Pharmacy Education (2024) 24(3) 111 - 115
https://doi.org/10.46542/pe.2024.243.111115

IGSCPS SPECIAL EDITION

RESEARCH ARTICLE

Zebrafish as a model for the study of wound healing in hyperglycemia

Lia Nurkhasanah, Farida Hayati, Rochmy Istikharah
Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia

Keywords
Animal model
Gene
Hyperglycemia
Regeneration
Wound healing
Zebrafish

Correspondence
Farida Hayati
Department of Pharmacy
Universitas Islam Indonesia
Yogyakarta
Indonesia
farida.hayati@uii.ac.id

Abstract

Background: For preclinical studies of diabetic wound therapy, zebrafish have many orthologous signalling pathways that play essential roles in wound healing and regeneration. **Objectives:** This study compares the expression of four essential wound-healing genes and caudal fin regeneration in hyperglycemic zebrafish (*Danio rerio*) to normal zebrafish. **Methods:** Hyperglycemia was induced by using the intraperitoneal injection method with 350 mg/BW streptozotocin on days one, three, and five. The regeneration of the zebrafish's caudal fin was observed on day 5 after amputation using a stereomicroscope, followed by sampling of the blastema to analyse gene expression. Caudal fin regeneration was analysed using Zeiss Zen 3.3 blue documentation; blood glucose levels were measured using a glucometer; and relative gene expression analysis of *sonic hedgehog (shh), insulin-like growth factor 2a (igf2a), bone morphogenetic protein 2b (bmp2b), collagen 1a2 (col1a2)* was performed by qRT-PCR method. **Results:** The glucose level in the hyperglycemia group was 203.2 mg/dL, and that in the normal group was 59.5 mg/dL. The authors found that i*gf2a, shh, bmp2b,* and *col1a2* expression were all downregulated in the hyperglycemia group and decreased in caudal fin regeneration. **Conclusion:** This novel adult zebrafish model of hyperglycemia significantly impairs gene expression and caudal fin regeneration.

Introduction

The development of natural medicine has the potential to discover effective, affordable, and safe therapies (Batubara & Prastya, 2020). Preclinical research is a key element of drug discovery and development. Animal models are important in studies of physiopathology and new alternative treatments for several diseases. Before trials on humans, animal studies on various species are required as part of the drug development process (Domínguez-Oliva *et al*., 2023). Based on prior research, animal model systems such as zebrafish are capable of demonstrating pathogenesis involving impaired wound healing (Intine *et al*., 2013; Zang *et al*., 2018; Chahardehi *et al*., 2020; Morikane *et al*., 2020; Sasmal & Chakraverty, 2020; Wibowo *et al*., 2021). Zebrafish have been widely used in various studies of diseases in humans (Goldsmith, 2004; Lieschke & Currie, 2007; Crisan *et al*., 2008; Goldsmith & Jobin, 2012). For example, zebrafish were involved in a study of diabetes mellitus, which reported that hyperglycemic zebrafish have the same secondary complications as humans. In addition, hyperglycemia-induced zebrafish showed disturbances in caudal fin regeneration, which can be used as a parameter in studying wound healing ability (Intine *et al*., 2013). In regulatory mechanisms linked to wound healing, the human gene has an orthologue in the zebrafish signalling pathway, including *Hedgehog* (*HH*), *Bone Morphogenetic Protein* (*BMP*), and *Wingless-related integration site* (*Wnt*) (Utami & Zebrafish, 2018; Wibowo *et al*., 2021). These signalling pathways have a vital role in wound healing and also in zebrafish fin regeneration stages. Differences in gene expression in these pathways can reflect the ability to heal wounds. In this research, we developed zebrafish as an animal model for the wound healing process.

Methods

Ethics approval

The Medical and Health Research Ethics Committee, Faculty of Medicine, Islamic University of Indonesia, Yogyakarta, has approved this study with letter number 8/Ka.Kom.Et/70/KE/III/2023.

