THE ROLE OF ABO ANTIBODIES AND MOLECULAR GENOTYPING OF BLOOD GROUP O IN HAEMOLYTIC DISEASE OF THE FOETUS AND NEWBORN

BY

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LIST OF ABBREVIATION

AHG	Anti human globulin
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
DAT	Direct antiglobulin test
DNA	Deoxyribonucleic acid
DTT	Diethiotretol
EDTA	Ethylene diamine tetraacetic acid
EIA	Enzyme immunosorbent assay
ET	Exchange transfusion
EtBr	Ethidium bromide
FBP	Full blood picture
G6PD	Glucose 6 phosphate dehydrogenase deficiency
Hb	Haemoglobin
HDFN	Haemolytic disease of the foetus and newborn
HUSM	Hospital Universiti Sains Malaysia
IAT	Indirect antiglobulin test
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVIG	Intravenous immunoglobulin
NICU	Neonatal intensive care unit
PCR	Polymerase chain reaction
RBC	Red blood cells
RFLP	Restriction fragment length polymorphism
SSP	Sequence specific primers

TBE	Tris borate EDTA
TSB	Total serum bilirubin
2ME	2 mecaptoethanol

MENILAI PERANAN ANTIBODI ABO DAN GENOTIP KUMPULAN DARAH O DALAM MENYUMBANG KEPADA PENYAKIT HEMOLITIK DIKALANGAN FETUS DAN BAYI BARU LAHIR

ABSTRAK

Selepas pengenalan profilaksis anti-D, kadar penyakit hemolitik Rh telah menurun dan buat masa sekarang ketidakserasian ABO di antara ibu dan fetus merupakan penyebab utama jaundis dikalangan neonatal yang berkaitan dengan ketidakserasian kumpulan darah. Ketidakserasian ABO neonatal terjadi terutamanya dalam neonatal kumpulan darah A/B yang dilahirkan oleh ibu yang berkumpulan darah O. Keterukkan kadar hemolisis adalah bermula dari ringan hingga teruk dan hanya beberapa neonat sahaja yang mengalami penyakit hemolitik ABO. Penilaian dan ramalan keterukkan dalam kalangan neonat dengan ketidakserasian ABO dan penyakit hemolitik sudah dijalankan sebelum ini. Kajian ini bertujuan untuk menentukan sama ada titer antibodi ABO dalam ibu dan genotip molekular kumpulan darah O boleh meramalkan hemolisis dalam neonat yang mengalami HDFN ABO. Keterukkan hemolisis juga dinilai dengan menilai penanda hemolisis dan ketidakserasian ABO. Sejumlah 96 neonat dengan jaundis didaftarkan untuk kajian ini. Tujuh puluh tidak serasi ABO (kumpulan kajian) dan 26 (kumpulan kawalan) adalah serasi ABO. Enam puluh ibu yang tidak serasi ABO dengan neonat dan 30 ibu yang serasi (ibu dan neonat berkumpulan darah O) dengan neonat telah dikaji titer anti A/B IgG dan genotip molekular kumpulan darah O. Tiga puluh empat individu lagi yang merupakan penderma darah juga telah dikaji untuk genotip molekular kumpulan darah O. Keputusan yang diperolehi menunjukkan perbezaan signifikan ($p<0.05$) diantara kumpulan ABO tidak serasi dan kumpulan yang serasi dengan ujian makmal yang digunakan semasa penilaian hemolisis. Neonat

ketidakserasian ABO mempunyai penanda hemolitik yang lebih tinggi berbanding dengan neonat yang serasi ABO. Cara kelahiran, jenis rawatan dan tahap hemoglobin berbeza secara signifikan di antara neonat yang mempunyai HDFN ABO dan tanpa HDFN. Neonat yang mempunyai HDFN ABO, titer ibu bagi anti A/B IgG merupakan peramal yang baik untuk hemolisis apabila karboksihemoglobin dan bilangan retikulosit mutlak digunakan sebagai penanda hemolisis. Tiada perbezaan significant ($p<0.05$) diperhatikan diantara titer IgG ibu yang serasi dan tidak serasi ABO. Daripada sejumlah 124 subjek yang sebagai genotip molekular kumpulan darah O, hanya satu yang mempunyai genotip yang berbeza iaitu O1O2 namun selebihnya mempunyai O1O1, ini menunjukkan frekuesi O1O1 yang tinggi. Kajian ini menunjukkan ketidakserasian neonatal ABO dikaitkan dengan lebih hemolisis apabila dibandingkan dengan neonat yang mengalami jaundis yang mempunyai keserasian ABO. Kepekatan antibodi IgG ibu tidak dipengaruhi oleh ketidakserasian kehamilan walaupun antibodi titer ibu boleh meramalkan hemolisis.

Molekular genotip kumpulan darah O yang paling kerap dikalangan orang Melayu adalah adalah O1O1.

