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The exercise-conditioned human serum and skeletal muscle cells secretome induce apoptosis in breast cancer cells

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Abstract

Background: Regular exercise training provides significant health benefits among cancer survivors and is associated with lower breast cancer mortality and reduced risk of recurrence. Both exercise-induced factors secreted into circulation (exerkines) and bioactive molecules contained in skeletal muscle secretome have been proposed to affect the tumor microenvironment and mediate some of the anti-carcinogenic effects of exercise. This study utilized exercise-conditioned human serum obtained from breast cancer patients during chemotherapy and skeletal myotubes' secretome after mechanical loading to investigate their effects on breast cancer cells *in vitro*.

Methods: Breast cancer patients participated in a 12-week exercise training program during their chemotherapy, and blood serum was collected immediately before and after an exercise session in the 2nd and 12th weeks of training. Skeletal myoblasts were differentiated into myotubes and subjected to mechanical stretching to collect their secretome (stretch medium (SM)). Hormone-sensitive Michigan Cancer Foundation-7 (MCF-7) and triple-negative M.D. Anderson-Metastatic Breast-231 (MDA-MB-231) breast cancer cells were treated with either human serum or with the skeletal myotubes' secretome to examine their metabolic activity, migration, cytotoxicity levels and apoptosis regulation.

Results: The exercise-conditioned serum obtained from breast cancer patients who were subjected to the 12-week training during chemotherapy resulted in reduced metabolic activity ($p < 0.001$) and increased lactate dehydrogenase activity (cytotoxicity) ($p < 0.001$) in both MCF-7 and MDA-MB-231 breast cancer cells when compared with the control condition. Moreover, incubation of breast cancer cells with the post-exercise serum induced apoptosis in MCF-7 and MDA-MB-231 cells, as indicated by increase in DNA damage and the percentage of necrotic cells ($p < 0.05$) when compared to pre-exercise condition. Similarly, a significant decrease ($p < 0.001$) was observed in the metabolic activity of MCF-7 cells treated with the SM, along with increased cytotoxicity ($p < 0.05$), compared to the cells cultured with the regular growth media. Comparable though not as profound effects were observed in MDA-MB-231 cells when treated with the SM secretome. Furthermore, the expression of apoptosis-inducing Caspase-7 ($p < 0.001$) and Caspase-8 ($p < 0.01$) proteins was increased, whereas cell survival-regulating factors interleukin-8 (IL-8) ($p < 0.001$), superoxide dismutase-2 ($p < 0.05$), Fas cell surface death receptor ($p < 0.05$), and vascular endothelial growth factor ($p < 0.01$) were downregulated in the SM-treated MCF-7 cells. In addition, the migrating behavior of MCF-7 cells was diminished, and higher levels of DNA damage were observed in cells treated with either SM or non-stretch media.

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Conclusion: Both exercise-conditioned serum of breast cancer patients and skeletal myotubes secretome after mechanical loading can reduce the metabolic activity, promote cell toxicity and DNA damage, modulate the protein expression of crucial cell survival-regulating factors, and lead to apoptosis in breast cancer cells. These findings suggest that even after cancer diagnosis, exercise may exert beneficial effects additive to chemotherapy against breast cancer prognosis.

Keywords: Anti-oncogenic; Exerkines; Exercise oncology; Muscle-derived factors; Myokines

1. Introduction

Solid evidence from epidemiological studies supports engagement in regular physical activity as conferring a substantial contribution to the primary and secondary prevention of several types of malignancies, such as breast, colon, lung, stomach, kidney, endometrial, bladder, and esophageal cancer.[1–3] Specifically regarding breast cancer, exercise lowers the risk of developing pre- and post-menopausal cancer by up to 21% and 16%, respectively.[4–6] Moreover, even after diagnosis, breast cancer survivors who meet physical activity guidelines exhibit not only fewer treatment-related side effects[7–11] but also a decreased risk of breast cancer recurrence by up to 35%[12] and of cancer-specific mortality by 38%.[13]

These encouraging epidemiological data could be partly attributed to the ability of exercise to modify key hallmarks of cancer, such as cell proliferation, evasion of growth suppressors, apoptosis, immune response, and persistent inflammation.[14] Thus, at the cellular and molecular level, the exercise-induced factors secreted into circulation (recently termed exerkines[15,16]) and in particular the skeletal muscle secretome, which is abundant in various bioactive molecules, are potentially key mediators of the beneficial effects of exercise against cancer.[17]

After treating various cancer cells with post-exercise human serum *in vitro,* a decrease of the proliferation rate and induction of apoptosis in those cells have been reported.[18–21] In breast cancer cells, it has been observed that incubation of the triple-negative breast cancer cell line M.D. Anderson-Metastatic Breast-231 (MDA-MB-231) with the human serum derived from healthy sedentary participants after a single bout of exercise resulted in reduced proliferative capacity and altered activity of the breast cancer-related Hippo and Wnt/β-catenin signaling pathways.[22] Similar results in terms of the *in vitro* growth and invasiveness of breast cancer cells have been reported when the cells were treated with exercise-conditioned serum derived from breast cancer survivors[23] or breast cancer patients during chemotherapy.[24] Additionally, cell growth, migratory capacity, and invasiveness of different types of breast cancer cells *in vitro* were inhibited upon treatment with a pulsed electromagnetic field-stimulated myotube secretome.[25]

In the present study, we investigated the potential biological effects of 2 different "vehicles" of exercise-delivered factors on breast cancer cells *in vitro*: (a) the exerkine-enriched post-exercise serum collected from breast cancer patients undergoing chemotherapy and (b) the secretome of mechanically loaded myotubes, which provides an efficient *in vitro* model for mimicking exercise-induced skeletal muscle loading and secretion *in vivo*.[26] Both models simulate *in vitro* the potential interactions between breast cancer cells and the exercise-induced factors, secreted from skeletal muscle cells or other cells and tissues. By utilizing these models, we aimed to shed light on the cellular and molecular processes through which exercise exerts its anti-carcinogenic effects on breast cancer, using different cancer cell lines that represent either the hormone-sensitive or triple-negative breast cancer clinical phenotype.

To the best of our knowledge, the post-exercise-collected serum of breast cancer patients undergoing chemotherapy has been utilized only once to examine its impact on breast cancer cells *in vitro*.[24] This is the first time that the impact of skeletal myotube secretome after mechanical loading *in vitro* is studied in terms of the survival, migration, and differential expression of apoptosis-regulating proteins on breast cancer cells.

2. Materials and methods

2.1. Ethical approval and informed consent

All experimental procedures of the present study were approved by the Ethics Committee of the Medical School of the National and Kapodistrian University of Athens (Approval No. 098/18.03.19) and sample collection and handling were carried out in accordance with all privacy law regulations. The female breast cancer patients, who voluntarily participated in the study, signed a consent form after being thoroughly informed regarding the aim and procedures of the study.

2.2. Cell cultures

The breast cancer cell lines Michigan Cancer Foundation-7 (MCF-7) and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC) and grown in high-glucose Dulbecco's modified minimum essential medium (DMEM) (#L0104; Biowest, Nuaillé, France) or Roswell Park Memorial Institute medium (RPMI 1640) (#L0498; Biowest), respectively, supplemented with 10% fetal bovine serum (FBS) (#S181H; Biowest) and 1% penicillin/streptomycin (#L0022; Biowest). The MCF-7 cell line retains several characteristics of hormone-sensitive breast cancers as it is both estrogen receptor (ER) and progesterone receptor (PR) positive, while the MDA-MB-231 cell line corresponds to triple-negative breast cancer (ER−, PR−, HER2−). C_2C_{12} myoblasts were also obtained from the ATCC and grown in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The differentiation of C_2C_{12} myoblasts to myotubes was attained by switching the 10%

FBS to 2% horse serum (#S0910; Biowest) when cells reached 80% confluency. All cell lines were cultured in a conventional incubator at 37˚C, in a humidified atmosphere of 5% CO_2.

