FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 7 No. 2, April, 2023, pp 235 - 239
DOI: https://doi.org/10.33003/fjs-2023-0702-1688

CRYOPRESERVATION OF PRIMARY SPLENOCYTES FROM ANTIBODY PRODUCED MICE AGAINST *Bitis arientans* SNAKE VENOM

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ABSTRACT

Snakebite is a neglected public health problem in Africa. In Nigeria, *Bitis Arientans* (Puff Adder) are regarded as the most venomous snake species. The most effective treatments against snakebite is antivenom. This antibody can be obtain by isolating antibody-secreting lymphocytes. These lymphocytes are found within the Splenocytes cells which can be used for hybridoma cells generation. Although development of hybridoma cell secreting monoclonal antibody is rare and tedious process. But valuable time and cell materials can be saved through cryopreservation. This research aimed at Cryopreservation of primary splenocytes from Antibody produced mice against *Bitis arientans* snake venom. The LD_{50} was determined according to World Health Organization guidelines. Antibody production was achieved after mouse immunization with adjuvant and phosphate buffer saline for six weeks and antibody titer were determined by indirect ELISA method. Myeloma cells were cultured and Mouse Splenocytes with the highest immune response were removed aseptically by mechanical method and cells viability was determined then the isolated splenocytes and myeloma cells were cryopreserved in cryovial tube stored at -80^{o}C. The LD_{50} was found to be 1.8 mg/kg and Elisa analysis shown mice 2 and 6 to elicited highest immune response with IgG Concentration 3.1 µg/ml and 4.6 µg/ml. isolated splenocytes were counted to be 6.5x10 and 1.2x10 cells for mice 2 and 6 and myeloma cells were $1.95x10^{5}$ cells. In this study, antibody against *Bitis Arientans* venom were produced and splenocytes and myeloma cells were cryopreserved. Thus, cryopreserved cells can be used as model for toxicological and immunological studies.

Keywords: Immunization, Antibody, *Bitis Arientans*, Myeloma Cells, splenocytes, cryopreservation

INTRODUCTION

Snakebite is a serious major neglected public health problem worldwide, especially in tropical and subtropical countries in Africa (Chippaux, *et al.*, 2017*)*. A recent global statistic estimates there were at least 1.8 million snake envenomations cause 81,000 deaths annually throughout the world (Gutiérrez *et al.*, 2017), with most bites occurring in regional and rural centres. In Nigeria, most especially northern region part *Bitis arietans* (Puff adder) causes number of morbidity and mortality are regarded as the most dangerous venomous snake species (Habib, 2013*)*.

Bitis Arietans envenomation is characterized by local reactions, including pain, blistering, edema, neutrophilia and tissue damage, aggravated by the systemic reactions such as fever, hemostatic and cardiovascular disturbances (Nghorn *et al.*, 2014). As a result, many victims can die or be left with permanent disabilities. The only specific treatment for snakebites is antivenom therapy, this antivenoms are scarce and expensive in under-developed countries (colo *et al.*, 2002).

Antivenoms both polyvalent and a few monovalent have been marketed for the treatment of envenomings caused by African snake species and several diagnosis strategies were developed (Habib and Brown, 2018). The beneficial effects of polyclonal antibodies come from its polyclonal nature and biophysical diversity. The polyclonality nature allows targeting multiple sites in a single window of the application and biophysical diversity provides greater stability in environmental changes (Ascoli and Aggeler, 2018). Polyclonal antibodies that is specific for a single epitope of an antigen are obtain by isolating antibody-secreting lymphocytes and fused them with a cancer cell line known as myeloma cells (Tabll *et al*., 2015). The fused cells are called hybridomas, and will continually grow and secrete antibody in culture. These Lymphocytes are found within the Splenocytes cells which can be used for a wide variety of immunology-based applications such as hyridoma technology (Wang *et al*., 2019). In addition, mouse splenocytes are also used as characterization of cell populations and generation of genetically modified cells through retroviral infection. Moreover, several research were carried out on mouse splenocytes as models for toxicological studies and hybridoma cells generation.

However, generation of hybridoma cell secreting monoclonal antibody is rare and tedious process especially when the experimental animal e.g mice are immunized with the antigens having low molecular weight. It is not also generally preferred to utilize all immunized mice at the same time because of its difficulty in screening procedures. Therefore, valuable time and cell materials can be saved and cells fusion can be delayed properly till time of usage through cryopreservation (Ibrahim *et al*., 2011). Therefore, this research work aimed at a cryopreservation of primary splenocytes from antibody produced mice against *Bitis arientans* snake venom.

