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Novel anti-aging herbal formulation Jing Si displays pleiotropic effects against aging associated disorders

Marthandam Asokan Shibu [a], Yu-Jung Lin [a], Chien-Yi Chiang [a], Cheng-You Lu [a], Debakshee Goswami [a], Navaneethan Sundhar [a], Surbhi Agarwal [a], Md. Nazmul Islam [a], Pi-Yu Lin [b], Shinn-Zong Lin [c,d], Tsung-Jung Ho [e,m,n], Wan-Ting Tsai [f], Wei-Wen Kuo [g,h,*,1], Chih-Yang Huang [a,i,j,k,l,**,1]

[a] Cardiovascular and Mitochondrial Related Disease Research Center, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien 970, Taiwan
[b] Buddhist Tzu Chi Charity Foundation, Hualien 970, Taiwan
[c] Bioinnovation Center, Buddhist Tzu Chi Medical Foundation, Hualien 970, Taiwan
[d] Department of Neurosurgery, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien 970, Taiwan
[e] Department of Chinese Medicine, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Tzu Chi University, Hualien, Taiwan
[f] Buddhist Tzu Chi Foundation Hospital, Hualien 97002, Taiwan
[g] Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichuang 406, Taiwan
[h] Ph.D. Program for Biotechnology Industry, China Medical University, Taichuang 406, Taiwan
[i] Graduate Institute of Biomedical Sciences, China Medical University, Taichung 404, Taiwan
[j] Department of Biological Science and Technology, Asia University, Taichung 413, Taiwan
[k] Center of General Education, Buddhist Tzu Chi Medical Foundation, Tzu Chi University of Science and Technology, Hualien 970, Taiwan
[l] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404, Taiwan
[m] Integration Center of Traditional Chinese and Modern Medicine, Hualien Tzu Chi Hospital, Hualien 97002, Taiwan
[n] School of Post-Baccalaure-ate Chinese Medicine, College of Medicine, Tzu Chi University, Hualien 97004, Taiwan

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ABSTRACT

Common characteristics of aging include reduced somatic stem cell number, susceptibility to cardiac injuries, metabolic imbalances and increased risk for oncogenesis. In this study, Pleiotropic anti-aging effects of a decoction Jing Si herbal drink (JS) containing eight Traditional Chinese Medicine based herbs, with known effects against aging related disorders was evaluated. Adipose derived mesenchymal stem cells (ADMSCs) from 16 week old adult and 24 month old aging WKY rats were evaluated for the age-related changes in stem cell homeostasis. Effects of JS on self-renewal, klotho and Telomerase Reverse Transcriptase expression DNA damage response were determined by immunofluorescence staining. The effects were confirmed in senescence induced human ADMSCs and in addition, the potential of JS to maintain telomere length was evaluated by qPCR analysis in ADMSCs challenged for long term with doxorubicin. Further, the effects of JS on doxorubicin-induced hypertrophic effect and DNA damage in H9c2 cardiac cells; MPP+-induced damages in SH-SY5Y neuron cells were investigated. In addition, effects of JS in maintaining metabolic regulation, in terms of blood glucose regulation in type-II diabetes mice model, and their potential to suppress malignancy in different cancer cells were ascertained. The results show that JS maintains stem cell homeostasis and provides cytoprotection. In addition JS regulates blood glucose metabolism, enhances autophagic clearances in neurons and suppresses cancer growth

Abbreviations: JS, Jing Si herbal drink; ADMSCs, Adipose derived mesenchymal stem cells; WKY, Wistar Kyoto; TERT, Telomerase Reverse Transcriptase; ROS, Reactive oxygen species; MSC, Mesenchymal stem cells; TCM, Traditional Chinese Medicines; FBS, Fetal Bovine Serum; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole; LVIDd, Left ventricular internal diameter at end diastole; LVIDs, Left ventricular internal diameter at end systole; EF, Ejection Fraction; FS, Fractional shortening; MPP+, 1-methyl-4-phenylpyridinium; TRPML1, The activation of Mucolipin TRP channel 1; EMT, Epithelial mesenchymal transition; CDK, Cyclin-dependent kinases.

* Correspondence to: Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichuang 406, Taiwan.
** Correspondence to: Cardiovascular and Mitochondria Related Diseases Research Center, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Tzu Chi University of Science and Technology, Hualien 970, Taiwan.
E-mail addresses: wwkuo@mail.cmu.edu.tw (W.-W. Kuo), cyhuang@mail.cmu.edu.tw (C.-Y. Huang).
[1] The authors share equal contribution.

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and migration. The results show that JS acts on multiple targets and provides a cumulative protective effect against various age-associated disorders and therefore it is a candidate pleiotropic agent for healthy aging.

1. Introduction

Aging is often characterized by progressive decline in the normal physiological functions of organelles, cells, tissues and organ units [1–5]. The loss of normal function and tissue homeostasis results in reduced adaptability to various stress conditions and thereby increases the vulnerability to aging disorders [6]. Aging is therefore one of the major risk factors for diseases such as cancer, diabetes, cardiovascular disease, Alzheimer's and Parkinson's disease [7–9].

Cellular senescence often involves damages to DNA and is associated with cellular response such as increase in Reactive oxygen species (ROS), mitochondrial dysfunction, increase in the expression of p53, p21 and $p16^{INK4a}$, increase in lysosomal β-galactosidase synthesis and reduction in antiaging proteins such as Klotho and TERT [10–14]. Adult somatic stem cells are crucial elements of rejuvenation and their persistence throughout the lifespan is essential for the maintenance of homeostasis against pathological assaults [15]. Mesenchymal stem cells (MSC) are prominent adult stem cells located in various tissue depots to assist tissue rejuvenation. MSCs, particularly those in the adipose tissue have been widely accepted to maintain various physiological processes and thereby contribute to physiological functions like immune modulations, paracrine effects, and metabolic regulation [16,17]. However, the functions of adult stem cells decline with age and a breakdown in stem cell homeostasis jeopardizes the regulatory mechanisms that act against age associated disorders [18]. The onset and the rate of aging process differ with individuals based on their adaptability to maintain physiological balance through various homeodynamic mechanisms [19] that mediate DNA repair, host immunity, clearance of damaged/dysfunctional proteins and organelles and synthesis and regulation of proteins and lipids [20]. Expression of anti-aging proteins and suppression of aging markers in MSCs are crucial for their maintenance and function and therefore they serve as effective targets for aging drugs [17,18]. In addition control of neurodegenerative diseases, oncogenesis, normal cardiac function and metabolic regulation are other targets for anti-aging drugs.

Therefore pharmacological interventions to promote healthy aging require a multi-pronged approach targeting various factors associated with aging disorders such as diabetes, neurodegeneration, cancer and simultaneously promote adult stem cells rejuvenation may help in providing an enduring aging care [21–26]. Traditional medicine based formulas, particularly those used in Traditional Chinese Medicines (TCM) have shown promising potentials of rejuvenation on various experimental models [27–29] In addition traditional medicine based herbal decoctions used for chronic diseases like diabetes have also shown to enhance lifespan [30]. Therefore TCMs are an excellent source to mine for anti-aging therapeutics with potentials to enhance stem cell function and rejuvenation [31,32].

In our quest for a potential pleiotropic drug for aging treatment we have formulated a TCM based decoction Jing Si herbal drink containing eight TCM herbs viz *Artemisia argyi*, *Ohwia caudate*, *Ophiopogon japonicas*, *Houttuynia cordata*, *Platycodon grandifloras*, *Glycyrrhiza uralensis*, *Perilla frutescens* and *Chrysanthemum* × *morifolium,* chosen based on their potential to regulate age associated disorders. The JS was evaluated for its anti-aging effects using suitable in vitro and in vivo models.

