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Human TNF-α Affects the Egg-laying Dynamics and Glucose Metabolism of Schistosoma Mansoni Adult Worms in Vitro

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Short report

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Abstract

Several studies described the effect of human TNF-α on *Schistosoma mansoni*. It affects the worm's development, metabolism, egg-laying, changes in the parasite´s gene expression and protein phosphorylation. Data available concerning the influence of hTNF-α on egg-laying are controversial and understanding the mechanism of egg-laying regulation is essential in combating schistosomiasis. We characterized the effects of *in vitro* treatment of *S. mansoni* adult worms with different doses of hTNF-α (5, 20 and 40ng/mL) for five days. We explored the effects on the egg-laying rate, glucose, ATP metabolism, mRNA expression levels of lactate dehydrogenase, of glucose transporters and of *Sm*TNFR, the parasite gene for hTNF-α receptor.

hTNF-α influenced egg-laying in a time and dose dependent manner: with 40ng/mL, egg-laying increased on day 2 and decreased on days 3 and 4; 20 ng/mL dose, egg-laying decreased on day 3, while at 5ng/mL dose, egg-laying decreased on day 4. The total number of eggs produced was not affected, but the egg-laying dynamic was altered; the median egg-laying time decreased significantly due to treatment. At 5 and 20ng/mL hTNF-alpha, lactate production diminished on days 3 up to 5, while glucose uptake increased on day 5. At 40ng/mL, glucose uptake diminished on days 1 up to 3, while ATP accumulation was detected on day 5. No significant changes in the mRNA expression were detected in all treatments. Crosstalk involving the hTNF-alpha and the parasite signaling play a role in the fine regulation of the worm´s metabolism and physiology and points to new strategies for disease control.

Introduction

Schistosoma mansoni is the major causative agent of schistosomiasis, which affects more than 200 million people worldwide, especially in developing countries. Schistosomiasis is a debilitating disease due to the parasite´s egg-laying and the consequent formation of periovular granulomas, which are the key events in schistosomiasis pathogenesis [1].

Understanding of the host-parasite interactions is important to increase our knowledge of the biology of schistosomes and the development of new strategies against schistosomiasis. Factors that are important for the parasite´s development and the signaling elements responsible for molecular crosstalk are key topics to be explored [2] . TNF-α is an early pro-inflammatory cytokine produced in mammalians [3]. The literature has described several effects of hTNF-α on the parasite. Amiri et al. [4] found that in schistosome-infected SCID mice, human cytokine increases egg-laying and restores granuloma formation. Controversially, Haseeb et al. [5] reported that *in vitro* treatment of *S. mansoni* with the human cytokine decreases egg-laying and increases tyrosine uptake in females. Similarly, Haseeb et al. [6] observed that males treated with TNF-α increase their uptake of tyrosine. Cheever et al. [7] found that in SCID immunodeficient mice (with lower production of TNF-α due to the absence of B and T cells), egg-laying is delayed but normal. A few years later, Davies et al. [8] reported the involvement of TNF-α in limiting liver pathology and helping the parasite to survive. Oliveira et al. [9] documented that human cytokine induces changes in the expression profile of *S. mansoni* schistosomula and adult worms *in vitro*;

later, Oliveira et al. [10] reported changes in the phosphorylation profile of male adult worms caused by hTNF-α. Recently, Haseeb et al. [11] showed a reduction in methionine uptake and egg-laying in females treated *in vitro* with hTNF-α. Over time, the effects of hTNF-α on the parasite have been shown to be more complex and interesting, but they are still controversial and difficult to understand.

In this work, we aimed to study the *in vitro* effects of hTNF-α on *S. mansoni* glucose metabolism, gene expression and egg-laying regulation.

For this purpose, *Mesocricetus auratus* were infected with 300 cercaria of *S. mansoni* (BH strain) through subcutaneous inoculation and were euthanized and submitted to portal perfusion 7-8 weeks post-infection. Adult worms were recovered from portal veins through perfusion with RPMI medium with Heparin (250 UI/L). Then the worms were washed in RPMI and then incubated in RPMI containing 10% bovine fetal serum with 1X antibiotic/antimycotic and Gentamicin (50mg/L), at 37ºC under 5% CO_2. Different concentrations of recombinant hTNF-α (5, 20, 40ng/mL, Sigma-Aldrich) were added to the culture medium; in parallel, the vehicle (Tris 10mM pH 8,0) was added as negative control. At least three biological replicates were performed for each parameter that was evaluated.

