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Hemp seed extract as a catalyst for cell proliferation via IGF-1 and growth hormone signaling: Increased natural regenerative potential of muscles

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Abstract. Hemp seeds provide a nutritious food source. They contain proteins and essential minerals such as phosphorus, potassium, magnesium, iron and zinc. These nutrients contribute to health benefits, including the support of cardiovascular health, blood pressure regulation and boosting immune function. To the best of our knowledge, however, their influence on cell proliferation has not been determined. The present study examined the biological effects of hemp seed extract on cell proliferation. The present study examined the biological effects of hemp seed extract on cell viability by evaluating its cytotoxicity in mouse muscle (C2C12) and embryonic (C3H10T1/2) cells, and identified 200 μg/ml as the optimal treatment concentration. At this concentration, growth factor protein levels were elevated, and the phosphorylation of the downstream JAK2/STAT5b signaling pathway was increased. The extract promoted phosphorylated STAT5b binding to insulin-like growth factor (IGF)-1 DNA without causing significant DNA damage. These results suggest that hemp seed extract supports muscle regeneration by stimulating IGF-1 signaling via the JAK2/STAT5b pathway.

Introduction

Insulin-like growth factor (IGF)-1 plays a crucial role in human growth and is related to the action of growth hormone (GH). Moreover, IGF-1 regulates growth, glucose uptake and protein metabolism (1). IGF-1 is released by the liver by the action of GH (2). IGF-1 promotes cell proliferation by mediating the effects of GH and, through a negative feedback mechanism, inhibits GH release by suppressing GH-releasing hormone (3). IGF-1 is particularly abundant during adolescence. The growth-promoting effects of GH are mediated by IGF-1 through activation of downstream signaling pathways via the IGF-1 receptor (IGF-1R) (4). Although rare, a lack of the IGF-1 gene or GH results in muscle development disorder and impaired growth (5). Thus, IGF-1 and GH have important functions as growth factors.

STAT proteins function as upstream transcriptional regulators of IGF-1 in-response to GH stimulation, while also serving as downstream effectors in IGF-1R-mediated signaling, and STAT is activated via phosphorylation by various factors and cytokines (6). This protein translocates to the nucleus and binds to its promoter site (7). Phosphorylated (p)STAT proteins bind in a dimeric form to the IGF-1 promoter, thereby regulating the expression of IGF-1 mRNA (8). In addition to STAT1, STAT2 and STAT5B bind and influence GH (9). STAT5B serves a pivotal role in the growth-promoting effects of GH, particularly in GH signaling, osteoblast differentiation and the maintenance of bone homeostasis (10,11).

Hemp seed (HS) was first cultivated in China and are nutritious, containing ~25% protein and 35% oil-based antioxidants (12,13). Previously, HS was crushed or consumed whole, as well as being used as an important grain in traditional food and medicines (12). In addition, the nutritional benefits of HS have long been recognized in Asia and Eastern Europe, where they have been used as food for both humans and livestock (12). Hemp is more commonly consumed as marijuana rather than as HS, and this has an association with addiction, which leads to negative perceptions (14,15). However, HS is increasingly recognized for benefits (16-18). HS is rich in fatty acids, minerals, vitamins and essential amino acids (12).

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Several studies have reported their positive effects on cardiovascular disease, cancer, inflammatory conditions, atopic dermatitis and antimicrobial activity (19-22). To the best of our knowledge, however, there are no studies on the effects of HS on GH or growth outcomes.

C2C12 cells were selected as they are a well-established *in vitro* model for skeletal muscle biology, capable of differentiating into myotubes and widely used for investigating the GH-IGF-1 axis, muscle hypertrophy and regeneration (23,24). C3H10T1/2 cells were selected for their multipotent differentiation potential into myogenic, osteogenic and chondrogenic lineages (25). These cells are used to assess early-stage proliferation and growth factor responsiveness prior to lineage commitment (26). HS extracts and their bioactive compounds promote myoblast differentiation (27). Recent studies have demonstrated that hemp extracts improve muscle health by regulating muscle protein degradation pathways (27,28). Hemp-derived compounds have been shown to promote the proliferation of hair follicle dermal papilla stem cells through genetic regulation, cell differentiation and modulation of the immune system (29). Based on this, it was hypothesized the active components in HS extract promote cell proliferation via cellular signaling pathways.