THE ROLE OF ABO ANTIBODIES AND MOLECULAR GENOTYPING OF BLOOD GROUP O IN HAEMOLYTIC DISEASE OF THE FOETUS AND NEWBORN

ABSTRACT

After the introduction of anti-D prophylaxis, the incidence of Rh haemolytic disease has decreased and ABO incompatibility between the mother and the fetus is now the leading cause of neonatal jaundice associated with blood group incompatibility. Neonatal ABO incompatibility occurs mostly in group A/B neonates delivered by group O mothers. The severity of haemolysis ranges from mild to severe with few neonates developing ABO haemolytic disease. Assessing and predicting the severity of haemolysis in neonates with ABO incompatibility and haemolytic disease has been carried out previously. This study was aimed at determining whether maternal IgG anti-A/B titre and blood group O molecular genotype can predict haemolysis in neonates with ABO HDFN. The severity of haemolysis was also assessed by determining the levels of haemolytic markers in neonates with ABO incompatibility. A total of 96 neonates with jaundice were enrolled in this study. Seventy were ABO incompatible (study group) and 26 (control group) were ABO compatible. Sixty mothers of the 70 ABO incompatible neonates and 30 mothers that were compatible (mother and neonate blood group O) with their neonates were studied for maternal IgG anti-A/B titre and ABO molecular genotype. Another 34 individuals who were blood donors were also studied for blood group O molecular genotype. The result obtained showed a significant difference ($p<0.05$) between the ABO incompatible and compatible groups in relation to the laboratory features that were used in assessing haemolysis. ABO incompatible neonates had higher levels of haemolytic markers compared to the ABO compatible neonates. Mode of delivery type of

treatment required and level of haemoglobin were significantly different between neonates thad had ABO HDFN and does without HDFN. In neonates that had ABO HDFN, maternal IgG anti-A/B titre was a good predictor of haemolysis when carboxyhaemoglobin and absolute reticulocytes count were used as markers of haemolysis. No significant difference ($p>0.05$) was observed between the maternal IgG titre of ABO incompatible and compatible mothers. Of the 124 subjects that were typed for blood group O molecular genotype, only one had a different genotype which is *O1O2* but the remaining had *O1O1*.

This study indicates that neonatal ABO incompatibility is associated with more haemolysis when compared with neonates with jaundice not caused by ABO incompatibility. Concentration of maternal IgG antibody titre is not affected by incompatible pregnancy even though maternal antibody titre can predict haemolysis. The major ABO molecular genotype of the O blood group in Malays is *O1O1*.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

ABO foeto-maternal blood group incompatibility in which a mother of blood group O delivered a newborn of either blood group A or B is associated with immune mediated neonatal hyperbilirubinaemia. The hyperbilirubinaemia is caused by maternal immunoglobulin G (IgG) anti-A or anti-B that passes through the placenta and attached to the corresponding antigen positive foetal red blood cells and cause haemolysis of the cells. This leads to increased plasma bilirubin in the circulation and equally carbon monoxide which is produced in equal amount with bilirubin during haem catabolism. ABO foeto-maternal incompatibility is the main cause of immune haemolytic disease of the foetus and newborn (HDFN). It occurs in 15-25% of all pregnancies and it is the leading cause of neonatal jaundice associated with blood group incompatibility. It therefore has an increase risk of causing Kernicterus, a permanent neurologic damage caused by bilirubin toxicity. The incidence of HDFN due to ABO incompatibility in Asian population (3.7%) is similar to that in blacks (Han *et al.*, 1988; Kaplan and Hammerman, 2005; Murray and Roberts, 2007).

The severity of HDFN is affected by several factors such as the concentration and specificity of the antibodies in the mother, and type of IgG subclass. However, this severity has reliably been predicted using several laboratory techniques that measures and identify the above listed factors and also techniques such as antibody screening and identification, and qualitative measurement of the antibody level in the

mother (antibody titration), although quantification of maternal antibodies correlates better than a titration with the severity of HDFN (Hadley, 2002; Armstrong and Smart, 2008). However, the severity of HDFN caused by anti-A or anti-B to some degree is determined by the number of A or B antigens on the foetal red cells.

Studies have shown that measurement of maternal antibodies and estimation of haemolytic markers such as bilirubin, carboxyhaemoglobin (COHb), and direct antiglobulin test (DAT) in neonates with blood group incompatibility may predict neonates at risk of significant hyperbilirubinaemia/severe haemolysis (Uetani *et al.*, 1989; Sarici *et al.*, 2002; Kaplan *et al.*, 2010).

Although data related to maternal IgG anti-A and anti-B and its relationship with severity of haemolytic disease caused by neonatal ABO incompatibility is scanty, contrasting literatures exist on the use of maternal antibody titre to predict the severity of HDFN. The incidence of ABO HDFN was reported to increase with the increase in maternal serum immune antibody titre, the difference between the titres was significant. However, the higher the titre the more likely it is that the infant will be severely affected by HDFN. The contribution of maternal anti-A and anti-B antibody titres in predicting newborns at risk of significant hyperbilirubinaemia was studied and it was found to be an important predictor of hyperbilirubinaemia in ABO incompatible neonates. A strong correlation was found between maternal IgG anti-A and anti-B titres with ABO HDFN (Usha and Sulochana, 1998; Armstrong and Smart, 2008; Bakkeheim *et al.*, 2009).