2.3. Exercise-training and physical performance assessments

Ten female breast cancer patients (age = 37.86 ± 1.73 years, body mass index: 23.70 ± 1.29 kg/m^2 (Mean and standard error of the mean ± SE)) participated in a 12-week supervised exercise training during their chemotherapy. The individualized exercise training program consisted of both resistance (elastic bands: 30 min) and aerobic training (treadmill: 25 min at 40%−60% heart rate reserve) and was carried out in person 3 times per week. Patients' exercise adherence was monitored, and the effects of exercise on the cardiorespiratory health of patients during chemotherapy was assessed every 4 weeks with the established Rockport 1-mile walk test;[27] muscle strength and functionality was evaluated with the 1-min sit-to-stand test[28] and 1-min half sit-up test.[29]

2.4. Exercise-conditioned (exerkines-containing) human serum

In the 2nd week of training, 14−18 days after the last chemotherapy dose and 48 h after the last exercise session, breast cancer patients performed a single bout of exercise, and blood samples were collected immediately before (PRE-EX) and after completion (POST-EX) (Fig. 1). Blood samples were allowed to clot at room temperature for 30 min, and serum was isolated following blood centrifugation at 1000 *g* and 4˚C for 10 min. The sampling procedure was repeated in the 12th week of training (trained PRE-EX; trained POST-EX) (Fig. 1). The isolated serum was utilized to treat breast cancer cells (MCF-7 and MDA-MB-231), which were cultured in their regular growth media supplemented with 10% human serum instead of 10% FBS.

2.5. Skeletal myotube (myokines-containing) secretome

C_2C_{12} myoblasts were grown in specialized culture plates (which had elastic membranes as culture surfaces), differentiated to myotubes, and then underwent cyclic stretching using the Flexcell FX-4000^{TM} Tension System (Flexcell International, Hillsborough, NC, USA). An optimized mechanical stretching protocol (anabolic and anti-apoptotic for myotubes) was utilized (i.e., duration = 12 h, frequency = 0.25 Hz, elongation = 2%), as we have previously characterized,[30] and 12 h after the completion of mechanical loading, the conditioned media were collected and filtered through a 0.20 μm filter to serve as the stretch-media (SM). The conditioned media of unloaded myoblasts were also collected and filtered, serving as non-stretch media (NSM). Breast cancer cells were treated with the myotube secretome, either the SM or the NSM, in a

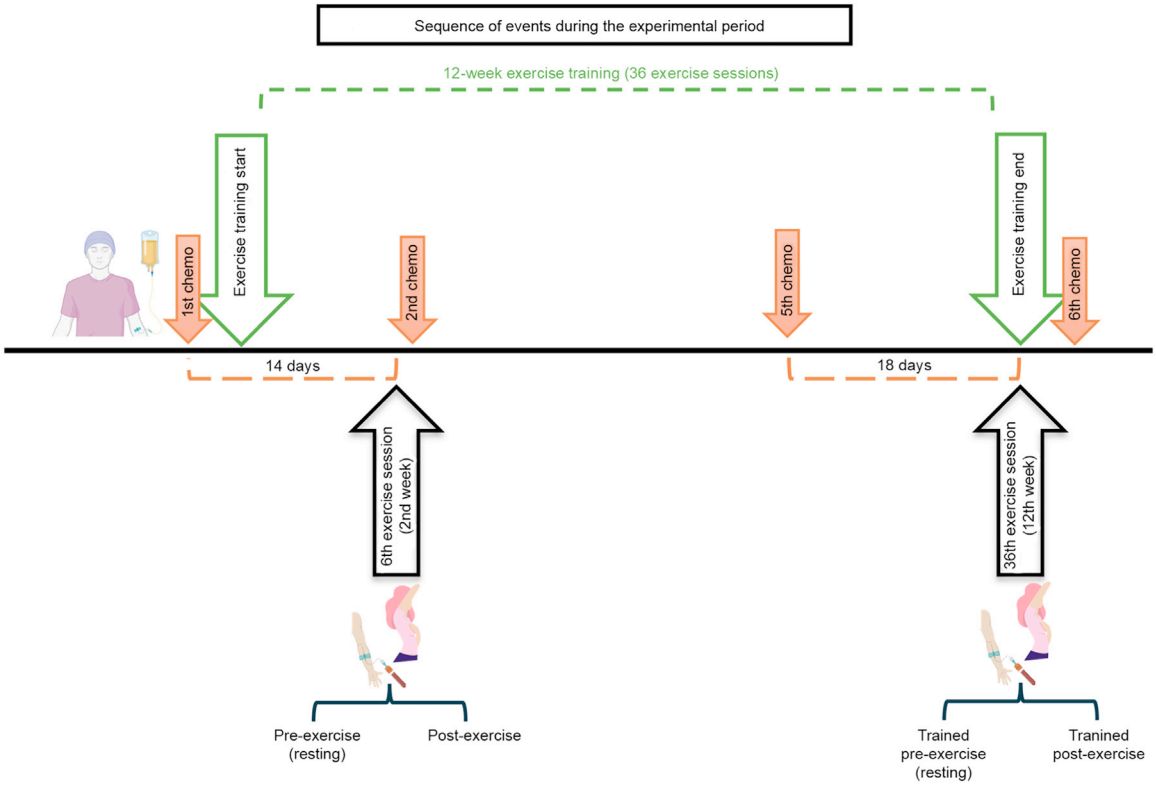

Fig. 1. Breast cancer patients participated in a 12-week supervised exercise training program during chemotherapy. Exercise training started directly after the administration of the 1st chemotherapy dose and was conducted 3 times per week (36 exercise sessions in total). In the 2nd and 12th week of training, patients performed a single bout of exercise (i.e., 2nd week: 6th exercise session at 40% heart rate reserve and 12th week: 36th exercise session at 60% heart rate reserve) and blood samples were collected before and immediately after the completion of the exercise bout. The 6th and 36th exercise sessions were conducted at least 14 days after the last chemotherapy dose in order to limit any potential interference of chemotherapeutic drugs with exercise effects.

1:1 ratio with regular culture media, supplemented with 10% FBS and 1% Penicillin/Streptomycin.

2.6. Metabolic activity

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) colorimetric assay was utilized to determine the metabolic activity of breast cancer cells as an index of their viability status. Based on the assay's principle, the succinate dehydrogenases of active mitochondria receive the yellow tetrazolium salt (MTT) and form purple formazan crystals. Specifically, MCF-7 and MDA-MB-231 breast cancer cells were seeded in 24-well plates (125.000 cells/well), and after 18−20 h they were pre-conditioned by a 5-h serum starvation (0.5% FBS in regular growth media) and then incubated with the myotube secretome (SM or NSM) or the exercise-conditioned serum for 24 h. After the treatment, the conditioning media were removed and cancer cells were incubated with MTT for 4 h at 37˚C at the concentration of 0.5 mg/mL (#A2231; ITW Reagents S.R.L., Monza, Italy). Then media were removed and 4% (v/v) HCl (1M) in isopropanol was added to dissolve the formazan crystals. Color formation in the supernatant was measured at 450 nm using the VersaMax Tunable Microplate Reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA), and calculations were performed using SoftMax Pro software (Molecular Devices).

2.7. Cell toxicity

MCF-7 and MDA-MB-231 breast cancer cells were seeded in 24-well plates (125.000 cells/well) and cultured for 18−20 h. Then, after a 5-h serum starvation (0.5% FBS in regular growth media), cancer cells were cultured with either the myotube secretome or exercise-conditioned human serum for 24 h or 8 h, respectively. To detect lactate dehydrogenase (LDH) activity, as an index of cell toxicity of breast cancer cells after treatment, a commercially available LDH cytotoxicity assay kit was used (MAK066; Sigma-Aldrich, Burlington, MA, USA). The principle of this assay is based on the release and detection of LDH in the extracellular environment after cell membrane damage. During the assay, the released LDH converts the oxidized form of nicotinamide adenine dinucleotide (NAD^+) to its reduced form (NADH), which is specifically detected by colorimetric assay using a NADH standard curve. Again, absorbance was measured at 450 nm, using the VersaMax Tunable Microplate Reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

2.8. DNA damage

A single-cell gel electrophoresis comet assay was performed under alkaline conditions to examine potential DNA damage[31] in breast cancer cells after their treatment with the myotube secretome or exercise-conditioned serum. Both MCF-7 and MDA-MB-231 breast cancer cell lines were seeded in 24-well plates (70.000 cells/well) and pre-conditioned with a 5-h serum starvation before their 18-h treatment. Afterwards, cancer cells were harvested and resuspended in 1% Low Melting Agarose in phosphate-buffered saline (PBS) at 37˚C to be spread in pre-treated comet assay slides. Cell membranes were lysed at 4°C for 2 h, and then slides were incubated in electrophoresis buffer for 40 min. A horizontal electrophoresis was conducted for 30 min at 1 V/cm, and slides with microgels were subsequently neutralized, washed with distilled H_2O, and left to dry overnight at room temperature. Slides were stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific, Waltham, MA, USA) and visualized under a fluorescence microscope (BioTek Cytation5 Cell Imaging System; Agilent Technologies, Santa Clara, CA, USA). Images were processed with the Fiji ImageJ software (v2.14.0; Fiji contributors, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), and the "tail moment" parameter was quantified using the OpenComet plug-in (National University of Singapore, Singapore).[32] For each condition, at least 150 cells were evaluated, and each experiment was performed 3 times in triplicate.

