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Research Paper

Comparative Study of Sickle Cell Diseases in Different Age Groups of Far-Western Terai Region of Nepal

Nitesh Kumar Sah[1]*, Yubraj Bhatta[2]

[1]*Assistant Professor
Faculty of Agriculture, Far West University, Nepal

[2]Medical Lab Technologist
Province Public Health Laboratory Sudurpashchim Province, Nepal

*Corresponding author Email: niteshkumarsah5@gmail.com

ABSTRACT

Sickle cell disease (SCD) is a group of genetically inherited autosomal disorders that results from the production of abnormal hemoglobin called as hemoglobin S (HbS). It is a recessive condition in which an individual possesses two copies of the gene to acquire SCD. SCD is characterized by different episodes of pain, hemolytic anemia, chest pain, a bout of fever, and cough which is prevalent in Mediterranean, sub-Saharian African, Indian, Bangladeshi, and Nepali. In Nepal majority of SCD cases have been reported in Tharu people who contract malaria seven times more than other Nepalese people. The molecular basis for the synthesis of hemoglobin S (HbS) in SCD is a single base pair point mutation occurring in the beta gene of hemoglobin A where amino acid valine is substituted for glutamic acid at position 6 of beta-globin chain. The present study is done to screen and diagnose SCD in the different age groups of Nepali Tharu people who are living near the forest areas of far west Nepal. A free health camp was organized at a different parts of the far-western Terai of Nepal for this study. Though different diagnostic methods are applied to screen the cases of SCD, this study is carried out by hemoglobin solubility test and hemoglobin electrophoresis test. All the positive cases detected by the hemoglobin solubility test were further confirmed by the hemoglobin electrophoresis method. After the confirmation of the entire test, it was found that the majority of the female were affected with hemoglobin disorders as compared to males. This study provides an encyclopedic idea of the current understanding of the sickle cell diseases inhabited by people of far west Terai and how this knowledge is exploiting to advance current and potential management in these areas.

KEYWORDS: Sickle cell diseases, Thalassemia, Hb trait, Genetic disorder, Haemoglobinopathies, Haemolysis.

INTRODUCTION

Sickle cell diseases are a group of an autosomal genetically inherited disorder that results from the mutation of human hemoglobin[1,2]. Hemoglobin is a protein that involves in the transport of oxygen and carbon dioxide in vertebrates and gives a red color to the red blood cells. It supplies oxygen from the lungs to the peripheral tissue of the body and carbon dioxide from different parts of the body to the lungs for an efficient metabolic process[3]. Heme is the iron-containing part and globins are the proteins of the hemoglobin. Therefore, hemoglobin is an iron-protein complex that contains more than 65 percent of the total iron content of the body[4]. Different types of hemoglobin exist in the human RBCs are hemoglobin A (HbA), hemoglobin A2 (HbA2), hemoglobin E(HbE), hemoglobin F (HbF), hemoglobin S(HbS), hemoglobin C(HbC), hemoglobin H(HbH), and hemoglobin M (HbM). Among these HbA,

HbA2, and HbF is the most common type of hemoglobin present in human RBCs. Normally there are three types of hemoglobin:

1. Hemoglobin A1 (HbA1 or HbA) - Also known as hemoglobin subunit alpha 1, is a hetero tetramer consisting of two alpha and two beta chains of globulin proteins. HbA1 is abundantly present in adults (about 95% to 98%).
2. Hemoglobin A2 (Hb A2) - It contains two alpha (α) and two delta (δ) chains of globulin proteins. This type of hemoglobin can be found in very little concentration (about 2%-3%) in adult hemoglobin.
3. Hemoglobin F- the production of HbF primarily occurs in the fetus and hence it is called as fetal hemoglobin. After birth, the production of HbF gradually decreases and reaches at adult level (γ-globin to β-globin) during postnatal period. It constitutes up to 1- 2% of adult hemoglobin and contains two alpha (α) and two gamma (γ) protein chains.

HISTORY

In 1910, the first case of SCD was reported in Chicago in a dental student who presented with a history of jaundice, leg ulcers, intolerance to exercise, and bouts of cough and fever[5,6]. Dr. John Herrick was the first who described sickle cell anemia in that patient. Four decades later, the molecular basis of SCDs was described by Ingram and his colleagues. They characterized the A to T substitution producing GAG to GTG codon in DNA and substitution of amino acid glutamic acid with valine in the sixth position of beta-globin chain. Establishment of Comprehensive Sickle Cell Centers (CSCC) in the United States in the 1970s is an important landmark to target the development of diagnosis and treatment strategies for sickle cell diseases[7,8]. As per the systematic analysis of the Global Burden of Disease Study, 3.2 million population in the world live with sickle cell disease (SCD), 43 million people are carriers of the mutation (i.e., they live with sickle cell trait), and around 176,000 people die per year because of complication related to SCDs[9].

Molecular Basis of Sickle Cell Diseases

The molecular basis of SCDs is a single-base mutation or short deletion at codon 6 of the HBB gene where there is a structural alteration in glutamine-valine substitution (GAG > GTG) in the chain of β-globin protein to produce sickle hemoglobin[10-12].

