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Cardiac blood sampling for glucose estimations in the laboratory zebrafish (*Danio rerio*)

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ABSTRACT

The zebrafish model has gained widespread popularity among the global research community due to its ever-increasing use in basic biology research, drug discovery, chemical safety assessment, and other areas. Several laboratory procedures, including the estimation of hematological, biochemical, and serological parameters in healthy and disease conditions, require blood sampling from laboratory animals. In zebrafish, blood is one of the least-explored tissues due to the organism's small size. The existing methods are tedious and difficult to obtain blood from this species. The researchers used several approaches to collect blood samples from different sites, including lateral incision, decapitation, tail amputation, and cardiac puncture. In all these techniques, researchers have used different numbers of animals and obtained varying volumes of blood. The scarcity of blood obtained by these methods also limits the analysis of many biochemical and cytological parameters. We adopted a cardiac puncture approach for blood sampling in anesthetized adult zebrafish. The blood sampling from adult male and female zebrafish was successfully carried out, and the collected blood volume ranged between 2-10 µl per adult fish. The blood specimens were used for the random blood glucose estimations. The mean blood glucose levels measured were lower in males than in females in all five lines, with a significant difference in the AB line. Due to the significant mortality anticipated with this approach, this method may not be suitable for repeated sampling; however, it can be considered for terminal bleeding.

Keywords: Blood collection, anesthesia, cardiac

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INTRODUCTION

The global research community witnessed a significant increase in the use of zebrafish (*Danio rerio)* in advancing biomedical research and assessing toxicity over the past few decades (Veldman & Lin, 2008; Choi et al., 2023; Ben-Zvi et al., 2023). The useful features, including a fully sequenced genome, robustness to ѕurvive in suboptimal conditions, high fecundity in females, transparent embryos, and visi-ble fertilization & development outside the mother's body, have popularized laboratory zebrafish among the research community (Teame et al., 2019). Having prominent advan-tages over other species, zebrafish has become a promising research model in biomedical studies to investigate verte-brate development, genetic analysis, cancer biology, myco-bacterium infection, and behavioural analysis (Teame et

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al., 2019). Moreover, its ex-utero fertilization and transparent embryos enable researchers to directly visualize the early events of embryogenesis (Westerfield, 2007).

Taking the advantage of fully sequenced and annotated zebrafish genome (Sprague et al., 2003; Sprague et al., 2006; Sprague et al., 2008), research community has explored zebrafish animal model as an excellent candidate in biomedical and preclinical, developmental (Kari et al., 2007. Keller and Murtha, 2004; Lieschke & Currie, 2007) immunological (Meeker & Trede, 2008; Novoa & Figueras, 2012; Trede, 2004) toxicological (Sipes et al., 2011), environmental pollution monitoring studies (Carvan et al., 2000; Scholz et al., 2008) and plasma proteome analysis (Babaei et al., 2013). Zebrafish is an interesting animal model in studying metabolic diseases because mechanisms associated with lipid metabolism, adipose biology, pancreas structure, and glucose homeostasis are functionally conserved. This model is also being explored to investigate novel targets linked to the risk and treatment of obesity and diabetes in humans (Zang et al., 2018).

In humans and animals, peripheral blood is analyzed in the laboratory to determine physiological and pathological status in healthy and diseased conditions, respectively. Variations in the fish haematological indicators, such as serum biochemistry and cellular composition, are strongly dependent on environmental factors and reflect health and reproductive cycles. These indicators are useful tools for monitoring fish responses to stressors or adverse conditions (Vazquez & Guerrero, 2007; Nordlie, 2009).

Knowledge of the zebrafish peripheral blood is scarce, despite the widespread popularity of this organism as an animal model. Blood sampling from laboratory animals is necessary for a wide variety of scientific studies; however, this common procedure is challenging in zebrafish due to their small size. In one study, cells isolated from the kidney, a hematopoietic organ of the fish, have been used in most zebrafish hematological studies (Traver et al., 2003).

