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Molecular Validation of Anna Bedhi Chenduram Effect on Hematopoiesis Using CD 34 Positive Hematopoietic Cell Expression

Gowri MA[1]*, Nandhini S[2], Lavanya A[3] and Baranidharan GR[4]

[1]Professor and Head, Madras Veterinary College, India

[2]Department of Animal Biotechnology, Madras Veterinary College, India

[3]Research Associate, Siddha Central Research institute, India

[4]Department of Clinics, Madras Veterinary College, India

***Corresponding author:** Gowri MA, Professor and Head, Centralised Instrumentation Laboratory, Madras Veterinary College, Chennai -7, India, Tel: 9444804033, Email: gowrivalavan1@gmail.com

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Abstract

The drug Anna bedhi Chenduram (ABC) is commonly used for prescribing in anaemia and its activity is unexplored on target cells10. The scientific validation of the drug would be ideal to exploit its potential in haematological disorders. The metronomic model of conventional chemotherapy suggests that advantages accrue to the administration of combinations of alternative drugs that interact with the multistep process of hematopoiesis and angiogenesis. The government of Tamil Nadu to address the issue and targeting the vulnerable population is recommending Anna bedi Chenduram during the second trimester of pregnancy. In the present study the objectives are designed to address the effect of drug through molecular multistep process of hematopoiesis in our standardized testing platforms of bone marrow stem cell culture using quantitative real time expression of specific self renewal genes.

Keywords: Effect of Anna Bedi Chenduram; Bone Marrow Stem Cells; Culture; Analysis

Abbreviations: ABC: Anna Bedhi Chenduram; FDA: Food and Drug Administration; CFC: Colony Forming Cell; BM: Bone Marrow

Introduction

Anemia affects nearly one third of the world's population and is associated with an increased rate of mortality and decreased physical functioning and quality of life. Anaemia is the most common nutritional problem in the world and mainly affects women of child-bearing age (especially during pregnancy and lactation) and young children. Globally 30% of the total world population is anaemic and half of them are suffering from Iron Deficiency Anaemia. Anaemia in pregnancy is present in very high percentage in India. The Food and Drug Administration (FDA) has issued a warning for increased risk of a thrombotic event in patients with CKD or cancer whose Hb is greater than 12 g/dL (2007). In 2011, the FDA published an additional safety alert warning on all ESAs of increased risk of death, cardiovascular

events, and stroke in CKD patients with Hb levels > 11 g/ dL. Owing to the gravity of the situation, a need for safe / effective oral dosage forms to improve the haemoglobin level in iron deficiency anaemia was felt. To address the public health needs scientific studies were initiated on Anna bedhi Chendooram, promising formulations, and is being successfully prescribed by Ayurvedic and Siddha physicians without any side effects since centuries. The formulations have been standardized after formulating SOPs besides safety / toxicity evaluation. The formulations are found safe and biological activity studies revealed significant haematenic effect. The multicentric clinical trials are in progress for which a validation is mandatory. The objective of the current study is to assess the efficacy through measurable objective parameters using molecular techniques for the activity of Anna bedhi chenduram (ABC) on hematopoiesis on invitro hematopoietic and mesenchyaml cell coculture.

Materials and Methods

Ethical Committee Permission

Dogs of both sexes were used in these studies. All dogs were vaccinated for common infectious disease and were in apparent normal health. Blood sample were collected in EDTA vacutainer tubes. The protocols were carried out as per the approval of the TANUAVS Institutional ethical committee for stem cell research and therapy N0.05/ICSCRT/2017 MVC, Chennai-7. The bone marrow samples collected from clinics from orthopaedic surgery were used for mesenchymal stem cell derivation as per our standardized and validated protocols.

Isolation, Enrichment of Cd34+ Progenitor Cells and Culture with Mesenchymal Cells

Bone marrow collection and processing from apparently healthy dogs from animal blood bank, Madras Veterinary College were collected and the isolation and enrichment of cd34+ progenitorcells were carried out [1]. The cells were subjected for culture and processed for assessment in annabedi chenduram (ABC) supplementation. The drug and control drug were obtained from The Indian Medical Practitioners Co-operative Pharmacy and Stores Limited Sales Depot (impcops). Elemental iron supplement 100 mg is used as control [2]. The tablets were dissolved in culture medium. Isolated enriched cells from CD 34 positive selective isolation cocktail (Stem cell technologies) were cultured in T25 culture flask at cell concentration of 1x10 [3] cells / ml in maintenance medium. The culture flasks were allowed to incubate at 37°C at 5% CO2 level without any disturbance for 1-2 days. Then the suspension cells were transferred in to 6 well plates with fresh growth medium containing a monolayer of bone marrow mesenchymal cells [4] to simulate bone marrow matrix in vitro. The cultures were allowed to grow for 7-14 days with change of medium at two days intervals. The confluent monolayer cells were subcultured, and third passage cells were used for proliferation analysis for a period of 24, 48 72 and 96 hrs with different concentrations such as0.5μM, 1.0μM, 1.5μM, and 2.0μM of ABC were used to test the system along with the control, and specifically, the number of viable cells was taken into consideration.

Dye-Based Proliferation Assay

The cells were cultured in a 96-well plate at a density of 1x10 [3] cells / mL. 20μL of MTT (5 mg/ ml) solution was added to each well and incubated for four hours. To each well, 200 μL of DMSO working solution was added, and the OD of the reaction product was evaluated in an ELISA reader at a 570-nm wavelength. At least three independent experiments from different sample were performed to examine proliferation [5].