2. Material and methods

2.1. Preparation and characterization of Jing Si herbal drink

The healthy, mature and fresh leaves of *Artemisia argyi, Ohwia caudate*, *Ophiopogon japonicas* and *Perilla frutescens*; *Roots of Houttuynia cordata* (*Ophiopogonis Radix*), *Platycodon grandifloras and Glycyrrhiza uralensis* (*Glycyrrhizae radix*); and flowers of *Chrysanthemum* × *morifolium* were collected locally in Taiwan. The plant materials were washed, dried under shade and mixed in the following composition- 6 g of *Artemisia argyi,* 6 g of *Ohwia caudata*, 4 g of *Ophiopogon japonicus*, 4 g of *Houttuynia cordata*, 4 g of *Platycodon grandiflorus*, 2 g of *Glycyrrhiza uralensis*, 2 g of *Perilla frutescens*, 0.2 g of *Chrysanthemum* × *morifolium*. The formulation was finely powdered and boiled with 600 mL water and concentrated into a 60 mL decoction. The preparation was characterized based on the concentration of prominent bioactive constituents of the respective herbs. HPLC-MS/MS analysis showed presence of 1104 ± 20.3 ng/mL of swertizin, 255.3 ± 31.5 ng/mL Isoliquiritigenin, 86.7 ± 21.4 ng/mL Eupatilin, 7.02 ± 2 ng/mL nerolidol.

2.2. Cell culture and maintenance

Human ADMSCs obtained from ThermoFisher, (Waltham, MA, United States) were cultured and maintained with 5% CO_2 in a humidified incubator at 37 °C in MesenPRO RS™ Basal Medium supplemented with MesenPRO RS™ Growth Supplement (ThermoFisher). ADMSCs with 70% confluence were subjected to subsequent passage and passage 8 was used for challenging with Doxorubicin. For senescence induction, low dose (0.1 μM) doxorubicin was added to 60% confluent cells and after 12 h the JS was supplemented in the media for the following 24 h. For DNA damage analysis and telomere length measurement, ADMSCs cultured in low dose doxorubicin for 3 passages were used. During analysis the cells were either challenged further with low dose doxorubicin or were cultured as controls without the challenge. The human colon cancer cell line LoVo was obtained from the American Tissue culture collection (ATCC). Gemcitabine-resistant cells were generated by exposing the CL1 cell line to incrementally increasing gemcitabine concentrations. As the cells adapted to the drug, the gemcitabine concentration was doubled. The oxaliplatin-resistance LoVo cells (LoVo-OXA) were established as described previously [33,34]. Human lung cancer cells A549 were cultured in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) with 10% Fetal Bovine Serum (FBS). Human colon cancer cell line LoVo and Oxaliplatin-resistance LoVo-OXA, SH-SY5Y neuroblastoma and H9c2 cardiomyoblasts were cultured in Dulbecco's minimum essential medium (D5523, Sigma, Saint Louis, Missouri, USA) containing 10% FBS (Fetal Bovine Serum, Hyclone, long, Utah USA), 1% penicillin-streptomycin (Invitrogen Corp., California, USA), and maintained at 37 °C in 5% CO.

2.3. MTT assay

The cell viability was measured using MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide Assay (Sigma) after treatment. Cells were plated in triplicate in a 96-well plate and treated with different concentrations of JS. After 24 h of incubation, 0.5 mg/mL of MTT was added to each well for at least 2–3 h. The blue MTT formazan crystals were then dissolved in 100 μL of DMSO. The absorbance at 570 nm was measured by ELISA reader.

2.4. Trans-well invasion/migration assay

The in vitro cell invasion (chamber coated with Matrigel 200 μL) and migration assay was performed using transwell chambers (8 μm pore size; SPLInsert™ hanging 24-well plate) [35,36]. A total of 4 × 104 cells for the invasion assessment and 2 × 104 cells for the migration assessment were plated in the upper chamber with serum-free medium. The medium containing 10% FBS in the lower chamber served as a

chemoattractant. After 48 h, non-invading and non-migrating cells were removed using cotton swabs from the upper face of the filters, and the migratory cells located on the lower side of the chamber were stained with crystal violet, air-dried, photographed, and counted. Images of four random fields at 20× magnification were captured from each membrane, and the number of invaded/migratory cells per field was counted.

2.5. Immuno-staining assay

For immunofluorescence staining, cells were fixed for 30 min with 4% of paraformaldehyde at room temperature and then washed three times with PBS. The samples were then incubated overnight at 4 °C with the respective primary antibody. The samples were subsequently rinsed with PBS three times and incubated for 1 h at room temperature with the appropriate dye-conjugated Alexa Fluor® 594 and Alexa Fluor® 488 secondary antibodies (1:100 dilution; Thermofisher). Finally, the samples were washed again with PBS and sealed with a mounting medium with DAPI (Abcam). All of the images were observed with the Axio Observer A1 digital fluorescence microscope (Olympus, Tokyo, Japan). Immunofluorescence and immunohistochemical staining of tissues were performed following methods reported previously [37,38].

2.6. High sucrose-fructose diet preparation

Diets containing around 60–70% (by energy) fructose or sucrose (which is a 50:50 molar mixture of fructose and glucose) are capable of elevating triglycerides and glucose production in the liver, ultimately leading to insulin resistance and hypertriglyceridemia relative to diets containing mainly glucose carbohydrate sources (i.e. dextrose, corn starch). The diet provided was prepared by mincing the diet in 1:1 ratio in a dry grinder and were then pelleted by drying in 60 ℃ for 3 h and were stored in 4 ℃. The treatment diets were prepared by mixing high sucrose–fructose diet (89247 and D12079Bi) in equal ratios.

2.7. Animal experiments

15 month old rats were maintained in the Animal center of Tzu Chi University, Taiwan. The animals were fed with normal chow and grouped into young (n = 10) and old rats (n = 9). The young were sacrificed at 15th week and the old rats were maintained up to 24 month and were then sacrificed. The adipose and heart tissue section were excised from the rats. For diabetic model, four groups (n = 8) of four week old Apoe$^{em1/Narl}$/Narl in a C57BL/6J background (RMRC13302, National animal research laboratory, Taiwan) were used. All of the mice were fed with either normal chow or Western diet (D12079Bi, Research Diet, NJ, USA) ad libitum. After 4 weeks of Western diet feeding, the animals that showed a fasting blood glucose level above 250 mg/dL were considered diabetic and chosen for treatment. The treatment group mice received 100 m/Kg JS by oral gavage for 10 weeks. Blood was obtained after a 12 h fast by tail tip nick and was analyzed using Acccu check glucometer (Roche, Basel, Switzerland). The body weight and diet uptake were monitored periodically.

2.8. Echocardiography

M mode heart echocardiography was performed to monitor the cardiac function of the rats with or without treatment. Rats sedated with 1.5% isoflurane were placed in supine position for the procedure. All ultrasound procedures were carried out using a commercial ultrasound scanner (GE healthcare, IL, USA) by an experienced echocardiographer. Based on the guidelines of the American Society of Echocardiography (ASE) quantitative measurements of echocardiography were performed. Fractional shortening (FS%) was calculated according to the following equation: [(LVIDd − LVIDs)/LVIDd] × 100, and Ejection Fraction (EF, %) was calculated as EF% = ((LVEDV − LVESV)/LVEDV) × 100 (%).