Hemoglobin Variants

Hemoglobin variants are the less common form of hemoglobin with altered oxygen affinity, also termed as hemoglobinopathies. It is estimated that about 7% of the world's population is affected by a mutation in the globin gene that affects hemoglobin leading to the formation of impaired globin protein subunit and Hb variants. Hb variants result in either increased or decreased affinity for oxygen, are most commonly associated with missense mutations, and are less commonly associated with substitutions of amino acids, deletion, and altered posttranslational modifications[13]. Benign lifelong erythrocytosis are generally associated with the mutation that increases oxygen affinity whereas Hb variants with reduced oxygen affinity are less common and are associated with cyanosis and mild anemia[14,15]. More than 1000 human hemoglobin variants have been discovered so far, that occur naturally with the substitution of single amino acid. The variants not only alter the structure of human hemoglobin but also affect the bio-physiochemical properties from normal to severe[16]. However most of the hemoglobin variants are supposed to be clinically silent and are unassociated with clinical phenotype, some of them like - HbS, Hb C, and Hb E are significant hemoglobin variants that are medically important for clinicians to diagnose hemoglobinopathies such as Thalassemia and Sickle Cell Anemia[17,18].

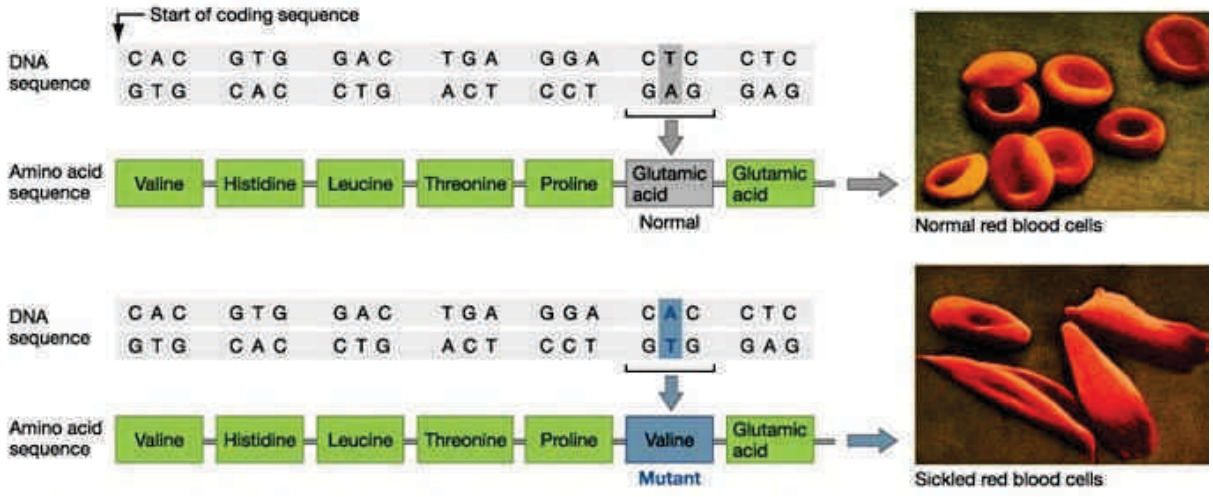

Figure 1: Change in Amino acid sequence in Hemoglobin molecules

Table 1: Examples of some Hb variants with molecular basis[19,16]

Mutation	**Example**	**Clinical aspects**	**Molecular basis**
Nucleotide base substitution for			
One amino acid	Hb S β6Glu → Val	Sickling	β:Cd 6 GAG → GTG
Two amino acids	Hb C-Harlem β6Glu → Val + β73Asp → Asn	Sickling	β:Cd 6 GAG → GTG & β:Cd 73 GAT → AAT
Termination	β145 Tyr → Termination	Increased oxygen affinity and polycythemia	β:Cd 145 TAT → TAA
Amino acid instead of termination	Hb Constant Spring α2:142 Termination → Gln	Decreased synthesis (thalassemia-like)	α2:Cd 142 TAA → AAA
Nucleotide base deletions			
Crossover	HbLepore δβ-fusion with segments of δ and β lost	Decreased synthesis (thalassemia-like)	δβ:7.4 kb deletion
Nucleotide base additions			
Two bases added →frame shift	Hb Cranston β144 Tyr-His → Ser-Ile-Thr	Unstable	β:Cd 144/145 +CT
Multiple codons	Hb Grady	Normal	α2 or α1:Cd 118/119 (+9 bp)

Hemoglobin Disorder

Along with the transport of oxygen and carbon dioxide in the tissue, hemoglobin also binds with physiologically important nitric oxide in the body. Moreover, the hemoglobin molecules limit some potential problems caused by its iron and free oxygen molecule, reactive molecules capable of damaging tissue through the production of reactive oxygen species (ROS).

Disorders related to hemoglobin can be classified as qualitative and quantitative. Quantitative hemoglobin disorders such as β-Thalassemia, α-Thalassemia, and acquired α-thalassemia are associated with the defect in the synthesis of the globin chain while qualitative disorders such as sickle cell disorders and Methemoglobinemia are associated with structural variants of hemoglobin[20].