2.9. Apoptosis

MCF-7 and MDA-MB-231 breast cancer cells were seeded in 24-well plates (80.000 cells/well) and cultured for 18−20 h. Cells were then starved (0.5% FBS in regular growth media) for 5 h and subsequently treated with the myotube secretome (SM or NSM) or exercise-conditioned serum (trained PRE-EX; trained POST-EX) for 24 h or 18 h, respectively. A specialized cell imaging kit (#R37601; Thermo Fisher Scientific) was utilized to distinguish live and dead cells (Live/Dead Assay) using a fluorescence microscope (BioTek Cytation-5 Cell Imaging System; Agilent Technologies). Calcein AM, a green cell membrane permeant dye, was used as a live-cell indicator, while BOBO-3 iodide, a cell-impermeant, red color dye that enters only damaged cell membranes, was used to stain the dead cells. Cells were imaged at emission wavelengths of 488 nm (green fluorescent protein, GFP) for live cells and of 570 nm (Texas Red) for dead cells. Quantification analysis included cell counts of calcein AM- and BOBO-3 iodide-positive cells in Fiji ImageJ software (v2.14.0; Fiji contributors).

2.10. Protein immunodetection

Protein expression in MCF-7 cells, after their treatment with the myotube secretome, was evaluated with a fluorescent confocal microscope (Leica SP8; Leica Microsystems, Wetzlar, Germany). MCF-7 cells were seeded in 8-well chambered culture slides (5.000 cells/well), subjected to starvation preconditioning for 5 h, and then treated with the myotube secretome for 48 h. Cells were then washed with PBS and fixed with formalin for 10 min. To allow the cell membrane to become permeable, cells were incubated for 10 min with 0.1% Triton-X in PBS, followed by 3 washes with PBS. Subsequently, a blocking solution (#50062Z; Thermo Fischer Scientific) was utilized for 2 h to prevent non-specific binding, and then cells were incubated with primary antibodies overnight at 4˚C for the immunodetection of Caspase 8 (#9746 CS; 1:300), Caspase 7 (#9492 CS; 1:300), Fas cell surface death receptor

(Fas) (#4233 CS; 1:300), vascular endothelial growth factor (VEGF) (#SC7269; 1:100), interleukin (IL)-8 (#SC 376750; 1:300), and superoxide dismutase 2 (SOD2) (#ab13533; 1:300). Afterwards, cell washes were repeated, and secondary antibodies were used for 1 h at room temperature in the dark (anti-rabbit #ab150079; 1:500, anti-goat #ab150129; 1:400, anti-mouse #ab150113; 1:500). Nuclear counterstain with 4',6-diamidino-2-phenylindole (DAPI) (#62248; 1:500) was used before imaging. Quantification of protein immunodetection was performed by measuring fluorescent intensity, which was normalized to the total number of cells per image using the Fiji ImageJ software (v2.14.0; Fiji contributors).

2.11. Random cell migration

To examine the potential effect of the myotube secretome on the migrating behavior of MCF-7 cells, they were seeded in 12-well plates, preconditioned with starvation (0.5% FBS in regular growth media) for 5 h, and then treated with the secretome derived from either stretched or non-stretched myotubes. Automated phase contrast image-acquisition was conducted every 1 h over a 24-h treatment period with an Incucyte® Live-Cell Analysis System (Sartorius AG, Göttingen, Germany). Quantitative assessment of the migrating potential of MCF-7 cells cultured under the different conditions was evaluated with the CellTraxx software (v4.6; SINTEF Industry and Oslo University Hospital, Oslo, Norway).[33] The CellTraxx program was utilized and executed through its interactive interface (tuning tool) provided within the respective macro plugin "celltraxx_ImageJ_macro.ijm", using Fiji ImageJ software (v2.14.0; Fiji contributors).

Briefly, at least 4 central sub-confluent culture regions per technical replicate were chosen upon image acquisition for an unbiased analysis of the MCF-7 migrating behavior. Single-frame TIFF-image sequences were formed for each analyzed culture region to further construct distinct .avi timelapse files in Fiji ImageJ software (v2.14.0; Fiji contributors), which were then executed by CellTraxx for a simultaneous multimodal image processing. The CellTraxx setting for performing interactive tuning was activated, while "wound healing mode", "flat-field correction", and "image shift correction" were not used. Pixel size was set to 1.240 μm, corresponding to Incucyte's 10x objective, which was utilized for image acquisition. Gaussian filter radius for enabling cell segmentation was set at 20.0 μm. Settings for geometrical properties of MCF-7 cells were optimized for "smallest cell diameter" = 12.0 μm, "largest cell diameter" = 40.0 μm, while the "cutting cell diameter" used for potential de-clumping of cell clusters was adjusted to 15.0 μm. "Time between images" was set at 60 min since individual frames represented per/h acquisitions. For the optimal and most reliable assessment of cell tracking, "highest cell velocity" was set at 2.0 μm/min to avoid false and/or unrealistic tracks upon testing different versions of processing as, for many, technical validity was observed. "Shortest cell track" was set at 18 frames (18 h), aiming to minimize the chance for falsely identified frame-to-frame matches.

The output results included visual interpretations of the identified and matched cells and valid tracks, as well as descriptive statistics on migrating properties of the valid tracks per studied culture region. Migrating velocities and accumulated migration distances were calculated automatically by CellTraxx for the distinct matched cells per frame and cumulatively for the distinct identified valid tracks of each culture region analyzed, producing an overall summary of velocity and migrating distance for all distinct regions.

2.12. Statistical analysis

Statistical analysis was conducted using the Graphpad Prism statistical package (Version 8.0.1; GraphPad Software, San Diego, CA, USA). Potential differences in physical performance of breast cancer patients over the 12-week period of exercise were assessed using the non-parametric Friedman test for repeated measures. Moreover, to examine the effect of the exercise-conditioned human serum on the behavior of breast cancer cells, the non-parametric Friedman test for repeated measures and multiple groups comparisons as well as the non-parametric Wilcoxon signed-rank test for comparing only 2 timepoints were used. The non-parametric Kruskal-Wallis test was utilized to assess potential differences in all examined parameters between the control condition and the myotube secretome-treated conditions (NSM and SM). Mean ± SE were used for descriptive statistics, and statistical significance was set at $p < 0.05$.

3. Results

3.1. The effect of exercise training on physical performance of breast cancer patients

Breast cancer patients' adherence to the exercise training program ranged from 94%−100%. The 12-week exercise training program during chemotherapy maintained, at least, patients' cardiorespiratory endurance, as the estimated maximal oxygen consumption (VO_{2max}) presented a trend to increase by 6.6% ± 4.7%, 7.0% ± 5.4%, and 8.6% ± 6.5% at the 4th, 8th, and 12th week, respectively, though without reaching statistical significance ($p > 0.05$; Supplementary Fig. 1A). The 1-min sit-to-stand performance was gradually improved by 28.7% ± 7.5% ($p < 0.01$; Supplementary Fig. 1B) in the 8th week of training and by 35.6% ± 9.4% ($p < 0.001$; Supplementary Fig. 1B) in the 12th week. Similarly, the number of repetitions in the 1-min half sit-up test was raised by 34.30% ± 8.8% ($p < 0.01$; Supplementary Fig. 1C) and 46.50% ± 10.3% ($p < 0.001$; Supplementary Fig. 1C) in the 8th and 12th week of training, respectively.