2.9. Statistical analysis

The results are shown as means ± SD obtained from at least 3 independent experiments. The differences among the groups were analyzed by one-way ANOVA analysis using Graphpad prism software (GraphPad Software Inc, San Diego, CA, USA) followed by Tukey's post hoc test and all results were quantified using ImageJ software (NIH, MD). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Aging reduces stem cell pools in adipose depots

The senescence effects in ADMSCs were analyzed in aging animals that were characterized by reduced cardiac function. Echocardiography analysis on the Cardiac function in 7 month and 24 month old WKY rats showed significant decline of the cardiac % EF and FS % in the old rats. While % EF and FS % in the young rats were respectively 83.62 ± 8 and 37.8 ± 6 and that of the aging rats were 68.30 ± 14 and 29.88. To comparatively assess the effect of aging on stem cell homeostasis, the histology of visceral adipose tissue from pericardial, perigonadal and retro peritoneal adipose tissue from 7 months old WKY rats and 24 months old WKY rats were examined. Immunohistochemical staining for the self-renewal marker nanog showed that young rats had a higher pool of adipose stem cells in pericardial, perigonadal and retro peritoneal adipose tissue when compared to older rats (Fig. 1A). Immunofluorescence analysis shows reduced levels of the antiaging protein klotho and the stem cell marker CD90 in the adipose tissue (Fig. 1B).

3.2. JS treatment ameliorates declining stem cell characteristics in adipose stem cells of aging rats

ADMSCs isolated and from 24 month old rats showed declining levels of the anti-aging protein klotho (Fig. 2A) self-renewal associated protein nanog and stem cell marker CD 90 expression (Fig. 2B). Klotho levels were much restricted to the nucleus of the aging ADMSCs. However, treatment with 100 and 200 μg/mL of JS showed effective improvement in the levels of klotho, CD90 and nanog in isolated ADMSCs.

3.3. JS treatment attenuates aging associated DNA damages in ADMSCs of aging rats

Immunofluorescence analysis showed an increase in the levels of p16^{INK4a} in the ADMSCs derived from 24 month old rats when compared to that derived from 16 week old adult SD rats (Fig. 3A). Therefore ADMSCs from 24 month old rats were under senescence however, treatment with 100 μg/mL and 200 μg/mL of JS showed an increase in the population of ADMSCs with reduced p16^{INK4a} level. The results show the anti-aging benefits of JS. To further confirm the anti-aging potentials of JS, their effects against aging associated cellular DNA damage in ADMSCs from 24 week old rats was evaluated. Immunofluorescence assay showed increasing pan localization of γ-H2AX in aging ADMSC along with reduction in the TERT levels (Fig. 3B). However, treatment with JS showed better reduction in the levels of γ -H2AX and increase in the nuclear accumulation of TERT in aging ADMSCs.

3.4. JS suppresses cellular senescence associated pathological manifestations in human ADMSC cells

In order to find the antiaging effect of JS in human ADMSCs different concentrations of JS was tested on a doxorubicin induced senescent human ADMSC cell model. Culturing ADMSCs in doxorubicin showed aging associated changes such as declining Klotho expression and increasing expression of p21, an indicator of replicative senescence. Treatment with 100 μg/mL and 200 μg/mL JS improved the levels of the antiaging protein Klotho that was correlated with decrease in p21

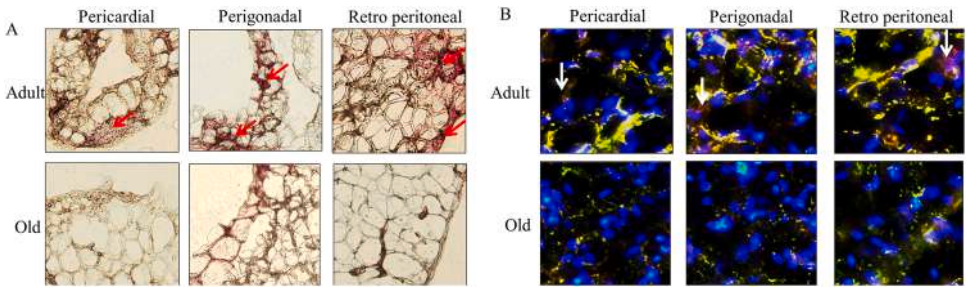

Fig. 1. Aging affects stem cell pool. (A) Immunohistochemical staining of nanog (Red arrow) in Adipose tissue derived from pericardial, perigonadal, retro peritoneal tissue. (B) Immunofluorescence staining shows klotho levels (green) and CD90 expression (red, white arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

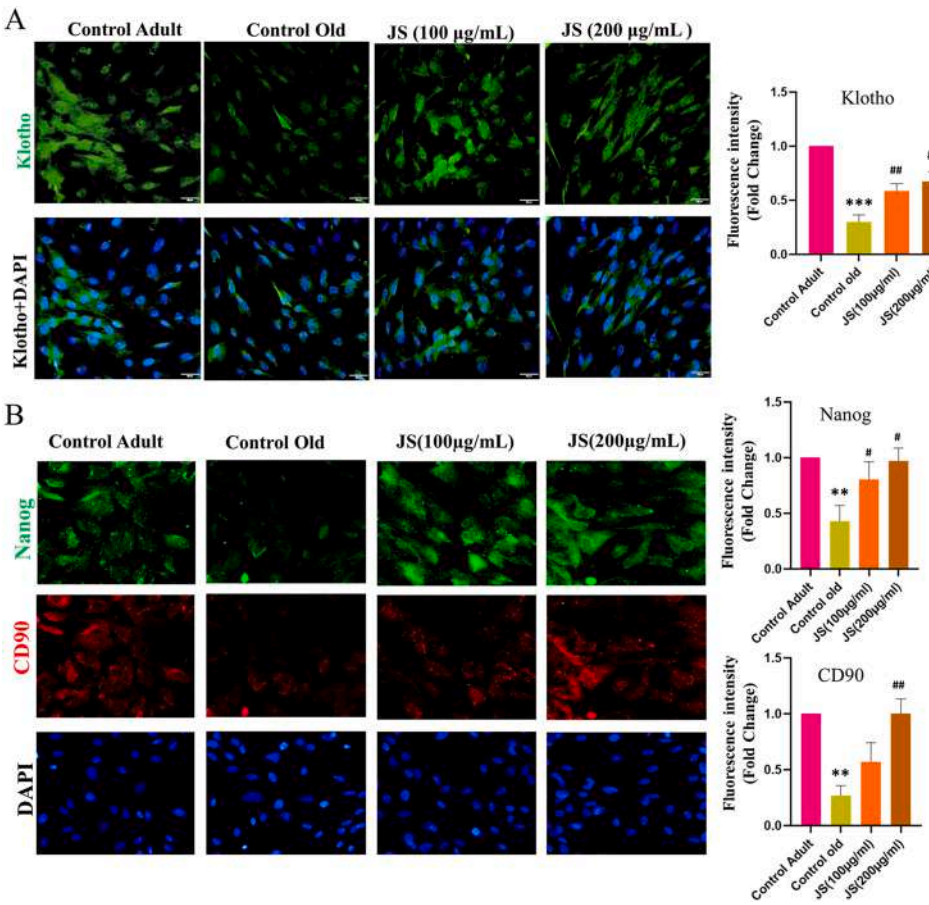

Fig. 2. Effect of JS on senescence characteristics of Adipose derived mesenchymal stem cells from old rats. (A) Immunofluorescence staining of Klotho (green) in ADMSCs from adult and old rats and those treated with JS. (B) Immunofluorescence staining for nanog (green) and CD90 (red) levels in ADMSCs from adult and old rats. Nuclei were stained with DAPI in blue. ***P < 0.001 indicates significant difference when compared to the control adult group and ## P < 0.01 indicates significant difference when compared to the control old group.

nuclear accumulation (Fig. 4A) and further improved the levels of Sox2, a stem cell self-renewal marker in the ADMSCs under doxorubicin challenge (Fig. 4B). Further 24 h pre-treatment with JS showed reduction in the mitochondrial ROS in senescent ADMSCs to levels similar to the control ADMSCs (Fig. 4C).