Sickle cell diseases result from the production of abnormal hemoglobin in the patients' RBCs. Production of abnormal hemoglobin is mainly related to a mutation that brings changes in the sequence of nucleotide molecules either by addition or by deletion of amino acids sequence within the globin gene. Rarely, crossing over between two like genes during the process of meiosis also results in the formation of abnormal hemoglobin because the crossing creates a fusion protein of both gene sequence[19]. Typically, hemoglobin variants are based on the point mutation either in the alpha or in the beta-globin chain[21].

Pathophysiology of SCDs

Clinical manifestations of SCD arise from complex path physiological process. The basis of SCD Pathophysiology is needed to improve the quality of life and survival of people with SCD. Sickle cell anemia is the most common form of SCD. 70 % of cases of SCD in African ethnicity have been reported with Sickle cell anemia caused by homozygosity of the beta-S (βS) allele located on chromosome 11p15.5. Homozygous inheritance, and coinheritance of the β^S mutation with other mutations such as β^C (HbSC), β^E (HbSE), or a β-thalassemia allele directs to different forms of SCD through multiple interconnecting pathways of molecular, cellular and biophysical mechanisms that include four major processes namely (a) polymerization of hemoglobin S, (b) impaired biorheology and micro-vascular vaso-occlusion, (c) haemolysis, and (d) sterile inflammation[22-24].

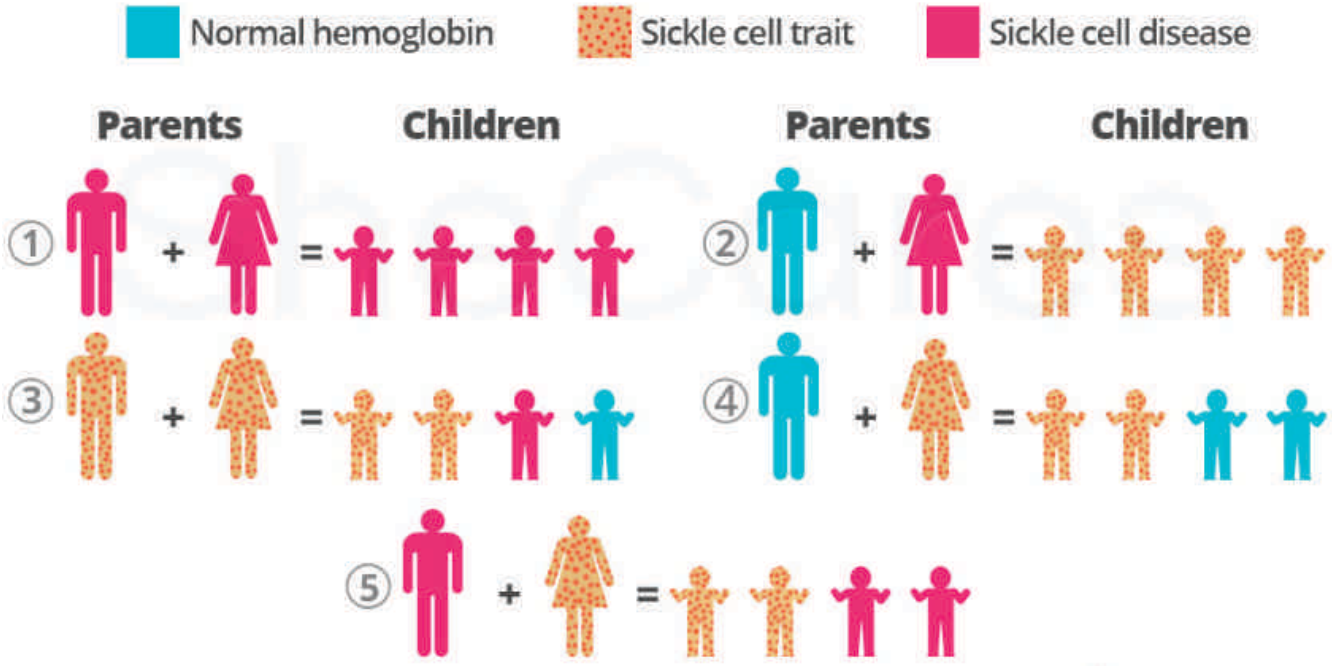

Figure 2: Inheritance of Sickle cell gene[33]