Treatment

The test subjects were zebrafish (*Danio rerio*) with predetermined inclusion and exclusion criteria. The zebrafish were divided into two groups (n=20): the normal group and those induced with hyperglycemia. The members of each group were calculated based on Festing's formula. The induction of hyperglycemia in the zebrafish was done by administering 350 mg/kg streptozotocin injected intraperitoneally on days one, three, and five (Abdullah *et al*., 2022). On day six, the zebrafish underwent caudal fin amputation by immersion anaesthesia with 0.01% tricaine. Then, the fish were observed until the 11th day with the maintenance conditions following the OECD (Economic Co-operation and Development) guidelines (OECD, 2013).

Observation

The caudal fin regeneration from each test group was observed under a stereo microscope (Zeiss, German). The percentage of caudal fin regeneration was calculated using the formula below:

$$\%\ Regeneration\ \text{percentage} = \frac{Regeneration\ area}{Amputated\ area}\ x\ 100\%$$

The fasting blood glucose levels were measured on day five after amputation. Zebrafish fasted for 12 hours, then decapitated, and blood was analysed using Easy Touch GCU(Bioptik Technology, Taiwan)

The caudal fin blastema was collected for gene expression analysis using qRT-PCR (Bio-Rad, United States). Primers were purchased from Macrogen (See Table I). Gene expression analysis began with RNA isolation of caudal fin blastema using a tissue total RNA kit (Geneaid), and cDNAs were synthesised using the cDNA synthesis kit (Thermo Fisher Scientific). Real-time qPCR was conducted using ExcelTaq 2X Q-PCR Master Mix (Smobio). Running was carried out for 50 cycles with a pre-denaturation temperature of 95°C, denaturation of 95°C, and annealing of 55°C. The independent t-test was used for statistical significance ($p < 0.05$).

Table I: Sequences of oligonucleotide primers

Name		Sequence	Accession number
actb1	F	TTCACCACCACAGCCGAAAGA	AF057040
	R	TACCGCAAGATTCCATACCCA	
shh	F	CTCTGTCCTTGGTGGTGTCC	BC162395
	R	CCCGTGTTCTCCTCATCCT	
bmp2b	F	AAAAGCCGAGGAGAAAGCAC	AF072456
	R	TGGGAATGTTGGAGTTGACC	
igf2a	F	GCCAGAACGCACATCAAAAG	BC085623
	R	CCACAGCCAGCCAACATT	
col1a2	F	TGAGGAGGGCAAGAGAGG	BC071278

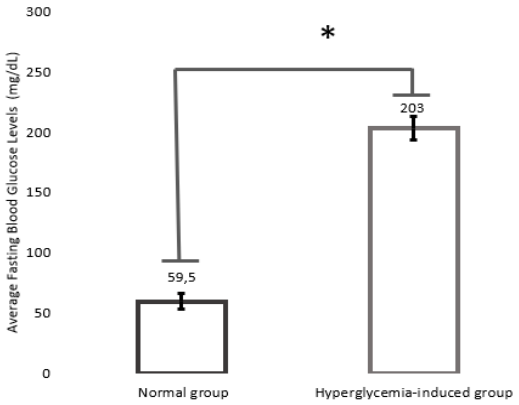
actin beta 1 (actb1), sonic hedgehog (shh), insulin-like growth factor 2a (igf2a), bone morphogenetic protein 2b (bmp2b), collagen 1a2 (col1a2)

Results

Induction of hyperglycemia in zebrafish

Developing animal models for wound healing in hyperglycemic conditions requires hyperglycemia-inducing agents. Streptozotocin has proved to selectively kill pancreatic β cells in zebrafish (Benchoula *et al*., 2019).

The tests on the normal group yielded an average fasting glucose level of 59.5 mg/dL. The range of the average glucose level in a normal control is 50-70 mg/dL; therefore, the control in this study was in the normal range (Benchoula *et al*., 2019; Sanapalli *et al*., 2021). Meanwhile, in the group induced with hyperglycemia, the average fasting glucose level was 203 mg/dL (See Figure 1). This result was expressed as hyperglycemia with a fasting glucose level P above 200 mg/dL (Benchoula *et al*., 2019). The results of statistical analysis showed that the normal and hyperglycemia groups had a significant difference ($p < 0.05$).

