Zebrafish Embryos Immune System induction with BSA as an initial Screening model for Covid-19 Treatment

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Abstract. The early phase of embryonic development involves a lot of signaling and growth factor proteins that will influence the interactions among cells. Administration of BSA (Bovine Serum Albumin) at the beginning of embryo development can interfere with organogenesis and causing abnormalities in embryo development. Administrations of BSA were expected to induce responses of the immune system, which be hoped mimicking the stress on immune system caused by infection such as of covid-19. This study aimed to screen the right BSA dose to influence the zebrafish immune system as model animal further study for covid-19 treatment. This research was conducted in the Laboratory of Animal Histology and Embryology, Faculty of Biology, Universitas Gadjah Mada. Eggs were immersed in 1%, 2.5% and 5% of BSA for immersed in 1 hours and 5 hours repeated 3 times. The Parameter of the study were embryo survival ability rate, morphology, and physiological performance of contraction muscle per minutes and also heart rate per minutes. The data obtained were tested by ANOVA and DMRT test with a confidence level of 95%. The result showed BSA (Bovine Serum Albumin) 1% and 5% has significant effect on normal development and organ function of zebrafish embryos, such as muscle contraction and heart rate of the embryos, as conclusion 1% BSA dose is the most effective responses to induce immune system as model animal further study for covid-19 treatment.

1. Introduction

Vaccines are antigens that play a role in the body's immune system [1]. The human immune system consists of the innate and adaptive immune systems [2]. The adaptive immune system is an immune system that can be induced [3], so that at the final stage it can form antibodies and memory cells [4]. Memory cells stimulate the formation of antibodies that are faster than normal before vaccine induction [5]. Vaccine induction can use several types of antigens, one of which is protein [6]. BSA protein or bovine serum albumin is a protein that can function as a stressor that is directly related to antibodies and memory cells [7].

The early phase of embryonic development involves a lot of signaling and growth factor proteins that will influence the interactions among cells [8]. Administration of BSA protein or bovine serum albumin at the beginning of embryo development can interfere with the formation of three germ layers [9], which can be causing abnormalities in embryo development. Administrations of certain doses at the beginning of zebrafish embryo development was expected to affect the formation of the immune system,
then treatment with ginger extract and its effect on the ACE-2 population as a Covid-19 receptor in future studies. Administrations of BSA were expected to induce responses of the immune system, which be hoped mimicking the stress on immune system caused by infection such as of covid-19. This study is a screening study to prepare the right BSA dose to influence the zebrafish immune system in the context of Covid-19 treatment.

Zebrafish (*Danio rerio*) is appropriate for use in embryo development research because zebrafish embryos (*Danio rerio*) get their oxygen supply through passive and transparent diffusion [10], so that the entire embryonic development process can be observed. Zebrafish also has various genes found in humans, namely At1, At2 and renin, angiotensinogen, ACE, and ACE2 receptors [11]. Zebrafish also has various genes found in humans, one of which is responsible for encoding angiotensin-converting enzyme 2 receptor (ACE2 receptor). The spike (S) protein of SARS-CoV-2 recognizes and binds to the host ACE2 receptor. Immune system response is important defend system, which react directly to the infectious agent such as virus attack to human body. In this study we use the protein of bovine serum albumin (BSA) to induce immune system respond mimicking virus protein for the study in search of Covid-19 treatment agent.

2. Materials and Methods

2.1 Fish setting and egg collection

This research was conducted in the Laboratory of Animal structure and Development, Faculty of Biology, UGM. Wild type Zebrafish (*Danio rerio*) was obtained from Leiden University and cultivated at the Faculty of Biology, UGM. Three female and six male wild type AB/TL zebrafish (*Danio rerio*) were maintained in a well-aerated aquarium for 2-week before matting. Fertilized fish eggs were collected and inspected under a microscope to select and ensure only normal and good quality zebrafish embryos, which reach 50% epiboly, were used in the experiment.

2.2 Preparation of BSA

Three concentrations of BSA were freshly prepared and used in this study, which were as follows 1%, 2.5% BSA, and 5% BSA on egg water respectively.

