasma-free cells.

Outcomes

The prespecified primary outcome was mean difference in LoVo cell proliferation between exercise and control conditions. Secondary outcomes, used to explore potential molecular mechanisms of action, included exercise-induced changes in serum cytokine concentration and LoVo cell protein expression.

Cell proliferation

Viability of the LoVo cells was assessed via quantification of the fluorescent signal by the resazurin assay (Sigma-Aldrich). Following trypsinization, cells were counted with a haemocytometer and seeded at $1x10^4$ cells.well^{-1} in quintuplets within opaque, clear-bottom 96-well plates (Greiner Bio One Ltd, Stonehouse, UK). Cells were not seeded in outer wells to avoid the potential influence of unequal evaporation on differences in fluorescent signal across the plate. Serum samples from each individual were used on the same 96-well plate to negate inter-plate variability. Cells were initially seeded in 100 µL of their normal growth medium for 24 hours to allow attachment to the bottom of the plate. The normal growth medium was then aspirated and replaced with 100 µL of DMEM containing 1000 mg/L glucose, 1% glutamine, and 10% serum from individual participants instead of FBS. Stimulated cells were incubated for 48 hours. Resazurin dye was then added to each well at a final concentration of 0.02%, mixed via orbital shaking, and incubated for a further four hours. Fluorescence was measured using a microplate reader at an excitation of 540 nm and emission of 590 nm. Background fluorescence was subtracted from each well and then values were normalised to fluorescence of control cells grown in 10% FBS, with the mean value five replicate wells used for analysis. LoVo cell proliferation following direct IL-6 stimulation was assessed by standard MTT assay after 48 hours of incubation.

Serum cytokines

Serum concentrations of seven cytokines/myokines were quantified using bead-based multiplex immunoassays (Milliplex, Merck Millipore, Burlington, USA). Interleukin 6 (IL-6), IL-8, IL-10, tumour necrosis factor-alpha (TNF-α) were assessed by a human cytokine 4-plex panel (HYCTA-60K), and irisin, osteonectin, and oncostatin M were quantified by a human myokine 3-plex panel (HMYOMAG-56K). All samples were run in duplicate and the assay was performed according to the manufacturer's instructions. Plates were read on the Luminex MAGPIX system (Merck Millipore). Median intra-assay coefficient of variations for each analyte ranged from 4.1% to 10.7%.

Western blot

LoVo cells are *KRAS* mutant but wildtype for several other colon cancer critical genes (e.g. TP53, BRAF, PTEN).[21] Thus, we mainly focused on components of the MAPK/ERK pathway, as well as the mTOR pathway, because of their relevance to colon cancer and potential to be modified by acute exercise. Phospho-specific antibodies MEK1/2 (#9154), ERK1/2 (#4370), CREB1 (#9198), ATF1 (#9198), RSK90 (#9344), NF-κB (#3033), Akt (#4060), mTOR (#5536), histone H2AX (γ-H2AX; #9718), β-actin (#8457) and α-Tubulin (#2144) were purchased from Cell Signalling Technology (CST; Beverly, MA, USA). Briefly, LoVo cells were trypsinised and seeded at $2x10^5$ cells.well^{-1} in 12-well plates. Upon reaching 80-90% confluency, cells were gently washed once in serum-free DMEM before incubation in serum-free DMEM supplemented with 0.2% bovine serum albumin (BSA) for two hours. Serum-starved LoVo cells were then treated with 10% participant serum samples for either 20 or 60 min before washing 3 times in cold phosphate-buffered saline (PBS) and lysis in 2% sodium dodecyl sulfate (SDS) lysis buffer supplemented with complete protease inhibitors (Roche, Mannheim, Germany). Protein concentration was determined by micro BCA assay according to the manufacturer's instructions (ThermoFisher, Cramlington, UK). 30-35 μg of total protein was separated on 5-17% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. Primary antibodies (1:2000 dilution) were prepared in TBST containing 0.5% BSA and 0.01% sodium azide and incubated overnight. Anti-rabbit (#7074) and anti-mouse (#7076) horseradish peroxidase (HRP) conjugated secondary antibodies were prepared in TBST at 1:5000 dilution and incubated for one hour. Western blots were detected using Clarity ELC substrate (Bio-Rad Laboratories, Hertfordshire, UK) and captured on an iBright CL1000 imaging system (ThermoFisher).

