

RESEARCH ARTICLE

Electric Field Stimulation of Stem Cell Culture Media Increases Oxygen Radical Uptake Capacity

Maki Yoshikawa¹, Masatoshi Oshita², Tomomi Gotoh¹, Yukina Tawa², Katsuaki Dan^{3,*}

¹Headquarters, RMDC Inc., 1-3-2 Kyobashi Chuoku, Tokyo 104-0031, Japan

²Toyama Laboratory, RMDC Inc., 16 Shimono Toyama-shi, Toyama 930-0871, Japan

³Research Organization of Biological Activity, 6-23-4 Jingumae, Shibuya-ku, Tokyo 150-0001, Japan

*Corresponding author. Email: kdan@iyakushigen.jp

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Abstract

BACKGROUND: Stem cell culture medium is garnering attention because it comprises growth factors and exosomes secreted by stem cells. In this study, stem cell culture medium was subjected to electric field stimulation using the cells alive system (CAS) to determine the antiaging effects of this medium on dermal fibroblasts.

METHODS: The medium was added to the monolayer cultures of human fibroblasts with or without 100 µg/mL advanced glycation end products (AGE), and the cells were incubated for 4 hours. Total RNA was extracted, and mRNA levels of several AGE receptors and heat shock proteins were measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

RESULTS: Similar amounts of exosomes were extracted from the supernatants of stem cells cultured with (CAS+) and without (CAS-) electric field stimulation. The

results demonstrated that CAS+ had a higher oxygen radical absorbance capacity than CAS-, indicating its high antioxidant activity. Furthermore, the expression of domain-containing link scavenger receptor-1 mRNA, which is involved in the degradation of cell surface receptors for AGE under glycation stress, significantly increased. The electric field stimulation also increased the expression of several heat shock proteins, which play important roles in cellular responses generated against glycation stress. The electric field stimulation of the stem cell culture medium effectively promoted the endocytosis and degradation of AGE, thereby exerting antioxidant effects and inducing glycation stress.

CONCLUSION: Electric field stimulation of stem cell culture medium enhanced the expression of Hsp and its antioxidant activity in fibroblasts.

KEYWORDS: AGE, electric field stimulation, exosome, fibroblast, oxygen radical absorbance capacity, stem cell

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Introduction

In regenerative medicine, studies have been conducted using stem cells and umbilical cord serum. Although they are considered effective in wound healing and aging, they may be involved in canceration.(1-4) Stem cell replacement therapy is also a modality of regenerative medicine. This therapy is based on the basic principle of the homing effect, which involves the transmission of SOS signals from

damaged tissues in the body to induce regeneration and consequent accumulation of stem cells upon receiving these signals at the damaged site and regenerate into that tissue.(5) However, the process of harvesting tissues from patients and reintroducing stem cells after cell isolation and cultivation is technically challenging and invasive. Previous studies have shown that biologically active liquid substances secreted by stem cells, such as various growth factors and cytokines, as well as exosomes play an important role in regenerative processes.(6-8)

Exosomes are secreted by almost every cell in the body. Tumor-derived exosomes have been suggested to regulate tumor metastasis sites.(9,10) Exosomes also play an important role in the regeneration of tissues, such as wound healing.(11)

The three major contributors of aging are oxidation, glycation, and chronic inflammatory reactions. Antiaging effects in skin can be simply achieved through delaying and preventing the aging of skin cells, such as fibroblasts, keratinocytes, and melanocytes. Furthermore, the suppression of oxidation and glycation can prevent wrinkles, dark spots, dullness, and sagging.(12) In a pilot study, the degree of oxidation in food was measured using the oxygen radical absorbance capacity (ORAC).(13)

Glycation is an important aging process. Since dietary carbohydrates are the main source of energy for humans, it is impossible to prevent the glycation of proteins caused by blood sugar.(14) Advanced glycation end products (AGEs) are produced by the nonenzymatic chemical reaction of amino acid residues during protein glycation.(15) AGEs are an important regulator of aging in animals and have been linked to a variety of diseases. The concentration of AGE in dermal collagen increases with age, and its concentration was found to be higher in diabetic patients than in age-matched healthy controls.(16) Skin aging (17), Alzheimer's disease (18), hypertension (19), arteriosclerosis (20), and osteoporosis (21) have all been linked to the formation and accumulation of intracellular AGEs.

