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ОРГАНОСПЕЦИФИЧНАЯ ЭКСПРЕССИЯ ГЕНОВ ВОСПАЛЕНИЯ В ОТВЕТ НА ВВЕДЕНИЕ ЛИПОПОЛИСАХАРИДА РЫБАМ *DANIO RERIO*

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Резюме. Известно, что системное воспаление является ключевым компонентом прогрессирования многих инфекционных и неинфекционных заболеваний и может приводить к полиорганной недостаточности, хроническому воспалению, иммуносупрессии, катаболическому синдрому и даже смерти. Важность этого состояния обуславливает необходимость создания релевантной *in vivo* модели воспаления, пригодной для изучения патогенеза многих заболеваний, а также для проведения скрининга эффективности фармакологических препаратов. Рыбы *Danio rerio* являются одними из наиболее важных моделей исследования биологических процессов *in vivo*. Целью настоящего исследования было создание модели системного воспаления *in vivo*, индуцированного внутрибрюшинным введением липополисахарида (ЛПС) у рыб *Danio rerio*, с последующей идентификацией органо-специфической провоспалительной активности генов. Было проведено исследование уровня экспрессии основных провоспалительных генов у *Danio rerio* после инъекции ЛПС. Сравнивая *18s, eef1a1l1, gapdh* и *actb* в качестве потенциальных генов домашнего хозяйства, мы пришли к выводу, что *eef1a1l1* с эффективностью 99% является наиболее перспективным для дальнейшей нормализации в этой модели. Активность генов была наиболее выраженной в сердце, где экспрессия *IL6, CXCL8α* и *CXCL18β* была повышена до 100 раз. Кроме того, почки были наиболее вовлечены в воспалительный процесс, поскольку там было активировано наибольшее количество из проанализированных генов: уровни экспрессии *CXCL18β, CXCL8α, IL1β, IL6, Mpeg1.2* и *TNFα* были значительно увеличены. Вероятно, это связано с активностью почек как иммунного и кроветворного органа. Самая низкая реактивность обнаружена в мышцах. Иммунные реакции демонстрируют дозозависимость, например, инфузия 20 мкг ЛПС приводила к снижению экспрессии *IFNγ*, *Mpeg 1.2* и *Mpeg 1.1* в печени и к увеличению экспрессии *Mpeg 1.2* в почках по сравнению с дозировкой 10 мкг. Таким образом, *Danio rerio* является релевантной моделью воспаления. Наша модель продемонстрировала, что исследование изолированных органов рыб может быть полезным и информативным для исследования воспалительных процессов.

Ключевые слова: Danio reri, воспаление, липополисахарид, экспрессия генов, сердце, почки

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ORGAN-SPECIFIC LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN ADULT ZEBRAFISH

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Abstract. Systemic inflammation is known to be a key component of infection and non-infection diseases progression and may lead to multiorgan failure, persistent inflammation, immunosuppression, catabolism syndrome or even indolent death. This importance dictates the need for relevant *in vivo* models of inflammation to investigate the pathogenesis of numerous diseases and to perform drug screening. *Danio rerio* (zebrafish) became one of the most important models to explore biological processes *in vivo*. The aim of the study was to generate a lipopolysaccharide (LPS) model of systemic inflammation *in vivo* using zebrafish and to identify organspecific proinflammatory genes activity after intraperitoneal LPS infusion. We performed organ specific analysis of main proinflammatory genes expression in zebrafish after LPS stimulation. Comparing *18s, eef1a1l1, gapdh,* and *actb* as potential housekeeping genes, we came to conclusion that eef1a1l1 with 99% effectiveness is the most promising for further normalization in this model. The genes activity was the most pronounced in the heart where the expression of *IL6, CXCL8α,* and *CXCL18b* was increased up to 100-fold. Moreover, the kidneys were the most involved in the inflammatory process since the highest number of analysed genes were up-regulated there: expression levels of *CXCL18β, CXCL8α, IL1β, IL6, Mpeg1.2*, and *TNFα* were significantly increased. This was probably related to the kidney activity as an immune and hematopoietic organ. The lowest reactivity was detected in the muscles. Immune reactions could be dose-dependent, for instance the infusion of 20 μg LPS led to decrease of expression of *IFNγ, Mpeg 1.2,* and *Mpeg 1.1* in the liver and to increase of *Mpeg 1.2* expression in the kidney comparing with 10 μg dosage. Thus, due to the high degree of the similarity and other unique properties, *Danio rerio* has the advantage of being relevant model of inflammation. Our model demonstrated that the investigation of isolated zebrafish organs could be useful and informative for the investigation of inflammatory processes.