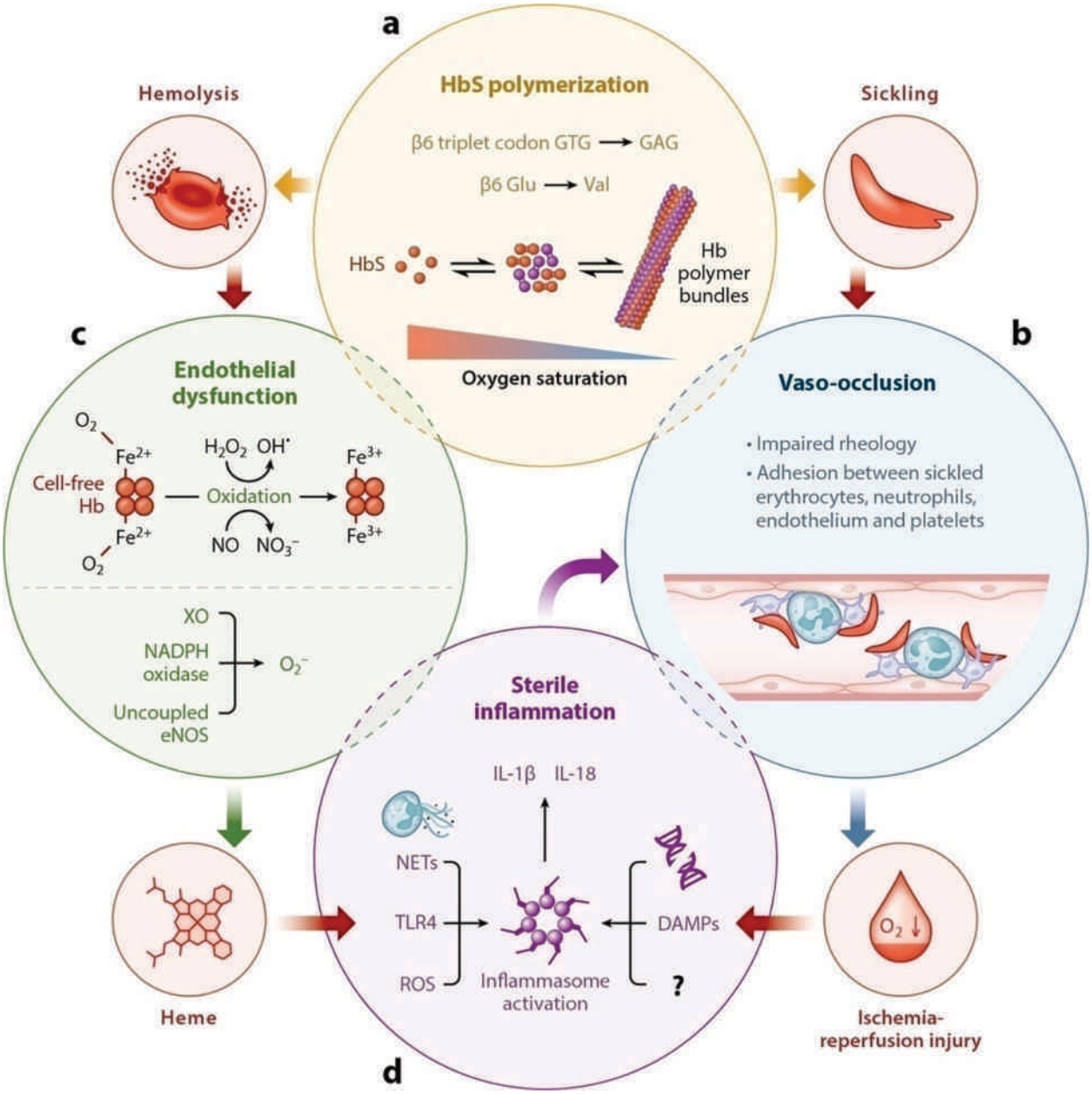

Figure 2: Schematic Diagram of the pathophysiology of Sickle cell disease[22]

A single-nucleotide polymorphism replaces valine amino acid for glutamic acid at the sixth position in the β-globin chain of the beta globin gene. Polymerizations of the muted HbS under deoxy conditions form bundles of hemoglobin polymer that result in sickling of erythrocyte and hemolysis followed by impaired rheology, accumulation of sickle erythrocytes and micro-vascular vaso occlusion. Vaso- occlusion promotes ischemia and organ injury. Hemolysis caused by Hemoglobin (Hb) polymer bundles is associated with the release of cell-free Hb into the blood circulation in the body.

Gelatinization of Hb S under deoxy conditions forms crystal gels known as tactoids. The crystals bring changes in the normal biconcave shape of red blood cells to a characteristic sickle shape when HbS are repeatedly exposed to deoxy condition for a prolonged period. Sickled blood cells are destroyed faster than normal blood cells causing anemia. In addition, sickled cells block or reduce blood flow in blood vessels. This can damage organs, muscles, and bones and may lead to life-threatening conditions25. Oxygenated hemoglobin either reacts with nitric oxide to form nitrate and methemoglobin or it can also react with hydrogen peroxide to produce hydroxy free radical and methemoglobin for endothelial dysfunction.