3.2. Treatment of breast cancer cells with exercise-conditioned serum

3.2.1. Post-exercise serum of breast cancer patients suppressed metabolic activity and increased cell toxicity in breast cancer cells in vitro

After 24-h incubation with the serum of breast cancer patients under chemotherapy, the metabolic activity of MCF-7

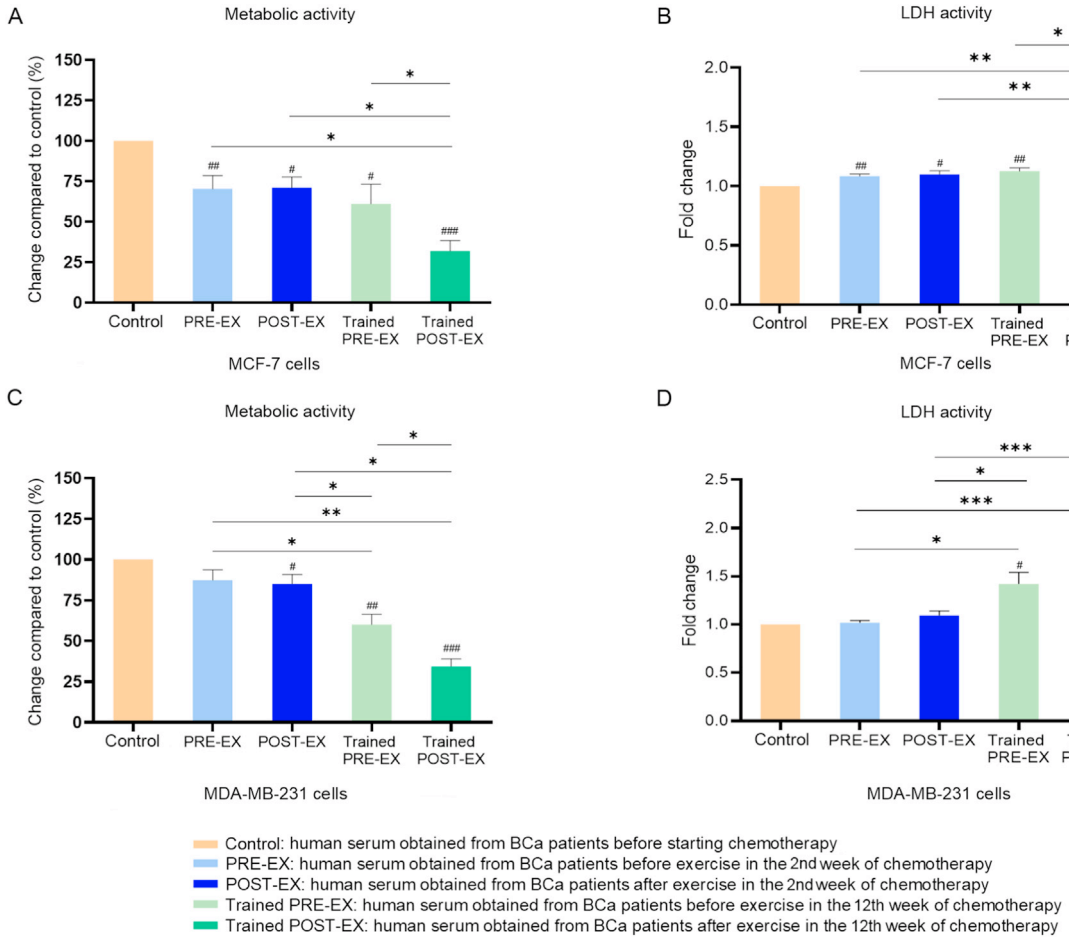

Fig. 2. Breast cancer cells were treated with the breast cancer patients' serum collected before chemotherapy (Control) and in various conditions during chemotherapy, at a 10% concentration. (A) Percentage changes in metabolic activity (MTT assay) of MCF-7 cells after the 24-h treatment. (B) Fold changes of lactate dehydrogenase (LDH) activity in the culture media of MCF-7 cells after the 8-h treatment. (C) Percentage changes in MTT assay of MDA-MB-231 cells after the 24-h treatment. (D) Fold changes of LDH activity in the culture media of MDA-MB-231 cells after the 8-h treatment. Results are presented as mean ± standard error; $n = 9$ biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences between conditions; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, significantly different compared to control. LDH = lactate dehydrogenase; MCF-7 = Michigan Cancer Foundation-7; MDA-MB-231 = M.D. Anderson-Metastatic Breast-231; BCa = breast cancer; POST-EX = human serum obtained from BCa patients after exercise in the 2nd week of chemotherapy; PRE-EX = human serum obtained from BCa patients before exercise in the 2nd week of chemotherapy; Trained POST-EX = human serum obtained from BCa patients after exercise in the 12th week of chemotherapy; Trained PRE-EX = human serum obtained from BCa patients before exercise in the 12th week of chemotherapy.

cells was decreased in every condition compared to the controls, in which cells were treated with the patients' serum obtained before chemotherapy (PRE-EX: 71.63% ± 9.19%, $p < 0.01$; POST-EX: 71.13% ± 6.48%, $p < 0.05$; Trained PRE-EX: 61.14% ± 12.14%, $p < 0.05$; Trained POST-EX: 32.00% ± 6.39%, $p < 0.001$) (Fig. 2A). Interestingly, when the acute effect of exercise-conditioned serum on the metabolic activity of breast cancer cells was examined, a significant reduction was found only when cells were treated with the Trained POST-EX compared to the Trained PRE-EX serum ($p < 0.05$), while no significant changes were revealed between the PRE-EX and POST-EX conditions ($p > 0.05$) (Fig 2A). Similarly, when MCF-7 cells were treated with the patients' serum for 8 h, LDH activity was increased in the POST-EX (1.10 ± 0.03-fold change, $p < 0.05$), Trained PRE-EX (1.13 ± 0.03-fold change, $p < 0.01$), and Trained POST-EX conditions (1.49 ± 0.04-fold change, $p < 0.001$), compared to the controls (Fig. 2B). Moreover, an acute effect of exercise-conditioned serum was revealed regarding MCF-7 cells' LDH activity, which was increased only in the Trained POST-EX compared to the Trained PRE-EX condition ($p < 0.05$; Fig. 2B).

In the MDA-MB-231 cells, treatment with the exercise-conditioned serum resulted in similar responses to those of the MCF-7 cells. Specifically, their metabolic activity was suppressed in the POST-EX (85.00% ± 5.93%, $p < 0.05$), Trained PRE-EX (60.00% ± 6.33%, $p < 0.01$), and Trained POST-EX (34.33% ± 4.64%, $p < 0.001$) conditions, in comparison with the control condition (Fig. 2C). Moreover, LDH activity exhibited a 1.42 ± 0.12- and 1.89 ± 0.09-fold increase in the Trained PRE-EX ($p < 0.05$) and Trained POST-EX ($p < 0.001$) conditions, respectively (Fig. 2D). As for the acute effect of exercise on metabolic and LDH activity, significant alterations were demonstrated only in the Trained POST-EX (metabolic activity: $p < 0.05$; LDH activity: $p < 0.01$; Fig. 2C and 2D) and not in the POST-EX condition,