Researchers have adopted a few techniques to withdraw blood from the zebrafish, including lateral incision (Pedroso et al., 2012; Jagadeeswaran & Sheehan, 1999), decapitation (Eames et al., 2010), tail ablation (Babaei et al., 2013), and cardiac puncture (Jenniferet al., 2009). However, these approaches for blood collection can sometimes be very lethal. Moreover, these methods are tedious and technically cumbersome, and the volume of blood collected is very small and unpredictable. In a typical experiment, only a few microliters of blood can be harvested from a zebrafish. This makes many biochemical and cytological analyses unfeasible. This technical obstacle limits the utilization of zebrafish in many applications, particularly in haematological research and plasma biomarker discovery.

Pedroso (2012) collected blood by making a diagonal incision with the steel blade just between the anal fin and the caudal fin. The blood oozing out from the incision wound was collected with the P20 pipette (pre-loaded with a low retention tip). The researchers noticed that the amount of blood collected using this approach depends on the size of the fish and on proper anesthesia, ranging from 5 to 20 µl. A similar approach to collecting blood was adopted by making an incision in the region of the dorsal aorta and the inferior vena cava, just posterior to the dorsal fin (Jagadeeswaran & Sheehan, 1999). In 2010, Eames and team attempted to withdraw blood from the decapitated fish by inserting a heparinized microcapillary collection tube into the pectoral articulation. As per the protocol adopted by Moss (2009), blood was collected by inserting a pulled capillary pipette into the atrium of the anesthetized zebrafish's beating heart. Approximately 50 µL of blood was collected for blood glucose estimation.

As reported in the protocol by Babaei et al. (2013), the blood withdrawal technique involves severing the caudal fin of anesthetized zebrafish with fine scissors. The amputated fish with the wound pointing down was inserted into a homemade 0.5 ml microcentrifuged tube (Eppendorf, Hamburg, Germany) having a perforation at the bottom. The tube carrying the amputated fish, was then placed into a 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) that contained 10 µl of heparin (500 i.u. mL^{-1}). The assembly was then put into a benchtop centrifuge (Eppendorf, Hamburg, Germany) and centrifuged at 40g for 5 min at 11 °C. In another research article, the team developed methods for collecting microsamples of whole blood and plasma to measure hematocrit and blood glucose (Stefani et al., 2010).The team of (Abdullah et al. 2024) fabricated a cheaper device comprising two main components: (i) the sampling syringe and (ii) the micro-capillary needle from the site along the body axis and posterior to the anus in the region of the dorsal aorta, requiring repeated blood samples. Zang et al. 2015) developed a novel, minimally invasive method to collect repeated blood samples from adult zebrafish. The site for blood collection was along the body axis, posterior to the anus, in the region of the dorsal aorta. The dorsal aorta (DA) and the posterior cardinal vein (PCV) are just ventral to the spine. This method minimizes trauma to the zebrafish and yields a low mortality rate of 2.3%. The maximum volume of blood that can be collected using this technique is approximately 2% of body weight.

In the present study, the aim was to collect blood samples by inserting a pulled glass capillary into the palpable heart of an anesthetized zebrafish, using gentle mouth suction via silicon tubing attached to the capillary. This blood sampling technique is one of the approaches adopted by the

previous researcher (Moss et al., 2009). The blood samples were used to estimate blood glucose levels in male and female adult zebrafish from five different lines, with the aim of increasing the utility of zebrafish as a model animal for hematological studies of human diseases.