Carboxyhaemoglobin (COHb) measurement has long been used to assess haemolysis in diseases that are frequently associated with increased bilirubin production such as G6PD deficiency and neonatal ABO incompatibility. As early as 1967 and 1968, (Fallstrom and Bjure, 1967; Fällström and Bjure, 1968) studied the role of COHb in

assessing haemolysis in neonates with Rh and ABO haemolytic diseases, they found that COHb correlated well with increased bilirubin production in both cases. In addition, they found an increased level of COHb in neonates with haemolytic disease when compared with the normal neonates. Their finding was supported by several studies that use COHb as an index of assessing haemolysis. Kaplan and colleagues found a significant correlation between COHb and total serum bilirubin in neonates with jaundiced caused by ABO incompatibility indicating the role of increase bilirubin production in the pathogenesis of hyperbilirubinaemia due to ABO blood group incompatibility (Kaplan *et al.*, 1998).

Traditionally, determination of ABO blood group of an individual was done by reactivity between antisera and the A, B and H antigens. In this era of genomics, determination of blood group antigens by molecular testing has been made possible. The basis for the determination of ABO molecular genotype is due to A and B genes nucleotide polymorphism. The A and B genes encode the A and B glycosyltransferases that confer specificity to the ABH antigens. The difference in specificity is due to substitution of four amino acid residues (176, 235, 266 and 286) (Fumi-ichiro Yamamoto *et al.*, 1990; Westhoff, 2006). The frequency of the ABO molecular genotypes of the known ABO alleles (A^1, A^2, B, O^1 and O^2) have been studied in different parts of the world and a number of genotypes were established from the alleles (*A1A1, A1A2, A1O1, A1O2, A2A2, A2O1, A2O2, A1B, A2B, BB, BO1, BO2, O1O1, O1O2,* and *O2O2*) (Hosseini-Maaf *et al.*, 2007; El-Zawahri and Luqmani, 2008). For the purpose of this research project, only O alleles (O^1 and O^2) are of interest because only O individuals were genotyped.

Treating neonates with ABO incompatibility is usually done with phototherapy, intravenous immunoglobulin (IVIG) administration and exchange transfusion (ET)

depending on the severity of the disease. Previous research focused on the use of total serum bilirubin as the main marker of haemolysis in neonates with hyperbilirubinaemia due to ABO incompatibility. To date there is no published report that correlates maternal IgG anti-A and anti-B with haemolysis using COHb and absolute reticulocytes count as the markers of haemolysis. Therefore, we would like to investigate whether maternal IgG anti-A/B titres can predict haemolysis in neonates with ABO HDFN using the above mentioned markers of haemolysis. However, due to the variation in the clinical presentation and severity of haemolysis, we determine the clinical and laboratory features in neonates with ABO incompatibility.

1.2 Objectives of the study

1.2.1 General Objective

The general objective of this study was to assess the role of ABO antibodies quantification and ABO molecular genotyping in ABO Haemolytic disease of the fetus and newborn (HDFN).

1.2.2 Specific Objectives

The specific objectives were:

1. To determine and compare the clinical and laboratory features of neonates with jaundice caused by ABO incompatibility and neonates with jaundice not caused by ABO incompatibility.
2. To determine the difference in titre of maternal IgG anti-A/B titres of ABO incompatible and compatible mothers.

3. To determine whether maternal IgG anti-A/B titre can predict haemolysis in neonates with ABO HDFN.
4. To determine the maternal blood group O genotypes in ABO HDFN
5. To correlate the maternal blood group O genotypes with the clinical presentation of ABO HDFN

1.3 Research hypothesis

It was hypothesized that:

1. Although the severity of ABO HDFN is mild, the clinical and laboratory features of newborns that are ABO incompatible with their mothers are different from ABO compatible newborns.
2. IgG anti-A and anti-B in group O individuals are naturally occurring, mothers that are ABO incompatible with their newborns have higher IgG anti-A and anti-B titres than mothers that are ABO compatible with their mothers.
3. The concentration of antibodies in the mother correlates with the severity of the disease in the newborn.
4. Maternal ABO genotype can predict the severity of ABO HDFN

1.4 Significance of the study

This study determined the role of maternal antibody titre relative to the severity of haemolysis in ABO incompatible newborns. The result obtained will help in resolving the controversial issues regarding the use of maternal antibody titre to

predict haemolysis in affected newborns. More so, the study has shed more light on the differences between the severity of the disease seen in blood group A and B newborns. The ABO genotypes of the mothers have also given a clue to the frequency of the different O alleles common in Malaysian population.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ABO blood group system

2.1.1 Historical background

Until the discovery of ABO blood group system in 1900 by Karl Landsteiner, the fatal outcome of blood transfusion was associated with bacterial antibodies. In 1900, Karl Landsteiner decided to carry out an experiment using the red blood cells and serum of his colleagues. He found that the sera of some individuals agglutinated red blood cells of other individuals. Questions were raised in response to these findings as to the reason responsible for the agglutination. Bacterial infection was suspected as the pathological cause whereas individual difference as the non-pathological cause. In 1901, Landsteiner found that antigens A and B, and anti-A and anti-B antibodies were responsible for the agglutination. He came up with three groups of individuals based on his findings. Individuals with only A antigen, only B antigen and neither A nor B antigen as group I, group II and group III respectively. He showed that an individual has antibody to the antigen that is lacking on his red blood cell. Anti-A, anti-B and anti-A,B were found in the sera of group I,II and III respectively. A fourth group was found by Decastello and Sturli in 1902. This rare group has both antigen A and antigen B on the red blood cell but has no antibody in the serum (Landsteiner, 1900; Landsteiner, 1901; von Decastello and Sturli, 1902).

