# Effect of chromium(III), estradiol and genistein on myotubes formation in mouse myoblast C2C12 cell line

## Wpływ chromu(III) estradiolu i genisteiny na formowanie się miotub w hodowli in vitro mięśniowych komórek linii C2C12

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**Wprowadzenie.** Różnicowanie w komórki mięśniowe jest wieloparametrowym procesem, w który zaangażowane są liczne białka (m.in. białka transkrypcyjne, białka cyklu komórkowego) i wiele innych czynników (m.in. reaktywne formy tlenu, miRNA lub miostatyna).

**Cel.** Zbadano wpływ suplementacji chromem III na formowanie miotub w modelu hodowli in vitro. W celu oznaczenia możliwości estrogenopodobnego działania chromu zbadano dodatkowo wpływ estradiolu i genisteiny (selektywny agonista receptora β-estrogenowego).

**Materiał i metody.** Badania przeprowadzono na mysich komórkach mięśniowych linii C2C12. Badane związki (chlorek chromu 10 µg $Cr^{3+}$/L, pikolinian chromu 10 µg $Cr^{3+}$/L, estradiol 0,1-100 nM lub genisteina 0,1-50 µM) zostały podane w pierwszym dniu różnicowania. Indeks fuzji były mierzony 2, 4, i 6 dnia różnicowania. Dodatkowo podano liczbę komórek posiadających 6-10 i więcej niż 10 jąder.

**Wyniki.** Suplementacja chromem (zarówno chlorkiem, jak i pikolinianem) spowodowała zależny od czasu wzrost procesu formowania miotub (mierzony zarówno jako indeks fuzji, jak i liczby komórek posiadających 6-10 i więcej niż 10 jąder w komórce), z największą różnicą obserwowaną 6 dnia różnicowania (wzrost o 40% w porównaniu do wartości kontrolnej). Efekt suplementacji estradiolem (0,1-100 nM) był krótkoterminowy. Maksymalny efekt stymulacji w porównaniu do warunków kontrolnych (ponad 100% zarówno indeks fuzji, jak i liczba komórek posiadających 6-10 jąder w komórce) obserwowany był drugiego dnia różnicowania. Genisteina (0,1-50 µM) nie wpływała na proces różnicowania.

**Wnioski.** Wykazano pozytywny wpływ suplementacji jonami chromu na procesy różnicowania komórek mięśniowych linii C2C12. Jednak nie dają one ostatecznej odpowiedzi na temat interakcji chromu i receptorów estrogenowych w procesie różnicowania.

**Słowa kluczowe:** *chrom, estradiol, genisteina, indeks fuzji, linia C2C12*

**Introduction.** Muscle cell differentiation is a multifactorial process engaging numerous proteins (e.g. transcription factors, cell cycle and signaling pathway proteins) and many other molecules (e.g. reactive oxygen species, miRNA, myostatin).

**Aim.** To examine the influence of chromium supplementation on the myotubes formation in vitro. To evaluate the possibility of estrogen-like mechanism of chromium action, the effect of estradiol and genistein on the differentiation process was also estimated.

**Material & Method.** The research was performed on the C2C12 cell line. The examined compounds (chromium chloride10 µg $Cr^{3+}$/L, chromium picolinate 10 µg $Cr^{3+}$/L, estradiol 0.1-100 nM or genistein: 0.1-50 µM) were added at the beginning of the differentiation process. The fusion index was measured at 2nd, 4th and 6th day of differentiation. Additionally the number of cells with 6-10 or more than 10 nuclei in the cell was counted.

**Results**. The chromium (chloride and picolinate) supplementation caused a time-dependent increase in the myotubes formation (measured both as the fusion index, and as the number of myotubes with 6-10 or more than 10 nuclei in the cell) with the highest differences at the 6th day of differentiation (about 40% of control value). The effect of estradiol supplementation (0.1-100 nM) was short-term. The maximum stimulatory effect (about 100% of the fusion index as well as number of myotubes with 6-10 nuclei in myotube) was observed at the 2nd day of the experiment. Genistein (0.1-50 µM) did not affect the differentiation process.