As a control, the activity of reconstituted TNF-α was evaluated by an apoptosis induction assay in HEp-2 cell line. The cells were cultivated in Eagles Medium containing 10% BFS with 1X antibiotic/antimycotic (Gibco) and Gentamicin (50mg/L), at 37ºC under 5% CO_2, in a plate with a cover glass slide at the bottom. After two days of culture and 90% cell confluence, the cells were treated with reconstituted hTNF-α (20ng/mL) in parallel with the respective negative control (treated with the same volume of Tris 10mM pH 8 – the vehicle) for 18h. After the treatment, the covered glass slides with attached Hep-2 cells were immersed in ethanol 100% for 2h for fixation. Colorimetric immunohistochemistry of HEp-2 cells was performed with Rabbit anti-cleaved-Caspase 3 antibody (PC679 Sigma-Aldrich) diluted 1:100 and anti-mouse IgG diluted 1:1000 according to the procedure described by Santos et al. [12]. Apoptotic cells were labeled with a brown color and were counted in sets of 100 cells in both TNF-α treated and control samples (three biological replicates). Student's t-test was used to compare the differences between the averages of apoptotic cells in treated and control samples [13]. The results are shown in **Additional file 1: Figure S1.**

Treatment with reconstituted hTNF-α induced apoptosis in a significantly higher number of treated cells when compared with the control. This finding indicates that reconstituted hTNF-α showed the expected activity for these experiments. The reconstituted cytokine was able to induce apoptosis in HEp-2 cell, which was expected since Goodkin et al. (2003) described that hTNF-α induces apoptosis in these cells via the caspase activation.

We evaluated the egg-laying of *S. mansoni* adult paired worms for 5 days at different concentrations of hTNF-α. Each couple was placed in a well of a 24-well plate, and eggs were counted every day for 5 days. We performed three independent experiments using a total of 27 adult couples for each condition. The

counting of eggs was performed using a blind assay, (the egg counter was blind as to the treatment condition in each well). On the third day, the medium containing hTNF-α (and controls) were replaced.

Data analysis was performed with GraphPad Prism 7.03, R Studio (RStudio, 2020). For the egg-laying, lactate, glucose, and RT-qPCR experiments, Shapiro-Wilk normality tests were performed on the data from all groups to evaluate the normality of the distributions. For data with normal distributions, analysis of variance (ANOVA) [14] was performed for four different conditions, and the Tukey test [15] was used for comparisons between two conditions. For data without a normal distribution, the Kruskal-Wallis test [16] was used to make multi-comparisons, and the Wilcox pairwise test [17] was used for comparisons between two conditions. The Benjamini-Hochberg correction [18] was used to adjust the p-values. Adjusted p-values <0.05 were considered significant.

The results are shown in **Figure 1**. We observed a significant increase in egg-laying on day 2 after treatment with 40ng/mL TNF-α, a significant decrease in oviposition on day 3 after treatment with 20 and 40ng/mL, and a significant decrease in egg-laying on day 4 after treatment with 5 and 40ng/mL (**Figure 1A**). When the total number of eggs produced across the five days was computed for each treatment condition, no significant difference in total egg output was observed among the treatments and the control (**Figure 1B**). **Additional file 2: Table S1** contains all raw data of egg-laying experiments and statistical analysis information. Our results allowed us to calculate the egg-laying decay time using the Weibull distribution [19] to evaluate the effect of hTNF-α on the scale parameter η of egg-laying time of each group. Each η of each group was compared by the Kruskal-Wallis test [16] (**Additional file 2: Table S1, Sheet C**). Considering this analysis, the half-life of egg-laying decreased significantly in comparison to the control in a dose-dependent manner as the TNF-α dose increased (**Figure 1C**).

The treatment with TNF-α increases the egg-laying dynamic for all doses along the 5 days in a dose-dependent manner (the highest the treatment dose produced the shortest egg-laying time).

We measured the concentrations of lactate, glucose, and ATP in the medium or in the adult couples that were treated with hTNF-α and the respective negative control for 1, 3 and 5 days. We used two approaches for the measurement of these metabolites on day 5: (i) replacing the medium on the day 3, and (ii) without replacing the medium during the 5-day experiment. Five *S. mansoni* adult worm couples were incubated in 1.5mL of medium with different concentrations of hTNF-α and incubation times, as described before. The culture medium was centrifuged, and the supernatant was sent to the Central Laboratory of São Paulo Hospital (UNIFESP). Lactate and glucose were measured in Cobas 6000 c501 equipment using the Lactate Gem.2 kit and Glucose HK Gen.3 kit (Roche), respectively.

