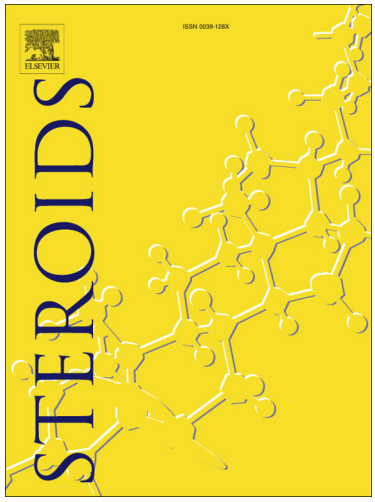

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Mechanical stretch enhances sex steroidogenesis in C_2C_{12} Skeletal Muscle Cells

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Abstract

Skeletal muscle contains estrogens and estrogen synthesis-related enzymes. However, it has not been reported whether myoblasts cultured *in vitro* also express these enzymes. The purpose of the current study was to address these issues and to explore the effects of mechanical stretch on the enzyme system. The *in vitro* cultured C_2C_{12} mouse myoblasts were divided into the control, stretch, testosterone and stretch plus testosterone groups. Cells in the stretch and stretch plus testosterone groups were mechanically stretched with the Flexercell cell stress loading device at an amplitude of 10% and in a frequency of 0.5 Hz for 8 hours. Cells in the testosterone and stretch plus testosterone groups were incubated with 100nM testosterone for 24 hours before distraction. Following the treatments, cell proliferation and estradiol levels, as well as the expressions of 17β-hydroxysteroid (17β-HSD), 3β-hydroxysteroid (3β-HSD) and aromatase were analyzed. Compared to the control, the cell proliferation in all experimental groups increased significantly, the estradiol levels in the mechanically stretched groups were significantly higher, and, moreover, the estradiol levels were positively correlated with the cell proliferation ($r = 0.615$, $p < 0.01$). Additionally, analyses of aromatase protein and mRNA showed that, compared to the control, their levels were significantly increased upon stretching and testosterone exposure. Similarly, the protein and mRNA levels of both 3β-HSD and 17β-HSD in the stretched cells differed significantly from the control. In the presence of aromatase and 5α-reductase inhibitors, the protein and mRNA levels of these enzymes altered significantly compared to the control. Conclusions: Steroid synthases were detected in the C_2C_{12} myoblasts cultured *in vitro*, the synthesized estrogen was closely related to the cell proliferation, and mechanical stretch was the external factor that affected the expression of the estrogen synthesis-related enzymes.

***Keywords*:** mechanical stretch, C_2C_{12}, estrogen, aromatase

1. Introduction

The capacity for estrogen synthesis varies throughout female's lifetime, especially when women enter menopause, uterine function and estrogen synthesis

ability decrease. Meanwhile, the strength and quality of the skeletal muscle are decreased, and the risk of developing metabolic syndrome is elevated [1]. A number of studies have demonstrated that the estrogen level is associated with muscle strength and metabolic disorders [2,3]. In human, the estrogen level is significantly lower in menopausal than in premenopausal women. Exogenous estrogen supplementation may improve the level of serum estrogen, ameliorate cardiovascular diseases, and improve cognitive performance. Likewise, animal studies have also shown that the estrogen concentration, as well as the quality of skeletal muscle, is significantly decreased in the ovariectomized than in the normal mice. Exogenous estrogen supplementation may reduce the loss and dysfunction of the muscle [4,5] and affect the metabolism of carbohydrates and lipids [6,7].

Aromatase, the rate-limiting enzyme for estrogen synthesis, is widely distributed in gonads, brain, kidneys, and other organs. It converts androgen to estrogen, maintaining, to certain extent, the homeostasis of sex hormones. Declined estrogen level usually precedes for women suffered from sarcopenia, indicating that the former is associated with the latter. Recent studies have found that aromatase is also expressed to synthesize estrogen in the skeletal muscle. Additionally, 17β-hydroxysteroid (17β-HSD), 3β-hydroxysteroid (3β-HSD) and 5α-reductase, enzymes involved in the synthesis of estrogen, have also been detected in the skeletal muscle. In animal and human studies, acute and chronic exercise may upregulate steroid synthases and increase the level of steroids [8,9]. While it is still controversial in terms of the impact of exercise on the level of circulating estrogen in human [10], experiments in mice have shown that treadmill training may promote the expression of androgen receptors in gastrocnemius [11]. *In vitro* studies have discovered that increasing DHEA, the precursor of androgen, in skeletal muscle significantly enhances the level of estradiol [12]. DHEA is catalyzed by 17β-HSD and 3β-HSD to be converted to testosterone which is further catalyzed by aromatase to produce androgen that targets at the estrogen receptor to exert its biological effects. Testosterone may also be catalyzed by 5α-reductase to generate DHT targeting at the androgen receptor [13,14].

Since skeletal muscle contains estrogen and the expression of the estrogen synthesis-related enzymes, and since preliminary evidence has shown that exercise and the related substrates, such as testosterone, may affect the metabolism of estrogen, it may be necessary to conduct *in vitro* cellular studies to further investigate the factors that may affect the enzymes that are associated with estrogen metabolism. Skeletal muscle cells are multinucleated cells generated by the fusion of myoblasts. Stimulated by various physical and chemical factors, the so-called satellite cell proliferates, migrates and eventually differentiates into myoblasts. Since C_2C_{12} myoblasts are morphologically and characteristically uniform and can be subcultured indefinitely, these cells offer an excellent model to study the development and metabolism of skeletal muscle cells.

In skeletal muscle, aromatase catalyzes the conversion of testosterone to estradiol. Previous studies have demonstrated that testosterone supplementation may stimulate the production of estrogen. Eriksen *et al*. have found that exposing *in vitro* cultured muscle cells to 100 nM testosterone for 7 days may increase the expression of aromatase. Therefore, not knowing whether the *in vitro* cultured C_2C_{12} cells express any enzymes related to the synthesis of estradiol, they provided the substrate for the aromatase by adding testosterone in the culturing media [15]. However, so far, it still has not been reported whether *in vitro* cultured C_2C_{12} myoblasts express all the enzymes required for estrogen synthesis and whether mechanical loading or stretching may stimulate the cells to express these enzymes and synthesize estrogen. On the other hand, it is known that stretching is an effective approach to imitate exercise for *in vitro* cultured cells and, moreover, appropriated stretching can effectively enhance the proliferation and metabolism of the cells [16-18].