Sample size

We used Monte Carlo simulations to estimate the sample size required to achieve at least 80% statistical power to detect a difference in the primary outcome (LoVo cell proliferation), assuming certain population parameters. Based on previous research,[18] we generated a random-sampled, normally-distributed data set assuming cell viability of 54 ± 3.5% at baseline, and a reduction in cell viability of 3 ± 3.5% following stimulation with exercise-conditioned serum. We then fit a linear mixed model to the data set and calculated power as the percentage of statistically significant *p*-values ($p \leq 0.05$) based on $1x10^4$ simulations.[24] A total of 15 participants were required to achieve approximately 89% power. Simulations were performed in R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) and reproducible code is available on the Open Science Framework (OSF) repository.[25]

Statistical analysis

Difference in serum-stimulated cell proliferation between exercise and control conditions was assessed with a linear mixed model. Change from baseline (delta) was the dependent variable, baseline values were entered as a covariate, condition was a fixed effect with two levels (exercise and control), and participants were a random factor with individual slopes.[26] Exercise-induced changes in serum markers and intracellular protein expression were assessed a using linear mixed model with timepoint as a fixed effect (pre and post) and participants as a random factor. Models were fit using the maximum likelihood method. Normality of model residuals were assessed via visual inspection of histograms and Q-Q plots. Positively skewed data were analysed with a generalised linear mixed model specifying a gamma distribution and log link function,[27] with goodness of fit compared between models using the Bayesian information criterion. When multiple comparisons were made in a 'family' of tests, we applied a Bonferroni correction to control the familywise error rate. Linear trend analysis was used to assess the dose-response effect of IL-6 on LoVo cell proliferation and γ-H2AX. Statistical significance was set at $p<0.05$. Analyses were performed in R. Data and code are available on OSF.[25]

RESULTS

Sixteen participants completed the exercise and control experiments (Table 1). Participant flow through the study is presented in Figure S1. All participants reported that they consumed the same meal 2-5 hours prior to laboratory visits and avoided physical activity and alcohol intake for ≥24 hours and caffeine intake for ≥12 hours prior.

Exercise bout

The median heart rate reserve during the aerobic intervals was 59.7% (IQR 57.6-61.8%), indicating high fidelity to the exercise protocol. The median RPE was 13.2 (IQR 12.7-13.8) and power output was 93.8 W (IQR 81.7-105 W). All participants completed all six aerobic intervals, apart from one participant who completed five intervals because of technical difficulties monitoring blood pressure, which is an indication for exercise termination.[28]

Acute exercise-conditioned serum reduces LoVo cell proliferation

We incubated LoVo cells for 48 hours with medium containing 10% human serum collected before and after the acute exercise bout and a non-exercise control experiment. Stimulating LoVo cells with post-exercise serum decreased cell proliferation compared with pre-exercise serum (-4.2%, 95% CI -6.8 to -1.5%; p=0.006; Figure 1A), whereas post-control serum led to an increase in cell proliferation compared to pre-control serum (5.4%, 95% CI 2.2 to 8.6%; p=0.003). After controlling for pre-values, exercise-conditioned serum reduced cell proliferation compared to control (-5.7%, 95% CI -8.8 to -2.6%; p=0.002; Figure 1B).

Acute exercise-conditioned serum reduces intracellular γ-H2AX expression

To explore potential mechanistic pathways by which exercise suppressed colon cancer cell proliferation, we quantified the expression of various candidate proteins in LoVo cells after incubation with exercise-conditioned serum for 20-60 min (Figure 2A). Exercise decreased levels of γ-H2AX compared with pre-exercise serum after 60 min of incubation (-24.6%; p=0.029; Figure 2B), indicating a reduction in DNA damage. In contrast, there was no evidence of an effect of exercise-conditioned serum on p-MEK1, p-ERK1, p-RSK90, p-ATF1, p-Akt, p-mTOR, p-CREB1, or p-NFκB (all p>0.05; Figure 2C-J).

Acute aerobic exercise increases serum IL-6

We then evaluated whether exercise modulated the serum concentration of seven cytokines to identify humoral factors that could be responsible for the exercise-induced suppression of cell proliferation and DNA damage. Serum IL-6 increased from pre- to post-exercise (24.6%, 95% CI 11.2 to 37.9%; p=0.002; Figure 3A), whereas there was no evidence of an effect of exercise on serum IL-8, TNF-α, osteonectin, or oncostatin M (all p>0.05; Figure 3B-E). IL-10 and irisin were undetectable in the serum samples.

IL-6 reduces LoVo cell proliferation and γ-H2AX expression in a dose-response manner

Given that acute exercise concomitantly increased serum IL-6 and reduced LoVo cell proliferation and intracellular levels of γ-H2AX, we proceeded to explore whether IL-6 could directly regulate LoVo cell proliferation and γ-H2AX. We stimulated LoVo cells with recombinant IL-6 (HumanKine human IL-6, Proteintech, UK) and demonstrated dose-dependent effects. Specifically, IL-6 doses of 10 pg/ml (-20.4%, 95% CI -36.8 to -4.0%; p=0.014) and 100 pg/ml (-32.1%, 95% CI -48.5 to -15.7%; p<0.001) reduced γ-H2AX expression compared to 1 pg/ml (Figure 4B). IL-6 doses of 0.1 pg/ml (-4.0%, 95% CI -7.9 to -0.1%; p=0.040), 1 pg/ml (-5.3%, 95% CI -9.2 to -1.4%; p=0.004), 10 pg/ml (-7.5%, 95% CI -11.4 to -3.6%; p<0.001), and 100 pg/ml (-5.8%, 95% CI -9.7 to -1.9%; p=0.002) also reduced LoVo cell proliferation compared with control (Figure 4C). Furthermore, there was evidence of a linear trend for cell proliferation (β=-5.3%, 95% CI -7.4 to -3.2%; p<0.001) and γ-H2AX expression (β=-22.7%, 95% CI -31.6 to -13.8%; p<0.001), demonstrating that as the IL-6 dose increased, LoVo cell proliferation and γ-H2AX levels decreased proportionately.

