The zebrafish as a model for tissue regeneration and bone remodelling

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Chapter 5: Acute exposure to dexamethasone in early-life is associated with enduring effects on wound healing in zebrafish larvae

Abstract

The goal of this study was to assess the impact of an acute but brief DEX exposure in early-life (*prim-6* stage) on the ability to repair damaged tissue following amputation of the caudal fin at later life stages including 5 and 14 dpf in zebrafish larvae. We show that prior history of DEX exposure, that is not concomitant with an injury sustained later in life, significantly impairs wound healing long after DEX has been washed out at both life stages studied. We also show that DEX-induced impairments in wound healing can however be fully restored to normal levels with longer post amputation recovery time.

Analysis of the several outcome measures taken in this study including cell proliferation, cell death (necrosis and apoptosis), gene expression, and leukocyte cell marker (L-plastin) suggest that DEX exerted its effects in the early phase (4 h) of wound healing rather than in the later phases of the tissue repair process. A main event include massive amount of cell death both by necrosis and apoptosis at 4h post amputation, which is not compensated, at least as far as 24 h post amputation. The concomitant significant reduction in the number of L-plastin positive/leukocyte cells observed at 4 h post amputation suggest that these cells may have been among the large amount of cells lost via necrosis and apoptosis. Therefore, it is likely that DEX-induced reduction in the number of L-plastin positive/leukocytes observed in this study may not only have altered the clearance of cell debris but also lowered levels of growth factors and cytokines at the injury site. Such events may simultaneously

contribute to impede the initiation of the blastema formation and its progression, and results in impaired wound healing.

In sum, this study provides evidence that DEX exposure during early sensitive periods of development appears to cause permanent alterations in the cellular/molecular immune processes that are involved in the early phase of wound healing in zebrafish. These findings are consistent with previous studies showing that antenatal course of DEX are associated with immediate and lasting alterations of the immune system in later life in rodents, non-human primates, and humans. Therefore, the findings presented in this study support the use of the larval zebrafish as a valid model to study the impact of stress and stress hormone exposure in immature organisms on health risks in later life.

Introduction

Stress and wound healing

A large core of epidemiological data shows the pervasive effects of both major and minor stressful life events on health including the worsening of existing illnesses [218-221]. One manifestation of this phenomenon is the impact of psychological stress on wound healing in humans. Several studies have clearly demonstrated the slowing of wound healing by psychological stressors including marital conflicts/hostility [221], examination stress [222], and caring for a relative afflicted with a debilitating disease [221]. Complex interplays between the neuroendocrine and immune systems mediate these effects [3][218,223]. One immediate function of the immune system during the early phases of wound healing is to sterilize the wound area and remove dead cells and bacteria. Once the wound has been cleared of debris, cells of the immune system (neutrophils, and leucocytes) play a key role in secreting a wide range of molecules (e.g. TGFα, TGFβ1, TGFβ2, TGFβ3, FGF, PDGF, IL-1,IL-4,IL-8,G-CSF,GM-CSF) that favour subsequent migration and division of cells involved in the proliferative phase of wound healing, just proximal to the injured site, and which results in tissue outgrowth replacing the damaged structure [1] (Table 1).

Progress in the understanding of the interplay between the stress and immune systems revealed that stress, via the stress hormone glucocorticoid (GC)

(cortisol in humans and fish, and corticosterone in rodents), exerts significant detrimental effects in the early phases of the inflammatory response [224]. Specifically, both stress-induced up-regulation of GCs as well as synthetic GC exposure have been shown to impacts the early phase of wound healing (within 4 h) by decreasing the number of immune cell types such as neutrophils, leukocytes, and macrophages recruited at the wound site as well as by down-regulating the expression of their secreted factors such as interleukin 1 (IL-1),interleukin-4 (IL-4),interleukin-5(IL-5), interleukin 8 (IL-8), chemokynes, cytokines and tumour necrosis factor- α (TNF-α) [225,226]. Globally, these GC-mediated events are believed to underlie the slowing of wound healing [218,227]. Conversely the stress-reduction intervention programs have been shown to up-regulate several components of the early immune response, and greatly accelerate wound healing in humans [218], thus confirming the importance of the GC pathway in wound and tissue repair.

Antenatal glucocorticoid exposure and enduring consequences on immunity

In parallel, exposure to various stressors and synthetic CGs in early life also trigger significant and enduring effects on both the neuroendocrine and immune systems [218,228-231]. One prominent example is the clinical practice consisting of administering the synthetic GC dexamethasone (DEX) during late gestation in situations where risks of premature deliveries are elevated [232-237]. This procedure is meant to promote foetal lung maturation and treat inflammatory complications of the respiratory system of newborn infants [238-240]. Though to be safe, this procedure was found to be associated with a host of side effects with far reaching impact on physical and mental health throughout the lifespan [5]. Relevant to the current study, antenatal DEX exposure has been associated with enduring consequences on immunity in two important ways: 1) long-lasting alterations of the stress-regulating system (referred to as the hypothalamic-pituitary-adrenal (HPA) axis leading to conditions of GC resistance (via decreased GC function or GR number and/or function) has been shown to impair immune responses in adulthood [223]. Immediate interference with thymus development leading to enduring changes in the structure of the thymus [241], T cell proliferation [228], cytokine

production [241,242], as well as predisposition to autoimmune disorders [242]. These findings suggest that exposure to stress and GCs during development exerts programming effects on the immune system, which may account to explain individual differences in the ability to deploy adequate immune responses in later life. In the present study, we have investigated the impact of DEX exposure during early development on later ability to repair tissue damage in zebrafish.

A novel model of wound healing in zebrafish: the caudal fin amputation paradigm

Because lower animals like urodeles and amphibians and many invertebrates have retained their regenerative capabilities to a remarkable extent throughout evolution, they have been widely used to deepen our understanding of the processes underlying wound healing and tissue regeneration. This is in sharp contrast with humans and other vertebrate species, which have retained a much less spectacular regenerative capacity, which is mostly restricted to the liver and fingertips [6,7]. One exception is the zebrafish, a vertebrate species endowed with a remarkable capacity to regenerate their fins, optic nerve, scales, heart, and spinal cord [2,243,244]. Over the last decade, the zebrafish has become a model of choice to investigate molecular pathways involved in regeneration and relevant to those of higher vertebrates, including humans [2,197,245]. One of the well-established zebrafish models of tissue regeneration is the caudal fin amputation model. This procedure consists of amputating the caudal fin at the neural keel, without touching the notochord. This procedure is typically performed on 2 day-old embryos [12]. By the third day post amputation (dpa), larval zebrafish can fully regenerate and replace their lost caudal fin including its shape, size, and structure, which become undistinguishable from that of intact larvae of an equivalent life stage [121]. Caudal fin regeneration involves three overlapping steps that are depicted on table 1 and including: 1) wound healing (0–3 h), 2) blastema formation (4–6 h), and regenerative outgrowth, which is divided into an early phase (6-24 h) and late phase (24-48 h). Within 10 min of the wound healing process, epithelial cells begin to migrate over the injured site, where the wound is sealed with actin-purse ring structure. During the blastema stage, cells proximal to the

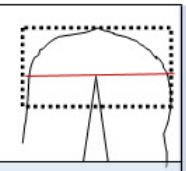
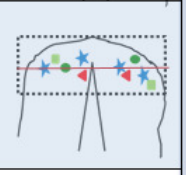
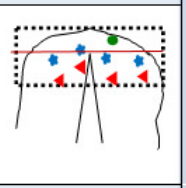
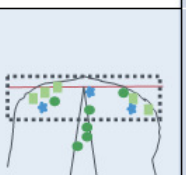
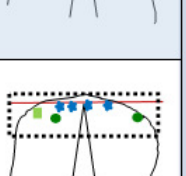
amputation site initiate a process of dedifferentiation and become momentarily pluripotent cells with the capability to proliferate and differentiate into the various cell types required to regenerate lost tissue [119,120]. The process of cell proliferation and differentiation, gradually leads to tissue outgrowth and eventually to a full recovery of the lost tissue by 3 dpa [121].