MATERIAL AND METHOD

Ethical Approval

This study was approved by the Ethical Committee of the Department of Biological Sciences, Bayero University Kano, Nigeria. All methods were carried out in accordance with the approved guidelines and regulations.

Experimental Animal

Six to 12 weeks old Balb/c mice were bought from the animal house of pharmacology department, Aminu Kano Teaching Hospital, kept inside the cage and were allowed to acclimatize for two weeks before the commencement of the research.

Venom Collection

Lyophilized *Bitis arietans* venom of 0.5 g was obtained from the Department of Pharmacology and Drug Development, Ahmadu Bello University, Zaria, Nigeria

Venom Toxicity Determination (LD_{50})

The LD_{50} of *Bitis Arientans* venom toxicity was determined in mice according to WHO guidelines 2010. The venom was reconstituted in normal saline and concentrations of 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml was made. Thirty mice were randomly allocated into 5 groups of 6 mice each (n = 6). Groups I served as the control group where mice were injected with normal saline (0.2 ml each i.v). Mice in groups II, III, IV and V were given the reconstituted venom at 0.2, 0.4, 0.8, 1.6 and 3.2 mg/kg, respectively through the intravenous route. The time of death was recorded over a period of 24 hours from venom administration. The animals were observed and compared with control for toxic symptoms such as weakness, loss of appetite, difficulty in movement, nose bleeding, mouse bleeding, and mortality for the first 2 h and 24 h post-envenoming.

Antibody Production

Animal Immunization

Six mice were immunized 4-5 weeks to enable a robust immune response to develop against the antigen of interest before proceeding to sacrifice. Serum sample was collected prior to immunization which was used as a baseline control for antibody screening and Stored at -20°C. After collection of the pre immune Serum, 10 µg of *Bitis Arientans* venom was stably emulsified with freunds complete adjuvant (FCA) was injected to six mice intraperitoneally (IP). AT Fourteen days interval after the primary immunization, Mice were injected with 10 µg of *Bitis Arientans* snake venom emulsified with freunds incomplete adjuvant (IFA). A Test bleed was collected from each mouse 10-14 days after the secondary immunization and serum from the sample was prepared and assessed to determine the antibody titre by ELIZA which was compared with the pre immune animal sample. Mouse with the highest antibody titre value was selected for further boosting with the antigen snake venom until a good titre value was obtained. Four days before sacrifice a final boost of the selected mice was performed intraperitoneally with 10 µg of *Bitis Arientans* snake venom in phosphate buffered saline (PBS) without advujant in a maximum volume of 200 µL.

Antibody Titre Estimation

All reagents and standards were prepared under standard conditions. Plate was washed 3 times prior to assay. Venom for the ELISA was then diluted to 1mg/ml solution (PBS). 50 µl of the antigen solution was transferred into each EISA well of 96 plate and incubated overnight at 4^0C.Plate was washed 3times with wash buffer. 100 µl of standard/samples was added to each well, sealed and incubated for 120 min at room temperature. Sample was aspirated and plate was washed 4times with wash buffer. 100 µl of working solution of HRP conjugate anti Mouse IgG to each well, sealed and incubated at room temperature for 45 minutes.it was aspirated and plate was washed 5times.100 µl of substrate solution was added to each well and incubated for 15 mins at room temperature. Protect from light**.** 50 µl of stop solution was added each well and plate was read at 430 nm within 5 mins.

Spleen Harvesting

The immunized mouse with the highest antibody titer were Sacrificed by CO2 inhalation and the spleen was excised. The cells was placed in 15ml tube for crushing after washing with PBS two times. A spleen cell suspension was prepared by pressing the spleen through a cell strainer (REF No:- 431751). Fill up to 15 ml with serum-free RPMI 1640. Centrifuge the spleen cell suspension at 167 centrifugal force for 10 mint. Resuspend the pellet in serum-free RPMI 1640. Discard the supernatant. Cell concentration was determining using Neubuer counting chamber. Where 8 µl of cell was added with 2 µl dye put in a tube mix up then place on counter for view on the microscope.