**Conclusion.** This study revealed a positive role of chromium supplementation on the C2C12 cell line differentiation process. However, it does not give a final answer about the interaction of chromium with estrogen receptors during the differentiation process.

**Key words:** *chromium, estradiol, genistein, fusion index, C2C12 cell line*



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## Introduction

The muscle repair mechanism is very complicated and precise. We can distinguish it in three major, subsequent processes: degeneration, regeneration, and remodeling of damaged tissue. The disruption of sarcomeres causes a release of cell content and induction of an acute inflammatory response. It results in an additional damage of cells around the place of injury

and allows for removal of the dead cells. Next, satellite cells (a myogenic stem cell population) start proliferation and differentiation into myofibers. During the final step, remodeling, myofibers are transformed into mature muscle fibers and recover the ability to contract [1]. The most important task in muscle regenerative medicine is how to modulate these processes to improve further recovery. It is rather complicated because e.g an acute inflammatory response causes cell damage around the wound, but it may also stimulate the influx of mesenchymal stem cells (MSC) from the body. MSC may help in the reconstruction process by their proliferation and differentiation into myofibers or production of growth factors/cytokines which may increase the regeneration intensity [2]. Therefore, only appropriate knowledge concerning muscle recovery will optimize and give adequate therapy results.

During muscle regeneration the myoblast fusion in the differentiation process is very important. Differentiation is a multifactorial process controlled by a whole set of interactions among specific transcription factors and cell cycle regulatory proteins. The main role in this process is played by tissue-specific transcription factors belonging to the MyoD family (MyoD, Myf-5, myogenin, and MRF4/Myf-6) which start and modulate the muscle cell differentiation [3]. There are numerous levels of the differentiation process regulation (e.g. epigenetic modifications, gene transcription and translation, posttranscription and posttranslation) of both smooth and skeletal muscle. This process involves several signaling pathways and molecules such as retinoid receptor, TGF family, reactive oxygen species, miRNA, myostatin, and many others [4-6].

Perhaps also chemical compounds like chromium(III) may affect this process. It has been demonstrated that chromium administration changed the level of mRNA of growth factors [7] as well as insulin pathway correlated genes [8, 9]. The possible mechanism of chromium action on gene transcription is still unknown, but it has been suggested that chromium may interact with estrogen receptors [10, 11]. Activated estrogen receptors could affect transcription by numerous independent ways, also by modification of other transcriptional factor activity.

## Aim

In this study we examined the influence of chromium supplementation (in the form of picolinate and chloride) on the myotubes formation. Furthermore, to evaluate the possibility of estrogen-like mechanism of chromium action, the effect of estradiol (activation mainly ER-α) and genistein (selective agonist of ER-β receptor) on the differentiation process was also estimated. The study was performed on the mouse myoblast cell line C2C12, differentiated by 2% of horse serum addition.

## Material and method

### *Experimental factors*

Chromium salts (chloride and picolinate, Sigma Aldrich Chemical Co.; St. Louis, MO, USA) were dissolved in deionized water to the concentration of 1 mg $Cr^{3+}$/ml (stock solution) and sterilized by syringe filtration (0.22 μm). The final chromium concentration in the medium (10 μg $Cr^{3+}$/L) was based on the results obtained in the previous studies [8].

Estradiol and genistein (Sigma Aldrich Chemical Co.; St. Louis, MO, USA) were dissolved in methanol to the concentration of 1mM (stocks) of both. Estradiol was tested at 0.01; 0.1; 1; 10 and 100 nM concentration and genistein at 0.1; 1 and 50 μM.

All compounds were stored frozen in -80°C until the analysis.

### *Cell Culture*

The experiment was performed on the mouse myoblast C2C12 cell line from the European Collection of Animal Cell Cultures (Porton Down, England). The cells were cultured in standard conditions (DMEM + 10% FBS, Gibco, Scotland) and differentiated by addition of 2% of horse serum (Gibco, Scotland). To eliminate the effect of estrogenic compound, horse serum was filtrated through the column filled with Lipidex-1000 according to the procedure described by Snochowski and Romanowicz [12].