It is noteworthy that the caudal fin amputation model can be as reliably performed in both larval and adult zebrafish since the molecular mechanisms of tissue regeneration comprising all cell types (e.g. four cell types in wound epidermis, three cell types in blastema and one *mmp-9* expressing cell type) [117] and key molecules (listed in Table 1), which are strikingly similar between larval and adult zebrafish. These qualities make the larval zebrafish model of caudal fin amputation especially attractive for forward genetic and high-throughput screening of molecules modulating the processes involved in tissue regeneration [121].

Goal of the study

Understanding the mechanisms by which stress and stress hormones slow the wound healing process and delay tissue repair is important. While GCs are known to be potent suppressors of tissue repair in acute exposure regimen concomitant with injury in several animal models [223], relatively little is known about the impact of acute GCs exposure prior (in early-life) but not concomitant with injury (later in life) on the individual's ability to repair damaged tissue. To address this issue, we assessed whether synthetic GCs (i.e. DEX) acutely administered prior but not concomitant with the caudal fin amputation procedure would also impair abilities to repair damaged tissue in the larval zebrafish model. Specifically, we performed the following steps: 1. We assessed the impact of early-life (stage *prim-6*) acute DEX exposure on the subsequent ability to repair damaged tissue following the caudal fin amputation procedure, which was performed later in life at two time points including 3 days

Table 1 Schematic representation of regeneration

schematic					
Time point	0-3hpa	4-24hpa	24hpa	48hpa	
Phases	Wound healing	Blastema formation	Early Regenerative outgrowth	Late Regenerative outgrowth	
Genes Involved	*β-catenin, lef1, wnt ligands,apoE (bmp2b), (shh), patched1, msxA and msxD, junb, junb*	**Wound epidermis** *apoE; β-catenin; msxa*[3]; *msxd* [3]*wfgf,fsdf-1a,b, Cxcr4a,b,junbl, junb,dlx5a,wnt3a* **Basal layer of wound epidermis** *apoE; bmp2b*[4]*, lef1; shh*[4]*, ptc1*[4]*, wnt5* **Forming blastema *bmp4***[5]*, eve1*[6]*, evx1*[7]*, evx2*[6]*, fgfr1*[8]*, hoxd11*[5]*, hoxd12*[5]*, msxb*[3 8]*, msxc*[3,8]*, RAR-gamma*[9] *hoxc13a,b*[10] **Differentiating scleroblasts** *bmp2b; ptc1; evx1*	**Wound epidermis** *apoE; -catenin; msxa; msxd; wfgf* [8]*,* dlx4 **Basal layer of wound epidermis** *apoE; bmp2b; lef1; fgfr1; ptc1; shh; wnt3a*[2]*; wnt5* **Blastema** *bmp4; eve1; evx2; fgfr1; hoxd11; hoxd12; lef1; msxb; msxc; RAR-gamma* **Scleroblasts** *bmp2b; evx1; ptc1, col2a1*	
Description	Actin purse ring forms/ Stump surface covers with epithelial cells	Cell proliferation increases, cell death occurs, necrosis occurs, Immune cells arrange at the amputation site	Epithelial cells are tightly packed Proliferation occurs, reduction in necrosis and cell death, immune cells still present		
	Actin purse ring forms/Stump surface covers with epithelial cells	Lesser cell proliferation , more cell death, More necrosis, Immune cells disrupted	Proliferation less than controls, more necrosis, cell death same as control, immune cell count not significantly different from controls		

References: 1, Mannat et al., 1999; 2, Poss et al., 2000a; 3, Akimenko et al., 1995; 4, Laforest et al., 1998; 5, Geraudie et al., 1998; 6, Enulfert et al., 1998; 7, Barday et al., 2001; 8, Poss et al., 2000b; 9, White et al., 1994.10,Ryan Thummel et al 2007

(Experiment 1) and 11 days (Experiment 2) following initial exposure to DEX. In both groups, a 2 day-post amputation recovery period was given after which the extent of tissue damage/repair was measured.

2. We also assessed whether the impairing effects of early-life DEX exposure on tissue repair processes observed in experiment 1 would be restored to levels comparable to that of controls if given longer post amputation recovery period. This was achieved by allowing longer recovery time following caudal fin amputation. In this group the extent of tissue damage/repair was assessed much later following amputation, i.e. 11 days (experiment 3). Note that this experiment could not be performed on individual older than 14 dpf (experiment 2) due to technical difficulties.

3. In order to get insights into the mechanisms underlying the lasting effects of DEX on tissue repair processes observed in experiment 1, we have taken the following endpoint measurements: 1) size of the regenerated area of the caudal fin, 2) analysis of a panel of regeneration-related genes (msxb, wnt3a, rarg, hoxd11, GR and MR) via qRT-PCR, 3) cell death (Tunnel and acridine orange), 4) cell proliferation assay (BrDU), and 5) quantitative analysis of immune cells invasion at the wound site using immunohistochemistry against L-plastin, a macrophage and leukocyte marker.

Materials and Methods

Statement of ethics on animal use

All experimental procedures were conducted in accordance with The Netherlands Experiments on Animals Act that serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, and were performed only after a positive recommendation of the Animal Experiments Committee had been issued to the license holder.

Zebrafish maintenance

Male and female adult zebrafish (*Danio rerio)* of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) who obtains

stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). Fish were kept at a maximum density of 12 individuals in plastic 7.5 L tanks (1145, Tecniplast, Germany) containing a plastic plant as tank enrichment, in a zebrafish recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands) on a 14 h light: 10 h dark cycle (lights on at 7h AM: lights off at 21 h PM). Water and air temperature were maintained at 24 ^{o}C and 23 ^{o}C, respectively. Fish were purchased at the juvenile stage and were allowed to adapt to our facility for at least 2 months before being used as adult breeders. The fish were fed daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen artemias (Dutch Select Food, Aquadistri BV, The Netherlands).

Zebrafish eggs were obtained by random mating between sexually mature individuals. Briefly, on the day (16h) before eggs were required, a meshed net allowing eggs to pass through but preventing adult fish from accessing/eating them, was introduced in the home tank of a group of 12 adult fish. Each breeding tank was only used once per month to avoid handling stress and ensure optimal eggs quantity and quality.

Selection of the dosage of DEX

The dosage (1 mM) of water soluble DEX (Sigma *Aldrich,* Switzerland) used in the present study was chosen based on a pilot study where four concentrations of DEX, including 100, 250, 500 and 1000 µM were tested on embryos at the life stage 25 hpf (equivalent to the *prim-6* stage [122] for a duration of 6 h. The caudal fin amputation procedure was performed on 3 dpf and assessment of regenerative capacity was done on 5 dpf. We found that dosages of 500 µM ($p< 0.01$) and 1000 µM ($p< 0.001$) of DEX significantly reduced tissue regeneration relative to controls (Fig.1 A). Note that no mortality or morphological abnormalities (Fig.1 B) were observed between the different treatment groups. Therefore, we selected the highest dosage of DEX (1 mM) because of its pharmacological safety and largest impact on tissue regeneration.