MATERIALS AND METHODS

Housing and husbandry of the zebrafish: Wild-type and mutant lines of zebrafish were maintained in a state-of-the-art zebrafish facility operated on the principle of continuous water recirculation (Aquatic Habitat, USA) established at the Department of Biological Sciences, TIFR, Mumbai. The UV-sterilized water is circulated in the fish aquaria continuously after filtration through a series of filters (biological, mechanical and chemical). The housing system provides a high-water exchange rate (5-30%) over 24 hours to keep the water healthy for housed zebrafish. The municipal corporation sourced raw water, was purified using a reverse osmosis (RO-100) system, and maintained the conductivity and pH of the aquarium water by mixing red sea salts (25 gm/L) and sodium bicarbonate (16 gm/L), respectively. The water temperature between 27 0C and 29 °C was maintained at all the times using built-in water heaters and a chiller. Photoperiod was maintained with a constant light: dark cycle (14:10 hrs) using high-quality timers, with illumination at 150-300 lux. Zebrafish of all age groups were fed with a highly nutritious and balanced diet. Four types of diets (larval diet, brine shrimp cyst, micro pellet and adult diet) were provided at four different timings (at 9.0, 12.0, 16.0 and 19.0 'O' Clock) to achieve proper growth and maintain them in healthy conditions (Kohale, 2021). Male and female zebrafish aged 12-24 months from 5 different lines were randomly selected for the experiments. A total of 50 adult fish (10 male & 5 female) from each of 5 lines were used for blood sampling.

Pulling the glass capillaries

The glass capillaries commonly used for embryo microinjection (Make: Borosilicate Sutter) were pulled using a pipette puller (Sutter-USA) and the tip diameter of the needle was adjusted between 100-200 µm (Zang et al., 2015). The blood-collecting needles were rinsed with heparin (5mg/ml) and stored in an incubator (28 °C) for two hours before the blood sampling (Fig.1).

Blood sampling

The adult fish were anesthetized using 0.02% Tricaine Methasulphonate solution before the blood sampling. The anesthetized fish were fixed in the slit on a wet sponge, and the palpation of the beating heart was located in the triangle ventral to the gills (Fig.2). A pulled capillary pipette attached with a mouth pipetting tube was inserted into the beating heart as per the protocol of Moss et al. (2009). The blood will flow into the capillary the moment it enters the heart (Fig.3). With gentle suction using mouth tubing attached to the pulled capillary, the blood was collected. The blood, with varying volumes, was obtained and transferred to the clean paraffin tape, ranging between 2 and 10 µl.

Blood glucose estimation

Using a commercial handheld glucometer (Accu-Chek Active), blood glucose levels were measured. The test strip was inserted into the glucometer and touched directly to the blood drop collected on the paraffin tape (Fig. 4). The blood drawn into the test strip was automatically observed, and the blood glucose results were displayed in 5 seconds. The results were recorded, and the used test strip was discarded. A new test strip was used for each measurement. The readings were used for further analysis (Zang et al., 2015; Moss et al., 2009).

Fig 1

Fig 2

Fig. 1: Materials arrangement for the blood withdrawal in zebrafish (Anaesthetic chamber, pulled capilary, silicon tubing, forcep, sciezer, glucometer).

Fig. 2: Anesthetized adult zebrafish fixed in the slit made on the wet sponge with ventral view on top. The moisture in the sponge helps to maintain the respiration in zebrafish. The site of the needle puncture is marked as a triangular area in the figure.

Fig 3

Fig 4

Fig. 3: Positioning of the anesthetized adult zebrafish on a wet sponge, the needle made of glass capillary is inserted into the palpating heart, and the blood is flowing through the capillary after pricking the heart.

Fig. 4: A blood drop collected on the paraffin tape obtained from cardiac blood sampling was touched on the strip for glucose estimation using a handheld glucometer.

STATISTICAL ANALYSIS

The paired t-test was used to compare mean blood glucose levels within and between the groups.