Materials and methods

Antibodies and reagents. Edible HS (origin: Canada) were purchased from a NongHyup market in the Republic of Korea and extracted with 50% ethanol for 48 h at room temperature to obtain the HS extract. DMEM, penicillin-streptomycin, trypsin-EDTA (0.05%) and fetal bovine serum (FBS; cat. no. A5670801) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Primary antibodies against pSTAT5 (cat. no. #9351), JAK2 (cat. no. #3230), pJAK2 (cat. no. #3776), pIGF-1Rβ (cat. no. #3012s) and cleaved caspase3 (cat. no. #9661s) were obtained from Cell Signaling Technology, Inc. IGF-1 antibody (cat. no. ab9572) was purchased from Abcam. Antibodies against β-actin (cat. no. sc-47778), GHR (cat. no. sc-137185), IGF-1Rβ (cat. no. sc-713) and STAT5b (cat. no. sc-1656), as well as HRP-conjugated secondary antibodies (goat anti-mouse and rabbit IgG; cat. nos. sc-516102, sc-2357), were obtained from Santa Cruz Biotechnology, Inc. JAK2 inhibitor AG490 (cat. no. 658411; Sigma-Aldrich, Merck KGaA) was used at 25 µM for 24 h at 37˚C in a humidified 5% CO_2 incubator during the western blot analysis to confirm the involvement of the JAK2/STAT5b pathway.

Cell viability assay. The mouse muscle and embryo cell lines C2C12 and C3H10T1/2 (cat. nos. CRL-1772 and KCLB 10226, respectively; both American Type Culture Collection) were seeded in 96-well plates ($5x10^3$ cells/well) and incubated for 24 h at 37˚C. HS extract (10-600 µg/ml) was added for another 24, 48 and 72 h at 37˚C in 5% CO_2. MTT reagent was applied and incubated for 4 h. The purple formazan was dissolved in dimethyl sulfoxide. Absorbance at 560 nm was measured to assess cell viability. All experiments were performed in triplicate.

Cell culture. The cells were cultured in DMEM with 10% FBS and 1% penicillin at 37˚C in 5% CO_2.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was conducted using the Imprint® ChIP kit (Sigma-Aldrich; Merck KGaA cat. no. CHP1) following the manufacturer's protocol at 4˚C. C2C12 and C3H10T1/2 cells were cross-linked with 1% formaldehyde and quenched with 1.25 M glycine for 5 min. Following lysis, chromatin was sonicated (25% amplitude, 30 sec on/off for 20 min on ice) and centrifuged at 21,000 x g for 10 min at 4˚C. The supernatant was diluted with the dilution buffer (1:1) and 5 µl aliquots diluted samples were used as internal controls. The supernatant was immunoprecipitated with 5 µl anti-STAT5b antibody (cat. no. sc-1656, Santa Cruz Biotechnology, Inc.), while controls used 1 µl normal goat IgG and 1 µl anti-RNA polymerase II. The amount of lysate used per immunoprecipitation reaction was ~100 µl, Bound DNA–protein complexes were treated with 1 µl Proteinase K for cross-link reversal and DNA release. Washing was performed with the wash buffers provided in the kit. Bound DNA was purified and analyzed by quantitative PCR using 10 µl TB Green Advantage Premix (Takara Bio, cat. no. 639676) using IGF-1 primers (forward: 5'-TGCTCACAAACCCACATCAA-3' and reverse: 3'-GCTAGGTTCTTCACAGCTCC-5'). Thermocycling was performed with 40 cycles at 95˚C (30 sec), 60˚C (30 sec) and 72˚C (40 sec), followed by final extension at 72˚C for 5 min. The calculations were performed using the 2-ΔΔCq values obtained (30).

Comet assay. C2C12 and C3H10T1/2 cells were seeded in 6-well plates at a density of $1x10^5$ cells/well and cultured for 24 h at 37˚C in 5% CO_2. Control cells were cultured under the same conditions without HS treatment. DNA damage was assessed using the Comet Assay kit (Abcam; cat. no. ab238544) according to the manufacturer's protocol. Cell morphology was analyzed using a fluorescence microscope (Olympus Corporation IX71/DP72).