DISCUSSION

Our results show that acute aerobic exercise-conditioned serum reduced colon cancer cell proliferation *in vitro*. This was accompanied by decreased levels of intracellular γ-H2AX, indicating a reduction in DNA damage. Acute exercise also increased serum IL-6, and stimulating colon cancer cells with recombinant IL-6 reduced intracellular γ-H2AX expression and cell proliferation in a dose-dependent manner, mimicking the effect of exercise. Thus, our findings suggest that the inhibitory effects of exercise on colon cancer cell proliferation may be partly driven by IL-6-induced regulation of DNA damage and repair.

Epidemiological evidence suggests that regular physical activity reduces the relative risk of primary and recurrent colon cancer.[1,3,4] However, the molecular mechanisms of action are poorly understood. Current dogma suggests physical activity reduces colon cancer risk through regulation of systemic insulin and pro-inflammatory cytokines,[5,6] but these effects seem to be tightly regulated by adiposity rather than physical activity *per se*.[29–31] Here, we have demonstrated that stimulating colon cancer cells with serum obtained immediately after exercise reduces cell proliferation by ≈6%. This finding suggests that bioactive molecules released into the systemic circulation during exercise elicit biological effects on colon cancer cells to regulate cell proliferation. This proposed mechanism of action may partly explain the epidemiological associations between regular physical activity and reduced colon cancer risk.

Our findings align with studies using *ex vivo* colorectal tissue samples. For example, a 12-month randomised controlled trial reported that aerobic exercise reduced the proliferative capacity of colon crypt cells in men who exercised at least 250 min·wk^{-1}.[32] A non-randomised controlled trial in rectal cancer patients also showed that six weeks of preoperative exercise following neoadjuvant chemoradiotherapy led to greater tumour regression grading at the time of surgery,[33] highlighting the potential of exercise to alter tumour morphology *in vivo* as well as *in vitro/ex vivo*.

γ-H2AX is a sensitive biomarker for DNA double-strand breaks (DSBs) and is formed following phosphorylation on the 139th serine residue of the histone variant H2AX.[34] The oncogene-induced DNA damage model for cancer development[23] proposes that DNA DSBs drive the early stages of carcinogenesis. According to this model, aberrant cell proliferation prompted by activated oncogenes induces DNA replication stress.[23] This replication stress results in the collapse of DNA replication forks, leading to the formation of DNA DSBs. LoVo cells harbour a mutation to the *KRAS* oncogene, and activated *RAS* has previously been shown to induce DNA DSBs in NIH3T3 fibroblasts within a single cell cycle.[35] The sustained formation of DSBs contributes to genomic instability, which is a hallmark of cancer cells and increases the propensity of acquiring additional genetic mutations favouring cancer progression.[36] Accordingly, the expression of γ-H2AX is positively correlated with the malignant progression of human colorectal carcinoma[37,38] and with colon cancer cell line (HCT15) proliferation.[38]

We found that the exercise-induced reduction in colon cancer proliferation was accompanied by decreased levels of intracellular γ-H2AX, indicating a reduction in DNA damage. The phosphorylation status of major signalling molecules downstream of the *KRAS* oncogene were unaffected by exercise stimulation (Figure 2). In line with the oncogene-induced DNA damage model for cancer development,[23] this suggests that exercise may not have prevented the initial formation of DNA DSBs, although we cannot rule out that signalling pathways downstream of other oncogenes (e.g., *APC*) were not altered by exercise. Instead, given that the disappearance of γ-H2AX following DNA damage indicates repair of DSBs,[39] it is possible that exercise facilitated DNA DSB repair. The capacity of exercise to enhance DNA repair has been highlighted previously,[40] with potential mechanisms including increased free radical scavenger enzyme activity,[41] increased DSB repair protein content such as Ku70,[42] or attenuation of telomere attrition.[43] Cancer cell lines are known to rapidly acquire new genetic variants in culture,[44] and LoVo cells have the hypermutator phenotype microsatellite instability[21] (a form

of genomic instability caused by a defective DNA mismatch repair system). Therefore, the exercise-induced repair of DNA DSBs may have shifted the cancer cells towards a more genetically stable phenotype,[45] reducing the acquisition of further genetic mutations in culture and subsequently reducing cell proliferation.

