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Highlights

Evaluation of Antimicrobial

Tertiary Hospital Experience

Malaria Infection in Ethiopia

Vivo Senescence of Erythrocyte

Discovering Thoughts, Inventing Future

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Microbiological Spectrum and Susceptibility Pattern of Clinical Isolates from Children Suspected of Urinary Tract Infection, Visiting Kanti Children's Hospital, Maharajgung, Kathmandu

By Bidur Aryal, Pappu Kumar Mandal & Prem Dev Tripathi
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Abstract- Background: The present study analyzes the clinical profile, identifies the pathogenic distribution and their antimicrobial susceptibility pattern in childhood urinary tract infections in order to provide standard reference for the optimal use of antibiotics in Nepal.

Methods: A hospital based cross section study was conducted among children suspected of urinary tract infection in Kanti Children's Hospital over a period of six months from August 2012 to November 2012. A total of 1890both sexes, ranging from post natal period to 14 years of age were studied. The modes of presentation, laboratory investigation reports, which included urine routine microscopy, bacterial isolation with colony count from urine culture, antibiotic sensitivity pattern and multidrug resistant profile, were documented. Data were analyzed by the Chi Square Test.

Results: Among 1890 urine samples, 300(15.88%) showed culture positive result: 256 gram negative organisms and 44 gram positive organisms.

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MICROBIOLOGICALSPECTRUMANDSUSCEPTIBILITYPATTERNOFCLINICALISOLATESFROMCHILDRENSUSPECTEDOFURINARYTRACTINFECTIONVISITINGKANTICHILDRENSHOSPITALMAHARAJGUNG
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Microbiological Spectrum and Susceptibility Pattern of Clinical Isolates from Children Suspected of Urinary Tract Infection, Visiting Kanti Children's Hospital, Maharajgung, Kathmandu

Bidur Aryal [α], Pappu Kumar Mandal [σ] & Prem Dev Tripathi [ρ]

Abstract- Background: The present study analyzes the clinical profile, identifies the pathogenic distribution and their antimicrobial susceptibility pattern in childhood urinary tract infections in order to provide standard reference for the optimal use of antibiotics in Nepal.

Methods: A hospital based cross section study was conducted among children suspected of urinary tract infection in Kanti Children's Hospital over a period of six months from August 2012 to November 2012. A total of 1890both sexes, ranging from post natal period to 14 years of age were studied. The modes of presentation, laboratory investigation reports, which included urine routine microscopy, bacterial isolation with colony count from urine culture, antibiotic sensitivity pattern and multidrug resistant profile, were documented. Data were analyzed by the Chi Square Test.

Results: Among 1890 urine samples, 300(15.88%) showed culture positive result: 256 gram negative organisms and 44 gram positive organisms. Among the positive growth samples 144(48%) were male patients and 156(52%) were female patients.; *Escherichia coli* was most common organisms isolated (52.33%), followed by *Klebsiella spp* (17.33%), *Staphylococcus aureus*(14%), *Pseudomonas aeruginosa* (4.33%), *Proteus spp* (3.67%), *Citrobacter spp* (3%), *Acinetobacter spp*(1.66%), *Enterobacter spp* and *Hafnia alvei* (1.33%), *Streptococcus spp* (0.66%) and *Salmonella paratyphi* B(0.33%). Nitrofurantoin was found to have the highest sensitivity (71.67%) amongst most bacteria. Amikacin, Norfloxacin and Gentamicin had sensitivity of 69%, 61.71% and 61.67% respectively. *Pseudomonas aeruginosa* was 100% sensitive to Tobramycin, Piperacillin and Imipenem. Though sensitivity to Vancomycin was tested to 44 cases it was 100% sensitive to *Staphylococcus aureus* and *Streptococcus fecalis*. Highest degree of resistance was noted with Ceftazidime (64%), Ofloxacin (61.33%), Ampicillin (60%), ciprofloxacin (55.67%), Cotrimoxazole (52%), Gentamicin (38.33%), Amikacin (28%) and Nitrofurantoin (23.67%).

Conclusion: Infected urine stimulates an immunological and inflammatory response leading to urinary tract injury and scarring, ultimately leading to end stage UTI. In a subtropical country like ours, there is a temporal relationship in the antibiotic sensitivity pattern of UTI. Hence, frequent large-scale studies are required from time to time to note the change in the sensitivity and resistance. Complicated UTI and subsequent renal failure continues to be one of the major causes of mortality in children. From this study, it can be concluded that *E.coli* still remains the commonest isolate in UTI. Nitrofurantoin, Amikacin, Norfloxacin, Gentamicin can be considered effective drugs. An emerging resistance has been noticed with Ceftazidime, Ofloxacin and Amikacin. Vancomycin may be reserve drug of choice in failed or multidrug resistance cases for gram-positive bacteria. Large-scale multicenter studies are required to generalize the data for the whole country.

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I. Introduction

Urinary tract infection (UTI) is common in pediatric practice and an important cause of morbidity and mortality in children. However, UTI is a common problem throughout the world, the microbial isolates and their sensitivity pattern need to be analyzed at regular interval to monitor the changing pattern of microbial flora and the development of resistance to drugs, which may help the physician to treat UTI in better way and to prevent further complications.

II. Materials and Methods

We conducted the prospective analysis ofthe cases attending pediatric OPD and those admitted in the ward of Kanti Children's Hospital, Kathmandu, Nepal. Study period was six months from August 2012 to November 2012. Children of both sexes up to the age of 14 years were included in the study. Their clinical presentation with associated condition and risk factors were noted. Approximately 1890 urine samples were screened and 300 urine samples showed positive culture result. Parents were explained about the study and professional care was taken to collect the urine sample for routine culture and sensitivity by sterile technique. Urine was sampled for culture by aseptic supra pubic bladder aspiration in infants. Sterile plastic receptacles were used for collection of urine in younger patients to avoid contamination with stool. Clean catch mid-stream urine was sampled in older children and

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adolescents after proper cleansing of urethra and under supervision. The samples were than processed for routine microscopy. Only samples with more than 5 WBC per high power field (hpf) were subjected for culture and antimicrobial susceptibility testing in the bacteriology laboratory of Kanti Children Hospital. Receptacle sample and mid-stream urine sample with culture with $>10^5$ colony forming units of bacteria/ml of urine in young infants and adolescents. Any colony count with supra pubic count in infants.

III. Observation and Result

Among the 1890 urine samples included in the study, 300(15.88%) showed positive culture result (fig. 1). Since, the study includes newly born babies up to 14 years of age. The high frequency of UTI was found in 0-2 years of age followed by 8-10 years of age. Among the 300 culture positive cases, 114(38%) were males and 186(62%) were females (Table 1).

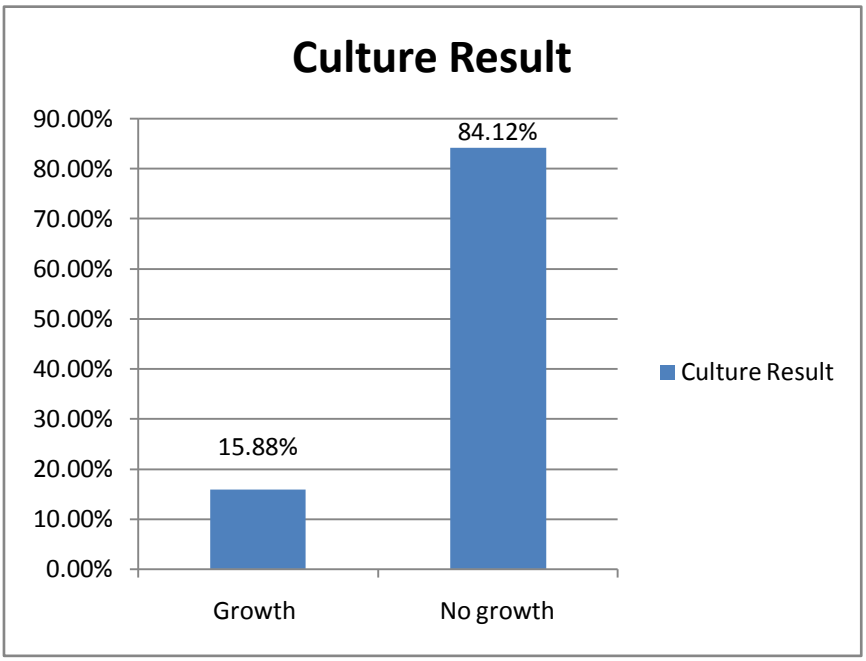

Figure 1 : Urine Culture Report

Table 1 : Age and sex distribution at Presentation (N=300)

Sex	Age(years)						
	0-2	2-4	4-6	6-8	8-10	10-12	12-14
Male	62	17	10	7	8	6	4
Female	90	31	25	13	10	10	7
Total	152	48	35	20	18	16	11

(14%) followed by Streptococcus fecalis (0.66%) of cases. (fig 2)

Among the gram negative isolates, the most common organisms isolated was *E. coli*(52.33%)followed by *Klebsiella pneumonia* (16%), *Pseudomonas aeruginosa* (4.33%), *Proteus mirabilis* (2.66%), *Citrobacter freundii* (2%), *Acinetobacter spp.*(1.66%), *Klebsiella oxytoca*, *Enterobacter spp.*, *Hafniaalvei* (1.33%), *Proteus vulgaris*, *Citrobacter koseri* (1%) and *Salmonella paratyphi* B (0.33%). *Staphylococcus aureus* (14%) followed by *Streptococcus fecalis* (0.66%) of cases. (fig 2).

Among the antibiotics used, Nitrofurantoin was found to have the highest sensitivity (71.67%) amongst most bacteria. Amikacin, Norfloxacin and Gentamicin had sensitivity of 69%, 61.71% and 61.67% respectively. *Pseudomonas aeruginosa* was 100% sensitive to Tobramycin, Piperacillin and Imipenem. Though sensitivity to Vancomycin was tested to 44 cases and it showed no resistance and it was 100% sensitive to *Staphylococcus aureus* and *Streptococcus fecalis*. Highest degree of resistance was noted with Ceftazidime(64%), Ofloxacin(61.33%), Ampicillin(60%) , ciprofloxacin (55.67%), Cotrimoxazole (52%), Gentamicin (38.33%), Amikacin (28%) and Nitrofurantoin (23.67%). The sensitivity pattern of various organisms was also studied. *E.coli* responded better with Nitrofurantoin, Aminoglycosides and Fluroquinolones but displayed a highresistance with most of thebeta lactams. Resitance was also noted with Ofloxacin, Nalidixic acid and Ciprofloxacin.

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The sensitivity pattern of *Klebsiella* and *Proteus* was similar with few minor differences like *Klebsiella*showing high sensitivity with Amikacin (77.09%), Nitrofurantoin (70.83%), Norfloxacin (68.75%), Ceftazidime (64.58%) as compared to *Proteusspp* showing sensitivity to Amikacin, Norfloxacin, Ciprofloxacin (62.5%), Ofloxacin, Ceftazidime (37.5%). Amongst the gram negative bacteria *P. aeruginosa*was 100% sensitive to Piperacillin, Imipenem and Tobramycin. Amikacin was 100% sensitive to *Citrobacterkoseri*, *Enterobacterspp.*, *Salmonella paratyphi* B.*Citrobacterfreundii*, *Acinetobacter spp.*, was sensitive to Amikacin. *Hafniaalvei* was 100% sensitive to Nalidixic acid.

*Klebsiella spp.*and *Proteus spp.* showed high degree of resistance with beta lactams, Fluoroquinolones, Sulfonamides and Nitrofurantoin. *Salmonella paratyphi* B was 100% resistant to Fluroqouinolone, Aminoglygosides and Nitrofurantoin. *Staphylococcus aureus*and *Streptococcus fecalis*showed high sensitivity to Sulphonamides, beta-lactams and Nitrofurantoin whereas similar type of resistance with beta-lactams and Fluroquinolone.