Moreover, ADMSCs cultured for 3 consecutive passages in low dose doxorubicin showed high levels of mitochondrial ROS and showed no further increase upon further addition of doxorubicin. In addition, as an indicator of senescence, long term doxorubicin challenge reduced the telomere length in ADMSCs as determined by qPCR analysis. Treatment with JS attenuated low dose doxorubicin induced mitochondrial ROS in ADMSCs challenged with low dose doxorubicin and in those cultured for 3 consecutive passages in doxorubicin in a dose dependent manner (Fig. 4D). The observation was also correlated with modulations in the telomere length in aging ADMSCs (Fig. 4E). JS maintained the telomere length in q dose dependent manner that suggest anti-aging benefits of JS.

3.5. JS on doxorubicin associated cardiac-cellular damages

Embryo derived cardiomyoblast H9c2 cells when challenged with 200 µM Angiotensin induced hypertrophy indicated by the increase in cell surface area (Fig. 5A). However treatment with JS showed reduction in the cell surface area in a dose dependent manner. Moreover, Doxorubicin challenge in H9c2 cells are also known to cause cellular hypertrophy and apoptosis. Our results show that JS treatment inhibits cellular hypertrophy (Fig. 5B) and apoptosis (Fig. 5C) induced by 1 µM doxorubicin.

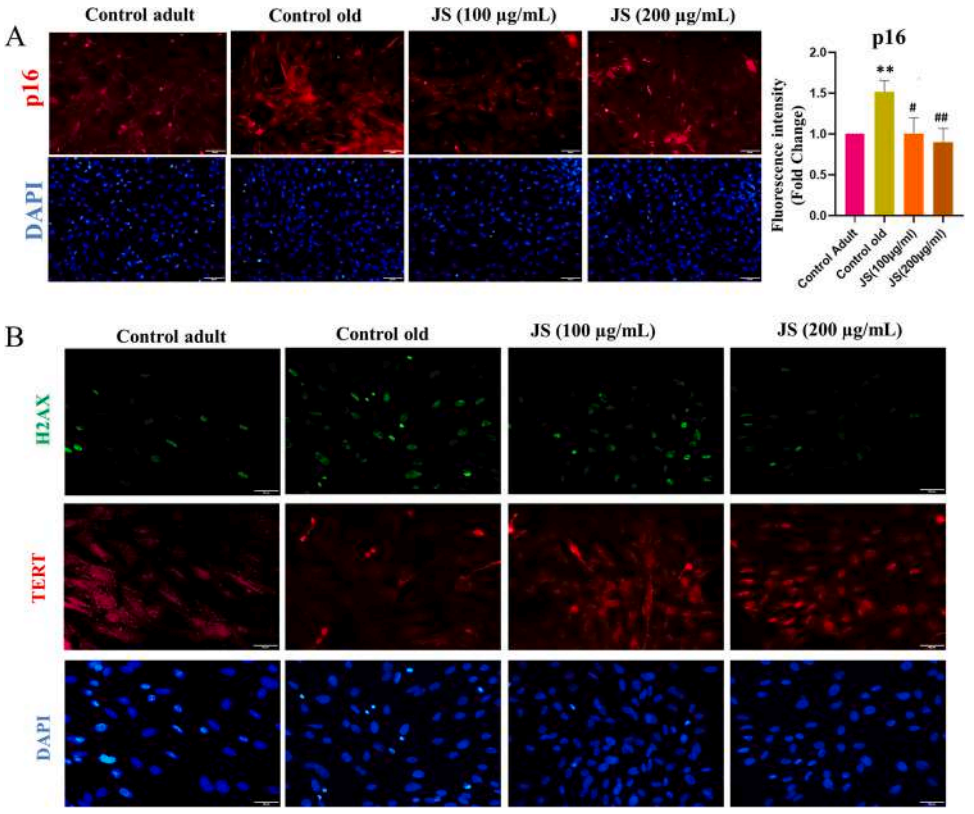

Fig. 3. JS attenuates aging associated-excessive DNA damage. (A) Immunofluorescence staining of $p16^{INK4a}$ (red) in ADMSCs from adult and old rats and those treated with JS. (B) Immunofluorescence staining for γH2AX (green) and TERT (red) levels in ADMSCs from adult and old rats. Nuclei were stained with DAPI in blue. **P < 0.01 indicates significant difference when compared to the control adult group and # P < 0.05 and ## P < 0.01 indicate significant difference when compared to the control old group.

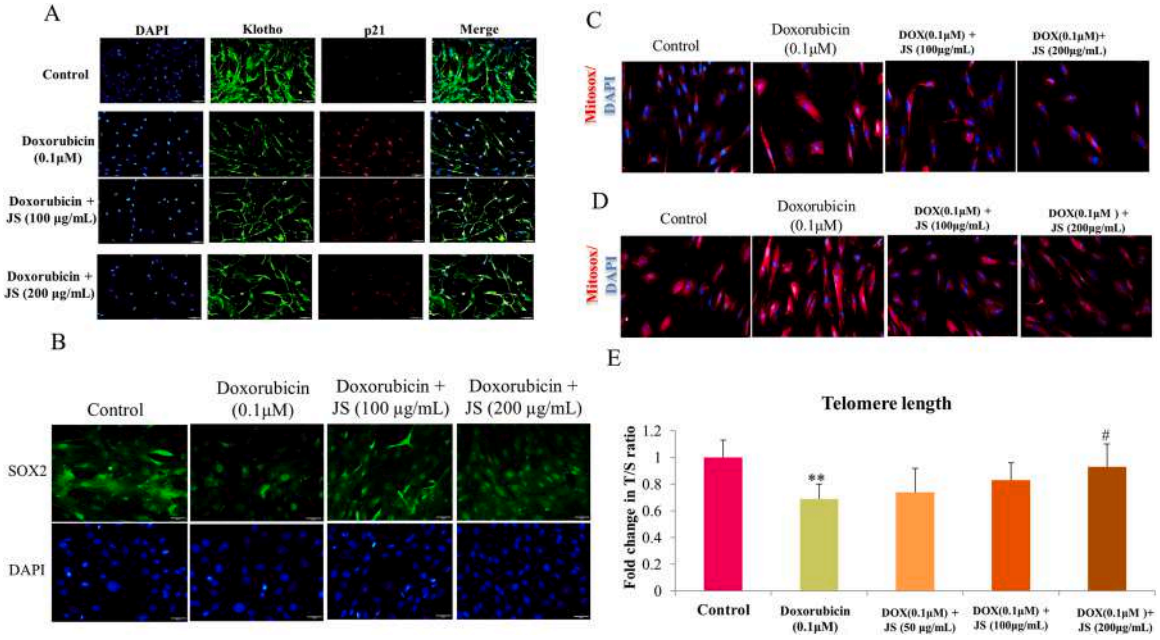

Fig. 4. JS treatment enhances stem cell homeostasis. (A) Immunofluorescence staining for klotho (green) and p21 (red) expression in low dose doxorubicin induced senescent ADMSCs and those pre-treated with JS. (B) Immunofluorescence staining for Sox2 (green) levels in aging induced ADMSCs and those pre-treated with JS. (C) Mitosox staining for mitochondrial ROS (red) in human ADSCs challenged with low dose doxorubicin and in those cultured in doxorubicin for longer time (D). qPCR analysis of telomere length in ADMSCs challenged with low dose doxorubicin for three continuous passages. Nuclei were stained with DAPI in blue. **P < 0.001 indicates significant difference when compared to control and # P < 0.001 indicates significant difference when compared to doxorubicin challenge.