Figure 2 summarizes the results. We observed a significant decrease in lactate levels in the medium with treatments of 5 and 20ng/mL on days 3 and 5 (without changing the media). An increase in lactate concentration was observed with treatment of 40 ng/ml hTNF-α on day 5, and it was statistically significant when the medium was changed (identified as day 5^{c}).

We also observed a significant decrease in glucose uptake with 40ng/mL hTNF-α treatment on days 1 and 3 (higher quantities of glucose remained in the treated medium when compared with the control medium). When the medium was replaced on day 3, an increase in glucose uptake was observed on the fifth day (see day 5^c) for worms treated with 5 and 20ng/mL hTNF- α.

Additional file 3: Figure S2 (A and B) shows the absolute mean concentration of lactate and glucose that were measured in the medium. An increase in lactate production and a decrease in glucose was observed along the days of culture. Extremely low amounts of glucose remain on day 5 if no change of medium occurred.

Since one of the ATP synthase subunits was found to be differentially phosphorylated upon adult *S. mansoni* treatment with hTNF-α [10] and differences in lactate and glucose concentrations were affected by hTNF-α in adult couples, we evaluated the ATP accumulation in the worms treated with hTNF-α. For this experiment, five adult worm couples were incubated in 5mL of media with different concentrations of hTNF-α and the respective negative control, as mentioned above, for 1, 3 and 5 days. After the incubation, the couples were transferred to microtubes containing 200μl of RPMI, macerated with a pistil for 5 minutes and centrifuged at full speed for 5 minutes. Then 100μl of the supernatant was pipetted in a white 96-well plate with 100μl of Cell Titer-Glo reagent (Promega). The plate was mixed for 2 minutes and luminesce was detected using an Enspire Multimode Plate Reader (PerkinElmer). Due to the variability in the luminescence detected in each experimental batch and/or replica and the arbitrary unit of measured luminescence, we calculated the ratio of the luminescence values of treated/control worms to perform a normalization among the different experiments (as an indirect estimation of ATP amounts). The one-sample t-test [20][20] with a theoretical mean=1.0 (control) was performed to evaluate the differences between averages of treated/control ratio of worms in the ATP assay.

We observed a significant increase in ATP levels on day 5 (without a change in medium) when worms were treated with 40ng/mL TNF-α (**Figure 2C**). The luminescence values are available in **Additional file 3: Figure S3C**. **Additional file 4 Table S2** contains all raw data for lactate, glucose, and ATP quantitation along with information about their statistical analysis.

We investigated the mRNA expression levels of *Sm*TNFR, LDH, and the glucose transporter genes for all TNF-α treatment conditions using the qPCR approach. We aimed to validate the result described in Oliveira et al. [9], i.e., up-regulation of LDH expression upon treatment with 20ng/ml hTNF-α for 24h, and to possibly explain the changes in lactate and glucose concentrations observed here.

Total RNA from TNF-α treated and control adult couples was extracted using RNeasy Micro kits (QIAGEN). Samples were quantified using a fluorometer (Qubit, Applied Biosystem), and RNA quality was evaluated via microfluidic electrophoresis using a Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. cDNA was synthetized with 200ng of RNA per sample using SuperScript IV Reverse transcriptase (Thermo Fisher Scientific). Real-time PCR was performed using Sybr green reagent (Roche), and gene expression levels of LDH (Smp_033040), *Sm*TNFR (Smp_168070) and glucose

transporters, SGTP1 (Smp_012440), SGTP2 (Smp_046790), SGTP3 (Smp_127200) and SGTP4 (Smp_103410) were measured. Primers' efficiencies were tested, and all of them had efficiencies between 90 and 110%. Smp_090920 and Smp_062630 were used as references genes [21] . The primers sequences are available in the **Additional file 4: Table S3**. Relative gene expression was calculated according to [22]. All RT-qPCR assays were performed according to the MIQE guidelines [23]

We verified that no significant changes in the mRNA expression levels of investigated genes was observed in any treatment condition. Although the expression mean of LDH increased due to treatment with 20ng/ml of hTNF-α, in accordance with Oliveira et al. [9], the result was not statistically significant (data not shown). We did not observe any other significant differences in the expression of SGTPs suggesting that regulation at the protein level or post-translational mechanisms may play a role on the parasite signaling in response to hTNF-α.