DAPI staining. C2C12 and C3H10T1/2 cells were seeded in 6-well plates ($1x10^5$ cells/well) and cultured for 24 h at 37˚C. The cells were then washed with PBS and fixed with 100% methanol for 10 min at room temperature. After fixation, the cells were washed twice with PBS and incubated with 1 ml of 5 µM DAPI staining solution for 15 min at room temperature. Cells were then washed twice with PBS and images were captured using a fluorescence microscope (Olympus Corporation IX71/DP72)

ELISA. C3H10T1/2 cells were cultured ($1x10^6$ cells/ml) for 24 h at 37˚C in DMEM containing 100 or 200 µg/ml HS extract. IGF-1 levels in the culture supernatant were measured using a mouse IGF-1 ELISA kit (Abcam, cat. no. ab100695) according to the manufacturer's instructions.

Total cell lysis and western blotting. Whole cell lysates were prepared from untreated cells or cells treated for 24 h at 37˚C with 100 or 200 µg/ml HS using RIPA buffer (Gibco; Thermo Fisher Scientific, Inc.) supplemented with protease and phosphatase inhibitors. Cell lysates were incubated for 30 min on ice and total protein concentration was measured using the Bradford assay (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 µg/lane) were separated by 6-15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes

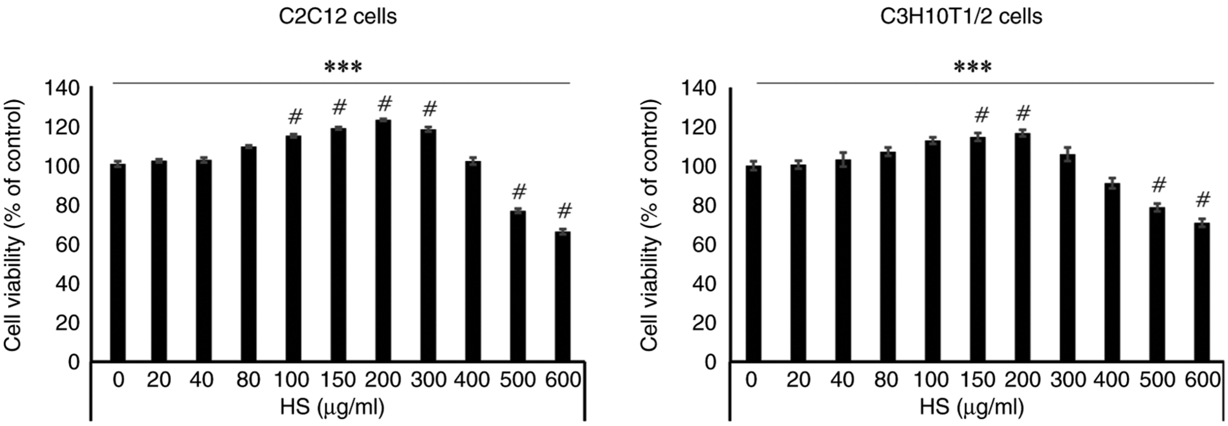

Figure 1. HS extract enhances the viability of C2C12 and C3H10T1/2 cells. MTT assay showed that HS extract increased cell viability by 20% over 24 h at a concentration of ~200 µg/ml. ***P<0.001; $^{\#}$P<0.001 vs. 0. HS, hemp seed.

were blocked with TBS-T buffer (0.1% Tween-20) and blocked with 5% skimmed milk (BD Biosciences; cat. no. 90002-594) prepared in TBS-T buffer for 1 h at room temperature. The membrane was incubated overnight at 4˚C with primary antibody diluted in 5% skimmed milk, followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Femto Clean ECL, GenDEPOT, LLC; cat. no. 77449) and captured with a LAS-4000 imaging system (FUJIFILM Corporation). Densitometric analysis was performed using ImageJ software (version 1.53; National Institutes of Health).

Statistical analysis. All data are presented as the mean ± SEM. All experiments were performed in triplicate and analyzed using one-way ANOVA followed by post hoc Tukey's test was used to evaluate differences among groups. Prior to analysis, data were tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. All statistical analyses were performed using SAS software (version 9.3, SAS Institute Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

HS enhances C2C12 and C3H10T1/2 cell viability in a concentration-dependent manner. HS was extracted in 50% ethanol and allowed to react for 48 h. Following filtration, only the pure liquid was used. To evaluate the effect of HS on cells, MTT assay was performed using C2C12 cells. There were significant differences between cells treated with HS concentrations ranging from 100 to 600 µg/ml. HS exhibited no cytotoxicity up to a concentration of 500 µg/ml. However, at concentrations >500 µg/ml, cell viability decreased, indicating cytotoxicity. Therefore, the optimal concentration for subsequent cell experiments was set at 200 µg/ml (Figs. 1 and S1).