### *Fusion Index*

The C2C12 cell line differentiates rather fast, producing extensive contracting myotubes and expressing characteristic muscle proteins. It is a well-known model to study the *in vitro* myogenesis and cell differentiation process. The C2C12 cells were seed on 8-chamber Lab-Tek slides in concentration of $2.5x10^4$ cells/$cm^2$ in grow medium (DMEM +10% FBS). 80-90% of the confluent cells were flushed twice with PBS and control medium (CM, contains 2% of horse serum) or modified medium (MM, contain 2% of horse serum without estrogenic compounds) with or without experimental factors added. The fusion index was measured at $2^{nd}$, $4^{th}$ and $6^{th}$ day of differentiation. 15 minutes before the end of experiment the Hoechst 3342 fluorescent stain (Sigma Aldrich Chemical Co) was added to the final concentration of 2 μg/ml. Then the cells were flushed twice with PBS and fixed with methanol/acetic acid solution (3:1, 3 min, RT). Blue stained nuclei were counted in a fluorescent phase-contrast microscope at the magnification of 200x (Fig. 1) from 10 randomly selected fields of view. Myotubes were considered as the cell with three or more nuclei. The fusion index was calculated as the ratio of the number of nuclei inside myotubes to the number of total nuclei × 100. In addition the number of myotubes containing 6 to 10

nuclei or more than 10 nuclei was counted. The results are presented as % mean (±SD) of control values from 5 independent experiments.

### *Statistical analyses*

In the case of normal distribution of values the parametric t-test was used. In the case of non-parametric distribution the Kruskal-Wallis test was used for the evaluation of significance of differences (GraphPad Prism software). P values <0.05 were considered significant.

## Results

The fusion index values of cells cultured in the control medium (CM) were: 2nd day – 3.4%, 4th day – 12.2% and 6th day – 20.6%. An addition of modified medium (serum without estrogenic compounds) caused a decrease of the fusion index by 10.9% (p>0.05), 28.4% (p<0.001) and 34.5% (p<0.001) respectively on the 2nd, 4th, 6th day in comparison to the control medium values. Moreover on the sixth day of differentiation a decrease in the number of myotubes with 6-10 and above 10 nuclei in the cell was observed (about 40%, p<0.05). The described results are presented in Fig. 2.

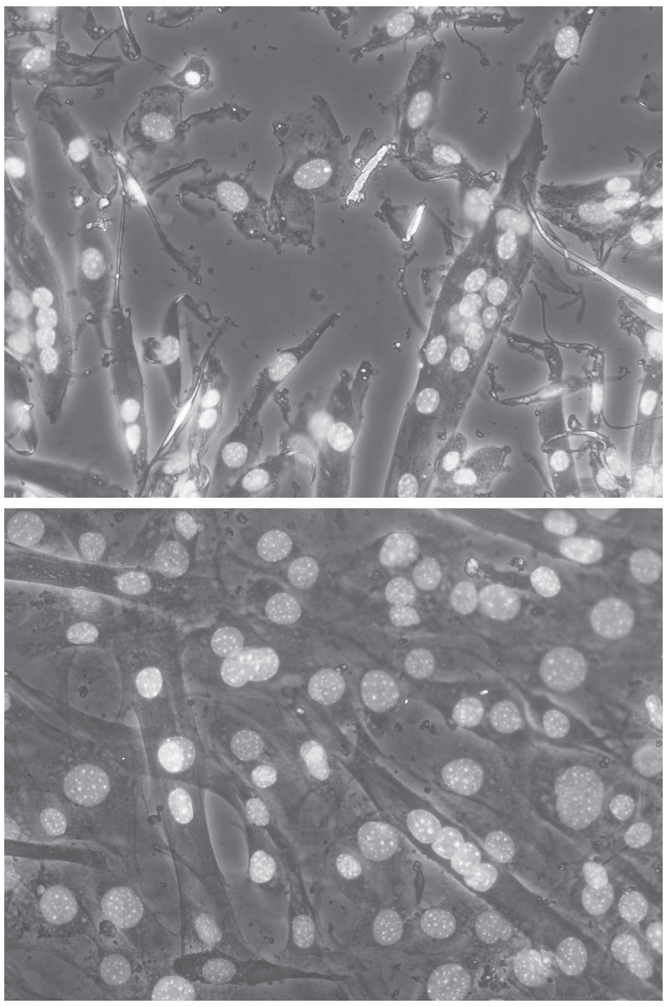

Fig. 1. Exemplary pictures of C2C12 cells in differentiation phase. Nuclei (bright) were stained with Hoechst 33342. Photo was taken at 4th day of differentiation of cells in fluorescent phase-contrast microscope at magnification of 200x

