

Hepatic Sinusoidal Obstruction Syndrome in children in Johannesburg

A Case-Series and Literature Review

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A research report submitted to the Faculty of Health Sciences, Johannesburg, in partial fulfilment of the degree of Master of Science in Medicine in Paediatrics.

University of the Witwatersrand

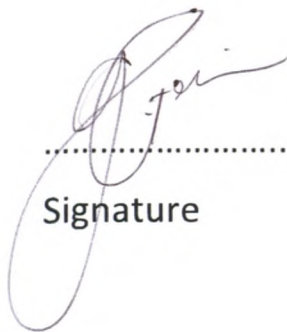
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1. ABSTRACT

Between January 2009 and October 2012, ten patients presented to Rahima Moosa Mother and Child Hospital (RMMCH), all diagnosed with Hepatic Sinusoidal Obstruction Syndrome (HSOS), which is also known as Hepatic Veno-occlusive Disease (HVOD). Hepatic Sinusoidal Obstruction Syndrome is a distinct clinical entity characterized by tender hepatomegaly, ascites and conjugated jaundice. It is caused by a toxin-mediated injury to the hepatic sinusoidal endothelium and hepatocytes which results in the occlusion of the hepatic sinusoids, liver dysfunction and may progress to cirrhosis. It can be due to a variety of toxins, one of them being pyrrolizidine alkaloids, one of the world's most common plant toxins. Common plant species known to contain this toxin are the *Senecio* and *Crotalaria* species. The cause of HSOS in this series of patients was never found but was strongly suspected to be due this plant toxin. The source of this toxin was most likely from traditional remedies, which are commonly used by South Africans. This study is a retrospective observational study, which will focus on the patients' details, including their demographic data, clinical and laboratory findings, management and short term outcomes.

2. DECLARATION

I, Aurelie Gerin, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine in Paediatrics in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



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Signature

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Date

3. ACKNOWLEDGEMENTS

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7. LIST OF ABBREVIATIONS

1. GC/MS.....Gas chromatography/ Mass spectrometry
2. HPLC.....High performance liquid chromatography
3. SCT.....Haematopoietic stem cell transplantation
4. HSOS.....Hepatic sinusoidal obstruction syndrome
5. HVOD.....Hepatic veno-occlusive disease
6. MMP.....Matrix metalloproteinase
7. MCC.....Medicines control council
8. PA.....Pyrrolizidine alkaloid
9. TAM.....Traditional African medicine
10. TMP.....Traditional medicine practitioners

8. OBJECTIVES

The objectives of this study include:

- 1) The presentation of a small group of children diagnosed with HSOS. This will be done as a descriptive analysis of the patients, looking at their demographics, clinical presentation and early outcomes
- 2) A literature review of
 - a. Hepatic sinusoidal obstruction syndrome
 - b. Pyrrolizidine alkaloids and their role in hepatic sinusoidal obstruction syndrome
- 3) A brief discussion of traditional practitioners and traditional African medicine use in the South African context
- 4) The patients will be compared to previously documented cases with hepatic sinusoidal obstruction syndrome in the literature, in terms of their presentation and acute outcomes

9. CASE SERIES PRESENTATION

9.1 STUDY DESIGN

This is a retrospective case-series done at Rahima Moosa Mother and Child Hospital comprising a total of ten patients with histologically confirmed HSOS on liver biopsy. Patient information was obtained from their files and records. Salient points on history, clinical examination, as well as laboratory and histological investigations were collected and presented in a tabulated format.

9.2 SELECTION CRITERIA

Inclusion criteria:

- 1) Children admitted to Rahima Moosa Mother and Child Hospital (RMMCH) Paediatric Unit
- 2) Period of admission between January 2009 and October 2012
- 3) Features of HSOS on liver histology

Exclusion criteria:

- 1) Previous exposure to chemotherapeutic drugs or radiation
- 2) No available liver histological features of HSOS
- 3) Presentation to Rahima Moosa Mother and Child Hospital outside of the above period

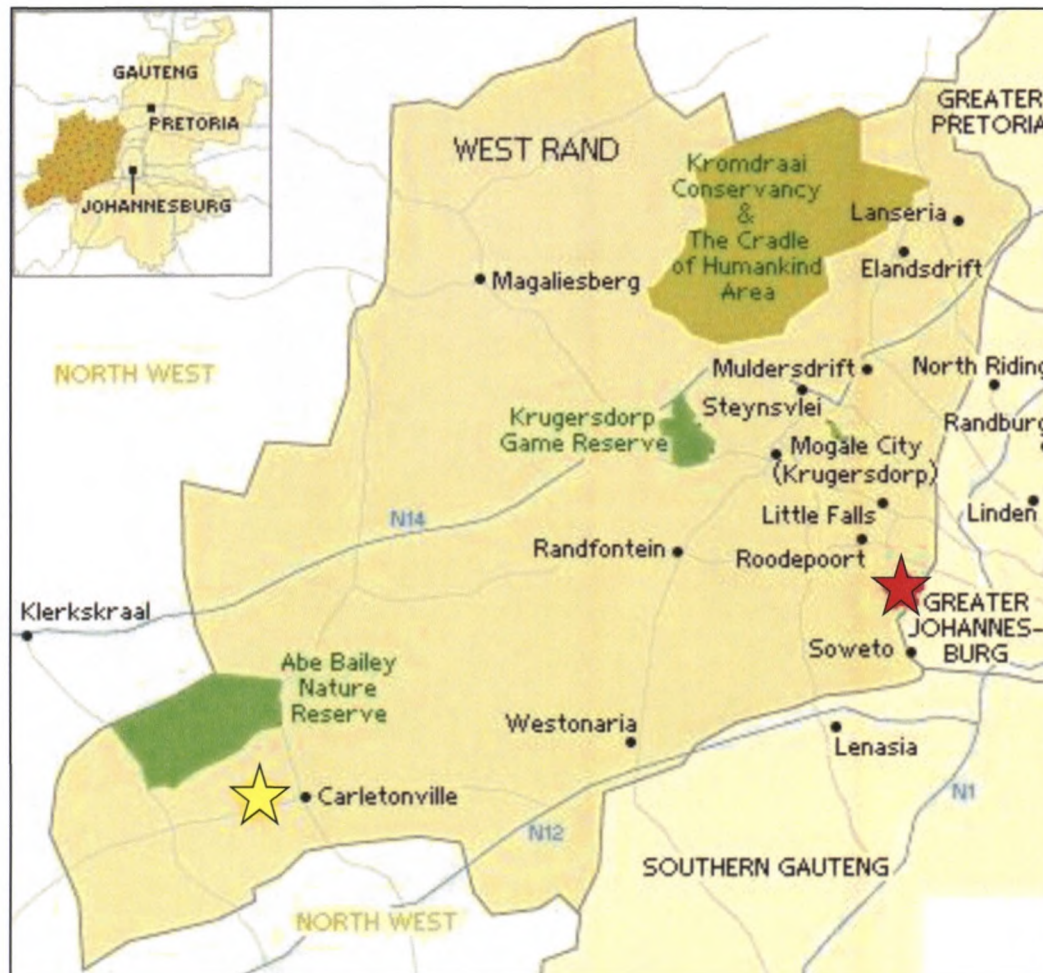
9.3 DEMOGRAPHIC DATA

All ten patients were of African ethnicity. Five of the patients were female and five were male. The mean age at presentation was 13 months old, (range 2-32 months). All but two of the patients originated from the Gauteng West Rand. All of these patients were from poor social backgrounds. A nutritional history was not available for all of these patients.