ABO antigens are present not only on red blood cells but also on tissues and in body fluids and secretions such as saliva. A previous study reported that ABO antigens

appeared earlier on epithelial tissues than on blood cells and should therefore be referred to as histo-blood group antigens (Fumi-ichiro Yamamoto *et al.*, 1990).

Detection of ABO blood group of an individual is routinely carried out based on serological testing. This involves the use of anti-A and anti-B antisera on red blood cells to determine the presence or absence of antigen A, B or both. A part from humans, only anthropoid apes and gorillas were reported to have ABO antigens on their red cells (Oriol R *et al.*, 1986; Svensson, 2011).

2.1.2 Genetics and biochemistry of the ABO system

The ABO gene is located at the long arm of chromosome nine. The specificity of the ABO blood group antigens are determined by the terminal sugar of the carbohydrate chains on the surface of the cells. Therefore, the properties of ABO antigens are not determined by the A, B or O gene but by the presence and activity of the glycosyltransferases genes. Mutations in these transferase genes form the basis of ABO molecular genotyping. They have seven exons and 1065 base pairs. Most mutations are located on exon six and seven.

ABO antigens are oligosaccharides antigens. Their biosynthesis starts with an addition of an L-fucose in α1-2 linkage on terminal galactose of a common precursor structure which is attached to lipids/proteins by α 1-2 - fucosyltransferase (H transferase) this leads to formation of H antigen. ABH antigenic structure on the surface of red cells are carbohydrates which are products of the ABO genes. The A and B allels encodes glycosyltransefarases (α1, 3-N- acetylgalactosaminyltransferase and α1, 3 – galactosyltransferase for A and B respectively) that catalyse the addition of specific sugar to the terminal galactose of the H antigen to produce A and B

antigens. For A antigen, α1, 3-N- acetylgalactosaminyltransferase add N-acetylgalactosamine to the H antigen. For B antigen, α1, 3 – galactosyltransferase add galactosamine to the H antigen. The O allele encodes an inactive glycosyltransferase that cannot catalyse the addition of any sugar to the H antigen. Therefore H antigen is the only ABO antigenic structure in blood group O (Hosoi, 2008; Svensson, 2011).

2.1.3 Molecular genotyping of the ABO blood group system

The use of molecular testing in transfusion medicine started more than two decades ago. Genotyping of most blood group antigens are not as complex as that of ABO blood group system. This is because over 100 glycosyltransferase genes are responsible for the production of A, B, and O blood groups including A and B subgroups. In addition, The nucleotide sequence of glycosyltransferases A and B are 99% similar (El-Zawahri and Luqmani, 2008). The A and B glycosyltransferase genes differ in seven base substitutions at positions 297, 526, 657, 703, 796, 803, and 930. Out of the seven base substitutions, four had amino acid changes at positions 526 (C to G: Arg to Gly), 703 (G to A: Gly to Ser), 796 (C to A: Leu to Met), and 803 (G to C: Gly to Ala). The DNA sequence of the A glycosyltransferase gene has been determined. The genes coding for other ABO phenotypes have been determined using the DNA sequence of A glycosyltransferse as the index because they possess a closely related sequence to the A glycosyltransferase gene except for few substitutions and deletions (Fumi-ichiro Yamamoto *et al.*, 1990).

The alleles for blood group O phenotype are O^1 and O^2. The coding region of Alleles O^1 and A^1 differ by a single deletion in the coding region of O^2 at nucleotide position 261 (deletion of G). This deletion results in a frame shift that creates a stop codon at

nucleotide 352-354 leading to production of enzymatically inactive protein. The O^2 allele differed from A^1 allele by nucleotide substitution at three positions: 297, 526, and 802. Substitutions at positions 526 (C to G: Arg to Gly) and 802 (G to A: Gly to Arg) produce amino acid changes. Studies have shown that more than 95% of O alleles are O^1 (Fumi-ichiro Yamamoto *et al.*, 1990; Seltsam *et al.*, 2005).

Most published data on determination of ABO genotypes employed different principles of PCR such as Restriction Fragment Length polymorphism (RFLM), use of radio labelled DNA probes and PCR using sequence specific primer (Gassner *et al.*, 1996).

2.1.4 ABO blood group antibodies

Antibodies are immunoglobulins produced in response to a foreign antigen. They are produced by B lymphocytes of the adaptive immune system. There are five types of immunoglobulins: IgG, IgM, IgA, IgD and IgE. Blood group antibodies are usually of the IgG and IgM type, but rarely IgA. Antibodies of the ABO system (anti-A and anti-B) are mainly IgM. They are naturally occurring antibodies that react best at lower temperatures than at 37^0C. Unlike the anti-A, B of group O individuals, the anti-A and anti-B antibodies of blood group B and A respectively, are almost entirely IgM.