Keywords: zebrafish, Danio rerio, inflammation, LPS, gene expression, heart, kidney

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Introduction

Since the 1960s, zebrafish (*Danio rerio*) has been used as a vertebrate model organism. Zebrafish became one of the most important models to study a variety of biological processes *in vivo* after establishing in 2013 that its genetic structure is similar to humans [21, 27]. Currently, *Danio rerio* is widely used in developing human disease models in oncology [4], neurology [10], endocrinology [62], cardiology [32] as well as being highly demanded in drug research and development [39]. Besides, *Danio rerio* was used as an *in vivo* model of inflammation triggered by chemical agents [8], microorganisms and their endotoxins [17, 61, 65], and extracellular membrane vesicles [1]. Traditionally, histological, cytological [1], and gene [17] analysis are among high priority assays. Among them, the most commonly used method is quantitative real-time PCR for detecting mRNA copy number [1, 29, 50, 61, 66].

Lipopolysaccharide (LPS) is an endotoxin and the major component of the outer membrane of all Gram-negative bacteria [7]. Directly or indirectly LPS elicits multiple pathophysiological processes *in vivo*, such as metabolic changes, fever, multiple organ dysfunction syndrome (MODS), endotoxic shock, and death in extreme cases [9, 34]. It has long been established that lower vertebrates, most notably fish and amphibians, are resistant to the LPS toxic effect. It has been demonstrated that the mechanism of LPS recognition in fish may differ from that of mammals. For example, the identification of TLR4 as a negative regulator of TLR signaling in fish along with its absence in most fish species, accounts for fish resistance to endotoxic shock [44]. The fish genes *TLR4α* and *TLR4β* appear to be paralogous rather than orthologous to human tlr4 but they probably play a role in zebrafish immunity, supporting the hypothesis that alternative LPS induction pathways predominate in fishes [49]. Nevertheless, zebrafish inflammatory models are suited to study the basic mechanisms of inflammation in human inflammatory diseases exerting a great translational impact potential [17].

By using microarrays G. Forn-Cun found that LPS injection modulated the gene expression in adult zebrafish and the most affected genes were inflammatory factors, such as interleukins (*IL1β, IL6*), chemokines (*CXCl8α, CXCL11.1, CXCL8*β) that were significantly up-regulated [17]. This increase was more profound in kidney than in liver and muscle [17]. Li-Ling Yang et al. found enhanced *IL1β, IL6* and *TNFα* expression in 3-dpf (day post-fertilization) zebrafish larvae injected with 0.5 mg/mL LPS [61]. Benard EL et al. demonstrated the anti-bacterial activity of the macrophage-produced perforins *Mpeg 1* and *Mpeg 1.2* in zebrafish [8].

Systemic inflammatory response syndrome (SIRS) is known to be an important pathogenesis factor of infection and non-infection diseases that may lead to multiorgan failure, chronic critical illness, persist inflammation, immunosuppression, catabolism syndrome or even indolent death [15, 43, 46]. Inflammation plays a central role in many cardiovascular diseases, including heart failure [13], myocardial infarction [18], arrhythmias [17], pericarditis [24], myocarditis [16], and sepsis-induced cardiomyopathy [26].

Therefore, an *in vivo* model of systemic inflammation involving the heart and other organs is of great importance for investigating a variety of human diseases. Use mice models of inflammation is time-consuming and costly, due to the long body development and maturation [22]. Thus, the need for a relevant but more accessible and cheap *in vivo* model of systemic inflammation is of great importance for further investigations of human diseases as well as for drug research and development. In addition, organ-specific gene expression profiling is absolutely crucial for model validation and needs to be identified to prove the effectiveness and reproducibility of chosen model system.

The aim of the study was to generate an *in vivo* modelof systemic inflammation using *Danio rerio* and to develop RT-qPCR based system for quantitative analysis of organ-specific proinflammatory genes expression activity after intraperitoneal LPS infusion.