HS (200 µg/ml) exhibits no cytotoxicity. When cells were treated with HS at a concentration of 200 µg/ml, there was a marked increase in the number of stained nuclei, indicating enhanced cell proliferation. By contrast, when cells were treated with HS at 500 µg/ml, the number of nuclei notably decreased, confirming cytotoxicity (Fig. 2A). Treatment with high concentrations of HS may lead to DNA damage (DNA unwinding) (31); therefore, comet assay was performed to determine whether HS induces DNA damage. Fluorescence microscopy revealed that treatment with 500 µg/ml HS increased the length of comet tails and the number of comet-positive cells, whereas no comet tails were formed in the untreated control and 200 µg/ml HS groups (Fig. 2B), consistent with the absence of detectable DNA damage at the optimal concentration. High concentrations of HS extract (500 µg/ml) increased cleaved caspase-3 levels, indicative of apoptosis induction (Fig. S2). These results indicated that HS at 100-300 µg/ml was not cytotoxic and promoted cell viability.

HS increases the expression of GHR and IGF-1 via the JAK2/STAT5b pathway. Western blotting was performed to investigate the cell signaling mechanisms. The protein levels of pJAK2 and pSTAT5 in C2C12 and C3H10T1/2 cells were upregulated by HS treatment in a concentration-dependent manner. Furthermore, the phosphorylation of JAK2 and STAT5b was induced, thereby activating the cellular proliferation signaling pathway (Figs. 3A and S3A and B). Furthermore, the protein levels of GHR, IGF-1, and pIGF-1Rβ were also increased by HS treatment in a concentration-dependent manner (Fig. 3B). The present study investigated the effect of HS on the binding of STAT5b to the IGF-1 promoter region. ChIP analysis using a STAT5b antibody revealed that HS at 100-200 µg/ml increased the binding of STAT5b to the IGF-1 promoter. STAT5b/IGF-1 complex formation was induced, resulting in the activation of downstream transcription (Fig. 3C). These findings suggest that pSTAT5 and IGF-1 are key targets of HS, with their expression enhanced to upregulate IGF-1 transcription. To validate that STAT5b activation is mediated via JAK2, cells were treated with JAK2 inhibitor. The inhibition of JAK2 markedly reduced STAT5 phosphorylation and downstream IGF-1 expression in C3H10T1/2 cells (Fig. S4), confirming that HS-induced STAT5b activation was dependent on JAK2 signaling. Overall, the results indicated that HS may function as a stimulator of key GH-associated cell signaling. ELISA further confirmed that HS treatment at 200 µg/ml significantly increased IGF-1 secretion in C3H10T1/2 cells (Fig. S5), supporting the upregulation of IGF-1 expression observed in the western blot analysis.