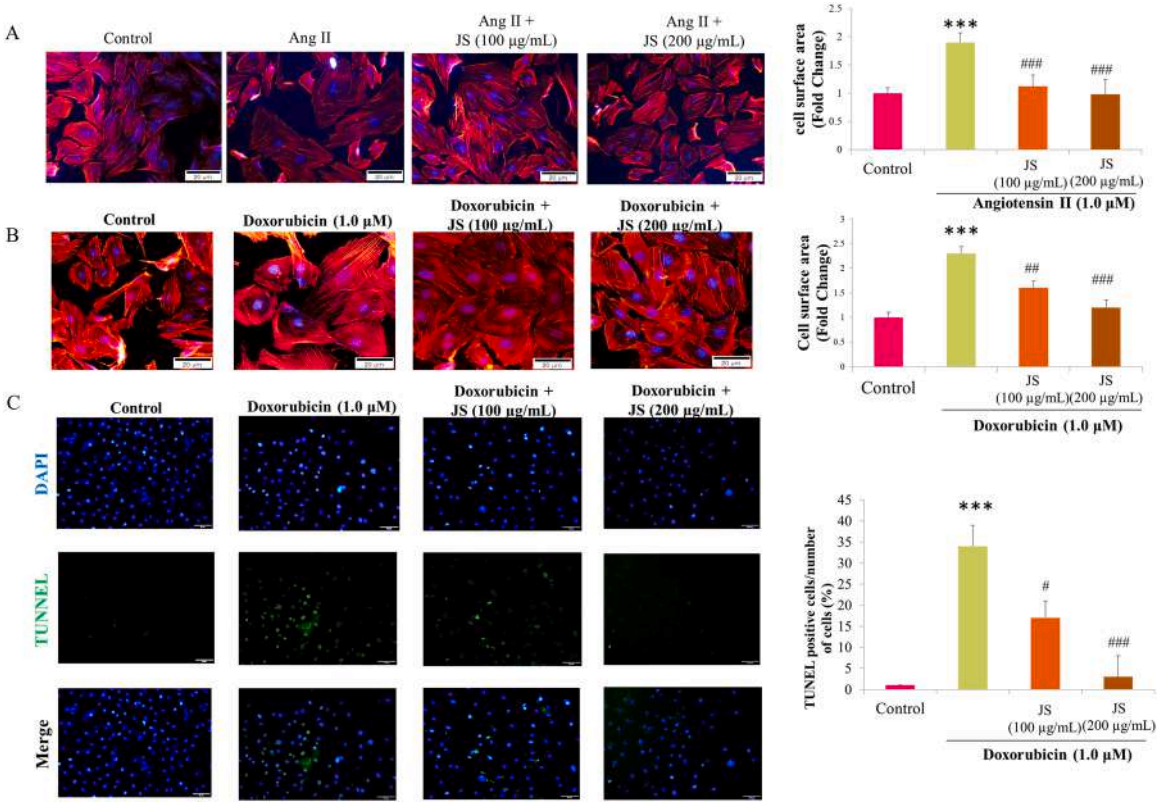

Fig. 5. JS protects from cardiac remodeling effects. Rhodamine-phalloidin staining in H9c2 cells challenged with 200 µM Angiotensin to determine cellular hypertrophy effects. H9c2 cells challenged with 1 µM of doxorubicin and stained with Rhodamine-phalloidin (B) and TUNNEL (C) staining. Nuclei were stained with DAPI in blue. **P < 0.01 indicates significant difference when compared to control and # P < 0.05 and ## P < 0.01 indicate significant difference when compared to challenged cells.

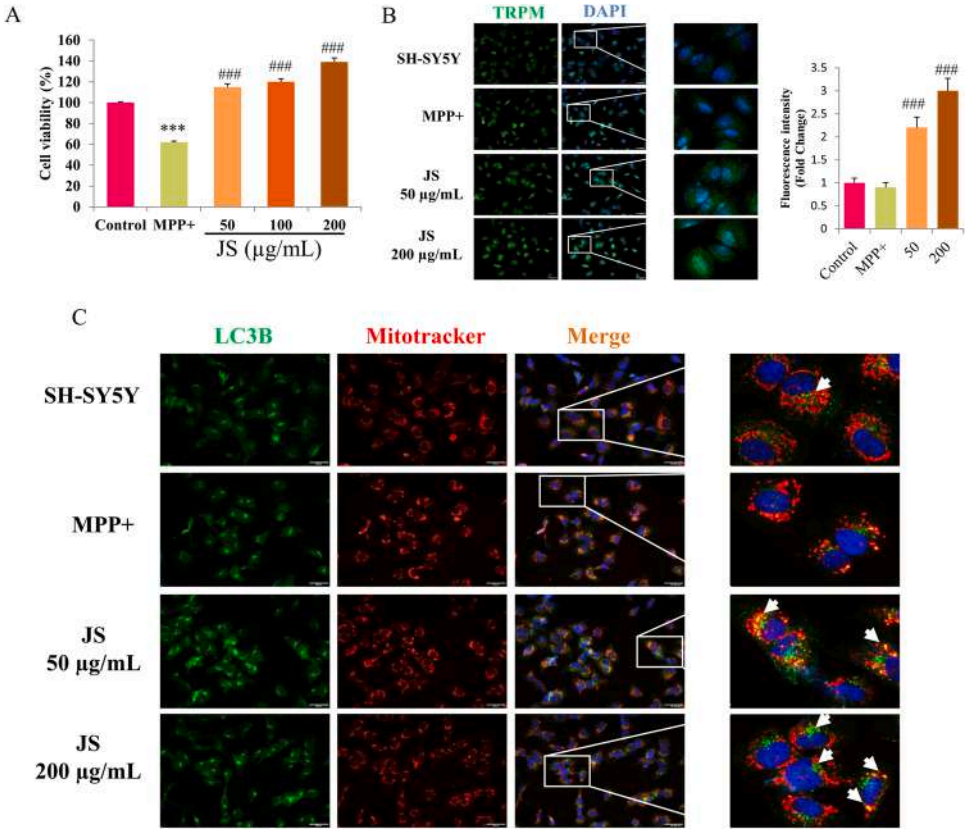

Fig. 6. JS triggers autophagic clearances in Neuroblastoma cells. (A) MTT assay showing cell viability upon MPP+ challenge and JS treatment with 50 µg/mL, 100 µg/mL, 200 µg/mL dosages. (B) Immunofluorescence staining for TRPM1 protein in SH-SY5Y cells challenged with MPP+ and those treated with JS. (C) LC3 immunofluorescence staining and mitochondrial staining with mitotracker to determine their co-localization. Nuclei were stained with DAPI in blue. ***P < 0.001 indicates significance when compared to control and ### P < 0.001 indicates significance when compared to MPP+ challenge.

3.6. JS enhances autophagic clearances in neuroblastoma cells

SH-SY5Y Neuroblastoma cells challenged with MPP+ are a commonly used in vitro model in Parkinson's disease research. MPP+ enters nigrostriatal neurons through dopamine transporters and it accumulates in the mitochondria that results in loss of mitochondrial functionality which triggers cell death [39]. MMP+ challenge in SH-SY5Y cells reduces the viability of the cells by almost 40% however treatment with JS significantly improved the cell survival. Administration of 50 μg/mL, 100 μg/mL and 200 μg/mL of JS suppressed the cytotoxic effects induced by MPP+ (Fig. 6A). Immunofluorescence assay for TRPML1, a lysosomal Ca^{2+} channel and a marker of autophagosome formation showed decrease in TRPML1 in cells challenged with MPP+, however upon treatment with JS enhanced the TRPML1 levels thereby promote autophagy (Fig. 6B). Further analysis on LC3B showed that JS administration increased their mitochondrial co-localization (Fig. 6C). Therefore, JS induces mitophagy in MMP+ challenged SH-SY5Y thereby maintains the mitochondrial quality in neuron cells and shows protective effects against neuro degenerative disease.