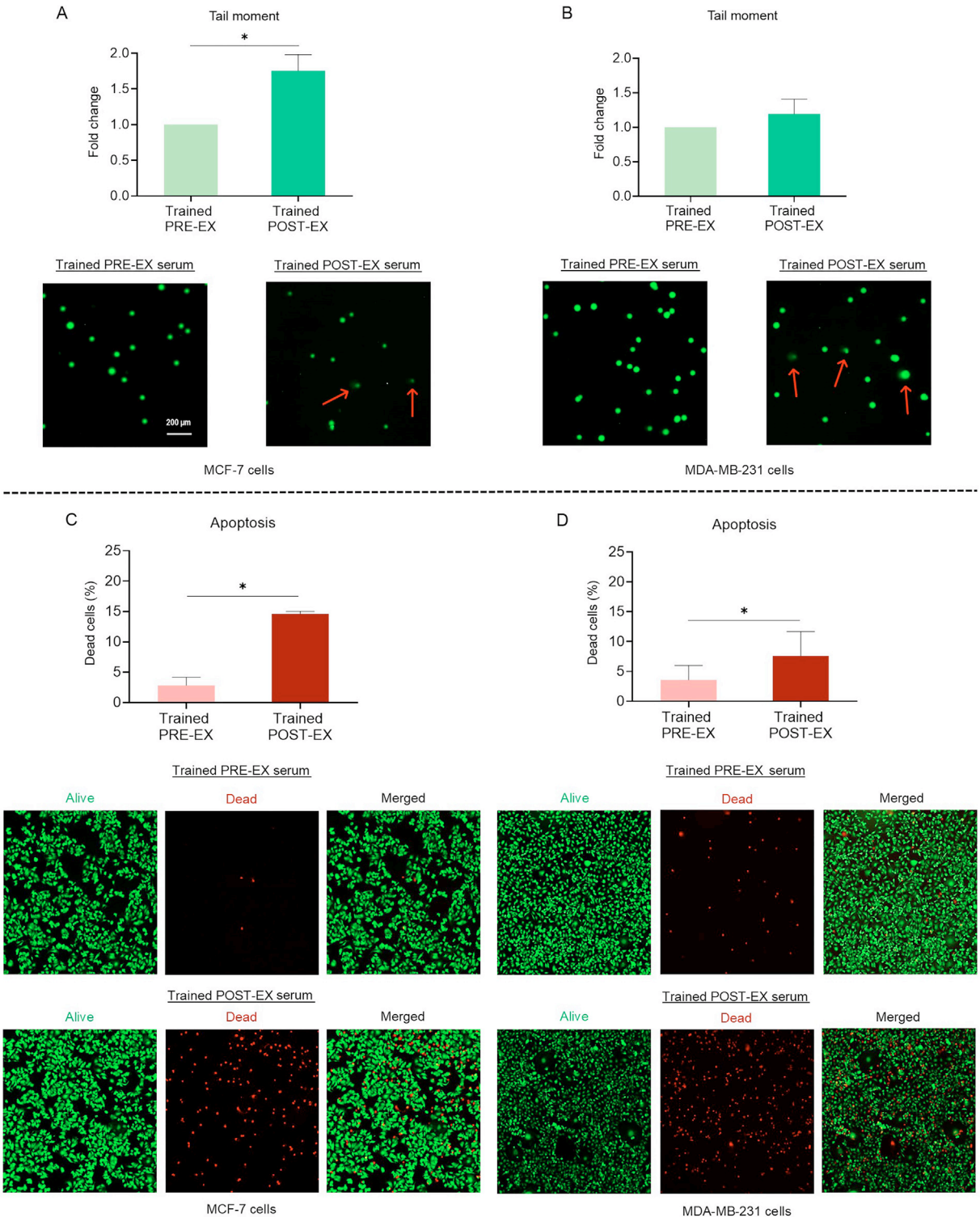

Fig. 3. Breast cancer cells were treated with the breast cancer patients' serum collected in the 12th week of exercise training, at a 10% concentration for 18 h, to detect possible early- and late-stage apoptosis. Specifically, DNA damage as an indicator of early-stage apoptosis was assessed by fold changes of "tail moment" compared to controls in (A) MCF-7 cells and (B) MDA-MB-231 cells. Late-stage apoptosis rate was examined with a double staining of Calcein AM (alive cells: green dye) and BOBO-3 iodide (dead cells: red dye). Alterations in the percentage of dead cells in (C) MCF-7 and (D) MDA-MB-231 cells are shown. Representative images are provided and quantified results are presented as mean ± standard error; $n = 7$ biological replicates. *$p < 0.05$, significant difference between conditions. MCF-7 = Michigan Cancer Foundation-7; MDA-MB-231 = M.D. anderson-metastatic breast-231; Trained POST-EX = human serum obtained from BCa patients after exercise in the 12th week of chemotherapy; Trained PRE-EX = human serum obtained from BCa patients before exercise in the 12th week of chemotherapy.

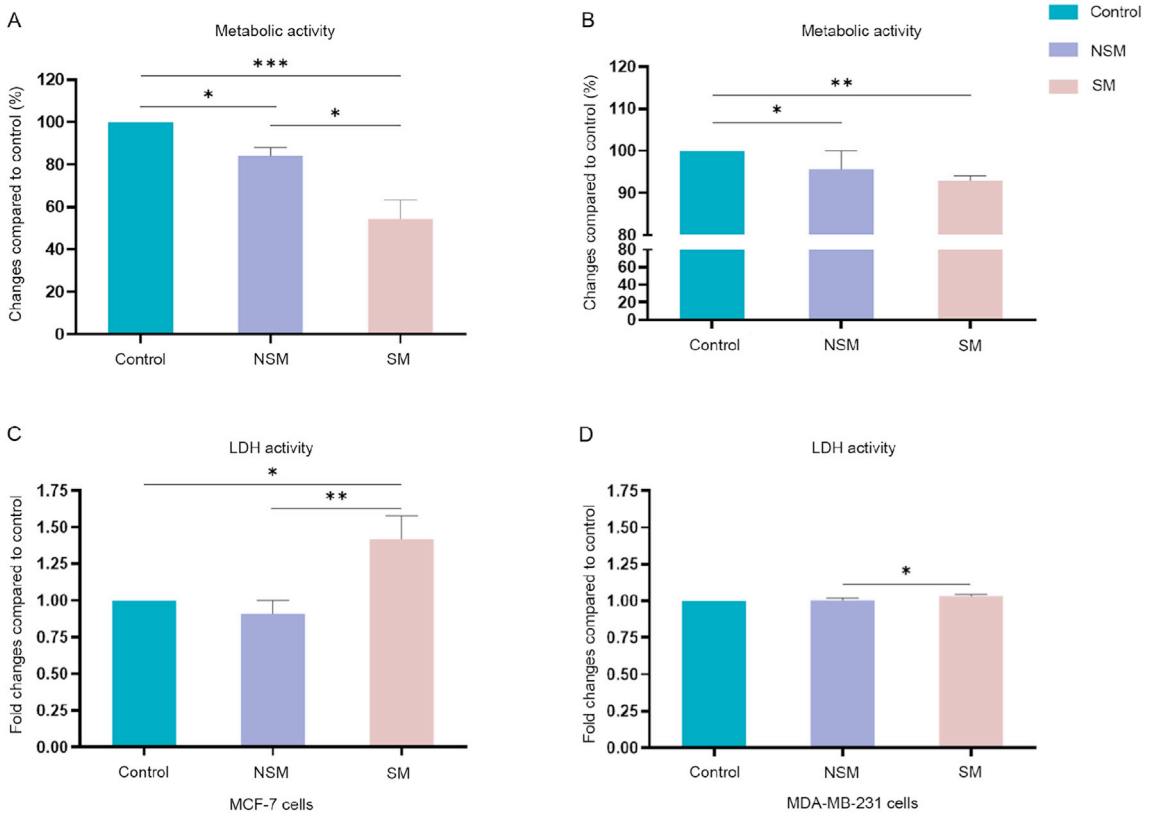

Fig. 4. Breast cancer cells were treated for 24 h with the myotube secretome obtained after mechanical loading (stretch media, SM) of the myotubes or without their mechanical preconditioning (non-stretch media, NSM). Percentage changes in metabolic activity (MTT assay) of (A) MCF-7 cells and (B) MDA-MB-231 cells, after treatment. Fold changes of LDH activity in the culture media of (C) MCF-7 cells and (D) MDA-MB-231 cells, after treatment. Results are presented as mean ± standard error of 3 independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences between conditions. LDH = lactate dehydrogenase; MCF-7 = Michigan Cancer Foundation-7; MDA-MB-231 = M.D. Anderson-Metastatic Breast-231; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zoliumbromide; NSM = non-stretch media; SM = stretch media.

compared to the corresponding pre-exercise conditions (i.e., the Trained PRE-EX and PRE-EX, respectively).

3.2.2. Post-exercise serum of breast cancer patients induced breast cancer cell death in vitro

Apoptosis was examined in breast cancer cells treated with the exercise-conditioned serum obtained in the 12th week of training (Trained POST-EX, i.e., the condition in which we observed the greater acute effects of post-exercise serum in terms of metabolic and LDH activity). Specifically, the comet assay technique was used to detect possible DNA damage in individual breast cancer cells as an indicator of early-stage apoptosis. It was found that the tail moment, the widely accepted index of DNA damage, was raised 1.75 ± 0.22 times following the 18-h treatment of MCF-7 cells with the Trained POST-EX serum compared to the Trained PRE-EX-treated cells ($p < 0.05$; Fig. 3A). However, in MDA-MB-231 cells an upward trend of DNA damage was observed; however, it did not reach statistical significance (1.20 ± 0.21-fold change; $p > 0.05$; Fig. 3B).