IgG antibodies are immune antibodies (they are known to be produced only when an individual have previously been exposed to the antigen in question). Exception to this are blood group O individuals that have naturally occurring IgG anti-A, B in addition to the IgM anti-A, B in their serum. The presence of these IgG anti-A, B form the basis of ABO HDFN because of their ability to pass through the placenta.

The reason behind the natural presence of ABO antibodies has not been found yet. But several literatures were of the view that the production of both IgM and IgG anti-A and anti-B is due to the presence of ABO like antigens on bacterial, viral and animal molecules (Hoffbrand *et al.*, 2005).

IgG subclasses also play a role in haemolysis caused by HDFN. There are four subclasses of IgG: IgG1, IgG2, IgG3 and IgG4. Although all the subclasses can cross the placenta, IgG1 and IgG3 are more associated with haemolysis compared to IgG2 and IgG4. The reason is the high affinity of IgG1 and IgG3 for the Fc receptors of the phagocytic cells. Some studies on the effect of IgG1 and IgG3 on haemolysis in neonates with positive DAT due to ABO incompatibility showed that there is no difference in terms of haemolysis between the subclasses (Kaplan *et al.*, 2009).

In HDFN caused by Rh incompatibility, newborns sensitized by IgG1 have more severe haemolysis than those with IgG3, and combination of both may give the most severe form of HDFN (Pollock and Bowman, 1990).

Production of antibodies starts at six month of age. The levels of these antibodies are said to reach their maximum in young adults and gradually decreases with advanced age (Mollison and Engelfriet, 1987). Der Maur et al studied the relationship between ABO antibodies titre and age. They found the same levels of antibodies in both old and young individuals. They however, suggested that decreased ABO antibody titre should not be associated with age but other factors should also be considered (der Maur *et al.*, 1993).

Determination of the concentration of the antibodies present in maternal serum by antibody titration has been used to determine foetuses and newborns at risk of severe hyperbilirubinaemia (Hadley, 2002; Bakkeheim *et al.*, 2009). The antibody titration

will determine the titre of the antibody, which is the reciprocal of the highest saline dilution that gives agglutination when AHG reagent is added.

Concentration of maternal antibodies can also be done using techniques such as auto analyser assay, ELISA, and immunofluorescence. This techniques give a more accurate concentration of the antibody compared to the titration although the titres measured by these techniques do not have much difference with that of titration (Hadley *et al.*, 1991; Ivanković, 2006).

2.1.5 Determination of IgG anti-A, B in maternal serum

Detection of IgG anti-A, in the serum may not give accurate result if naturally occurring IgM anti-A, B are not deactivated. There are many techniques described in the literature which inactivate the IgM antibodies. These include using Dithiotretol (DTT), 2 Mecaptoethanol (2ME), using the stability of IgG antibodies at 70^0 C, and use of some animal substances. Of all the techniques, DTT and 2ME are said to be more advantageous than the others techniques. This is because the other techniques usually leave some active IgM or deactivate some IgG antibodies. DTT and 2ME act by dissociating the 19S IgM molecule into 7S subunit which does not have antibody activity. However, the IgM antibodies are usually left unaltered by these techniques (Knight, 1978).

2.2 Haemolytic disease of the foetus and newborn

2.2.1 Definition and background

Haemolytic disease of the foetus and newborn (HDFN) was first reported in 1609 by a French midwife. Levine explained the cause of the HDFN after the discovery of Rh blood group system by Landsteiner and Weiner (Levine *et al.*, 1941).

Haemolytic disease of the foetus and newborn (HDFN) is a condition in which the red blood cells of the foetus or neonate are destroyed by maternal alloantibodies against an antigen the foetus or neonate inherited from the father. HDFN is commonly caused by Rh (anti-D, anti-c, and anti-E) and ABO (IgG anti-A and anti-B) blood group antibodies. However, antibodies to other blood group systems such as Kell, kidd, duffy and MNSs are also associated (but rarely) with HDFN (Murray and Roberts, 2007).

Haemolytic disease of the foetus and newborn (HDFN) was once upon a time the major cause of foetal and neonatal demise. As stated earlier, the condition was first described by a French Midwife in 1609, who observed a set of twins. The leading twin was swollen and died soon after birth, while the other twin became icteric and died days later. Subsequently, many cases with similar presentations were described in which the neonates failed to survive.

In the middle of the last century, there was a better understanding into the underlying cause of HDN. It was described that the newborn's red blood cells (RBCs) are being attacked by maternal antibodies transferred to the baby from the mother. This attack starts during the period of gestation (in-utero) and is due to an incompatibility between the maternal and foetal antigens present on their respective red blood cells. These antigens include ABO, Rh and other red cell antigens.

There were therapeutic trials in the United States and the United Kingdom in the 1960s that tested the use of antibodies against the proteins that cause HDN from the

mother's circulation. The trials showed that administering therapeutic antibodies to susceptible women during their pregnancy succeeded in preventing HDFN.