Acute exercise increased serum IL-6, which is consistent with evidence showing that IL-6 is expressed and secreted by contracting skeletal muscle during exercise.[46] We also found that directly stimulating LoVo cells with recombinant IL-6 reduced γ-H2AX expression and cell proliferation in a dose-dependent manner, mimicking the effect of exercise. Taken together, the findings suggest that IL-6 signalling may have driven exercise-induced regulation of DNA damage. In support of this, direct IL-6 treatment has been shown to reduce γ-H2AX expression in oral squamous cell carcinoma cells[47] and promote DNA repair in CD133-positive cancer stem-like cells after irradiation.[48] IL-6 has also been shown to cause cell cycle arrest, reduce proliferation, and activate DNA repair enzymes following partial hepatectomy in mice.[49]

IL-6 is a pleiotropic cytokine and appears to play a dual role in cancer progression, which may depend on the duration of exposure. Lee and colleagues[50] reported that acute exposure to IL-6 (less than 28 passages) reduced prostate cancer cell growth, but long-term exposure (more than 42 passages) increased cancer cell growth and IL-6 mRNA expression. Thus, short-term IL-6 exposure may inhibit cancer growth through endocrine/paracrine signalling, whilst chronic exposure may lead to autocrine cell growth stimulation by inducing cells to acquire endogenous IL-6 production.[50] Acute exercise transiently increases circulating IL-6 and prolonged exercise training reduces resting IL-6 levels.[46] Evidence of the dual role of IL-6 indicates that these opposing systemic responses to acute and chronic exercise may both contribute to the inhibition of cancer progression.

Whilst other serum markers were unaltered by exercise in this study, it is unlikely that the growth-inhibitory effect of exercise was driven exclusively by IL-6. Exercise elicits widespread effects on multiple organ systems and leads to the secretion of thousands of metabolites, peptides and RNA species.[14] Thus, the preventive effect of acute exercise with respect to colon cancer is likely to result from a coordinated response of many diverse humoral factors and intracellular signalling pathways. Multi-omic profiling of exercise-conditioned serum and colon cancer cell lines following serum stimulation, combined with bioinformatics analytic tools, may help elucidate the complex molecular networks involved.

This study has several strengths. In contrast to previous research,[18] we incorporated a non-exercise control condition in a randomised crossover design to determine the effects of exercise-conditioned serum on cancer cell proliferation. The inclusion of a control experiment accounts for biological variation and potential regression to the mean effects,[19] thus providing a more accurate estimate of the true treatment effect. We also controlled for physical activity and dietary intake prior to the experimental sessions. Moreover, we prospectively registered the protocol and primary outcome and have made the data and code available on OSF,[25] allowing researchers to computationally reproduce our results.

A limitation of this study is that the two-dimensional cell culture model used does not fully reflect *in vivo* tumour morphology or microenvironment. Moreover, in contrast to the assay used in this study, bioactive molecules released during exercise *in vivo* must travel a potentially long distance in the systemic circulation to reach aberrant colonic epithelial cells. Ligands lacking a secretory signal sequence and those with a low molecular weight (and hence short residency time in serum) may be transported in small membranous extracellular vesicles (EVs).[14] Further research is warranted to investigate whether plasma EVs mediate inter-organ crosstalk during exercise by transporting bioactive molecules to distant aberrant cells. Furthermore, future research could use a colonic adenoma model to explore the effects of exercise on the growth of premalignant colorectal lesions, which is perhaps more relevant to cancer prevention than cancer cell-based assays. A further study limitation is that most of the secondary outcomes were not prespecified, although all deviations from the pre-registered protocol have been documented and justified (Table S1). Deviations included the measurement of some serum markers and the assessment of intracellular signalling pathways following stimulation with exercise serum.

To conclude, aerobic exercise-conditioned serum reduced colon cancer cell proliferation *in vitro*, which appeared to be driven by IL-6-induced regulation of DNA damage and repair. This mechanism of action may at least partly underlie epidemiological associations linking regular physical activity with reduced colon cancer risk.

DISCLOSUERS

Author Contributions. STO: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation; Writing - Original Draft, Visualization. **ARJ**: Methodology, Investigation, Resources, Writing – Review & Editing. **AO**: Methodology, Validation, Investigation, Resources, Writing – Review & Editing, Visualisation. **OK**: Investigation, Resources, Writing – Review & Editing. **KMH**: Methodology, Investigation, Writing – Review & Editing. **TE**: Investigation, Writing – Review & Editing. **ST**: Methodology, Resources, Writing – Review & Editing, Supervision. **JMS**: Conceptualization, Methodology, Writing – Review & Editing, Supervision. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

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Conflict of Interest. The authors have no conflicts of interest to declare.

Data Availability Statement. All data and code are available on the Open Science Framework project page (https://osf.io/trw78/). Further information is available from the corresponding author upon request.

Ethics Statement. The study was approved by the Faculty of Health and Life Sciences Ethics Committee at Northumbria University (ref: #17596) and informed consent was obtained from all participants before taking part in this research. The study was prospectively registered at ClinicalTrials.gov (ID: NCT04057274).