Cryoconservation of Splenocytes

Harvested B cells were prepared for cryopreservation for long time storage. Cells were washed by adding 10 ml PBS in 50 ml sterile tubes and were centrifuge at 167 rcf for 5mins. Above step was repeated twice for a total of three washes. After the final wash, cells were transferred into a sterile cryotube and suspended with 800 µL RPMI 1640, 100 µL FBS and 100 µl of DMSO and then stored at -80 degrees for storage.

Preparation of Myeoloma Cell Culture

The myeloma cell line using is X63 Ag8.653 was -80^0C store at**.** All work was performed under sterile conditions. A fresh culture medium will be prepared for cell cultivation. 500 ml of RPMI 1640 with 10% fetal calf serum (50 ml), and pen-strep are to be supplemented. A vial of murine cells was thawed in warm water in which the cell solution was transferred into 10 ml tube and 10 ml of RPMI 1640 culture medium was added and centrifuge for 5 mins at 167 centrifugal force so as to remove DMSO and separate cells. Discard the supernatant and resuspend the pellet in 10ml of fresh culture medium. The cells was transferred in a 25 cm3 flask and incubated at 37 degree and 5% Co2 in an incubator. The cells were expanded to two 75 cm flasks before fusion. But before storage the number of cells was determined by direct counting using Neubeur counting chamber.

RESULTS

Table 1: Median Lethal Dose (LD_{50}) of *Bitis Arientas* Venom

Mice	Dose (mg/kg)	Log Conc	Mortality	(%) Mortality	Probit-Value
1	0.2	0.30103	0/6	0	3.25
2	0.4	0	0/6	0	3.25
3	0.8	0.30103	5/6	83	5.95
4	1.6	0.6206	6/6	100	6.75
5	3.2	0.90309	6/6	100	6.75

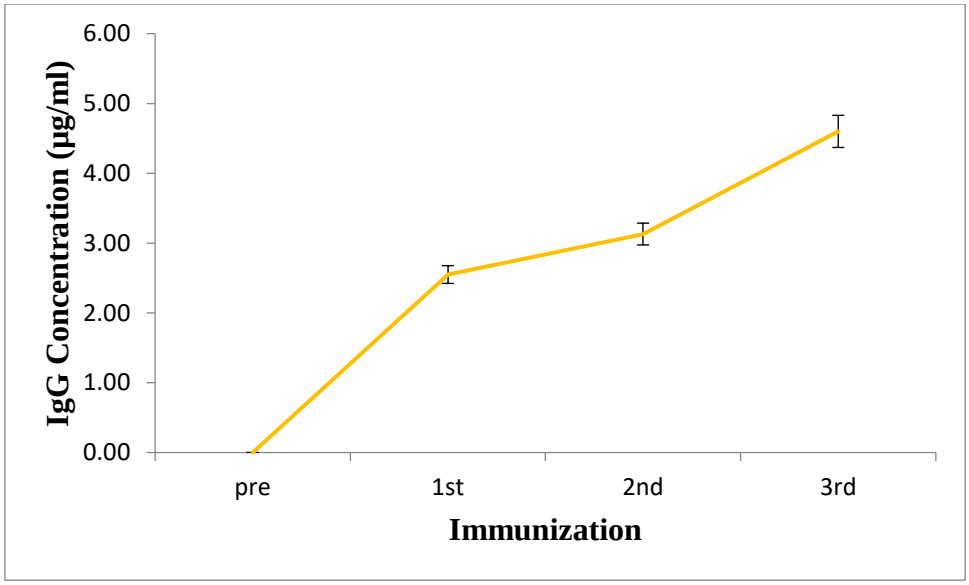

Figure 1: Different level of Antibody concentration produced against *Bitis Arientans* at different stage of immunization. Values are expressed as mean and standard deviation