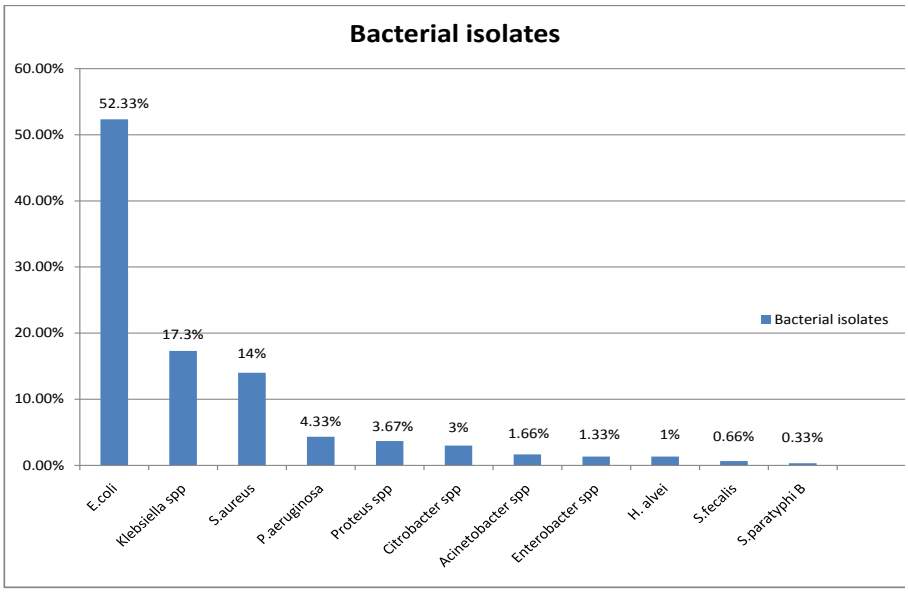

Figure 2 : Bacterial isolates of culture positive cases

Table 2 : Resistant Pattern of Bacterial Isolates Against Commonly used Antibiotics and Observation of MDR Bacterial Isolates

Organisms	Total no. of isolate	Resistant to				
		0 Drug (%)	1 Drug (%)	2 Drug (%)	MDR strains	
					>2drug	%
E. *coli*	157	9(5.73)	14(8.91)	29(18.47)	105	66.87
K. *pneumonia*	48	5(10.4)	3(6.25)	13(27.08)	27	56.25
K. *oxytoca*	4	0(0)	1(25)	1(25)	2	50
P. *mirabilis*	8	0(0)	2(25)	1(12.5)	5	62.5
P. *vulgaris*	3	0(0)	0(0)	1(33.34)	2	75
P. *aeruginosa*	13	0(0)	0(0)	0(0)	13	100
C. *koseri*	3	0(0)	0(0)	1(33.34)	2	75
C.*fruendii*	6	0(0)	1(16.67)	2(33.34)	3	50
Acinetobacterspp	5	1(20)	0(0)	0(0)	4	80
Enterobacterspp	4	0(0)	0(0)	1(25)	3	75
S. *aureus*	42	0(0)	2(4.76)	5(11.90)	35	83.33
S. *fecalis*	2	0(0)	0(0)	0(0)	2	100
H. *alvei*	4	0(0)	1(25)	0(0)	3	75
S. *paratyphi B*	1	0(0)	0(0)	0(0)	1	100
Total	300	15(5)	24(8)	54(18)	207	69

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IV. Discussion

Urinary tract infection (UTI) is common cause of febrile illness in young children. In the first year of life. Urinary tract infection(UTI) is one of the most important causes of morbidity in the general population and the second most common cause of hospital visits(Das et al., 2006). Urinary tract infection(UTI) is not uncommon cause of bacterial illness in children, 4-8% of children have had an UTI from a population-based study(Suresh kumaret al., 2009). The prevalence and incidence of is higher in female than in male children, which are likely the result of several clinical factors including anatomical differences, hormonal effects and behavior pattern(Griebling, 2009). The prevalence of UTIs is quite different between two gender and age with high incidence in girls (1% in male and 3% in female), except the male infants with an incidence of 0.7% compared to the 0.1-0.4% of female infants (Foxman, 2002), which is due to bacteria harboring in prepuce of young infants.

Among the growth positive samples, 144(48%) were male patients and 156(52%) were female patients. Among 1890 urine samples, 1094(57.88%) were symptomatic, in which 166(15.18%) was culture positive. Urinary symptoms like dysuria, burning urine, increased frequency, haematuria, oliguria, bed wetting, chills and rigors, abdominal pain, vomiting, loose stool, etc. The first and the most critical step in establishing the diagnosis of UTI in infants and young children is the method by which the urine is collected. In the young infants care must be taken to prepare carefully the perineum and periurethral area for placement of sterile plastic receptacle for collection of urine. In the infants, the purest way to obtain urine for culture aseptically is by precutaneoussuprapubic aspiration. Older children and adolescents can be instructed to collect the midstream urine specimen after proper cleansing of the urethral area. These steps were strictly followed for collection of urine in our study. The presence of 105 organisms or more per ml of urine is diagnostic of UTI. If 103 to 105 colony forming units of a single genus and species per ml are recovered from two successive urine culture of a child, a diagnosis of UTI should be made6.

In our context, such cases were not included in our study as it was difficult to call the patient for repeated urine culture though they were empirically treated as suspected UTI. The suprapubic bladder aspiration or by catheterization contain fewer than 105 organisms because the organisms have not had sufficient time to multiply before the removal of urine from the bladder(Griebling TL.,2009).

In this study, 300(15.88%) resulted a positive culture in urine with significant colony count of ≥105rest 1590(84.12%) were culture negative or they had colony count <105 (fig.1). E.coli(52.33%) was found to be predominant organism in this study which resembles with the study done by Raiet al., (2008); Maliangoet al., (2012); Beyene and Tsegaye (2011);Daoud and Afif (2011); Elkehiliet al., (2010); Aypaket al., (2009); Hawn et al., (2009). Our distribution of pathogens wereE.coli(52.33%)was predominant organism isolated followed by Klebsiella pneumonia (16%), Staphylococcus aureus (14%), Pseudomonas aeruginosa (4.33%), Proteus mirabilis (2.66%), Citrobacterfruendi (2%), Acinetobacterspp (1.66%), Klebsiellaoxytoca (1.33%), Enterobacterspp (1.33%), Hafniaalvei (1.33%), Proteus vulgaris (1%), Citrobacterkoseri (1%), Streptococcus fecalis (0.66%) and Salmonella paratyphi B (0.33%).E.coli is by far the most common bacteria isolated from urine samples in both outpatients and inpatients of both sexes in children. This finding is also in agreement with findings ofTanejaet al., (2010); where E.coli (47.1%), Klebsiellaspp(15.6%), Enterococcus fecalis(8.7%), Proteus spp(5.9%), P. aeruginosa(5.9%) and others 17.1%.Yet in another study, the findings were consistent with ours where the pathogens were E.coli (47%), Klebsiellaspp (18%), S.aureus (13.4%), Proteus spp (9%), E.fecalis (5.3%),P.aeruginosa (5%), and others 2.3%.

In our study,Nitrofurantoin was found to have the highest sensitivity (71.67%) amongst most bacteriawhereas Proteus, Salmonella paratyphi B and P. aeruginosa was resistant to the same.Amikacin, Norfloxacin and Gentamicin had sensitivity of 69%, 6

1.71% and 61.67% respectively. Pseudomonas aeruginosa was 100% sensitive to Tobramycin, Piperacillin and Imipenem. Though sensitivity to Vancomycin was tested to 44 cases it was 100% sensitive to Staphylococcus aureus and Streptococcus fecalis. Highest degree of resistance was noted with Ceftazidime (64%), Ofloxacin (61.33%), Ampicillin (60%), Ciprofloxacin (55.67%), Cotrimoxazole (52%), Gentamicin (38.33%), Amikacin(28%) and Nitrofurantoin (23.67%).

In this study, 69% (207/300) were found to be Multidrug resistant (MDR) i.e. they were resistant to more than two drugs which is similar to the result of Pokhrelet al., 2006 in which 60.40% were MDR. The MDR in E.coli was found to be 66.87% (105/300). Although multidrug resistance was shown 100% by P. aeruginosa,Enterococcus fecalis and S. parathypi B, these were low in number and considered insignificant. In a study done by Tuladharet al., 2003 at TUTH, MDR bacterial strains were detected in 35.2% cases in which the most predominant was E.coli(22.2%) followed by Klebsiellaspp (6.1%) and Staphylococcus aureus (2.2%).

V. Conclusion

As UTI is the significant problem in the children and still continues to be a major threat for morbidity and mortality in subtropical parts of the world, larger scale studies must be carried out at a regular intervals in order

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to identify the changing trend in the pathogenic organisms and update on its changing antibiotic susceptibility. Based on the sensitivity patterns we recommend empirical use of Nitrofurantoin, Amikacin, Norfloxacin and Gentamicin for patients with UTI.Vancomycin showed 100% sensitivity to gram-positive bacteria. Gram-negative bacteria like Proteus spp, P. aeruginosa and S.paratyphi B was 100% resistant to Nitrofurantoin. P.aeruginosa was 100% resistant to Imipenem, Piperacilin and Tobramycin. So, Vancomycin should be kept as reserve drug for gram positive organisms and Tobramycin, Imipenem and Piperacilin for P. aeruginosa.

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High Grade Infiltrative Urothelial Carcinoma- Sarcomatoid Variant, A Rare Entity: Case Report and Review of Literature

By Manas Madan, Tejinder Singh Bhasin, Mridu Manjari, Manisha Sharma & Bhawna

SGRD Medical College, India

Abstract- In the urinary bladder, majority of the neoplasms are of pure epithelial origin. In contrast pure mesenchymal tumors and biphasic epithelial-mesenchymal neoplasms, although documented, rarely occur at this site Heterologous carcinosarcomas (also called metaplastic carcinomas or sarcomatoid carcinomas with heterologous differentiation) are defined as biphasic tumors made up of a varying mixture of carcinomatous and heterologous sarcomatous components with abrupt or gradual transition between one component and the other. These are rare tumors and account for approximately 0.3% of all bladder malignancies with a poor prognosis. Because of its unfavorable histopathologic nature and also its rarity, not much is known about the various treatment options in these tumors. The overall 5 year cancer specific survival rate after cystectomy is only 20.3%.

Keywords: heterologous, carcinosarcomas, biphasic.

GJMR-C Classification : NLMC Code: QZ 365

HIGHGRADEINFILTRATIVEUROTHELIALCARCINOMASARCOMATOIDVARIANTARAREENTITYCASEREPORTANDREVIEWOFLITERATURE

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Keywords: heterologous, carcinosarcomas, biphasic.

I. Introduction

Heterologous carcinosarcomas (also called metaplastic carcinomas or sarcomatoid carcinomas with heterologous differentiation) are defined as biphasic tumors made up of a varying mixture of carcinomatous and heterologous sarcomatous components with abrupt or gradual transition between one component and the other [(1)]. These are rare tumors and account for approximately 0.3% of all bladder malignancies with a poor prognosis[(2,3)].

The histological composition of these tumors is variable but requires the presence of both epithelial and mesenchymal malignant components [(4)].

Treatment of bladder carcinosarcomas should be aggressive and multimodal but optimal treatment is still unknown [(5)].

Herein, we report a case of sarcomatous carcinoma with chondrosarcomatous differentiation of the urinary bladder.

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II. Case History

A 77 year-old male was admitted to our hospital, who presented with complaints of hematuria and abdominal pain. Ultrasonography (USG) revealed a mass in the bladder involving the posterior wall and projecting into the lumen. Computed Tomography (CT) scan showed a hyper dense mass in the bladder. Transurethral resection of the bladder tumor (TURBT) was done and the biopsy submitted for histopathology.

Histologically, the tumor showed features of infiltrative high grade urothelial carcinoma with tumor cells arranged in sheets, having high nucleo-cytoplasmic ratio, severe anisonucleosis along with spindling of the nuclei in some of the cells.

In addition, there were also present heterologous elements in the form of groups and islands of cartilaginous tissue. Individual chondrocytes were atypical, pleomorphic and showed binucleation. Areas of necrosis and hemorrhage along with tumor giant cells were also appreciated. The deep muscle included in the biopsy was also involved by the tumor. (Fig 1, 2, 3, 4, 5)

So a diagnosis of infiltrative urothelial carcinoma– high grade, sarcomatoid variant was made.

III. Discussion

In the urinary bladder, majority of the neoplasms are of pure epithelial origin. In contrast pure mesenchymal tumors and biphasic epithelial-mesenchymal neoplasms, although documented, rarely occur at this site [(4)].

Carcinosarcomas are defined by the World Health Organization as tumors composed of intimately admixed malignant epithelial and mesenchymal elements [(4,6)]. The epithelial components are squamous, glandular or high grade transitional carcinoma, whereas the mesenchymal components may be chondro-sarcoma, malignant fibrous histiocytoma, osteo-sarcoma, leiomyosarcoma, fibrosarcoma or rhabdomy-osarcoma [(2)]. In our case, histopathology showed infiltrative high grade urothelial carcinoma along with areas showing chondrosarcomatous elements.

The exact histogenesis and pathogenesis of these neoplasms remained enigmatic for many decades, and even today remains one of the

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controversial issues in tumor pathology. Previous studies have suggested that the sarcomatous component of the sarcomatoid carcinoma is derived from urothelial carcinoma [4,7]. Interestingly, Sung et al. have recently suggested the monoclonal nature of sarcomatoid bladder carcinoma with the use of molecular techniques. This finding strengthens the fact that bladder sarcomatoid carcinoma is a monoclonal lesion derived from urothelial carcinoma [8].

Kikuchi and colleagues suggested that the frequent location of these tumors in the trigone is evidence of origin from the Wolffian body. In recent studies, commonest location of these tumors was the lateral wall [9]. In our case, the tumor was located on the posterior wall.

Most commonly, these tumors are found in the female genital tract, where they have historically been termed malignant mixed müllerian tumors[4]. These tumors commonly occur in older individuals, display an aggressive biologic behavior with poor prognosis and are at advanced stage disease at initial diagnosis [2,4]. The carcinosarcoma of the bladder is more commonly seen in males, and male/female ratio is nearly 2 : 1. The disease appears in the seventh decade of life. The most common symptoms are macroscopic hematuria and dysuria. Generally more than 70% of cases present with advanced stage and have a worse prognosis than conventional urothelial carcinomas [9]. The patient in this case was a 77 year old male who presented with complaints of hematuria and abdominal pain.

The mesenchymal element of carcinosarcoma lacks epithelial markers and patients with carcinosarcoma present at a more advanced stage and are at greater risk for death compared to patients with high-grade urothelial carcinoma [9].

Because of its unfavorable histopathologic nature and also its rarity, not much is known about the various treatment options in these tumors. The overall 5 year cancer specific survival rate after cystectomy is only 20.3% [2].