Table 1. Demographic data

Patient number	Age (months)	Sex	Area origin
1	32	M	Bekkersdal
2	10	F	Carletonville
3	5	M	Carletonville
4	29	M	Carletonville
5	2	F	Carletonville
6	4	M	Carletonville
7	13	F	Vrededorp
8	4	F	Carletonville
9	5	F	Carletonville
10	30	M	Westbury

Figure 1: Map of the West Rand



★ Rahima Moosa Mother and Child Hospital

★ Carletonville

9.4 HISTORY AND CLINICAL PRESENTATION

A definite history of Traditional African medicine (TAM) ingestion was present in four out of the ten patients, but suspected in all of the patients. The TAMs were not made available for testing in any of the

patients, despite requests. The predominant presenting complaint was abdominal distention found in all of the cases. Other gastrointestinal symptoms were found in half of the patients.

Table 2: History of TAM ingestion and main presenting complaints

Patient number	TAM ingestion	Main complaints
1	Suspected	A fourteen day history of abdominal distention
2	No history given by caregiver	A two day history of abdominal distention
3	No history given by caregiver	A four day history of abdominal distention, vomiting after feed and constipation
4	Confirmed	A ten day history of abdominal distention, vomiting and diarrhea
5	Confirmed	A fourteen day history of abdominal distention and diarrhea
6	Suspected	A four day history of abdominal distention and malaise
7	No history given by caregiver	A four day history of abdominal distention and diarrhea
8	Confirmed	An eighteen day history of abdominal distention and irritability
9	No history given by caregiver	A two day history of abdominal distention, diarrhea and haematemesis
10	Confirmed	A fourteen day history of abdominal distention, vomiting, jaundice and cough

Two of the patients had failure to thrive. Jaundice was clinically observed in five of the patients. The most striking clinical feature in all of the patients was severe ascites. Hepatomegaly was palpable in seven of the patients, and difficult to appreciate in three due to massive ascites, but subsequently seen on ultrasound. Splenomegaly was found in six of the patients. Pedal oedema was present in nine patients. None of the patients were noted to be encephalopathic. None of the patients had digital clubbing on first presentation.

Table 3: Clinical Presentation

Clinical feature	Prevalence
Failure to thrive	2/10
Jaundice	5/10
Ascites	10/10
Hepatomegaly	10/10
Splenomegaly	6/10
Pedal oedema	9/10
Encephalopathy	0/10

9.5 LABORATORY FINDINGS AND LIVER HISTOLOGY:

Eight of the patients were found to have anaemia. Thrombocytopenia was present in four of the patients. Eight of the patients had a conjugated hyperbilirubinaemia. Hypoalbuminaemia was found in all of the patients. The gamma glutamyl transferase enzyme was raised in nine of the patients. Seven patients had a transaminitis. Eight of the patients were coagulopathic. Two of the patients had bacterial sepsis. One patient had spontaneous bacterial peritonitis. All of the patients were HIV negative. Serology for the viral hepatitis A, B, C and cytomegalovirus were negative in all of the patients. The ascitic fluid was found to be transudative in nature in all of the patients.

Table 4: Laboratory findings

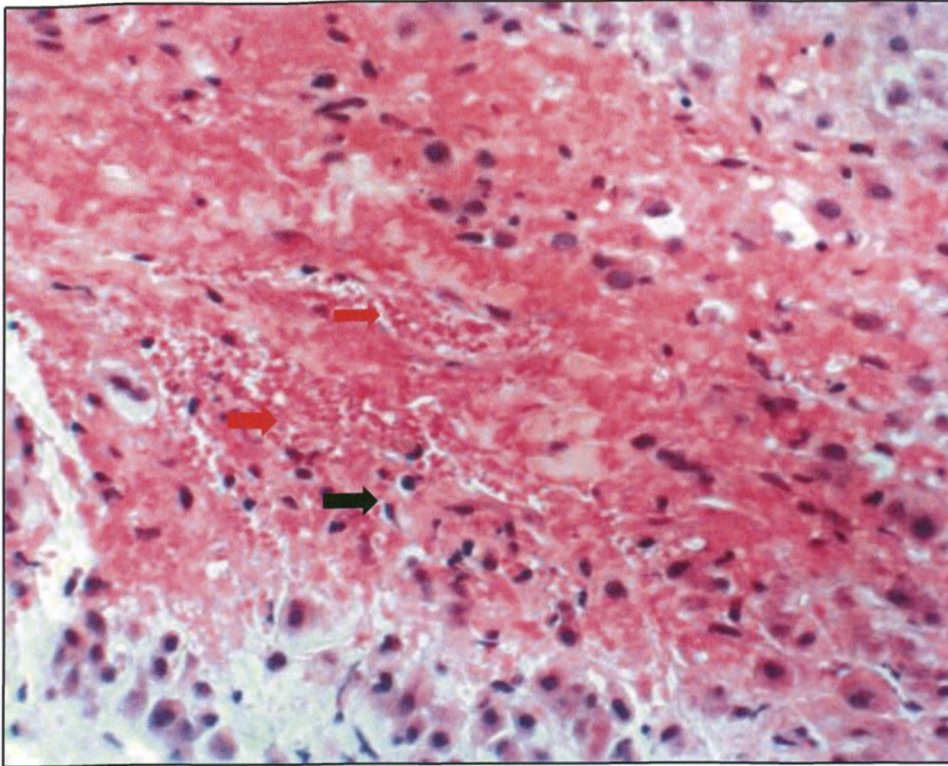
Laboratory finding	Prevalence
Anaemia	8/10
Thrombocytopenia	4/10
Conjugated bilirubinaemia	8/10
Hypoalbuminaemia	10/10
Transaminitis	10/10
Coagulopathy	7/10
Sepsis	3/10

Closed liver biopsy was performed in nine of the patients, whilst case 6 underwent an open wedge liver biopsy. Centrilobular ischemia and sinusoidal dilatation and congestion were found in all of the cases. Hepatocyte changes included hepatocyte drop out, necrosis and balloon cell degeneration. Fibrosis of varying degrees was found in nine of the patients, and central venule obliteration was noted in seven. Lobular disarray was found in five of the patients.

Table 5: Liver histology

Case no	No. of portal tracts	Centrilobular sinusoidal dilatation and congestion	Hepatocyte changes			Fibrosis	Central venular obliteration	Lobular disarray	Steatohepatitis	Central vein thrombosis
			Ischemia or necrosis	Balloon cell degeneration	Nuclear atypia					
1	6	+	+	-	-	early	+	mild	mild	-
2	2	+	+	-	-	early	-	mild	-	-
3	10	+	-	-	-	early	+	-	-	-
4	13	+	+	+	-	early	+	+	mild	-
5	17	+	+	-	-	early	early	mild	mild	-
6	(wedge biopsy)	+	+	-	-	+	+	-	-	-
7	15	+	+	+	+	+	+	+	-	-
8	17	+	+	-	-	early	+	-	-	-
9	14	+	+	-	-	early	-	+	mild	-
10	(wedge biopsy)	+	+	-	-	-	-	+	mild	-

Figure 2: Liver biopsy histology slide of Case 2



Masson trichrome stain showing conspicuous vascular ectasia and centrilobular congestion (red arrows) as well as necrosis and focal sclerosis (black arrow).

9.6 MANAGEMENT

All of the patients were admitted and managed supportively whilst being investigated.