Additionally, apoptosis was further quantified utilizing the live/dead assay, distinguishing live from necrotic cancer cells. After the 18-h treatment with the Trained Post-EX serum, the percentage of dead MCF-7 cells was increased to 14.60% ± 0.40%, in contrast to the Trained PRE-EX condition, in which only 2.8% ± 1.36% dead cells were detected ($p < 0.05$; Fig. 3C). Similar though not so pronounced differences were found in MDA-MB-231 cells, in which the percentage of dead cells increased from 3.60% ± 2.40% in the Trained PRE-EX condition to 7.60% ± 4.09% in the Trained POST-EX condition ($p < 0.05$; Fig. 3D).

3.3. Treatment of breast cancer cells with skeletal myotube secretome

3.3.1. Skeletal myotube secretome reduced breast cancer cell metabolic activity and increased their toxicity in vitro

Apart from the treatment of breast cancer cells with the exercise-conditioned human serum, cells were also treated for 24 h with the myotube secretome either after mechanical loading of the myotubes (SM) or without their mechanical preconditioning (NSM). Compared to the control condition, the metabolic activity of MCF-7 cells was significantly diminished, by 15.90% ± 3.94% ($p < 0.05$; Fig. 4A) in the NSM-treated cells and by 45.50% ± 8.77% ($p < 0.001$; Fig. 4A) in the SM-treated cells. Moreover, LDH activity exhibited a 1.42 ± 0.16-fold increase ($p < 0.05$; Fig. 4C) in the SM condition over the control condition, in contrast to the NSM-treated

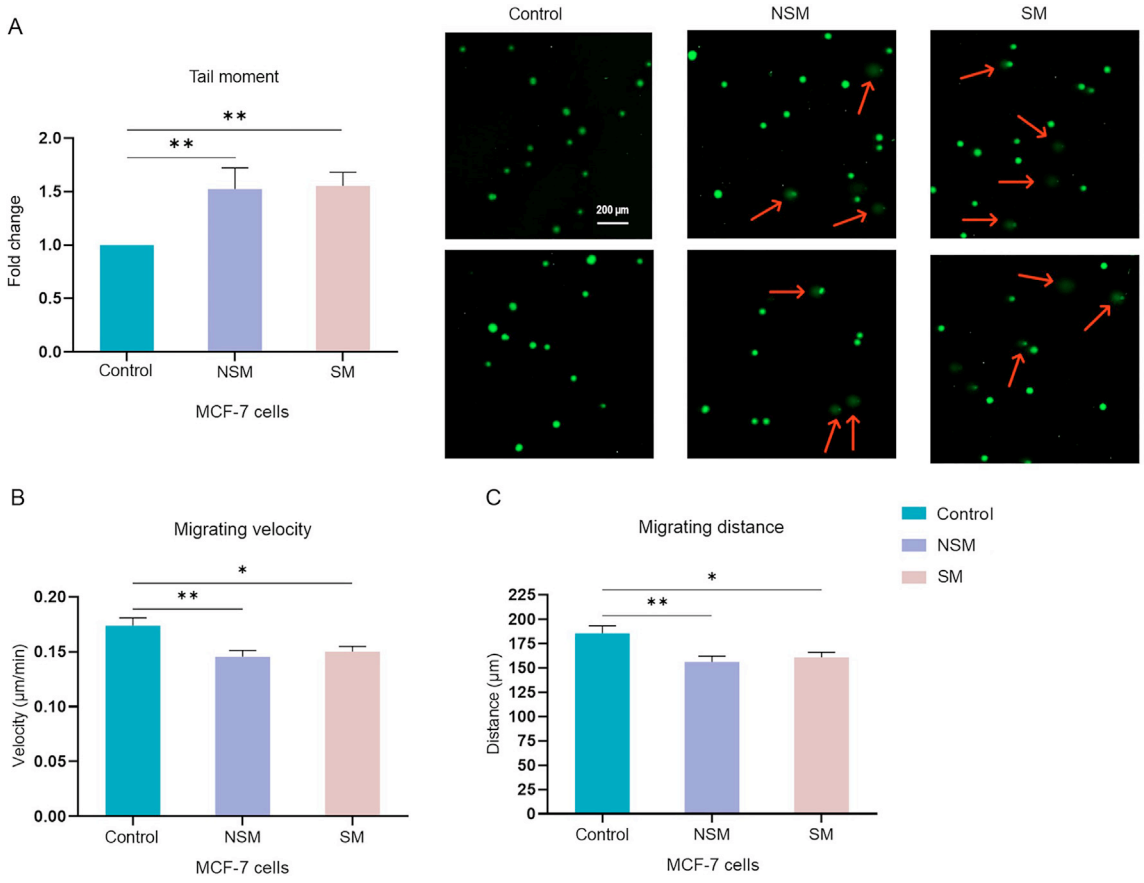

Fig. 5. The hormone-sensitive breast cancer cells MCF-7 were treated with the myotube secretome obtained after mechanical loading (SM) of the myotubes or without their mechanical preconditioning (NSM). (A) Fold changes of "tail moment" compared to controls and representative images after the 18-h treatment with the myotube secretome are shown. (B) Alterations in the migrating velocity and (C) migrating distance of MCF-7 cells after their 24-h incubation with the myotube secretome, as observed with the automated Incucyte® Live-Cell Analysis System. Results are presented as mean ± standard error of 3 independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, significant differences between conditions. MCF-7 = Michigan Cancer Foundation-7; MDA-MB-231 = M.D. Anderson-Metastatic Breast-231; NSM = non-stretch media; SM = stretch media.

cells, which demonstrated no differences from the controls ($p > 0.05$; Fig. 4C). Both metabolic and LDH activity were significantly different in the SM compared with the NSM condition ($p < 0.05-0.01$; Fig. 4A and 4C).

In the MDA-MB-231 cells, metabolic activity exhibited a similar pattern of responses to that of MCF-7 cells, though to a lesser extent, showing a reduction of 4.33% ± 4.35% ($p < 0.05$; Fig. 4B) and 7.11% ± 1.12% ($p < 0.01$; Fig. 4B) when subjected to treatment with the NSM and SM, respectively, compared to the control condition. Additionally, a mild increase in LDH activity was found only in the SM compared to NSM (1.03 ± 0.01 *vs.* 1.00 ± 0.01-fold change; $p < 0.05$, Fig. 4D), while no significant differences were observed with the control condition ($p > 0.05$; Fig. 4D).

3.3.2. Treatment of MCF-7 cells with skeletal myotube secretome triggered their apoptosis in vitro

Focusing specifically on the MCF-7 cells, which were found to be more susceptible to treatment with the myotube secretome compared to the MDA-MB-231 cells, the comet assay technique was again utilized to quantify potential DNA damage as an indicator of early-stage apoptosis of those cells. DNA damage was detectable in both myotube secretome-treatment conditions, compared to control (i.e., after incubating the cells for 18 h with either the NSM (1.53 ± 0.20-fold change; $p < 0.01$) or the SM (1.56 ± 0.13-fold change; $p < 0.01$) (Fig. 5A). Interestingly, mechanical loading preconditioning didn't trigger significant changes, and the observed DNA damage after myotube secretome treatment was independent of the mechanical stretching ($p > 0.05$, Fig. 5A).

Subsequently, MCF-7 cells were subjected to treatment with the myotube secretome for 24 h to evaluate possible late apoptosis using the live/dead assay. Conventional fluorescence microscope imaging showed that the ratio of live to dead cells was not significantly different between the 3 conditions, indicating no detectable changes in the apoptosis rate after treating the cells with either SM or NSM for 48 h ($p > 0.05$; Supplementary Fig. 2).

3.3.3. Skeletal myotube secretome restricted the migrating potential of MCF-7 cells

The migratory behavior of MCF-7 cells was also examined after their exposure to either NSM or SM myotube secretome, using a single-cell tracking method for 24 h. The migration

velocity of cancer cells decreased from 0.17 μm/min in the control condition to 0.15 μm/min in both conditions that received the muscle secretome ($p < 0.05-0.01$; Fig. 5B). Similarly, the accumulated migrating distance significantly declined to 156 μm ($p < 0.01$) and 160.87 μm ($p < 0.05$) in the NSM- and SM-treated cells, respectively, compared to the 185.6 μm distance covered in the control condition (Fig. 5C).