DEX Treatment

The eggs were harvested 30 min after the onset of lights at 7 h AM and age was set as dpf 0. This is based on the staging system employed in the zebrafish text book untitled *Zebrafish: practical approach* [43]. Approximately 150 eggs were transferred in 10 cm Petri dishes filled with 50 ml of egg water (0.21 g/l Instant Ocean Sea Salt and 0.0005% (v/v) methyl blue) and housed in a separate climate room maintained at a temperature of 28 °C and 50% humidity and under a light-dark cycle of 14h: 10h (lights on at 7h AM/lights off at 21 h PM). When all embryos reached the stage *prim-6* (25 hpf) they were then transferred to 6-well plate (25 embryos/well) and immediately exposed to an acute pulse of DEX. DEX treatment consisted of exposing the embryos for 6 h to water-soluble DEX (1 mM). At the conclusion of the treatment, the embryos were then washed 3 times with egg water and transferred to new clean 6-well plates (30 embryos/well), where they remained undisturbed for 2 days (experiment 1, see details below) and 11 days (experiment 2, see details below). The control group was treated according to the same procedure as described above except that the DEX exposure step was replaced by egg water only. The larvae were placed in the fish facility at 25°C in large tank in egg water till 7 dpf and later on in regular water as for adult fish. The tanks were cleaned every day. The larvae were fed on baby fish food 2x a day.

Experimental Procedure

The timeline and the experimental steps involved in the caudal fin amputation procedure are depicted in Fig. 2. Also see section 2.6 for details regarding the caudal fin amputation method. In the current study, we have performed three separate experiments, which are described below:

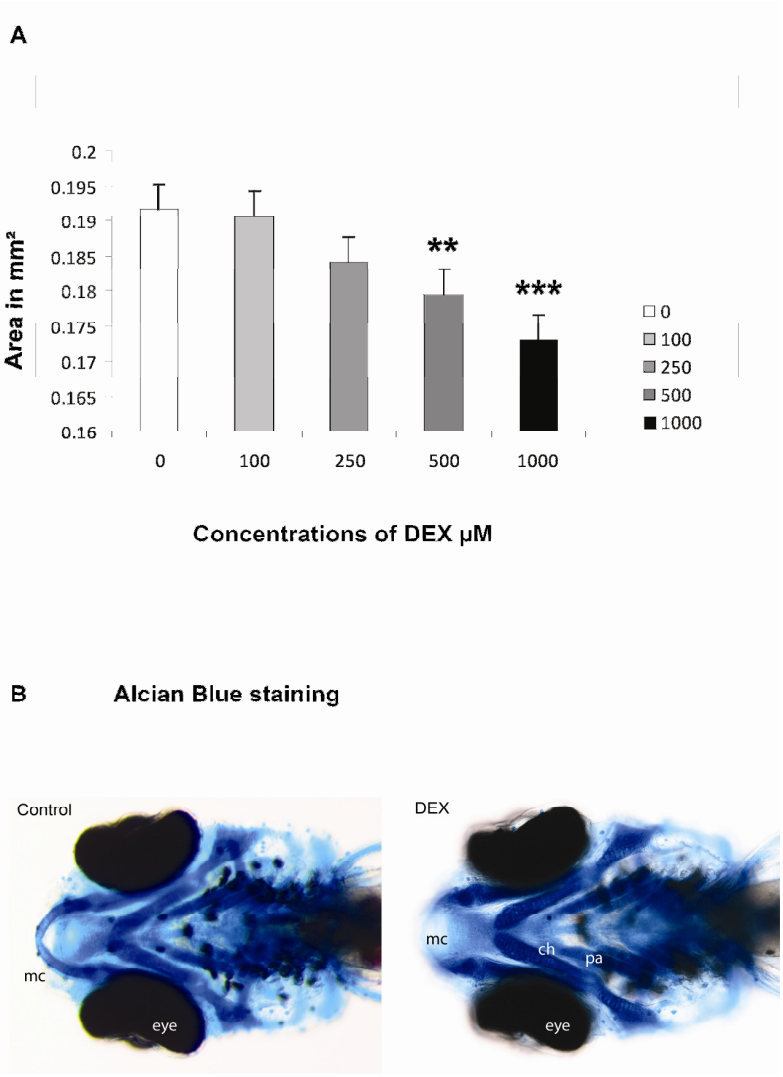


Fig. 1 (A) Effect of varying dosages of dexamethasone (DEX) on tissue regeneration. DEX treatment was administered at the life stage *Prim-6* and lasted 6h. Results show that dosages of 500 µM and 1000 µM significantly affected tissue repair. (**B**) Alcian Blue staining shows that DEX treatment (dose 1000 µM) did not cause any morphological alterations relative to control treatment. All measures presented in panel A and B were performed on 5 dpf larvae. Abbreviations: mc (Meckel's cartilage), ch (ceratohyal) pa (pharyngeal arches), and eye.

Experiment 1: The detailed methodology is presented in Fig. 2A. Briefly, embryos were exposed to DEX (1 mM) at 25 hpf (prim-6) for 6h. On 3 dpf (protruding mouth stage), the embryos were anesthetized with 0.04% Tricaine (MS222, Sigma) after which amputation of the caudal fin posterior to neural keel was performed with a sharp scalpel (see section 2.6). Immediately after amputation larvae were washed 3 times with egg water and then gently transferred to new 6-well plates using a Pasteur pipette. A post amputation recovery period of 2 days was given after which the extent of tissue damage/repair was assessed by measuring a number of outcome measures on 5 dpf-old larvae (see section 2.7).

Experiment 2: The detailed methodology is presented in Fig. 2 B. Embryos were treated exactly according to the procedure described in experiment 1 with the exception that the caudal fin amputation procedure was performed 11 days following DEX exposure (i.e. on 12 dpf-old larvae). Following amputation, larvae were transferred to new tanks (plastic 7.5 L tanks from 1145, Tecniplast, Germany). The post amputation recovery period was the same as in experiment 1, i.e. 2 days. For this experiment, only the size of the regenerated area was measured (see section 2.7.1)

Experiment 3: The detailed methodology is presented in Fig. 2 C. Since findings obtained from experiment 1 showed that acute DEX treatment (1 mM) caused significant reduction in tissue regenerative capacity 2 days post amputation (measured in 5 d old larvae), we assessed whether the impairing effects of DEX on tissue regeneration would recover to a level comparable to that observed in DEX-untreated animals with longer post amputation recovery period. To achieve this goal, larvae were treated exactly as described in experiment 1 with the exception that a longer post amputation recovery period was given, i.e. 11 days. For this experiment, only the size of the regenerated area was measured on 14 dpf-old larvae.