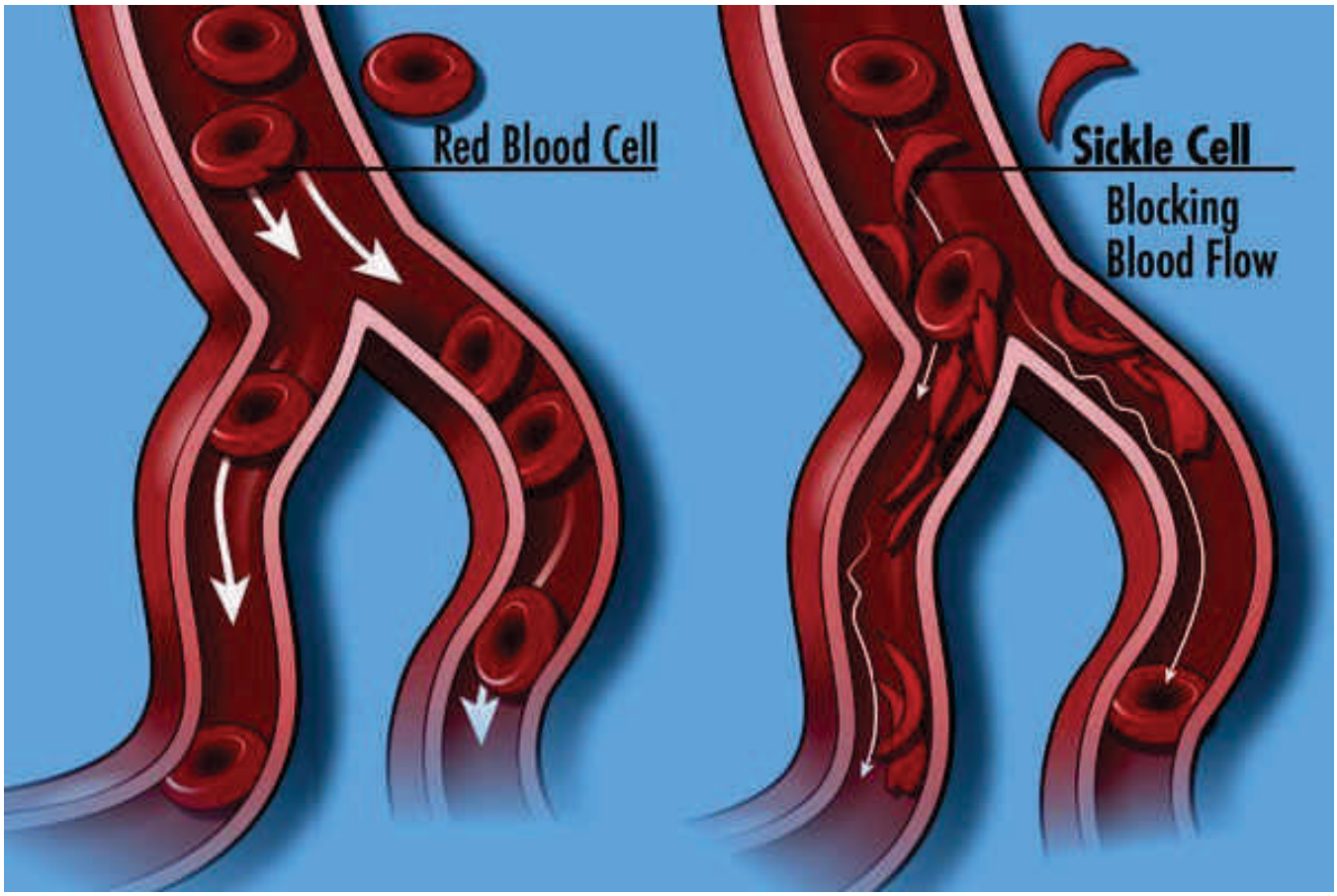

Figure 4: Normal free floating Donut-shaped RBCs and Sickle celled RBCs blocking blood flow[4]

Moreover, enzymes such as NADPH oxidase, xanthine oxidase (XO), and NO synthase facilitate the formation of oxygen-free radicals to promote endothelial dysfunction. Degradation of Methemoglobin release cell-free heme, is a prime damage-associated molecular pattern (DAMP). Production of reactive oxygen species (ROS), activation of Toll-like receptor 4 (TLR4), formation of neutrophil extracellular trap (NET), the release of tissue or cell-derived DAMPs, DNA, and other hidden factors set off by cell-free heme or I-R injury may contribute to sterile inflammation through the inflammasome pathway in vascular and inflammatory cells. Finally, sterile inflammation further promotes vaso-occlusion through a feedback loop by promoting the adhesiveness of neutrophils, platelets, and endothelial cells[22].

Clinical Picture

Clinical manifestations of SCD include chronic hemolysis, vaso - occlusion, organ damage, and pain[26]. Chronic hemolysis leads to hemolytic anemia, jaundice and delayed growth, and maturation .Vaso- occlusion results in tissue ischemia which leads to acute and chronic pain together with organ injury that can damage any organ such as the liver, spleen, lungs, kidneys, brain, and bones. Most often, dactylitis is the earliest manifestation of Sickle cell diseases in adults. In case of children, spleen becomes filled with red blood cells resulting in asplenia[27].

MATERIALS AND METHODS

The whole blood sample was collected from the sickle cell camp organized by Dhangadhi Diagnostic and Research Centre private limited, Hasanpur-5 Dhangadhi at different locations in Kailai and Kanchanpur districts of the Far west province of Nepal. The camp was continued for six successive days.