3.3.4. Treatment of MCF-7 cells with myotube secretome modulated the expression of determinant, apoptosis-regulating proteins

Myotube secretome, with or without mechanical preconditioning, was administered to MCF-7 cells for 48 h to examine potential changes in the expression of apoptosis-related proteins detected by immunofluorescence. As compared to the control MCF-7 cells, expression of the well-characterized, apoptosis-regulating Caspase-8 was increased to a 1.39 ± 0.07-fold change ($p < 0.01$; Fig. 6A) in the SM-treated cells, and Caspase-7 was upregulated to a 1.63 ± 0.14-fold ($p < 0.01$; Fig. 6B) and 1.89 ± 0.09-fold ($p < 0.001$; Fig. 6B) change in the NSM and SM conditions, respectively. Moreover, along with the upregulation of caspases compared to the control condition, the myotube secretome resulted in the downregulation of Fas (NSM: 0.83 ± 0.08-fold change, $p < 0.01$; SM: 0.82 ± 0.06-fold change, $p < 0.05$; Fig. 6C), VEGF (NSM: 0.67 ± 0.06-fold change, $p < 0.001$; SM: 0.66 ± 0.05-fold change, $p < 0.01$; Fig. 6D), SOD2 (SM: 0.82 ± 0.05-fold change, $p < 0.05$; Fig. 6E), and IL-8 (NSM: 0.82 ± 0.08-fold change, $p < 0.001$; SM: 0.75 ± 0.04-fold change, $p < 0.001$; Fig. 6F).

4. Discussion

Evidence from epidemiological studies, systematic reviews, and meta-analyses indicates that there is a link between regular exercise and both primary prevention of breast cancer and reduction of post-diagnosis complications. However, the cellular and molecular processes that explain the anti-oncogenic effects of exercise remain elusive and have been only partially described.[34,35] The findings of the present study show that the exercise-induced secreted factors (exerkines), even after cancer diagnosis, can provoke several cellular perturbations to breast cancer cells *in vitro* that ultimately lead to their apoptosis, regardless of the cell type (i.e., hormone-sensitive or -insensitive). Importantly, these anti-cancer effects of exercise-conditioned human serum were not masked by the established benefits of chemotherapy treatment but instead were found to be additive against breast cancer cells. Moreover, and interestingly, the post-exercise serum induced the apoptotic effects on the different breast cancer cells at the completion as opposed to the beginning of the 12-week progressively intensifying exercise training. Furthermore, this study characterized for the first time the anti-cancer effects of the bioactive molecules derived from skeletal muscle cells (muscle secretome) distinctly from the impact of the sum of factors secreted into blood circulation by several other organs during exercise.

Different research groups have previously examined the effects of exercise-conditioned serum on either MCF-7 or MDA-MB-231 cells, though focusing mainly on the proliferative capacity and micro-tumor formation.[22–24,36] To the best of our knowledge, only a single study to date has evaluated the exercise-conditioned serum from breast cancer patients during chemotherapy to examine the effect on the regulation of apoptosis of breast cancer cells *in vitro*.[24]

Our findings are that the beneficial effects of exercise-conditioned serum against cancer progression appear to be mediated by the ability of exercise-induced secreted factors to suppress metabolic activity of breast cancer cells, increase cell toxicity (as showcased by the increase in LDH activity), and cause considerable DNA damage, ultimately leading to cell demise. Indeed, the percentage of apoptotic cells was significantly elevated in both MCF-7 and MDA-MB-231 cells after their incubation with the post-exercise breast cancer patients' serum, which was collected acutely after an exercise bout in the 12th week of training. Interestingly, a similar detrimental effect on breast cancer cells was not observed when the cells were treated with the post-exercise serum collected in the 2nd week of training. It can be speculated that this late effectiveness of the post-exercise serum in the 12th week of training might be attributed to the increased exercise intensity that the patients could achieve over time, as during the 2nd week of training, aerobic exercise was performed at 40% of the patients' heart rate reserve, while exercise intensity had been gradually increased to 60% by the 12th week of training. Thus, the observed improvements in patients' muscle strength during the 12-week exercise training not only allowed them to achieve a higher exercise intensity but might also result in a higher responsiveness to exercise bout(s) and/or increased secretion of anti-cancer bioactive molecules triggered by the (36th) exercise session. In other words our study showed that, although breast cancer patients exercised regularly during chemotherapy, their pre-exercise serum did not provoke similar effects on cancer cells at the 12th week of training, suggesting that it was the acute response to an exercise bout of a higher intensity rather than a systemic training effect that regulated efficacy of exercise in inducing the detrimental effects on the cancer cells. This is in agreement with a previous study, which showed no effect on apoptosis regulation of cancer cells when they were treated with a resting human blood serum, even if individuals had an established exercise training history.[37] Overall, it can be assumed that breast cancer patients benefit from each exercise bout performed regularly above a threshold intensity, which triggers acutely the secretion of anti-cancerous bioactive molecules into circulation.

To further characterize the exercise loading-induced cellular and molecular responses of breast cancer cells *in vitro*, we utilized an exercise-mimicking model to distinguish, for the first time, the effects of exerkines from the alterations caused purely by muscle-derived factors, such as myokines, chemokines, microRNAs, and exosomes.[17,38] Specifically, we used a model previously established by our group, a protocol of mechanical loading of terminally differentiated myotubes *in vitro*[30] that mimics mechanical strain applied to skeletal muscle cells *in vivo*, to collect the secretome produced upon mechanical loading.