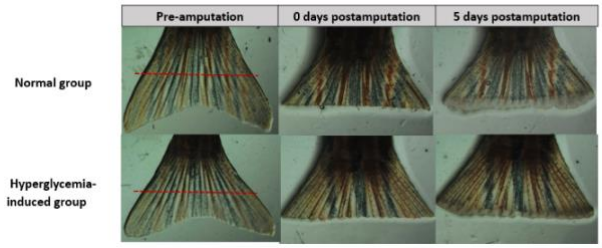


The asterisks (*) show represent significance.

Figure 1: The average of fasting blood glucose levels (mg/dL)

Observation of caudal fin regeneration

The average (n = 20) regeneration percentage of the caudal fin in healthy zebrafish was 41.46%, compared to 26.55% in hyperglycemic zebrafish. The independent t-test was used to examine the statistical significance of the regeneration percentage; the results showed a significant difference in regeneration ($p < 0.05$). Caudal fin regeneration is shown in Figure 2.



The red dashed line shows the amputation lane.

Figure 2: Observation of zebrafish caudal fin regeneration

Observation of gene expression

The tracing of *shh, igf2a, bmp2b, and col1a2* with housekeeping gene *β-actin* expression for zebrafish caudal fin regeneration was carried out by using the RT-PCR technique, with the results of the sample RNA reading being in the form of amplification curves and cycle threshold (Ct) values with beta-actin as a housekeeping gene. The beta-actin gene is a set of continuously expressed genes needed to maintain basic cell functions; therefore, they always exist in living things. From the tracing of gene expression with the RT-PCR technique, the melting temperature (Tm) value or melting temperature (melting point) was also obtained. Tm is related to the nitrogenous bases that make up the primer and the length of the primer, and Tm can, therefore, be used to confirm whether the amplified DNA, which is the product of RT-PCR amplification, is a target gene or not. As a marker of the product being analysed, the Tm value obtained from the three replications was close to ±5°C.

From the Ct values obtained, calculations were made to obtain the value of $2^{-(\Delta\Delta Ct),}$ which represents the expression value of the four gene markers. A comparison of the expression of marker genes in the regeneration of the normal and hyperglycemia groups can be seen in Figure 3.

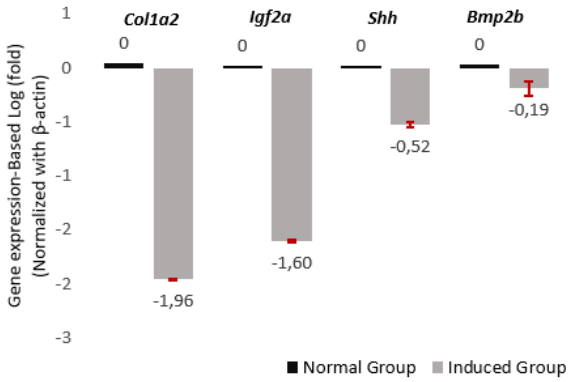


Figure 3: Comparison of gene expression

Discussion

Zebrafish (*Danio rerio)* are one of the experimental animals that have been developed for in-vivo testing, one of which is as an animal model for hyperglycemia. The advantage of zebrafish is the faster onset of diabetic phenotype compared to rodents (Goldsmith & Jobin, 2012). Other advantages of using zebrafish are the lower maintenance cost, easy-to-control experimental condition, shorter testing period, and, most notably, genetic similarity of zebrafish with humans (Intine *et al*., 2013). Zebrafish have a large number of orthologous signalling pathways (Khan & Alhewairini, 2018; Wibowo *et al*., 2021). The four marker genes *shh, igf2a, bmp2b, and col1a2* all have a significant impact on the healing of wounds. The *shh* and *twhh* genes are expressed during the development of fish fin shoots. The HH signalling pathway is involved in the regeneration of the caudal fin, including the formation and maintenance of blastema and fin patterning (Avaron *et al*., 2013; Howe *et al*., 2013). The *Igf2a* gene is one of the genes in the HH signalling pathway that plays a role in tissue repair when injury occurs (Sumbayak, 2015). This gene is in the same signalling pathway as *Sonic Hedgehog* (*shh-a*). The *shh-a* gene acts as a transcription factor for other genes, while *igf2a* is a gene whose expression is influenced by a transcription factor, which will cause an increase in fibroblast migration to make the repair process in wound tissue more effective (Xin *et al*., 2017). The *col1a2* gene has an important role in homeostasis by stopping bleeding and becoming the main component of the extracellular matrix (ECM), which is essential in the reconstruction or building of new tissues, where the proliferation of epithelial cells will thicken the epidermal layers (Valluru *et al*., 2011). The *BMP-2* gene is part of the *TGF-β1* superfamily and has a major role in the development of hypertrophic chondrocytes (cartilage), genital development, and radial fins of zebrafish until maturation (Halloran *et al*., 2020). The