2.3 Experiment for BSA treatment

Two sets of experiments were implemented in these trials, as follows: 1). dechorionated and non-dechorionated egg. Eggs were immersed in 1%, 2.5%, and 5% of BSA for the period of 1 and 5 hours with 3 replication each. After BSA treatment, eggs were carefully rinsed with egg water and transferred into a new well plate filled with egg water as the medium. The collected eggs were immersed in 5 ml of egg water medium containing several concentrations of each sample BSA (1 – 5 %). The well plates were covered to avoid evaporation of test solution and incubated at room temperature.

2.4 data collection Preparation of Zebrafish Eggs and Screening

The treated and control eggs were then observed with a Leica Microscope for their morphological appearances, somite formation, muscle contraction, blood flow, and heartbeat frequency. Observations were conducted until the embryos hatched. The data obtained were tested by ANOVA and DMRT with a 95% confidence level.

3. Results and Discussion

BSA (*Bovine Serum Albumin*) is a biologically stable globular protein that is used in a variety of biochemical applications [12]. BSA is a plasma protein of cattle having 76% similarity with HAS (*Human Serum Albumin*) and composed of a single polypeptide chain consisting of more than 500 amino
acid residues [13]. BSA affects the sarcoplasmic reticulum channel importantly on lipid bilayer which affects muscle contraction [14].

3.1 BSA effect on early-stage embryo
The early stage of embryo exposure to BSA didn’t suddenly affect the embryo at the first 24 hours of embryo age. Morphological changes weren’t observed on the embryo exposed to various BSA concentration of 1%, 2.5% and 5%, respectively (Fig. 1).

![Figure 1. Early stage of embryo expose to different concentrations of BSA on 24 hpf (hour post fertilization) (A: BSA 1% 5h; B: BSA 2.5% 5h; C: 5% 5h; D: Control): Yo (Yolk); Ch (Chorion); Eb (embryo).](image)

We also conducted the exposure of embryo with and without de-chorionation of the embryo. Dechorionated embryo showed a higher influence of BSA to the embryo, even on lower concentrations compared to non-dechorionated counterparts especially after a long time exposure period (Fig 1, and Fig. 2).

3.2 BSA effect on embryo survivability
Embryo exposure to BSA affects embryo survivability, especially after a 24-hour post-exposure period, in a dosage-dependent manner. Embryo survivability was decreased drastically from the concentration of 1% to 5%. The lowest survivability of embryo on BSA 5% 5 hours in 48 hpf (Fig.2).
The result suggests that the 5 hours of BSA exposure caused a permanent change in embryonic development since it was exposed on the period of the early time of gastrulation stage, which possibly affected the critical period of cellular differentiation and organ formation of the embryo, caused defect on organ structure and function, leading to fatality.

3.3 BSA effect on embryonic muscle contraction
The BSA exposure to the embryos were caused an adverse effect on the muscular contraction of the embryo, especially at the first 18 to 24 hours of embryo age. At this initial age of embryo development, BSA didn’t cause remarkable morphological changes in all of the treatments. However, muscular contraction frequency was reduced on the period of 36 to 48 hpf especially on the embryo exposed to BSA concentration of 2.5% and 5%, respectively (Fig.3 and Fig. 4).
Interestingly, 1-hour exposure to BSA on the concentration of 1% and 2.5% reduced muscle contraction of zebrafish embryos, which didn’t show by the exposure of BSA on the same concentration at 5 hours treatment period. Early development of the zebrafish embryo relies on communication between cells [15]. This communication cell to cell communication mode were mainly conducted by chemical signal [16], such as protein. BSA is bovine serum albumin protein was made from bovine cow’s embryo that rich in growth factor [17], BSA treatment in the early development stage will affect cell to cell communication easily and cause certain developmental defects [18].