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Figure captions

Figure 1. Effect of acute aerobic exercise-conditioned serum on colon cancer cell growth. (**A**) LoVo cell viability following 48 hours of incubation with medium containing 10% human serum collected before and after an acute bout of aerobic exercise and a non-exercise control experiment. (**B**) Change in serum-stimulated cell viability in the exercise and control experiments (mean ± 95% confidence interval). $^{**}p<0.01$.

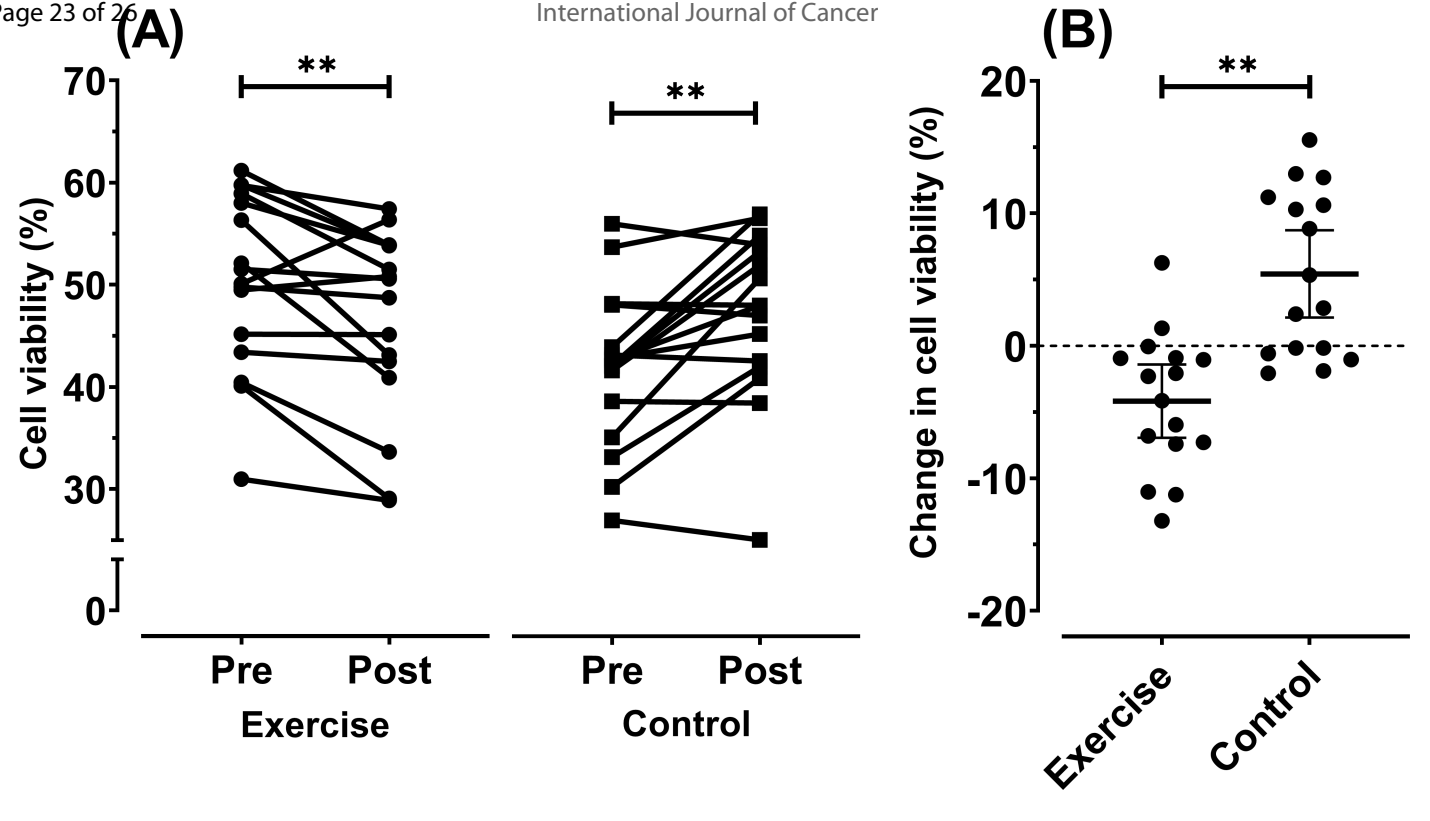
Figure 2. Effect of acute aerobic exercise-conditioned serum on protein expression in LoVo cells. (**A**) Representative immunoblots of p-MTOR, p-NFκB, p-MEK1, p-ERK1, p-CREB1, p-ATF1, γ-H2AX, p-RSK90, p-Akt, and α-tubulin in LoVo cells following 20-60 minutes of incubation with medium containing 10% human serum collected before and after an acute bout of aerobic exercise. (**B-J**) Quantification of protein expression in LoVo cells (mean ± SEM). Bonferroni corrections were applied to adjust for multiple comparisons across the two timepoints (20 and 60 min). FBS = fetal bovine serum; POST = post-exercise; PRE = pre-exercise; SF = serum free; $^{*}p<0.05$.