Gene Expression Analysis by qRT-PCR

Quantitative real time PCR was performed using the SYBER Green (TaKaRa) reagent on a Biorad CFX connects Real Time PCR system4. Data were analysed using Biorad CFX Manager Software Version 2.1. Gene expression was normalized according to the expression of the housekeeping gene β-actin. Hox b3 was targeted to analyse the expression in isolated and cultured and cultured progenitor cells in drug supplementation. Melting curve was determined and gene amplification was done in triplicate within a single instrument run. Non template control was run for each template and primer pair to avoid false positives. Fold change was calculated relative to the control using the relative quantification for the experimental genes and Beta actin (internal control). The mRNA abundance was determined by analyzing the resultant Cq values for each sample (gene of interest), normalized to the level of Beta actin mRNA abundance for the same RNA sample. Relative expression of HoxB3 and HoxB4 mRNA was determined using the (2-ΔΔCt) method.

Result and Discussion

The colony forming cell (CFC) assay is used to study the proliferation and differentiation pattern of hematopoietic progenitors by their ability to form colonies in a semisolid medium. The CFC assay has been used extensively to determine the proliferation and differentiation patterns of hematopoietic progenitors and to study the effects of oncogenes [3,5]. In these study Bone marrow stem cells, from the higher animal model dogs was isolated and expanded in vitro. To test the effect of ABC on hematopoiesis, in vitro stem cell culture system from bone marrow with, CD 34+ HSC and MSC was used. Progenitor cells proliferation was checked on alternate days. There was also a significant difference in the proliferative ability between the cells grown at different time points. There was an increase in the number of viable cells with increasing concentration of ABC supplementation (Figure 1).

This signifies that the enhancement in cellular proliferation and its support in hematopoiesis. The confirmation on proliferation was checked with quantitative gene expression for self renewal genes in progenitor cell proliferation. HSC, the primitive HPC, is defined as cells that have the properties of pluripotent differentiation and permanent self-renewal [3]. Hematopoietic progenitor cells can be isolated based on the expression of cell surface markers such as CD34, CD38 [6]. Different mammalian CD34 cell subpopulations express at least 22 of the 39 Hox genes [7,8]. Hox genes are highly expressed in the most primitive HSCs and progenitors, while their expression is not present in differentiated bone marrow (BM) cells [9,10]. Real–time expression of HoxB3 and HoxB4 in canine hematopoietic progenitor cells was observed. The amplification of self renewal genes HoxB4 and HoxB3 showed that the amplification started after 25th cycle it has amplification up to 35 cycles. The optimum annealing temperature was found to be 55˚C which has been checked in gradient PCR with a range of 50˚C to 65˚C. The quantitative gene expression of self renewal genes as an indication of proliferation in ABC supplementation in hematopoietic progenitor cultured cells were shown as 2-ΔΔct value were HoxB3 0.19, HoxB4 0.28 respectively. The amplification was approximately 1.5 folds in HoxB3 1.2 folds in HoxB4 when compared with references β-actin gene (Table 1).

Gene Name	Primer Forward (F) and Reverse (R)
HoxB3	F: TCAGGGTAGAATCCAAGAAG
	R: CACAGGTGTGTTAATTTGGG
HoxB4	F: CCAGAAATTAATGGCTATGAG
	R: TGGTCGCTGGGTAGGTAATC
Beta actin	F: GACAGGATGCAGAAGGAG
	R: TGATCCACATCTGCTGGAG

Table 1: Primer sequences of self renewal genes in dog CD 34 + cells.

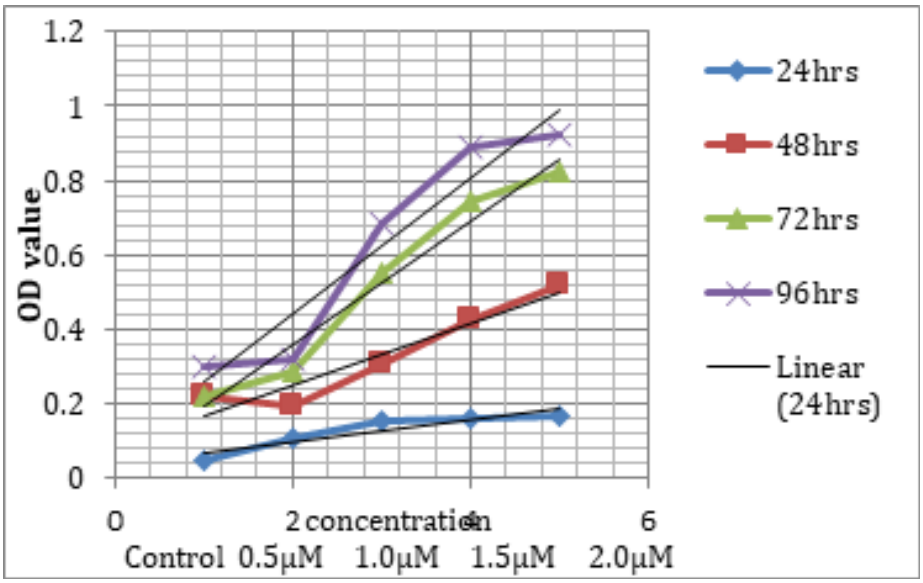

Figure 1: Proliferative Effect of Anna Bhedi Chenduram on Cocultured Cd 34 + Cells with Mesenchymal Stem Cells.

Conclusion

The scientific validation of ABC, siddha drug being used for anemia was scientifically validated on cultured cells using stem cell culture system. There was significant cellular proliferation in drug supplementation and the effect was shown relatively increased during 72 and 96 hrs. Real time-PCR analyzed expression of genes specific for self- renewal such as HoxB3 and HoxB4 showed amplification that was approximately 1.5 folds in HoxB3, 1.2 folds inHoxB4 in the CD 34 positive hematopoietic cells in ABC supplementation.

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