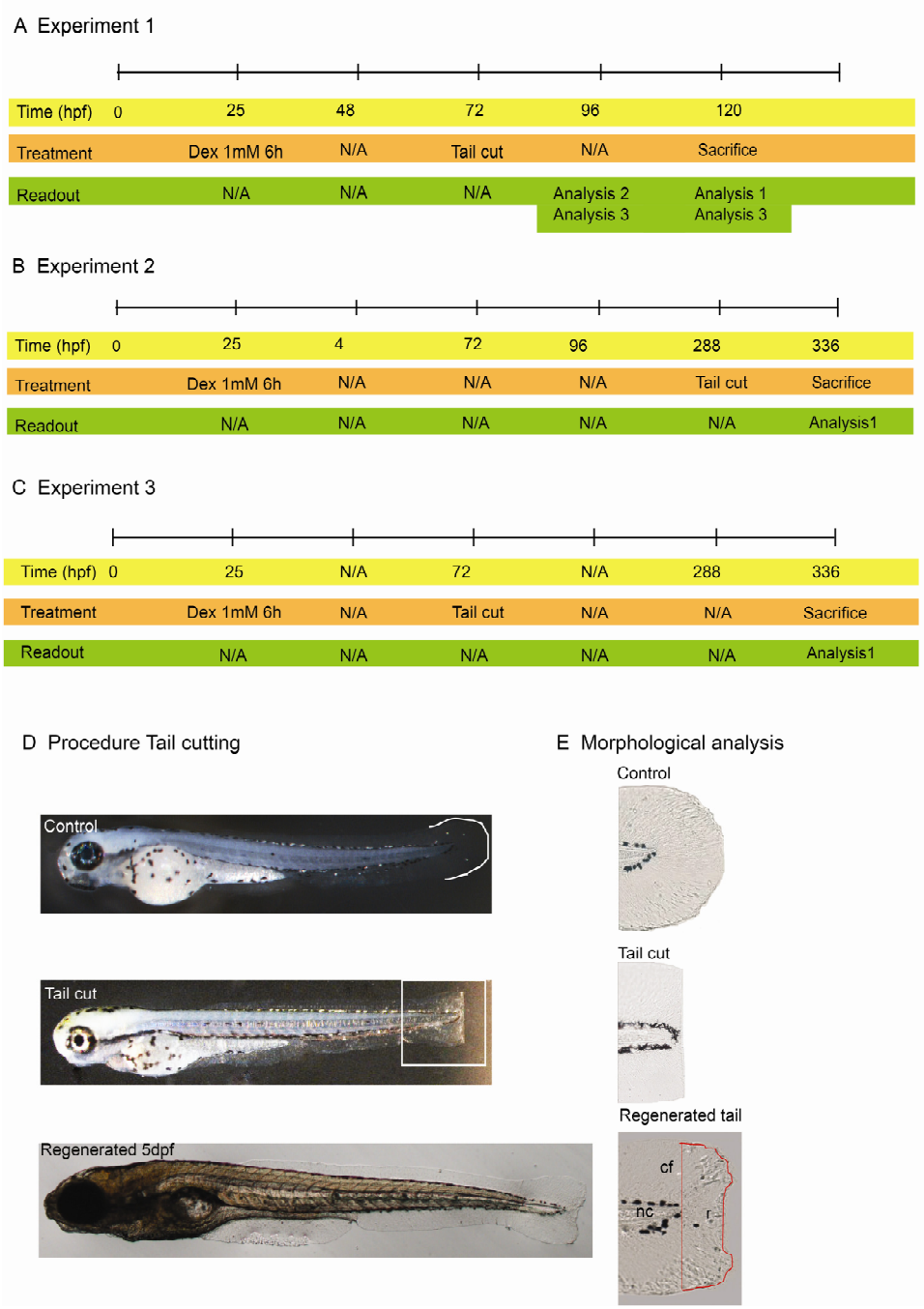


Fig. 2: Overview of the experimental procedure: **(A)** Time line of the experimental procedures performed in experiment 1. The procedures include DEX (1 mM) treatment

at 25 hpf, which lasted 6 h, caudal fin amputation at 72 hpf and termination of the experiment at 120 hpf. Analyses 2 and 3 were performed on 96 hpf while analyses 1 and 3 were done at 120 hpf. **(B)** Time line of experiment 2 is identical to that of experiment 1 with the exception that the caudal fin amputation was performed later on 288 hpf. Termination of the experiment occurred at 336 hpf. Note that only analysis 1 was performed at this time point. **(C)** Time line of experimental 3 was similar to the time line of experiment 1 with the exception that the end of the experiment and analysis 1 were performed later, i.e. at 336 hpf. (**D**) Caudal fin amputation procedure. The top photograph (control) depicts a typical larva with an intact caudal fin while the photograph (tail cut) below depicts a representative example of a larva, which underwent the caudal fin amputation procedure. Note that both larvae are 3 dpf. The amputation plane was kept near the caudal end of neural keel (boxed area). (**E**) Morphological analysis. The top photograph (Control) depicts a high magnification image of normal tail while the middle photograph (Tail cut) depicts a high magnification image of the amputated caudal fin. The bottom photograph depicts a representative example of a regenerated caudal fin at 5 dpf. Analysis of the regenerated tail was performed by outlining the area of the regenerated caudal fin as shown in the photograph (see red line). This area is referred to area 'r'. Measurement of the volume of the regenerated area 'r' was automatically calculated using image J. Analysis 1 includes the measurement of regenerated area of amputated caudal fin with image J. Analysis 2 includes the necrosis, cell proliferation, and apoptosis assays. Analysis 3 includes qRT PCR analysis for expression of GR, MR, hoxd11, msxb, wnt3a and RARγ. Abbreviations: DEX: dexamethasone, GR: glucocorticoid receptor, MR: mineralocorticoid receptor, nc: notochord, cf: caudal fin, N/A: not applicable, hpf: h post fertilization.

Caudal Fin Amputation Procedure

Caudal fin of 3dpf zebrafish embryos were amputated after anesthetising with 0.04% MS-222 (tricaine methanesulfonate). The embryos were placed in the 9cm Petri dish filled with egg water on their lateral side. With a sharp scalpel the caudal fin was carefully cut off under a dissecting microscope along the amputation plane depicted in Fig 2 C and D. After amputation the embryos were transferred 2 x through fresh egg water in separate Petri dishes and then to 6well plate till outcome was measured until 5 dpf (experiment 1) and 14 dpf (experiment 2 and 3), where the experiment was terminated. The larvae were sacrificed by an overdose of anesthesia (tricaine), and were fixated in 4% PFA at 4°C overnight.

Outcome Measures

Assessment of Tissue Repair Capacity

In order to prepare the tissue for morphological analyses, larvae were anesthetized with tricaine and then fixed in chilled 4% PFA over night. This procedure was equivalently performed for experiments 1 and 2 but at different time points depending on the specifics of the experiment, i.e., at 5 dpf (experiment 1), and 14 dpf (experiments 1 and 2). Following fixation, the area of the caudal fin was imaged under a Nikon eclipse E800M equipped with DSF1 camera. (Fig.2D). Area of the regenerated caudal fin was outlined as performed in Fig. 2D and referred to area 'r'. Measurement of the regenerated area 'r' was automatically calculated using image J (National institute of health. See: http://rsbweb.nih.gov/ij/).

Cell Proliferation

To perform a quantitative assessment of cell proliferation, we used the well-established cell proliferation marker BrDU [246]. Briefly, larvae were treated with BrDU beginning 24 h post amputation until 30 h post amputation according to a protocol described previously [121]. At the conclusion of the 6h-BrDU treatment, larvae were immediately fixed in 4% PFA at 4 ^{o}C over night. The tissue was prepared for confocal imaging by undergoing a series of washes with phosphate buffers and dehydration steps with methanol (25%, 50%, 75%, and 100%). Subsequently, the tissue was stored at -20^{o}C until the next day where labelling with fluorescent antibodies was performed. The next day, prior to immunohistochemical staining the larvae were labelled with anti-BrdU antibody (Roche) at a dilution of 1:200 and Alexa flour 534 conjugated anti mouse IgG (Molecular probes Eugene) for 2h at room temperature. Tissue was maintained at 4^{o}C while confocal images were taken for morphological assessments. All samples (Veh n=25 and DEX-treated n=25) were processed within the same day.