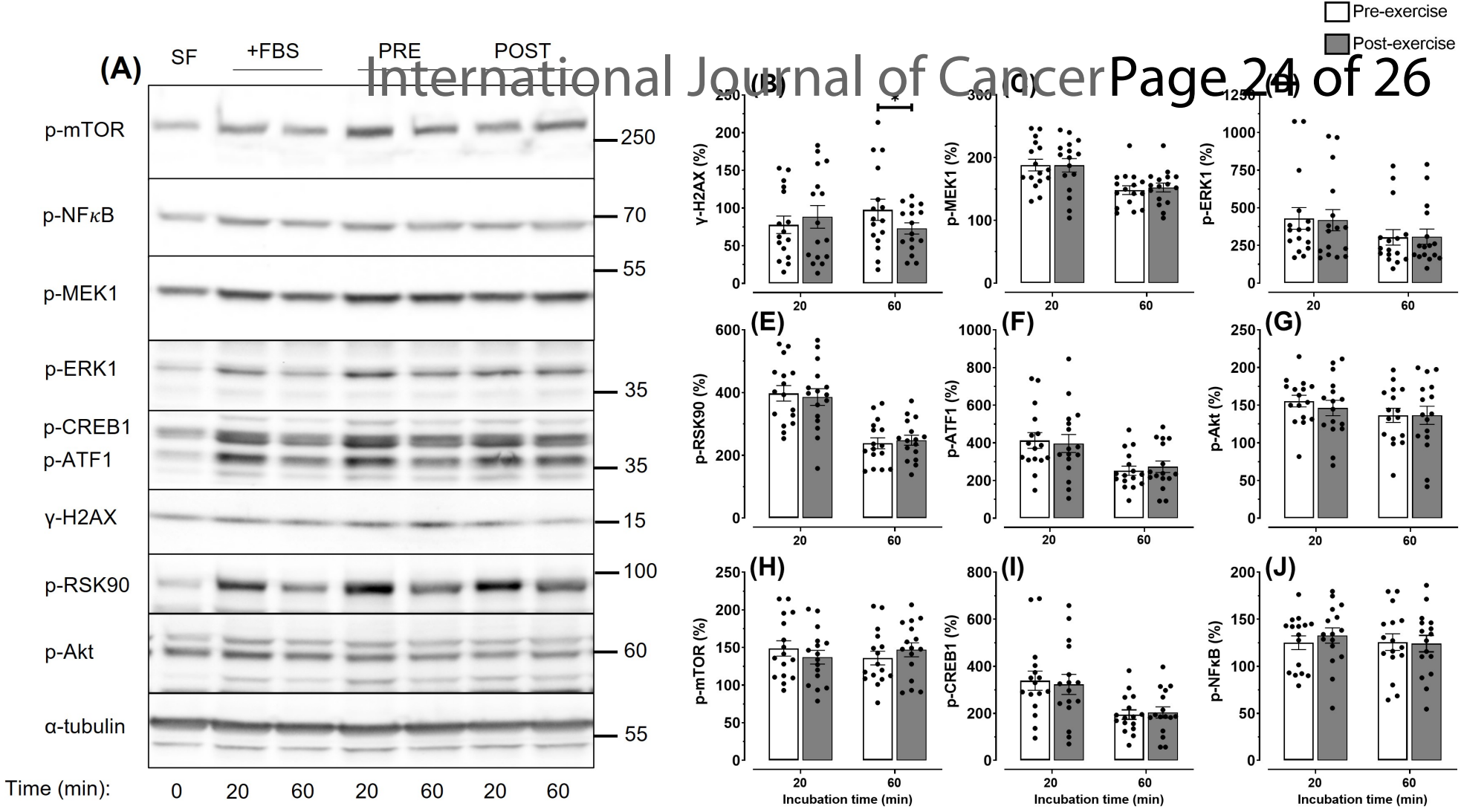
Figure 3. Effect of acute aerobic exercise on serum cytokine concentration. (**A-E**) Concentrations of interleukin 6 (IL-6), IL-8, tumour necrosis factor-alpha (TNF-α), osteonectin and oncostatin M in pre- and post-exercise serum (mean ± SEM). $^{**}p<0.01$.