four marker genes have proved to decrease gene expression in hyperglycemia. This outcome was further supported by a decrease in the regeneration percentage of caudal fin in induced-hyperglycemic zebrafish. This proves that zebrafish have regeneration disorders in hyperglycemia conditions, which can be used as a parameter for observing wound healing.

Diabetic wounds in humans include barrier disruption and infection, high oxidative stress, neuropathy, microvascular complications, and suboptimal chronic inflammatory response. As an animal model, zebrafish may not accurately represent the environmental complexity of the diabetic wound. However, they can be used as animal models in the process of new drug discovery for wound healing therapy in hyperglycemia with many advantages. The development of zebrafish animal models for wound healing is expected to be an initial screening tool for new drug candidates.

Conclusion

The authors report that caudal fin regeneration and expression genes severely decreased in this newly developed adult zebrafish model of hyperglycemia. The zebrafish model appears to be promising for the prospective discovery of novel drugs for wound healing therapy in hyperglycemia.

Acknowledgements

The authors would like to express their gratitude to the Department of Pharmacy at Universitas Islam Indonesia for their support. This research was funded through the Master's Thesis Research Grant of 2022 by the Ministry of Education, Culture, Research, and Technology.

Conflicts of Interest

The authors declare no conflicts of interest.

References

Abdullah, N. R., Mohd Nasir, M. H., Azizan, N. H., Wan-Mohtar, W. A. A. Q. I., & Sharif, F. (2022). Bioreactor-grown exo- and endo-β-glucan from Malaysian Ganoderma lucidum: An in vitro and in vivo study for potential antidiabetic treatment. *Frontiers in Bioengineering and Biotechnology,* **10**, 960320. https://doi.org/10.3389/fbioe.2022.960320

Avaron, F., Smith, A., & Akimenko, M.-A. (2013). Sonic hedgehog signalling in the developing and regenerating fins of zebrafish. In: *Madame Curie Bioscience Database*. Landes Bioscience. https://www.ncbi.nlm.nih.gov/books/NBK6368/

Batubara, I., & Prastya, M. E. (2020). Potential use of Indonesian medicinal plants for cosmetic and oral health: A review. *Jurnal Kimia Valensi*, **6**(1), 120–134. https://doi.org/10.15408/jkv.v6i1.16252

Chahardehi, A. M., Arsad, H., & Lim, V. (2020). Zebrafish as a successful animal model for screening toxicity of medicinal plants. *Plants*, **9**(10), 1–35. https://doi.org/10.3390/plants9101345

Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P. N., Traas, J., Schugar, R., Deasy, B. M., Badylak, S., Buhring, H. J., Giacobino, J. P., Lazzari, L., Huard, J., & Péault, B. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell stem cell*, **3**(3), 301–313. https://doi.org/10.1016/j.stem.2008.07.003

Domínguez-Oliva, A., Hernández-Ávalos, I., Martínez-Burnes, J., Olmos-Hernández, A., Verduzco-Mendoza, A., & Mota-Rojas, D. (2023). The importance of animal models in biomedical research: Current insights and applications. *Animals*, **13**(7), 1223. https://doi.org/10.3390/ani13071223

Goldsmith, J. R., & Jobin, C. (2012). Think small: Zebrafish as a model system of human pathology. *Journal of Biomedicine and Biotechnology*, **2012**. https://doi.org/10.1155/2012/817341