Necrosis

For quantitative assessment of necrotic cell death induced by caudal fin amputation and/or antenatal exposure of DEX, acridine orange staining was

performed to label necrotic cells at 4 h and 24 h post amputation in both control and DEX-treated groups. Acridine orange staining consisted of soaking living larvae in a freshly prepared solution of acridine orange stain (200 µM/L distilled water) (Schmid and Co, Stuttgart-Unterturkheim, Germany) for 1 hr at 28^{o}C. After several washes with egg water, living larvae were placed into a cavity glass slide and covered with a thin cover slip, and then imaged under a Zeiss Observer LSM 500. Since acridine orange is fluorescent, necrotic cells were visible at an excitation wavelength of 488 nm. Total number of fluorescent cells was counted with image J.

Apotosis

For assessment of cell death by apoptosis, terminal deoxy-nucleotidyl-transferase-dUTP nick end labelling (TUNEL) was used. The staining procedure consisted of taking fixed (4% PFA) larvae and rehydrating them through graded series of methanol, including 75%, 50%, 25% and phosphate buffer (15 min/solution). Embryos were further washed for 3 time 5 min in phosphate buffer (pH 7.4). We used a commercially available TUNEL labelling kit (TACS® TdT *in situ* – Fluorescein, R&D Systems, Inc. 614 McKinley Place NE) and followed the manufacturer's instructions. Images of the caudal tail area for all larvae were taken using a Zeiss observer LSM 500 microscope using excitation wavelength 488nm. Total number of tunnel-stained cells was counted manually from the images.

Gene Expression

Gene expression levels were measured by qRTPCR [247]. The protocol, methodology and primers (Invitrogen) used for each gene measured in the current study are derived from previous work [10,112,137,140,248] (See Table 1 for sequences).

Table 1. Primer sequences used for qRT-PCR.

Primer	Nucleotide sequence 5`-3`	Reference

wnt3a dr F	TGGGCTCACGCGTGTCATGG	Shimizu et al 2004 [137]
wnt3a dr R	GCGGAGCTGTTTGGGGACCA	
ß-actin F	TGTCCCTGTATGCCTCTGGT	
ß-actin R	AAGTCCAGACGGAGGATGG	Alsop et al 2007 [249]
GR F	ACAGCTTCTTCCAGCCTCAG	Alsop et al 2007 [249]
GR R	CCGGTGTTCTCCTGTTTGAT	
MR F	CCCATTGAGGACCAAATCAC	Alsop et al 2007 [249]
MR R	AGTAGAGCATTTGGGCGTTG	
RARγ F	GCTTGGACCGGGACAGCCAG	White et al 1994 [112]
RARγ R	GAGGTGGCGGGGAAGGGGAA	
Msxb F	GAA TTC TCC TAA GGG ACC C	NM131260 [140]
Msxb R	GATTCAACACTGAACGGGAGG	
Hoxd11 F	AATCCTCCGTTTCAACCTGCGATGG	Van der Hoeven 1995 [248]
Hoxd11 R	GCATCTCTTCTTTCTGGACTTTGTCG	

Total RNA was extracted using Trizol® reagent (Invitrogen Life Technologies, Belgium) according to the manufacturer's protocol. Note that for all genes studies, the cDNA was taken from the amputated tails and not from the whole animal. The following categories of gene were measured: 1) GC receptors (MR and GR mRNA) [129], tissue differentiation markers associated with tissue patterning (wnt3a and RARγ mRNA) [16,17], and cell proliferation markers

(msxb and hoxd11 mRNA) [18]. All genes were measured at two points including 24 h and 48 h post amputation.

Immune Cell Staining

The L-plastin immunostaining procedure used in the current study was derived from a previous study from Cui and colleagues [191]. Briefly, caudal fin tissue was incubated over night at 4°C in blocking buffer containing an antibody directed against L-plastin [final concentration 1:500] [181]. Embryos were incubated for 2 h at RT in Alexa Fluor 405 Goat-anti-Rabbit (Invitrogen) 1:200 and were then stored at 4°C and imaged using Zeiss observer LSM 500.

Statistical Analysis

Statistical analyses and graphs were performed using GraphPad Prism software (version 5.0). One-Way ANOVA analysis was performed to analyze the effects of DEX treatment with varying dosages including 100 µM, 250 µM, 500 µM and 1000 µM on tissue regeneration. A Dunnett's multiple comparison post-hoc test was applied to further decompose group comparisons, where each dosage of DEX was compared to the vehicle treatment (DEX 0 µM/egg water). Student T-tests (two-tailed) were performed to analyze the impact of DEX (1 mM) treatment on tissue regeneration (Experiments 1, 2 and 3) as well as to analyze the impact of DEX (1 mM) on gene expression and cell proliferation (BrdU). Two-Way ANOVA analyses were performed to analyze the data from the acridine orange staining, tunnel staining, and L-plastin immunohistochemistry. Tukey's multiple comparison post-hoc tests were applied to further decompose group comparisons. Data are presented as mean ± SEM, and a probability level of 5% was used as the minimal criterion of significance.

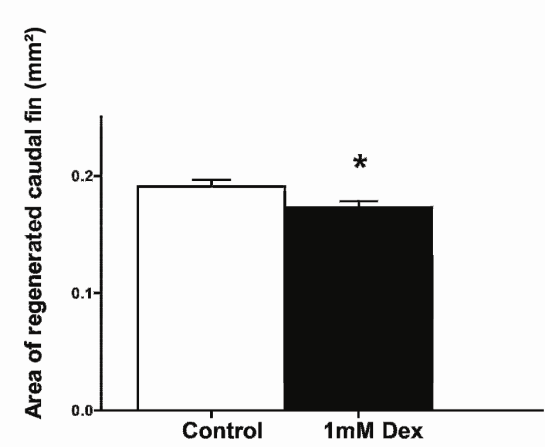


Fig. 3 Area of caudal fin (mm^2) amputated at 3 dpf and regenerated for 48 h. Measurements were done with Image J.

Results

Experiment 1

Relative to the control group, DEX-treated embryos displayed significant decreases in the total area of the regenerated caudal fin tissue at 2 days post amputation (Fig. 3) ($p< 0.05$). In order to gain insights into the possible mechanisms underlying DEX effects, we have measured the following outcome measures including, cell proliferation, necrosis, apoptosis, gene expression, and macrophage markers. The results are presented below:

Cell Proliferation

Relative to controls, we observed a significant reduction in the number of proliferating cells in the regenerating caudal fin in DEX-treated embryos 24 to 30 hrs post amputation (Fig. 4 A) ($p< 0.001$). Confocal images confirm that only the amount and not the appearance and/or distribution of proliferating cells is altered by prior exposure to DEX (Fig. 4 B).