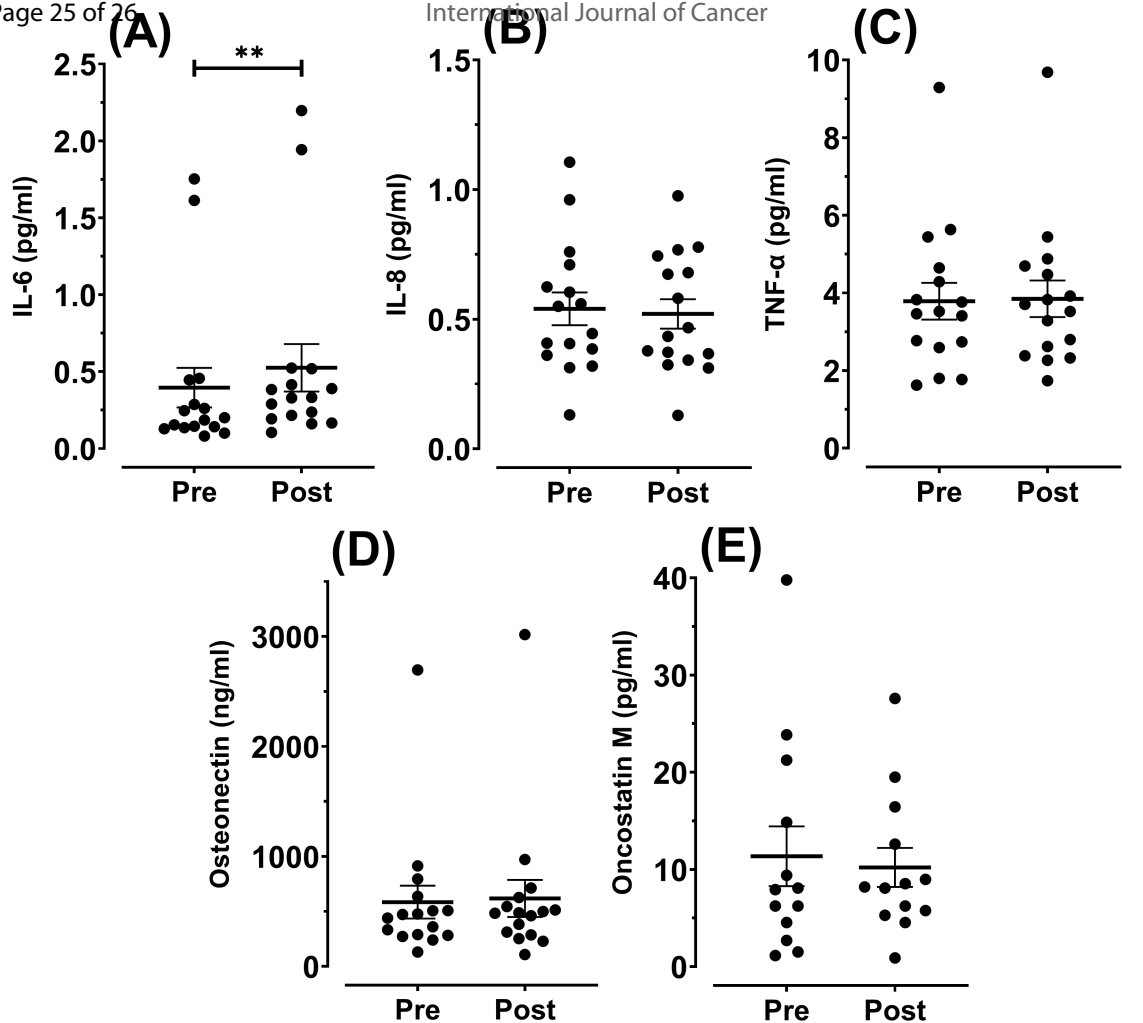
Figure 4. Effect of interleukin-6 (IL-6) on LoVo cell proliferation and intracellular γ-H2AX expression. (**A**) Representative immunoblots of γ-H2AX expression in LoVo cells after stimulation with actin and 0, 10 and 100 pg/ml of recombinant IL-6 for 45 minutes. (**B**) Quantification of γ-H2AX expression in LoVo cells showing a dose-response effect of IL-6 (mean ± SEM of five repeat experiments). (**C**) Quantification of LoVo cell proliferation 48 hours after direct stimulation with recombinant IL-6, showing a dose-response effect (mean ± SEM of eight repeat experiments for control and four repeat experiments for IL-6 doses). Bonferroni corrections were applied to adjust for multiple comparisons. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