We previously found that mesenchymal stem cell-derived exosomes could provide defense against oxidative stress-induced epidermal and cellular senescence.(22) In addition, we reported that the expression level of several Hsps increased when skin fibroblasts were subjected to glycativ stress induced by AGEs.(23)

In this study, we collaborated with a company that owns the cells alive system (CAS), a high-voltage generator, and investigated the effects of cell culture fluids in which electric field stimulation was applied to the stem cell culture environment on skin aging. The results suggested that electrofield-stimulated stem cell cultures exerted an antiaging effect on the skin.

Methods

Cell Culture

Human mesenchymal stem cells from adipose tissue (HMC, C-12977) and normal human dermal fibroblasts from juvenile foreskin (NHDF, C-12300) were purchased

from PromoCell (Heidelberg, Germany) as dissociated and established cells. Cells were cultured at 37°C in a 5% CO₂ incubator using the corresponding dedicated medium (PromoCell), HMC medium (C-28019), and fibroblast growth medium (C-23010). DL-glyceraldehyde and hydrogen peroxide (H₂O₂) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and bovine serum albumin (BSA) of Fraction V was purchased from Sigma (St. Louis, MO, USA).

Preparation of BSA-AGE (AGE)

AGEs were prepared using a previously described method. (24) The experiments were performed using BSA (25 mg/mL) and 0.1 M DL-glyceraldehyde dissolved in phosphate-buffered saline (PBS, pH 7.4), and the solution was incubated at 37°C for 7 days.

Electric Field Stimulation of Culture Medium of Human Adipocyte-derived Stem Cells

Cells were incubated at 37°C in 5% CO₂. An electric field was created within the incubator using a high-voltage generator, CAS (Wi-Free, O's & Tec., Tokyo, Japan). Then cells were exposed to a voltage of approximately 1,500 V.

Quantification of Exosome

The amount of exosomes was measured using an enzyme-linked immunosorbent assay (ELISA) kit (EXH0102EL, Cosmo Bio, Tokyo, Japan). This kit can quantify exosomes in the cell culture supernatant having both CD9 and CD63 molecules on their surfaces. Exosomes were measured using the procedure mentioned in the kit, which was based on the principle of sandwich ELISA method.

ORAC Assay on Cultured Supernatants

ORAC values were measured using the H-ORAC measurement kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). According to the kit instructions, the fluorescence intensity (excitation, 485±20 nm; detection, 530±25 nm) was measured every 2 minutes for 90 minutes. A standard curve was constructed using Trolox solution. The ORAC value for each sample was determined by measuring, the Trolox equivalent antioxidant capacity using the standard curve. ORAC values (µM Trolox equivalent (TE)/g) were expressed as the amount (µM) of Trolox per gram of sample extracted with 30% ethanol.

Experimental Design

The culture medium was collected by culturing stem cells (50,000 cells/mL, 5 mL) with (CAS+) or without (CAS-)

electric field stimulation for 18 hours, and the amount of exosomes was measured. Culture media containing no stem cells (exosome-negative) with or without electric field stimulation served as controls. The obtained culture media were added to a single-layer culture of human fibroblasts with or without AGE (100 µg/mL), and cells were incubated for 4 hours. Total RNA was extracted to measure mRNA levels using quantitative RT-PCR (qRT-PCR).

Quantification of Exosomes in CAS+ and CAS- Supernatants

The PS Capture TM Exosome ELISA Kit (FUJIFILM Wako Pure Chemical Corporation) was used to quantify the amount of exosomes based on the kit protocol, which was based on the detection of the anti-CD63 antibody.

Total RNA Extraction

Total RNA was extracted using the Trizol® Reagent (ThermoFisher Scientific, Waltham, MA, USA.) protocol. The culture medium was removed from the petri dish, and the cell surface was washed with PBS. The solution was pipetted into a 1.5-mL test tube containing 1 mL of Trizol, and 200 µL of chloroform was added to the tube, which was then agitated and the tube was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was then transferred to a 1.5 mL test tube containing 0.5 mL of isopropyl alcohol, and the tube was then stirred. The tube was centrifuged again before the supernatant was discarded, and after adding 1.0 mL of ethanol, the contents were mixed by inversion. The tube was centrifuged again before the supernatant was discarded and 50 µL of ultrapure water (RNase-free) was added to the precipitate, and it was completely volatilized using ethanol to obtain a total RNA sample. One µL aliquot of this sample was quantified for RNA levels using a NanoDrop (ThermoFisher Scientific).

qRT-PCR

Somatic mRNA levels of AGE receptors (25-29), such as RAGE, FEEL-1 (Stabilin-1), FEEL-2 (Stabilin-2), CD-36, AGE-R1, AGE-R2, and AGE-R3, and heat shock proteins in NHDF, including Hsp104, gp96, Hsp90, Hsp70, Hsp60, and Hsp32, were measured using a one-step qRT-PCR method (Table 1). Further, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured as an internal control.