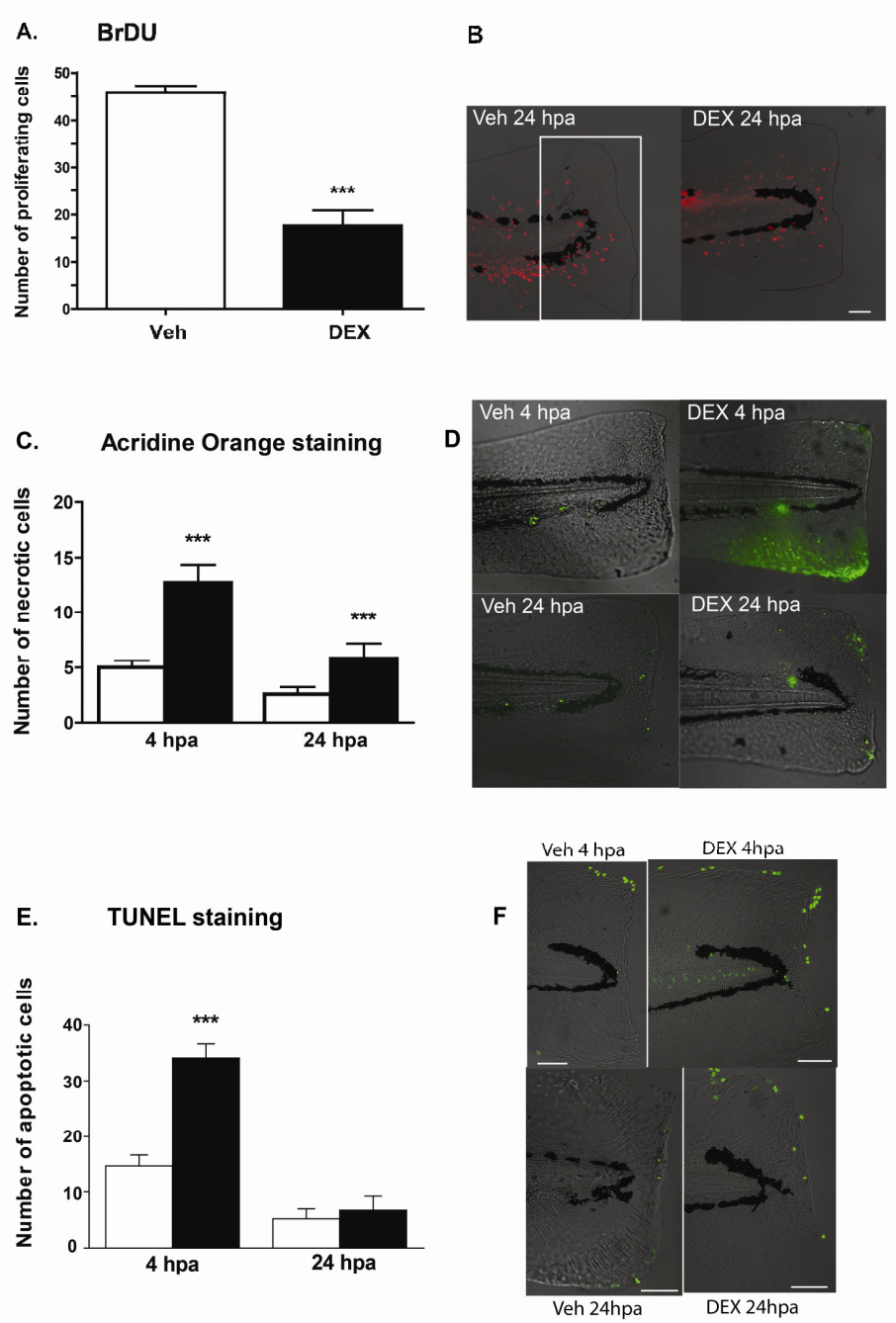


Fig. 4 (**A**) Number of proliferating cells in the regenerated caudal fin after 24-30 hpa is significantly reduced in DEX treated larvae. (**B**) Confocal images showing proliferating

cells (red) in the caudal fin region of the regenerating tails of larvae. Number of the cells was counted within the boxed area. (**C**) Number of necrotic cells labelled with acridine orange staining after 4h and 24h of amputation. The results show a significant increase in cell death in DEX treated larvae as compared to controls. (**D**) Confocal images of (upper left) veh 4hpa and DEX 4hpa (upper right), veh 24hpa (lower left) and DEX 24hpa (lower right) showing necrotic cells (green) in the regenerating tail region in living embryos. (**E**) Number of apoptotic cells labelled with Tunel staining at 4hpa and 24hpa. (**F**) Confocal images of Veh 4hpa (upper left) and DEX 4hpa (upper right), 24hpa Veh (lower left) and 24hpa dex (lower right) dead cells (green).

Cell Death

Analysis of necrotic cell death reveals significantly higher amount of necrotic cells in DEX-treated embryos relative to controls both 4 h ($p< 0.001$) and 24 h ($p< 0.001$) post amputation (Fig. 4 C). Confocal image analysis showed that, for both time points studied, necrotic cells were not aligned within the amputation plane but were rather accumulated at the dorsal and ventral corners of the regenerating fins (Fig. 4 D). Analysis of apoptotic cell death showed significant increases in the number of apoptotic cells at 4 h ($p< 0.001$) but not 24 h post amputation (Fig. 4 E). Confocal image analysis showed that in general the apoptotic cells were aligned with the amputation plane but were also present in the notochord region. Note that this was only observed at 4 h post amputation and appear to have returned to control levels by 24 h post amputation (Fig. 4 F).

Gene Expression

We observed that, while the expression of the GR receptor mRNA (MR and GR) (Fig. 5 A and B) and proliferations markers mRNA (msxb and hoxd11) (Fig. 5C and D) were not significantly altered by prior exposure to DEX, significant changes in the expression levels of genes involved in tissue patterning (wnt3a and RARγ) were observed in DEX-treated embryos relative to controls (Fig. 5E and F). Specifically, we observed a significant up-regulation of wnt3a in DEX-treated embryos relative to controls at 24 h ($p< 0.05$) but not 48 h post amputation (Fig. 5 E). We also observed a significant up-regulation of RARγ mRNA levels in DEX-treated embryos at 24 h post amputation (Fig. 5 F) ($p< 0.05$). We observed an opposite effect of DEX on RARγ mRNA levels at 48 h post amputation, which were significantly down-regulated (Fig not shown).

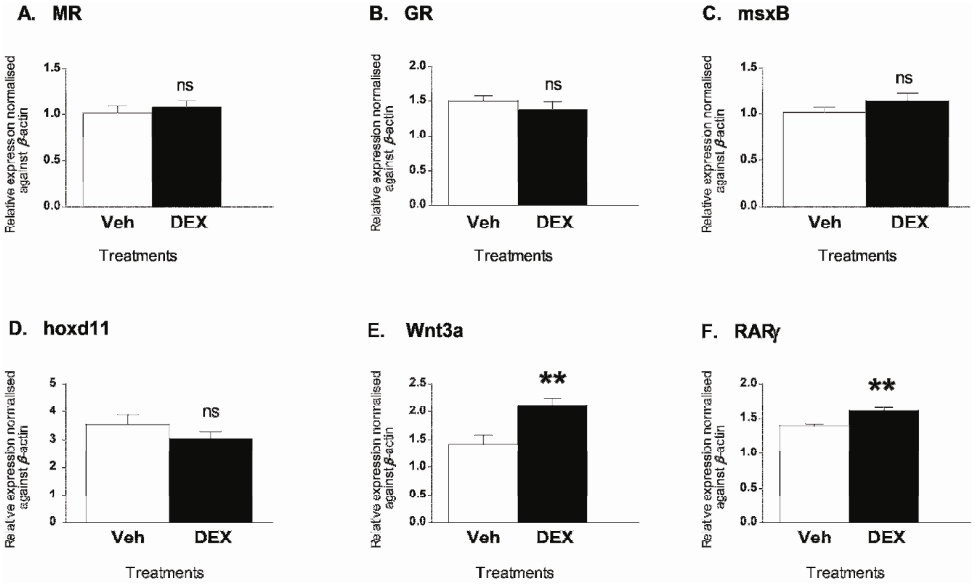


Fig. 5 Column graph showing gene expression relative to β-actin in the regenerated caudal fins of veh and DEX treated zebrafish larvae 24 hpa. Note the significant increase in expression levels of Wnt3a and RARγ in Dex-treated larvae relative to controls.