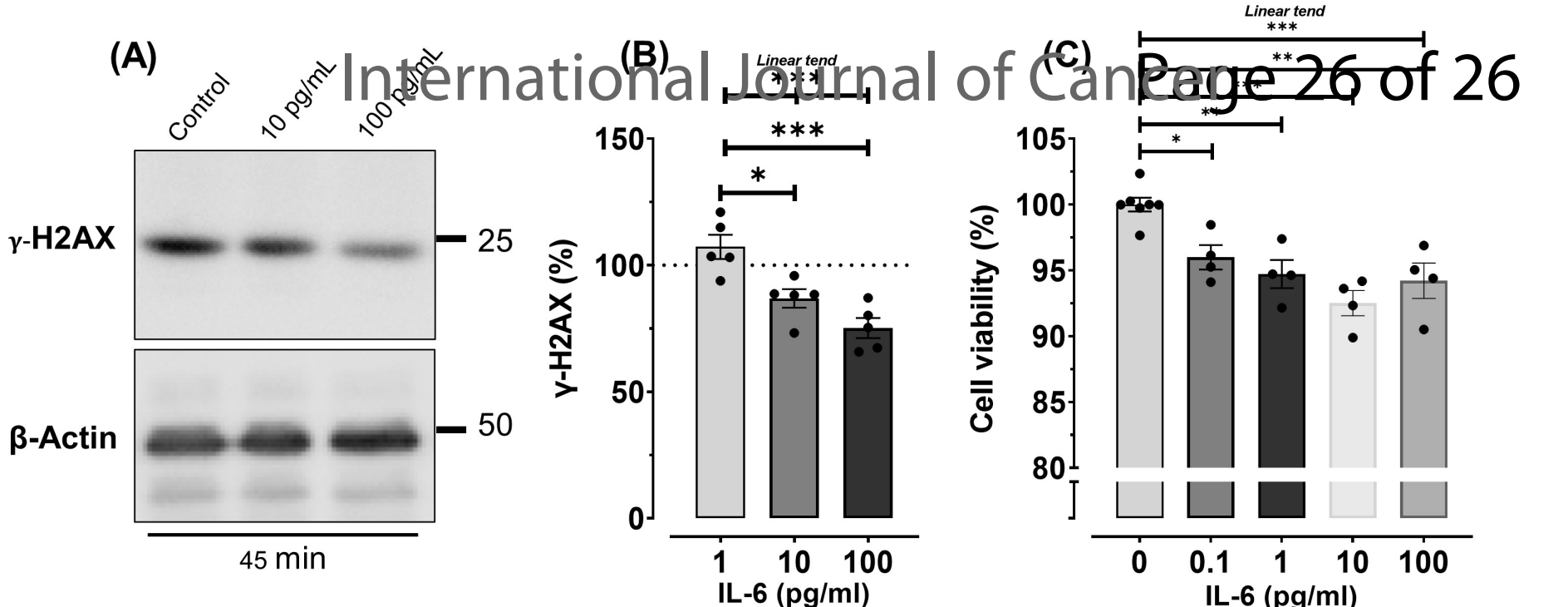
Table 1. Participant characteristics (n = 16)

Characteristic	Mean ± SD or number (%)
Age (years)	60.0 ± 8.0
Body mass (kg)	93.2 ± 7.7
Height (cm)	177 ± 6.6
BMI (kg/m^2)	29.9 ± 2.4
Waist circumference (cm)	101 ± 6.3
Hip circumference (cm)	109 ± 10.6
Waist to hip ratio	0.93 ± 0.07
Systolic blood pressure (mmHg)	132 ± 11.7
Diastolic blood pressure (mmHg)	80.2 ± 8.0
Peak flow (l/min)	498 ± 100
Smoking status	
Current smoker	0 (0)
Previous smoker	3 (13)
Ethnicity	
White British	16 (100)
Marital status	
Married	11 (69)
Single	3 (19)
Cohabiting	1 (6)
Divorced	1 (6)
Highest education	
High school	4 (25)
College	5 (31)
Undergraduate	0 (0)
Postgraduate	5 (31)
Doctorate	2 (13)
Employment status	
Employed full-time	4 (25)
Employed part-time	1 (6)
Self-employed	4 (25)
Retired	7 (44)


(A)
**
**
70
60
50
40
30
0
Cell viability (%)
Pre
Post
Exercise
Pre
Post
Control
(B)
**
20
10
0
-10
-20
Change in cell viability (%)
Exercise
Control


(A)
SF
+FBS
PRE
POST
p-mTOR
p-NFκB
p-MEK1
p-ERK1
p-CREB1
p-ATF1
γ-H2AX
p-RSK90
p-Akt
α-tubulin
250
70
55
35
35
15
100
60
55
Time (min):
0
20
60
20
60
20
60
Pre-exercise
Post-exercise
(B)
γ-H2AX (%)
(C)
p-MEK1 (%)
(D)
p-ERK1 (%)
(E)
p-RSK90 (%)
(F)
p-ATF1 (%)
(G)
p-Akt (%)
(H)
p-mTOR (%)
(I)
p-CREB1 (%)
(J)
p-NFκB (%)
Incubation time (min)
Incubation time (min)
Incubation time (min)


(A)
**
IL-6 (pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post
(B)
IL-8 (pg/ml)
1.5
1.0
0.5
0.0
Pre
Post
(C)
TNF-α (pg/ml)
10
8
6
4
2
0
Pre
Post
(D)
Osteonectin (ng/ml)
3000
2000
1000
0
Pre
Post
(E)
Oncostatin M (pg/ml)
40
30
20
10
0
Pre
Post


(A)
Control
10 pg/mL
100 pg/mL
γ-H2AX
25
β-Actin
50
45 min
(B)
Linear tend
***
***
*
150
100
50
0
γ-H2AX (%)
1
10
100
IL-6 (pg/ml)
(C)
Linear tend
***
**
**
*
105
100
95
90
85
80
Cell viability (%)
0
0.1
1
10
100
IL-6 (pg/ml)