3.7. JS on body metabolic homeostasis

Defects in the Lipoprotein metabolism and accompanying insulin resistance are often associated with the aging process [40]. Polymorphisms in the Apolipoprotein-E (ApoE) gene affect lipoprotein transport and their metabolism and thereby alter serum lipid profile. The metabolic disorders of apolipoproteins are associated with the pathophysiological progression of Type-II diabetes. In order to find the effect of JS on metabolic homeostasis, an ApoE knockout mouse C57BL/6-Apoe^{em1Narl}/Narl generated with CRISPR/Cas9 technology was used. ApoE defective mice are known to develop Type-II diabetes upon feeding with high carbohydrate and lipid containing Western diet formula indicating their susceptibly to metabolic dysregulation [41]. Our results showed that 14 weeks of Western diet increased the fasting blood glucose significantly in C57BL/6-Apoe^{em1Narl}/Narl mice, however treatment with JS for 10 weeks improved blood glucose homeostasis and thereby showed reduction in fasting blood glucose levels (Fig. 7A). In addition, the diabetic rats showed reduction in the body weight whereas the treatment groups showed improvements in the body weight (Fig. 7B). In order to find if the increasing body weight in treatment groups is correlated with high amounts of diet intake, weekly amounts of diet consumption was recorded. Even though there was a considerable change in body weight among the diabetic mice groups the treatment groups showed no difference in the amount of diet uptake. The results therefore indicate the defective metabolic rate in the diabetic mice JS treatment effectively improved the metabolism in the diabetic mice and reversed the hyperglycemic effects.

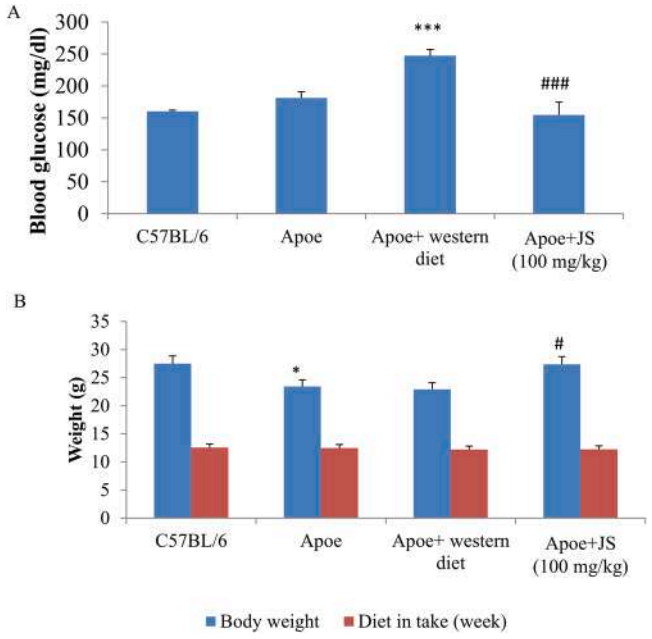

Fig. 7. JS regulates blood glucose levels. (A) Fasting blood glucose levels in control C57BL/6 mice and in Apoe$^{em1/Narl}$/Narl mice on western diet feeding and with JS treatment. (B) Weekly average of Body weight and diet uptake. * $P < 0.01$ and ***$P < 0.001$ indicate significance when compared to control and # $P < 0.01$ and ### $P < 0.001$ indicate significance when compared to diabetes challenge.

3.8. JS on cancer cells

While JS promotes stem cell population in adipose depots, experiments in cancer cells show inhibition of cancer cells, particularly their metastatic effects. JS showed a dose dependent inhibition on the viability of LoVo colon cancer cell lines. MTT assay showed that treatment with JS for 24 h showed reduction of viability in both Wild type LoVo cells and Oxaliplatin resistant LoVo cells (Fig. 8A). Interestingly JS showed a lower IC50 in resistance cells than the parental cells. The inhibition was also correlated with reduction in cell migration as observed from the trans-well assay particularly in higher doses (Fig. 8B). Moreover, treatment with JS increased the epithelial marker E-cadherin thereby shows an inhibitor effect on epithelial mesenchymal transition

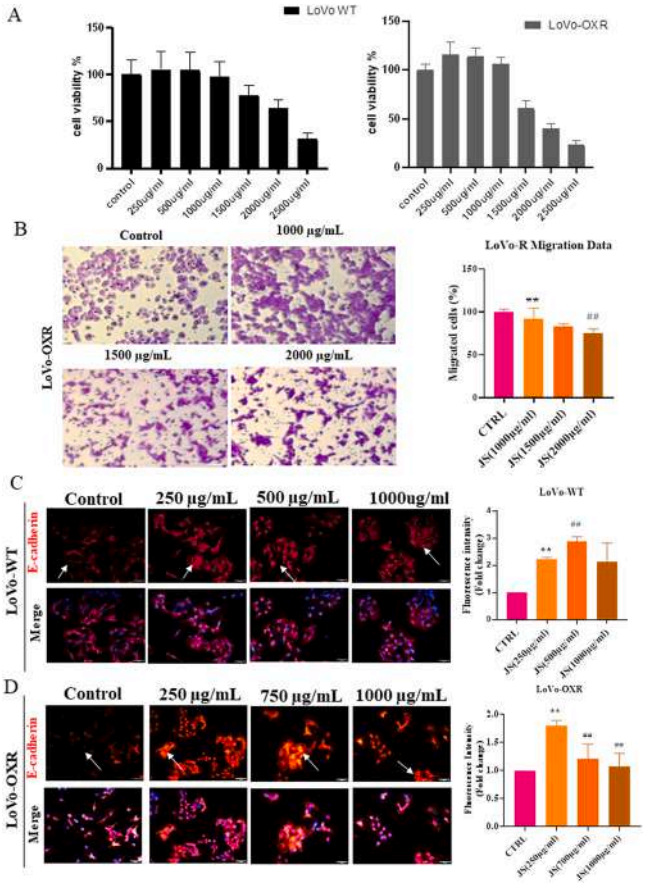

Fig. 8. JS attenuates cancer resistance and inhibits the EMT in LoVo cancer cells. (A) MTT assay was performed to calculate the growth inhibition rate of LoVo-WT and LoVo-OXR cells treated with JS for 24 h. (B) LoVo-OXR Cell migration ability was analyzed by trans-well migration assay. (C) Presence of E-Cadherin was detected in LoVo-WT and LoVo-OXR by Immunofluorescence staining. Nuclei were stained with DAPI in blue.