Even though transurethral resection, radical cystectomy, neoadjuvant chemoradiotherapy were the proposed treatment protocols of these tumors, radical cystectomy with adjuvant chemotherapy was the most appropriate treatment option due to the patient's poor prognosis. Nevertheless mean survival rates of these patients are very poor and metastatic disease occurs in 66% of patients within 1 year [2,4].

IV. Conclusion

Sarcomatoid variant of urothelial carcinoma tends to be high grade, exhibit aggressive biologic behavior, and has a poor prognosis as compared to usual urothelial carcinoma. More studies are needed regarding the exact histogenesis of this tumor to ascertain the radical therapy needed for this rare neoplasm

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Figure Legends

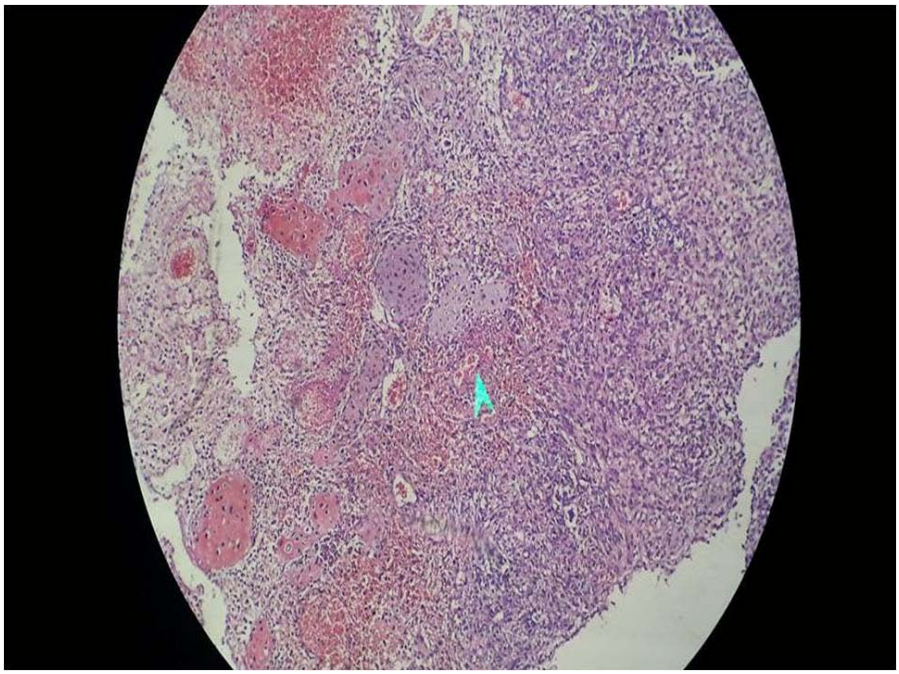

Figure 1 : Scanner view showing Chondrosarcomatous, carcinomatous foci along with hemorrhage (H&E 40X)

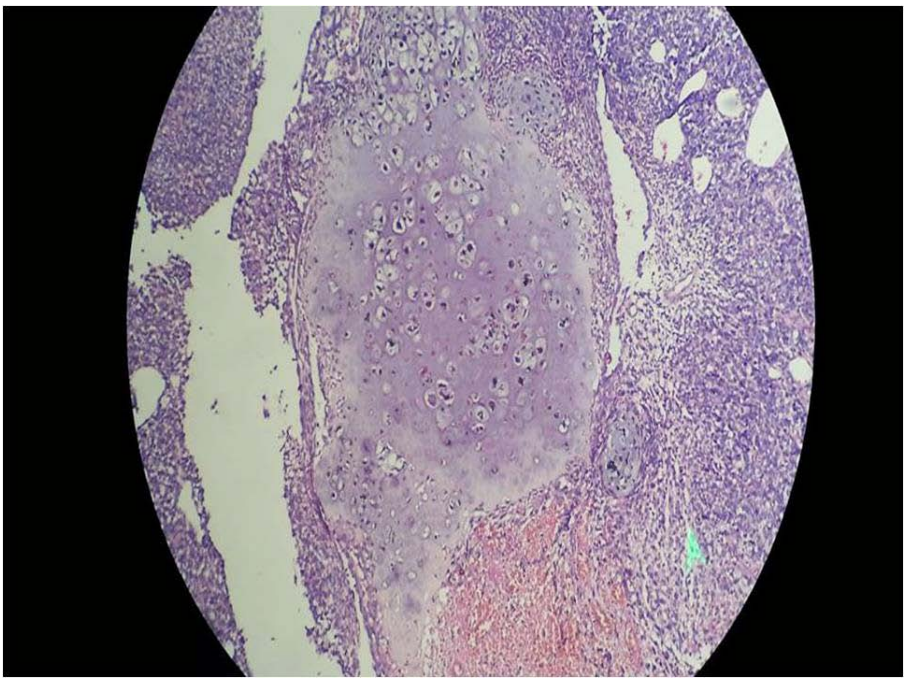

Figure 2 : Low power view showing mixture of chondrosarcomatous and carcinomatous foci (H&E 100X)

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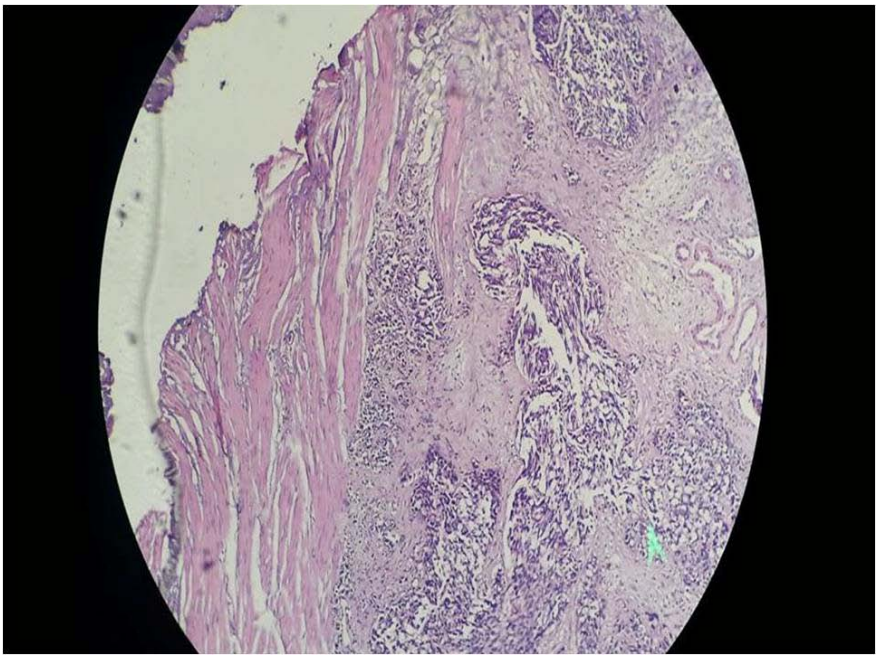

Figure 3 : Low power view showing Muscularis Propria invasion (H&E 100X)

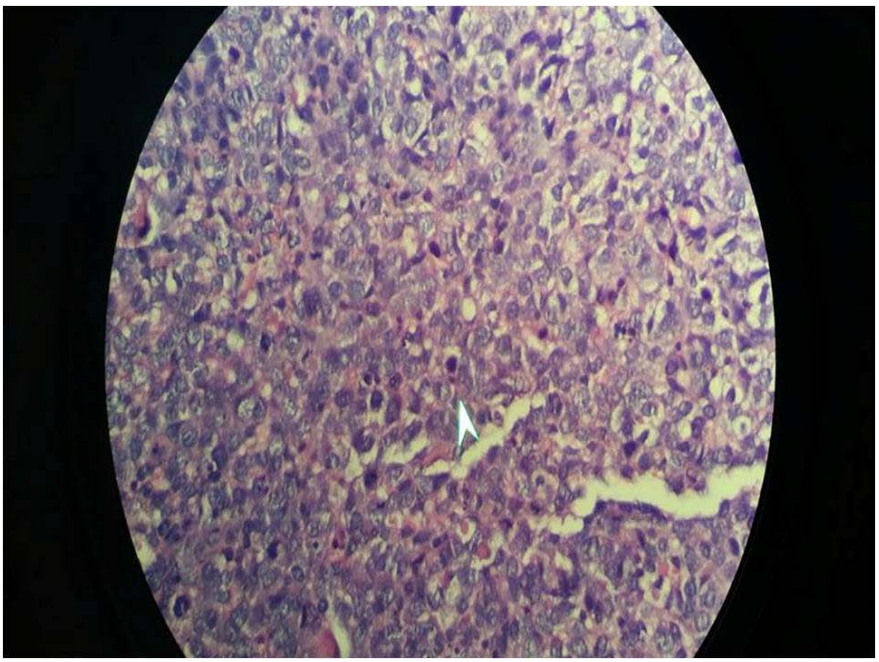

Figure 4 : High power view showing the carcinomatous component (H&E 400X)

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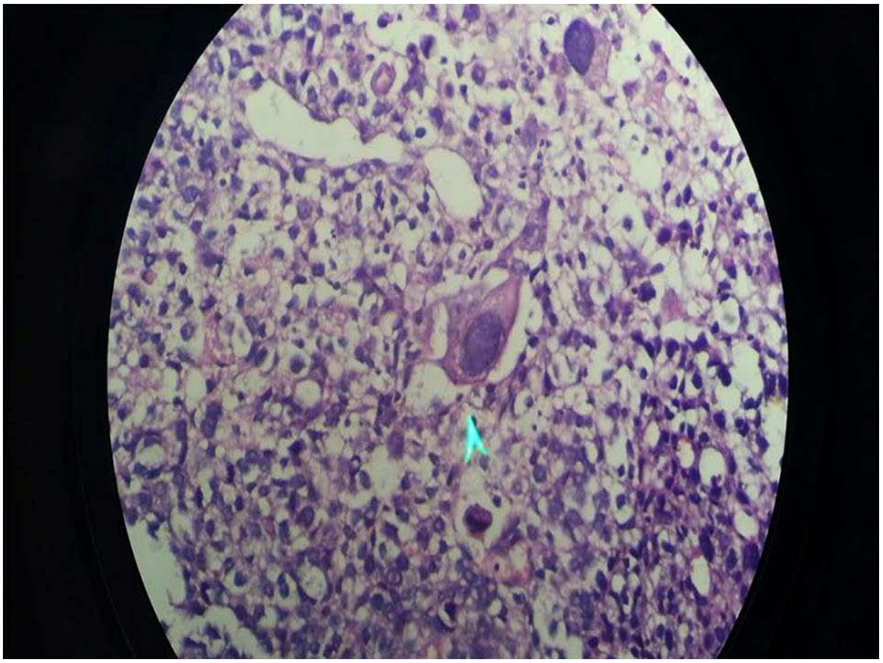

Figure 5 : High power view showing large bizzare tumor cells (H&E 400X)

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Magnitude of Malaria Infection in Ethiopia

By Belayneh Regasa
Arba Minch University, Ethiopia

Abstract- Background: Malaria is a major public health problem in worldwide and causes high morbidity and mortality. Studying its prevalence is necessary to implement effective control measures. Therefore, the aim of this study was to determine the prevalence of malaria in Arba Minch hospital, Ethiopia.

Method: A cross sectional study was conducted from January to April, 2010. A well designed and structured questionnaire and Laboratory investigation were used to collect data. Data was processed and analyzed with SPSS version16.0.

Results: A total of 400 patients clinically suspected to have malaria were examined with overall prevalence of malaria was 7% (28 malaria cases out of 400 patients) of which 18 (64.3%) were positive for Plasmodium falciparum and 7 (25%) for Plasmodium vivax; the remaining 3 (10.7%) showed mixed infections of Plasmodium falciparum and Plasmodium vivax. Males 16 (4%) were more infected than females 12 (3%). Gender had statistically significant association with malaria infection ($p<0.005$). All age groups were infected but high prevalence observed in age groups 15–19, followed by 20–29 years old.

Conclusion: This study showed that high prevalence of malaria was observed. Therefore health professionals and administra- tors have to focus on giving health education on prevention and control of malaria.

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MAGNITUDEOFMALARIAINFECTIONINETHIOPIA

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Conclusion: This study showed that high prevalence of malaria was observed. Therefore health professionals and administrators have to focus on giving health education on prevention and control of malaria.

I. Introduction

Malaria is a life-threatening infectious disease caused by the protozoan parasite called Plasmodium. The World Health Organization (WHO) estimated 660,000 deaths in 2011 directly attributed to malaria, approximately half of the world's population being at risk of infection [1]. Four main species of malaria infect humans: *Plasmodium falciparum* (P. falciparum), *Plamodium vivax, Plasmodium malariae* and *Plasmodium ovale*. *Plasmodium falciparum* is the most highly virulent species and is responsible for almost all of the 1.7–2.5 million deaths worldwide caused by malaria [2,3]. It is a leading public health problem in Ethiopia where an estimated 68% of the population lives in malarious areas and three-quarters of the total land mass is regarded as malarious [4] This makes malaria the number one health problem in Ethiopia with an average of 5 million cases per year [5]. The disease causes 70,000 deaths each year and accounts for 17% of outpatient visits to health institutions [6]. The aim of this study is to asses prevalence of malaria in Arba Minch hospital.