Characterization of the effects of hTNF-α on *S. mansoni* physiology and metabolism is important for understanding the molecular basis of host-parasite interactions and platyhelminths´ biology and, therefore, it could point to new targets for the development of strategies against schistosomiasis. It is also particularly relevant because, recently, Bertevello et al. [24] described 29 parasitic platyhelminth genes homologous to *Sm*TNFR.

There is no consensus in the literature regarding the effect of hTNF-α on *S. mansoni* egg-laying. However, egg-laying is the central event for the pathogenesis and spread of schistosomiasis. Amiri et al. [4] found that the *in vitro* treatment of females with different doses of TNF-α (10, 20 and 40ng/mL) proportionally increased the number of eggs over three days. Haseeb et al. [5] showed that the *in vitro* treatment of females with 20, 40 and 100ng/mL of hTNF-α induces a significant decrease in the number of eggs. Additionally, Cheever et al. [7] showed *in vivo* that SCID mice have fewer eggs in their tissues in the early infection stage than Balb/c mice; however, after nine weeks of infection, no difference was observed.

Here, we used paired adult couples *in vitro* to maintain an adequate assay environment since male parasites provide a signal for female worms to access key nutrients [25]. The median egg output per couple in all treatment conditions was higher on the first day, but there were no significant differences when compared to the control; on the second day of treatment, we observed a significant increase at 40ng/mL hTNF-α. On the third day we observed a significant decrease in the median egg/couple measurements for treatment with 20 and 40ng/mL, which corroborates Haseeb et al.'s [5] findings and contradicts Amiri et al [4]. The most important observation is that over five days of treatment, the total number of eggs was not different among the treatments and the control, which means that although the dynamics of egg-laying were affected, the fecundity was not. The presence of host TNF-α makes the couples lay eggs faster (**Figure 1C**) but not in higher or lower quantities (**Figure 1B**). In general, our results can be interpreted in a similar as those described by Cheever et al. [7], in which a delay in egg-laying was observed while performing *in vivo* experiments with SCID mice. It is apparent that hTNF-α accelerates egg-laying, while in diminished doses it delays oviposition.

To understand which mechanisms are involved in the egg-laying regulation by hTNF-α, we measured glucose/lactate and tried to correlate these measurements with the expression of related genes. We did observe that when the lactate or glucose concentration changed significantly, there was also a significant change in egg-laying. On day 3 of treatment, when lactate production or glucose uptake was diminished, the egg-laying decreased, indicating that energetic metabolism is a relevant player regulated by hTNF-α and interferes in the egg-production dynamics.

Egg production is limited in an *ex vivo* environment due to the exhaustion of metabolic resources (glycogen and fat) [25]. Female adult worms uptake more than three-fold their dry weight in glucose per day, with glucose most probably directly used in worm metabolism (Pearce and Huang, 2015). The hTNF-α effect on glucose uptake by *S. mansoni* couples is subtle and not related to the mRNA expression of SGTPs. The changes in lactate production are not related to the expression level of LDH mRNA; the regulation of this enzyme is probably at the protein expression level and/or is a post-translational modification (as suggested by Oliveira et al., 2016). The observed increase in ATP on day 5 could be explained by the compromised egg-laying at this time. The high demand for ATP is for oogenesis, and when it is not necessary, this molecule can accumulate. **Graphical abstract** summarizes the proposed *in vitro* effect of this host cytokine on the parasite.

In this present study, we concluded that hTNF-α did not affect the worm´s fecundity but affected the dynamics of egg production; lactate production and glucose uptake may be relevant for the regulation of this event. Here, we demonstrated that molecular crosstalk between host and parasite is relevant for the fine regulation of worm physiology and is important for the successful adaptation of this ecological relationship. Most parasite platyhelminth species have homologous genes for *Sm*TNFR but not for hTNF-α [24] and may have established a homologous relation with their hosts through evolution.