Goldsmith, P. (2004). Zebrafish as a pharmacological tool: the how, why and when. *Current opinion in pharmacology*, **4**(5), 504–512. https://doi.org/10.1016/j.coph.2004.04.005

Halloran, D., Durbano, H. W., & Nohe, A. (2020). Bone morphogenetic protein-2 in development and bone homeostasis. *Journal of Developmental Biology*, **8**(3), 19. https://doi.org/10.3390/jdb8030019

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrerr, J. C., Koc, R., Rauch, G. J., White, S., Chow, W., Kilian, B., Quintais, L. T., Guerra-Assuncao, J. A., Zhou, Y., Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, **496**(7446), 498–503. https://doi.org/10.1038/nature12111

Intine, R. V., Olsen, A. S., & Sarras, M. P. (2013). A zebrafish model of diabetes mellitus and metabolic memory. *Journal of visualized experiments: JoVE*, **72**, 1–7. https://doi.org/10.3791/50232

Khan, F. R., & Alhewairini, S. S. (2018). Zebrafish (Danio rerio) as a model organism. *Intechopen*. https://doi.org/10.5772/intechopen.81517

Lieschke, G. J., & Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*, **8**(5), 353–367. https://doi.org/10.1038/nrg2091

Morikane, D., Zang, L., & Nishimura, N. (2020). Evaluation of the percutaneous absorption of drug molecules in zebrafish. *Molecules*, **25(**17), 1–12. https://doi.org/10.3390/molecules25173974

OECD. (2013). *OECD guidelines for testing of chemicals*. Dermatotoxicology, 509–511. https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

Sanapalli, B. K. R., Yele, V., Singh, M. K., Thaggikuppe Krishnamurthy, P., & Karri, V. V. S. R. (2021). Preclinical models of diabetic wound healing: A critical review. *Biomedicine and Pharmacotherapy*, **142**, 111946. https://doi.org/10.1016/j.biopha.2021.111946

Sasmal, S., & Chakraverty, R. (2020). The zebrafish model of diabetes mellitus: a re-appraisal, **7**(1), 1–3. https://austinpublishinggroup.com/endocrinology-diabetes/fulltext/ajed-v7-id1074.php

Sumbayak, E. M. (2015). Literature review fibroblasts: Structure and role in wound healing. *Jurnal Kedokteran Meditek*, **21**(6), 1–6. [http://ejournal.ukrida.ac.id/ojs/index.php/Meditek/article/view/1169]

Utami, N.. (2018). Zebrafish *(Danio rerio*) as an animal model for diabetes mellitus. *BioTrends*, **9**(1). https://terbitan.biotek.lipi.go.id/index.php/biotrends/article/download/224/194]

Valluru, M., Staton, C. A., Reed, M. W. R., & Brown, N. J. (2011). Transforming growth factor-β and endoglin signaling orchestrate wound healing. *Frontiers in Physiology*, **2**, 1–12. https://doi.org/10.3389/fphys.2011.00089

Wibowo, I., Utami, N., Anggraeni, T., Barlian, A., Putra, R. E., Indriani, A. D., Masadah, R., *et al*. (2021). Propolis can improve caudal fin regeneration in zebrafish (*Danio rerio*) induced by the combined administration of alloxan and glucose, *Zebrafish*, **18**(4), 1–8. https://doi.org/10.1089/zeb.2020.1969

Zhu, Z. X., Sun, C. C., Ting Zhu, Y., Wang, Y., Wang, T., Chi, L. S., Cai, W. H., Zheng, J. Y., Zhou, X., Cong, W. T., Li, X. K., & Jin, L. T. (2017). Hedgehog signaling contributes to basic fibroblast growth factor-regulated fibroblast migration. *Experimental cell research*, **355**(2), 83–94. https://doi.org/10.1016/j.yexcr.2017.03.054

Zang, L., Maddison, L. A., & Chen, W. (2018). Zebrafish as a model for obesity and diabetes. *Frontiers in cell and developmental biology*, **6**, 91. https://doi.org/10.3389/fcell.2018.00091