Thieme

Comprehensive Analytics of Actovegin® and Its Effect on Muscle Cells

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ABSTRACT

The ingredients of Actovegin® were analyzed and its effects on the muscle cell proliferation were investigated. C2C12 myoblasts were cultured in medium. Actovegin® was added in five different concentrations (1, 5, 25, 125, and 250 µg) to the differentiation medium. The formations of proliferation factor Ki67 and myosin heavy chains were measured by immunofluorescence. The first primary antibody was anti-Ki67 and anti-Mf20. Cells were washed and treated with the second fluorochrome. Thirty-one Actovegin® ingredients were found to contain significantly higher concentrations and twenty-nine ingredients were found to contain significantly lower concentrations, compared to the mean ranges as described in the literature for the normal physiological concentrations in human adult serum/plasma. A significant increase in the formation of Ki67 was observed in Actovegin® groups, compared to controls. The mean area of myotubes was significantly increased in Actovegin® groups. A significant decrease in the number of myotubes was observed. An increased myotube size (fusion) was observed. The intensity of Mf20 was significantly increased in Actovegin® groups. It could be demonstrated that Actovegin® contains many physiological substances in significantly higher and some in lower concentrations compared to human adult serum. Furthermore, it could be shown that Actovegin® improves muscle cell proliferation.

Introduction

Actovegin® is a biological drug manufactured from a natural source. For more than sixty years, Actovegin® has been used for many medical indications, e.g., as an intravenous infusion to treat acute stroke [9, 16] or postpartum hemorrhage [2], as a topical form to treat skin ulcers [8], and as an intra-arterial infusion to treat long bone fractures [11], malfunction of the blood circulation in the brain and trophic disturbances (i.e., ischemic insult, canio-cerebral injury) [47], impairment of peripheral blood circulation (e.g., angiopathy and ulcus cruris) [11, 13, 27], wound healing problems (e.g., torpid wounds, decubitus) [11, 32, 34, 45] and mucosal lesions after radiation [4–6, 11, 49].

The treatment of muscle tears with intramuscular Actovegin® was first described by Pfister and Koller 1990 [40]. Their partially blinded case control study with 102 patients showed a reduction in recovery time in a treatment group of 5.5 weeks, compared with 8.3 weeks for the control group [40]. However, in this study, the diagnosis of specific muscle injuries was only based on clinical findings and was not graded according to imaging, e.g., Magnetic Resonance Imaging (MRI). Furthermore, Actovegin® was mixed with anesthetics before injection resulting in pharmacodynamic and pharmacokinetic alterations [40].

Muscle injuries are one of the most common sports-related injuries, and their incidence varies from 30–55 % [20, 26, 50]. Twelve per-

cent of all injuries are hamstring injuries, which are 2.5 times more frequent than, for example, quadriceps injuries [3, 51]. Studies have shown that the administration of anti-inflammatory drugs promotes muscle healing by reducing degeneration and inflammation [1]. However other studies demonstrated that anti-inflammatory drugs are detrimental to the entire healing process [35, 46]. Moreover a recent systematic review describes the potential myotoxicity of local anesthetics and non-steroidal anti-inflammatory drug injection, while no evidence is given that Actovegin® has such a side effect [43]. A variety of treatments such as growth factor injection therapy is still very experimental and has shown initial results in some pilot studies, however, due to their performance enhancing and anabolic properties, they are prohibited by the World Anti-Doping Agency (WADA) [World Anti-Doping Agency (WADA), Prohibited List (2017). In Internet: https://www.wada-ama.org/en/media/news/2016-09/wada-publishes-2017-prohibited-list; (March 2017)].

Therefore, Actovegin® injection therapy could be important in the treatment of muscle injuries [37]. Actovegin® is a deproteinized calf blood hemodialysate. Clinicians in Europe, China and Russia have used Actovegin® for a long time [6, 41, 52]. It can be administered as tablets, topical formulations, injections or infusions via intramuscular, intravenous or intra-arterial routes. Many researchers have tried to identify the active ingredients in this mixture, but have been unsuccessful. Although the active ingredients within Actovegin® are yet to be identified, there are some clinical studies for Actovegin® to confirm its safety [30, 41, 56]. There is much media attention and there are many anecdotal beliefs regarding Actovegin® injection therapy. In the lay press, controversial discussions between proponents and opponents have been published in recent years regarding the use of Actovegin® in high performance athletes.

In vivo and in vitro studies suggest that Actovegin® contains some active components, although they are yet to be identified [8, 41, 52, 53]. In a previous in vitro study, an increase in the mitochondrial oxidative phosphorylation was demonstrated in permeabilized human muscle fibers (obtained from overweight and untrained subjects) acutely exposed to Actovegin® [48]. But there have been no in vivo and/or in vitro studies investigating the effect of stand-alone Actovegin® therapy in muscle precursor cells highly relevant in skeletal muscle regeneration. C2C12 muscle cells are a useful tool to investigate effects of various substances/solutions on muscle precursor cell proliferation [53].