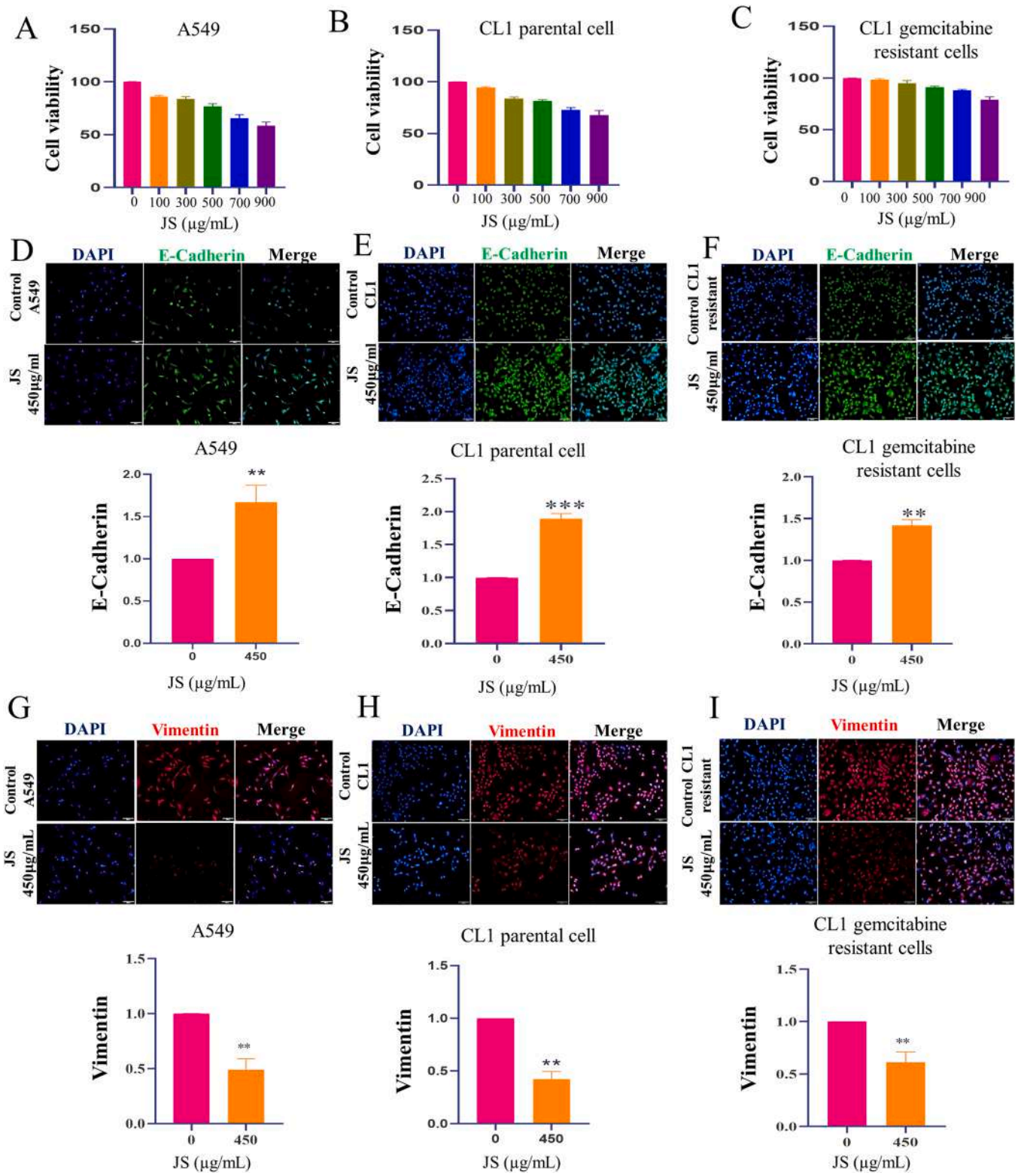

Fig. 9. JS inhibits the viability of Lung cancer cells and inhibits the EMT in lung cancer cells. Lung cancer cell lines A549 (A), CL1 (B), and Gemcitabine resistant CL-1 cells (C) were treated with different concentrations of JS for 36 h, and the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay was used to measure cell viability. (B) Immunofluorescence microscopy of E-cadherin (D–F) and vimentin (G–I) in human lung cancer A549, CL1 parent and CL-1 Gem Resistance cells. Lung cancer cells treated with JS (450 μg/mL) exhibited increased E-cadherin expression and decreased vimentin in all cell lines compared to the control cells. E-cadherin (green), vimentin (red), 4′,6-diamidino-2-phenylindole (DAPI, blue), and merged images (original magnification, ×200). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

(EMT) in parental LoVo-WT (Fig. 8C) as well as LoVo the resistant LoVo-OXR (Fig. 8D).

Similarly, JS administration in A549 cells, CL1–5 lung cancer parental cells and in gemcitabine resistant CL1–0 cells showed reduction in cell viability in a dose dependent manner (Fig. 9A–C). Similarly JS enhanced E-cadherine level (Fig. 9D–F) and reduced levels of mesenchymal marker vimentin in (Fig. 9G, I) A549 (Fig. 9D, G), CL1–5 lung cancer parental cells (Fig. 9E, H) and in gemcitabine resistant CL1–0 cells (Fig. 6F, I).

Further to investigate the effect of JS against formation of new metastatic growth in vitro, the anchorage-dependent colony formation and anchorage-independent embedded colony growth in soft agar was determined using parental and gemcitabine resistant CL1–0 lung cancer cells. The results show that the resistant cells showed higher colony formation ability compared to the parental cells. However treatment with JS effectively suppressed the colony formation effects in a dose dependent manner. Treatment with 500 μg/mL of JS suppressed the colony formation in the parental and the resistant cells in an identical level. In addition, the effect of JS on spheroid formation was determined in the CL1–0 cell model. JS suppressed the aggregation of both parent and resistant lung cancer cells. The area of the aggregated lung cancer cells was increased to 162% and 144% respectively in parent and gemcitabine resistant CL1–0 cell lines compared to that of controls (100%). However, the density of the cells was reduced to 24% and 35%, respectively in parental and the resistant cells compared to that in the control group (100%). Therefore, data show that the clonal spheroid formation and aggregation of the gemcitabine resistant lung cancer cells was effectively suppressed by JS treatment (Fig. 10).

4. Discussion

Global increase in life expectancy has attracted a major research focus on aging care. In this regard, it is imperative to formulate strategies that improve the healthy span of life among the aging population. As aging is a multi-facet process, pleiotropic drugs with multiple functions are ideal for anti-aging strategies. In this study, JS herbal extract, consisting of Eight Chinese herbal constituents, was found to possess multiple effects against aging associated disorders. The deterioration of tissue homeostasis in organs increases with aging which is potentially attributed to declining adult stem cell pool and their function [42]. In this study the anti-aging effects of JS against various aspects of aging such as loss of stem cell number, damaged protein and organ clearance, metabolic homeostasis and organ damages was investigated. While JS effectively enhanced stem cell potentials to overcome senescence-associated functional loss, it exhibited inhibitory effects on cancer cells. Moreover JS enhanced autophagy in neurons challenged by MPP+ induced mitochondrial damage, to improve the clearance of damaged organs and proteins. In addition JS attenuated aging associated cardiac modulations and suppressed metabolic imbalance in diabetic condition.

Aging is associated with increased risk factors for various diseases including diabetes and cancer [43]. While stem cells possess strong ability for self-renewal and differentiation into various different mature cell lineages upon certain physiological potentiation, they tend to lose the self-renewal and culminate terminal differentiation during aging. The loss of stem cell pool is also attributed to an increase in the demand to replace damaged cells in aging condition. Therefore enhancing the regenerative and survival potentials of stem cells in aging conditions equip the repair mechanisms against aging related loss of tissue homeostasis and function [44]. Moreover, Herbal extracts and phytochemicals exert beneficial effects by targeting diverse signaling mechanisms [45–47] and thereby provide wide biological benefits such as antioxidant [48], antimicrobial [49], anti-inflammatory [50], cytoprotective [37,51–56], cardio-protective, clearance of defective proteins [57], antiatherogenic [58] and anti-cancer effects [59,60]. Various studies have also revealed that phytochemicals promote stem cells to exert such beneficial effects against degenerative disorders [17,61–63]. Therefore phytochemical approaches to reverse aging stem cells may potentially enhance health promoting effects during aging conditions [64]. In this study a decoction of eight Chinese herbs show anti-aging effects in stem cells which could play better health promoting effects against various degenerative disorders. Presence of diverse dietary