L-Plastin

We observed a significant decrease in the number of L-plastin positive macrophages/leukocytes in DEX-treated embryos relative to controls at 4 h but not 24 h post amputation (Fig. 6 A) ($p < 0.001$). Interestingly, we also observed that the spatial arrangement of the L-plastin positive cells differed between DEX-treated and control groups at 4 h but not 24 h post amputation. Specifically, confocal image analysis revealed that, while L-plastin positive cells are aligned with the amputation plane in controls, they were rather irregularly distributed across the regenerated area of the caudal fin in DEX-treated embryos (Fig. 6 B).

Experiment 2

To assess if prior history of early-life DEX exposure would also affect tissue repair capacity at later life stages than that tested in experiment 1, we assessed the effect of DEX after initial exposure. Similar to what is observed in

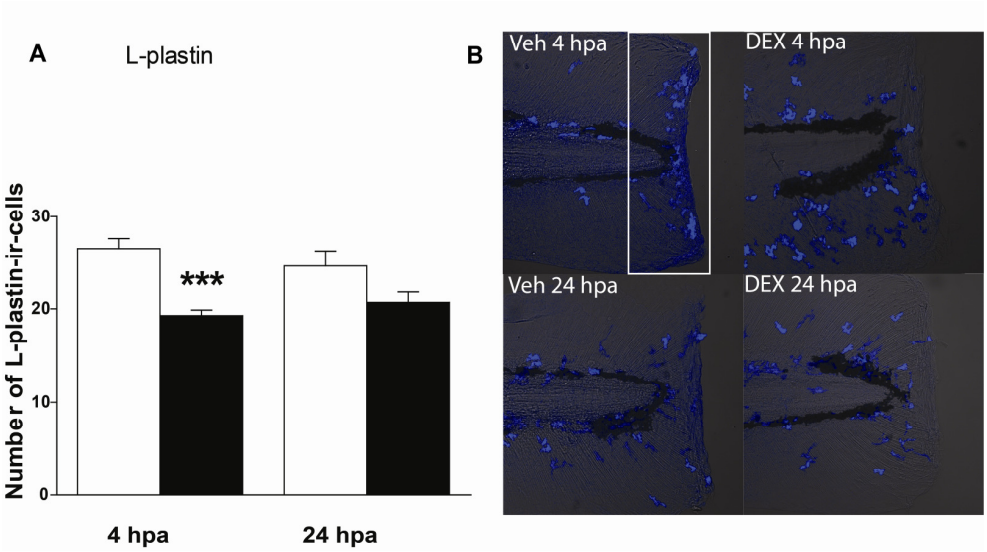


Fig. 6 Graph showing total number of L-plastin immunoreactive (ir) leukocyte cells counted from the area demarcated with a box in panel B. Cells were counted at 4 hpa and 24 hpa in veh and DEX treated larvae. Note the significant decrease in L-plastin positive cell in DEX-treated larvae relative to controls 4hpa but not 24 hpa (although a trend towards a decrease is also observed at 24 hpa). (**B**) Overlay of confocal and transmitted image showing leucocytes (blue cells) in the regenerating caudal fin region in both controls and DEX-treated larvae at both 4 hpa and 24 hpa.

Experiment 1, we also observed that relative to the control group, DEX-treated embryos displayed significant decreases in the total area of the regenerated caudal fin 11 days post exposure to DEX (Fig. 7 A) ($p< 0.5$).

Experiment 3

To assess whether the effects of DEX are permanent or transient, we allowed a longer recovery time following caudal fin amputation and assayed the embryos at 11 days post amputation. We observed that, relative to controls, DEX-treated embryos were no longer significantly different from the control group in terms of size and shape of the total regenerated area of caudal fin at 11 days post amputation (Fig. 7 B).

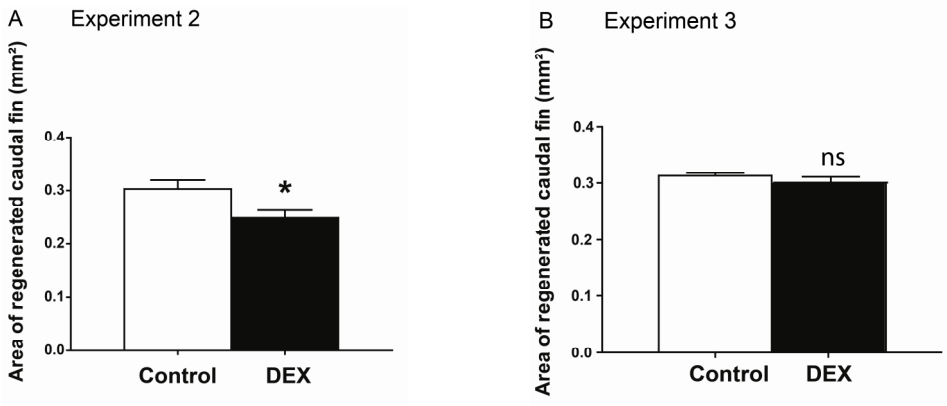


Fig. 7 (**A**) Experiment 2. Graph showing area of regenerated caudal fin in control and DEX-treated larvae amputated after 11 days of initial DEX treatment and allowed to regenerate for 48h. The result show a significant reduction in the are of the regenerated caudal fin in DEX-treated larvae relative to controls. (**B**) Experiment 3. Graph showing the area of the regenerated caudal fin in larvae that were amputated at 3 dpf and allowed to regenerate until 14 dpf. The results show no significant differences in the area of the regenerated caudal fin between controls and DEX-treated larvae suggesting that full recovery occurs despite initial impairment with longer post amputation recovery period.

Discussion

This study investigated the influence of DEX exposure during a sensitive period of development (*prim-6*) on the ability to repair damaged tissue following amputation of the caudal fin in the post-hatching period in larval zebrafish. We report that early-life history of DEX exposure impairs ability to repair and regenerate caudal fin tissue across the first 2 weeks of life, and that significantly longer post-amputation periods are required for full recovery. The possible mechanisms underlying these effects as well as the implication of these results in the field of medicine are discussed below.

Possible mechanisms

The wound healing phase (0-3 hpa)

Analysis of the several outcome measures taken in this study including cell proliferation, cell death (necrosis and apoptosis), gene expression, and

leukocyte cell marker (L-plastin) suggest that DEX exerted its effects in the early phase (4 h) of wound healing rather than in the later phases of the tissue repair process. This is based on our observation of a massive amount of cell death both by necrosis and apoptosis at 4 h post amputation. This cell loss does not appear to have been compensated, at least as far as 24 h post amputation, since cell proliferation was found to be drastically decreased in DEX-treated embryos relative to controls at that time. The large amount of cell death may be responsible for the significant reduction in the number of L-plastin positive/leukocyte cells observed at 4 h but not 24 h post amputation. These results are consistent with previous studies showing significant reductions in immune cell types (e.g. leukocytes) at the injury site few h following acute exposure to synthetic GCs (e.g. DEX) [110,225,226].