In the present study, the effect of a stand-alone Actovegin® addition on the proliferation of C2C12 muscle cells was investigated. Furthermore, there are no data available in the literature regarding the detailed qualification and quantification of Actovegin® ingredients. As a result, in this study the ingredients of Actovegin® were qualified and quantified with various modern analytical methods. In the null-hypothesis it is assumed that Actovegin® does not have an effect on the proliferation of muscle cells.

Materials and Methods

C2C12 cell culture and treatment

C2C12 mouse myoblasts [53], obtained from the DSZM (Braunschweig, Germany), were cultured in cell culture flasks (BD Falcon, Bedford, USA) at 37 °C and 5 % CO_2 in proliferation medium (PM) consisting of DMEM, 1 % panicilin-streptomycin, 4 mM glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate (all from Invitrogen, Karlsruhe, Germany) and 20 % fetal calf serum (FCS) (PAA, Pasching, Austria). Cells were plated on gelatin-coated (0.1 % in DPBS) (Sigma-Aldrich, Steinheim, Germany) (Life-Technologies, Carlsbad, CA, USA) glass cover slips at a density of 10,00 cells per cm^2. After plating, the cells were kept in proliferation medium until the cells reached 80–90 % of confluence. Thereafter, the medium was switched to a differentiation medium (DM) containing DMEM, 1 % penicillin/streptomycin, 4 mM glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 4 % horse serum (all from Life-Technologies, Carlsbad, CA, USA). Actovegin® (Takeda Pharmaceutical, Ōsaka, Prefecture Osaka, Japan) (LOT Number 10946788) was added in five different concentrations (1, 5, 25, 125, and 250 µg) to the DM. The control group was only treated with the normal DM. All concentration groups and control group had six days to differentiate, with a medium change every day. When the medium was changed, Actovegin® was also added to the different concentrations.

Immunofluorescence

After five days of differentiation, cells were fixed for 20 min incubation with 4 % paraformaldehyde (Merck KgaA, Darmstadt, Germany) followed by three washing steps with DPBS. Thereafter, cells were stored at 4 °C until undergoing immunofluorescence staining. Cells were then permeabilized with 0.25 % Triton X-100 in TBS for 10 min followed by four washing steps with TBS. In order to avoid unspecific binding, cells were blocked with 5 % bovine serum albumin (BSA) in TBS for 30 min. The first primary antibody was diluted in 0.8 % BSA, and cells were incubated at 4 °C overnight. The first primary antibody was anti-Mf20 (1:220; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and anti-Ki67 (1:250; Novus Biological, Littleton CO, USA). On the following day, cells were washed four times with TBS, and then treated with the first secondary antibody solution against the first primary antibody for one hour. This was followed by four washing steps with TBS and the treatment with the first fluorochrome (Streptavidin-Alexa 488) (Life-Technologies, Carlsbad, CA, USA) for one hour. Thereafter, washing with TBS and a blocking step with 5 % BSA in TBS for 30 min, followed by treatment with the second primary antibody (anti-Ki67;1:250; Novus Biological, Littleton CO, USA) at 4 °C overnight. On the third day, cells were washed four times with TBS followed by the second secondary antibody solution against the second primary antibody for one hour. Thereafter, cells were washed four times with TBS and treated with the second fluorochrome (Streptavidin-Alexa 555) (Life-Technologies, Carlsbad, CA, USA). After incubation, cells were washed four times with TBS and incubated with a mixture of DAPI (Life-Technologies, Carlsbad, CA, USA) including a 1:2000 dilution of DRAQ5 (Biostatus Ltd, Shepshed, UK) for 10 min. This was followed by two times of washing with TBS and one time of washing with de-ionized water. Cells were then mounted onto a microscope slide using Aquapolymount (Polysciences Inc., Warrington, PA, USA).

Analysis for Mf 20 and Ki 67

Cover slips were analyzed using the Zeiss LSM-meta 510 (Carl Zeiss AG, Jena, Germany). For every condition, 10 pictures with a 10x ob-

jective were photographed. The area, intensity of both antibodies, and the number of myotubes were measured in these pictures using the Image J software (National Institute of Health, Bethesda, MD, USA).

Laboratory diagnostics

Most relevant clinical chemistry parameters were determined on an automated AU 5800 clinical chemistry analyzer (Beckman Coulter). Osmolarity was measured using an osmometer Model 2020 (Kreienbaum). Homocystein was determined on a Centaur XP automated immunoassay analyzer (Siemens). Cortisol, estradiol, progesterone, vitamin B12, folate and testosterone were measured on an Cobas 8000 e602 automated immunoassay analyzer (Roche). 25-OH vitamin D3 was determined on an ISYS immunoassay analyzer (IDS). Vitamin B1 and B6 were measured by HPLC. Zinc and copper were determined by atomic absorption spectrometry (Analytik Jena). Free hemoglobin was measured by spetrophotometry (Hitachi). Fibrinogen was measured on a BN Prospec analyzer (Siemens). 17-OH progesterone was measured by ELISA. Protein electrophoresis was performed using a Capillarys 2 instrument (Sebia). Aminoacids were determined using a Biochrom 30 + analyzer (Onken).