RESULTS AND DISCUSSION

In zebrafish, random blood specimens were collected from the palpating heart of 50 adult zebrafish using pulled glass micro-capillary needles under the influence of 0.02% Tricain Methasulphonate anesthetic agent. The site of the needle insertion used for blood withdrawal marked as triangular area located ventral to the gills was shown in Fig.2. The blood flow was clearly visible in the capillary needle after inserting it inside the palpating heart (Fig.3). The blood volume obtained using this route ranged between 2-10 µl which was further used for the estimation of blood glucose levels using Accu-Chek Instant glucometer. The mean blood glucose levels measured were 31.6, 32.2, 35.2, 40.4, and 60.4 mg/dl in AB, Albino, Cld-GFP, Tubingen, and DBS male fish, respectively. Whereas the mean blood glucose levels measured were 45, 48, 48.2, 57, and 83 mg/dl in Albino, AB, Tubingen, Cld-GFP, and DBS female fish, respectively. The mean blood glucose level was lower in male fish than in female fish across all lines. The lower levels of blood glucose (31.6 mg/dl) and (45 mg/dl) were measured in AB male and Albino female fish, respectively. Whereas higher levels of blood glucose (60.4 mg/dl) and (83 mg/dl) were measured in DBS male and females respectively (Table.1, Fig.5). The statistical analysis using t-test for the pair wise comparison showed significant difference between male and female of AB line, however, in all other lines there was no significant difference (Fig. 6). The group wise comparison showed no significant difference in all the zebrafish lines (Fig.7). The mean random blood glucose (31.6±6.73 mg/dl) measured in male AB strain in the present study was found lower than the fasting blood glucose (FBG) 46 ± 5 mg/dl in male AB strains reported by Zang et al. (2017). Eames et al. (2010) and Olsen et al. (2010) reported blood glucose levels of approximately 50–75 mg/dl in adult zebrafish. We demonstrate that the handheld glucometer designed for human diabetics returns valid results when used with zebrafish blood. The blood collected using this approach was sufficient for the blood glucose test using a commercial glucometer. The regular glass capillary (Borosilicate, Sutter) used for cell/embryo microinjection can be pulled using a pipette puller (P-97, Sutter, USA) to adjust the tapering end's internal diameter to 100-200 um and its length to 1-2 mm.

Blood sampling from laboratory animals is regularly required for experimental or clinical procedures, such as the estimation of hematological, biochemical, and serological parameters in healthy and disease conditions. Although zebrafish have gained wide popularity in biomedical research, drug discovery, and chemical toxicity studies, the blood is the least-explored tissue of this species due to the tedious, difficult methods for obtaining blood samples. Few sites, such as lateral incision, decapitation, tail ablation, and cardiac puncture, have been adopted by the researchers for obtaining blood samples. In all these techniques, researchers have used different numbers of animals and obtained varying volumes of blood (Babaei et al., 2013; Pedroso et al., 2012; Jagadeeswaran & Sheehan, 1999; Eames et al., 2010). The scarcity of blood obtained by these methods also limits the analysis of many biochemical and cytological parameters (Babaei et al., 2013). We adopted a cardiac puncture approach for blood sampling in anesthetized adult zebrafish, as described by Moss et al. (2009). The blood sampling from adult male and female zebrafish was used for the glucose estimation and succeeded in collecting blood samples ranging from 2 to 10 µl per adult fish. We have observed that mortality can

be anticipated in this approach to heart-blood sampling in zebrafish. This method may not be a suitable approach for experiments requiring repeated blood sampling; however, it can be advocated for terminal bleeding.

Table 1: Glucose estimation from five different zebrafish lines (mg/dl)

Sr. No.	AB		Albino		Cld-GFP		Tubingen		DBS	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	33	68	36	32	41	27	29	36	60	122
2	42	39	33	45	38	39	33	70	46	155
3	24	53	33	61	28	159	44	47	68	48
4	28	46	27	55	38	40	51	45	104	54
5	31	34	32	32	31	20	45	43	24	36
Mean±SD	31.6±6.73	48±13.28	32.2±3.27	45±13.17	35.2±5.44	57±9.67	40.4±9.09	48.2±12.87	60.4±19.27	83±9.16