### Table 1. The Effect of BSA Concentration on Zebrafish Embryo Muscle Contraction

| Treatment          | Average |
|--------------------|---------|
| BSA 0% 18 hpf      | 17\(^a\) |
| BSA 1% 5h 18 hpf   | 16\(^a\) |
| BSA 2.5% 5h 18 hpf | 12\(^a\) |
| BSA 5% 5h 18 hpf   | 7\(^b\)  |

The Zebrafish embryos treated with 5% BSA for 5 hours period, showed a significant reduction of muscle contraction compared to other treatment, based on ANOVA with 95% (0.05) confidence level and DMRT analysis (Table 1). BSA has the ability for ligand binding also as various transporter [19]. BSA's ability to ligand impact on non-specific binding protein enhancement [20]. BSA effect on protein channel opening, it causes enhancement \(Ca^{2+}\) concentration in the cell. The increase of \(Ca^{2+}\) concentration cause muscle contraction enhancement [13]
3.4 BSA effect on Heart rate
Embryo exposure to BSA suddenly affects the embryo heart rate, especially at the concentration of 2.5% and 5%, which started at the first 24 hours of embryo age. (Fig.5).

The result showed that the highest heart rate of zebrafish embryos was on 18 hpf (hour post-fertilization) BSA 5% in 5 hours immersion. The lowest heart rate of zebrafish embryos was 48 hpf BSA 5% in 5 hours of immersion. The data showed that embryos of zebrafish on BSA 2.5% and 5% 5 hours immersion have died. The BSA exposure at several concentrations could affect and inhibit the early phase of embryonic development.

| Treatment          | Average |
|--------------------|---------|
| BSA 0% 18 hpf      | 48a     |
| BSA 1% 5h 18 hpf   | 50a     |
| BSA 2.5% 5h 18 hpf | 42a     |
| BSA 5% 5h 18 hpf   | 38b     |

Result showed that exposure with 5% BSA caused a significant different of heart rate level compared to other treatment based on ANOVA with 95% (0.05) confidence level and DMRT. The 18 hpf embryo that has been treated with BSA 5% for 5 hours showed the lowest heart rate compared to other treatments. The result also showed that maximum BSA concentration tolerance of zebrafish larvae was 1.5% [21]. BSA induced the secretion of interferon-y which activated heart rate. the BSA causes a difference in osmotic pressure [22], which caused defect on embryo’s development at several stages of embryo. In this research, we also conducted the BSA exposure on the dechorionated zebrafish embryo. The results of the study on decorated eggs and normal showed in Figure 6.
Figure 6. Zebrafish embryos exposed in different concentrations on 48 hpf (hour post fertilization) of BSA (A: BSA 1% 5h; B: BSA 2.5% 5h; C: 5% 5h; D:Control)

Figure 7. The Effect of BSA Concentration on the Zebrafish Embryo (A: Early stage of the dechorionated egg expose of BSA; B: Dechorionated egg 24 hpf expose of BSA; C: Dechorionated egg expose of BSA; D: Early stage of egg without dechorionated; E: Egg 24 hpf expose of BSA without dechorionated; F: Egg 24 hpf expose of BSA without dechorionated; G: Dechorionated egg embryos on 48 hpf exposed of BSA; H: Zebrafish embryos on 48 hpf exposed of BSA)
Figure 7 showed the chorion function on the Zebrafish embryo as an effect of BSA. The dechorionated Zebrafish embryos that have been immersed in BSA show a lethal effect which resulted in embryonic death after the somite formation phase, while the non-dechorionated Zebrafish embryo can survive until hatching. This indicates that the chorion has an important protective function to filter out non-permeable substances for the embryos.

The result suggest that the BSA 2.5% 5h and BSA 5% 5h exposure caused permanent change on embryonic development, since it was exposed on the period of early time of gastrulation stage, which possibly affected the critical period of cellular differentiation and organ formation of the embryo, caused defect on organ structure and function, leading to fatality. We address to induce immune response of the zebrafish without lethal effect, therefore the 1% BSA showed sign induction of immune system without caused lethal effect to the fish.

4. Conclusion
BSA (Bovine Serum Albumin) could induce physiological stress on zebrafish embryo, which could be assumed as stress induce by virus protein, mimicking stress caused by virus, it is potential setup for further research on covid treatment. 1% BSA dose was chosen as immune system inducers because it has higher survivability rate, muscle contraction and heart rate.

Acknowledgment
Authors acknowledge for all people involved in this research, Universitas Gadjah Mada and financial support provided by RISTEKDIKTI.

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