Materials and methods

Adult wild-type zebrafish AB strain were obtained from a local commercial distributor (Axolotl Co., St. Petersburg, Russia) and housed in the ZebTec Active Blue Stands equipped with Water Treatment Unit (Tecniplast, West Chester, USA) in the Aquatic Facility of the Almazov National Medical Research Center (St. Petersburg, Russia) in groups of 10 fish per standard 3.5-L tanks. Tanks were filled with filtered system water maintained at 27±0.5 °C and pH 7.4. Water was prepared by reverse osmosis and subsequent enrichment with Heka Marine Reef Crystals (Germany). The microbiological purity of the water has been tested and demonstrated to be free from bacteria. Illumination (950–960 lux) in the holding and testing rooms was provided by 18-Wt fluorescent light tubes with a 14/10-h light/dark cycle according to the standards of zebrafish care [46]. All fish were fed twice daily with Neon Micro Granules for fish size 1-2 cm long (Dajana Pet, Bohu ovice, Czech Republic). The nutrition for Zebrafish was chosen and applied according to recommendations by Watts et al. [57].

Fish experiments were approved by the Institutional IACUC at the Almazov National Medical Research Centre (Protocol No. 19-2, dated of January 25, 2019) and fully adhered to the National guidelines and regulations on animal experimentation. The fish used in the experiments belonged to the same baseline population, and were allocated randomly to the experimental groups. The animals were acclimated at least two weeks prior to the experiments. Thirty-six adult fish of both sexes above four months of age were enrolled in the study.

Before procedures, every fish was anesthetized by immersion into the 0.032% (wt/vol) tricaine solution (MS-222; Sigma-Aldrich) until immobilization. Two groups were injected using sterile single-use syringe into the midline of the cavity, posterior to the pectoral fins with 10 μg of LPS (lipopolysaccharide) (Sigma, L2630) (12 fish) and with 20 μg LPS injection (12 fish). LPS was dissolved in sterile PBS. Each injection dose was 10 μl. Twelve fishes were injected with 10 μl of sterile PBS and were included in the control group. After the injection, each fish was returned to the tank with water prepared as described above and remained under the surveillance for 5 h. Fishes from different groups were reared in separate tanks. Then, fishes were euthanized with a tricaine overdose (0.16%). After euthanasia, heart, liver, kidney, and skeletal muscles were dissected under the microscope (without blood removing). Samples were snap frozen and stored at -80 °C.

Total RNA was isolated from tissues using ExtractRNA reagent (Evrogen, Moscow, Russia) according to the manufacturers recommendations. as previously described [28]. RNAse-free glycogen was used as co-precipitate to increase visibility and recovery of low amounts of RNA as previously described [28]. The RNA pellet was air dried, dissolved in 10-50 μl of RNase-free water and stored at -80 °C.

The quantity and quality of RNA isolation were measured using NanoDrop spectrophotometer (ThermoFisher Scientific, Massachusetts, USA). The efficiency (Eff) of each primer was calculated by using formula Eff = 10^(-1/slope)-1. In our experiments

efficiency of all primers was within the range of 90-100%. Reverse transcription was performed by using MMLV Reverse Transcriptase (Evrogen, Moscow). Amplification was performed by using qPCRmix-HS SYBR + LowROX (Evrogen, Moscow) according to the manufacturer's recommendations as previously described [34]. PCR was performed by using 400 ng of total RNA in 25 μl reaction mix (cDNA 5μl, forward primer (2μM) 1 μl, reverse primer (2 μM) 1μl, SYBR + LowROX 5 μl). Conditions for a routine PCR were as follows: 45 cycles 95 °C for 15 sec, 60 °C for 30 sec, 70 °C for 30 sec. The PCR primers were designed by using the BLAST program. All primers are listed in SupTable 1.

GraphPad Prism 8.00 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform statistical analysis and visualizations. Gene expression heatmaps were generated using the Phantasus Web tool (https://artyomovlab.wustl.edu/phantasus/). Differences between groups were calculated by using Mann–Whitney test and detected as statistically significant if a p-value was less than 0.05. Correlations were calculated by using Spearman test, a p-value less than 0.01 was considered as statistically significant.

GeNorm [54] and NormFinder [2] were used to compare stability of reference genes expression. For geNorm, raw Ct values were transformed to relative non-normalized quantities (Q), according to geNorm. The relative levels of gene expression (RQ) in each sample were calculated as RQ = 2^(Cqmax-Cqsample). NormFinder software (https://www.moma.dk/normfinder-software/) performed by GenEx Standard software (bioMCC, Freising, Germany) was used to compare targeted genes expression and reference genes. We tested housekeeping genes most commonly used in zebrafish, such as *18s* (*18s* ribosomal RNA), *eef1a1l1* (eukaryotic translation elongation factor 1 alpha 1, like 1), Gapdh (glyceraldehyde-3-phosphate dehydrogenase) and *actb* (actin, beta 1 [35]. Finally, *eef1a1l1* was selected as reference gene for normalization, since it was constitutively expressed and not affected by the treatment. Next, fold changes in target gene expression in stimulated tissues were normalized to the non-treated controls.