As a result, the current studies are intended to analyze whether the *in vitro* cultured C_2C_{12} myoblast expresses estrogen synthesis-related enzymes, including aromatase, 17β-HSD and 3β-HSD, and synthesizes estrogen, and investigate how mechanical stretch may affect the expression of the enzymes and the synthesis of the hormone.

2. Material and Methods

2.1. Reagents and instruments

The reagents and instruments were purchased from various companies as follows: Dulbecco's modified Eagle medium, fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin from Gibco, USA; trypsin from Sigma, USA; BCA protein assays, antibody dilution buffer from Beyotime Biotechnology, China; primary antibodies from CST, USA; 17β-HSD and 3β-HSD antibodies from Abcam, England; HRP-conjugated goat anti-rabbit IgG and β-actin antibodies from CST, USA; aromatase antibodies and HRP-conjugated donkey anti-goat IgG from Santa Cruz, USA; Immobilon™ Western chemiluminescent HRP substrates from Merck Millipore, Germany; CCK-8 kit from Beyotime Biotechnology; estradiol ELISA kit and testosterone from Shanghai Lanpai Biotechnology, China; anastrozole and finasteride from Selleck, USA; CryoSure-DMSO from WAK, Germany; SYBRGreen fluorescent dye from Roche, Switzerland; Maxima H Minus First Strand cDNA Synthesis kit from Thermo, USA; Trizol from Thermo, USA; CO_2 incubator from Sanyo, China; 6-well plates from Flexcell, USA; fluorescence plate reader from Biotek, USA; Gel DocTM EZ imager EZ-170-8270 from Bio-Rad, USA; Trans-Blot electrophoresis tank and Trans-Blot transfer tank from Bio-Rad, USA; inverted microscope from Leica, Germany; Multiskan from CLARIOstar, Germany; fluorescence image analysis system from Tanon, China; Flexercell 5000 from Flexercell, USA; real-time fluorescence quantitative PCR instrument from Applied Biosystems, USA.

2.2. Cell Culture

Mouse C_2C_{12} myoblasts were cultured in Dulbecco's modified Eagle medium supplemented with 10 % fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5 % CO_2 in the humidified CO_2 incubator. The growth

medium was changed every other day. When the cells reached 80% confluence, they were digested with 0.25 % trypsin and passaged at a split ratio of 1: 2. $3.5x10^5$ cells in the growth medium and then seeded into each well of the 6-well plates.

2.3. Experimental Protocol

Testosterone, anastrozole and finasteride were solubilized in DMSO. When these compounds were used, DMSO was added to all experimental conditions at the same 0.1 % concentration. C_2C_{12} myoblasts were seeded into 6-well plates. For sex hormone-treatment groups, cells were cultured for 36 hours, before stretching, with (1) 100 nM testosterone, (2) 400 μg/mL aromatase inhibitor anastrozole, (3) 100 nM 5α-reductase inhibitor finasteride. For mechanical stretch groups, cells were stretched at a 10 % amplitude and in 0.5 Hz frequency for 8 hours.

2.4. CCK-8 Assay Analysis

CCK-8 assay was conducted according to manufacturer's instructions. After cells were cultured to reach 80% confluence, they were detached, seeded into the 96-well plates ($2x10^3$/well), and kept quiescent for 24 hours. 10 μL CCK-8 solution was then added to each well and the cells were incubated at 37 °C for 2 hours. Optical density was measured using the fluorescence plate reader at an absorbance wavelength of 450 nm.

2.5. Total Protein Extraction

Cells were lysed with 70 μL lysis buffer per well, and the protein was transferred into the 1.5 mL centrifuge tubes, and the sample was centrifuged at -4 ℃ for 15 minutes at 12000 rpm. The supernatants were collected. A BCA assay kit was used to quantify the total protein content.

2.6. Estradiol Concentration Test

Estradiol concentrations were analyzed using the ELISA kit according to manufacturer's instructions. 10 μg samples, standards or controls were added to each well, followed by 50 μL labeled antibodies. The mixtures were incubated at 37 ℃ for 1 hour. After washing for 3 times, 80 μL labeled HRP-conjugate was added to each well and the mixtures were incubated for 30 minutes at 37 ℃. The TMB substrate solution was then added, the plates were incubated for 10 minutes, and the stop solution was added afterwards. The optical density was read at 405 nm.

2.7. Western Blot Analysis

Protein extracts (25 μg/well) were separated by SDS-PAGE (5 % stacking gel and 10 % separating gel) before blotting onto PVDF membranes. The membranes were blocked with 5 % nonfat dried milk for 1 hour at room temperature and incubated with the primary antibodies (1: 1,000 dilution) at 4 °C for overnight. The membranes were then washed with TBST for 3 times and incubated with the secondary antibodies (1: 3,000 dilution) for 1 hour. The protein bands in the membranes were visualized by the enhanced electrochemiluminescence detection reagents. The immunoreactive bands were scanned by the Gel DocTM EZ imager EZ-170-8270 and densitometric values were counted using LabWorks.

2.8. Quantitative real-time PCR (qPCR)

Total RNA was isolated with Trizol reagent, transferred into the 1.5ml centrifuge tube containing chloroform and incubated for 10 minutes. The mixture was then centrifuged, followed by addition of isopropanol for 10 minutes and centrifugation. Total RNA was washed by 75 % ethanol and then dried in the air. DEPC-treated water was added to the RNA. RNA concentration was quantified by microplate reader. 2μg RNA was reversely transcribed using 10 μg oligo dT primers and 10 mM

dNTPs. The mixture was incubated at 65 °C for 5 minutes, and cDNA was produced by incubating at 4 2°C for 50 minutes with the first strand buffer, 100 mM DTT, RNase inhibitor, and Superscript II reverse transcriptase. PCR was performed using the QuantiTect SYBR Green PCR kit according to manufacturer's instructions. The mixture was evaluated with ABI7300 fluorescence quantitative detection system and the data were analyzed with software Excel. The expression of 3β-HSD, 17β-HSD and aromatase genes was monitored by qPCR, using primers listed in Table 1. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as the reference gene. All primers were synthesized by Sangon Biotech Co., Ltd. Differences in mRNA expression were determined using the $2^{-\Delta\Delta Ct}$ method.