Specimen Collection, Preparation, Handling, and Analysis

The people were explained orally about SCDs screening test procedures and were requested to have screening for SCD. The name, ages, sex, and community of the participants were registered by the lab authorities. Special patient preparation was not recommended before specimen collection. At first ring

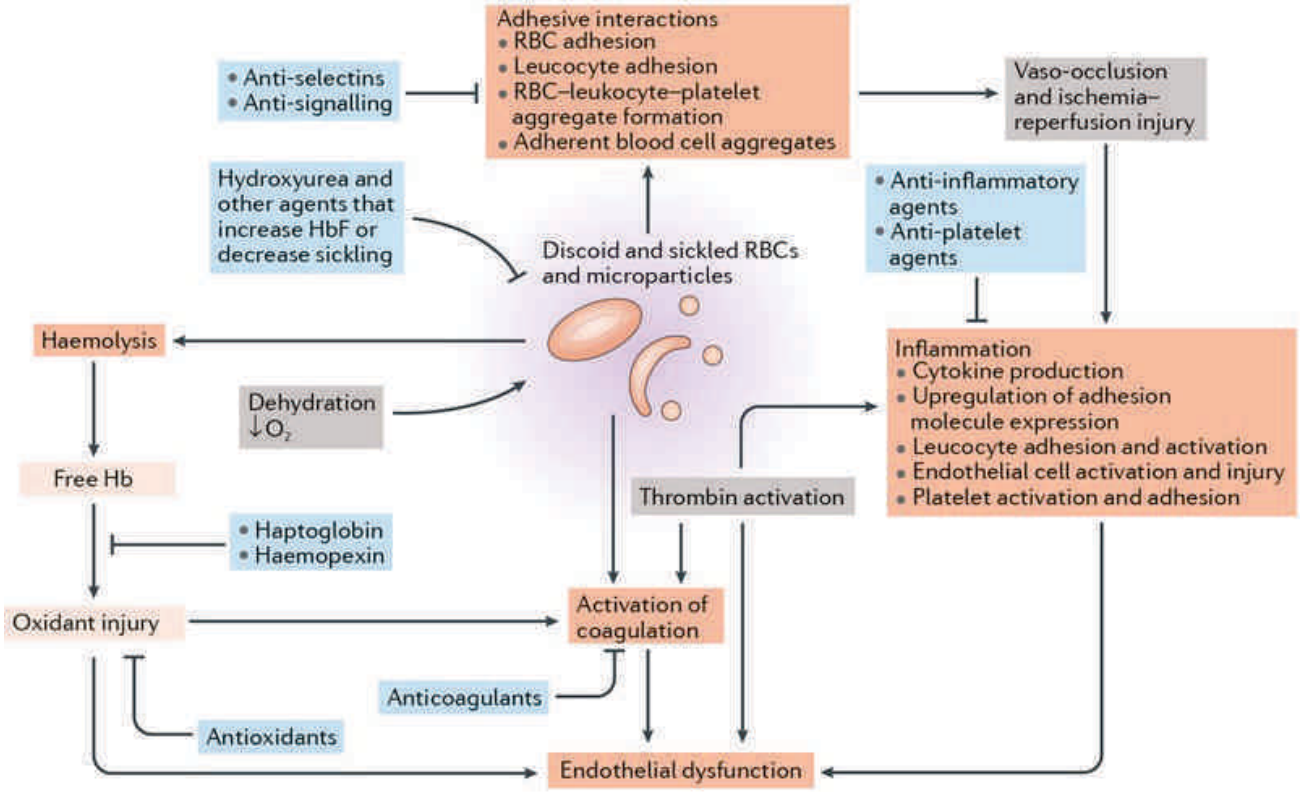

Figure 5: Pathophysiological pathway of SCDs[26]

finger of the patient was selected and sterilized with 70% alcohol before pricking. Then the finger was pricked with the help of a disposable lancet to collect a fresh blood samples for the Hb solubility test. To confirm all positive results obtained from the Hb solubility test, the vein puncture method was used to collect the whole blood sample for Hb-electrophoresis. Whole blood specimens collected in BD vacutainer EDTA blood collection tubes were processed for Hb electrophoresis. The remaining samples were stored at 2° C to 8° C in case of delay. Long-term storage and thawing were avoided to prevent pre-analytical errors.

Analytical Methods

Sickle cell solubility test followed by Hb- electrophoresis were employed to screen Sickle cell diseases in this study.

Sickle Cell Solubility Test Principle:

Method

20 µL of fresh blood sample (obtained from finger prick) was mixed with a 2 mL working solution of concentrated phosphate buffer and sodium dithionite in a reaction tube so as to release and reduce hemoglobin from red blood cells. Turbidity results from the precipitation of HbS was read by holding the result reading stand against a dim illumination and viewing a marked line on the background of the stand, through the solution in the reaction tube. Further, Hb-AA (Normal) Hb-AS (Sickle cell trait) Hb-SS (sickle cell Anemia) were differentiated by repeating the procedure with an increased volume of the test sample (100 µL) followed by incubation for 10 minutes at room temperature and centrifugation at 1200 rpm for 5 minutes.

Table 2: Differentiation method by Hb solubility test

Type	Lower Layer	Upper Layer
Hb-AA (Normal)	Clear and dark red in color	Grey Precipitate
Hb-AS (Sickle cell trait)	Clear and light red to pink in color	Red Precipitate
Hb-SS (sickle cell Anemia)	Clear and colorless	Red Precipitates.