Calculations and statistics

Data are presented as mean ± standard deviation (SD). A one-way ANOVA was performed for C2C12 cell experimental analyses to compare the effects of control group between each Actovegin® dosage group. Tukey's HSD post hoc analyses were then performed to establish where differences apply. A P value of < 0.05 was considered statistically significant. All statistical analysis were performed using SPSS Version19 (IBM statistics Inc. USA).

Ethical standards

This study meets the ethical standards of IJSM [23].

Results

Analysis data

The following Actovegin® ingredients were found in similar mean ranges as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)] for the normal physiological concentrations in human adult serum/plasma: folate, estradiol and free hemoglobin (Hb).

Thirty-one Actovegin® ingredients were found to contain significantly higher concentrations, compared to the mean ranges as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)] for the normal physiological concentrations in human adult serum/plasma (▶ **Fig. 1**). Highest levels were found for cystathionine (▶ **Fig. 2a**), o-phosphoserine (▶ **Fig. 2b**), asparagine acid (▶ **Fig. 2c**), and glutamic acid (▶ **Fig. 2d**).

15	cystathionine	3	osmolality in serum
15	phosphoserine	3	inorganic phosphate
14	glutamic acid	3	valine
14	asparagine acid	3	sodium
10	potassium	3	phosphoethanolamine
7	citrulline	3	phenylalanine
6	ornithine	3	tyrosine
5	leucine	3	β-alanine
5	glycine	3	serine
4	chloride	2	methionine
4	creatinine	2	threonine
4	isoleucine	2	arginine
4	urea	2	taurine
4	glucose	2	histidine
4	alanine	2	α-aminobutyric acid
4	lysine		

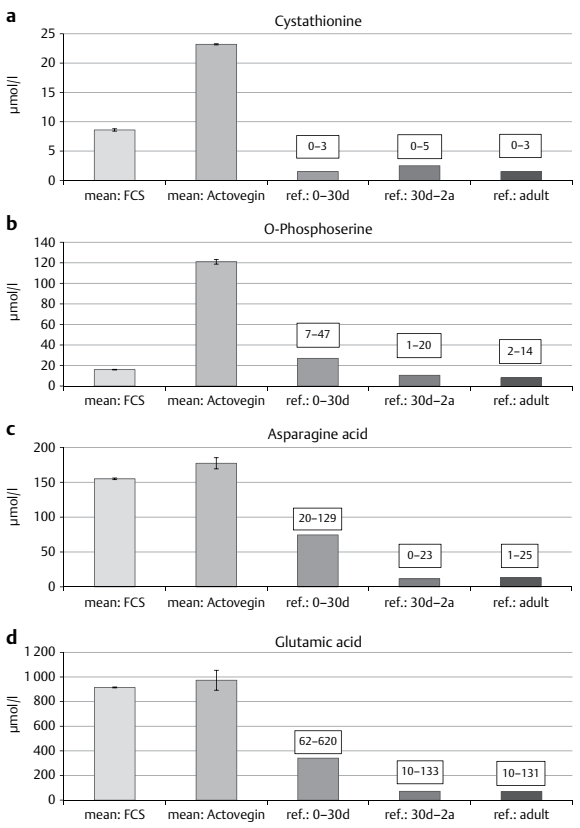
▶ **Fig. 1** Factors of increased values of Actovegin® ingredients, compared to the mean values of the corresponding substance in adult serum/plasma, as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)].

Twenty-nine Actovegin® ingredients were found to contain significantly lower concentrations, compared to the mean ranges as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)] for the normal physiological concentrations in human adult serum/plasma (▶ **Fig. 3**). Lowest levels were found for vitamin B1 (▶ **Fig. 4a**), uric acid (▶ **Fig. 4b**), testosterone (▶ **Fig. 4c**), and cortisol (▶ **Fig. 4d**).

Muscle cell experiments

Following the addition of Actovegin®, a significant increase in the proliferation of Ki67 in C212 myoblasts (▶ **Fig. 5**) was observed in 1 µg ($p < 0.001$), 5 µg ($p < 0.001$) and 250 µg ($p < 0.001$) Actovegin® concentrations compared to controls. The mean area of myotubes (single myotubes) (▶ **Fig. 6**) was significantly increased in 5 µg ($p < 0.05$), 25 µg ($p < 0.05$), 125 µg ($p < 0.001$) and 250 µg ($p < 0.001$) Actovegin® concentrations compared to controls. A significant decrease in the number of myotubes was observed following the addition of Actovegin® compared to controls (▶ **Fig. 7**). The intensity of Mf20 (▶ **Fig. 8**) was significantly increased in 1 µg ($p < 0.01$), 5 µg ($p < 0.001$), 25 µg ($p < 0.05$) and 250 µg ($p < 0.001$) Actovegin® concentrations compared to controls.

Actovegin® induced an increased cell proliferation in myoblasts (▶ **Fig. 9b**) compared to controls (▶ **Fig. 9a**). A higher fusion tendency was revealed by the decrease in myotube number and an increased myotube size (▶ **Fig. 9d**) compared to control (▶ **Fig. 9c**).