Ryc. 1. Przykładowe zdjęcia zróżnicowanych komórek C2C12. Jądra komórkowe (jasne pola) zostały zabarwione barwnikiem Hoechst 33342. Zdjęcie zostało wykonane w mikroskopie kontrastowo-fazowym (powiększenie 200x) w 4 dniu różnicowania

### *Chromium*

The chromium addition (10 µg $Cr^{3+}$/L) causes a significant increase in the myotubes formation in time-dependent manner. For chromium chloride salt it was: 2nd day – 21.4% (p<0.01), 4th day – 31.5% and 6th day – 36.4% (p<0.001) and for the chromium picolinate respectively: 21.5% (p<0.01), 38.4 and 43.5% (p<0.001) in comparison to the medium modified (MM) value. The kinetic value of the myotubes formation after the chromium supplementation (both chloride and picolinate) was decreasing in time. The highest stimulatory effect was observed at 2nd day (about 20%), then a slight slowdown was seen (4th day – about 10%, 6th day – about 5%). The chromium ions (both chloride and picolinate) also affected the number of myotubes with 6-10 and more than 10 nuclei in the cell. The highest difference between the modified medium (MM) at the 6th day of differentiation was observed (about 100% for myotubes with 6-10 nuclei and about 70% for more than 10 nuclei in the cell). Interestingly, slightly higher results were noted for picolinate than chloride chromium salts, but they were statistically insignificant. The described results are presented in Fig. 3.

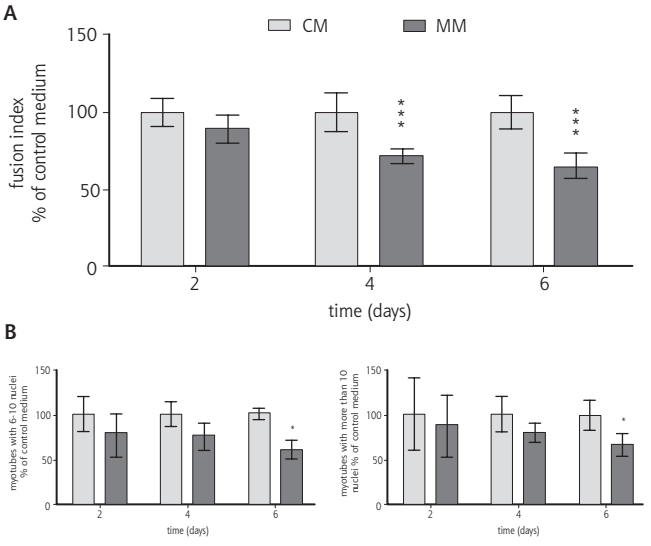


Fig. 2. Effect of modified medium supplementation (MM) on myotubes formation at 2nd, 4th and 6th day of differentiation {A – fusion index; B – number of cells with 6-10 (left) or more than 10 nuclei (right). Results are presented as percent of mean (±SD) obtained in control medium (CM)*, p<0.05***; p<0.001; N=50}

Ryc. 2. Wpływ medium modyfikowanego (MM) na formowanie miotub w 2., 4. i 6. dniu różnicowania. „A" – indeks fuzji, „B" – liczba komórek z 6-10 (lewa strona) lub więcej niż 10 jądrami w komórce (prawa strona). Wyniki zostały przedstawione jako średni procent (±SD) wyników uzyskanych w medium kontrolnym (CM) *p<0,05; ***p<0,001; n=50

### *Estradiol*

The estradiol administration caused a significant increase of the fusion index at the second day of differentiation in all tested concentrations (0.1- 00 nM) of this hormone. The highest values in the range of 0.1-10 nM concentration (about 100% of increase as compared to untreated cells) were noted. The stimulatory effect of estradiol was reduced in time. At the 4th day of differentiation a significant increase of the fusion index was observed only at 0.1 and 1 nM concentration (about 40% of medium modified value), and at the sixth day no significant changes were noted.