Results

On the first stage, four genes were used as potential housekeeping genes. We found that qRT-PCR results of these genes (*18s, eef1a1l1, gapdh,* and *actb*) showed high diversity in different tissues both with or without LPS injection (SupFigure 1A, B, C, D). *Actb* demonstrated the peak baseline, whereas *18s* – the lowest level. Moreover, *18s* exhibited the most stable expression according to the geNorm (SupFigure 1E) data and NormFinder analysis (SupFigure 1F). However the effectiveness of *18s* was 85%. The relative M-values were defined as a measure of gene expression stability whilst increasing M-value correlated with less stability. However, the high abundance of rRNA compared to mRNA complicated subtraction of the

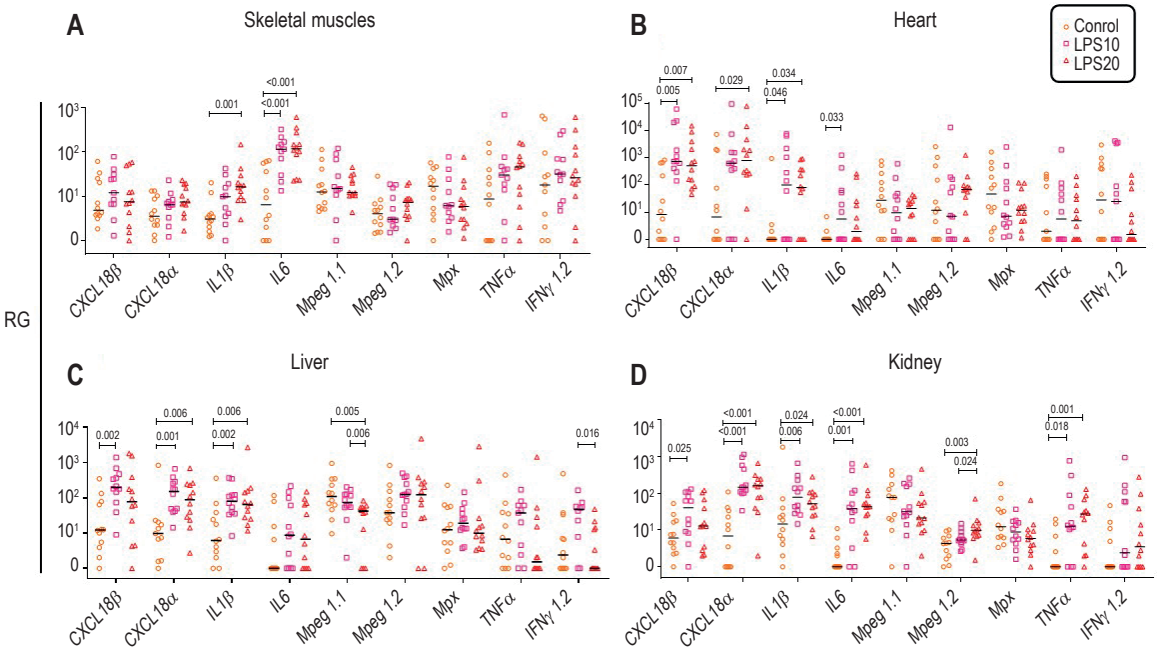

Figure 1. Expression of genes in different tissues of *Danio rerio* after LPS injection
Note. A, skeletal muscles. B, heart. C, Liver. D, kidney. RQ, relative quantification

baseline value in qPCR analysis [38, 39]. Thus, taking into account the data obtained we chose *eef1a1l1* with 99% effectiveness as a reference gene for the further normalization.