▶ **Fig. 2** Highest increased values of Actovegin® ingredients (**a-d**) (mean ± sem, n = 4), compared to the mean values of the corresponding substance in adult serum/plasma. Values in solid squares represent the minimum and maximum range of the corresponding substance as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)].

–141	vitamin B1
–54	uric acid
–51	testosterone
–26	cortisol
–24	IgA
–14	IgM
–12	albumine immunological
–12	IgG
–11	copper
–10	iron
–9	cholesterine
–8	tryptophan
–7	17-OH-progesterone
–6	aspartate transaminase
–6	alanine transaminase
–6	25-OH-vitamin D
–6	vitamin B12
–5	Zinc
–5	triglyzeride
–5	lactate dehydrogenase (310 K)
–5	progesterone
–4	magnesium
–4	albumine
–3	homocysteine
–3	hs-CRP
–3	bilirubin total
–2	albumen total
–2	calcium corrected (albumen)
–2	fibrinogen immunological
–2	Calcium

▶ **Fig. 3** Factors of decreased values of Actovegin® ingredients, compared to the mean values of the corresponding substance in adult serum/plasma, as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)].

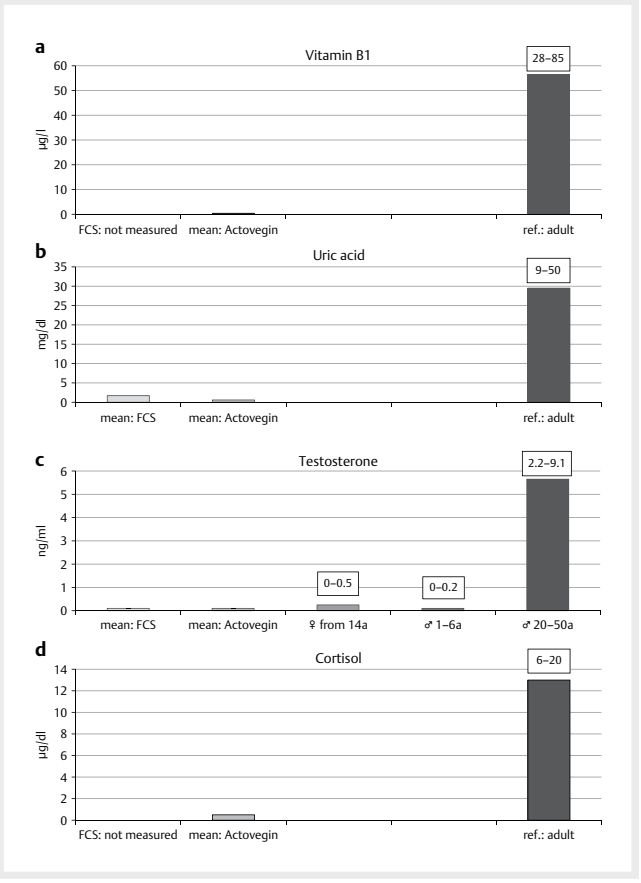
P-values of the statistical analysis for myotube area and Mf20 expression is provided in ▶ **Table 1**.

Discussion

C2C12 cells are a useful tool to study the differentiation and proliferation of myoblasts, to express various proteins, and to explore mechanistic pathways [53]. A myoblast is a type of progenitor cell that can differentiate to skeletal muscle tubes [39]. Differentiation is regulated by various myogenic regulatory factors [54, 55]. Skeletal muscle fibers are made when myoblasts fuse together. The fusion of myoblasts is specific to skeletal muscle (e.g., biceps brachii) and not cardiac muscle or smooth muscle. Myoblasts in skeletal muscle that do not form muscle fibers de-differentiate back into myosatellite cells [33]. These satellite cells remain adjacent to a skeletal muscle fiber, situated between the sarcolemma and the basement membrane [12] of the endomysium (the connective tissue investment that divides the muscle fascicles into individual fibers). To re-activate myogenesis, the satellite cells must be stimulated to differentiate into new fibers, a key step in muscle regeneration after relevant injury [19, 33].

Muscles are predominantly powered by the oxidation of fats and carbohydrates, but anaerobic chemical reactions are also used, particularly by fast-twitch fibers. These chemical reactions produce adenosine triphosphate (ATP) molecules that are used to power the movement of the myosin heads [10]. Mf20 is part of the Myosin protein heavy chain. Myosin is a protein that forms macromolecular filaments composed of multiple myosin subunits. Similar filament-forming myosin proteins were found in muscle cells and non muscle cells. In our experiments a significant increase of Mf20 was found after addition of Actovegin® to C2C12 cells, compared to controls (▶ **Fig. 6**). These results indicate that the addition of Actovegin® may lead to increased sarcomeric myosin expression, compared to controls. This is in line with an increased differentiation of the myotubes [28].

