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CHARACTERIZATION OF KIDNEY MARROW IN ZEBRAFISH (DANIO RERIO) BY USING A NEW SURGICAL TECHNIQUE

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Abstract: Zebrafish kidney marrow (ZKM), which is equivalent to the haematopoietic bone marrow of mammals, produces all major blood cell types, which morphologically resemble their mammalian counterparts. To be able to exploit the advantages of zebrafish genetics for analysis of the general mechanisms controlling self-renewal, proliferation and lineage decisions of vertebrate haematopoetic cell populations, it is essential to develop a simple surgical technique in order to identify, dissect and take out the ZKM without contamination with other surrounding tissues and cells. However, the size of adult zebrafish is small (average size: 2.5 cm) and the ZKM is an extremely protected organ and not easy to localize, which makes this procedure a great microsurgical challenge. Here we report a new microsurgical technique to identify, localize and dissect ZKM in adult zebrafish using a new approach. The potential advantages of this technique are summarized here: it allows purity of the sample, which is critical for performing flow cytometry analysis and/or cell number count; it enables visualization of the ZKM without a parenchimal incision, which simplifies the further dissection; the learning curve is short, requiring only basic microsurgical skills, and it is reliable and highly reproducible. To further characterize the kidney marrow cells obtained by this technique, we performed histology, flow cytometry, cytospin experiments and cell counts.

Key words: zebrafish, kidney marrow, microsurgery, technique.

Introduction

As a vertebrate organism, the zebrafish (*Danio rerio*) presents many organs and cell types similar to those of mammals [1]. The zebrafish was recently described as 'the canonical vertebrate', due to the similarities between zebrafish and mammalian biology [1]. As a result of these shared features, many laboratories have begun to exploit the unique advantages of the zebrafish system to study human disease. The zebrafish is particularly suited for studying early haematopoiesis because of the wide variety of manipulations that can be accomplished with it. It represents also an ideal genetic system for haematopoietic developmental studies due to the fact that haematopoiesis in zebrafish is very similar to mammals both with respect to cell types and haematopoietic genes. Zebrafish kidney marrow (ZKM), which is equivalent to the haematopoietic bone marrow of mammals, produces all major blood cell types, which morphologically resemble their mammalian counterparts [2]. Many orthologues of mammalian genes required for blood development are expressed in the ZKM [3]. Furthermore, the key regulatory genes responsible for generating the first haematopoietic cells during development are also conserved between zebrafish and mammals [2].

To be able to exploit the advantages of zebrafish genetics for analysis of the general mechanisms controlling self-renewal, proliferation and lineage decisions of vertebrate haematopoetic cell populations, it is essential to develop a simple surgical technique in order to identify, dissect and take out ZKM without contamination with other surrounding tissues and cells. However, adult zebrafish are of a very small size (average size: 2.5 cm) and the ZKM is an extremely protected organ, which makes this procedure a great microsurgical challenge. Most of the published reports use an approach involving a ventral, midline incision without giving further details [2, 4]. We have developed a new microsurgical technique to identify, localize and dissect ZKM in adult zebrafish using a new and different approach. By removing the zebrafish`s integumentary system, we were able to observe and identify the inner anatomy and to detect the ZKM without a parenchymal incision, which greatly simplifies the further dissection. To further characterize the kidney marrow cells obtained by this technique, we performed histology, flow cytometry, cytospin experiments and cell counts, confirming the existence of at least 4 distinct cell populations.

Materials and Methods

Maintenance and microsurgery of zebrafish

Adult zebrafish (India strain) were housed in tanks in recirculating dechlorinated tap water at 26˚C (water temperature). For our experiments, we used