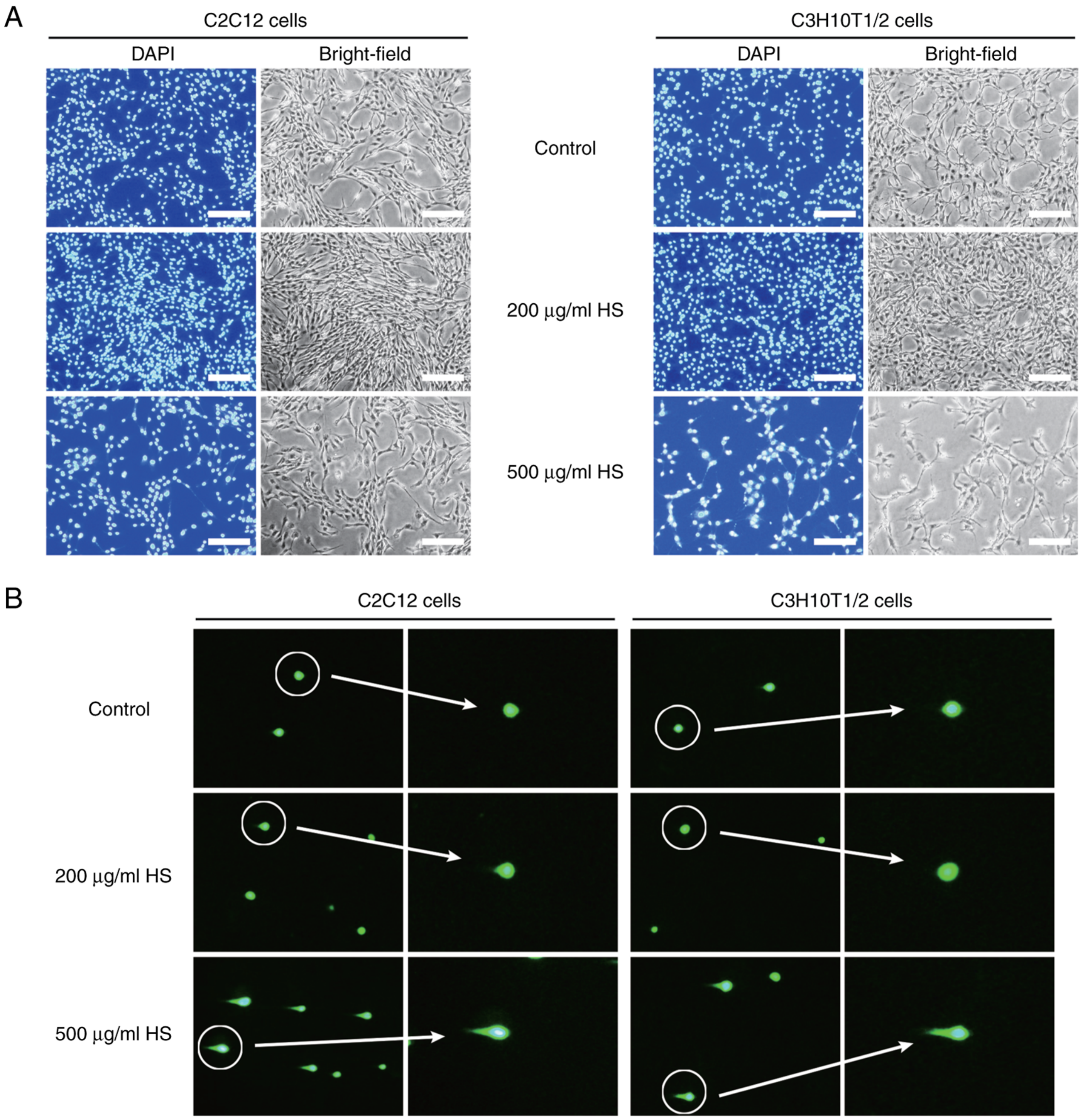

Figure 2. HS extract does not cause cytotoxicity at a concentration of 200 μg/ml. (A) Normal and abnormal nuclear staining induced by 24 h treatment with HS extracts at concentrations of 200 or 500 μg/ml in C2C12 and C3H10T1/2 cells, respectively. Scale bar, 100 μm. (B) Comet assay images from fluorescence microscopy analysis at X10 and X40 magnification showed fragmented DNA migration from the nucleoid body that formed a comet tail. Circle, cells with fragmented DNA forming comet tails in the comet assay. Cells treated with HS extracts at a concentration of 200 μg/ml showed no DNA migration. HS, hemp seed.

Discussion

Cell proliferation studies have primarily focused on GH (32,33). One of the key upstream regulators of GH is IGF-1, which binds IGF-1R to enhance GH activity, thereby influencing growth and development (34). This phenomenon occurs in numerous types of tissue and involves the continuous secretion of these factors to maintain homeostasis, although differences arise depending on the specific receptors involved (35,36). The benefits of IGF-1 include building muscle and bone mass, preventing muscle wasting, helping with growth, regulating blood sugar levels and protecting against neurological disorders. IGF-1 increases skeletal muscle protein synthesis via the PI3K/Akt/mTOR and PI3K/Akt/glycogen synthase kinase 3 β pathways (37). The dangers of IGF-1 include potentially increasing the risk of developing certain types of cancer (including breast, prostate, colorectal and thyroid cancer) and reducing life span (38).