2.9. Statistic Analysis

All results were expressed as means ± SD. One-way ANOVA was used for multiple comparisons and the association between cell proliferation index and estradiol concentration was calculated using the Pearson correlation analysis and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mechanical stretch promoted the proliferation of C_2C_{12} myoblasts

CCK-8 Kit was used to measure the cell proliferation. WST-8 in the kit can be reduced by mitochondrial dehydrogenase to produce a product, the orange-colored formazan. The quantity of formazan is positively proportional to the number of the cells: the more the proliferation of the cell, the darker the cell plasma. As illustrated in Fig. 1, the cell proliferations in stretch, testosterone and stretch plus testosterone groups were not significantly different among themselves. However, they were all significantly higher than that in the control group ($p < 0.05$).

3.2. Mechanical stretch enhanced the levels of estradiol in C_2C_{12} myoblasts

Estradiol ELISA kit was used to analyze the levels of estradiol. The level of estradiol in the stretch group was significantly higher than that in the control group ($p < 0.05$). Additionally, the estradiol level in the stretch plus testosterone group was also significantly higher than that in the control group ($p < 0.05$). However, no significant difference in the level of estradiol was observed between the testosterone group and the control group ($p > 0.05$) (Fig. 2).

3.3. Mechanical stretch increased the expression of 3β-HSD enzyme in C_2C_{12} myoblasts

Estrogen is synthesized from testosterone *in vivo* and 3β-HSD is the key enzyme for the synthesis of testosterone. Western blot and real-time quantitative PCR were applied to measure, respectively, the protein and mRNA levels of 3β-HSD in the mouse myoblasts. As shown in Fig. 3, the band of 3β-HSD protein in the stretch group was significantly denser (Fig. 3a) and, quantitatively, the level of 3β-HSD protein in the stretch group increased 188 % ($p < 0.05$), compared to that in the control group. No significant difference was found between the control group and testosterone or testosterone plus stretch group ($p > 0.05$). On the other hand, the levels of 3β-HSD mRNA in testosterone and testosterone plus stretch groups were 58 % ($p < 0.05$) and 43 % ($p < 0.05$), respectively, of that in the control group.

3.4. Mechanical stretch upregulated the expression of 17β-HSD protein and mRNA in C_2C_{12} myoblasts

17β-HSD and 3β-HSD cooperatively catalyze the synthesis of testosterone. The expressions of 17β-HSD protein and mRNA were analyzed. As shown in Fig. 4, comparing to that in the control group, the level of 17β-HSD protein in the stretch group increased 121 % ($p < 0.05$). However, there was no significant difference ($p > 0.05$) in 17β-HSD protein between the control group and the testosterone or

testosterone plus stretch group. On the other hand, as shown in Fig. 4c, the mRNA level was 4.42 times ($p < 0.05$) in the stretch group as that in the control group. While the mRNA level in the testosterone group was 2.19 times as that in the control group, they were, statistically, not significantly different.

3.5. Mechanical stretch promoted the expression of aromatase protein and mRNA in C_2C_{12} myoblasts

Aromatase is the rate-limiting enzyme that regulates the synthesis of estradiol. The protein and mRNA levels of this enzyme in C_2C_{12} myoblasts were analyzed by Western blot and real–time quantitative PCR, respectively. As shown in Fig.5, compared to the control, the levels of aromatase enzyme increased 82 % and 123 % ($P<0.05$), respectively, in the testosterone group and testosterone plus stretch group. Although a slightly increase in mRNA in the stretch group was observed, it was statistically not significant ($p > 0.05$). In terms of aromatase mRNA, the levels were 12.7, 8.1 and 12.6 times, respectively, in the stretch, testosterone and stretch plus testosterone groups as that in the control group. Statistical analyses showed that only the increases in the stretch group and stretch plus testosterone group were significant ($p < 0.05$) (Fig. 5c).

3.6. Pearson analysis

Pearson analysis found that the cell proliferation was positively correlated with the level of estradiol and the correlation coefficient was 0.615 ($p < 0.01$) (Fig. 6).

3.7. Addition of aromatase and 5α-reductase inhibitors reduced stretch-promoted levels of mRNAs and proteins of 3β-HSD and 17β-HSD

As shown in Fig.7 and Fig.8, simultaneous exposure of the stretched cells to both inhibitors of 5α-reductase and aromatase significantly decreased both protein and

mRNA levels of 3β-HSD and 17β-HSD ($p < 0.05$). However, compared to the control, treatment of the cells with inhibitor of 5α-reductase alone only significantly lowered the mRNA ($p < 0.05$), but not protein ($p > 0.05$), level of 3β-HSD and 17β-HSD.

3.8. Addition of aromatase and 5α-reductase inhibitors reduced stretch-promoting levels of mRNAs of aromatase

As shown in Fig.9, compared to the stretched cells treated with only 5α-reductase inhibitor, simultaneous exposure of the stretched cells to both inhibitors of 5α-reductase and aromatase only significantly decreased the mRNA level of aromatase ($p < 0.05$). Although the protein level of aromatase was also reduced, the reduction was not statistically significant ($p > 0.05$).

4. Discussion

Earlier investigation has discovered that skeletal muscle not only contains aromatase, the key enzyme in the synthesis of estrogen, but also can synthesize estrogen [12,19]. Skeletal muscle is an organ that is highly sensitive to the stimulation of exercise or mechanical stretch. Exercise improves the quality of the muscle. Does exercise also affect the synthesis of local estrogen? The current study was intended to explore whether skeletal muscle cultured *in vitro* might express the enzymes related to estrogen synthesis. Our results showed that the protein expression of a number of steroid synthesis enzymes, 3β-HSD, 17β-HSD and aromatase, was detected in the *in vitro* cultured mouse myoblasts. Moreover, mechanical stretch could significantly stimulate the expressions of the enzymes, suggesting that the *in vitro* cultured mouse myoblast could express the enzymes.