The average duration of admission was 3.5 weeks, range of 2-5 weeks. Supportive management for liver dysfunction, including vitamin supplementation, were instituted. One patient was severely coagulopathic and required the transfusion of fresh frozen plasma. Sepsis was managed with the appropriate antibiotic. As all of the patients had ascites and the majority were oedematous, at least one diuretic was required in all the cases. The use of diuretics was to reduce extracellular fluid accumulation, maintain intravascular volume and improve renal perfusion. Spironolactone was the initial diuretic used. A dose of 1-5 mg per kilogram per day was used, with doses increased gradually to effect. In four of the patients, a second

diuretic was required, and hydrochlorothiazide was used. The diuretics were later tapered to a lower dose as the ascites and oedema improved. The ascites caused significant discomfort and respiratory embarrassment in seven of the patients, therefore requiring serial therapeutic paracentesis. The infusion of albumin was required in eight of the patients who had severe hypoalbuminaemia and ascites.

Table 6: Treatment given

Treatment given	Prevalence
Spironolactone	10/10
Hydrochlorothiazide	4/10
Paracentesis	7/10
Albumin infusion	8/10
Vitamin supplementation	10/10

9.7 OUTCOMES AND FOLLOW-UP

In all except for one patient, the symptoms and clinical picture showed initial improvement during admission with diuretics and supportive management. These patients were discharged on diuretics and vitamin supplementation and followed up as outpatients. The diuretics were progressively weaned to stop in most cases. Several of the patients developed complications after discharge. The mean duration of follow-up was 8 months (range 1-36 months).

The residual clinical signs of the patients included hepatomegaly in one patient, hepatosplenomegaly in six of the patients, and portal hypertension in three of the patients. Three of the patients developed recurrence of acute ascites after discharge, probably due to the development of portal hypertension. These

relapses occurred 1 month after discharge in two of the patients and 8 months after discharge in one patient. Long term outcomes were unknown in 40% of the patients.

Clinical cirrhosis developed in all of the patients who followed up for a period longer than six months. These comprised 40% of the patients. Haemorrhagic gastritis and hepatopulmonary syndrome occurred as a complication of chronic liver disease in two of these patients.

Case 6 demised from the acute complications of HSOS during initial admission. He was found to have *E. coli* sepsis, for which he was treated. His initial abdominal sonar showed gross ascites as well as mildly dilated left calyceal system. His renal abnormalities were not further investigated. Although his renal function was normal on admission, it began to worsen two weeks after admission and he developed severe electrolyte imbalances. He also became coagulopathic. He was treated for these disorders but deteriorated over a few days and demised. The cause of his death was probably due to Gram-negative sepsis compounded by liver and renal dysfunction.

Case	Age (Mo)	Wasting	TBR	ALT	Sepsis	Central vein obliteration	Short term outcome	Relapse	Other	Follow-up duration (Mo)	Long term outcome
1	31	No	13	557	Nil	Present	Well	Yes	Nil	4	Unknown
2	10	Yes	21	72	Nil	Nil	HM	No	Nil	3	Unknown
3	6	No	91	28	Nil	Present	HSM	No	Nil	6	Unknown
4	27	No	91	1150	<i>S. aureus</i>	Present	HSM	No	Hematemesis	13	Cirrhosis
5	2	No	31	141	Nil	Nil	Well	No	Nil	1	Unknown
6	4	No	185	136	<i>E. coli</i>	Present	Death	N/A	HRS Liver failure	N/A	N/A
7	13	Yes	100	25	<i>S. aureus</i>	Present	HSM PHT	Yes	Liver failure	12	Cirrhosis
8	4	Yes	40	39	Nil	Present	HSM PHT	Yes	Nil	6	Cirrhosis
9	5	No	25	43	Nil	Nil	HSM	No	Nil	3	Unknown
10	2	No	158	128	Nil	Present	HSM	No	HPS	36	Cirrhosis

Key:

ALT = Alanine transaminase
TBR = Total bilirubin
HM= Hepatomegaly
HSM= Hepatosplenomegaly
HRS = Hepatorenal syndrome
HPS = Hepatosplenomegaly

S. aureus = *Staphylococcus aureus*
E.coli = *Escherichia coli*
Mo = Months

Table 7: Outcomes and follow up

10. LITERATURE REVIEW

10.1 DISEASE DEFINITION

Hepatic sinusoidal obstruction syndrome (HSOS) or hepatic veno-occlusive disease (HVOD) is a rare clinical syndrome presenting with a clinical triad of ascites, tender hepatomegaly and jaundice. It has characteristic findings on liver biopsy of sinusoidal congestion and centrilobular haemorrhage, necrosis and fibrous tissue deposition. It may cause acute or, more commonly, chronic liver failure with progression to cirrhosis. The management of HSOS is supportive as there is no effective cure. Thus, primary prevention is an important focus.

Hepatic sinusoidal obstruction syndrome may occur in the following circumstances:[1]

- 1) Following the ingestion of phytotoxins called pyrrolizidine alkaloids (PAs)
- 2) As an iatrogenic adverse effect of the following
 - Preconditioning regimens used in haematopoietic stem cell transplantation
 - Chemotherapeutic drugs
 - High dose irradiation
 - Immunomodulators (azathioprine and cyclophosphamide)

In children, most documented cases of HSOS in the current literature have been in the setting of haematopoietic stem cell transplantation, and as an adverse effect of the chemotherapy used in the management of Wilms' tumour and rhabdomyosarcoma [2]. Occasionally, cases of pyrrolizidine alkaloid-associated HSOS are reported on.

Up until 2002, the disease had been known only as *Hepatic veno-occlusive disease* (HVOD) when it was proposed by DeLeve et al to rename it *Hepatic Sinusoidal Obstruction Syndrome* (HSOS) [1]. However, the terms hepatic veno-occlusive disease and hepatic sinusoidal obstruction syndrome are synonymous and

are used interchangeably in the literature to describe the condition. For the purposes of this thesis, the condition will henceforth be referred to as hepatic sinusoidal obstruction syndrome (HSOS).

10.2 HISTORICAL SIGNIFICANCE OF HSOS

Until the 1970's, before the advent of chemotherapy and stem cell transplantation, HSOS was mostly linked to PA toxicity. Reports of suspected or proven PA-associated HSOS have been reported internationally in humans and animals. Cases affected have varied from isolated individuals to devastating mini-epidemics.

Below is a table summarizing some important publications and landmark discoveries of HSOS from the 19th to the 21st century.