The present study used C2C12 cells as a committed myogenic model responsive to GH/IGF-1 via the JAK2/STAT5b/IGF-1 pathway (35). C3H10T1/2 cells, as multipotent mesenchymal progenitors capable of differentiating into osteogenic, chondrogenic or, under certain conditions, myogenic lineages, were used to examine early

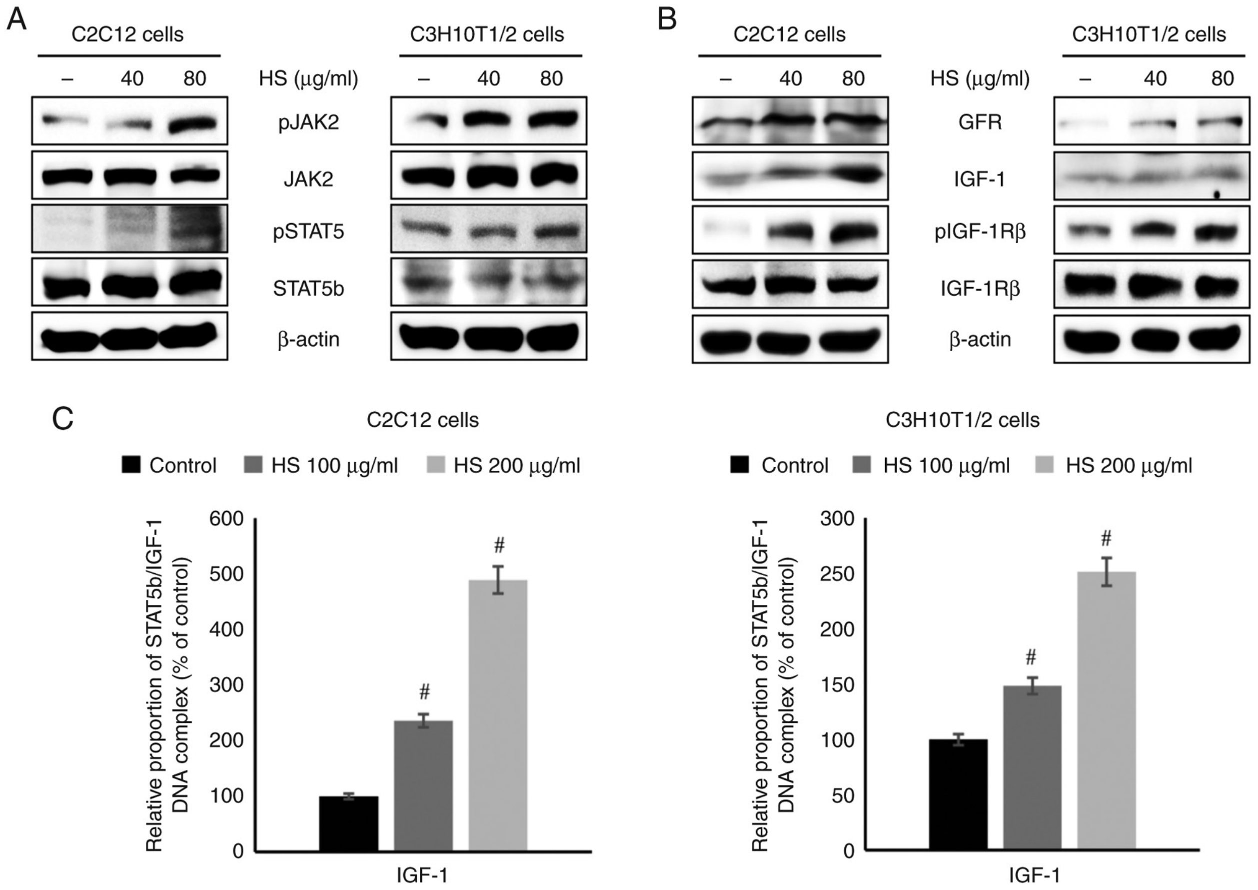

Figure 3. HS extracts enhances expression of nuclear pSTAT5b and its binding to the IGF-1 promoter. (A) Western blotting showing the protein expression of JAK2, pJAK2, STAT5b and pSTAT5 following the treatment of C2C12 and C3H10T1/2 cells with 100 and 200 μg/ml HS extract for 24 h. (B) Western blotting showing the protein expression of GHR, IGF-1, pIGF-1Rβ and IGF-1Rβ following the treatment of C2C12 and C3H10T1/2 cells with 100 and 200 μg/ml HS extracts for 24 h. (C) Relative STAT5b/IGF-1 complex binding in C2C12 and C3H10T1/2 cells treated with 100 and 200 μg/ml HS extracts for 24 h by chromatin immunoprecipitation assay. $^{\#}$P<0.001 vs. control. HS, hemp seed; p, phosphorylated; IGF-1R, insulin-like growth factor-1receptor; GHR, growth hormone receptor.