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II. Methods and Materials

During the period January to April 2010 a total of 400 patients with typical symptoms of the disease, such fever (>37.8°C), headache and back and joint pain consistent with malaria was included in this study.

a) Sample collection

Two milliliters of venous blood samples were collected into an Ethylene diamine tetra acetic acid (EDTA) containing bottles for the study, using vein puncture technique [7].

b) Blood examination

Laboratory analysis was carried out after thin and thick blood films prepared according to technique out lined by Cheesbrough [7] and examined microscopically for malaria parasites under the microscope.

III. Result

A total of 400 patients clinically suspected to have malaria in Arba Minch Hospital were participated, of these, 238 (59.5%) were males and 162 (40.5%) were females (Table1).

Table 1 : Socio-demographic characteristics of study participants (n=400)

Variables	Frequency (percent)
Gender	
Male	238 (59.5)
Female	162 (40.5)
Age	
15-19	30 (7.5)
20-29	190(47.5)
30-39	110 (27.5)
40-49	50(12.5)
50-60	20(5)

The overall prevalence of malaria was 7% (28 malaria cases out of 400 patients) of which 18 (64.3%) were positive for *Plasmodium falciparum* and 7 (25%) for *Plasmodium vivax*; the remaining 3 (10.7%) showed mixed infections of *Plasmodium falciparum* and *Plasmodium vivax* (Table 2).

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Table 2 : Prevalence of malaria parasite among study participants

Malaria parasite	Frequency	Percent (%)
Plasmodium falciparum	18	64.3
Plasmodium vivax	7	25
Mixed infections (*Plasmodium falciparum* and *Plasmodium vivax)*	3	10.7

Males 16 (4%) were more infected than females 12 (3%). Gender had statistically significant association with malaria infection ($p<0.005$) [Table 3].

Table 3 : Prevalence of malaria parasite in relation to sex (Gender) among study participants

Sex	Number of examined	Number infected	Percentage	P-value
Male	238	16	4	0.001
Female	162	12	3	
Total	400	28	7	

In this study all age groups were infected but high prevalence observed in age groups 15–19, followed by 20–29 years old (Table 4).

Table 4 : Prevalence of malaria parasite in relation to age groups among study participants

Age	Number of examined	Number infected	Percent (%) infected
15-19	30	12	3
20-29	190	8	2
30-39	110	4	1
40-49	50	3	0.75
50-60	20	1	0.25
Total	400	28	7

IV. Discussion

Malaria is a major public health problem in Ethiopia. Over the past years, the disease has been consistently reported as the first leading cause of outpatient visits, hospitalization, and death in health facilities across the country [8]. In this study the overall prevalence rate of malaria was 28 (7%). This result was lower than similar studies done in Ethiopia [9, 10, 11] but higher than study conducted in other area [12]. This difference might be due to altitude variation and climatological differences that may contribute to a great role for breeding of Anopheles vector. The predominant Plasmodium species detected was *Plasmodium falciparum*, followed by *Plasmodium vivax*. This was in agreement with other previous studies [13-17]. But other a studies reported that the most prevalent species was *Plasmodium vivax*, followed by *Plasmodium falciparum* [18, 19].

Males were more infected than females, which was statistically significant ($p<0.005$). This is in line with the other previous studies [9, 20, 21]. The higher prevalence rate might be due to the fact that males engage in activities which make them more prone to infective mosquito bites as compared to females' counterparts which are mostly at home and protected from such infective bites.

In all age groups, malaria was reported in the study area. However, significantly affected age groups were 15–19 years old, followed by 20–29 years old. This might be associated with their daily activities. Farming is extensive in Arba Minch. Because of high temperature in this area, daily activities are accomplished especially during night. This may expose them to the bite of mosquitoes.

The occurrence of malaria depends on adequate rainfall and temperature. In areas with a temperate climate, transmission of malaria is commonly limited to months in which the average temperature is above the minimum required for sporogony[22].

V. Conclusion

This study showed that there is high prevalence of malaria. Malaria has statistically significant association with sex and age. Therefore, health planners and administrators need to give intensive health education for the community on prevention and control of malaria.

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VII. Competing Interest

The author declared that there is no any relevant competing interest to disclose in this research.

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Evaluation of Antimicrobial Susceptibility Pattern of Pseudomonas Aeruginosa with Special Reference to MBL Production in a Tertiary Care Hospital

By Dr. B. Anuradha, Uzma Afreen & M. Praveena

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Abstract- Background: Pseudomonas aeruginosa is emerging as a nosocomial pathogen by producing Metallo Beta lactamases and acquiring resistance to many antimicrobial agents.

Materials and methods: 132 isolates of Pseudomonas aeruginosa from various clinical samples were tested for MBL production by double disk synergy method. Antibiotic susceptibility pattern was done by comparing non-MBL producers and MBL producers.

Results: Pseudomonas aeruginosa was isolated from Pus-39.39%, from urine-37.87%, sputum-3.03%. MBL producers were 44(33.34%), non MBL producers were 88(66.6). all MBL producers were 0% sensitive to Imipenem and Meropenem.

Keywords: bacterial infection, carbapenem resistance, carbapenemase, mbl producers, multi drug resistance, pseudomonas aeruginosa, routinely used antibiotics, nosocomial infection, and antibiotic susceptibility.

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EVALUATIONOFANTIMICROBIALSUSCEPTIBILITYPATTERNOFPSEUDOMONASAERUGINOSAWITHSPECIALREFERENCETOMBLPRODUCTIONINATERTIARYCAREHOSPITAL

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Evaluation of Antimicrobial Susceptibility Pattern of Pseudomonas Aeruginosa with Special Reference to MBL Production in a Tertiary Care Hospital

Antibiotic susceptibility and MBL production in Pseudomonas aeruginosa

Dr. B. Anuradha [α], Uzma afreen [σ] & M. Praveena [ρ]

Abstract- Background: Pseudomonas aeruginosa is emerging as a nosocomial pathogen by producing Metallo Beta lactamases and acquiring resistance to many antimicrobial agents.

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Conclusion: our study showed that MBL producing Pseudomonas was isolated from all clinical samples and resistant to routinely used antibiotics and also to Imipenem and Meropenem.

Keywords: bacterial infection, carbapenem resistance, carbapenemase, mbl producers, multi drug resistance, pseudomonas aeruginosa, routinely used antibiotics, nosocomial infection, and antibiotic susceptibility.

I. Introduction

Pseudomonas aeruginosa is a Gram negative motile bacillus, belongs to the family Pseudomonaceae. It is found in moist environment, disinfectant solutions, water due to its ability to utilize many different organic compounds and survive in nutrient deficient conditions (Nadeem etal 2009). It is a leading cause of nosocomial infection especially critical ill and immune-compromised patients (Hugbo etal 1992). It has been implicated in diverse nosocomial pneumonia, urinary tract infection, surgical site infection, severe burns and infection of patients undergoing chemotherapy for neoplastic diseases or those on antibiotics therapy (Erdem 1999). It has intrinsic resistance to many antimicrobial agents and show resistance to many antibacterial agents. The mechanism of resistance is due to cell wall permeability, production of extracellular chromosomal and plasmid mediated β-lactamases (Livermore 1989), aminoglycoside modifying enzymes, cephalosporinases (Prince 1986), and active multidrug efflux mechanism (Li et al 1994). This multi-drug resistant Pseudomonas aeruginosa causes nosocomial infections which is a global health care problem as it prolongs the duration of hospitalisation and increases the cost of the patient care..

The role of Carbapenems in the treatment of serious bacterial infections caused by β - lactamase resistant bacteria is a great advancement. The Carbapenems available for use in India are Imipenem and Meropenem (Gupta et al 2006). However Carbapenem resistance has been observed frequently in *Pseudomonas aeruginosa* which is due to decreased outer membrane permeability, increase efflux system, alteration of penicillin binding protein and Carbepenem hydrolysing enzyme – carbepenamase (Gladstone et al 2005). They have potent hydrolyzing activity not only against carbepanamase but also against other β lactamase antibiotics (Bush 1998 and Bennet 1999). These MBL producing *P. aeruginosa* strains have been reported to be important cause of nosocomial infection associated with clonal spread (Bush etal 1995).

Therefore detection of MBL producing Gram negative bacilli especially *Pseudomonas aeruginosa* is crucial for the optimal treatment of patients particularly in critical ill and hospitalized patients to control the spread of resistance (Richet etal 2001). Studies about resistant organisms, their impact on health care and cost are important. Detection of emerging resistance to various antibiotics and proper guidelines for empirical therapy are important. Hence the present study is taken up to detect Metallo- beta- lactamase production in pseudomonas aeruginosa in various clinical isolates

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and also to know the susceptibility pattern of MBL and non MBL producers to various antibiotics.

II. Materials and Methods

A cross sectional study was conducted on 132 Pseudomonas aeruginosa strains isolated from different clinical specimens like pus, wound swabs, urine, sputum, body fluids, endotracheal tube secretions, and stool and ear swabs. The following parameters were noted such as age of patient, sex, type of clinical specimen, antibiotic usage and duration of hospital stay, history of Diabetes mellitus, pregnancy, malignancy and alcoholism, history of lung disorders, smoking, any immunosuppressant condition and history of urinary catheterization. The Pseudomonas aeruginosa isolates were confirmed by biochemical reactions as per the standard conventional methods. Standard strain of Pseudomonas aeruginosa ATCC 27853 was used as control.

Antibiotic Sensitivity was performed by Kirby – Bauer Disc Diffusion method and the results are recorded as per CLSI recommendation (David Greenwood etal 2008) The antibiotics used were Gentamicin(10ug), Azithromycin(50ug), Ciprofloxacin (5mcg), Cefepime(30ug), Ceftazidime(30ug), ceftriaxone (30mcg), Piperacillin,/Tazobactom(100/10ug), colistin (10ug), Aztreonam(10ug), Meropenem(10ug), Imipenem (10ug). Sensitivity pattern was determined by measuring the zones of inhibition with a calibrated ruler and comparing with the standard reference chart (supplied by Hi media Laboratories).

All Pseudomonas aeruginosa isolates were tested for MBL enzyme production by Double Disk Synergy Test (DDST) as per CLSI guidelines using 10 μg imipenem discs with EDTA- On a plate of Mueller Hinton agar two Imipenem discs (10mcg) were placed and 10mcl of 0.5M EDTA solution was added to one of the discs and incubated over night at 37 degrees. The zones of inhibition around Imipenem and Imipenem-EDTA discs were noted and compared. In case of MBL producers the zone of inhibition around Imipenem and EDTA disc was more than 7mm compared to Imipenem disc alone.

III. Results

Out of 132 Clinical isolates of Pseudomonas aeruginosa 52 (39.39%) were isolated from pus, 50 (37.87%) were isolated from urine, 10 (7.57%) were isolated from sputum, 4 (3.03%) from endotracheal (ET) secretions, 2 (1.54%) from Pleural fluid, 4 (3.03%) from stool sample, 2(1.54%) from Ascitic fluid (ASF), and 6(4.54%) from Broncho alveolar lavage fluid (BAL). (Table: 1)

Table 1 : Clinical sample wise Distribution of Pseudomonas aeruginosa

Type of sample	Number of samples
Pus	52 (39.39%)
Urine	50 (37.87%)
Sputum	10 (7.57%)
Stool	04 (3.03%)
BAL	06 (4.54%)
ASF	02 (1.54%)
ET	04 (3.03%)
PLF	04 (3.03%)
Total	132 (100%)

BAL: Broncho alveolar lavage, ASF: Ascitic fluid, ET: Endotracheal tube, PLF: Pleural fluid

Out of 132 isolates Pseudomonas aeruginosa was isolated from 120 (90.50%) inpatients and 12 (9.50%) out patients.

Age wise distribution of Pseudomonas aeruginosa was as follows. 32 (24.24%) isolates were in the age group of 21 to 30, 11 (16.66%) isolation were in the age group of 31 to 40, 20 (15.15%) isolates were from the age group of 41 to 50, 14 (10.62%) isolates were in the both in 0 to 10 and 51 to 60 years, 10 (7.57%) isolates were in the age group of 61 to 70 and the rest of the 6 (4.54%) isolates were seen in above 70 years of age. This shows that Pseudomonas aeruginosa infections are not confined to particular age group but distributed in all age groups with slight variation.

Out of 132 isolates MBL enzyme production was seen in 44 (33.34%) cases and in 88 (66.66%) cases there was no MBL production. The ATCC 27853 *Pseudomonas aeruginosa* did not show any zone size enhancement by Double Disk Synergy Test (DDST). (*Table 2)*

Table 2 : MBL production in P.aeruginosa

MBL	Number of cases
MBL positive	44 (33.34%)
MBL Negative	88(66.66%)
Total	132 (100%)

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Antibiotic sensitivity pattern of both MBL and non MBL producers to various antibiotics is shown in table-3, MBL producers were showing less sensitivity to routinely used antibiotics including imipenem and Meropenem compared to non MBL producers (p value-0.002).