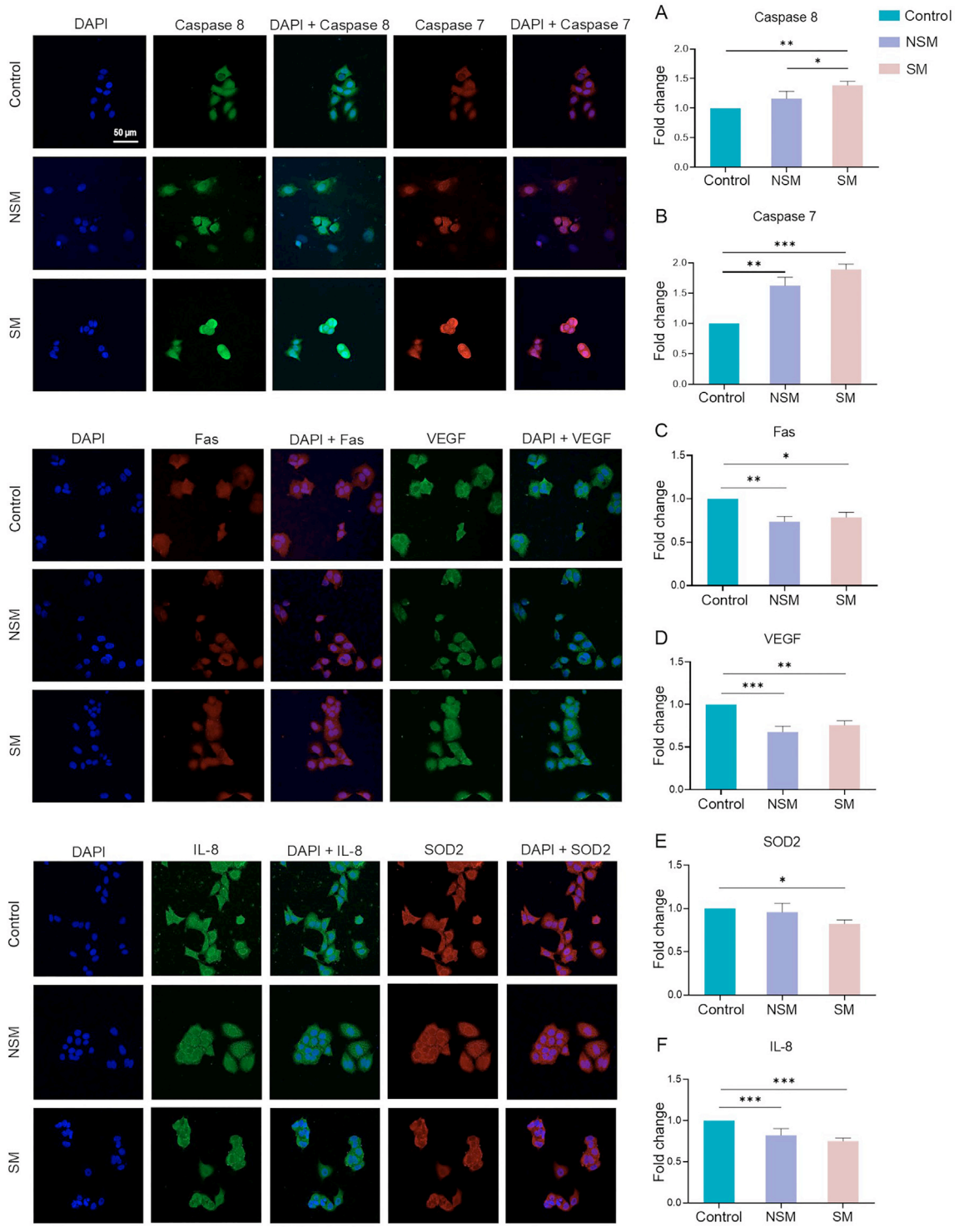

Fig. 6. Fold changes in the protein expression of (A) Caspase-8, (B) Caspase-7, (C) Fas, (D) VEGF, (E) SOD2, and (F) IL-8 in MCF-7 breast cancer cells after their treatment for 48 h with the skeletal myotube secretome with (SM) or without mechanical preconditioning (NSM) compared to controls. Protein expression was determined by confocal fluorescence microscopy and the fluorescent intensity was normalized to the number of cells (representative images are shown). Results are presented as mean ± standard error of 3 independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences between conditions. DAPI = 4′,6-diamidino-2-phenylindole; Fas = Fas cell surface death receptor; IL-8 = interleukin 8; MCF-7 = Michigan Cancer Foundation-7; MDA-MB-231 = M.D. Anderson-Metastatic Breast-231; NSM = non-stretch media; SM = stretch media; SOD2 = superoxide dismutase-2; VEGF = vascular endothelial growth factor.

The findings from this series of experiments suggest that, mainly in the hormone-sensitive breast cancer cells, the skeletal myotubes secretome exerts important anti-oncogenic effects. Specifically, it appears that the myotube secretome, after mechanical loading, influenced various cellular processes in MCF-7 cells as it reduced their metabolic activity and increased their toxicity. On the other hand, in the (hormone-insensitive) triple-negative breast cancer cells, the skeletal muscle cell-derived factors may only partly orchestrate apoptosis induction in MDA-MB-231 cells, while causing only a mild decrease in the cells' metabolic activity. Similar suppressive effects on breast cancer cell proliferative capacity have been recently reported after treating MCF-7 cells with the secretome of myotubes stimulated by pulsed electromagnetic fields.[25]

MDA-MB-231 cells were found to be less susceptible to the myotube secretome, so we further analyzed potential detrimental effects only on MCF-7 cells, which displayed the most pronounced responses regarding their metabolic and LDH activity. Specifically, DNA damage, as an early stage of apoptosis,[39] was detectable after treating MCF-7 cells with the myotube secretome. Through the comet assay we observed a significant increase in DNA damage in both conditions that received the myotube secretome, regardless of the mechanical loading preconditioning. Overall, this is the first study showing the induction of DNA damage in breast cancer cells after their exposure to either the exercise-induced human serum during chemotherapy or the muscle cell secretome *in vitro*.

We further examined the migratory capacity of MCF-7 cells, which constitutes a major hallmark of breast cancer,[40] after their treatment with the myotube secretome. More specifically, the *in vitro* single-cell tracking method allowed us to monitor the hourly changes in cell motility before breast cancer cells started to rapidly proliferate. We found that incubation of MCF-7 cells with the myotube secretome, both with and without loading preconditioning, led to a significant suppression of their migration potential, in terms of the migratory distance and velocity of individual breast cancer cells, switching them to a less invasive phenotype. The reduced migrating activity of the breast cancer cells found in this study is in line with the results of previous studies that had investigated the effects of pulsed electromagnetic field-stimulated myotube secretome on MCF-7 cells[25] or the effects of myotube secretome after electric pulse stimulation on pancreatic cancer cells.[41]

Furthermore, changes in the expression of apoptosis-regulating proteins verified that the myotube secretome, especially after mechanical loading, could modulate the apoptotic responses of breast cancer cells *in vitro*. Indeed, treatment of MCF-7 cells with the secretome led to the upregulation of Caspase 7 and 8, indicating the potential induction of different apoptosis-related signaling pathways.[42] Moreover, the expression of VEGF and IL-8, both implicated in tumor angiogenesis and recently studied as emerging therapeutic targets,[43,44] also exhibited a significant decrease in breast cancer cells after their incubation with the myotube secretome. Furthermore, SOD2 and Fas, which play key roles in cell proliferation and invasion,[45,46] were also downregulated in MCF-7 cells after their exposure to mechanical loading-induced muscle cells secretome.

Taking all the above into account, we can conclude that the high adherence of patients to the specific exercise program suggests its applicability and the possibility of integrating physical exercise as an adjunct therapy alongside chemotherapy regimens. Indeed, the progressive increase in exercise intensity during chemotherapy was well tolerated by breast cancer patients and led to the maintenance of cardiorespiratory fitness and improved muscle strength. The improved muscle strength in combination with a higher intensity (60% heart rate reserve) of aerobic training probably enhanced the responsiveness and/or secretion of bioactive molecules due to muscle contraction, resulting in greater anticarcinogenic effects. Several bioactive molecules, which could potentially be used as serum biomarkers, have been proposed to possess antitumorigenic properties, including the myokines secreted protein acidic and rich in cysteine (SPARC), Oncostatin M, Decorin, Irisin, IL-6, IL-7, and IL-15.[17] These muscle-derived factors, along with other exerkines, can trigger various cellular and molecular effects that mediate long-term exercise-induced suppression of cancer cells.[14,47,48] Thus, future studies should particularly focus on the mechanistic analysis of factors found in muscle secretome or systemic circulation in response to exercise that can orchestrate the observed anticarcinogenic properties of exercise.

This study has some limitations. The potential influence of patients' individual characteristics (e.g., age, fitness level, tumor subtype) on exercise response cannot be excluded. However, this may represent a consistent bias throughout repeated measurements, as comparisons were made between different time points in the same individuals within the same group. In this light, it can be assumed that these anticarcinogenic benefits of exercise were not affected by potential inter-individual differences between different (exercising *vs.* non-exercising) groups. Moreover, the escalation of exercise intensity from 40% to 60% of heart rate reserve, based on the patients' current functional ability during the aerobic exercise training, does not allow clear conclusions to be drawn regarding the optimum combination of intensity and duration of the combined (aerobic and resistance) exercise protocol used in these patients for maximizing anti-cancer effects. In addition, the extent to which the exercise effects were additive to chemotherapy cannot be determined due to the absence of a non-exercising control group of breast cancer patients undergoing chemotherapy. The incorporation of a non-exercising chemotherapy group in this study could provide more information about the net effect of chemotherapy, better isolating the effects of exercise-conditioned serum (trained PRE-EX condition) with respect to the observed apoptotic and metabolic effects on cancer cells.

5. Conclusion

Physical exercise provides remarkable benefits against cancer onset and progression, which are potentially mediated

through exerkines and myokines. The bioactive molecules, the secretion of which is triggered by exercise and muscle loading, modulate basic hallmarks of cancer, such as cancer cell metabolism, proliferation, apoptosis, angiogenesis, and migration. In this study we found that both exercise-conditioned serum of breast cancer patients and the skeletal myotubes secretome after mechanical loading can reduce metabolic activity, promote cell toxicity and DNA damage, modulate the protein expression of crucial cell survival-regulating factors, and lead to apoptosis in breast cancer cells. Thus, we suggest that even after cancer diagnosis, exercise is not only well-documented to preserve physical performance and mitigate treatment- and disease-related side effects but also exerts beneficial effects additive to chemotherapy to improve breast cancer prognosis. However, understanding the exact impact of exercise during chemotherapy requires the addition of a non-exercising control group, which would help isolate the net effect of chemotherapy from the effects of exercise-conditioned serum, providing clearer insights into the distinct effects of exercise and chemotherapy overall. Moreover, future studies should focus on the mechanistic characterization of specific factors that are secreted in response to exercise and muscle loading and can drive the anti-carcinogenic effects through specific molecular pathways.

Authors' contributions

APa collected and analyzed the data, performed the statistical analysis, and wrote the original draft of the manuscript; AG contributed to data collection, analysis, and interpretation of results; TG, AM, and MP contributed to data collection; FZ and SD contributed to data collection and coordination; MM participated in the design, coordination, and supervision of the study; MK participated in the design and supervision of the study; APh participated in the design, coordination, and supervision of the study as well as revised the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests

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Supplementary materials

Supplementary materials associated with this article can be found in the online version at doi:10.1016/j.jshs.2025.101051.

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