Table 8: Timeline of HSOS

<u>TIMELINE OF HSOS</u>	
1800'S FIRST REPORTS IN ANIMALS	
1880	"... Pictou Cattle disease", Derbyshire, Canada 1880
1900	"Winton disease", Gilruth, New Zealand 1902 "Molteno Cattle disease and Dunsiekte", South Africa ~ 1907
1910	"Walkabout disease in horses", Australia ~ 1910 "...Senecio Alkaloids and the causation of Hepatic cirrhosis in cattle", Cushny, 1910, UK
1900'S FIRST REPORTS IN HUMANS	
1920	"Senecio Disease and cirrhosis of the liver due to Senecio poisoning", Willmot et al, South Africa
1930	"Outbreak of camel belly..." Uzbekistan 1931 "...types of liver cirrhosis in Egyptian children." Hashem, Egypt 1939
1940	"Enlarged liver with ascites in children..." McFarlane and Branday, Jamaica 1945
1950	"Senecio poisoning exhibiting as Chiari's syndrome" Seltzer and Parker, South Africa, 1951 "Serous Hepatosis", Hill and Rhodes, Jamaica, 1953
1954 COINING OF TERM: HEPATIC VENO-OCCLUSIVE DISEASE	
1950	"Veno-occlusive disease of the liver..." Bras and Hill, Jamaica, 1954 "Bush teas cause of HVOD" Bras et al 1956
1960	"Veno-occlusive disease of the liver" Stein and Isaacson, South Africa, 1962 "Hepatic vein occlusion disease in Egyptian children" Safouh, Egypt, 1965 "Veno-occlusive disease of the liver" Freiman, South Africa, 1968
1970	"An epidemic of veno-occlusive disease...in central India" Tandon, India, 1976 "An epidemic of veno-occlusive disease ... in Afghanistan" Tandon, Afghanistan, 1978
1979 FIRST REPORTS AFTER BONE MARROW TRANSPLANTION	
1980	"Veno-occlusive disease of the liver after allogenic bone marrow transplantation..." Berk, USA 1979
1990	"An outbreak of Heliotrope food poisoning in Tajikstan" Chauvin, Tajikstan, 1994
2000'S PROPOSED NAME CHANGE TO HEPATIC SINSUOIDAL OBSTRUCTION SYNDROME	
2000	"Toxic injury to hepatic sinusoids: sinusoidal obstruction syndrome..." deLeve 2002 "Clinical and analytical aspects of pyrrolizidine alkaloid poisoning ..." Steenkamp et al, South Africa, 2000 "Hepatic veno-occlusive disease as a result of a traditional remedy..." Zuckerman et al, South Africa, 2002

2010	"An outbreak of Hepatic Veno-occlusive disease in Western Afghanistan..." Kakar et al, Afghanistan, 2010
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10.2.1 REPORTS IN LIVESTOCK

The first account of HSOS was in livestock at the turn of the nineteenth century. The animals affected included mostly cattle and horses. These presented with gastrointestinal symptoms, abdominal distention and emaciation. The outcomes were often fatal. Post mortem liver histology showed hepatic congestion and cirrhosis.

Initially, this disease was described in cattle in Canada as Pictou disease in 1880, where thousands of cattle were lost [3], then in New Zealand in 1889 as Winton disease [4], and South Africa as Molteno cattle disease in 1907 [5]. Contamination of grazing pastures by species of the *Senecio* plant proved to be the cause of the outbreaks, using feeding experiments on cattle.

In Australia, from as far back as 1897, hundreds of horses and cattle were reported to have died from a condition which is still known as "Walkabout" disease. The affected animals would appear to be blind and aimlessly wander about, probably as a consequence of hepatic encephalopathy, hence the name Walkabout disease. It is now known that this condition is due to PA-associated HSOS from the ingestion of *Crotalaria* species [6].

In 1910 Cushny, a Scottish doctor and researcher, took into account the above veterinary reports and became one of the first researchers to analyse the suspicious plants. He isolated certain alkaloids in the plants, then known as *Senecio* alkaloids, and studied their effects in experimental animals, and confirmed the link to human alkaloid poisoning [7].

10.2.2 REPORTS IN HUMANS

The first known documented human cases of HSOS were from South Africa in 1920, as documented by Willmot et al [8]. They reported on a series of eleven cases from the Western Cape who presented with abdominal pain, nausea and vomiting, as well as abdominal distention. The clinical disease had been known to exist in this region for at least a decade prior, affecting some eighty people, although these had never been reported on. It was

found to occur mostly in underprivileged Europeans households, whose staple diet was bread, and occurred mostly in children. Prognosis was poor with many fatalities and management was supportive. Post mortem liver biopsy showed liver congestion, central hepatic venular dilatation and cirrhotic changes in advanced cases. Of note, gastric ulcers were found in some patients. Inadequately winnowed wheat, contaminated with *Senecio* species proved to be the cause of the outbreak. This was demonstrated by conducting feeding experiments on rodents, in which the disease was reproduced. The authors tentatively called the condition "Senecio disease".

Later studies observed that other species of plants, namely *Crotalaria* and *Boraginaceae*, caused the same disease. "Seneciosis" thus came about as a generic term for the condition caused by the above plant genera [9]. This term is still used today to describe the disease in animals.

In Jamaica from the 1940's to the 1960's, liver disease progressing to cirrhosis among Jamaican children was recognized as a significant public health problem [10]. This prompted many researchers in this Caribbean island to investigate the aetio-pathophysiology of cirrhosis in these children. Numerous case-series were investigated during this time and contributed to the understanding of the condition. The researchers noticed trends in all the children studied: the condition affected young children (18-36 months old); they came from lower socio-economic groups; their diets were devoid of protein; and the majority routinely ingested traditional Jamaican "bush teas". Relevant studies that emanated from this Jamaican cohort included:

- 1) In 1945, McFarlane et al. studied eighteen children affected by the condition, causing hepatic enlargement with ascites, and suspected it to be inflammatory or toxin mediated [11].
- 2) In 1953, Hill et al. investigated one hundred and fifty Jamaican children with liver disease [12]. They performed liver biopsies on half of these patients. The histological picture showed a sequence of events, beginning with a serous exudate and sinusoidal congestion in the peri-sinusoidal space of Disse and centrilobular venules, and progressing to cirrhosis. The researchers called the condition "Serous Hepatosis" because of these histological features [12]. The condition was suspected to be due to a protein deficient diet, with toxins from the bush teas perhaps contributing a role.

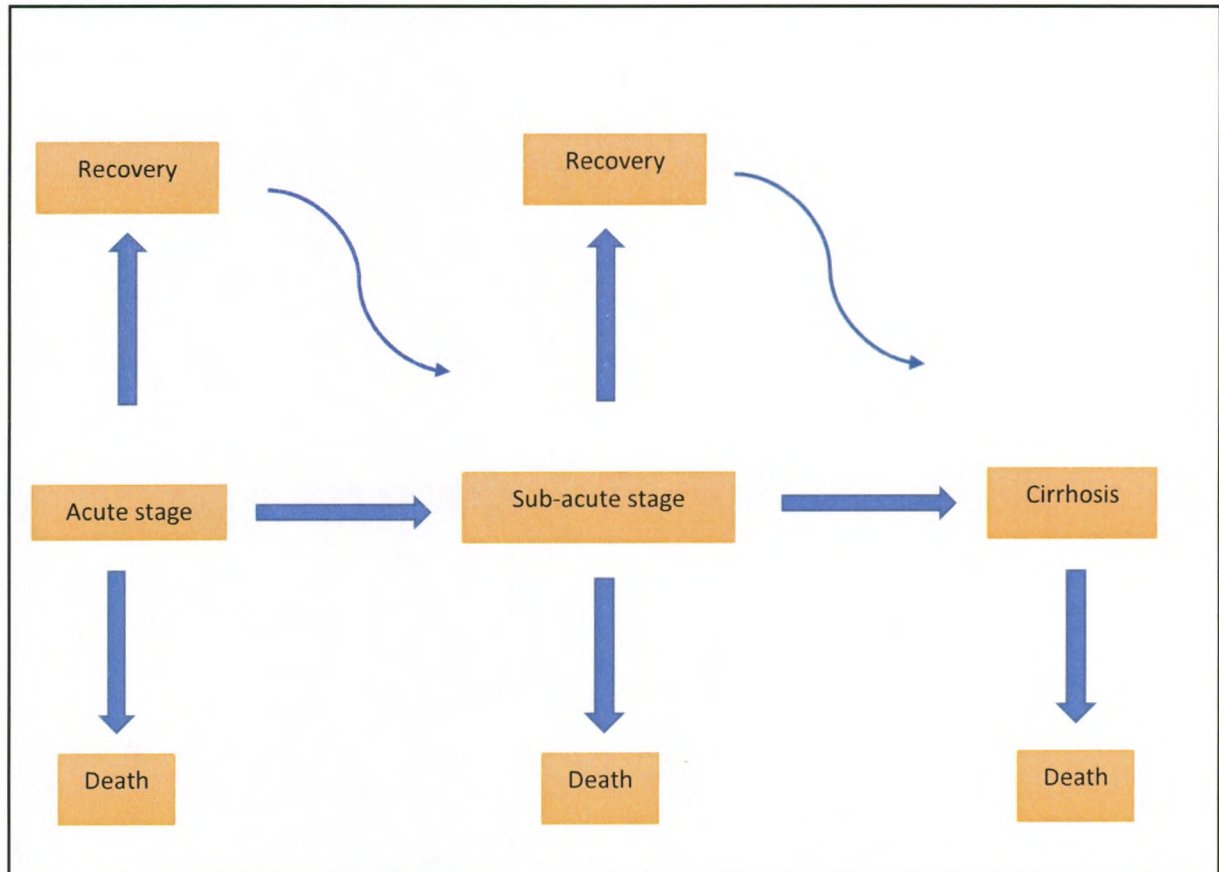
- 3) From 1954 to 1956, various authors, including Bras, Hill and Stuart added to the growing knowledge on HSOS.