Total RNA (500 ng) was used as a template, and one-step RT-PCR for a simultaneous reverse transcriptase (RT) reaction and cDNA synthesis in a single tube were performed using the Luna Universal One-Step RT-qPCR Kit. The PCR

was conducted using the Thermal Cycler Dice Real Time System II (Takara Bio, Shiga, Japan). The PCR reaction was performed using 10 mL of the Luna Universal One-Step Reaction Mix (2×), 1 µL of the Luna WarmStart RT Enzyme Mix (20×), 0.8 µL of a 10 µM forward primer, 0.8 µL of a reverse primer, and template RNA; the final volume was adjusted to 20 µL using nuclease-free water. Each reaction involved 1 cycle that was performed at 95°C for 30 seconds, followed by 50 cycles performed at 95°C for 5 seconds and at 60°C for 30 seconds. qRT-PCR was performed using target and housekeeping genome (GADPH) primers (Table 1). Changes in mRNA expression were considered significant when there was at least a fourfold change (two PCR cycles).

Statistical Analysis

Data analysis was carried out using SPSS software (IBM Corporation, Armonk, NY, USA). The results are presented as the mean±standard deviation. Statistical analysis was performed using one-way analysis of variance and Student's *t* tests, and *p*<0.05 was considered significant.

Results

Measurement of the Amount of Exosome in Culture Medium

We quantified exosomes in the culture medium where stem cells (50,000 cells/mL, 5 mL) were cultured with (CAS+) or without (CAS-) electric field stimulation for 18 hours. The amount of exosome was 122±8.3 pg/mL and 125±10.8 pg/mL in CAS+ and CAS- supernatants, respectively, and there was no difference between the two.

ORAC of Cultured Supernatants

ORAC values were expressed as the amount of Trolox (µM) in supernatants, which was calculated using a standard curve of Trolox solution. The ORAC of CAS+ supernatant (3.0 mM TE/L) was 0.1 mM TE/L higher than CAS- supernatant (not significant).

Expression of AGE Receptor mRNA

The effects of glycation stress were examined by adding AGE to human fibroblasts treated with CAS+ and CAS- supernatants and measuring changes in mRNA levels of cell surface AGE receptors. The results revealed that FEEL-1 expression was upregulated in human fibroblasts treated with CAS+ supernatant, and glycative stress significantly increased FEEL-1-mRNA level (Figure 1).

Table 1. Primers for PCR.

Gene	Primer	References or NCBI Reference Sequence
RAGE:	Forward; GAA ACT GAA CAC AGG CCG GA Reverse; CAC GGA CTC GGT AGT TGG AC	24
FEEL-1 (Stabilin-1)	Forward; AGG ACT GCC GCT ACG AAG TA Reverse; CAC TGC CCT GCT GTG TGT AG	24
FEEL-2(Stabilin-2)	Forward; TCT GAA GGC AGG TCT CAC CTA Reverse; CTG GGG AGC AGA AAT TTT GTA	25
CD-36	Forward; GAG AAC TGT TAT GGG GCT AT Reverse; TTC AAC TGG AGA GGC AAA GG	26
AGE receptor-1	Forward; GTG GGA AAA TGG CAC AAC TT Reverse; CTG GCC ACG TCC CTA TTT TA	27
AGE receptor-2	Forward; AGG GCC GTA AGG AGA GAG AG Reverse; GTG GCG TCT GTC TGT GTG TC	28
AGE receptor-3	Forward; GAT AAC AAT TCT GGG CAC GG Reverse; TGG AGC ACT GGT GAG GTC TAT G	28
Hsp-104	Forward; TCA TCG ACA AGG ACA GCA AG Reverse; GGC TGT GAG GAG GTA AGC AG	NM_0012583
gp96	Forward; TGG GAA GAG GTT CCA GAA TG Reverse; GTT GCC AGA CCA TCC GTA CT	AJ890084.1
Hsp-90	Forward; TGG ACA GCA AAC ATG GAG AG Reverse; AGA CAG GAG CGC AGT TTC AT	BC121062
Hsp-70	Forward; AGT GGT GGC CAC TAA TGG AG Reverse; CAA TCC TTG CTT GAT GCT GA	BC112963
Hsp-60	Forward; GCA ATG TGT CCA GAG CAA GA Reverse; AAG CTC AAC AGC TGG GAA AA	AF143723.1
Hsp-32	Forward; TCC GAT GGG TCC TTA CAC TC Reverse; TAA GGA AGC CAG CCA AGA GA	NM_002133.2
GAPDH 161-162	Forward; AGG GCT GCT TTT AAC TCT GGT Reverse; CCC CAC TTG ATT TTG GAG GGA	24