Distribution of Pseudomonas aeruginosa in various clinical samples is shown in table-4

Table 3 : showing antibiotic sensitivity pattern of MBL producers and non producers

Antibiotics (μg)	MBL Positive n=44(33.34%)	MBL Negative n=88 (66.6%)
Gentamicin (10)	0	52(59%)
Ciprofloxacin (5)	5 (11.3%)	29 (65%)
Cefepime (30)	3 (6.8%)	40 (45.4%)
Ceftazidime (30)	5 (11.3%)	52 (59%)
Ceftriaxone (30)	5 (11.3%)	25 (28%)
Azithromycin (50)	3 (6.8%)	10 (11.3%)
Piperacillin/Tazobactam (100/10)	20 (45.4%)	44 (95.4%)
Imipenem (10)	0	20(22%)
Meropenem (10)	0	21(23%)
Colistin (10)	100 (100%)	100 (100%)
Aztreonam (10)	3(6.8%)	23 (25%)

P<0.05

Graph : showing antibiotic sensitivity pattern for both MBL and non MBL producers

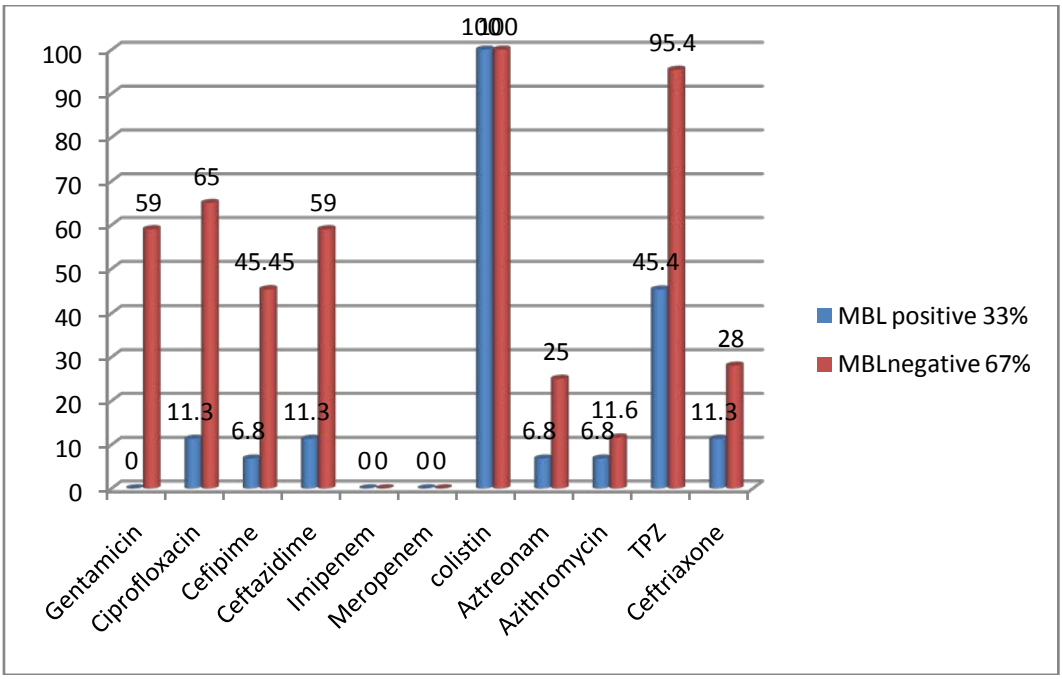

Table 4 : Distribution of MBL producing P.aeruginosa according to clinical specimen

Clinical specimen	P.aeruginosa (n-132)	MBL Producers(n=44)
Pus	52	18 (40.90%)
Urine	50	16(36.36%)
Sputum	10	04(9.09%)
ASF	02	02 (4.45%)
PLF	04	02 (4.45%)
ET	04	02 (4.45%)
Stool	04	00
BAL	06	00
Total	132	44(100%)

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IV. Discussion

Out of 132 clinical isolates of Pseudomonas aeruginosa 52 (39.39%) were from pus samples followed by 50 (37.87%) urine samples. In one study by Bashir etal (Bashir etal 2011), among MBL producers 27.3% from urine, followed by 24.2% from wound infections. In 2008 Javiya et al(Javiya etal 2008) reported the highest number of Pseudomonas infection was found in urine followed by pus and sputum which indicates that wound infections and Urinary tract infections are the most common hospital acquired infections. These are the most important cause of morbidity in general population and also in hospitalized patients. In our study 90.5% of isolates were from inpatients and 9.5% were out patients, all MBL producers were from in-patients which show that Pseudomonas aeruginosa mainly causes nosocomial infections. Our study is correlating with Bashir etal 2011. Pseudomonas aeruginosa infections were seen almost equally in both males and females with 53.03% and 46.96% respectively. Among the isolates of Pseudomona*s* aeruginosa 24.24%cases were in the age group of 21 to 30 followed by 16.66% were in the age group of 31 to 40 only 4.54% were above 71 years. There was a slight variation with other studies in age but pseudomonas infections are distributed in all age groups.

MBL producing Pseudomonas aeruginosa is emerging as nosocomial pathogen and cause of concern for clinicians. It has the ability to acquire resistance to broad spectrum β - lactam antimicrobial agents which include 3rd generation Cephalosporins, Cephamycin, Carbepenems, Gentamicin and Fluoroquinolones. This MBL enzyme production has a potential to spread rapidly and to different Gram negative bacilli such as E.coli, Klebsiella and no suitable antimicrobial agents are available. In our study among the 132 isolates of Pseudomonas aeruginosa 44(33.34%) were MBL producers and 88 (66.66%) were non producers. Among the MBL producers majority were Pus samples 18 (40.90) followed by urine samples 16(36.36%) and 4 (9.09%) sputum. This indicates that MBL production among the isolates of Pseudomonas aeruginosa significant problem in wound infection followed by urinary tract infection. In our study prevalence of MBL producing Pseudomonas aeruginosa strains was 33% as the studies from other parts of India showing variable prevalence rate. In one study by Attal Ro in 2010 showed 11.4%, Navaneeth et al in 2000 12%,[Mendiratta et al in 2005 8.2% ,[Hemalatha et al in 2005 14%, Agraval et al in 2008 8.5%. Since then the incidence of MBL producing by Pseudomonas aeruginosa has been reported to be 10 to 30% from various clinical specimens across the country (Taneja etal 2003). Mary et al reported 42% MBL production; Sarkar et al reported 54.54%. This shows that prevalence of MBL producing Pseudomonas aeruginosa is increasing day by day.

Among the MBL non producers maximum sensitivity was seen to Colistin (100%), Piperacillin/Tazobactam (95.4%) followed by Ciprofloxacin (65%), Ceftazidime (59%) and Gentamycin (59%). Imipenem(22%) and Meropenem (23%) and Aztreonam (25%). Among MBL producers majority were sensitive to Colistin (100%), Piperacillin/Tazobactam (45.4%) and none of them were sensitive to Imipenem and Meropenem (0%) Gentamicin (0%), Cefepime and Azithromycin (6.8%), ciprofloxacin, Ceftazidime, ceftriaxone (11.3%) and Aztreonam (6.5%). The reason for Carbapenem resistance would be due to reduced uptake of drug. Our study is correlating with Bashir etal 2011 where MBL producers were resistant to gentamicin and 9.1% sensitive to Ciprofloxacin, 18.1% to Piperacillin/Tazobactam. Similar results were observed by Navneet etal 2002 and Agarwal etal 2006 in one study by Rakesh Kumar etal 2014 showed that very low sensitivity was observed to 3rd generation Cephalosporins, Ceftazidime 5%, Amikacin 10%, Ciprofloxacin 20%. They also observed that MBL producing Pseudomonas aeruginosa strains were 95% sensitive to Colistin. Our results are nearer to the above study. Our results are similar to Seema etal 2012 where both MBL and non MBL producers were 100% sensitive to colistin. , MBL producers were 0% sensitive and non MBL producers were 20% sensitive to Aztreonam. This shows that MBL producing Pseudomonas aeruginosa is developing resistance to routinely used antibiotics compared to non MBL producers. A 'p' value <0.05 was considered to be significant. This emerging drug resistance in Pseudomonas aeruginosa is causing problems in treatment, increasing the mortality rates and prolonged hospitalization.

V. Conclusion

Pseudomonas aeruginosa continues to be leading cause of serious infections particularly nosocomial infections mainly effecting the inpatients. The predominant infections observed were wound infections and urinary tract infections. The most common age group affected was between 21 to 30 years and Pseudomonas aeruginosa infections were equally distributed between males and females. The present study has demonstrated that 33% of pseudomonas aeruginosa are MBL producers and developing resistance to commonly prescribed antimicrobial agents such as Azithromycin, Cefepime, Ciprofloxacin, Gentamicin and Ceftazidime. MBL producers were resistant to Imipenem and Meropenem.
The emergence of multidrug resistant Pseudomonas aeruginosa due to the indiscriminate use of antibiotics is a challenging clinical problem which leads to the

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development of resistance to the routinely used antibiotics. There is a need to do surveillance to know the susceptibility pattern and to detect the MBL producers. MBL producers cause problems in treatment and in infection control. Revised guidelines on rational antibiotic usage are to be reinforced. Infection control measures must be intensified to minimize costs of patient care.

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Oxidative Stress Induced Carbonyl Group Incorporation to Human RBC Membrane: Role in Vivo Senescence of Erythrocyte

By Dr. Arghya Sur, Dr. Hemontika Chakraborty, Dr. Arindam Basu & Dr. Sasanka Chakraborty

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Abstract- This study was focused to identify the link between oxidative stress and senescence of erythrocytes in vivo. To elucidate this mechanism, various modification of RBC membrane proteins and lipids were analyzed invitro, exposing them to oxidative stress and the results were compared with the changes observed in erythrocytes undergoing senescence in vivo. The other objective was to confirm the mechanism of autoantibody mediated removal of aged RBCs. Our results established that increased lipid peroxidation products, followed by the enhanced damage of RBC membrane protein caused increased RBC membrane protein carbonylation, to normal red cells exposed to the in vitro Fe^{2+} & ascorbate induced oxidative stress. It was presumable that these changes were mediated by hydroxyl (ȮH) radicals. Further, similar changes were also seen in percoll gradient age fractionated high density aged RBCs.

Keywords: human RBC membrane, protein carbonylation, oxidative membrane damage, senescence of RBC, oxygen free radical, ROS, autoantibody to RBC.

GJMR-C Classification : NLMC Code: WH 150

OXIDATIVESTRESSINDUCEDCARBONYLGROUPINCORPORATIONTOHUMANRBCMEMBRANEROLEINVIVOSENESCENCEOFERYTHROCYTE

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Oxidative Stress Induced Carbonyl Group Incorporation to Human RBC Membrane: Role in Vivo Senescence of Erythrocyte

Dr. Arghya Sur [α], Dr. Hemontika Chakraborty [σ], Dr. Arindam Basu [ρ] & Dr. Sasanka Chakraborty [ω]

Abstract- This study was focused to identify the link between oxidative stress and senescence of erythrocytes in vivo. To elucidate this mechanism, various modification of RBC membrane proteins and lipids were analyzed invitro, exposing them to oxidative stress and the results were compared with the changes observed in erythrocytes undergoing senescence in vivo. The other objective was to confirm the mechanism of autoantibody mediated removal of aged RBCs. Our results established that increased lipid peroxidation products, followed by the enhanced damage of RBC membrane protein caused increased RBC membrane protein carbonylation, to normal red cells exposed to the in vitro Fe^{2+} & ascorbate induced oxidative stress. It was presumable that these changes were mediated by hydroxyl (ȯH) radicals. Further, similar changes were also seen in percoll gradient age fractionated high density aged RBCs. Both in vitro oxidative stress & in vivo studies with high density red cells showed no changes in membrane protein thiol oxidation and protein cross-linking. It was presumable that the enhanced bound IgGs to the red cells represented auto antibodies directed against carbonylated proteins of RBC membranes playing a central role in the senescence of RBCs followed by in vivo removal of such antibody coated RBCs from the circulation.

Keywords: human RBC membrane, protein carbonylation, oxidative membrane damage, senescence of RBC, oxygen free radical, ROS, autoantibody to RBC.

I. Introduction

The term 'senescence' in the context of normal blood cells implies that the cells are removed from the circulation in an age dependent manner. Life span of erythrocyte varies between species, is exceptionally constant within a species[1]. Over the last few decades several age related alterations of erythrocyte membrane and mechanisms of damaged or aged RBCs have been investigated [1,2]. Out of these oxidative damage to erythrocyte membrane proteins and lipids is presently thought to play key role during senescence of normal RBSs as well as accelerated senescence of pathological red cells seen in thalassemia , sickle cell anaemia etc. The oxidative damage is initiated probably by oxygen free radicals (ROS) and other oxidants produced endogenously [3, 4, 5, 6, 7, 8]. In vitro characterization of density separated red cells also has provided cumulative oxidative damage [3].

Dense human red cells show moderately increased amounts of methaemoglobin [9]. Exposing intact erythrocytes or ghosts to oxidizing systems in vitro showed various modifications e.g. lipid peroxidation, cross-linking and fragmentation of membrane cytoskeletal proteins, binding of hemichrome and heinz body to the inner surface of the membrane, clustering of band-3 protein, loss of membrane free sulphydryl groups, incorporation of carbonyl content in the membrane protein etc [5, 8, 10, 11, 12, 13, 14, 15, 16, 17]. Some of these changes have also been studied in vitro density separated or biotinylated aged erythrocytes and also in pathological red cells of accelerated ageing [1, 2, 4, 18, 19, 20, 21]. Many of these changes are seen in stored RBC in blood bank [22, 23]. A possible reason for cumulative oxidative damage to the red cell membrane would be a reduction in the activity of oxidative defense enzymes like, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase etc [24, 25].