Bras et al concluded that obliteration and inflammation of the central hepatic venules was fundamental to the disease, so they proposed to name it *Hepatic Veno-Occlusive Disease (HVOD)* [13]. They developed a clinical and histological staging system for HSOS, which is still applicable in PA-associated HSOS [14]:

- a. **Stage one (The acute stage)** consisted of a sudden onset of gastrointestinal symptoms and abdominal distention, with hepatomegaly and ascites lasting four to six weeks. This correlated histologically with centrilobular congestion, sub-endothelial intimal thickening and partial occlusion of the central hepatic venules.
- b. **Stage two (The sub-acute stage)** consisted of persistent firm hepatomegaly, and recurring episodes of ascites. Splenomegaly could be present. The ascites could subside with the development of a collateral circulation. Histologically, central hepatic venule congestion and obliteration by necrotic hepatocytes and fibrous tissue were present.
- c. **Stage three (The chronic stage)** manifested clinically with features of cirrhosis and correlated histologically with centri-lobular cirrhosis

These researchers described the progression of HSOS as having a progressive downhill course over months or years. Some patients showed complete recovery after the acute stage, whilst some showed initial recovery then progression to the sub-acute or chronic stage. Some died during the acute stage from fulminant liver failure. The patients in the sub-acute stage could progress directly to the chronic stage or recover. Some presented for the first time in the sub-acute or chronic stage, with no history of any preceding illness. Of note, jaundice was rarely found in the acute and sub-acute stages.

Figure 3: Natural history of PA-associated HSOS as described by Hill et al: [14]



As mentioned, the aetiology of HSOS in Jamaican children had long been postulated to be due to toxins found in Jamaican “bush teas”, particularly in undernourished children. The use of such teas was known to be a part of Jamaica’s cultural heritage, consumed for traditional and medicinal reasons. At the same time, Stuart was involved in the pharmacological analysis of these infusions, and found that these commonly contained *Crotalaria* extracts. Then, Bras et al. looked at veterinary literature of livestock poisoning by *Senecio* plants and found a close similarity in the liver histology of the human cases and the animals [15]. They produced hepatic lesions identical to HSOS in a calf by feeding it *Crotalaria fulva*, thus proving that bush teas were an aetiological factor of HSOS. Public education campaigns stressing the toxicity of bush teas were undertaken in Jamaica, and in the next decade there was a dramatic fall in the number of HSOS cases [16].

10.2.3 SOUTH AFRICA'S CONTRIBUTION TO HSOS IN HUMANS

South Africa has contributed several advances and case-series on PA-associated HSOS to the literature from the 1920's to the 21 century.

In 1951, three decades after Willmot described Senecio disease, Selzer and Parker published a case-series report of twelve patients from the Western Cape region [17]. These patients presented with features typical of acute HSOS. Half of the patients were children between the ages of four to thirteen years old. As the condition occurred in households, it was suspected, but not proven, that the consumption of improperly winnowed wheat with *Senecio* plants was the cause. Only the acute outcomes were studied, and half of the patients demised. Post mortem liver biopsies were performed on these patients, showing hepatomegaly and liver congestion in most cases and thrombi in the hepatic venules in three cases. Central hepatic vein congestion was found in all cases. The researchers thought that the disease originated from a lesion obstructing the hepatic venules, and concluded that their patients were examples of Budd Chiari's Syndrome, although given the history of contaminated bread, these patients were probably cases of PA-associated HSOS.

In 1962 and 1968 at Chris Hani Baragwanath hospital, Stein et al. [18] and Freiman et al. [19] respectively presented two case-series of African children with typical clinical and histological HSOS. The first series comprised of twelve patients, and the second of fifteen patients. The researchers compared these patients to the Jamaican series of HSOS and suspected that they were dealing with the same condition. They found that their patients were similar to the Jamaican series in terms of socio-economic standing, diet and nutritional status. The cause of the condition was unknown, but suspected to be from toxins in (traditional African Medicines) TAMs, which were commonly used in South Africa. The patients' clinical presentation and acute outcomes were studied. The case fatality rate was 42% in the first series and 67% in the second series. They found that the deaths tended to occur in younger patients who were jaundiced and presented in the acute stage of illness.

Furthermore, in 2000, Steenkamp et al. presented a series of twenty children with clinical and histological HSOS [20], all presenting in the acute stage of the disease. TAMs were suspected to be the cause of their illness. Nine

of the patients demised, and severe jaundice at presentation was once again noted to be a poor prognostic feature. Portal hypertension and cirrhosis developed in all of the surviving patients who were followed up over an unspecified time period. The researchers commented that, in the setting where HSOS is endemic, the clinical presentation of the acute phase of HSOS is virtually diagnostic of the disease. They also suspected that PA-associated HSOS in South Africa occurred more frequently than estimated.

Additionally in this study, Steenkamp et al. performed a colorimetric screening test on urine to prove the presence of PA intoxication in the patients. On admission, urine samples were performed on four of the patients. The test consisted of colorimetric testing and demonstrated the presence of PA's on all four of the samples, confirming that the condition was due to PA poisoning.

Similarly in 2002 at RMMCH, Zuckerman et al. described a fatal case of PA-associated HSOS in an infant from TAM ingestion [21]. Of note, the infant was septic which contributed to her deterioration. The TAM was analysed using colorimetry to detect PA's followed by gas chromatography/mass spectrometry (GC/MS) to identify the PA subtypes. *Senecio latifolius* was isolated in the mixture. In addition, *in-vitro* studies demonstrated that hepatocytes treated with the TM extract underwent necrosis and apoptosis.

10.2.4 OUTBREAKS OF HSOS IN HUMANS

PA-associated HSOS of epidemic proportions have been documented from Central and South Asia from the 1930's to 2010, due to the PA contamination of wheat and cereal. In Uzbekistan in 1939, one thousand five hundred people were affected by a condition then known as "Camel Belly", which was in fact, HSOS due to poisoning with *Heliotropium* species [22]. In India in 1976, Tandon et al. reported on a series of a hundred cases of HSOS, all due to contamination of cereal with *Crotalaria* species [23]. In Afghanistan, two outbreaks were reported, both due to contamination of wheat with *Heliotropium* species, the first in 1975 by Tandon, where almost eight thousand people were affected, and then in 2010, with sixty seven people reportedly affected [24]. In Tajikistan in 1992, a further outbreak occurred, affecting three thousand nine hundred people [25].