When subjected to a centrifugal force the precipitated haemoglobin (HbS) forms a red precipitate on the top layer leaving the lower solution clear and colorless. The soluble haemoglobin (HbA) produced a clear red lower solution with a grey precipitate on the top layer and most HbAS which contains both precipitated and soluble hemoglobin impart a red precipitate ring on the top layer with a red to a pink color lower solution. All the positive samples were further confirmed by hemoglobin electrophoresis test.

Hemoglobin Electrophoresis Test Principle:

Method

0.5 µL of Whole blood samples collected in BD vacutainer EDTA blood collection tubes were mixed with 1500 µL of diluents provided by the supplier and introduced to Bio-Rad D-10 analyzer for the fractionation of hemoglobin species to identify Hb-AA (Normal), Hb-AS (Sickle cell trait) Hb-SS (sickle cell Anemia). Hemoglobin electrophoresis utilizes an electric current in a blood sample. Hemoglobin electrophoresis differentiates normal and abnormal types of hemoglobin based on rate of movement to the anode and cathode in an electric field. The rate of movement is dependent upon the molecular weight and size of the normal and abnormal hemoglobin to be separated.

Methodological Investigations

The screening was done by two separate procedures. One by hemoglobin solubility test method and another by hemoglobin electrophoresis method. Hemoglobin electrophoresis was done to confirm the positive cases. Positive results obtained from Hemoglobin solubility test were confirmed in Bio- Rad D-10 hemoglobin testing system which is based on ion exchange HPLC. The details of the assay protocols of the instrument are mentioned as follows:

Specification of Hemoglobin Testing System (Bio- Rad D-10)

Table 3: Specification of Hb testing instrument

Dimensions (W x H x D)	15.8 in x 19.5 in x 21 in / 402 mm x 495 mm x 534 mm
Weight (uncrated)	75 lbs / 34 kg
Operating altitude (maximum)	6,652 ft. / 2000 m
Operating temperature	15-30° C
Operating humidity	20-80% relative humidity, non-condensing
Overvoltage category	II
Pollution degree	2
Electrical supply voltage fluctuation	10% max
Ambient temperature	0-50 °C
Storage humidity	10-95%
Power input requirements	100-240 V ~ at 50-60 Hz
Power consumption	220 VA max
Thermal power	1010 BTU/h max
Fuses	T 2.5 A/250 V (2 fuses)
Sound level	<70 dBA
Sample requirements	1505µL
Analytical device	Cartridge: Application dependent
Detector	Visible wavelength detector
Printer	Graphic thermal, 112 mm (4.4 in) wide
User interface	Integrated LCD touchscreen
Data export	USB flash drive, RS232 or LAN
Ethernet/LAN connection	RJ-45
Waste tank volume	10 L

Figure 6: : Bio- Rad D-10 Hemoglobin Testing System

RESULTS AND DISCUSSION

Sickle cell anemia is a widespread disorder reported in Africa, Asia, and Middle East[28,29]. Sickle cell diseases are very common among the Tharu people of Nepal who lived in the jungle side of the Terai region. The majority of them are live in Midwestern and Far-western Terai regions of the country. In a study, it has been reported that 4.5% of Tharu people of mid-western Nepal tested CKD positive[30]. A separate study from India has shown that 9.30-10.6% of Indian people were confirmed with sickle cell trait, 0.21-0.6% were confirmed SCD positive, and the ratio of males to females confirmed with sickle cell trait and the sickle cell disease was 1:131 . In our study, sickle cell trait male-to-female ratio was 0.4:2 and the sickle cell disease female to male ratio was 2.3:1. Our study Suggest that the prevalence of sickle cell disease in female was slightly higher as compared to male.

Sickle cell diseases are one of the most common genetically inherited diseases of interest diseases have always been a disease of interest all around the world. Though the diseases are most common in Africans, It has been found that sickle cell diseases are very common in the Tharu community people of Nepal[32]. Lack of awareness and limited and impassable healthcare facilities in Nepal has made the ethnic group people suffer from sickle cell disease. There for Screening Sickle cell disease is important to manage and treat the SCDs to reduce the morbidity and mortality rate. Various studies have been carried out to assess sickle cell disease. This study is based on the qualitative analysis for the screening of sickle cell diseases in the different age groups in the Tharu community people. Among the various test available for the screening of sickle cell anemia, only hemoglobin solubility test and hemoglobin electrophoresis was employed to carry out this study. A camp was organized in different location of the far west Terai regions where the majority of the people belong to the Tharu community. The study was initiated after oral explanation and registration by laboratory authorities from Dhangadhi Diagnostic center. The study population consisted of 1077

samples collected from different age groups who visit the camp for screening purpose.