7-month-old (adult) male zebrafish (n = 60). the adult zebrafish were anaesthetized with 0.02% tricaine and killed by exanguitransfusion before starting the surgery. Due to the opacity of the zebrafish`s integumentary system and skin, it is impossible to see and detect its inner anatomy (Figure 1A). The characteristic adult pigmentation pattern of the zebrafish consists of three distinct classes of pigment cells arranged in stripes (Figure 1A): black melanophores, reflective iridophores and yellow xanthophores [5]. Under microscopic magnification (× 2.5), the superficial layer (outer epidermis and an underlying dermis) were carefully dissected out on both sides of the fish using two micro-forceps with 0.3 mm tips (World precision instruments, Sarasota, Fl, USA) taking care not to enter into or damage the inner structures. Thereafter the fish was placed under a microscope (Olympus SZX16, USA) provided with a back light and an attached Olympus DP71 camera (Olympus, Japan). Using this procedure, we were able to observe and identify the inner anatomy and in particular the ZKM without a parenchymal incision (Figure 1B). The kidney is located between the vertebral column and the gas bladder extending longitudinally from cranium to anus. It is positioned in the retroperitoneum along the dorsoposterior aspect of the body cavity. The organ is divided into two portions, an anterior smaller head kidney composed of haematopoietic, lymphoid, and endocrine tissue (ZKM), and a posterior trunk kidney composed of numerous nephrons surrounded by interstitial lymphoid tissue. In the microscopic assisted resection, using small microsurgical super-fine Vannas scissors (World precision instruments, Sarasota, Fl, USA), the incision is performed 2 mm down to the vertebral column under microscopic magnification taking care to avoid damage to the dorsal aorta, elements of the abdomen and the swim bladder (Figure 1C). The incision should follow the plane between muscles that encounter the vertebral column and muscles covering the abdominal and retroperitoneal cavity in order to enter directly into the retroperitoneum and to avoid entering into the abdominal cavity. In order to expose the kidney marrow, the ribs should be cut following the same direction of the incision. This incision should be precise to the millimetre developing a surgical plane that will directly expose the kidney marrow. The parenchymal resection should be performed using more blunt than sharp dissection since any damage to the inner anatomy and surrounding structures will make the further procedure more difficult. Figure 1C shows the exact location of the incision. In this picture, a long incision was made for a better demonstration of the technique. Usually, the procedure can be performed through a small incision 1 mm in above the swim bladder. Furthermore, the parenchyma should be gently divided in order to expose the ZKM (Figure 1D). The presence of a dark red coloured tissue with black dots under the frontal part of the vertebra confirms its location.

The ZKM location is marked by 2 bone extensions on both sides and one in the middle originating from the vertebra. These bone extensions represent important

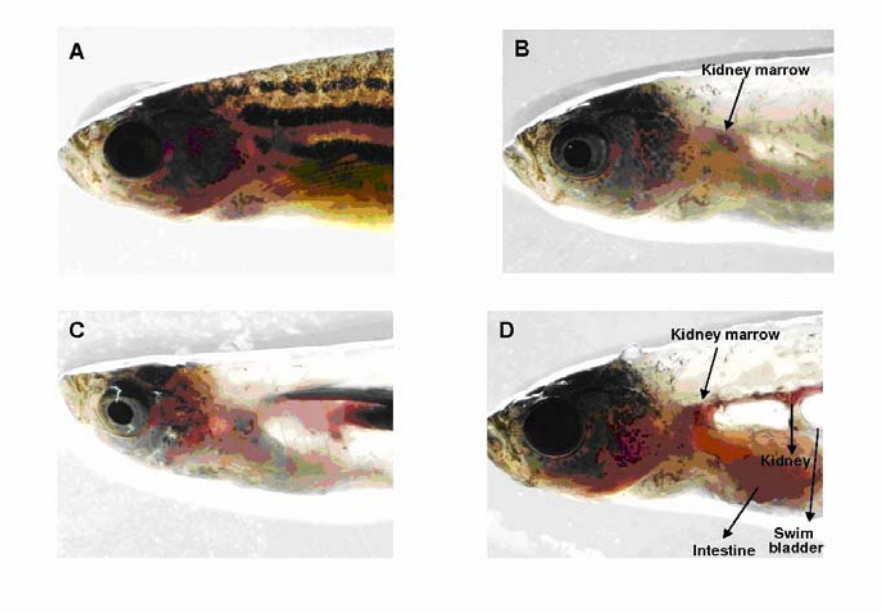

Figure 1 – A. Wild type 7-monthsold adult male zebrafish. Please note the opacity of the adult fish. B. The zebrafish`s integumentary system was dissected out and the fish was placed under a microscope (Olympus SZX16, USA) provided with a back light. The location of the kidney marrow is marked with a flash. C. The exact location of the incision. This incision should be precise to the millimetre, developing a surgical plane that will directly expose the kidney and head kidney. D. Dissected zebrafish. The presence of a dark red coloured tissue with black dots positioned in the retroperitoneum along the dorsoposterior aspect of the body cavity confirms the location of the kidney marrow