Beginning in the 1970s, researchers began to discover new myosin genes in simple eukaryotes [42] encoding proteins that acted as monomers and were therefore entitled Class I myosins. These myosins were collectively termed “unconventional myosins” [14] and have been found in many tissues other than muscle. These superfamily members have been grouped according to phylogenetic relationships derived from a comparison of the amino acid sequences of their head domains, with each class being assigned a Roman numeral [7, 15, 21, 25]. The unconventional myosins also have divergent tail domains, suggesting unique functions [36]. Analysis of the amino acid sequences of different myosins shows great variability among the tail domains, but strong conservation of head domain sequences. Presumably this is so the myosins may interact, via their tails, with a large number of different cargoes, while the goal in each case – to move along actin filaments – remains the same and therefore requires the same machinery in the motor. For an intact myosin synthesis, various aminoacids are necessary and must be available in muscle cells. The significantly increased proliferation of C2C12 muscle cells compared to control



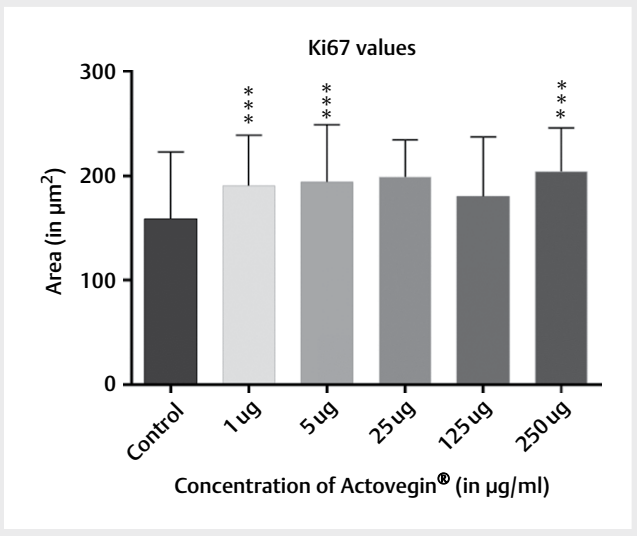
▶ **Fig. 4** Lowest decreased values of Actovegin® ingredients (**a–d**) (mean ± sem, n = 4), compared to the mean values of the corresponding substance in adult serum/plasma. Values in solid squares represent the minimum and maximum range of the corresponding substance as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)].

cells in this study, may be explained by the high availability of aminoacids, found in Actovegin® up to the factor 14 (e.g., for cystathionine, o-phosphoserine, asparaginic acid and glutaminic acid), compared to the corresponding aminoacids, as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)] for the physiological adult human serum/plasma. Further explanation for this increase may be provided by the increased Ki67 values (marker for proliferation), after the addition of various concentrations of Actovegin®, compared to controls. Furthermore, a significant increase of the area of the myotubes was observed, compared to controls. This may be explained by an increased fusion of myoblasts towards myotubes, with the highest effect observed in 125 µg of Actovegin®. For this, muscle fusion energy is necessary (e.g., ATP), which may be formed from increased availability and uptake of glucose. In the Actovegin® analysis a four-fold higher glucose level was detected, compared to the glucose level in the adult human physiological serum/plasma [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)]. Furthermore, most of the sarcoplasm is filled with myofibrils, which are long protein cords composed of myofilaments. The sarcoplasm is also composed of glycogen, a polysaccharide of glucose monomers, which provides energy to the cell with heightened exercise, and myoglobin, the red pigment that stores oxygen until needed for muscular activity [44].

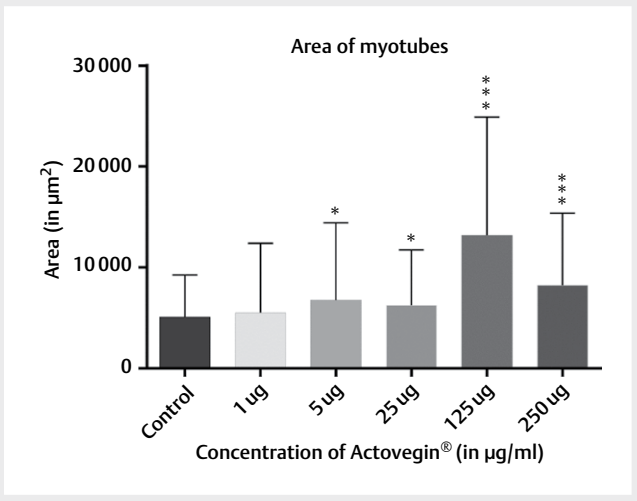
For an intact myosin function phosphorylations are necessary with high levels of phosphate, which have to be also available in the muscle cells. Also in these processes, myosin phosphatase target subunit, MYPT is involved [24]. Various isoforms of MYPT exist and the relatively minor distinctions are in the C-terminal leucine zipper motifs and with inserts in the central region. In addition, these isoforms all contain the four residue PP1c-binding motif (Arg/Lys-Val/Ile-Xaa-Phe) [24]. These aminoacids and inorganic phosphate were detected in high levels in the Actovegin® solution in our study, therefore high availability and functionability may result for these isoforms. Furthermore, after skeletal muscle injury a regeneration process takes place to repair muscle. Skeletal muscle recovery is a highly coordinated process involving cross-talk between immune and muscle cells [18]. Studies have demonstrated that amino acids and phosphate/potassium/sodium can improve also skeletal muscle regeneration by targeting key functions of muscle cells [18]. These results are also in agreement with the results in our study, because higher levels of aminoacids and phosphate/potassium/sodium were found in Actovegin®, compared to controls. Furthermore, in other studies Actovegin® is described for enhancement of aerobic oxidation in mammals [22] and an increase in mitochondrial respiratory capacity in human muscle cells [48]. This improves absorption of glucose and oxygen uptake in (muscle) tissue [22], which may also enhance physical performance and stamina [22].