10.2.5 HSOS IN THE ONCOLOGY SETTING

Hepatic sinusoidal obstruction syndrome was first described as an adverse effect to the preconditioning chemotherapeutic regimens used in haematopoietic stem cell transplantation (SCT) by Beck in 1979 [26]. Since then, chemotherapy used in this setting has become the most common cause of HSOS in the Western hemisphere, contributing significantly to patient morbidity and mortality [27].

Other than in the setting of SCT, HSOS has also been described sporadically in association with certain chemotherapeutic drugs, radiation therapy, and immunosuppressants.

The incidence of HSOS in children following SCT varies and was found to be between 11-20% by Cesaro in 2005, depending on the treatment regimen used and the risk factors present in the patient [28]. In 2013 at Chris Hani Baragwanath hospital, Andrade reported on a series of children treated for Wilm's tumour, and found that 23% of these patients developed clinical HSOS [29].

HSOS has been widely researched since it was found to be associated with chemotherapeutic agents. This has expanded current knowledge of HSOS in terms of understanding its pathophysiology; identifying risk factors for its development; developing diagnostic clinical criteria to recognize it and stage it clinically, and in developing novel therapy for its management [30].

A landmark in the pathophysiology of HSOS was described by deLeve et al. in 2002, when they concluded that HSOS is a disorder of the hepatic microcirculation originating in the hepatic sinusoids rather than the central venules. This brought about the conditions' name-change to HSOS [1].

Novel therapy in the setting of iatrogenic HSOS include the use of defibrotide and N-acetylcysteine, which have shown to be beneficial in drug trials [31].

10.3 PYRROLIZIDINE ALKALOIDS AND PYRROLIZIDINE ALKALOID ASSOCIATED HEPATIC SINUSOIDAL OBSTRUCTION SYNDROME

10.3.1 INTRODUCTION TO PYRROLIZIDINE ALKALOIDS

Pyrrolizidine alkaloids are phytotoxins occurring in three percent of the world's flowering plants and an estimated six thousand plant species [32, 33]. Alkaloids are nitrogen containing compounds, and PA's are alkaloids that typically contain a necine base. The main sources of PAs are from three plant families: *Boraginaceae*, *Compositae* (genus *Senecionae*) and *Leguminosae* (genus *Crotalaria*). These plants are widely distributed, growing in most climates all over the world [34, 35].

Certain *Senecio* and *Crotalaria* species are indigenous to South Africa [34]. Around five hundred species of *Senecio* are indigenous to South Africa, and most of these are regarded as weeds.

Figure 4: *Crotalaria capensis*

Indigenous to South Africa and known to contain Pyrrolizidine alkaloids.



10.3.2 EFFECTS OF PYRROLIZIDINE ALKALOID INTOXICATION

In animals, PA intoxication is known to produce HSOS, and other systems have also been proven to be affected. These include pulmonary toxicity and pulmonary hypertension, neurotoxicity, and nephrotoxicity [22]. Pyrrolizidine alkaloids have been shown to be carcinogenic and teratogenic in animals [36]. In addition, PAs have also been shown to have an immunosuppressant effect in animal studies.

Pyrrolizidine alkaloid intoxication in humans primarily causes hepatotoxicity in the form of HSOS as will be discussed below. Rarely, cases of pulmonary hypertension have been reported in humans following the ingestion of PAs [22, 37]. In Uzbekistan in the 1940's, the contamination of grain by *Trichodesma* species, known to contain PAs, is believed to have caused encephalitis in two hundred patients [22]. Pyrrolizidine alkaloids have not been proven to be carcinogenic in humans. However, they have been shown to induce cell changes [38], which may play a role in carcinogenesis, especially hepatocellular carcinoma.

10.3.3 TOXICOKINETICS

Pyrrolizidine alkaloid intoxication may occur after ingestion of PAs or absorption of PAs through broken skin. PAs are metabolized by the hepatic cytochrome p450 enzymes within a few hours after ingestion [37]. This results in the formation of intermediates that are toxic to hepatocytes and sinusoidal endothelial cells as will be discussed under the next section.

The majority of PA metabolites are excreted in the urine and faeces within two to three days. However, PA metabolites can persist in hepatocytes long after exposure by forming adducts with DNA, RNA and cellular proteins [38]. These can have an anti-mitotic effects. *In vitro* studies of cultured human hepatocytes treated with PAs show mutagenicity such as necrosis and apoptosis [38].

Exposure time and dosage amount affect PA toxicity. Animal studies have shown that a single large dose may cause acute liver failure. Recurring small doses of PA have a more subtle presentation but ultimately can lead to cirrhosis [33, 39].

Certain host factors, such as young age and protein deficiency, has been shown to increase the toxicity of PA's in animal studies [33].

10.3.4 IDENTIFICATION OF PYRROLIZIDINE ALKALOIDS

Pyrrolizidine alkaloids are one of the few phytotoxins in South Africa that can be specifically identified in grains, foodstuffs, complementary medicines and TAMs [40].

Various laboratory techniques may be used to detect PA's in plant material or body fluid. Colorimetry may be used as a screening test to detect the presence of PA's [22]. Gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) are more sensitive and specific methods used to identify specific PA's in a sample [22]. These are not widely available in South African laboratories, and clinicians are ignorant of the services available, and as to what specimens are required for testing.

10.3.5 ROUTES OF EXPOSURE OF PYRROLIZIDINE ALKALOIDS

In humans, PA toxicity has been described mostly through the ingestion of plants containing these toxins. Weeds containing PA's may contaminate wheat or maize crops through inadequate winnowing practices [34]. Honey, eggs and milk have also been shown to contain PA's, although in very small amounts [22][35]. Topical application of pastes containing PA's on broken skin may also result in PA intoxication.

As discussed previously in the Jamaican and South African experience, some cultures and alternative practitioners use plants in traditional remedies and infusions that may contain PA's [14, 33]. Plants of the *Senecio* species have been used by traditional healers across the world for their specific medicinal effects. Examples of these in South Africa include the topical use of *Senecio latifolius* for the treatment of burns and its use as an abortifacient when given orally [33]. There has also been a growing interest in herbal remedies in the developed world, resulting in a growing market for complementary medicine, and the risk of PA exposure [33, 41].

10.3.6 REGULATION OF EXPOSURE TO PYRROLIZIDINE ALKALOIDS

As PA's are widespread and well-known for their toxic effects, efforts have been made in many countries to ensure that foodstuffs, agricultural crops, and complementary medicines are free of, or contain a safe minimum amount of PA's.

1. In foodstuffs: Comfrey (*Symphytum officinale*), a well-known medicinal herb, is known to contain PA's. The Foodstuffs, Cosmetics and Disinfectants Act of 1972 has prohibited the sale and use of this herb in South Africa since 2003 [42].
2. In agriculture: International guidelines have been drawn up by the Codex Alimentarius Commission to prevent the contamination of staple grains by PA's in agriculture. South Africa is a co-signatory in these guidelines. These state that staple grains produced for human consumption may not contain more than a threshold concentration of certain toxic seeds. *Crotalaria* seeds are considered as one of these [41].
3. In complementary medicines: Certain countries, including Germany, the UK and the USA, have set up regulations limiting exposure to PA's in herbal remedies [41].