Слика 1 – А. Див сој на 7-месечна возрасна риба-зебра. Обрнете внимание на опацитетот на надворешниот слој на рибата, Б. Надворешниот слој е внимателно дисециран и рибата е поставена на микроскоп со позадинско светло. Стрелката ја означува точната локација на бубрежната медула. Ц. Точната локализација на инцизијата. Оваа инцизија треба да е точна до милиметар создавајќи слој кој директно ќе ги прикаже бубрегот и бубрежната медула. Д. Дисецирана риба-зебра. Присуството на темноцрвено обоено ткиво со црно пребоени точки сместено во ретроперитонеумот ја потврдува точната локација на бубрежната медула

landmarks in the dissection since they precisely mark the ZKM location (Figure 2 A, B, C). After cutting out the bone extensions, the ZKM was removed for further

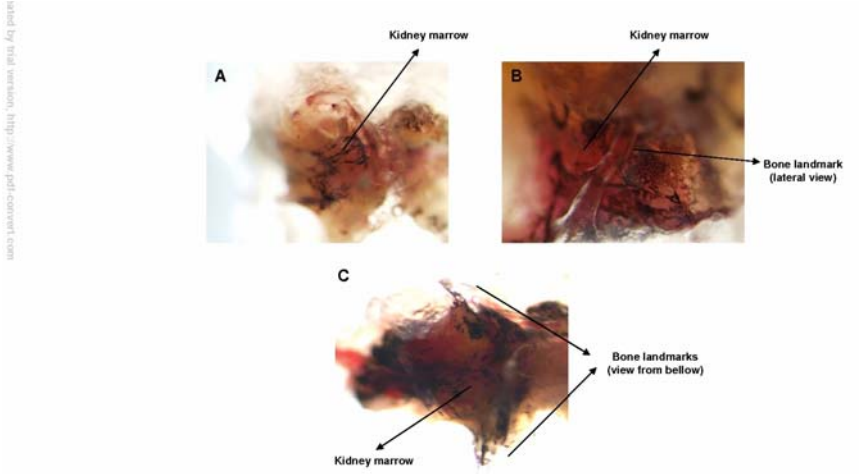

Figure 2 – The kidney marrow is marked by bones on both sides originating from the vertebra which represents a landmark for location of the kidney marrow. A. Kidney marrow from below. B. Lateral view. C. View from below
Слика 2 – Бубрежната медула е маркирана со 2 коскени продолжетоци од двете страни кои потекнуваат од 'рбетниот столб. А. Поглед одоздола. Б. Латерален поглед. Ц. Поглед одоздола

analyses (see below). It should be noted that the spleen is in close proximity with the ZKM and we can distinguish it by a slightly lighter red colour when compared to the ZKM. Dissection was generally performed under 2.5–3 times magnification using a Leica MZ6 dissecting microscope (Leica Microsystems, Germany). Pictures were taken using an Olympus SZX16 microscope with attached Olympus DP71 camera (Olympus, Japan). Adequate instruments are mandatory. Minor mistakes can lead to failure in dissecting out the ZKM.

Histology and cytology

For the histological analysis, ZKMs were fixed in 4% paraformaldehyde overnight. After washing 3 times in PBS(-) and once in 20% sucrose in PBS(-), the ZKMs were embedded into OCT compound and frozen. Ten µm sections were cut in transverse sections. Staining was done with haematoxylin and eosin.

Cytospin preparations were performed using 1×10^4 to 2×10^4 kidney cells cytocentrifuged at 550 rpm for 6 min using a Shandon Cytospin 3 centrifuge (Thermo Electron Corporation, Pittsburgh, PA) onto glass slides (Matsu-

nami, Japan). Cytospin preparations were processed through May-Grünwald and Giemsa stains (Muto PureChemicals, Japan) for morphologic analyses by light microscopy. Total cell counts were performed by using a cell counter. Values were expressed as a mean from 3 experiments. Visible light imaging was performed on a Leica DMIL microscope (Leica Microsystems, Germany) using 10 × and 100 × oil objectives.