The significant increase of the proliferation of C2C12 muscle cells after addition of Actovegin® may be explained by the high availability of many Actovegin® ingredients, which were higher, compared to the corresponding substance concentrations in human adult serum/plasma. But it is emphasized that various Actovegin® ingredients were detected in a significantly lower concentration (possibly depending on the ultrafiltration of the Actovegin® manufacturing process), compared to the corresponding substance concentration as described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)] in human adult serum/plasma. Whether an additional regulatory effect towards inhibitors/factors by these substances may also result in an increase of the proliferation, must be investigated in further experiments.

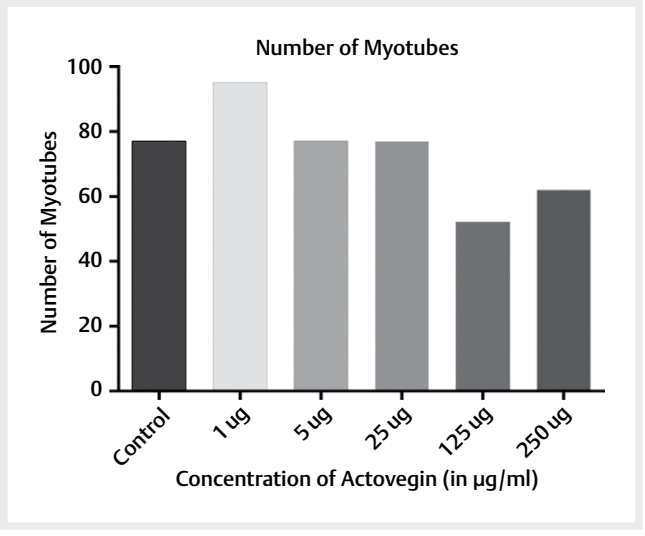
Actovegin® is a deproteinized calf blood hemolysate. Therefore, in this study ingredients were also compared as well to the serum/



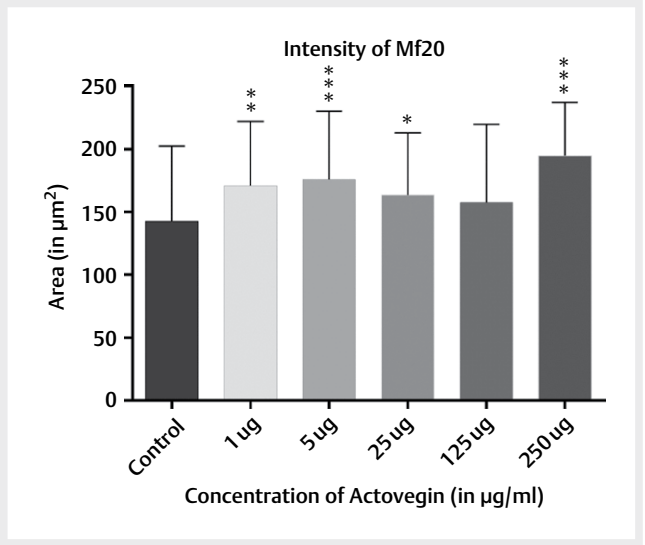
▶ **Fig. 5** Ki 67 values. Intensity of Ki 67 was significantly higher in 1 µg (P < 0.001), 5 µg (P < 0.001) and 250 µg (P < 0.001) Actovegin® concentrations than in control. Values are presented as means ± sem, (* p < 0.05 vs. Control, * * p < 0.01 vs. Control, * * * p < 0.001 vs. Control).



▶ **Fig. 6** Area of myotubes. Mean area of myotubes was significantly higher in 5 µg (P < 0.05), 25 µg (P < 0.05), 125 µg (P < 0.01) and 250 µg (P < 0.01) Actovegin® concentrations than in control. Values are presented as means ± sem, (* p < 0.05 vs. Control, * * p < 0.01 vs. Control, * * * p < 0.001 vs. Control).