The primers mentioned in Table 1 were designed to investigate the changes in AGE receptors present on the cell surface and corresponding intracellular heat shock protein-mRNA when fibroblasts were subjected to AGE-induced glycation stress. RAGE: receptor for advanced glycation end products; FEEL-1 (Stabilin-1): a homeostatic scavenger receptor with multiple functions; FEEL-2 (Stabilin-2): hyaluronan receptor for endocytosis; CD-36: cluster designation-36; AGE receptor-1: advanced glycation end products receptor-1; AGE receptor-2: advanced glycation end products receptor-2; AGE receptor-3: advanced glycation end products receptor-3; Hsp-104: heat shock protein-104; gp96: glycoprotein 96; Hsp-90: heat shock protein-90; Hsp-70: heat shock protein-70; Hsp-60: heat shock protein-60; Hsp-32: heat shock protein-32; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Hsp mRNA Expression

The effects of electric field stimulation on stress responses were investigated by measuring the expression of Hsp mRNA. We specifically measured the levels of Hsp that react in cells exposed to stress such as heat, ultraviolet rays, and active oxygen. Hsp104, gp96, Hsp70, and Hsp32 levels were significantly higher in CAS⁺ cells than in CAS⁻ cells (Figure 2).

Discussion

To stimulate stem cells, the stem cell culture medium was subjected to an electric field generated by a high-voltage generator, CAS. Although the difference between CAS⁺ and CAS⁻ supernatants was not significant, the ORAC of CAS⁺ was higher because that of onions as an antioxidant food,

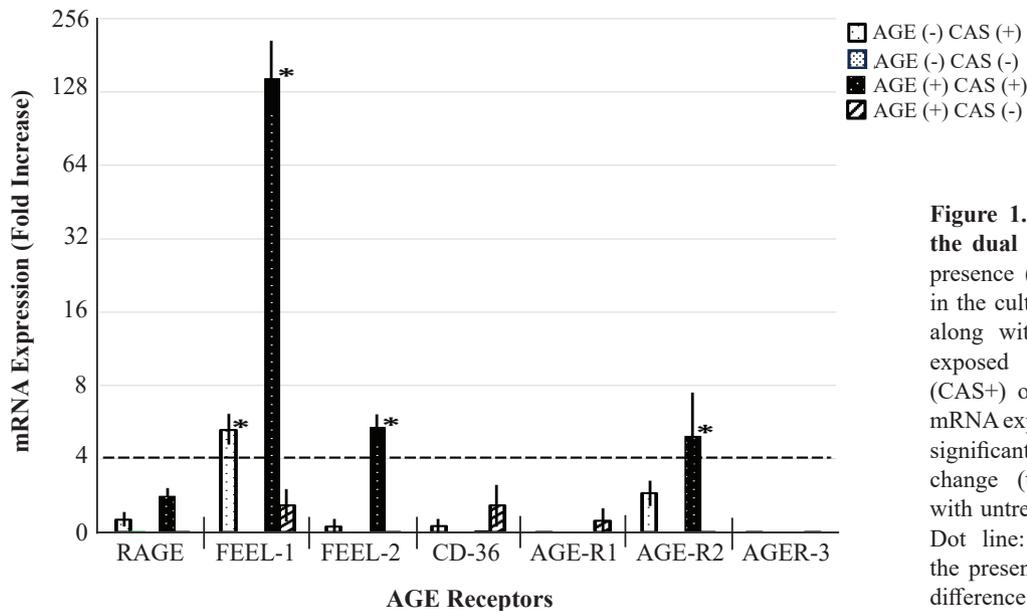


Figure 1. mRNA expression levels of the dual roles of AGE receptors. The presence (+) and absence (-) of AGEs in the culture medium have been shown along with whether the medium was exposed to electric field stimulation (CAS+) or not (CAS-). *: Changes in mRNA expression levels were considered significant if there was at least a 4-fold change (two PCR cycles) compared with untreated mRNA expression levels. Dot line: A borderline that separates the presence or absence of a significant difference (4 or more is significant).