We analyzed the relationships between cell proliferation and estrogen before and after mechanical stretch in the mouse C_2C_{12} myoblasts. We found that mechanical stretch promoted the proliferation of C_2C_{12} myoblasts and, moreover, the level of intracellular estradiol increased significantly. Statistical analysis showed that the cell

proliferation and the estradiol level correlated positively in group C and S (Fig. 6). Animal studies have also demonstrated that exercise may enhance not only the estrogen level in gastrocnemius muscle of the male rats but also muscle cross-sectional area [20]. Earlier studies have also found that lack of estrogen may lead to the decrease in muscular quality [21] and affect, to certain extent, the synthesis of contractile proteins of the muscle [22]. Exogenous estrogen supplementation can increase the quantity of skeletal muscle [23], as well as the proliferation of the satellite cell of the skeletal muscle in rats [24]. However, the impact of mechanical stretch on estrogen is unknown. Our results showed that mechanical stretch promoted not only the proliferation of the cell but also the synthesis of estrogen.

To avoid the impact of the lack of substrates on the synthesis of estradiol, we added testosterone to the *in vitro* cultured myoblasts. We did not find that 100nM testosterone increased the synthesis of estradiol, which differed from the observation reported by Aizawa *et al*. [12] who found that supplementation of 300μM testosterone to the *in vitro* primary cultured SD rat myoblasts significantly increased the synthesis of estradiol. The differences between our and their results may be due to the differences in the species of the cells or the types of the cells, i.e. the primary cultured cell vs. an established *in vitro* cultured cell line. The relationships between the estrogen and the cell proliferation following the addition of testosterone were analyzed. The results showed that the mechanical stretch stimulated the synthesis of estrogen but the cell proliferation were not increased. Overall, statistical analysis showed that the cell proliferation and the estradiol level correlated positively ($r=0.615$, $p<0.01$). The positive correlation between E2 and cell proliferation suggested the stimulative effects of E2 on cell proliferation. However, only when the level of testosterone was low, stretching could promote cell proliferation via estrogen. The results are consistent with those in the similar studies conducted by Tanideh *et al*, who found that estrogen supplementation and endurance exercise could increase the number of muscular satellite cells in the rats. On the other hand, our results showed that in the presence of exogenous testosterone, stretching stimulated the synthesis of E2 but the

cell proliferation was not increased. As a result, testosterone is likely to play a significant stimulative role in cell proliferation, which is supported by a large number of studies [26-27].

3β-HSD and 17β-HSD are required for the synthesis of testosterone from DHEA. We found that the expression of both enzymes were increased for the cells that were being stretched, indicating that both enzymes play roles in the biosynthesis of testosterone. Moreover, testosterone suppressed the expression of both enzymes in the mechanically stretched cell. However, it did not markedly change the level of the enzymes in the un-stretched cells. It is likely that stretching could promote the usage of testosterone by the C_2C_{12} myoblasts.

Aromatase is the rate-limiting enzyme in the bioconversion to estrogen. We found that when the cells were cultured in the presence of testosterone, the expressions of both aromatase mRNA and protein were significantly increased, suggesting that mechanical stretch and testosterone had a stimulating effect on aromatase [28]. On the other hand, the level of estrogen did not change significantly. Whether it is related to the dosage of the exogenously added testosterone remains to be investigated.

Another purpose of the current studies was to explore the impact of mechanical stretch on the expression of steroid synthases. 3β-HSD, 17β-HSD and aromatase are important steroid synthases [29]. 3β-HSD and 17β-HSD are involved in the conversion of DHEA to testosterone, which is further converted to estradiol catalyzed by aromatase. Testosterone may also be converted to DHT by 5α-reductase. In other words, testosterone can be metabolized in two different metabolic pathways to produce either DHT or estrogen (Fig. 10). Our results also demonstrated that, in the cultured C_2C_{12} cell, mechanical stretch could promote the expression of aromatase and the synthesis of estrogen, regardless whether substrate testosterone was supplemented. To further explore the impact of mechanical stretch on the enzymes related to the synthesis of estrogen, we chose mechanical stretch without the supplementation of substrate testosterone. The results showed that mechanical stretch promoted the expression of 3β-HSD, 17β-HSD and aromatase. Moreover, as illustrated in Fig. 10, the mRNAs of 17β-HSD and aromatase were also significantly

increased, which was consistent with the observation reported by Aizawa *et al.* [20]. However, in the presence of both inhibitors, anastrozole and finasteride, the expressions of 3β-HSD, 17β-HSD and aromatase proteins were significantly suppressed.

In summary, we found that the *in vitro* cultured mouse C_2C_{12} myoblast expressed steroid synthases, including 3β-HSD, 17β-HSD and aromatase. Moreover, mechanical stimulation promoted the expression of estradiol synthesis-related enzymes, which provides an important experimental basis for exploring the metabolism of estrogen in myoblasts by exercise or mechanical stimulation in the further.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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Table 1. Primers used for RT-PCR

Primer		Sequence	Length
GAPDH	Forward	5’-ACTCCACTCACGGCAAATTC-3’	20bp
	Reverse	5’-TCTCCATGGTGGTGAAGACA-3’	20bp
Aromatase	Forward	5’-ACTACATCTCCCGATTCGGCA-3’	21bp
	Reverse	5’-GGGTCAACACATCCACGTAGC-3’	21bp
3β-HSD	Forward	5’-CCTCCGCCTTGATACCAGC-3’	20bp
	Reverse	5’-TTGTTTCCAATCTCCCTGTGC-3’	21bp
17β-HSD	Forward	5’-TTAGTCGGACACTGGAAAAGC-3’	21bp
	Reverse	5’-CCAGGACTGCCTTCTGATCC-3’	20bp

Figure legend

Fig. 1. Cell proliferation of C_2C_{12} myoblasts upon stretch or testosterone addition

C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST: stretch plus 100 nM testosterone addition group. * indicates $p < 0.05$ compared to the control

Fig. 2. Estradiol concentrations in C_2C_{12} myoblasts upon stretch or testosterone addition

C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST: stretch plus 100 nM testosterone addition group. * indicates $p < 0.05$ compared to the control

Fig. 3. 3β-HSD protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or testosterone addition

C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST: stretch plus 100 nM testosterone addition group. * indicates $p < 0.05$ compared to the control

Fig. 4. 17β-HSD protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or addition of testosterone

C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST:

stretch plus 100 nM testosterone addition group. * indicates $p < 0.05$ compared to the control

Fig. 5. Aromatase protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or testosterone addition

C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST: stretch plus 100 nM testosterone addition group. * indicates $p < 0.05$ compared to the control

Fig. 6. Pearson's correlation analysis between cell proliferation and estradiol concentration of C_2C_{12} myoblasts.