Similarly to the fusion index, the estradiol supplementation increased the number of myotubes with 6-10 nuclei in the cell at the 2nd day of differentiation (over 100%) at 0.01-10 nM concentrations. In the next days (4th and 6th) there was no impact of estradiol on the number of myotubes with 6-10 nuclei in the cell. Estradiol did not affect the number of myotubes with more than 10 nuclei in the cell at the 2nd day of differentiation; however a significant decrease for 10 and 100 nM of the estradiol concentration at the 4th and 6th day was noted (over 50%), in comparison to modified medium. The described results are presented in Fig. 4.

### *Genistein*

The genistein addition (0.01; 1 and 50 μM) did not affect the myotubes formation in both the fusion index and in the number of cells with 6-10 or more than 10 nuclei in comparison to the modified medium values (Fig. 5).

## Discussion

Mononucleated myoblast cells are capable of fusion which leads to creation of multinucleated myotubes – the primary structure of the muscle fiber formation. The fusion ability is a one of the most important processes of muscle tissue recovery following damage. This process involves several proteins, wherein the most significant role is played by the proteins of MyoD family.

This study evaluated the effect of chromium, estradiol and genistein supplementation on the myotubes formation *in vitro* in the mouse myoblast cell line C2C12. It is considered that the chromium diet supplementation has no apparent effect on muscle tissue growth, although the role of the element in construction of muscle fibers has been suggested [13]. Unfortunately, the literature presents only a few studies which confirm the influence of chromium on the differentiation process. In 1986 the Uyeki group demonstrated a stimulatory effect of the chromium addition (1 mM) on chondrogenesis in primary culture of mesenchymal stem cells isolated from chicken legs, however chromium used in this study was on +6 oxidation state [14]. In turn, the researchers from Thailand did not observe *in vitro* the effect of chromium(III) addition (chromium picolinate, 0.96

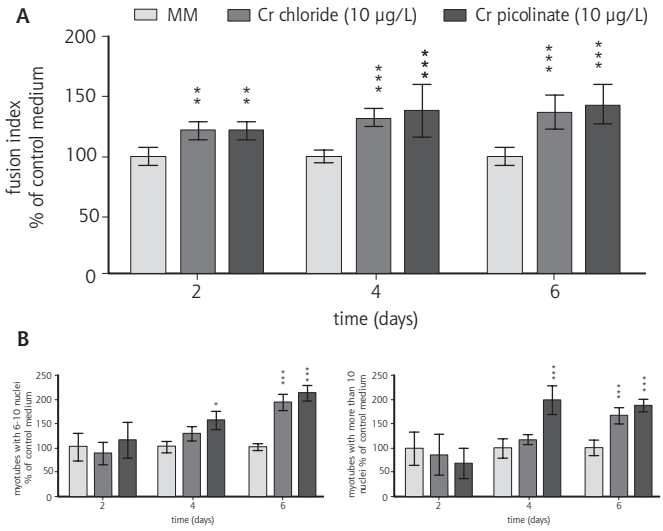


Fig. 3. Effect of chromium addition (chloride or picolinate, 10 μg $Cr^{3+}$/L) on myotubes formation at 2nd, 4th and 6th day of differentiation {A – fusion index; B – number of cells with 6-10 (left) or more than 10 nuclei (right). Results are presented as percent of mean (±SD) obtained in modified medium (MM)*; p<0.05***; p<0.001; N=50}

Ryc. 3. Wpływ suplementacji jonami chromu (chlorku lub pikolinianu chromu, 10 μg $Cr^{3+}$/L) na formowanie się miotub w 2., 4. i 6. dniu różnicowania. „A" – indeks fuzji, „B" – liczba komórek z 6-10 (lewa strona) lub więcej niż 10 jądrami w komórce (prawa strona). Wyniki zostały przedstawione jako średni procent (±SD) wyników uzyskanych w medium modyfikowanym (MM) *p<0,05; ***p<0,001; n=50