Flow cytometry analysis of kidney marrow cells

In a separate group of 23 zebrafish, we performed flow cytomertry analysis to identify cell populations of ZKM obtained with this technique. Zebrafish were sacrificed and kidney marrow obtained as above. Haematopoietic cells were processed as above, washed and resuspended in ice-cold PBS(-) containing 5% foetal bovine serum (FBS) and passed through a 40-μm nylon mesh filter with a plunger from a 2.5-ml syringe. Propidium iodide (PI; Sigma, St Louis, MO) was added at 1 μg/ml to exclude dead cells. Flow cytometry analysis and sorting was performed based on PI exclusion, forward scatter and side scatter using a FACSCalibur (BD Biosciences, San Jose, CA). Flow cytometry data were analysed using FlowJo software (Tree Star, Ashland, OA).

Results

After dissecting out the outer epidermis and an underlying dermis of the zebrafish using our new approach, the inner anatomy and in particular the ZKM could be observed and identified without a parenchymal incision as shown in Figure 1B. Haematopoietic tissue is situated between the renal tubules (Figure 3A), similar to the placement of developing mammalian blood cells among the fat and stromal elements of bone marrow. Figure 3A shows a robust population of haematopoietic cells (labelled "H") intertwined with normal kidney tubules (labeled "T"). The major blood lineages can be isolated from the ZKM by flow cytometry based on differences in light-scattering characteristics reflecting variations in cell size and cellular granularity [4]. Forward scatter is directly proportional to cell size and side scatter is proportional to cellular granularity [6]. Using this technique and flow cytometry analyses of the cells we were able to identify at least 4 populations, which is consistent with the data published in the literature [4] (Figure 3B). By cell counting we calculated that from one zebrafish we can obtain 2.45×10^5 cells (n = 29). Using kidney marrow cell cytospin, we were able to observe the morphology of the haematopoietic cells. We observed a heterogeneous population of mature haematopoietic cells and their precursors (Figure 3C). We also observed mature erythrocytes, lymphocytes and eosinophils as well as a robust population of immature haematopoietic cells (imamture precursors).

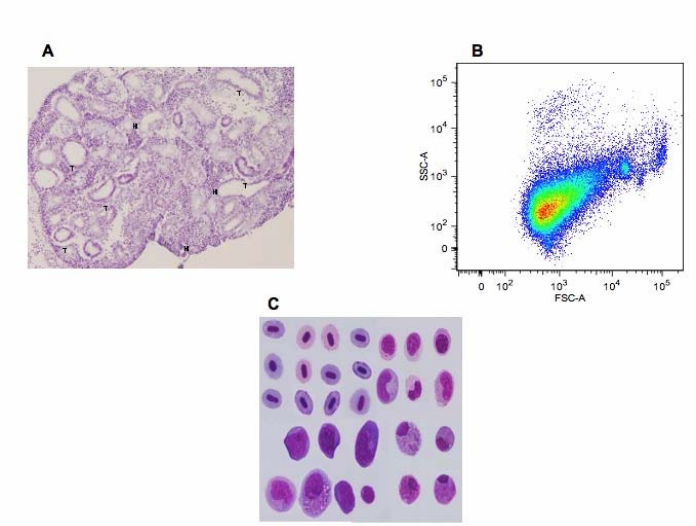

Figure 3 – A. Micrographs of adult zebrafish kidney tissue sections stained with haematoxylin and eosin. Note the robust population of haematopoietic cells (labelled "H") intertwined with normal kidney tubules (labeled "T") (Magnification × 10). B. Flow cytometry performed on whole-kidney marrow. The analysis and sorting was performed based on PI exclusion, forward scatter, and side scatter. FSC is directly proportional to cell size and SSC is indicative of cellular granularity. At least 4 cell populations are observed. C. The morphology of haematopoetic cells in zebrafish by cytospin (Magnification × 100)

Слика 3 – А. Репрезентативни хистолошки препарати (боење со хемотаксилин, еозин). Се забележува обилно присуство на хематопоетски клетки (Х) помеѓу кои се наоѓаат нормални бубрежни тубули (Т) (зголемување (× 10), Б. Флоу цитометрија на цела бубрежна медула. Анализата и сортирањето се базираше на ПИ ексклузија како и forward и side scatter. Првото е пропорционално со големината на клетките, додека второто со нивната гранулираност. Најмалку 4 клеточни популации можат да се идентификуваат. Ц. Морфологија на хематопоетските клетки на рибата зебра издвоени со методата на цитоспин

Discussion

This technique enables precise dissection and identification of the ZKM at both sides of the vertebra, avoiding contamination with other structures or cells. The mean operative time of microscope-assisted ZKM dissection was 23 min. On the other hand, resection using the transabdominal approach is technically more difficult, and requires increased experience with microsurgical techniques. After getting used to this new approach, the operating time for these resections was 15 min.