Cell proliferation was positively correlated with the level of estradiol of each group . The correlation coefficient was 0.615 ($p < 0.01$). C: the control group; S: the stretch group; T:100 nM testosterone addition group; ST: stretch plus 100 nM testosterone addition group.

Fig. 7. 3β-HSD protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or anastrozole and finasteride addition

S: the stretch group; SF: stretch plus100 nM finasteride addition group; SAF: stretch, 100 nM finasteride and 400 μg/mL anastrozole addition group

* indicates $p < 0.05$ compared to the control; # indicates $p < 0.05$ compared to the SAF group

Fig. 8. 17β-HSD protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or testosterone addition

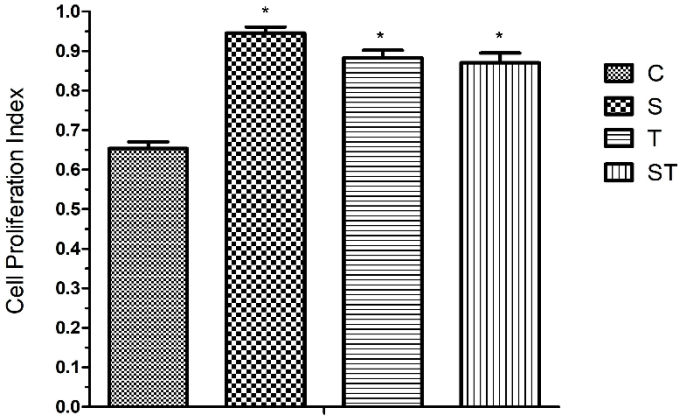
S: the stretch group; SF: stretch plus 100 nM finasteride addition group; SAF: stretch, 100 nM finasteride and 400 μg/mL anastrozole addition group. # indicates $p < 0.05$ compared to the SAF group

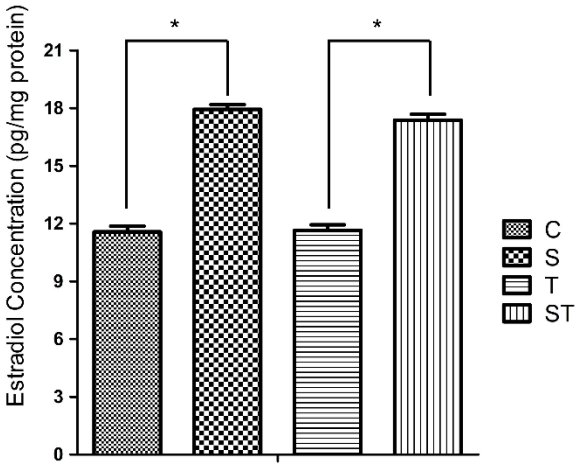
Fig. 9. Aromatase protein expression and mRNA contents in C_2C_{12} myoblasts upon stretch or testosterone addition

S: the stretch group; SF: stretch plus 100 nM finasteride addition group; SAF: stretch, 100 nM finasteride and 400 μg/mL anastrozole addition group. # indicates $p < 0.05$ compared to the SAF group.

Fig.10. Estrogen synthesis pathway in muscle cell.

DHEA is converted to 5-diol by the effect of 17β-HSD1, and The biosynthesis of testosterone from DHEA is achieved by 3β-HSD. Estrogens are converted from testosterone catalyzed by aromatase. In addition, testosterone is also converted to DHT by 5-reductase. Our results showed that mechanical stretch increased 17β-HSD, 3β-HSD and aromatase protein expression, as well as promoting estradiol synthesis. DHEA: dehydroepiandrosterone, 5-diol: 5-Androstenediol, 17β-HSD1: 17β-Hydroxysteroid Dehydrogenase Type 1, 3β-HSD: 3β-Hydroxysteroid Dehydrogenase, DHT: Dihydrotestosterone.





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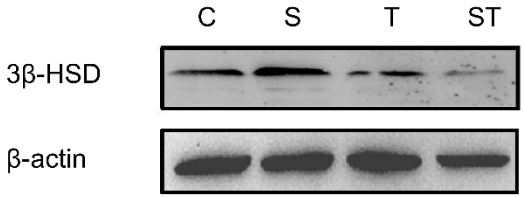

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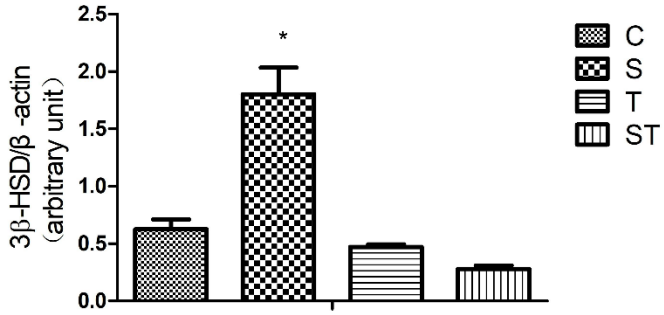

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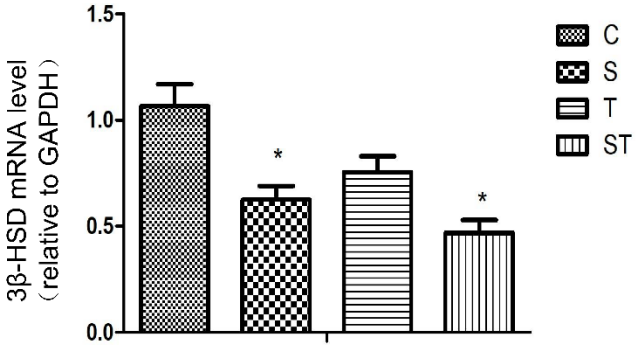