In South Africa, too, PA's are regulated. The South African Medicines Control Council (MCC) has banned the import, sale and human use of products containing *Senecio* species since 2012 [43]. In 2013, the South African Health Department amended the Medicines Act of 1965 to further regulate the use of complementary medicines in South Africa. The amendment included the decision to ban complementary medicines containing PAs from the South African market [44].

4. In Traditional African Medicines: As TAM's in South Africa are not regulated as yet, their PA content as well as other potential toxins, is unknown. Details related to traditional medical practitioners (TMP) will be discussed later.

10.3.7 PYRROLIZIDINE ALKALOID ASSOCIATED HEPATIC SINUSOIDAL OBSTRUCTION SYNDROME

10.3.7.1 PATHOPHYSIOLOGY AND CLINICAL PRESENTATION

Pyrrrolizidine alkaloid associated HSOS is a toxin-mediated disease of the hepatic microvasculature and hepatocytes. The centrilobular hepatic sinusoidal endothelium is primarily injured by PA metabolites, leading to a complex cascade of events, ultimately causing sinusoidal obstruction syndrome. PA metabolites are also directly toxic to hepatocytes and have the potential to alter the DNA in these cells [38].

The pathophysiology of HSOS at a cellular level has been studied using the PA, Monocrotaline [31]. The pathogenic role of matrix metalloproteinases (MMP's), nitric oxide and glutathione have been noted to be of importance.

- 1) MMP's are enzymes produced by hepatic sinusoidal endothelium and act to digest surrounding basement membrane. These enzymes have been shown to play a role in initiating HSOS. Monocrotaline induces the production of MMPs [45]. The loss of basement membrane in the sinusoid causes rounding up and detachment of the endothelial cells and gaps in the sinusoidal endothelial cell layer. This allows the extravasation of red cells into the space of Disse and embolization of endothelial cells downstream, resulting in obstruction of blood flow and sinusoidal congestion. The obstruction of blood flow leads to ischemia and necrosis of the centrilobular hepatocytes. Sloughing of the endothelial cells and hepatocytes creates cellular debris, contributing to the obstruction, which may extend to involve the central venules. The above leads to lobular disarray, the deposition of connective tissue and eventual centrilobular fibrosis [46, 47].
- 2) Nitric oxide is produced by hepatic endothelial cells and Kupffer cells. Nitric oxide is suspected to inhibit the production of MMP's in the liver. Monocrotaline reduces the production of nitric oxide, and its depletion plays a pathogenic role in HSOS [48].

- 3) The antioxidant, glutathione, maintains integrity of sinusoidal endothelium and hepatocytes. Monocrotaline depletes glutathione in hepatic endothelial cells, causing endothelial cell death [49].

Coagulation pathways possibly play a role in PA-associated HSOS [47]. Cytokines have been shown to be released in murine models of PA-associated HSOS, which may activate the coagulation cascade and induce fibrin deposition and haemostatic disorders [50].

The presentation of PA-associated HSOS has been described historically by Bras et al. as having three stages as previously discussed [13]: the acute stage of ascites, tender hepatomegaly and edema; the sub-acute stage of persistent hepatosplenomegaly; and the chronic stage of clinical cirrhosis. This clinical staging is still used by certain researchers today [20].

HSOS in the oncology setting can be diagnosed clinically using the Seattle, modified Seattle and Baltimore clinical criteria, which look at jaundice, tender hepatomegaly, ascites and weight gain post stem cell transplantation. These criteria may be applicable to the acute stage of PA-associated HSOS, while awaiting liver biopsy.

Associated laboratory findings in HSOS include direct hyperbilirubinaemia, transaminitis, raised alkaline phosphatase and gamma glutamate transferase, and hypoalbuminaemia. Coagulopathy may occur from liver dysfunction. Thrombocytopenia may be present [27].

The complications of PA-associated HSOS include: acute liver failure; the development of portal hypertension and its complications such as hypersplenism and oesophageal varices; and the development of cirrhosis.

10.3.7.2 DIAGNOSIS

The definitive diagnosis of PA-associated HSOS requires three steps [40]:

- 1) Having a clinical suspicion of PA-associated HSOS based on the typical clinical presentation of ascites, hepatomegaly and jaundice, together with a history of possible exposure to PA's
- 2) Proving intoxication with PA's by collecting samples of suspicious plant material or body fluid on admission, and performing laboratory testing on these
- 3) Making a histological diagnosis of HSOS on liver biopsy

It is often difficult to establish a definitive diagnosis of PA-associated HSOS in South Africa, as:

- 1) Clinicians may be unaware that screening tests exist in some South African laboratories and fail to collect samples for testing
- 1) Traditional African medicines are rarely made available for testing as patients may not admit to their use due to the stigma associated with TMP use
- 2) The laboratory testing for PA identification is not widely available in many South African laboratories, as mentioned previously
- 3) Patients may be too unstable for liver biopsy

As previously demonstrated by Steenkamp and Zuckerman, PAs can be detected in TAM's as well as in urine using the above methods [20, 21]. Serological testing for PA-protein adducts has proved to be a reliable confirmatory test for the definitive diagnosis of PA intoxication, as shown in a Chinese study by Gao et al. in 2012 [51]. These researchers developed an ultra-performance liquid chromatography-mass spectrometry test to detect PA in blood samples of patients who had ingested the traditional Chinese herb, *Tusantqi*.

10.3.7 USE OF ADJUNCTIVE TESTING

Laboratory tests are non-specific for HSOS, but may assist in assessing the degree of liver impairment and thus prognosticate on outcomes. Direct hyperbilirubinaemia, transaminitis and coagulopathy represent hepatocellular injury [27]. Severe hyperbilirubinemia may indicate a poorer prognosis in HSOS [19, 20, 52].

Imaging investigations such as abdominal ultrasound, CT and MRI are not diagnostic for HSOS [31]. They may be used to support a diagnosis of HSOS, to exclude other disorders with similar clinical presentations, and to detect complications of HSOS. Doppler sonography may show attenuation or reversal of venous flow from portal hypertension, which is a complication of HSOS [31, 53]. CT and MRI findings in PA-associated HSOS were looked at by Zhou et al. in 2014 [54]. They found that ascites, patchy liver enhancement and narrowing of the main hepatic veins were the most common radiological findings.

10.3.8 MANAGEMENT

Therapeutic strategies for the management of acute PA-associated HSOS are largely supportive [46, 50].

These include:

- 1) Paracentesis should the ascites be tense and causing respiratory compromise.
- 2) The infusion of albumin to alleviate ascites and improve renal perfusion.
- 3) Salt restriction and diuretics to treat third spacing and maintain renal perfusion.
- 4) Supportive measures for liver dysfunction including vitamin supplementation.
- 5) Screening for sepsis. This is essential as sepsis is common and needs to be rigorously treated.

Novel therapy includes the use of corticosteroids, which has been shown to reduce liver fibrosis in mice with PA-associated HSOS and in the oncology setting [55]. Defibrotide, an antithrombotic, has also shown good results in small groups of patients with PA-associated HSOS [50].

Long term follow-up to manage chronic liver disease and its complications is essential in the management of PA-associated HSOS [20]. These include vitamin supplementation; vigilance for the development of portal hypertension and oesophageal varices and the management of these; and the possible eventual need for liver transplantation.