Reduction in immune cells at the injury site has important implications for the wound healing phase. Specifically, the invasion of immune cells including leukocytes, macrophages, and neutrophils occurs early (within 4 h) during the wound healing process and is essential for clearance of cell debris and foreign molecules by phagocytosis [250]. It has been shown that alterations in the clearance process may hinder and even prevent subsequent steps to take place (e.g. blastema formation) [251]. Therefore, it is possible that a decrease in the number of immune cell types such as L-plastin positive/leukocytes may have altered the clearance process, which in turn interfered with the initiation of the subsequent steps resulting in impaired tissue repair and regeneration. Following clearance of cell debris, another important function of the immune cells is the secretion of growth factors and cytokines. The presence of sufficient amount of growth factors and cytokines is also essential for the initiation of the blastema formation and its progression [110]. Therefore, it is likely that DEX-induced reduction in the number of L-plastin positive/leukocytes observed in this study may not only have altered the clearance process but also lowered levels of growth factors and cytokines at the injury site. Such events may simultaneously contribute to impede the initiation of the blastema formation and its progression, and results in impaired wound healing.

Interestingly, we also observed an aberrant arrangement (i.e. misalignment with the amputation plane) of the L-plastin positive/leukocytes at the injury site when compared to control situations. These findings suggest that DEX also

affected the migration pattern of immune cells towards the injury site, which may further contribute to delay the initiation of the blastema formation and eventually impair tissue repair. However, the fact that full recovery is possible with longer post amputation period suggests that the molecular components of the immune system are functional but that the natural time course of their actions appears delayed.

The blastema formation (4-24 hpa)

Alterations in gene expression patterns in the early phase of the blastema formation (24 h) reveal that DEX exposure also impedes the natural progression of this phase, although perhaps to a minor extent than the wound healing phase (see above). Specifically, we observed a significant up-regulation of wnt3a and RARγ genes in the DEX treated embryos after 24 h of amputation at the injury site. Both of these genes are actively involved in the Remodelling of the epidermal or blastemal tissue. Retinoic acid is a signalling molecule for vertebrate pattern formation both in developing and regenerating tissue [136]. RARγ mRNA is the prominent RAR transcript found in normal regenerating tissue. RARγ is expressed in the distal ends of blastema of regenerating adult fin tissue [112]. Wnts are secreted glycoprotiens that play an important role in body patterning, cell proliferation, cell differentiation and tumour formation [137]. Wnt signalling plays a role in the maintenance and renewal of stem cells which are cells that help repair the tissue damage [137]. It is possible that up-regulation of these genes may have served the purpose of compensating for the massive cell death and decreased proliferation observed at earlier phases of the wound healing process. However, we did not observed any significant differences between groups in the expression levels for gene coding for cell proliferation marker (msxb and hoxd11). This may be due to our selection of a later time point (24 h) than reported in the literature since a previous study showed an upregulation of these genes at an earlier time point (16-24 h) [117]. Taken together, these findings confirm that the impact of DEX is more prominent in the early phase (4 h) of wound healing and extending to the early phase of the blastema formation (4-24 h) since no further differences were observed between groups in any of the outcome measures studied at 48h post amputation (data not shown).

Possible mediators

A possible mediator of the impairing effects of DEX on the wound healing process may be the immunotoxic properties of DEX on developing organs. For instance, lymphoid organs (spleen and thymus) and lymphocytes have been shown to be particularly sensitive to GCs, especially in immature organisms [230]. A main toxic effect of DEX includes apoptosis of immune cells in thymus and spleen. A significant reduction in the size of both immune organs has been previously reported in response to antenatal DEX exposure in mammal models [229,252]. While both thymus and spleen are not developed before 60 hpf in zebrafish, the caudal hematopoetic tissue (CHT) serves as lymphoid organ in young larvae as early as 24 hpf [253]. Although not measured in the current study, it is possible that a brief exposure to high dose of DEX during early development (*prim-6,* 25 hpf) may have affected the development and/or function of the CHT, which in turn may have impacted the production of immune cells (e.g. macrophages and leukocytes) in the initial phase of wound healing. This possibility is also consistent with our observation of a massive cell death (via apoptosis and necrosis) within 4 h post amputation.

Another possible mediator of the impairing effects of DEX on wound healing may be via DEX effects on GR expression levels. DEX is known (even brief exposure) to cause enhanced activity of the adrenal glands, which causes both lower lymphocytes proliferation response and decrease sensitivity of immune cells to GCs [228-230]. This type of insensitivity is usually related to a down regulation of GR. A reduced sensitivity to GCs has been shown to impair immune processes in several species including humans, and therefore may have contributed to the effects observed in the present study [229,230]. It is noteworthy that GR is expressed on immune cells such as macrophages and leukocytes in fish [110,254]. Since we did not observe any significant changes in both MR and GR expression levels between groups at both 24 h and 48 h post amputation (data not shown), this possibility is unlikely to play a major role in the current study. However, we do not reject the possibility that changes in GR expression levels may exist between groups at the level of certain types of immune cells such as macrophages and leukocytes. This possibility remains to be tested.

Implications

The findings presented here are in agreement with previous studies reporting a link between psychological stress and impaired wound healing in human in adulthood [218,220,221]. Transient psychological stressors, like academic examinations, marital troubles, caring for a sick relative and depression can also slow wound healing [110,218,220,221,228]. Our findings also suggest that not only stress (or stress hormones) experienced in adulthood but also during early-life may influence wound healing at later time points during the lifespan. We provide evidence that DEX exposure during early sensitive periods of development appears to cause permanent alterations in the cellular/molecular immune processes that are involved in the early phase of wound healing in zebrafish. In support to this claim, previous studies have shown that antenatal course of DEX are associated with immediate [255] and lasting alterations of the immune system in later life in rodents, non-human primates, and humans [228-230,256]. The current study extends these findings by showing, for the first time, a similar phenomenon in zebrafish. Our study also provides a demonstration of the immediate functional consequences of a DEX-rendered malfunctioning immune system on tissue repair in the juvenile organisms.

These findings have implications for the field of medicine, especially with regard to the clinical practice consisting of administering antenatal course of DEX to pregnant women at risk for premature delivery. The results obtained in this study contribute to enrich the repertoire of information related to the side effects of such procedure, which are needed by medical authorities to assess the risks/benefits of these extreme intervention methods for the welfare of the developing foetuses and infants.

Future Studies

Further studies are required to assess whether blocking the effect of DEX using pharmacological antagonism or morpholino against GR would have prevented the effects observed in the current study. It may also be possible that the genes selected here may have been up or down-regulated at earlier time than the time points assessed here. Furthermore, whether early-life DEX-induced impairment of wound healing will endure throughout the lifespan remain to be

shown. The results presented in this study argue in favour of this idea since we observed that not only the impairing effects of DEX are observed relatively early (5 dpf) but also at a much later life stage (14 dpf) suggesting that DEX effects on wound healing may endure throughout the lifespan. However, further studies are required to ascertain the last claim.

Concluding Remarks

Our findings are in agreement with the view that GC exposure during early development can influence the embryonic/larval immune system resulting in prolonged effects on the immune response related to functional impairment of tissue repair after hatching. Therefore, similar to other species like rodents and non-human primates, zebrafish larvae also appear particularly sensitive to GC treatment in early development. Therefore, the findings presented in this study support the use of the larval zebrafish as a valid model to study the impact of stress and stress hormone exposure in immature organisms on health risks in later life.