Abbreviations

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

BFS: Bovine fetal serum

HEp-2: Human Epithelial type 2

hTNF-α: Human tumor necrosis factor alpha

LDH: Lactate dehydrogenase

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

RPMI: Roswell Park Memorial Institute medium

RT-qPCR: Reverse transcription followed by quantitative polymerase chain reaction

SCID: Severe combined immunodeficiency

SGTP: *Schistosoma mansoni* glucose transporter protein

*Sm*TNFR: *Schistosoma mansoni* tumor necrosis fator receptor

Declarations

Ethical approval

All procedures in animals were performed with the approval of the animal ethical committee of UNIFESP (process 2252221119).

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Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that have no competing interest

Authors' contributions

KCO, EHLJ and SVA conceived, designed the study, wrote, and critically revised the manuscript. EHLJ, GOS, CBG, GDR, VSR, MSA, CTK, PLSP and RFK performed the experiments, the data analysis and were involved in the discussion of results in this manuscript. All authors read and approved the final manuscript

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Figures

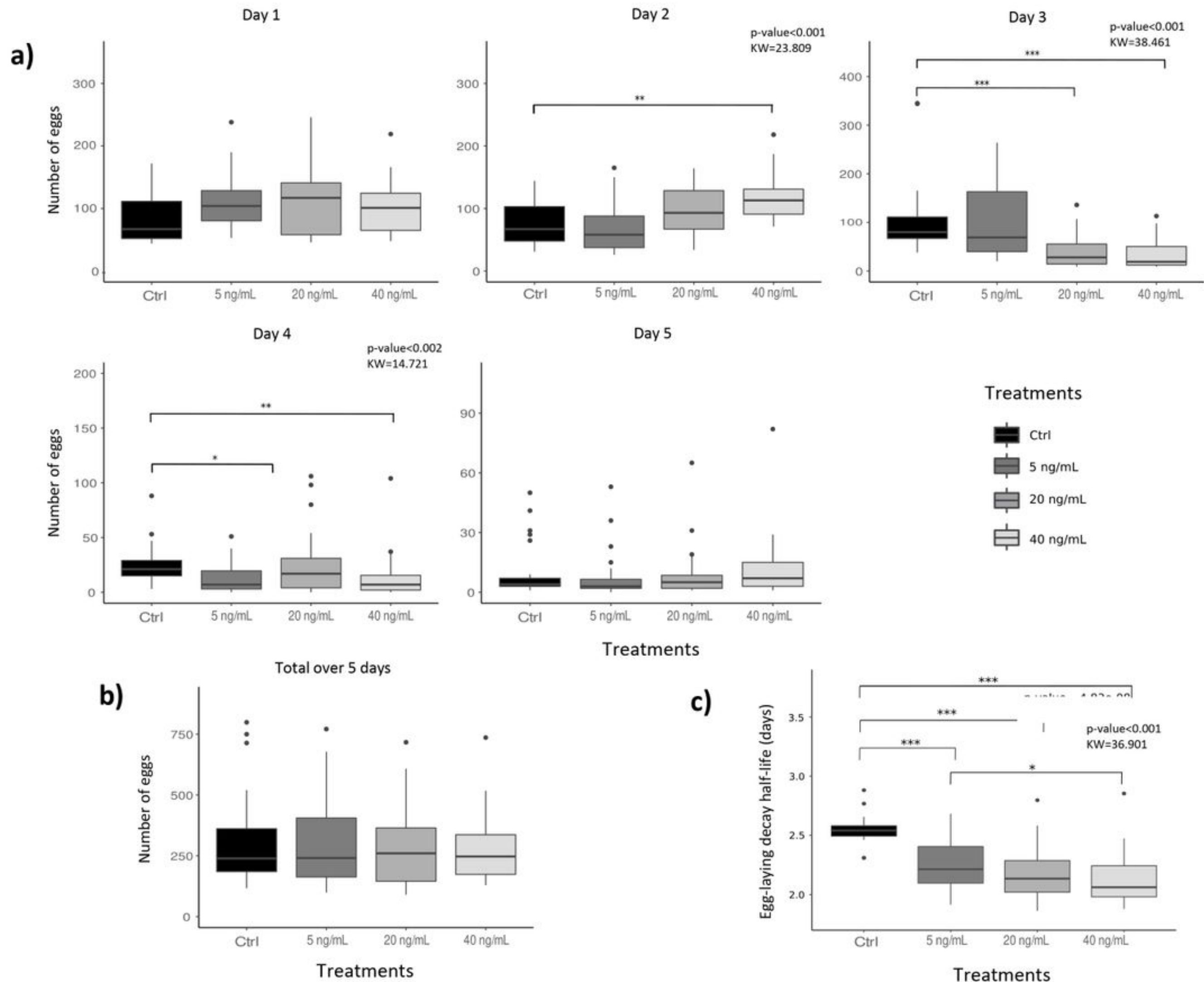

Figure 1

Effect of hTNF- α on S. mansoni egg-laying. A) Box plots showing the number of eggs laid by paired couples treated with TNF-α (5, 20 and 40ng/mL) and the respective control at each of the 5 days of experimentation, as indicated at the top of each panel. The different treatment conditions are indicated in the legend on the right. B) Total number of eggs laid over the 5-day period, for each treatment condition. C) Box plots of egg-laying decay half-life (days) for each treatment condition. The different conditions are indicated on the x-axis. *P-value< 0.05, **P-value<0.001; ***P-value< 0.0001.

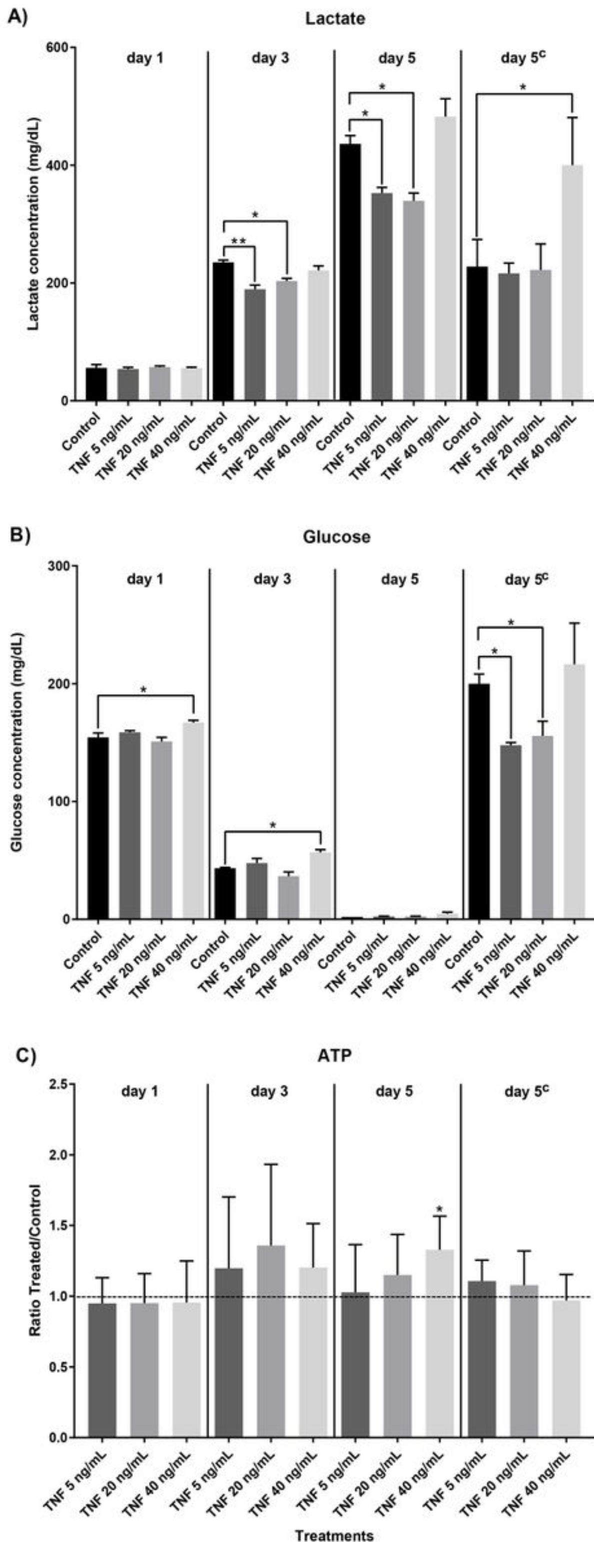

Figure 2

Concentrations of lactate (A), glucose (B) and ATP (C) in the culture media of adult worms treated with hTNF-α (5, 20 and 40ng/mL) on days 1, 3, 5 and 5c. Mean SEM readings are plotted. *P-value<0.05; **P-value< 0.005. Day 5c: the culture medium was replaced on day 3. The y-axis indicates the concentration (A and B) or the ratio of the luminescence values of treated/control worms (C); the x-axis indicates the experimental condition, with the treatment days on top.

Supplementary Files

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