Nine genes associated with inflammation (*CXCl18*β, *CXCL8*α, *IL1β, IL6, Mpeg 1.1, Mpeg 1.2, MPX, TNFα, IFNγ1.2*) were selected as targets. The injection of LPS into the peritoneum of *Danio rerio* resulted in upregulated expression of *IL1β* and *IL6* in the muscles (Figure 1A), heart (Figure 1B), and kidney (Figure 1D). Additionally, there was a significant increase of *IL1β* but not *IL6* in the liver (Figure 1C). Moreover, expression of *CXCL18β* and *CXCL8*α in the heart (Figure 1B) and liver (Figure 1C) was upregulated. Kidneys appeared to be the most responsive organ to the LPS infusion since it contained the majority of up-regulated genes studied here: expression levels of *CXCL18β*, *CXCL8*α, *IL1β*, *IL6*, *Mpeg 1.2*, and *TNF*α were significantly increased (Figure 1D). Furthermore, the lowest reactivity was detected in the muscles.

Interestingly, the significant increase of *CXCL18β* in the liver and kidney was detected only after 10 μg but not 20 μg LPS injection. Besides, the usage of 20 μg LPS resulted in decreased *Mpeg 1.1* expression in the liver.

The heart tissue demonstrated the peak reactivity after LPS stimulation. The gene expression was increased by around 100-fold and 80-fold for *Il1β* and for *CXCL8β*, respectively. The liver and the muscles were less reactive demonstrating up to an 18-fold gene expression change. Interestingly, the change in *IL6* expression in the liver was not significant despite that this is the main cytokine to stimulate C-reactive protein synthesis in the liver. Meanwhile, it had marked about a 40-fold increase in the kidney.

Immune reactions could be dose-dependent, for instance the infusion of 20 μg LPS led to decreased expression of *IFN*γ, *Mpeg 1.2*, and *Mpeg 1.1* in the liver and to increase of *Mpeg 1.2* expression in the kidney (Figure 1) comparing with lower dosage.

Discussion

The study of the systemic inflammatory response requires whole-organism experiments. *Danio rerio* seems to be a very promising model, since zebrafish immune system has almost the full repertoire of lymphoid organs and types of immune cell found in mammals [36, 52, 63]. The innate immune system is detectable and active at day 1 of zebrafish embryogenesis [20], whereas the adaptive immune system is morphologically and functionally maturate only at 4-6 weeks after the egg fertilization when the lymphocytes become functional [11, 31, 52, 58]. Zebrafish as a vertebrate are genetically closer to mammals than invertebrate models. About 71% of human genes have at least one corresponding gene found in the zebrafish genome, known as an ortholog [21]. The genetic similarity between humans and zebrafish enables to use them for modeling human diseases [3]. Moreover, the zebrafish genome is fully sequenced and was the third high-quality sequenced genome after mouse and human [21]. Experiments with fish are easy to perform to be carried out in the same conditions. Zebrafish have high fecundity and short maturation times. It makes possible getting a large number of homologous offspring in a short time, which in turn shorten the experiment cycle and also reduce the experiment-related genetic heterogeneity. All these features lead us to the decision to generate the experimental model of systemic inflammation by using *Danio rerio*.

The intraperitoneal injection of LPS provoked the changes in the proinflammatory genes expression in different organs (Figure 2). We suggest that this gene expression profile could become a specific marker of the systemic inflammatory response. The highest number of genes with increased expression was found in the kidney but the most prominent reactions were found in the heart. Moreover, we observed that different concentrations of LPS have different effects on organ inflammation process, which might be due to potential for lower LPS concentrations to stimulate the immune system by activating monocytes-phagocytes, endothelial cells etc., releasing inflammatory factors, and causing systemic inflammation. At higher concentrations LPS not only exhausts pro-inflammatory factors but also provokes a systemic toxic effect, directly or indirectly inducing apoptosis of immune cells, thereby suppressing the immune system [59].

Previous studies have already shown that after different types of stimulation zebrafish leukocytes can produce a variety of cytokines orchestrating inflammatory reactions [47, 48, 49, 50]. In our study, we found significant changes in tissue-specific expression of cytokine genes. Interestingly, the proinflammatory *IL1β* gene expression was most prominent in the heart. The relationship between IL-1 and cardiovascular disease (CVD) was shown in recent studies [51, 56]. IL-1 is one of the first cytokines to be recognized as a pivotal mediator of cell-to-cell communication within the immune system and a key driver of local and systemic immune responses in many diseases including atherosclerotic CVD [53]. Now, *IL1β* blockade is the most promising approach for autoinflammatory disease therapy [52]. Our results demonstrate the involvement of different organs in the LPS-induced inflammatory process.