proliferative and signaling effects prior to full myogenic differentiation. This dual-cell line approach enabled investigation of distinct stages of muscle-associated cellular responses and provided a robust platform for evaluating the effects of HS extract.

GH levels peak during the pubertal growth period, serving a crucial role in adolescent growth spurts, and decline progressively with aging. Because GH serves a crucial role in muscle and bone metabolism, maintaining its secretion is important in old age (39). In older adults aged ≥70 years, muscle loss, or sarcopenia, occurs because of decreased levels of GH and IGF-1, which notably affects muscle mass and strength (40). Increasing and activating GH and IGF-1 levels, as well as muscle-strengthening exercises, are key to mitigate the development of sarcopenia. In addition, as digestive efficiency declines with age, nutrient-dense foods such as HS may help support muscle health by enhancing the action of IGF-1 or mimicking its effects (41,42). HS shows promise as a potential dietary strategy to delay the onset and progression of sarcopenia.

Recent studies have demonstrated that plant-derived compounds regulate the GH-IGF-1 signaling axis and modulate downstream STAT pathways (43,44). Clinical investigations have identified GH and IGF-1 as key regulators of metabolic activity, immune response and hepatic stellate cell function in non-alcoholic fatty liver disease (45,46). In pediatric populations, researchers have described the key role of GH and its hepatic effector IGF-1 in normal growth processes and have emphasized the regulatory involvement of SIRT1 in GH secretion and IGF-1 expression (47). Daucosterol, a phytosteroid derived from walnut meat, is a plant-based compound that promotes IGF-1 protein expression and enhances cell proliferation (48). Similarly, researchers have evaluated the effects of *Epimedium* extract, particularly icariin, and have shown that it increases IGF-1 expression in skeletal muscle cells (23). In C2C12 myotubes, icariin has been reported to activate the IGF-1/PI3K/Akt pathway, induce muscle hypertrophy and suppress catabolic atrophy markers (49). Furthermore, studies have demonstrated that the targeted deletion of STAT5a/b in skeletal muscle decreases local IGF-1 expression by ~60%, thereby impairing muscle growth and function (50,51).

Consistent with previous studies (45-47,49,50), the present results demonstrated that STAT5b served as a key transcriptional mediator of IGF-1 in peripheral and muscle-related cells. Previous studies have examined the effects of individual plant-derived compounds (daucosterol, icariin) or employed genetic knockout models to demonstrate the role of STAT5a/b in muscle growth (45-47,49). The aforementioned studies established that STAT5 activation is key for transcriptional regulation of IGF-1 target genes. The present study demonstrated that HS extract, a commonly consumed nutritional source, pharmacologically enhances IGF-1/STAT5b signaling in muscle-associated cells, demonstrating a dietary approach to modulate this pathway. Targeted deletion of STAT5a/b decreases IGF-1 expression and impairs muscle growth (50). In agreement, the present data showed that increased STAT5b activation was associated with upregulated IGF-1 signaling, supporting the functional relevance of STAT5b in muscle regeneration. Thus, the present findings not only corroborate the established role of STAT5b in muscle biology but also demonstrated that a nutritional extract can modulate this pathway pharmacologically.

HS is a highly nutritious food source rich in protein, essential amino acids, polyunsaturated fatty acids (including linoleic acid and α- and γ-linolenic acid), tocopherols such as vitamin E and essential minerals (phosphorus, potassium, magnesium, iron and zinc), which collectively contribute to cardiovascular health, blood pressure regulation, antioxidant defense and immune function (12). However, despite these benefits, little is known about their effects on cell proliferation, and the underlying mechanisms remain unclear.