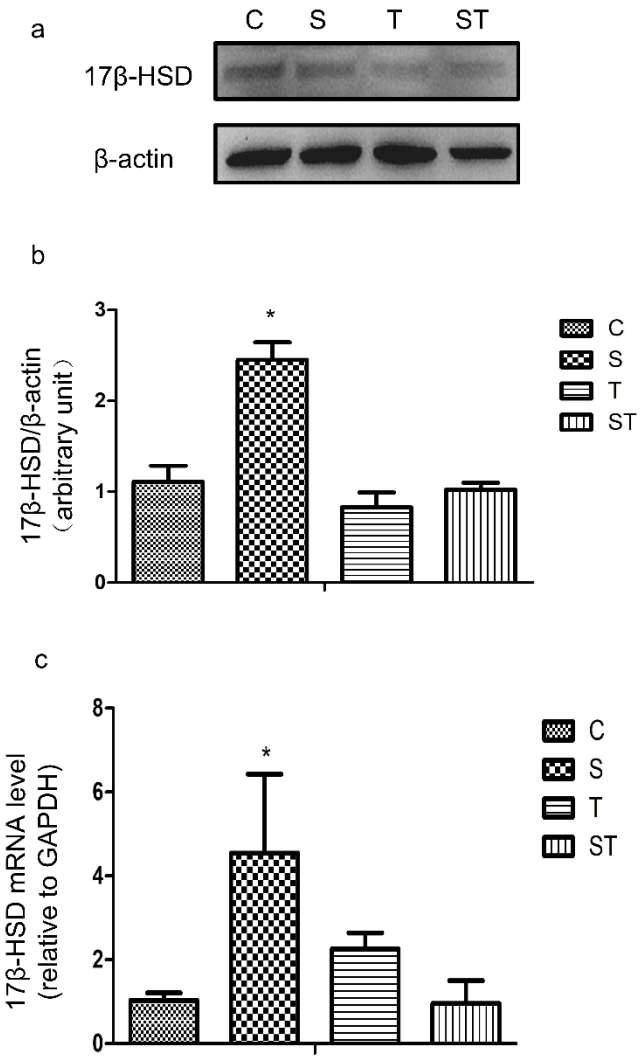

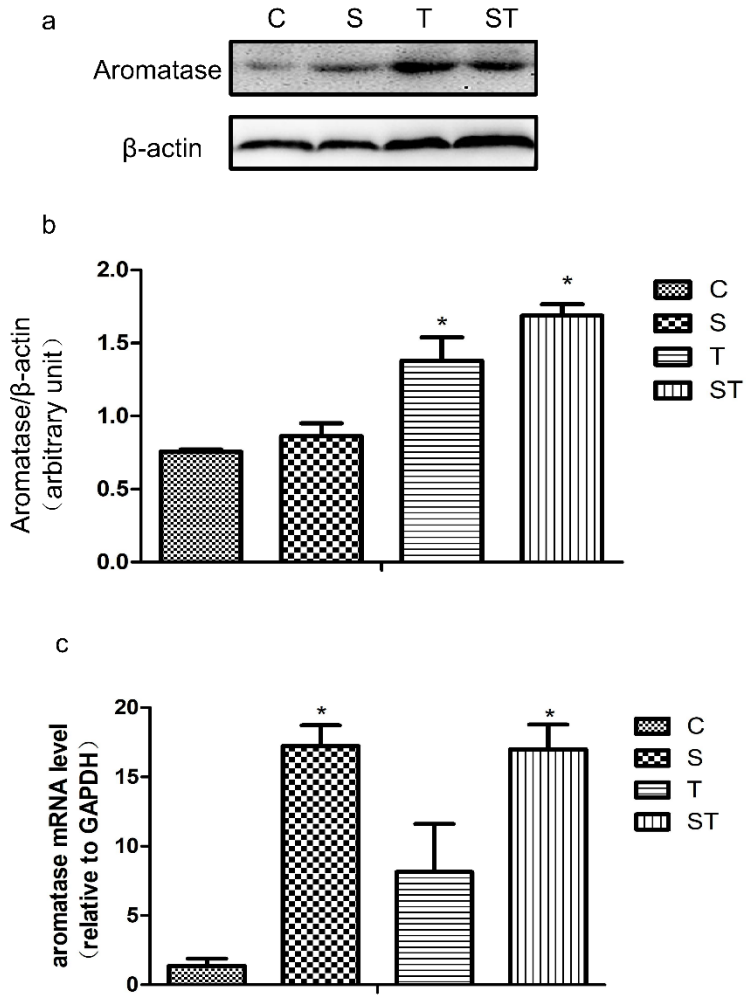

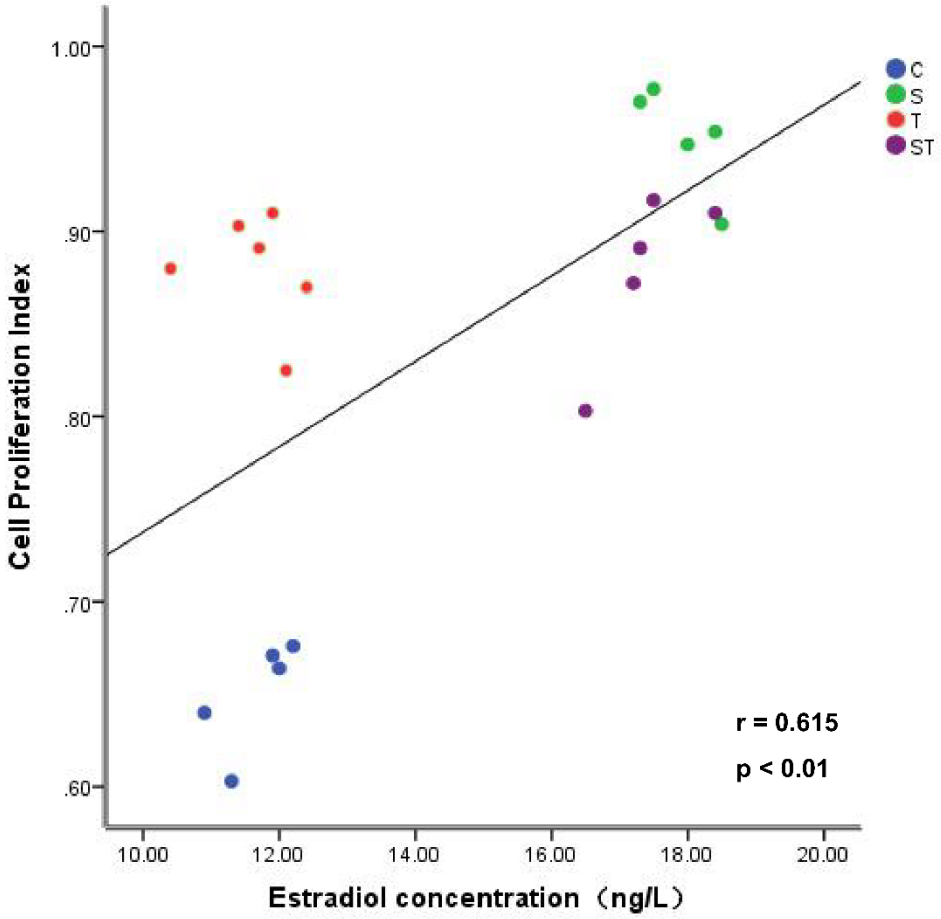

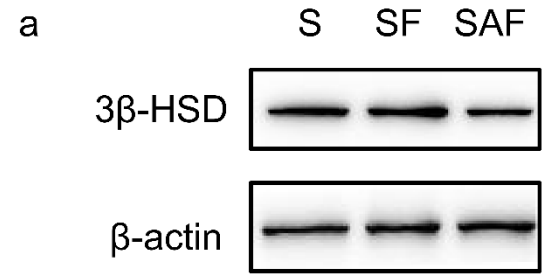

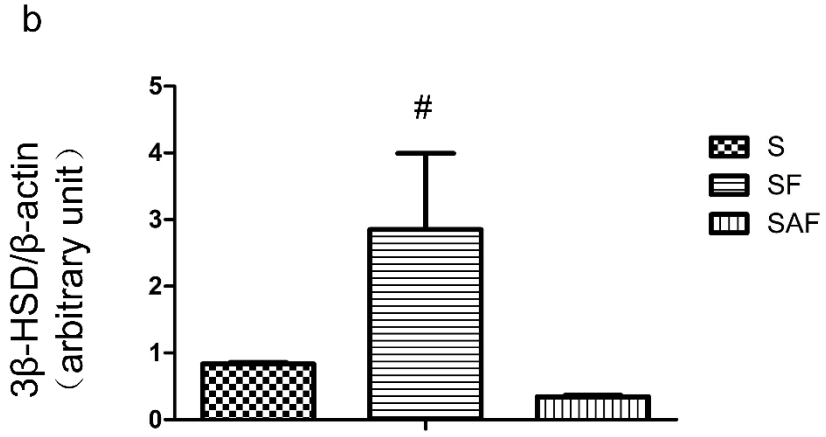

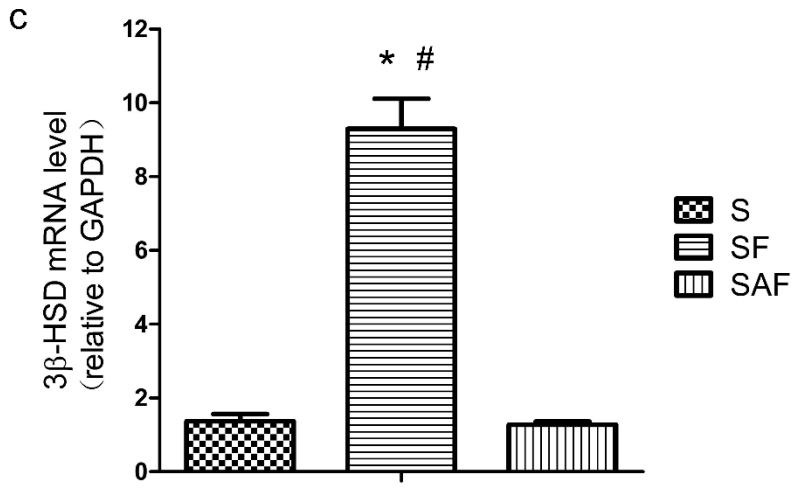