In this study, Out of 1077 samples, 114(10.58%) had sickle cell traits. Among them, 2 (1.65%) patients had beta-thalassemia trait, 93 (76.83%) had HbS trait, 13 (10.74%) had Sickle cell trait with beta-thalassemia minor, 1(0.83 %) had sickle cell diseases with minor beta-thalassemia homozygous, 1(0.83%) had sickle cell diseases with minor beta-thalassemia, 3 (2.48%) had sickle cell diseases with beta-thalassemia homozygous, 1 (0.83%) had sickle cell diseases with beta-thalassemia heterozygous and 7 (5.79%) people had normal hemoglobin peak. 1077 samples were collected from the camp include 1049 (97.4%) from the Tharu Nepalese and 28 (2.6%) from other than the Tharu community of Nepal. Among 114 sickle cell trait confirmed, 112 (98.25%) samples were found to be from Nepalese Tharu Community and remaining 2(1.75%) was detected in other than the Tharu people.

The total sample collected from the camp was categorized into the different age groups and gender. In which it was found that 400 (62.79%) were female and 237 (37.21%) were male among the 637 samples collected from people below 20 years. Accordingly, 181 (81.53%) females and 41 (18.47%) males among 222 samples collected from people between 21-40 years, 116 (76.32%) female and 36 (23.68%) male among 152 samples collected from people between 41-60 years, 26(61.90 %) female and 16 (38.10 %) male among 42 samples collected from people between 60-70 years and 13(54.17%) female and 11 (45.83 %) male among 24 samples collected from people over 70 years. 736 (68.34%) people were female and 341 (31.66%) were male among 1077 total samples.

Table 4: Age and gender-wise distribution of the sample

Age Group (Years)	Total	Males No.	Male %	Females No.	Female %
<20	637	237	37.21	400	62.79
21-40	222	41	18.47	181	81.53
41-60	152	36	23.68	116	76.32
60-70	42	16	38.10	26	61.90
>70	24	11	45.83	13	54.17
Total	1077	341	31.66	736	68.34

Table 5: Age group & Hb solubility

Age Group (Years)	Total	SCD Negative		SCD Positive		HbS Trait	
		No.	%	No.	%	No.	%
<20	637	579	90.89	2	0.31	56	8.79
21-40	222	191	86.04	1	0.45	30	13.51
41-60	152	127	83.55	0	0.00	25	16.45
60-70	42	39	92.86	0	0.00	3	7.14
>70	24	24	100.00	0	0.00	0	0.00
Total	**1077**	**960**		**3**		**114**	

HB Solubility test result was analyzed based on different age groups. 637 samples collected from people below 20 years were found to be 579 (90.89% Negative, 2(0.31%) Positive, and 56 (8.79%) HbS trait. Similarly, 222 samples collected from people between21-40 years was found to be 191 (86.04% Negative, 1(0.45%) Positive, and 30 (13.51%) HbS trait, 152 samples collected from people between 41-60 years was found to be 127(83.55%) Negative, no Positive and 25 (16.45%) HbS trait, 42 samples collected from people between 60-70 years was found to be 39 (92.86) negative, no positive and 3 (7.14%) HbS trait and 24 samples collected from people above 70 years were found all negative. Age group-wise analysis of sickle cell disease shows the higher the trait in the age group 41-60 years.

Table 6: Age and gender-wise distribution of Sickle cell trait

Age Group (Years)	Total	Males	%	Females No.	%
<20	56	28	50.00	28	50.00
21-40	30	2	6.67	28	93.33
41-60	25	2	8.00	23	92.00
60-70	31	2	66.67	1	33.33
Total	14	34	29.82	80	70.18

The sample population tested for sickle cell trait was categorized based on their age and sex. Among 56 samples collected from people below 20 years 28 (50%) were male and 28(50%) were female. Accordingly, and 2 (6.67%) male and 28 (98.33%) female were found among 30 people between 21-40 years, 2 (8%) male and 23(92%) female among 25 people between 41-60 years and 2 (66.67%) male and 1(33.33%) female among 3 people between 60-70 years were found with sickle cell trait. As a whole 34 (29.82% male and 80(70.18%) female were tested with sickle cell trait among 114 samples.

SUMMARY & CONCLUSION

With an increase in population, the prevalence of Sickle cell disease is widely distributed and screening of SCD for every individual is necessary for the management and treatment of Sickle Cell Disease. The unavailability and limited source of healthcare facilities in the Far West region is a burden to screen and diagnose sickle cell disease in this area. The majority of the patient with SCD left untreated earlier before 2014AD because healthcare providers always get confused with the sign and symptoms shown by the patient. The disease was often misdiagnosed as arthritis or jaundice. Though the healthcare facilities are somewhat improved and accessible now, but people are not aware of Sickle Cell Disease. In this study whole blood sample had tested against SCD in the far west Terai region which showed that the majority of the people from the Tharu community were confirmed SCD Positive than other community people of Nepal. Among the entire tested sample, the ratio of female to male was higher. This indicates that sickle cell diseases are higher in females than the male.

CONCLUSION

From this study, we conclude that people in the 41-60 years age group are highly affected by sickle cell disease and other haemoglobinopathies, among which female is the major victim. The responsible stakeholder should extend the facility of diagnosing Sickle cell disease in the access of the affected community to prevent its further inheritance, and timely management and control measures taken into action will help to reduce the morbidity and mortality rate of the patient in the affected area.

CONFLICTS OF INTEREST: None

FINANCIAL SUPPORT: None

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