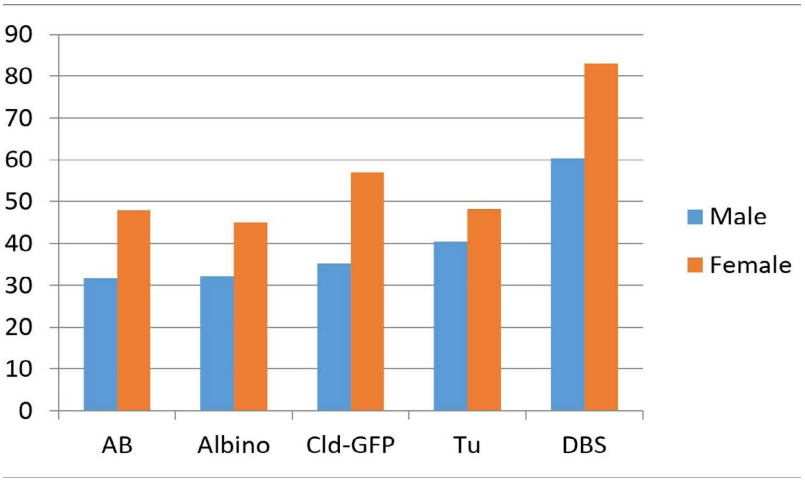

Fig. 5: Bar diagram showing blood glucose levels in male and female animals from five different zebrafish lines.

Fig 6

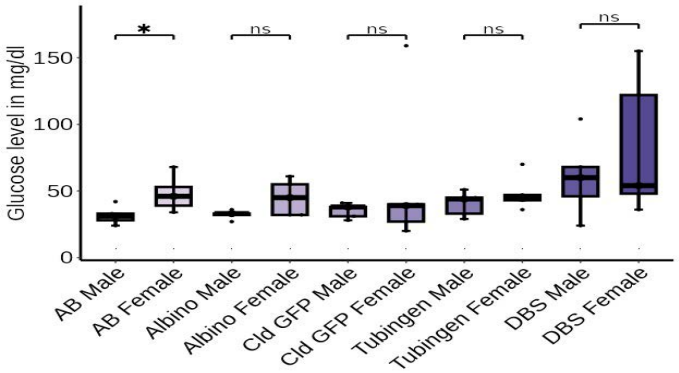

Fig 7

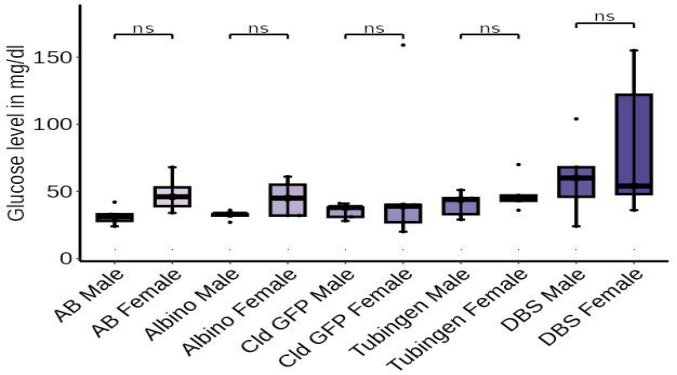

Fig. 6: Mean blood glucose in males and females of AB, Albino, Cld-GFP, Tubingen and DBS lines. *Pairwise comparison showed a significant difference ($p<0.05$) in blood glucose levels between males and females of the AB line (*).

Fig.7: Mean blood glucose in males and females of AB, Albino, Cld-GFP, Tubingen and DBS lines. *Group-wise comparison showed NO significant difference ($p<0.05$) in blood glucose levels between males and females of all the lines.

CONCLUSION

In the present study, a total of 50 adult fish from 5 different zebrafish lines were used for the blood samplings. The blood specimens were successfully collected from the palpating heart using pulled glass micro-capillary needles attached to the silicon tubing using 0.02% Tricain Methasulphonate anesthesia. The blood volume (2-10ul)

obtained from the adult zebrafish using cardiac puncture was enough to carry out the blood glucose estimation. The mean blood glucose level was found to be lower in male zebrafish when compared with the female zebrafish in all five lines. Pairwise comparisons showed a significant difference ($P<0.05$) in mean blood glucose levels between males and females in the AB lines; however, there was no significant difference ($P>0.05$) across all lines.

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