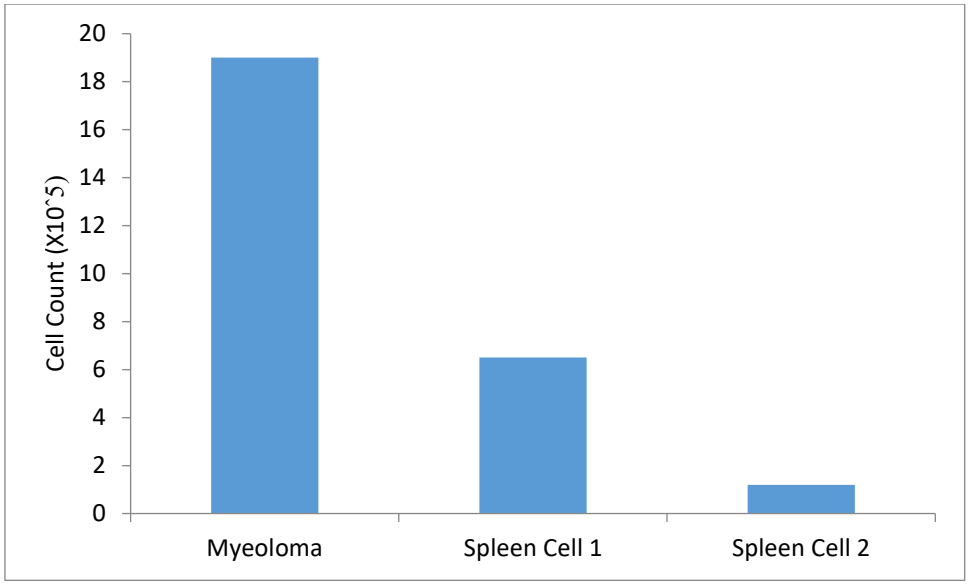

Figure 2: Cell concentrations of myeloma and spleen cell count stained with trypan blue dye (Mg×100).

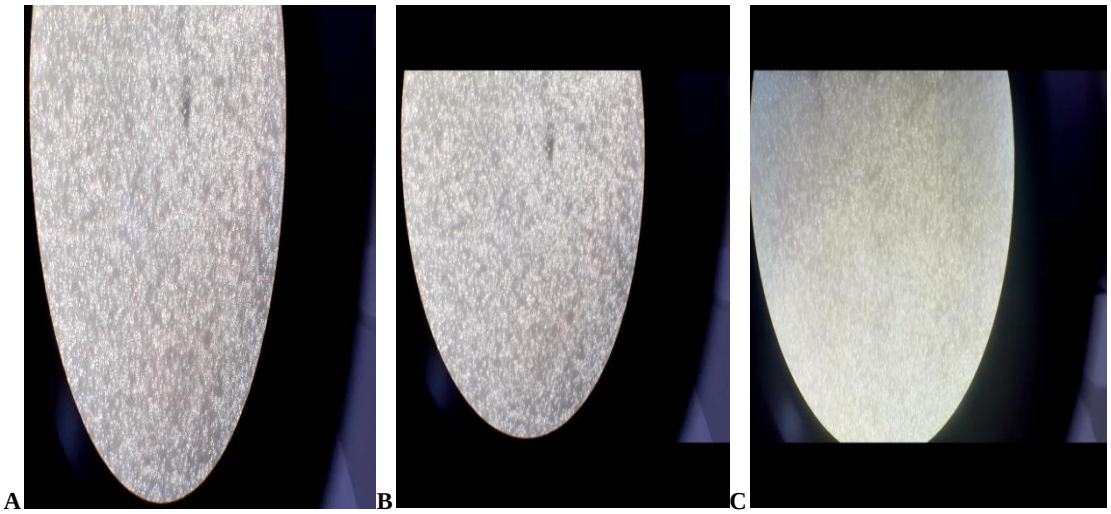

Figure 3: Show myeloma cell viability from day 1 to day 3 and it was examined using microscope. A) Day 1 cells confluency B) Day 2 cells confluency C) Day 3 cells confluency

DISCUSSION

Bitis arientans is responsible for more snakebite fatalities than any other African snakes, due their it wide distribution, common occurrence, large size, potent venom that is produced in large amounts, long fangs, and their habit of basking by footpaths and sitting quietly when approached (Spawls *et al*., 2004). LD_{50} of *Bitis arietans* was calculated because the lethality of the same snake venom varies from place to place due to factors such as dietary, geographical, ontogenetic and gender associated changes (Daltry *et al*., 1996a). The LD_{50} was found to be 1.8 mg/kg body weight Table 1. From the result, 100% survival was observed from the mice immunized with the dose of 0.2 and 0.4 mg/kg showing less toxicity but at the dose of 0.8 mg/kg, 80% mortality was recorded. However, all the experimental animal died within 48 hours of immunization at the higher doses of 1.6 and 3.2 mg/kg. This LD_{50} correspond to the LD_{50} value range published by Mallow *et al*., 2003 administered through intra peritoneal route (0.9 – 3.7 mg/kg). This shows that the *Bitis arietans* venom is very potent and has not been tempered during the course of the research and is toxic to the experimental animal. However, mortality was observed from the *B. arietans* envenomed mice with toxic symptoms such as weakness, loss of appetite, difficulty in movement, nose bleeding.