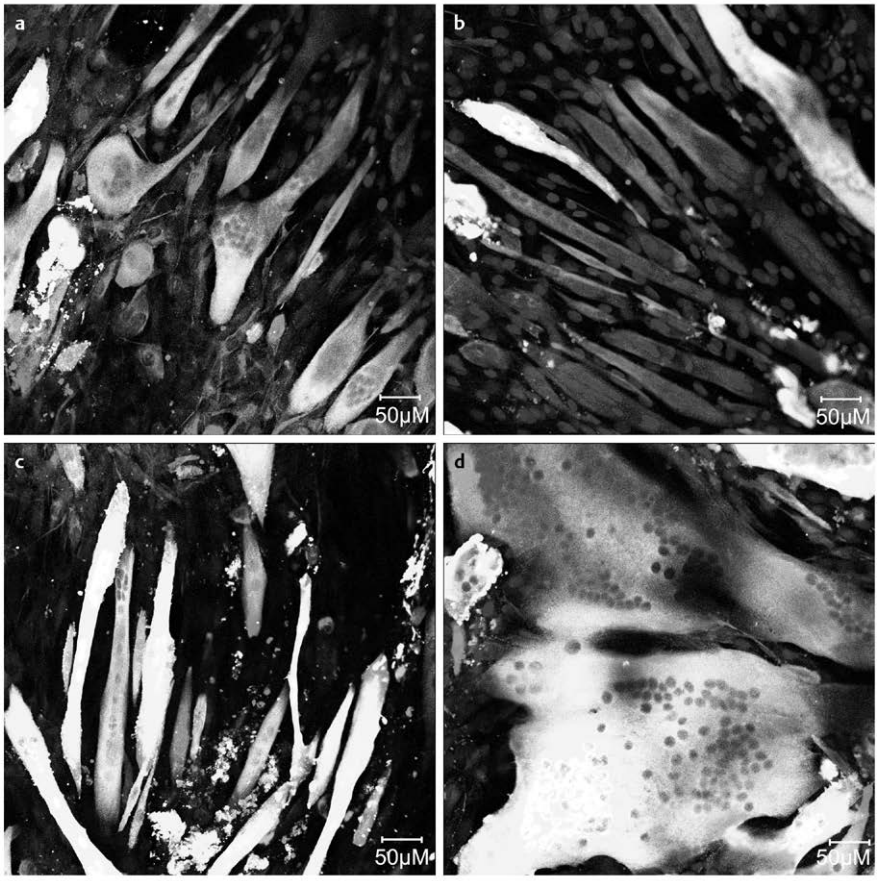
▶ **Fig. 7** Number of myotubes. The total number of myotubes observed for control and each Actovegin® concentration. Values are presented as means.



▶ **Fig. 8** Intensity of Mf20. The intensity of Mf20 was significantly higher in 1 µg (P < 0.01), 5 µg (P < 0.001), 25 µg (P < 0.05) and 250 µg (P < 0.001) Actovegin® concentrations than in control. Values are presented as means ± sem, (* p < 0.05 vs. Control, * * p < 0.01 vs. Control, * * * p < 0.001 vs. Control).

plasma concentrations to age dependent younger human beings, who are still in a growing phase as to normal calf serum (not deproteinized) (FCS). Many Actovegin® ingredients were detected in significantly higher concentrations, compared to the serum/plasma concentrations to age dependent younger human beings and to normal calf serum (not deproteinized). Therefore, these substances may play a more important role in the proliferation of muscle cells, compared to the Actovegin® ingredients which were detected in lower concentrations.

There is an anecdotal belief that Actovegin® can increase the performance of athletes [29]. One reason might be, because it is used sometimes in conjunction with prohibited substances. However Actovegin® was tested by anti-doping laboratories and no growth hormone or prohibited hormones were found since it is ultrafiltered to 6,000 Daltons [World Anti-Doping Agency (WADA), Prohibited List (2017). In Internet: https://www.wada-ama.org/en/media/news/2016-09/wada-publishes-2017-prohibited-list; (March 2017), World Anti-Doping Agency (WADA), Prohibited List (2011). In Internet: http://www.wada-ama.org/en/World-Anti-Doping-Program/Sports-and-Anti-Doping-Organizations/International-Standards/Prohibited-List/QA-on-2011-Prohibited-List/#; (May 2011)]. In a study with 567 diabetic patients, no improvement of muscle strength or condition was found after treatment with the maximum recom-



▶ **Fig. 9** **a–d**: C2C12 muscle cells exposed to Actovegin®, compared to controls. Representative light microscope (x10) images after cell culture between control and Actovegin®. Actovegin® (250 µg) induced an increased cell proliferation in myoblast **b**, compared to controls **a**. A higher fusion tendency was revealed by the decrease of myotubes and increase of myotube size with Actovegin® (250 µg) **d**, compared to controls **c**, respectively (Bar = 50 µm).

▶ **Table 1** P-values of the one-way-ANOVA test comparing myotube area and Mf20 expression in C2C12 muscle cells with and without Actovegin® addition.