Superoxide radicals ($\dot{O}_2$) undergo dismutation reaction spontaneously ($2\,\dot{O}_2 + 2H^+ \rightarrow H_2O_2$) or catalyzed by SOD, produces H_2O_2 ($2\dot{O}_2 + 2H^+ \xrightarrow{SOD} H_2O_2$) [26]. Again, $\dot{O}_2$ and H_2O_2 in presence of Fe^{2+} / Cu^{+} / Mn^{2+} can produce more reactive $\dot{O}H$ radical by Haber Weiss reaction ($\dot{O}_2 + H_2O_2 \xrightarrow{Fe2+\ /\ Cu+\ /\ Mn2+} \dot{O}H + OH^-$). In biological system, simple salt of Fe^{2+} / Cu^{+} / Mn^{2+} and H_2O_2 can also produce $\dot{O}H$ radical via Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \dot{O}H + OH^-$) [27]. Now, H_2O_2 can be removed from cells by the action of catalase ($2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$) or by the action of selenium dependent glutathione peroxidase ($H_2O_2 + 2GSH \xrightarrow{glutathione\ peroxidase} GSSGH + 2H_2O$) [24, 27, 28].

In vitro studies showed that ascorbic acid by virtue of its property of causing reductive recycling of

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Fe^{3+} to Fe^{2+} may promote metal catalyzed Haber Weiss reaction, to produce more damaging ȯH radicals [28, 29]. Hence, Ascorbate has dual role - anti oxidant and pro-oxidant, where latter is exhibited in presence of transition metals [27, 30, 31, 32, 33]. Again, it has been shown that membrane of high density red cell is highly susceptible to peroxidative damage exclusively to poly unsaturated fatty acids of membrane in a chain reaction [27, 5, 34, 35, 36, 15].

The signals that trigger the removal of aged erythrocytes and the actual mechanisms of such removal, i.e. elucidation of link between oxidative membrane damage and removal of aged cells from circulation are still controversial. However it has been suggested that recognition and removal of aged, infected or damaged RBC involve multiple pathways; mainly antibody independent phagocytosis, antibody dependent phagocytosis and removal by splenic sinuses [37]. In antibody independent removal, oxidatively damaged erythrocytes are phagocytosed by macrophages through scavenger receptors for low density lipo protein (LDL) in absence of opsonizing antibodies [38, 39]. In antibody dependent removal of aged RBC, auto antibodies (anti band-3, antigalactosyl antibodies or antibodies to malonaldehyde-protein adduct) bind to the surface of aged RBCs leading to phagocytic removal of aged, oxidatively damaged and pathologically damaged red cells by macrophages [40, 41, 42, 2, 7, 43, 37, 23]. But the nature of such antibodies has been subjected to debate [40, 41, 42, 37, 18, 2].

It has been proposed that impaired deformability provides a major route of the destruction of senescent red cells [44, 45]. Poor deformability or increased rigidity due to oxidatively modified red cells as well as aged RBC membrane proteins and lipids retard the red cells' movement in the spleen and they may be entrapped in splenic sinuses for destruction [14, 18, 46, 47].

It was clear that although oxidative damage to RBC membrane protein and lipid play key role in the mechanism of senescence of erythrocyte; but there were important lacunae in our present state of knowledge. This investigation led to fill up that lacunae and which in the long run might lead to identification of suitable agents to prolong the survival of endogenous or transfused RBCs in the circulation.

II. Aims and Objectives

The specific objectives of this work were-a) study of various oxidative modifications of human erythrocyte membrane lipids and proteins exposed in vitro to physiologically relevant oxidizing system like iron and ascorbate. b) study of effects of free radicals and metal chelators on the above system. c) to investigate if oxidative damage to RBC membrane like increased carbonylation led to enhanced binding of immunoglobulins (IgG) to cell surface. d) to study whether the oxidative damage to RBC membrane observed in vitro were also present in aged erythrocytes obtained from the circulation by density gradient method.

III. Materials and Methods

Blood obtained from healthy human male and female volunteers of age group 20 to 50 years was collected in 3.2% sodium citrate solution. Institute's research ethical committee approval was obtained. All the healthy blood donors were informed the purpose of the study prior to drawing of blood.

Materials

Percoll, Alpha cellulose, Microcrystalline cellulose, Catalase, Superoxide dismutase (SOD), Diethylene triamine penta acetic acid (DETAPAC), Butylated hydroxy toluene (BHT), Bovine serum albumin (BSA), Polyvinyl difluride (PVDF) membranes, Alkaline phosphatase conjugated goat anti rabbit IgG, Polyclonal anti-DNP antibody, 4-(2-hydroxy ethyl)-1-piperazine ethane sulphonic acid (HEPES), Phenyl methyl sulphonyl fluoride (PMSF), Tween20(Polyoxyethylene sorbitan mono laurate, Sigma ultra) and Ferritin (Type1: from horse spleen) were purchased from Sigma Chemical Co., USA., Biogel P-6 were obtained from Bio Rad, USA., 5-bromo-4 chloro-3-indolyl- phosphate/ nitro blue tetrazolium (BCIP/NBT), Tetra methyl benzidine (TMB), Protein-A- horse radish peroxidase conjugate, protein –A- agarose column and Human IgG purification kit were obtained from Bangalore Geni Pvt. Ltd., India. Thiobarbituric acid (TBA), Trichloro acetic acid (TCA) were from E.Merck, Germany. 2- Deoxy ribose (DR), Mannitol, Dimethyl sulphoxide (DMSO), L-Ascorbic acid, 2,4- dinitrophenyl hydrazine (DNPH) and all chemicals for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sisco Research Laboratory, Mumbai, India. All other chemicals used were of analytical grade.

Methods

a) Preparation of human erythrocyte ghosts

Human blood collected in 3.2% sodium citrate solution made free from WBC and Platelets by passing through a column bed of microcrystalline cellulose and alpha cellulose (1:3) as adopted from Beutler et al (1975) [48]. . Briefly, microcrystalline cellulose (mean size 50µm,Sigma cell-50) and α-cellulose were taken in 1:3 ratio by weight and suspended in phosphate buffered saline (PBS), pH 7.4 and packed in a 10 ml plastic syringe with an inner diameter of 1.5 cm, so that 5ml column was 1.5 cm by 5 cm. The packed column was washed with PBS, pH 7.4. The blood (approx 5 ml) was centrifuged at 1,000 x g for 5 min and the

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supernatant plasma was removed along with the buffy coat. The packed red blood cells were poured over the cellulose column and washed down with ice cold isotonic PBS, pH 7.4. The eluate was collected (approx. 40-50 ml for 5 ml of blood) and centrifuged at 1,000 x g for 10 min to obtain the erythrocyte pellet. The erythrocytes were routinely recovered from the column up to 80% and the preparations were practically free of leucocytes (> 99.5%) and platelets (> 95%) as verified microscopically. The erythrocyte ghosts were prepared by hypotonic lysis [49]. The pelleted erythrocytes were lysed in 60 volumes of 5 mM sodium phosphate buffer, pH 8.0 followed by centrifugation at 20,000 x g to obtain haemoglobin free white ghosts. The creamy white ghosts were suspended in 50 mM phosphate buffer, pH 7.4 and kept frozen at -20°C for subsequent experiments.

b) Estimation of protein

Protein content of erythrocyte membrane ghosts was estimated by the method of Lowry et al (1951) [50] after solubilizing in 1% SDS and using BSA as standard.

c) 2-Deoxy ribose (DR) degradation assay

Hydroxyl radical generation in the incubated mixture of ascorbate and/or Fe^{2+} has been measured by DR degradation assay [51]. The reaction mixture in 50 mM phosphate buffer, pH 7.4 contained 1mM DR, 0.5 mM ascorbate, 0.2 mM $FeSO_4$ or 5.6 µM Ferritin with or without other additions like mannitol (20mM) or 20 mM DMSO in a total volume of 600 µl. Incubation was terminated after 1 h by addition of 1.4ml of 2.8% TCA to each tube followed by addition of 0.6ml of 1% (w/v) TBA. Then all the tubes were heated for 10 min in a boiling water bath. The tubes were cooled briefly and absorbance taken at 532 nm.

d) SDS-PAGE of erythrocyte membrane ghosts

Freshly prepared erythrocyte ghosts with Fe^{2+} (0.2mM) and ascorbate (0.5mM) were incubated at 37°C for 2 h with or without other additions. Incubation was terminated by addition of electrophoresis sample buffer containing 3% SDS, and 5% β-mercapto ethanol followed by heating in boiling water bath for 3 min. The samples were immediately applied for discontinuous SDS-PAGE in reducing condition using 10% separating gel following the method of Laemmli (1970) [52]. Slab gels were stained with coomassie brilliant blue-R and destained in 5% methanol & 7.5% glacial acetic acid mixture.

e) Lipid peroxidation study

Lipid peroxidation in incubated ghosts was estimated by measuring the production of malonaldehyde (MDA) as described by Ohkawa et al (1979) [53, 51]. Erythrocyte ghosts incubated with or without ascorbate (0.5mM) and /Fe^{2+} (0.2mM) at 37°C up to 2 h with other additions like 0.5 mM BHT, mannitol (20mM) , DMSO (20mM) or catalase (50 µg/ml) in a total volume of 200 µl. To each 200 µl of incubation mixture were added 100 µl of 8.0% SDS, 750 µl of 20% acetic acid (pH 3.5) and 750 µl of 8.0% aqueous TBA. The samples were heated at 100°C for 15 min. The tubes were briefly cooled and 2.5 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added to each tube and vortexed thoroughly. The samples were centrifuged at 4,000 r.p.m. for 10 min. The organic layer was collected and absorbance measured at 532 nm. Amount of MDA was expressed in n moles/mg protein using a molar extinction co-efficient of 1.56 x 10^5 $M^{-1.}$ cm^{-1} [54].

f) Estimation of carbonyl content

Erythrocyte ghosts were incubated with or without ascorbate (0.5mM) and Fe^{2+} (0.2mM) at 37°C up to 2 h with other additions in a total volume of 200 µl. To each tube was added 500 µl of TCA followed by centrifugation at 3,000 r.p.m. to obtain a precipitate. To each precipitate 500 µl of 10 mM DNPH in 2 M HCl was added. The tubes were allowed to stand at room temperature for 10 min with occasional vortexing. Then to each sample 500 µl of 10% TCA was added followed by vortexing and centrifugation at 10,000 x g for 5 min. The supernatant was discarded and the pellet was washed 3 times with 1ml ethanol-ethyl acetate mixture (1:1) to remove excess reagent. Precipitated protein as pellet was solubilized in 1ml protein dissolving solution containing 2% SDS, 0.05% EDTA in 0.8 M sodium phosphate buffer, pH 8.0. Appropriate sample and reagent blanks were kept for this assay. Absorbance was taken at 370 nm and the carbonyl content of each sample was calculated using a molar absorption co-efficient of 22,000 $M^{-1}.cm^{-1}$ and the results were expressed in terms of n moles/mg protein [55, 56, 57, 58].

g) Estimation of sulphydryl group

Erythrocyte ghosts, in presence or absence of ascorbate (0.5 mM) and Fe^{2+} (0.2mM) with other additions were incubated for 2 h at 37°C in 50 mM phosphate buffer, pH 7.4 in a total volume of 200 µl. At the end of the incubation, erythrocyte membrane proteins were solubilized by the addition of 1 ml of protein dissolving solution containing 2% SDS, 0.05% EDTA in 0.8 M sodium phosphate buffer, pH 8.0. To each sample 50 µl of DTNB solution (40mg DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 8.0) was added. A set of reagent blanks were made and absorbance taken at 412 nm after 15 min, The thiol content was estimated using a molar absorption co-efficient of 13,600 $M^{-1}.cm^{-1}$ and expressed as n moles of of thiols/mg of protein [13, 57].

h) Purification of autologous IgG

Human autologous IgG was purified by affinity chromatography on a protein–A agarose column using a commercial kit of Bangalore Genei, India. The eluate

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containing IgG was passed through a Bio-gel P-6 column equilibrated in PBS, pH 7.4. IgG was stored in aliquots in presence of 0.1% sodium azide at 4°C.

i) Binding of autologous IgG to oxidatively damaged red blood cells

Erythrocyte ghosts were incubated with or without Fe^{2+} (0.2 mM) /ascorbate (0.5mM) in presence or absence of mannitol (20 mM), DMSO (20 mM) or catalase (50 µg/ml). After the incubation at 37°C for 2h the red cell ghosts were pelleted down. The supernatant from each tube was aspirated and the pellet suspended in PBS, pH 7.4 containing 1% BSA. Samples (100 µl) of red cell ghost suspension (control, oxidized and oxidized with inhibitors) were taken in micro centrifuge tubes pre-coated with 1% BSA. To each tube was added 100 µl of IgG in PBS -1% BSA and the tubes were left overnight at 4°C for IgG binding. Appropriate blanks (without membrane suspension) and negative controls (without added IgG) were kept. After the incubation, to each tube was added 0.8 ml of PBS-0.2% BSA followed by centrifugation at 10,000 x g for 10 min. The supernatant was removed and the pellet was washed twice with 1 ml of PBS-0.2% BSA. 100 µl of protein- A-HRP conjugate (diluted 1:10,000) was added to each tube and kept at 4°C for 1 h followed by centrifugation at 10,000 x g for 10 min after addition of 0.8 ml of PBS-0.2% BSA. The pellet was washed twice with PBS-0.2% BSA to remove completely the excess unbound Protein-A-HRP. Subsequently, 100 µl of the substrate solution TMB/H_2O_2 was added to each tube and mixed thoroughly. The tubes were kept in dark for 30 min during which a blue colour was developed. The reaction was stopped by addition of 200 µl of 2 M H_2SO_4 which also changed the colour of the reaction mixture from blue to yellow. The absorbance was taken at 450 nm. The absorbance of the blank and the appropriate negative control were subtracted from the absorbance of each test sample. The net absorbance of each test sample was a measure of IgG bound to red cell ghosts. IgG bound to oxidized ghosts with or without inhibitors was expressed as ratio to IgG bound to control unoxidized ghosts after appropriate corrections for differences in protein content in individual samples [13, 43].