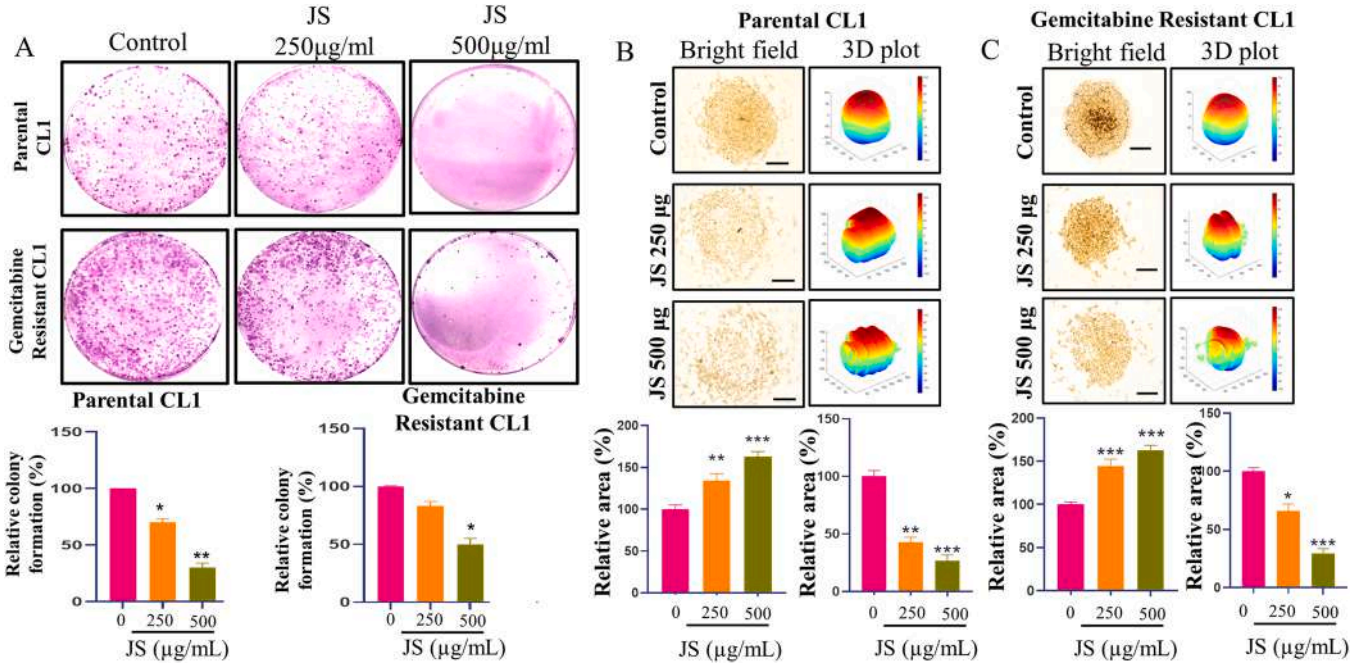

Fig. 10. Inhibition of cancer cell aggregation by JS in both parent and gemcitabine resistant human lung cancer cells. (A) Colony formation assay shows colony clones in parental CL1 and Gemcitabine resistant CL-1 cells treated with 250 μg/mL and 500 μg/mL JS. (B) Changes in the tumor spheroid morphology following JS treatment shown in bright field images and 3D plots. The total area and density of the spheroid were calculated using the ImageJ software. The asterisks indicate statistically significant differences compared to the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Scale bar indicates 100 μm.

phenolic compounds in herbal extracts has been known to play a major role for their potential pleiotropic effects against different chronic disorders associated with oxidative stress, inflammation process or aging [65]. Similarly the phenolics in the JS potentially deliver the observed pleiotropic effects. Moreover, specific effects of JS on hub genes such as AMPK or on anti-inflammatory and antioxidant mechanisms may contribute to their pleiotropic effects, as they activate and regulate various downstream effectors and regulatory proteins. Various herbs such as *Silybum marianum*, *Lippia citriodora*, *Hibiscus sabdariffa*, *Theobroma cacao* and *Olea europaea* have been previously known to display such multi targeting effects [66].

Cyclins are regulators of cyclin-dependent kinases (CDK) that control the phase transition in the cell cycle. In addition cyclin-dependent kinase inhibitors like p16 and p21 interrupt the CDK complex formation resulting in cell-cycle arrest and apoptosis. They are considered as markers of senescence associated DNA damage which triggers their overexpression [67,68]. In this study, the effect of JS in suppressing DNA damage response associated senescence mediator $p16^{INK4a}$ and damage response protein H2AX and its ability to enhance TERT expression and accumulation were determined by immunofluorescence staining. JS effectively attenuated the increasing levels of CDK inhibitors in ADMSCs in the old rats thereby counteracting age associated cell cycle arrest and apoptosis in stem cells.

Moreover, shortening of telomere length is considered as a major hallmark of aging. Telomeres consist of TTAGGG repeats and recruit proteins like the shelterin complex that maintains the telomere length [69,70]. The enzyme telomerase in humans is capable of maintaining the telomere length, particularly its catalytic subunit TERT. Somatic cells do not express telomerase and therefore are subjected to shortened telomeres with every replication cycle and are therefore a reason for their growth arrest or replicative senescence. Chemotherapeutic drugs such as doxorubicin inhibit the binding of TERT to their targeted region [71,72]. In our study doxorubicin administration in ADMSC resulted in aggravated decrease in telomere length in senescent ADMSCs. The phenomenon was the same in cultured human ADMSCs. However, treatment with JS restored the senescence effect.

For their differentiation abilities, synthesis of various paracrine factors and ease of access, these ADMSCs are considered for pharmacological applications. But ADMSCs, while being phenotypically similar among different age groups, their function deteriorates with aging [73,74]. However, improving their functions by preconditioning is seen to be a safe and effective strategy to improve rejuvenation and counter age associated diseases like Parkinson's disease, obesity, cardiac damages and cancer [75–83]. However, ADMSCs are often associated with elevated risk of malignancy [84–86], therefore a streamlined rejuvenating strategy to enhance the function of ADMSCs function with reduced risk is essential. In the present study we show that JS treatment enhances the self-renewal in adult stem cells however JS inhibits cancers therefore JS uptake may not be associated with any risks of oncogenesis.

Adult stem cells play a major role in maintaining the tissue homeostasis of an organism, exhaustion of stem cells is therefore a major hallmark of aging. Some of the hallmarks of aging in stem cells include telomere shortening, mitochondrial dysfunction, cellular senescence, loss of proteostasis, etc. In our study the JS treatment enhanced the autophagic removal of damaged mitochondria to maintain homeostasis. Further JS suppressed attenuated cardiac remodeling effects against hypertension associated Ang II challenge and doxorubicin challenge. Therefore JS may protect organs from stress associated damages thereby potentially help in tissue rejuvenation and homeostasis in aging conditions. In addition, JS restores normal glucose levels in $Apoe^{em1/Narl}$/Narl mice that are susceptible to diabetes.

This study mainly demonstrates the multi-pronged anti-aging effects of JS however it is not comprehensive in identifying the proper mechanism and the targets. Moreover, the pharmacokinetics of JS uptake and the metabolic transformations should be analyzed in order to find the bioavailability and to identify metabolites that actually exert the effect of JS. Further studies to demonstrate the pleiotropic effects in an appropriate aging model exhibiting multiple pathological phenomena are necessary to establish the available evidence.

In conclusion, JS exhibits pleiotropic effects to maintain stem cell quality in adipose depots and acts against organ damages and dysregulation in metabolism and oncogenesis. Therefore it is a potential agent for rejuvenation effects in aging treatment and to promote healthy aging.

CRediT authorship contribution statement

M.A.S., Y.-J.L., C.-Y.C., C.-Y.L. and C.-Y.H. conceptualized the study. D.G., N.S., S.A. and M.N.I. performed the experiments. M.A.S., Y.-J.L., C.-Y.C., C.-Y.L., B.-Y.L., S.-Z.L., T.-G.H., W.-T.T. and C.-Y.H. analyzed and interpreted the data. M.A.S. wrote the initial draft of the manuscript. W.W.K. and C.-Y.H. provided resources and validated the data.

Conflict of interest statement

None to declare.

Data availability

All the data associated with the manuscript is included in the manuscript and the supplementary files.

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