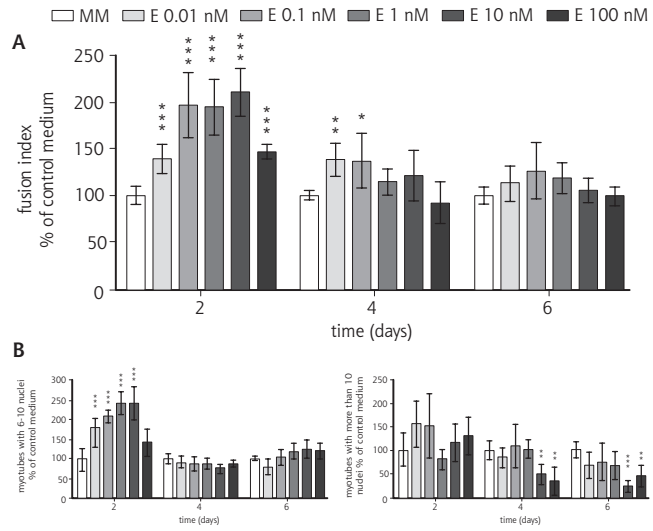


Fig. 4. Effect of estradiol addition (0.01-100 nM) on myotubes formation at 2nd, 4th and 6th day of differentiation {A – fusion index, B – number of cells with 6-10 (left) or more than 10 nuclei (right). Results are presented as percent of mean (±SD) obtained in modified medium (MM)*; p<0.05***; p<0.001; N=50}

Ryc. 4. Wpływ suplementacji estradiolem (0,01-100 nM) na formowanie się miotub w 2., 4. i 6. dniu różnicowania. „A" – indeks fuzji, „B" – liczba komórek z 6-10 (lewa strona) lub więcej niż 10 jądrami w komórce (prawa strona). Wyniki zostały przedstawione jako średni procent (±SD) wyników uzyskanych w medium modyfikowanym (MM) *p<0,05; ***p<0,001; n=50

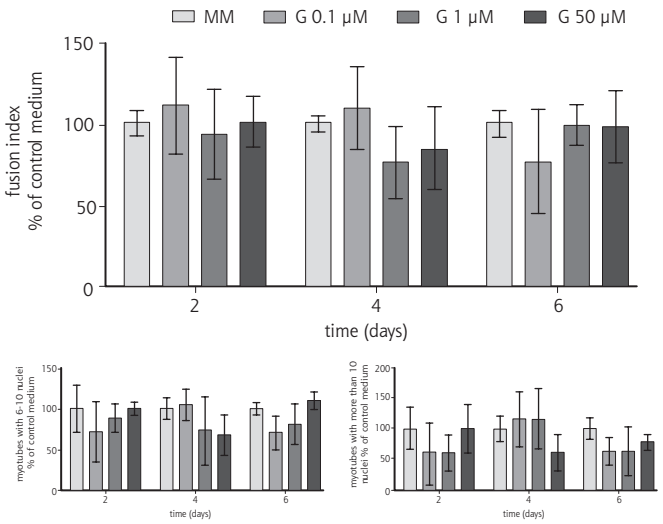


Fig. 5. Effect of genistein addition (0.1-50 µM) on myotubes formation at 2nd, 4th and 6th day of differentiation {A – fusion index, B – number of cells with 6-10 (left) or more than 10 nuclei (right). Results are presented as percent of mean (±SD) obtained in modified medium (MM); N=50}

Ryc. 5. Wpływ suplementacji genisteiną (0,01-100 nM) na formowanie się miotub w 2., 4. i 6. dniu różnicowania. „A" – indeks fuzji, „B" – liczba komórek z 6-10 (lewa strona) lub więcej niż 10 jądrami w komórce (prawa strona). Wyniki zostały przedstawione jako średni procent (±SD) wyników uzyskanych w medium modyfikowanym (MM) *p<0,05; ***p<0,001; n=50

µM) on the differentiation of mouse preadipocyte 3T3-L1 and myoblast C2C12 cell line [15, 16]. In contrast to these results, we observed a positive effect of chromium addition on the fusion index {about 40% higher at the 6th day of differentiation in comparison to the control value (MM)}. Perhaps this differences are associated with the concentration of chromium used in the experiment. In this study approximately a 5-fold lower concentration of chromium was used (0.19 µM). Additionally it has been previously demonstrated that a higher concentration of chromium(III) decreased the number of cells [17]. The chromium administration also increased the number of myotubes containing 6-10 and more than 10 nuclei in the cell. It is an interesting observation, especially in view of the treatment of muscle injuries. An increasing number of start-differentiation places and the fusion speed may be an important factor in reduction of recovery time. Therefore the use chromium as a candidate for the treatment of muscle tissue reconstruction should be considered.