j) Purification of band-3 protein from human erythrocyte

Human erythrocyte ghosts obtained were suspended in a solution containing 1 mM EDTA, 5 mM β-mercapto ethanol, 0.03 mM PMSF, which was adjusted to pH 7.5 with 1 M NaOH and stirred gently for 18 h at 4°C. The membranes were recovered by centrifugation at 1,00,000 x g for 1 h and re suspended in 50 mM sodium phosphate buffer, pH 7.4. The membrane pellet was subjected to SDS-PAGE as described earlier. The protein visible in the gel was predominantly band-3 and this alkali treated pellet was used as partially purified band-3 protein. Autologous IgG (1,5 mg/ml) was mixed with an equal volume of suspension of partially purified band-3 protein (alkali treated erythrocyte pellet, protein content 2 mg/ml) and kept at 4°C for 2 h followed by centrifugation at 1,00,000 x g for ½ h. The supernatant recovered was used as autologous IgG, depleted of anti band-3 antibodies (IgG-depleted) and used for IgG binding assay [43].

k) Separation of young and aged erythrocytes

Young and aged erythrocyte of human were isolated by isopyenic centrifugation through gradient of "Percoll", a commercially available polyvinyl-pyrrolidone-coated colloidal silica following the method of Rennie et al (1979) [59]. A continuous density gradient of "Percoll" (0-100%) was prepared with the help of a gradient maker and a peristaltic pump and using two stock solutions: one containing 5.263% BSA in water and another containing 5.263% BSA (w/v) in percoll. 19 volumes of each of these solutions were separately mixed with 1 volume of solution (final pH 7.4) containing 2.66 M NaCl, 0.09 M KCl and 200 mM HEPES, pH 8.5. Thus, resultant mixtures were isotonic containing 5% BSA. 0.8 ml of red cell suspension was brought to room temperature and carefully layered on the top of the continuous percoll gradient (8 ml). Age fractionation of red cell was achieved by centrifugation at 1,100 x g for 9 min at room temperature followed by slow deceleration of the rotor. The cell fractions were then sequentially aspirated from the top of the gradient using a Pasteur pipette attached to the peristaltic pump. Top 20% of the fractionated cells were taken as young cells and bottom 20% of the cells used as aged red cells. The cells were finally washed to free percoll by 3 washes in PBS, pH 7.4 [59].

l) After preparation of young and aged erythrocyte membrane, protein carbonyl content, free thiol groups, protein cross-linking etc were measured by previously mentioned methods.

m) Immunoblot analysis for protein carbonyls in young and aged erythrocyte membrane ghosts

Freshly prepared young and aged erythrocyte membrane ghosts were taken in two separate tubes each containing 100 µl of RBC membrane ghosts and to each of them was added 50 µl of 18% SDS to solubilized the protein followed by addition of 200 µl 20 mM DNPH in 10% TFA. A yellow colour was developed after incubating each mixture for 10 min. The samples were then neutralized to orange red colour by slow addition of 2 M Tris in 30% glycerol and 19% β-mercapto ethanol. The samples were subjected to SDS-PAGE followed by electroblotting in a semi dry electroblotter unit, using a constant current of 2 m.A./cm^2 for 45 min. The transfer of proteins to PVDF membrane was confirmed by staining one portion of the blotted

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membrane after it was dried. The other half of the blotted membrane was incubated with PBS containing1% BSA and kept for 2 h for blocking the non-specific sites. The blocked membrane was incubated at 37°C with shaking in presence of anti-DNP antibody (diluted 1:1000) for 1 h. After the incubation, the PVDF membrane was washed in a washing solution containing 0.1% (v/v) Tween-20 in PBS followed by further incubation for 1 h with alkaline phosphatase conjugated goat anti rabbit IgG. The bands in the PVDF membrane were detected by the addition of BCIP/NBT [55, 32].

n) Quantitation of cell bound IgG of young and aged erythrocytes

The young and aged red cells were washed thoroughly in PBS followed by preparation of corresponding membrane ghosts as described earlier. Membranes of young and aged erythrocytes were taken in separate microcentrifuge tubes pre-coated with 1% BSA. To each tube was added autologous IgG in PBS-1% BSA and the tubes were left overnight at 4°C for IgG binding. Bound IgG was quantitated as mentioned earlier. IgG bound to aged RBC membrane ghosts was expressed as a ratio of IgG bound to control young RBC membrane ghosts [43].

o) Statistical Analysis

All experiments were checked for reproducibility and statistical significance worked out. All the values in Tables and Charts of result section were presented as mean ± standard error of mean (SEM). Significance of difference between groups were determined following Students't' test for paired and unpaired observations [60].

IV. Results

a) Results presented in Table-1 showed that the mixture of Fe^{2+} (0.2 mM) and ascorbate (0.5 mM) produced hydroxyl radicals by Fenton reaction as shown by 2- deoxyribose (DR) degradation assay. The phenomenon was inhibited by hydroxyl radical scavenger like mannitol (20mM) upto 62.4% and DMSO (20 mM) up to 77.4% or by antioxidant enzyme catalase (50 µg/ml) upto 58.9%. Again, it was also observed that ferritin (5.6 µM) can also produce ȯH radical when incubated with 0.5 mM ascorbate (Table-1). Hence iron and ascorbate were physiologically relevant to erythrocyte in vivo and mixture of these components was used as a model system to oxidative stress in vitro.

b) Fig-1 showed that SDS-PAGE analysis of membrane proteins of erythrocyte ghosts incubated with Fe^{2+} and ascorbate. In control sample (Lane-a) different protein bands of red cell membranes were visible and named according to Fairbanks et al (1971) [49]. Incubation of erythrocyte membranes with Fe^{2+} (0.2 mM) and ascorbate (0.5 mM) did not however produce any cross-linking or protein fragmentation (Lane-b, c & d).

c) As presented in Table-2, no significant change in protein thiol content of red cell ghosts were observed during incubation with Fe^{2+} (0.2 mM) and ascor bate (0.5 mM) for 2 h at 37°C.

d) A significant increase in protein carbonyl content in red cell membrane ghosts exposed to iron and ascorbate for a period of 2 h was observed (Fig-2). The phenomenon was inhibited by hydroxyl radical scavengers like mannitol, DMSO and also by antioxidant enzyme, catalase (Fig-2). The inhibition was statistically significant.

e) When erythrocyte ghosts (protein content 0.6 to1.0 mg/ml) were incubated with Fe^{2+} (0.2 mM) and ascorbate (0.5 mM) at 37°C , MDA produced per mg protein per 2 h were significantly higher with respect to control erythrocyte ghosts. Lipid peroxidation was significantly inhibited (about 90%) by 0.5 mM of BHT, while mannitol (20 mM), DMSO (20 mM) and catalase (50 µg/ml) did not inhibit MDA production (Table-3).

f) Table-4 showed that a very significant increase (about 4 fold) in the binding of auto logous IgG occurred in oxidized ghosts compared to control. The binding of auto logous IgG to ghosts was quantitated by an immunoassay using Protein-A-HRP conjugate and TMB (substrate). Since it was difficult to estimate the number of IgG molecules bound per ghost cell, the net absorbance at 450 nm for each sample normalized to a protein concentration of 1 mg/ml was calculated and taken as a measure of bound IgG. The net absorbance for each sample was derived by subtracting from the total absorbance, the values for the blank and the appropriate negative control (ghost incubated without IgG). This increased binding of oxidized ghosts was however not seen when anti-band-3 depleted IgG was used for binding assay.

g) In order to elucidate the link between oxidative stress and enhanced IgG binding to red cell ghosts several radical scavengers and antioxidant enzymes were used in the binding assay. Table-5 showed that catalase and hydroxyl radical scavengers like mannitol (20 mM) and DMSO (20 mM) prevented significantly enhanced binding of autologous IgG to oxidized erythrocyte ghosts.

h) The parameters of oxidative stress were then measured in density separated erythrocytes in a percoll gradient. There was a statistically significant rise (1.5 fold) in protein carbonyl content in aged RBC membrane ghosts with respect to young red cell membrane ghosts (Fig-3).

i) Immunoblot analysis of young and aged erythrocyte ghosts using anti-DNP antibody also showed increased carbonylation of proteins in aged erythrocyte membrane (Fig-4).

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j) Fig-5 showed that there was no significant decrease in protein thiol content in aged RBC membrane ghosts compared to young RBC membrane ghosts.

k) SDS-PAGE of young and aged erythrocyte ghosts showed that there was no such remarkable difference in the band pattern of membrane proteins (Fig-6), and especially no cross-linked high molecular weight proteins or evidence of protein fragmentation was apparent in aged erythrocyte.

l) As presented in Table-6, autologous IgG binding to aged erythrocyte ghosts was more than 2.5 fold higher than that of young erythrocyte ghosts, when incubation was carried out with IgG concentration of 1 mg/ml. Raising IgG concentration to 2.5 mg/ml, IgG binding to aged red cell ghosts was about 3.7 times more than that of young erythrocyte ghosts.

V. Discussion

Erythrocyte membrane encounters oxidative stress both from cell interior and exterior [3]. Different investigators have studied red cell membrane alterations by subjecting intact RBC or RBC membrane ghosts to different oxidizing systems like ascorbic acid and iron /copper, H_2O_2/iron, ADP/Fe^{3+}, phenyl hydrazine, xanthine / xanthine oxidase etc have been reported [47, 31, 10, 43, 11, 8]. Many of these changes have also been noticed in aged red cells [1, 2, 3, 14, 15] or in damaged RBCs undergoing accelerated senescence in some pathological conditions [19, 4, 6, 21, 15, 16] or in stored RBC in blood bank [22, 23].

In this study, mixture of ascorbic acid and iron (Fe^{2+}) has been used to induce oxidative stress, because both these components are physiologically relevant in the context of oxidative injury to erythrocytes in vivo. Several discrete iron compartments in red cell have been suggested [21]. This iron is bioactive and can valance-cycle between Fe^{3+} and Fe^{2+} states is capable of generating hydroxyl radicals [31, 33, 27, 29]. Reducing agents like ascorbate can potentiate this mechanism and thereby promote the formation of hydroxyl radicals via Fenton's mechanism [30, 29]. The elaborate transport and recycling of ascorbate by red blood cells in vivo have been highlighted by many investigators [28, 33].

Results from Table-1 indicate that a mixture of ascorbate and iron (Fe^{2+}) is an active source of hydroxyl radicals generated by Fenton's reaction as evident from the inhibition of 2-deoyribose degradation by catalase and hydroxyl radical scavengers like mannitol and DMSO. The oxidation of Fe^{2+} to Fe^{3+} produces $\bar{O}_2$ radicals, which by dismutation produces H_2O_2. H_2O_2 further reacts with Fe^{2+} to give rise to $\dot{O}H$ radicals by Fenton's mechanism [31, 26, 33]. Ascorbate potentiates the reaction by reductive cycling of iron from Fe^{3+} to Fe^{2+} [29]. Further ascorbate undergoes autoxidation to produce H_2O_2 [33]. This autoxidation of ascorbate is $\bar{O}_2$ mediated and metal dependent as evident from its inhibition by SOD and DETAPAC [33]. The fact that ascorbate can interact with ferritin to generate $\dot{O}H$ radicals as shown in Table-1 implies that a similar reaction between ascorbate and native or denatured haemoprotein in red cells may also lead to the formation of toxic reactive oxygen species(ROS).

ROS can induce various forms of protein damage such as cross-linking of polypeptide chains, oxidation of protein thiol (-SH) groups, incorporation of carbonyl groups into protein etc had been reported [57, 43, 55, 22]. In hydroxyl radical generating system like iron/ascorbate, we have examined some of these modifications which are considered as hallmark for oxidative modification [55, 57, 32]. Under our experimental conditions protein cross-linking or fragmentation were not observed (Fig-1). Some earlier studies however, showed cross-linking of isolated cytoskeletal proteins of erythrocyte membrane when incubated for prolonged time with haemoglobin /H_2O_2 [46] or with xanthine/ xanthine oxidase [11] etc.

Oxygen free radicals also cause aggregation of proteins, converting the side chains of cysteine, methionine, histidine and tyrosine and form disulphide bonds as the consequence of oxidation of free thiol groups [13, 22]. The present results showed no significant decrease in protein thiol content, which was in conformity with the result of Beppu et al (1990) [43]. Beppu et al (1989) also observed from their result of SDS-PAGE of RBC membrane proteins in reducing condition no significant cross-linking of RBC membrane protein exposed to oxidative stress with respect to control ghosts[13] which was in conformity of our result (Fig-1). However, it has been observed by the same observers that SDS-PAGE of RBC membrane proteins in non-reducing condition formed disulphide bond and cross-linking of protein during oxidative stress [13, 43].