In this study, animal were immunized three stages and serum from immunized mouse was collected 2 weeks after each stage of immunization to analyze the presence of polyclonal antibodies using Elisa kit. Antibody titer increased significantly across the stages of immunization. However, the immunized mice were bleed and ELISA analysis conducted on the blood sample taken from the six mice shown the presence of antibody titer but mice 2 and 6 elicited the highest immune response titer concentration 3.1ug/ml and 4.6ug/ml Figure1. Therefore, the spleen cells of the two mice that elicited highest immune response were excised for further study.

Safety and accurate detection in venom immunotherapy have also been improved by application of enzyme-linked immunosorbent assay (ELISA) to snake venom research and diagnosis. Theakston *et al* first described the used of this assay in detection of antigen and antibody in snake Bitten patients. Subsequently, ELISAs have been used for detection of venom antigens from different species of venomous animals. (Barral-Netto *et al*., 1990).

The spleen is the largest lymphoid organ of the body and has been as models toxicological and immunological functions. The spleen also provides a critical function for the immune system by mounting a primary immune response to antigens in the blood and synthesizing antibodies (Wang *et al.*, 2019). We isolated and cryopreserved splenocytes as a primary cell culture and cultured myeloma cells. Figure 2 a showed Cell concentrations of myeloma and spleen cell count stained with trypan blue dye used to determined cell viability which can be used in the hybridoma process and it was found the cultured myeloma cell to be $1.95x10^5$ cells and spleen cells 1 and 2 were found to be $6.5x10^5$ and $1.2x10^5$ cell respectively. With these data obtained revealed cells continue to grow in full cell culture media and myeloma cell reached more than 50% confluency within three days figure 3. Previous research reported, Spleen cells were confluent after 72 hours and their viability was measured by trypan blue assay (Faw zi *et al*., 2020). However, the use of viability days for estimation of cell viability has been commonly relied upon in experimental procedure using cell culture *in vitro* (Hutz *et al.*, 1985).

Although Cryopreservation of cells is a well-known technique which has wide application in the field of medicine and biology. In cryobiology, the cryopreservation of an entire tissue and organs is still challenging (Keros *et al.*, 2005). Cryopreservation is an efficient and effective way to transport biological samples over long distances, properly delayed cells fusion, store samples for prolonged periods of time, and create a bank of samples for users (Cavender *et al*., 2005).

Dimethylsulfoxide (DMSO) is commonly used for cell and tissue cryopreservation and is also used in this study as a cryoprotectant because of its low molecular weight and penetration ability (Pegg, 2006). In the present study, we report that splenocytes of mice were cryopreserved as a whole tissue in cryovial tube then stored at -80 degrees for storage. Thus the cryopreserved spleen and myeloma cells can be used for the successful generation of antibody producing hybridoma cells and can also be used as model for pharmaceutical research.

CONCLUSION

In this study, antibodies against *Bitis Arientans* were produced and the lethal toxicity of *Bitis Arientans* was found to be 1.8 mg/kg. Thus, the LD_{50} value obtained from this study can effectively utilized to determine neutralization capacity of produced antivenom serum before release for consumption. However successful isolation and cryopreservation of the spleen and myeloma cells can be used for the successful generation of monoclonal antibody.

RECOMMENDATION

i. There is need to produce monoclonal antibodies against *Bitis arientans* due their specificity against antigens.
ii. However further immunological and pharmacological investigation should be carry on the cryopreserved primary splenocytes.
iii. Future research must aimed to generate humanized monoclonal antibodies

ACKNOWLEDGMENTS

We wish to thank Auwal Adam Bala (Department of Pharmacology, College of medicine and Health Sciences, Federal University Dutse, Nigeria), Abduljabbar Imam, Aliyu Ahmad and Abdullahi Tela (Department of Pharmacology, Bayero University Kano) For their technical assistance. This work was partially funded by Centre for Biotechnology Research Bayero University kano State Nigeria.

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