During the 70s, there was introduction of routine screening of pregnant women during antenatal care to detect the pregnancies at risk of HDFN, so as to offer timely preventive treatment. This had succeeded in reducing the occurrence of the disease significantly, particularly the severe forms of its presentation that cause stillbirths and neonatal mortality (Ross and de Alarcón, 2013; Dean, 2005b).

2.2.2 Allo-antibodies from mother cross the placenta and attack foetal red cells in-utero

At the time of pregnancy, maternal antibodies against fetal red cell antigens diffuse across the placenta to reach the foetal circulation. This may be beneficial in a way, as by the time of birth, newborns have an immature immune system, so the presence of these maternal antibodies serves a protective function and enhances their survival while their immune system undergoes maturation. The negative effect of this phenomenon is that by targeting the foetal red cells, the maternal antibodies can also cause HDFN.

A major cause of HDFN is an incompatibility between the Rh blood group of the mother and foetus. Of the Rh system, the most antigenic component that commonly causes HDFN is the D antigen, although other Rh antigens, such as c, C, E, and e, can also cause the disease, in order of reducing antigenicity strength.

The people at risk of being involved in HDFN are the Rh D-negative mothers who become pregnant with an Rh D positive foetus that inherits the D antigen from the father. The mother mounts an immune response to the foetal D antigen by forming anti D antibodies against it. The antibodies produced are usually of the IgG type that

can diffuse across the placenta and get transported to the foetal circulation in which they target the foetal red cell D antigen.

HDFN can also arise due to incompatibility between maternal and foetal/neonatal ABO blood group. This occurs when an ABO blood group O mother becomes pregnant with a foetus carrying a different blood group, say A, B, or AB. The serum of blood group O mothers contains naturally occurring anti-A and anti-B, which are usually of the IgG type that can cross the placenta and attack their corresponding antigens on the foetal red cell, causing haemolysis (Hoffbrand *et al.*, 2005).

HDFN that is caused by ABO mismatch usually has a milder clinical presentation than Rh incompatibility. An explanation to this could be due to low expression of the ABO blood group antigens in foetus and neonates compared with adult levels. Another possible reason is, in contrast to the Rh antigens, the antigens in ABO blood group system are present in many foetal tissues, such as kidneys lungs, heart, bone marrow, serum; hence, they serve as many targets for the maternal anti-A and anti-B, reducing the chances of the anti-A and anti-B binding their target antigens on the foetal/neonatal red cells.

Rare causes of HDFN include maternal antibodies directed against Kell blood group system antigens such as anti-K and anti-k, against the Kidd blood group system antigens such as anti-Jka and anti-Jkb, against Duffy blood group system antigens such as anti-Fya, and against MNS and 's' blood group system antigens. To date, there is no known association between antibodies directed against the P and Lewis blood group systems and HDFN (Dean, 2005a; Dean, 2005b).

2.2.3 Mechanisms of Maternal Exposure

It has been known that individuals with blood group O have a natural expression of antibodies to blood group A and B red cell surface antigens. In these people, no previous exposure to blood group antigens is necessary for antibodies to develop. The antibodies are of the immunoglobulin G type and can pass to the foetus across the placenta.

Individuals with blood group A exhibit antibodies against blood group B individuals and vice versa. However, these antibodies are predominantly immunoglobulin M class, and being multimeric, they do not cross the placental barrier.

For all other blood group antigens, there must be maternal exposure and sensitization to the blood group antigens in order for antibodies to develop. The notable cause of maternal sensitization is via mixing of foetal blood with the mother's blood (foeto-maternal haemorrhage, or FMH). This can occur during the first trimester of pregnancy, but is most common during the third trimester or at delivery. Usually, this sensitization occurs during the first pregnancy (Hadley, 2002).

The first encounter of the immune to an antigen causes sensitization and mounting of response by the immune system. If the HDFN is caused by Rh incompatibility, an Rh D-negative mother may be likely sensitized following her first encounter to D antigen while being pregnant with an Rh D-positive child, or via a blood transfusion of Rh D-positive red cells. Once the sensitization to the D antigen occurs, the mother's serum will develop anti-D antibodies. A test for the confirmation of a prior sensitization in the mother is the direct Coombs test (DCT), which shows the presence of anti-D that indicates that the mother has been sensitized.

A small quantity of foetal blood, for example about 0.1 ml that enters the maternal circulation is enough to cause the maternal sensitization. Usually, this foeto-maternal

exchange takes place during the first delivery of an Rh D-positive baby. The possibility of the exchange is enhanced by a prolonged or complicated labour, which in turn increases the risk of sensitization. It can also occur during the pregnancy, for instance, in the event of an abdominal trauma, prenatal bleed or an abortion. It may also be as an iatrogenic cause during medical procedures, such as chorionic villous sampling, amniocentesis or intra-uterine transfusion.

The probability of becoming sensitized to Rh D antigen is less if the foetus is ABO incompatible. The reason for this could be due to the fact that the foetal cells carrying D antigen that gain access into the maternal circulation are rapidly destroyed by maternal anti-A and/or anti-B antibodies, thereby reducing the chances of maternal exposure to the D antigen (Hadley, 2002).