Area	Control	1 ug mean	5 ug mean	25 ug mean	125 ug mean	250 ug mean
Control	1	0.528452222	0.017779022	0.03710327	1.0047E-06	8.07234E-06
1 ug mean	0.528452222	1	0.106949493	0.2679651	1.4068E-06	0.00087289
5 ug mean	0.017779022	0.105815942	1	0.50084423	0.00026226	0.107613694
25 ug mean	0.037103268	0.267965096	0.500844227	1	3.7905E-05	0.01028146
125 ug mean	1.00467E-06	1.40684E-06	0.000262262	3.7905E-05	1	0.008751209
250 ug mean	8.07234E-06	0.00087289	0.107613694	0.01028146	0.00875121	1
MF20	Control	1 ug mean	5 ug mean	25 ug mean	125 ug mean	250 ug mean
Control	1	0.001128198	0.000432408	0.01914552	0.18711663	5.35093E-08
1 ug mean	0.001128198	1	0.527677249	0.35663921	0.16119997	0.002831994
5 ug mean	0.000432408	0.527677249	1	0.14538706	0.0760394	0.028937188
25 ug mean	0.019145516	0.356639208	0.145387056	1	0.52112711	0.000147449
125 ug mean	0.187116633	0.161199975	0.076039401	0.52112711	1	0.000274381
250 ug mean	5.35093E-08	0.002831994	0.028937188	0.00014745	0.00027438	1
KI67	Control	1 ug mean	5 ug mean	25 ug mean	125 ug mean	250 ug mean
Control	1	0.000295793	0.000292236	0.5892932	0.05013726	4.016E-06
1 ug mean	0.000295793	1	0.617047189	0.00045433	0.26626772	0.069231471
5 ug mean	0.000292236	0.617047189	1	0.0003562	0.1678515	0.248753532
25 ug mean	0.589293205	0.000454328	0.000356199	1	0.07689084	9.60411E-07
125 ug mean	0.050137259	0.266267724	0.167851499	0.07689084	1	0.012316997
250 ug mean	4.016E-06	0.069231471	0.248753532	9.6041E-07	0.012317	1

mended dose of Actovegin® for 160 days [29, 56]. Currently, Actovegin® is not prohibited in sport in or out of competition, according to the WADA List of Prohibited Substances and Methods, the IOC, German, and many other National Anti-Doping Agencies (NADA), except if it is used by intravenous infusion [World Anti-Doping Agency (WADA), Prohibited List (2017). In Internet: https://www.wada-ama.org/en/media/news/2016-09/wada-publishes-2017-prohibited-list; (March 2017), World Anti-Doping Agency (WADA), Prohibited List (2011). In Internet: http://www.wada-ama.org/en/World-Anti-Doping-Program/Sports-and-Anti-Doping-Organizations/International-Standards/Prohibited-List/QA-on-2011-Prohibited-List/#; (May 2011)]. However, intravenous infusions are prohibited with any medical substance, even with saline. Intravenous injections with a simple syringe are permitted, if the injected substance is not prohibited, the volume does not exceed 50 ml, and the injections are given at intervals equal to or greater than six hours [World Anti-Doping Agency (WADA), Prohibited List (2011). In Internet: http://www.wada-ama.org/en/World-Anti-Doping-Program/Sports-and-Anti-Doping-Organizations/International-Standards/Prohibited-List/QA-on-2011-Prohibited-List/#; (May 2011)].

In this analytical Actovegin® study, significantly decreased concentrations of sexualic (anabolic) substances (e.g., testosterone, cortisol) were detected in the adult human physiological serum/plasma, compared to the corresponding substances described in the literature [Institut für Laboratoriumsmedizin Klinikum der Universität München/Teupser D., Einsenderhandbuch – Verfahrensliste – Campus Großhadern (February 2017); In Internet: http://www.klinikum.uni-muenchen.de/Institut-fuer-Laboratoriumsmedizin/download/de/untersuchungsverfahren/Verfahrensliste.pdf; (March 2017)]. These results indicate that Actovegin® cannot be classified as an anabolic solution, and Actovegin® is not (and cannot) be listed on the currently valid doping list published by the WADA [World Anti-Doping Agency (WADA), Prohibited List (2017). In Internet: https://www.wada-ama.org/en/media/news/2016-09/wada-publishes-2017-prohibited-list; (March 2017)]. It is also emphasized that Actovegin® is always administered locally to injured muscle tissue, therefore a systemic effect of Actovegin® is unlikely and only of minor relevance.

In the recent past, there has been more and more attention placed on autologous serum products (e.g., platelet rich plasma, PRP). They are being marketed aggressively as a treatment for almost all sports injuries. For a PRP preparation, venous blood must first be drawn from a patient. Platelets are then separated from other blood cells by centrifugation and injected with plasma into the injured area. However, there are many different preparation protocols, with different concentrations of PRP [31], each one is a biologically different product, with different potential effects [17]. A recent meta-analysis showed that PRP injection has no effect on acute hamstring injury [38]. Furthermore, PRP is a high concentration of platelets, related growth factors and plasma. Thus, it might contain elevated levels of substances (e.g., testosterone or cortisol), compared to the substance concentrations measured in Actovegin®.

So far, many studies confirmed the safety and effectiveness of Actovegin® [41, 52, 56]. However, further objective evidence in form of clinical studies on the treatment of muscle injuries is needed before any definitive conclusions can be drawn.

Conclusion

It could be demonstrated that Actovegin® contains many physiological substances in significantly higher and some in lower concentrations compared to human adult serum. Furthermore, it could be shown that Actovegin® improves muscle cell proliferation. These findings may help to explain the positive effects of Actovegin® on muscle injuries that were shown in previous studies.

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Conflict of interest

The authors have no conflict of interest to declare.

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