10.4 TRADITIONAL MEDICAL PRACTITIONERS AND TRADITIONAL AFRICAN MEDICINE USE IN SOUTH AFRICA

Traditional medical practitioners (TMP's) play an integral role in South African culture and primary health care. Up to eighty percent of South Africans consult TMP's. Traditional medical practitioners outnumber conventional medical practitioners by a factor of one in eighty in South Africa. [56].

Western medicine and African traditional medicine are not well integrated in South Africa. Traditional medical practitioners are generally viewed in a negative light by Western doctors [57]. This may have a detrimental effect on patient care. Patients may be unwilling to disclose to conventional medical practitioners that they have consulted TMP's and have used TAM's.

TAM's have been used for generations by TMP's. Traditional African medicines are not regulated, their formulations are variable, their dosages are not fixed and no formal testing regarding their effectiveness or toxicity has been done [58]. The regulation of TAM's remains a great challenge in South Africa. Currently, TAM's are not regulated, although efforts have been made by the Medicines Control Council to do so. The South African Traditional Medicine Regulation Group is in the process of creating monographs for widely-used TAM's, in an attempt to study the contents of these [57].

Traditional African medicines may contain toxic substances, including phytotoxins, such as PA's [49, 50]. In 2001, Du Plooy et al. found that TAM's contributed 4.7% of all admissions for acute poisonings over a five year period in a South African hospital [59].

Young children are especially vulnerable to the potentially toxic effects of TAM's, due to the immaturity of their liver enzymes and the risk of over-dosing [33]. Young children in South Africa are commonly given TAM's. In 2008, a study by Ayibor in Johannesburg screened a hundred children treated by traditional healers. This study revealed that 45% of the children treated were less than a year of age, and that 64% of these patients had been given oral TAM's [60].

In 2003, Steenkamp et al. investigated seventy-seven children admitted to hospitals in Johannesburg with definite TAM ingestion. They found that 66% were less than a year of age. The mortality rate in this series was 33%, and the researchers suspected that TAM's may have contributed to these poor outcomes.

11. DISCUSSION AND CONCLUSIONS

This is a descriptive analysis of a case-series of ten African patients presenting with clinical and histological HSOS, most likely due to intoxication with PA's in TAM's. A literature review of HSOS, PA's and TMP's in South Africa has assisted in understanding the presentation and outcomes of these patients.

These patients are comparable in many ways to cases of PA-associated HSOS found in the literature from Jamaica and South Africa. Their demographics are similar as all of the patients in this series came from underprivileged backgrounds as described in the literature. The patients in this series had a mean age of 13 months, which concurs with previous series'.

A difficulty in confirming the aetiology of HSOS, but a strong suspicion of PA-associated HSOS caused by TAM ingestion, was found in this group of patients. This has been a trend in previously reported South African case-series. In this series, a confirmed history of TAM ingestion was found in only 40% of cases, but suspected in all cases. As discussed in the literature review, South African TMP's are stigmatized by conventional practioners. This may have contributed to the caregivers in this series denying the use of TMs in these patients.

Malnutrition was present in 20% of cases in this series. The Jamaican series and a few South African series have also shown that only a minority of cases with PA-associated HSOS are malnourished.

The clinical picture of all the patients in this series included the presence of ascites (100%), hepatomegaly (100%) and clinical jaundice (50%). These are typical of the acute stage of HSOS, as defined by Bras et al. and is comparable to the clinical staging criteria used in the oncology setting. These clinical features have been described as essentially diagnostic of PA-associated HSOS in the appropriate setting by Zuckerman et al.

The laboratory findings included hypoalbuminaemia in all of the patients. This has been a common finding in most cases of HSOS, and is of multifactorial aetiology. The presence of sepsis has a possible association with poorer outcomes in HSOS. In this series, sepsis was present in three of the cases, and contributed to a poor outcome in one patient. Sepsis has been documented to occur in the setting of PA-associated HSOS and seemed to be associated with poor outcomes in previous series' from South Africa.

On histology, central vein obliteration was found in 70% of patients and fibrotic changes were found in 90% of patients. This is of significance as not all patients had central venule obliteration, in keeping with HSOS, and in contrast to HVOD. Also of significance is the presence of fibrosis in 90% of the patients during the acute phase of illness. This may indicate a poorer prognosis with the development of cirrhosis. Cell changes, including hepatocyte balloon cell degeneration, hepatocyte drop out, nuclear atypia and necrosis, were present in up to 50% of patients. These cell changes were likely due to be due to ischemia from HSOS and possibly from direct PA-hepatotoxicity. They concur with other studies of HSOS.

Acute outcomes were good overall, with resolution of acute symptoms in 90% of patients over 2 -5 weeks. These outcomes are better than those in previously recorded case-series of PA-associated HSOS, where more fatalities were documented. This may be because the patients presented in the early stages of HSOS. One patient demised in the acute stage of illness, due to liver failure compounded by sepsis and renal failure. His poor prognostic features were young age (4 months old), severe jaundice and sepsis.

Three of the patients had a relapse of ascites following the acute stage. Two of these were severely malnourished, which may have contributed to their relapse. Sub-acute HSOS developed in 70% of the patients on short-term follow up.

Long term follow-up was poor in this series, with only 40% patients following up for longer than a 6 month period. Thus no conclusions could be drawn about long term outcomes in this group of patients. Clinical cirrhosis developed in all of the patients that did follow up on a long term basis, suggesting that long term outcomes are poor, which concurs with the literature. This places emphasis on the importance of long term follow-up in HSOS, as chronic liver disease requires specialist review and extensive supportive care. Pyrrolizidine alkaloids are suspected to be carcinogenic in humans. Therefore, vigilance for the development of tumours is needed in the long term follow-up of PA-associated HSOS.

An unexpected and intriguing finding in this group of patients was that 80% of cases originated from the Gauteng West Rand, which could be coincidental or be because of the referral pattern of the patients or indicate a common source of PA's, or may point towards a yet unrecognized etiology. The presumed source of PA's in these patients was most likely from the ingestion of TAM's containing PA's. It is unlikely that the source was from contaminated bread, or foodstuff, as the cases were quite sporadic rather than indicating a mini-epidemic.

A review of PA's has shown their historical importance in HSOS, and their significance in South Africa. They are currently one of the few identifiable toxins found in TAM's. Pyrrolizidine alkaloid associated HSOS is a rare condition in South Africa, but does occur, and is probably underdiagnosed as it is difficult to prove. Clinicians in South Africa need to have a high index of suspicion for PA-associated HSOS where the history and clinical presentation are suggestive. Definitive diagnosis of HSOS by liver biopsy does not prove causality of PA intoxication. Thus there is a need to make diagnostic tests for PA intoxication more easily available in South African laboratories.

Traditional medical practitioners form an integral part of health care for the majority of South Africans. Therefore, communication and collaboration between TMP's and conventional practitioners should be encouraged. The rational and safe use of TAM's and the regulation of TMP's in South Africa needs to be developed. Traditional medical practitioners need to be informed that TAM's may contain phytotoxins, including PA's. Traditional medical practitioners should be taught to recognize and avoid the use of plant species known to contain PA's in their TAM's. The dangers of TAM use in children should be highlighted to TMP's.

This study has highlighted the high prevalence of TAM use by South Africans and the dangers of TAM's in children. South African clinicians should be aware of HSOS and its relation to PA's in TAM's. Enquiring about TAM use in a non-judgemental way is essential in clinical practice. Clinicians should also make an effort to obtain TAM's for laboratory testing. This would greatly assist in proving causality of PA-associated HSOS. Education of caregivers about the dangers of TAM's, especially in children, needs to be emphasized, as prevention is often the only effective treatment of their toxic effects.

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