2.2.4 HDN occurs in subsequent pregnancies

The first antibody that is formed against D antigen during sensitization is of the IgM type, which is a multimer and cannot pass through the placenta. Later exposure to D antigen in subsequent pregnancies stimulates the rapid production of IgG anti-D antibodies, which can cross the placenta and enter the foetal circulation. Once in the foetus, the anti-D attaches to the Rh D antigens present on the foetal red cells, causing them to be haemolysed. Less common causes of HDFN (non RhD) include antibodies directed against antigens of the Kell blood group (e.g., anti-K and anti-k), Kidd blood group (e.g., anti-Jka and anti-Jkb), Duffy blood group (anti-Fya, anti-Fyb), and MNS and s blood group antibodies (Basu *et al.*, 2011) .

Women with history of previous transfusion represent only a small proportion of all pregnant women. However, they also represent half of the pregnancies affected by

non-RhD HDFN. The current practice involves routine blood typing and cross-match screen for ABO and RhD blood types, but none is carried out for the other blood antigens involved in HDFN. Survivors of serious childhood illnesses such as cancers and congenital heart diseases are likely to have been sensitized by a prior blood transfusion. As they become mothers, we are likely to see an increase in HDFN (Ross and de Alarcón, 2013).

2.2.5 Mechanisms of Maternal Exposure to Red Blood Cell Antigens

1. ABO group - Innate antibody production, no previous exposure needed

2. Known foetal-maternal haemorrhage

    a. Placental abruption

    b. Other placental bleeding or injury

    c. Foetal surgery

    d. In utero transfusion

    e. Delivery of previous infant

    f. Delivery of previous infant affected by HDFN

3. Risk for unappreciated foetal-maternal haemorrhage

    a. Ectopic pregnancy

    b. Abnormal placental insertion

    c. Spontaneous abortion

    d. Induced abortion

e. Foetal demise

f. Amniocentesis

g. Cordocentesis

h. Chorionic villous sampling

i. Maternal abdominal trauma

j. Foetal version manoeuvres

k. Delivery of previous infant requiring exchange transfusion or phototherapy

4. Known maternal transfusion

5. Maternal history with potential for unappreciated maternal transfusion

a. Prolonged hospital stay as an infant

b. Survivor of childhood cancer

c. Repair of craniosynostosis in childhood

d. Correction of congenital heart defect

e. Major surgical procedure in childhood or adulthood

f. Abdominal surgery in childhood or adulthood

g. Splenectomy for unclear indication (may suggest maternal red cell defect)

h. Return to operating room within 7 days of delivery of an infant

The degree of the haemolysis gives an insight about the nature of the HDFN-whether it is mild, moderate, or severe. Usually in mild cases, the rate of the haemolysis is well tolerated by the foetus. At birth and neonatal period, presentation

includes mild anaemia and jaundice, both of which can spontaneously resolve without medical intervention.

If the rate of haemolysis is high, there may be an elevation of serum bilirubin level. However, it may still remain low during the pregnancy due to the ability of the placenta to filter out the bilirubin from the foetal circulation. After birth, the neonate may not tolerate high bilirubin levels due to liver immaturity, which is unable to metabolize the increased amount of bilirubin, which instead accumulates in its blood as a result of which clinical features of the HDFN become obvious. Within the first day of post natal life, the level of bilirubin may rise dramatically. If levels continue to rise, the bilirubin may cross the blood-brain barrier, enter the brain and cause kernicterus, a potentially fatal condition that can lead to severe sequelae such as permanent neurological damage in the neonates that survive.

A more prolonged and rapid haemolysis of red cells can cause severe anaemia in the foetus/neonate. The liver, spleen, and other organs undergo extra-medullary haematopoiesis and increase their production of red cells to compensate for the anaemia. This extra-medullary haematopoiesis causes the liver and spleen to increase in size (hepatosplenomegaly), and a derangement in liver function can occur. In addition, immature red cells (erythroblasts) are released into the peripheral blood circulation, giving rise to the alternative name of this disease, erythroblastosis fetalis. A notorious complication of severe HDFN is known as Hydrops Foetalis, in which the foetal tissues become oedematous. This condition is mostly fatal, either in-utero or soon after birth.

2.2.6 Red Blood Cell Antigens Most Frequently Involved in Haemolytic Disease of the Foetus and Newborn

Among the known causes of HDFN, RhD incompatibility is the best described. Despite the world wide attempts towards the prevention of HDFN in infants of Rh-negative mothers, to date, Rh still remains the most commonly identified red cell antigen known to be related to HDFN. Individuals who are RhD-negative are said to be lacking the D antigen on their red cell membrane. As reported in literature, 11 percent to 35% of the population of white subjects are RhD-negative. This is due to deletion of the D antigen on their red cell membrane. In contrast, majority of subjects from Eastern countries of Asia as well as Africans that lack red cell membrane expression of RhD have a grossly intact RhD gene. In Africans, a 37-base pair insertion results in the insertion of a stop codon leading to a premature shortening of the protein product (Murray and Roberts, 2007).