It is well known that steroid hormones, especially in combination with growth hormone and insulin like growth factor, enhance muscle gains [18]. In this study a maximum stimulatory effect of the estradiol supplementation (0.1-100 nM) was seen at the 2nd day of differentiation (mainly myotubes up to 6 nuclei in the cell). The stimulation efficiency decreased during the experiment time. A similar time-dependent effect on the number of myotubes with more than 6 nuclei was noted. It was shown that the estradiol supplementation repressed myogenic differentiation in the C2C12 cell line on the 8th day of experiment [19]. Also Montague and co-authors revealed a decrease of isolated aortic smooth muscle differentiation following the estradiol treatment (10 nM) [20]. It is known that apoptosis is a necessary process in the myoblast differentiation. Apoptosis allows to control the number of proliferating cells and their differentiation into mature myotubes [21]. Estradiol is an anti-apoptic factor. The hormone abrogates the $H_2O_2$-induced apoptosis in C2C12 muscle cells. Blockage of the estrogen receptor (ER) isoforms α and β by specific antibodies abolish the 17β-estradiol inhibition [22]. It may explain the results of this study – a high number of small myotubes observed in the first two days and lack of stimulation of this process in further days of incubation.

Genistein did not affect the myotubes formation during the whole experimental period. The results obtained for phytoestrogen were similar to the control values (modified medium) or were slightly lower (not significant). This observation is in agreement with Ji and co-authors' results which demonstrated a decreased differentiation potential of the rat myotube L6 cell line after 10 or 100 nM genistein supplementation [23]. However, a negative effect of genistein addition *in vitro* was not observed in other tissue cultures. It was shown that phytoestrogen had a positive influence in osteogenesis and adipogenic (human primary bone marrow stromal cells), osteogenesis (MG63 human osteosarcoma osteoblasts) as well as keratinocytes differentiation [24-26].

The comparison of results obtained for the chromium (chloride and picolinate) and estradiol supplementation revealed some similarities in the myotubes formation process. Both chromium and estradiol stimulated fusion of mononucleated cells into myotubes with the highest rates observed after 2 days of incubation. However, the chromium addition had a long-lasting effect – at the end of the experiment it amounted to 40% of modified medium values and estradiol addition caused short-term changes – at the end of the experiment the results were similar to control (modified medium). Also the numbers of myotubes with 6-10 nuclei and more than 10 nuclei in the cell were different. The chromium supplementation increased the myotubes formation (with 6-10 and more than 10 nuclei) during the entire experiment period and estradiol stimulated the myotubes formation (merely with 6-10 nuclei) only at the 2nd day of differentiation.

The comparison of the genistein and chromium supplementation results on the myotubes formation did not show any similarity in the mechanism of action. Genistein in the tested concentration (0.1-50 µM) had no influence on this process and chromium positively affected the fusion process.

The results presented in this paper did not give the final answer about the interaction of chromium action with estrogen receptors on the differentiation process in the mouse myotube C2C12 cell line but it seems that such interaction may exist. However if such dependency exists it is more probable that chromium regulates the myotube formation through the estrogen receptor α.

## Conclusion

1. Chromium(III) (chromium and picolinate, 10 μg $Cr^{3+}$/L) cause a time-dependent increase in the myotubes formation (both the fusion index, and the number of myotubes with 6-10 or more than 10 nuclei in the cell).
2. The effect of estradiol supplementation (0.1-100 nM) was short-term. The maximum stimulatory effect was observed at the 2nd day of experiment, then it was reduced to the control level (MM).
3. Genistein (0.1-50 μM) did not affect the differentiation process.

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