which exhibit antioxidant activity, is 1.7 mM TE/L.(13) It was thought that it enhanced the ability to take in oxygen radicals. There are two types of AGE receptors: those that activate intracellular signaling and cause the accumulation of reactive oxygen species (ROS) during inflammation and those that cause the degradation and removal of AGE (Figure 3). To delay and prevent cell aging, it is crucial to overcome the accumulation of ROS and promote the degradation and removal of AGE. AGE receptor (RAGE) and AGE receptor 2 (AGE-R2) are involved in the former type of receptors, whereas ink domain-containing scavenger receptor-1 (FEEL-1), FEEL-2, AGE-R1, AGE-R3, and CD36 are the latter type of receptors.

Our results showed that FEEL-1-mRNA level in fibroblasts was elevated in CAS+, and the level was further elevated under glycativ stress. FEEL-1 is involved

in the endocytosis of extracellular AGE for lysosomal degradation.(30) An increase in FEEL-1-mRNA level can promote the intake of extracellular AGEs by cells, which are then sent to lysosomes and degraded. This finding indicated that electric field stimulation exerts an antiaging effect by reducing the glycativ stress in the skin.

Hsp104 is involved in the disaggregation of proteins (31), gp96 is involved in antigen presentation (32), and Hsp90 promotes the maturation of specific proteins (33). Similarly, Hsp70 can control the quality suppressing protein folding, transport, and degradation (34,35), Hsp60 maintains protein folding in mitochondria and regulates the membrane permeability of proteins in mitochondria (36), and Hsp32 is involved in heme degradation, which generates antioxidant products (37). Our results showed

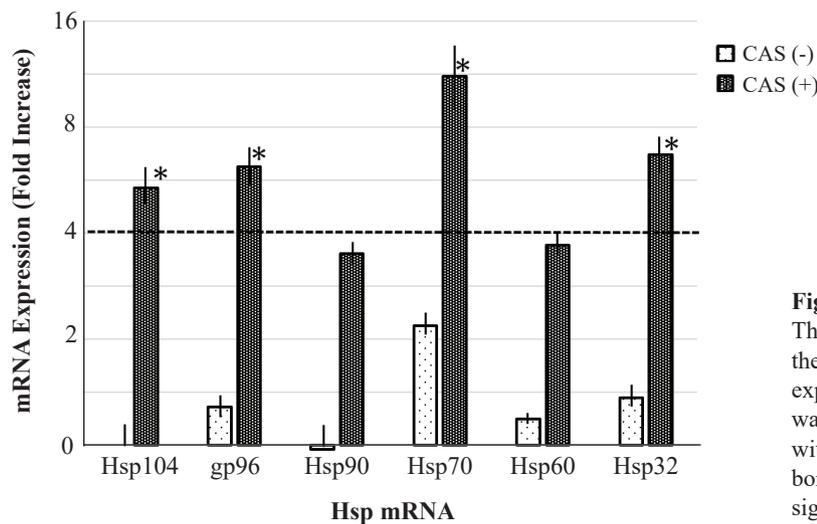


Figure 3. mRNA expression levels of several Hsp. They are shown with (CAS+) or without (CAS-) during the electric field stimulation. *: Changes in mRNA expression levels were considered significant if there was at least a 4-fold change (two PCR cycles) compared with untreated mRNA expression levels. Dot line: A borderline that separates the presence or absence of a significant difference (4 or more is significant).