A special situation exists where there is an RhD variant called weak D. "Weak D" is an Rh phenotype that is usually present in about 1% of Caucasians and is only slightly more common in African Americans (Westhoff, 2004). It is said to arise as a result of a single amino acid switch in the transmembrane region of the RhD protein. This causes a disruption in the pattern of insertion of the RhD protein into the RBC membrane, causing a decrease in the level of expression of RhD antigen. Even though serologic testing will identify these individuals as RhD-negative, in most cases, adequate levels of D antigen are present and because there has been no change in D epitopes, the formation of anti-D is prevented even if they are transfused with RhD-positive blood.

ABO blood group antigens are the second most common red cell antigens associated with HDFN. In individuals with ABO antigens, HDFN due to the ABO incompatibility is seen most of the occasions in infants of mothers with blood group O. Haemolysis is said to be commoner with anti-A than with anti-B, though the severity of the disease overall is said to be more with anti-B in Blacks. The overall picture of HDFN due to ABO blood group mismatch is hyperbilirubinaemia without severe anaemia. Treatment is by phototherapy, which is sufficient for most of the affected infants (Kaplan and Hammerman, 2005; Ziprin *et al.*, 2005).

Kell antigens of the Kell blood group system are another set of important cause of HDFN. There are many antigens in the Kell blood group systems that are highly immunogenic. They are said to be complex antigens and are known to be the third most potent antigens in triggering an immune reaction.

Antibodies against the Kell blood group antigens can cause hemolytic transfusion reactions as well as hemolytic disease of the foetus and newborn. Kell protein is a glycoprotein that has 15 antigens with their associated variants. It is said to be the first erythroid-specific antigen that is expressed during erythropoiesis. The cases of HDFN that result from anti-Kell are known to suffer severe foetal anaemia because maternal antibodies produced against Kell antigens target fetal red cell precursors (proerythroblasts), suppressing foetal erythropoiesis (Dean, 2005b).

The clinical picture of HDFN caused by anti-Kell is characterized by a more severe anaemia and reticulocytopaenia. Elevation of serum bilirubin is not as markedly seen with the HDFN caused by other RBC antigens. Antibodies against Kell blood group system are rare and are found in only about 0.1% of pregnant women, which mostly

became sensitized following a previous blood transfusion that exposed them to the Kell antigens they lacked (Moise Jr, 2000).

2.2.7 Pathophysiology of HDFN

Immunoglobulin M (IgM) antibodies are the initial antibodies produced by the maternal immune system upon exposure to a foreign antigen. This occurs in almost all cases of HDFN except that which is caused by ABO antibodies. This is because of the natural presence of IgG anti-A and anti-B in group O mothers. Upon exposure to the same antigen, maternal memory B lymphocytes will be stimulated to differentiate in to plasma cells that can rapidly proliferate and produce IgG antibodies. These IgG antibodies will pass through the placenta and attach to foetal red blood cells carrying the corresponding antigen. The cells are then taken to the spleen by macrophages where they undergo extravascular haemolysis. This leads to foetal anaemia. Erythroblasts (immature red blood cells) are released into the circulation to compensate for the anaemia; this is called erythroblastosis foetalis (a term that was previously used to describe HDFN). The bilirubin produced as a result of the haemolysis, is transported across the placenta for excretion by the mother. Continuous anaemia leads to oedema of the foetal spleen and liver which in severe cases leads to hydrops foetalis.

After delivery, maternal system is no longer available for the excretion of bilirubin. Because the liver of newborns is not mature enough to conjugate bilirubin, there will be increased level of unconjugated bilirubin in the circulation which if left untreated can deposit on the basal ganglia of the brain leading to kernicterus (Moise, 2004; Armstrong and Smart, 2008; Ross and de Alarcón, 2013).

2.2.8 Clinical Management of HDFN:

Identifying at risk pregnancies

The clinical monitoring and management of HDFN depend upon events during previous pregnancy, whether the neonates from the past pregnancy were affected by the haemolysis, the type of the red cell antigen involved in the disease, the paternal phenotype and genotype, the presence and quantity of the titre of the maternal antibody involved, and the presentation and severity of the anaemia in the foetus/neonate (Roberts, 2008).

The initial monitoring is by serial indirect Coomb's test (or indirect agglutination test, IAT). An antigen negative woman that is carrying her first pregnancy needs to be monitored by performing a serial anti–RBC antibody titres. A continuous monitoring is recommended even if the titre remains low, until the foetus is delivered at term. If the antibody titre rises above a critical level, then foetus should be monitored by performing serial foetal middle cerebral artery (MCA) velocities. It has been proven that increased MCA velocity correlates well with foetal anaemia. For women with history of HDFN in the past pregnancies, the current pregnancy should be monitored with serial foetal MCA velocities by using Doppler ultrasound (Mari, 2005).

Management of Intrauterine HDFN

Any at risk pregnancy (with positive IAT, rising antibody titre) should be monitored with serial measurement of foetal MCA velocities (using Doppler ultrasound) to monitor for foetal anaemia. Foetuses noted to have mild anaemia should continue to