The analysis of ZKM has been limited to embryogenesis due to the opacity of the adult fish. The data presented here demonstrate that the optical properties of the transparent adult fish offer a unique combination of high resolution, sensitivity and amenability to deep tissue imaging with commonly available laboratory equipment. Using this technique and flow cytometry analyses of the cells, we were able to identify at least 4 cell populations which is consistent with the data published in the literature [4, 8]. By performing haematopoietic cell transplantation for each of the light-scatter populations, it was shown that the haematopoetic stem cells (HSCs) are contained only in the lymphoid fraction [4]. However, the resolution of this procedure is not sufficient to achieve purification of specific cell populations such as HSCs.

A large number of haematopoietic mutants in zebrafish have been generated and characterized to date. The strength of the zebrafish system is illustrated by the fact that these haematopoietic mutants represent many of the proposed steps of both the primitive and definitive haematopoietic programmes. In principle, it should be possible to purify HSCs using monoclonal antibodies selective for these cells. However, such reagents have not been developed in zebrafish due to the general difficulties of generating monoclonal antibodies to non-mammalian vertebrates [4, 9]. As a consequence of these limitations, isolation and characterization of HSCs from zebrafish have not yet been reported.

Conclusion

We developed a new technique of identifying and dissecting ZKM that can be mastered by a microsurgeon through practice. To the best of our knowledge, this is the first report describing a simple technique of localizing ZKM. The potential advantages of this technique are summarized here: it allows purity of the sample, which is critical for performing flow cytometry analysis and/or cell number; it enables visualization of ZKM through the skin even without incision, which simplifies the further dissection; the learning curve is short, requiring only basic microsurgical skills, and it is reliable and highly reproducible. We believe that this technique will provide an excellent experimental tool to analyse different cellular, molecular and genetic mechanisms of haematopoiesis.

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Резиме

КАРАКТЕРИЗАЦИЈА НА БУБРЕЖНАТА МЕДУЛА КАЈ РИБАТА-ЗЕБРА (DANIO RERIO) КОРИСТЕЈЌИ НОВА ХИРУРШКА ТЕХНИКА

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Бубрежната медула на рибата-зебра, која е еквивалентна на коскената срцевина кај цицачите, ги продуцира сите главни крвни клетки, кои морфолошки

се слични со истите најдени кај цицачите. За да ги искористиме предностите на генетскиот код на рибата-зебра за анализа на основните механизми кои ја контролираат самообновливоста, пролиферацијата и видовите на хематопоетски клеточни популации кај 'рбетниците, од есенцијално значење е да се развие една едноставна хируршка техника за идентификација и дисекција на бубрежната медула без контаминација од други ткива и клетки. Како и да е, димензијата на рибата-зебра е многу мала (2,5 см) додека бубрежната медула е екстремно протектиран орган кој не е лесно да се пронајде што ја прави оваа процедура голем микрохируршки предизвик.

Во оваа студија презентираме нова хируршка техника со цел: локализација, идентификација и дисекција на бубрежната медула. Потенцијалните предности на оваа техника се следните: – Овозможува прочистеност на примерокот што е од клучно значење за изведување на flow цитометрија како и број на клетки. Овозможува визуализација на бубрежната медула без паренхимна инцизија. Кривата на учење е кратка и бара само основни микрохируршки способности. Техниката е високо репродуцибилна. За да ги карактеризираме понатаму клетките на бубрежната медула обезбедени со оваа техника, направивме хистологија, flow цитометриски анализи, цитоспин анализа, како и клеточен број.

Клучни зборови: риба-зебра, бубрежна медула, микрохирургија, техника.

Работен наслов: Хирургија на бубрежната медула кај рибата-зебра
Running title: Surgery of kidney marrow in zebrafish

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