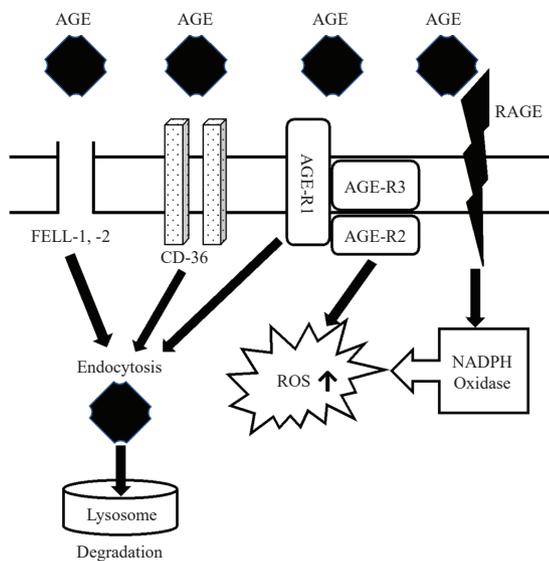


Figure 3. AGE receptors expressed in normal human dermal fibroblasts and cell signaling pathways. It has two roles: endocytosis of extracellular AGEs into cells for subsequent lysosomal degradation (AGE-R1, FEEL-1 and -2, CD-36, etc.) and direct or indirect increase of intracellular ROS levels through activation of NADPH oxidase (receptors such as AGE-R2, AGE-R3 and RAGE).

that mRNA levels of Hsp104, gp96, Hsp70, and Hsp32 were significantly elevated in CAS+ supernatant.

These results suggest that electric field stimulation increased the level of stress responses in fibroblasts, especially those involved in protection of cells and control of proteins. In the field of antiaging, it is now being considered that, rather than supplementing stem cells, treatments should be performed using exosomes derived from stem cells. Although we could not show whether electric field stimulation can promote the growth of fibroblasts and wound healing, which have been reported as the benefits of using stem cells and cord blood serum (1,3,4), we demonstrated the possibility that electric field stimulation can exert antiaging effects, independent of the amount of stem cells and exosomes. Electric field stimulation may have caused changes in stem cells, stem cell-derived extracellular vesicles represented by exosomes, and small molecules (amino acids, growth factor, mRNA, microRNA, peptides etc.) (Figure 4). Further study to clarify the effects of oxidative stress such as H₂O₂ stimulation and the effects of electric field simulations is necessary.

Conclusion

The current findings demonstrated that electric field stimulation of stem cell culture media can have antioxidant

effects. The analysis of mRNA levels also revealed that CAS+ stem cell culture media induced endocytosis and subsequent degradation of AGE, which causes glycation stress, in dermal fibroblasts. This study indicated that electric field stimulation enhances these effects and increases the level of stress response by inducing the expression of Hsp.

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Authors Contribution

All authors contributed to this work. MY designed and planned the research. The experiments were carried out and the results were analyzed by all of the authors. Furthermore,

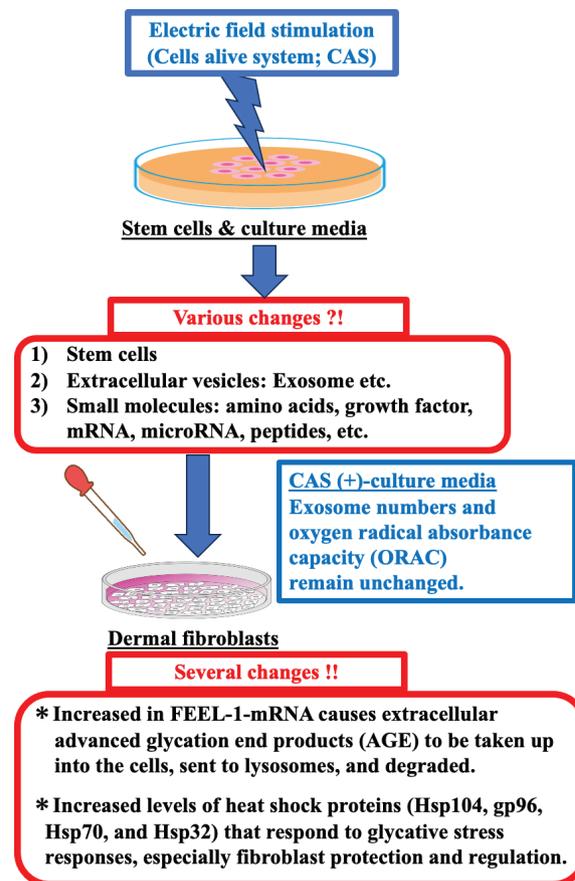


Figure 4. A scheme representing electric field stimulation of stem cell culture medium improves oxygen radical uptake capacity.

all authors interpreted the results and designed the research strategy. The manuscript was primarily prepared by KD and MY. The final manuscript was read and approved by all authors.

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