Chemokines are a family of small cell-signaling proteins that direct the migration of cells expressing corresponding receptors along a ligand concentration gradient. Chemokines and their receptors are widely presented on vascular cells, such as endothelial cells (ECs) and smooth muscle cells (SMCs) [55]. As the main subfamily of chemokines, CXC (Cysteine-X-Cysteine) exists in both mammalian and teleost species. CXCL8 is the best-studied neutrophil chemoattractant in humans [54] and is conserved in zebrafish [57, 58], but lacked in rodents [59, 60]. Contrary to humans, *CXCL8α* in zebrafish requires no CXCR1 receptor and recruits neutrophils essentially via CXCR2 [58].

CXCL18β is an inflammatory marker which was found in zebrafish and other piscine and amphibian species. Similarly to *CXCL8α*, *CXCL18β*-dependent recruitment required the chemokine receptor CXCR2 [58]. Meanwhile, it activity was independent of the expressed CXCR1 and CXCR4b. Like other inflammatory chemokines, *CXCL18β* transcription was found to rely on activated Myd88-dependent innate immunity signaling pathway [61, 62]. It was also demonstrated that *CXCL18β* is up-regulated in response to treatment with toxic and pro-apoptotic compounds, which highlights this gene as a marker of inflammation [63]. In our model of LPS-induced acute inflammation, we also found significant changes in these two chemokines. Moreover, the changes in the heart exceeded those found in the kidney, which is the main fish immune organ. It may be related to the presence of high number of endothelial cells in the heart.

Danio rerio have 3 copies of *Mpeg 1* (Macrophage-expressed gene 1): *Mpeg 1.1* and *Mpeg 1.2* expressed in macrophages and exhibit antibacterial activity [21], whereas *Mpeg 1.3* could be a pseudogene. The *Mpeg 1.1* and *Mpeg 1.2* genes demonstrated differential expression during infection of zebrafish embryos with the bacterial pathogens Mycobacterium marinum and Salmonella typhimurium. *Mpeg 1.1* was down-regulated during infection with both pathogens, *Mpeg 1.2* was up-regulated. Up-regulation of *Mpeg 1.2* is partially dependent on identified functional *Mpeg 1* and requires the Toll-like receptor adaptor molecule MyD88 and the transcription factor NF-κB [21]. Similar to these results, we observed trends in down-regulation of *Mpeg 1* and up-regulation of *Mpeg 1.2* in all analyzed organs and tissues. These findings demonstrate the mechanisms for compartmentalized inflammation.

Mpx are the molecular markers of zebrafish heterophil granulocytes which play a key role in acute inflammation resembling human neutrophils. Zebrafish heterophils show strong histochemical myeloperoxidase activity, suggesting about presence of existing intracellular peroxidase activity [64]. Zebrafish myeloblasts, promyelocytes, myelocytes, and metamyelocytes are found in the kidney hematopoietic tissue [65]. It was noted that kidneys in zebrafish are the main immune and hematopoietic organ similar to the human bone marrow [66, 67]. This could partially account for as to why the LPS stimulation most prominently affected proinflammatory genes expression in the kidneys (Figure 3). Meanwhile simultaneous up-regulation of proinflammatory genes was detected in the liver and skeletal muscles but not in the heart presumably related to detected innate immune cells therein.

Due to the high degree of the similarity and other unique properties, *Danio rerio* has the advantage of being relevant model of inflammation that could be