The present study evaluated the cytotoxicity of HS extract on mouse muscle (C2C12) and embryonic (C3H10T1/2) cells and determined the optimal treatment concentration (200 μg/ml). The primary objective was to assess whether HS extract exerts any biological effects before conducting detailed phytochemical analyses. At 200 μg/ml, the protein levels of growth factors were elevated, and there was increased phosphorylation of the downstream Jak2/STAT5b signaling pathway members. Furthermore, HS extract did not notably affect the levels of damaged DNA, and enhanced the binding of the transcription factor pSTAT5b protein, which acts on IGF-1 DNA. Therefore, HS may support cell proliferation and aid in muscle recovery by stimulating IGF-1 and GH via the JAK2/STAT5b signaling pathway.

Effective muscle regeneration requires not only activation of signaling pathways but also viability and proliferative capacity of muscle cells. The present data demonstrated that HS promoted viability of C2C12 and C3H10T1/2 cells, providing a cellular basis for regenerative effects. While PI3K/AKT and MAPK/ERK pathways are recognized regulators of IGF-1-mediated muscle hypertrophy, their primary roles are typically associated with downstream events such as protein synthesis and cell proliferation. By contrast, STAT5b serves as a direct transcriptional mediator of IGF-1 target genes (51). In the present study, HS extract consistently activated the JAK2/STAT5b pathway, providing a mechanistic link to IGF-1 transcriptional regulation. However, the present study did not exclude the possibility of crosstalk with PI3K/AKT or MAPK/ERK.

In addition to STAT5b, the IGF-1 signaling axis also engages the PI3K/Akt and MAPK pathways, which are major downstream effectors regulating muscle cell function. The PI3K/Akt pathway is key for normal muscle growth, survival and hypertrophy (35,52). Following IGF-1 stimulation, PI3K activation leads to Akt phosphorylation, which promotes muscle hypertrophy, supports cell proliferation and differentiation and conveys anti-apoptotic signals that preserve muscle integrity (53,54). The MAPK pathway is critical, particularly in controlling myoblast proliferation and differentiation. Activation through Ras triggers the ERK cascade, which drives myoblast proliferation but can transiently suppress differentiation by inhibiting key regulators such as MyoD. Conversely, other MAPK branches, including p38 MAPK and myocyte enhancer factor 2C, are key for initiating and maintaining myogenic differentiation (55,56). Together, these pathways complement STAT5b by regulating the balance between proliferation and differentiation.

Beyond the GH/JAK2/STAT5b/IGF-1 signaling pathway, HS contains bioactive compounds that may influence muscle cells more generally. The ω-3 fatty acid α-linolenic acid and the ω-6 fatty acid γ-linolenic acid decrease muscle inflammation and protect against cell injury, while L-arginine contributes to anti-inflammatory effects and supports cell repair processes (57-59). Together, these components maintain muscle cell membrane integrity, attenuate inflammatory responses and provide essential substrates for recovery. These actions suggest that HS extract supports overall muscle function through diverse mechanisms, rather than acting solely on a single growth-associated pathway.

Future work should identify and characterize the active components responsible for these effects. Based on previous reports, essential amino acids such as arginine and lysine, together with storage proteins such as edestin, may contribute to the increased cell proliferation (60-62). In addition, polyunsaturated fatty acids (γ- and α-linolenic acid and linoleic acid) and minerals such as methylsulfonylmethane modulate GH signaling and underlie the enhanced JAK2/STAT5b activation and IGF-1 production (16,63-65). Although further experimental validation is needed, these bioactive components provide a mechanistic basis for the present findings. Due to potential cytotoxic effects at lower concentrations, the clinical application of HS extract may be limited, underscoring the need for further research to evaluate tissue-specific safety *in vivo*.

In summary, HS induces expression of IGF-1 via the Jak2/STAT5b signaling pathway to promote proliferation in C2C12 and C3H/10T1/2 mouse cells. Moreover, pSTAT5b bound to IGF-1 DNA as a direct transcription factor, serving a key role in enhancing cell signaling. Therefore, HS may deliver specific growth signals for cell proliferation and muscle recovery.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JC and KJJ designed the experiments. DYK, HTK and YK performed experiments. DYK and KJJ analyzed the data. DYK wrote the manuscript. JC and KJJ edited and reviewed the manuscript. DYK and KJJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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