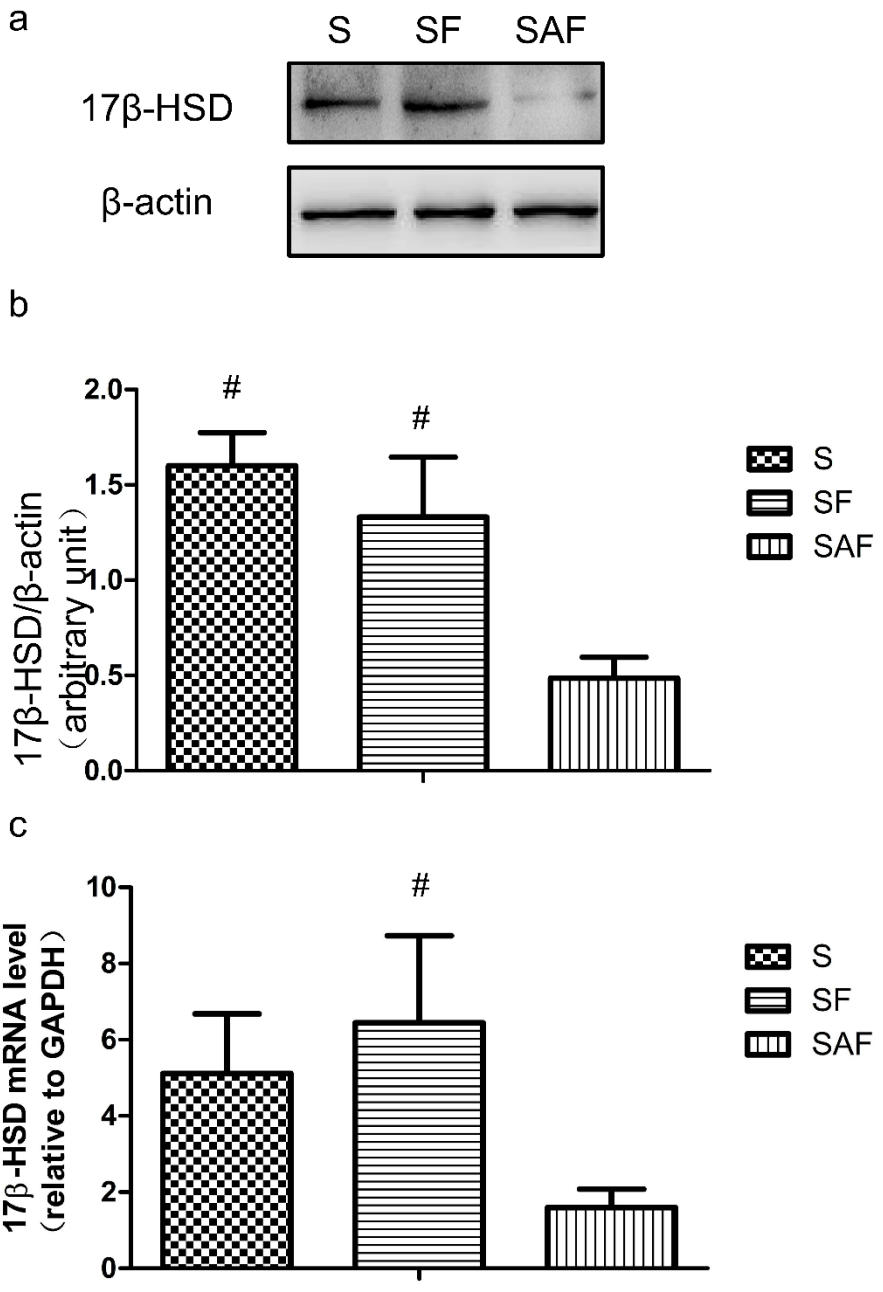

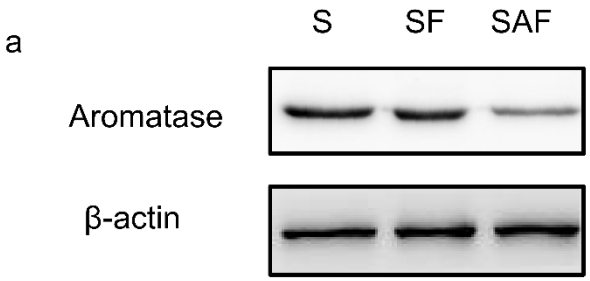

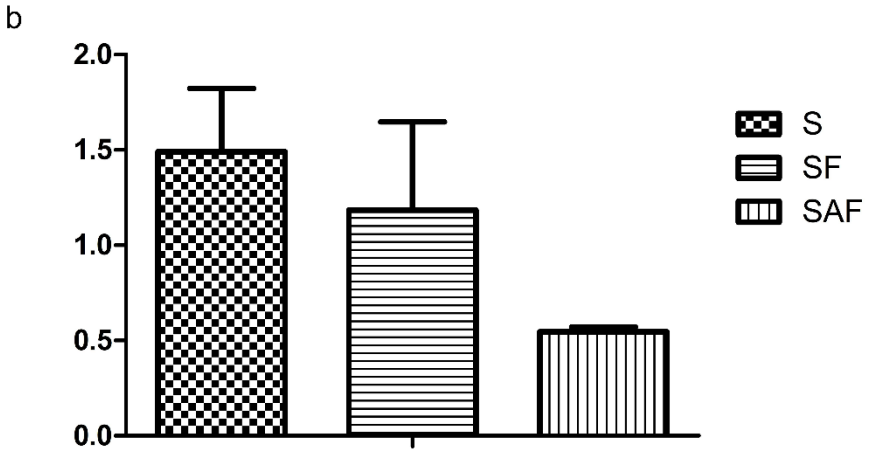

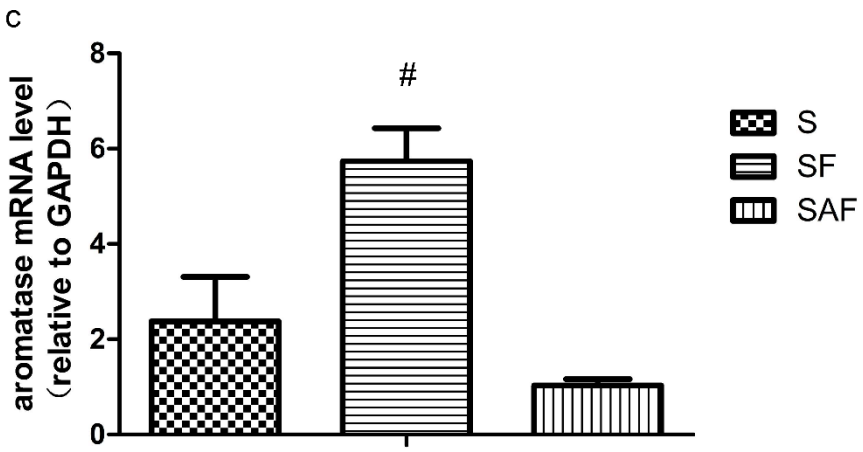

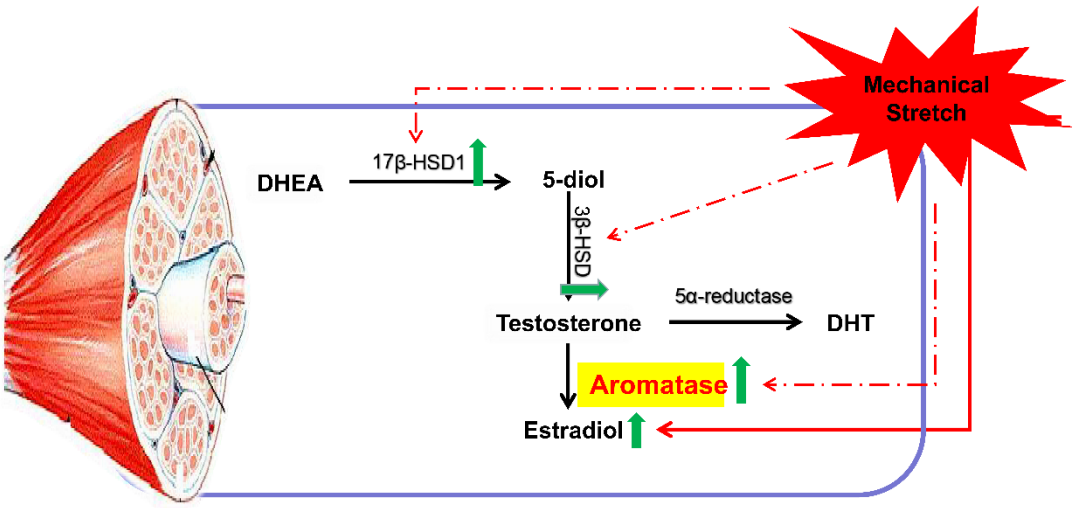

Highlights:

- Steroid synthases were detected in the C_2C_{12} myoblasts cultured in vitro.
- Mechanical stretch was the external factor that affected the expression of the estrogen synthesis-related enzymes.
- Cyclic mechanical stretching promotes cell proliferation and estrogen production.