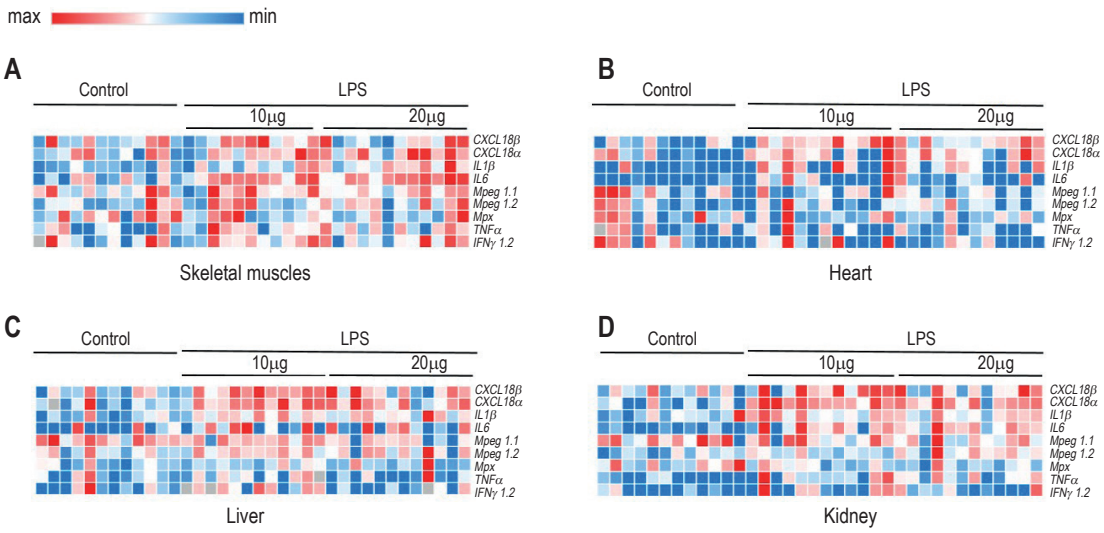

Figure 2. Heatmap of gene expression levels in organs from individual Zebrafishes of different groups
Note. A, muscles. B, heart. C, liver. D, kidney.

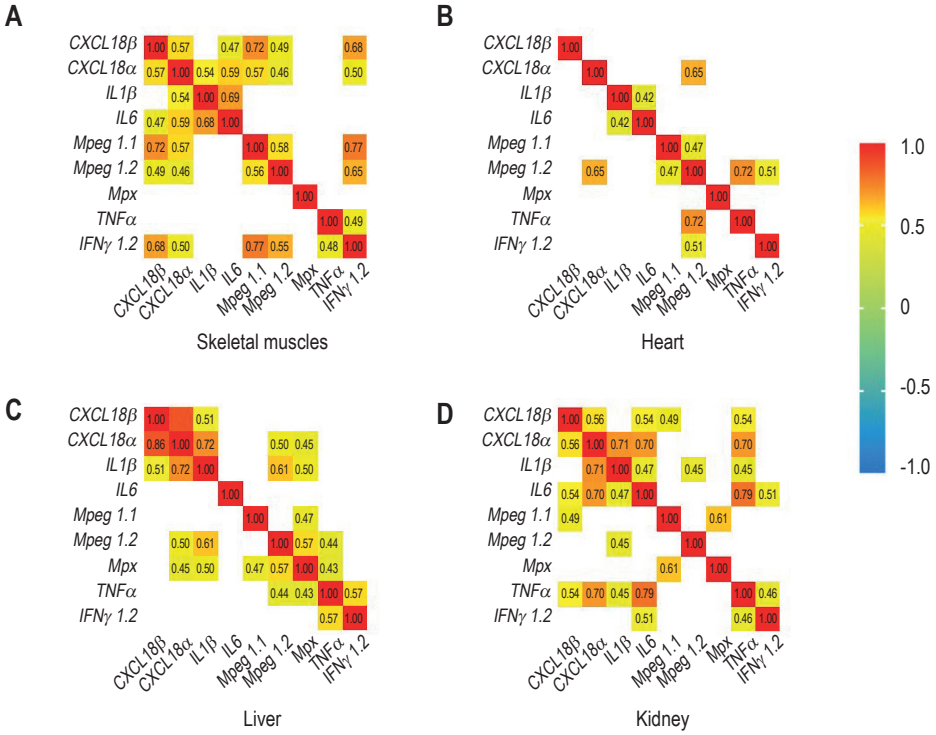

Figure 3. Correlations between levels of genes expression

Note. A, skeletal muscles. B, heart. C, liver. D, kidney. The correlations are presented if $p < 0.01$.

used in different fields, e.g. investigation of disease pathogenesis or drug discovery. Our model demonstrated that the investigation of isolated zebrafish organs could be useful and informative for the investigation of inflammatory processes.

Conclusion

It was found that after generation of lipopolysaccharide induced model of systemic inflammation in adult zebrafish the genes activity was the most pronounced in the heart where the expression of *IL6*, *CXCL8a*, and *CXCL18b* was increased up to 100-fold. The kidneys were the most involved in the inflammatory process since the highest number of analysed genes were up-regulated there: expression levels of *CXCL18β, CXCL8a, IL1β, IL6, Mpeg1.2*, and TNFα were significantly increased. The lowest reactivity was detected in the muscles.

Author contributions

Conceptualization, writing, project administration A.G.; methodology A.F. and K.K., investigation M.Y. and A.K.; resources M.V. All authors have read and agreed with the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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