The metal catalyzed oxidation of proteins can introduce carbonyl group at lysine, arginine, proline and threonine residues in a 'site-specific' manner [61]. Free radical damage to amino acid residues and/or reaction with aldehydes or both derived from lipid peroxides could contribute towards generation of protein carbonyl [61]. The carbonyl bearing residues have not been completely identified but gamma-glutamyl semialdehyde appeared to be the major residue [55]. In different purified and crude extracts of protein several workers have reported changes in carbonyl content as a consequence of oxidative modification [55, 62.57], but on erythrocyte membranes such reports are scanty. The results obtained from this study (Fig-2) showed that iron/ ascorbate induced oxidative stress to RBC membranes caused a significant rise in red cell membrane protein carbonyl content which was inhibited by catalase and hydroxyl radical scavengers-mannitol, and DMSO (Fig-

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2) which indicated that the increase in protein carbonyl content was mediated by hydroxyl radical attack.

In crude tissue preparation increased carbonyl content as a result of oxidant stress may also be secondary to associated lipid peroxidation [57]. During lipid peroxidation, lipid peroxide or hydroperoxide or peroxyl radicals are formed [27]. These preformed lipid peroxidation products which broke down to yet more radical species by reacting with transition metal like iron [27] in turn caused oxidative damage to membrane proteins [57]. This type of damage could not be inhibited by ȯH radical scavengers like mannitol, DMSO or anti oxidant enzyme catalase; but lipid soluble chain breaking antioxidants like α-tocopherol and BHT were effective[57]. In this study however, protein carbonylation has been inhibited significantly by hydroxyl radical scavengers like mannitol, DMSO and catalase (Fig-2); but lipid peroxidation of RBC membrane was not inhibitible by these agents (Table-3). This implied that in this system protein oxidation was not secondary to lipid peroxidative damage. The enhanced production of MDA in red cell membranes by Fe^{2+} and ascorbate during in vitro incubation was presumably the result of breakdown of pre-formed lipid peroxides or hydro peroxides by Fe^{2+} (Table-3). Ascorbate potentiated such iron catalyzed break down of peroxides and hydro peroxides keeping the iron in the reduced (Fe^{2+}) state [57]. That has been observed in other systems also [27, 5, 10, 53]. However, the consequence of such peroxidative damage to membrane was controversial [8, 34].

Autologous IgG binding to red cell surface has been studied by many investigators [40, 18, 42], but there were several controversies regarding the binding site and antigenic specificity of bound autologous antibody [37, 18, 42]. The signal that led to enhanced IgG binding to red cell surface also were controversial. [3]. Clustering of band-3 protein or proteolytic cleavage of band-3 have been suggested by different workers as trigger for enhanced IgG binding to RBC membrane [40, 18, 41, 23, 62]. Beppu et al (1990) [43] and later on other scientist [7] have shown that in vitro enhanced binding of autologous Ig G to red cell surface occured following an oxidant stress. We have tried to confirm that finding. Our results confirmed that increased binding of anti band-3 immunoglobulins to red cell surface following an oxidative stress by iron and ascorbate (Table-4). However that was prevented by hydroxyl radical scavengers like mannitol, DMSO and anti oxidant enzyme catalase (Table-5).

Results presented in Fig-1 indicated that oxidant stress to red cell ghosts under our experimental conditions did not lead to protein cross-linking /fragmentation or any change in protein thiol content (Table-2). On the other hand ȯH radical scavengers like mannitol, DMSO and anti oxidant enzyme catalase prevented both the increased incorporation of carbonyl groups to membrane protein (Fig-2) and enhanced binding of autologous IgG to red cell surface following an oxidative stress (Table-4). That obviously implied that the two phenomena were inter-related.It was presumable that the bound IgG in our in vitro assay systems were auto antibodies generated earlier in vivo against oxidatively modified proteins with increased carbonylation.

Various types of membrane alterations have been reported to aged red cells, which were linked to oxidative damage [2, 3, 18, 19]. In the present study increased carbonyl content to aged RBC membrane, observed both in spectrophotometric and immune-detection assay system (Fig-3, Fig-4) with respect to that of young red cell membrane ghosts represented that the aged red cells have been subjected to oxidative stress in vivo which had been considered as the hall mark of oxidative protein damage [55]. That result seemed interesting, as no such information was available in aged erythrocytes except for an isolated study on stored RBC in blood bank [22].

No cross-linking or polymerization of red cell membrane polypeptides were noticed in reducing condition discontinuous SDS-PAGE pattern of aged red cell membranes in comparison to young red cell membranes (Fig-6). These results were also in accordance with the *in vitro* data where oxidant treatment of RBC membrane ghosts failed to produce any cross-linking or fragmentation of proteins (Fig-1). However Rettig et al (1999) [2] have reported a covalent, non-reducible, non disulphide cross-linking of globin subunits of denatured haemoglobin with membrane at the end of the RBC's life span. Our failure to observe any cross-linking of membrane proteins in SDS-PAGE of aged erythrocytes membrane might be related to running the electrophoresis of aged RBC membrane ghosts in reducing condition [13] or species difference or method of isolation of aged red cells.

Again, we have observed no significant alteration in membrane protein thiol content of aged red cells compared to young cells (Fig-5). That was in conformity with our in *vitro* result, where oxidative stress by Fe^{2+} and ascorbate failed to cause measurable change in protein thiol content of RBC membrane ghosts after incubation up to 2 h (Table-2). Our result was in agreement with the result of Piccinini et al (1995) [63].

Increased binding of autologous IgG to aged red cell membrane have been well documented [40, 18, 37], although disputes were there regarding precise nature of the auto-antibodies binding to RBC surface [37]. Our results have confirmed this (Table-6). Enhanced IgG binding to human aged red cells as seen in our study supported the observation of Kannan et al (1991) [64] and the result was also consistent with elevated IgG binding seen in red cells of other species like mouse, dog etc, using different methods for

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separating aged red cells from young red cells [65,2]. Since oxidant stress in *vitro* led to enhanced binding of autologous IgG to red cell surface (Table-4), it might be implicated that the increased binding of autoantibodies to aged erythrocyte membrane was the result of oxidative injury to red cell membrane during in vivo ageing. This had also been suggested by Beppu et al (1990) [43] and Fujino et al (2000) [7]. The nature of the auto antibody binding to aged red cells have not been elucidated in our study. However, since increase in lipid peroxidation products followed by increased damage of RBC membrane protein as increased carbonyl group incorporation was the only noticeable change in the membrane proteins of aged erythrocytes compared to that of control young erythrocyte, it would be tempting to speculate that auto-antibodies had been directed against such altered membrane proteins. The antibody dependent phagocytosis of red cells had been considered as an important mechanism of removal of old and damaged red cells from the circulation [40,18, 2, 7]. The oxidative modification of membrane protein during ageing of erythrocytes was therefore, directly linked with the subsequent removal of those cells from circulation.

VI. Conclusion

From the results and discussion of our study, it can be concluded that in vitro oxidant stress to red blood cell ghosts by iron and ascorbate, led to increased lipid peroxidation products followed by enhanced damage to RBC membrane protein, caused increased membrane protein carbonylation; presumably mediated by hydroxyl radicals and that phenomenon was directly linked to enhanced binding of autologous IgG to oxidized ghosts under similar condition of incubation. It was presumable that bound autologous immunoglobulins to oxidized red blood cell membranes represented auto-antibodies generated in vivo against carbonylated proteins. Further, in vivo senescence of red cells was associated with increased membrane protein carbonylation and enhanced binding of autologous IgG to red cell surface. Since removal of aged red cells from the circulation in large measure depended upon antibody dependent phagocytosis of red cells by macrophages, our results indirectly pointed out that oxidative stress induced modification of RBC membrane carbonylated proteins triggered the latter mechanism for removal of RBC by subsequent formation of auto-antibodies.

VII. Acknowledgement

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Table 1 : Hydroxyl radical detection by 2-deoxy ribose (DR) degradation assay

Incubation mixture	Absorbance at 532 nm after 2 h	% Inhibation
DR + Buffer	0.012 ± 0.000	-
DR + Ascorbate + Fe^{2+}	0.402 ± 0.005	-
DR + Ascorbate + Fe^{2+} + Mannitol	0.151 ± 0.002	62.4
DR + Ascorbate + Fe^{2+} + DMSO	0.091 ± 0.006	77.4
DR + Ascorbate + Fe^{2+} + Catalase	0.165 ± 0.006	58.9
Dr + Ferritin	0.072 ± 0.001	-
Dr + Ferritin + Ascorbate	0.243 ± 0.005	-

Reactions carried out with incubation mixtures containing 2-deoxyribose (1 mM) with or without $FeSO_4$ (0.2 mM) or Ferritin (5.6 µM) and ascorbate (0.5 mM) in presence or absence of inhibitors like mannitol (20 mM), DMSO (20 mM) or catalase (50 µg/ml) as described in the methods.
The values presented were mean ± SEM of 6 observations (N=6).

Table 2 : Protein thiol (-SH) content of erythrocyte membrane

Incubation mixture	**n moles/mg protein**
Erythrocytes ghosts + Buffer	36.00 ± 1.0
Erythrocytes ghosts + Fe^{2+} + Ascorbate	34.09 ± 1.101
Erythrocytes ghosts + Fe^{2+} + Ascorbate + Mannitol	34.59 ± 1.0
Erythrocytes ghosts + Fe^{2+} + Ascorbate + DMSO	35.55 ± 1.079

Incubation of erythrocyte ghosts was carried out for 2 h at 37°C in phosphate buffer (50mM, pH 7.4) with or without addition of $FeSO_4$ (0.2 mM), ascorbate (0.5 mM) in presence or absence of mannitol (20 mM) and DMSO (20 mM) followed by estimation of protein thiol content as described in methods.
The values presented were the mean ± SEM of 10 observations (N=10).
Values were not significantly different from corresponding control values as observed from Student's 't' test (paired).

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Table 3 : Iron and ascorbate induced lipid peroxidation in erythrocyte membrane

Incubation mixture	n moles MDA per mg protein after 2 h
Erythrocyte ghosts + Buffer	1.27 ± 0.000
Erythrocyte ghosts + Fe^{2+} + Ascorbate	6.53 ± 0.112*
Erythrocyte ghosts + Fe^{2+} + Ascorbate + BHT	1.79 ± 0.033†
Erythrocyte ghosts + Fe^{2+} + Ascorbate + Mannitol	6.52 ± 0.112#
Erythrocyte ghosts + Fe^{2+} + Ascorbate + DMSO	6.52 ± 0.113#
Erythrocyte ghosts + Fe^{2+} + Ascorbate + Catalase	6.53 ± 0.115#

Incubation of erythrocyte ghosts was carried out at 37°C for 2 h in phosphate buffer (50 mM, pH 7.4) with or without addition of $FeSO_4$ (0.2 mM) and ascorbate (0.5 mM) in presence or absence of other additions like BHT (0.5 mM), mannitol (20 mM), DMSO (20 mM) or catalase (50 μg/ml) followed by measurement of lipid peroxidation as described in the methods.
Results presented were the mean ± SEM of 5 observations (N=5), followed by Student's 't' test (paired).
Value marked '' indicated significant rise of lipid peroxidation with respect to control (erythrocyte ghosts incubated alone with buffer). *$p < 0.001$.*
Value marked '†' showed significant inhibition by BHT. †$p < 0.001$.
Value marked '#' showed no significant inhibition when compared with the value marked ''.*

Table 4 : Autologous IgG binding to red blood cell ghosts

Incubation mixture	Autologous IgG binding (ratio to control)
Oxidized ghost + Unfractionated IgG	4.26 ± 0.175
Oxidized ghost + Anti band-3 depleted IgG	0.98 ± 0.188

Erythrocyte ghosts incubated with or without $FeSO_4$ (0.2 mM) and ascorbate (0.5 mM) for 2 h at 37°C and binding of unfractionated autologous IgG (1mg/ml) and anti band-3 depleted autologous IgG (0.8 mg/ml) were measured as described in the methods.
Results presented were mean ± SEM of 6 observations (N=6).

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Table 5 : Effects of Catalase, Mannitol and DMSO on autologous IgG binding to red blood cell ghosts

Incubation mixture	Autologous IgG binding (ratio to control)
Oxidized ghost + IgG	4.26 ± 0.176
Catalase treated oxidized ghost + IgG	0.85 ± 0.155*
Mannitol treated oxidized ghost + IgG	0.91 ±0.159*
DMSO treated oxidized ghost + IgG	0.85 ± 0.149*

Erythrocyte Ghosts incubated with or without $FeSO_4$ (0.2 mM) and ascorbate (0.5 mM) for 2 h at 37°C in presence or absence of catalase (50 µg/ml), mannitol (20 mM) and DMSO (20 mM) and binding of unfractionated autologous IgG (1 mg/ml) was measured as described in the methods.
Results presented were mean ± SEM of 6 observations (N=6) followed by Student's 't' test (paired).
Values marked '' showed significant inhibition of IgG binding when compared with the IgG binding of oxidized ghost alone. *p < 0.001.*

Table 6 : Autologous IgG binding to young and aged red blood cell ghosts

Incubation mixture	Concentration of IgG	Autologous IgG binding (ratio to control)
Aged RBC membrane ghost + IgG	1 mg/ml	2 .70 ± 0.174†
Aged RBC membrane ghost + IgG	2.5 mg/ml	3.72 ± 0.183*

Young and aged erythrocyte ghosts were prepared and used for autologous IgG binding assay as described in the methods.
IgG binding to aged erythrocyte ghosts was expressed as ratio to that of young erythrocyte ghosts (control).
The values presented were the mean ± SEM of 5 observations (N=5), followed by Student's 't'test (unpaired).
Value marked '' is significantly higher than the value marked '†'; *p < 0.001.*

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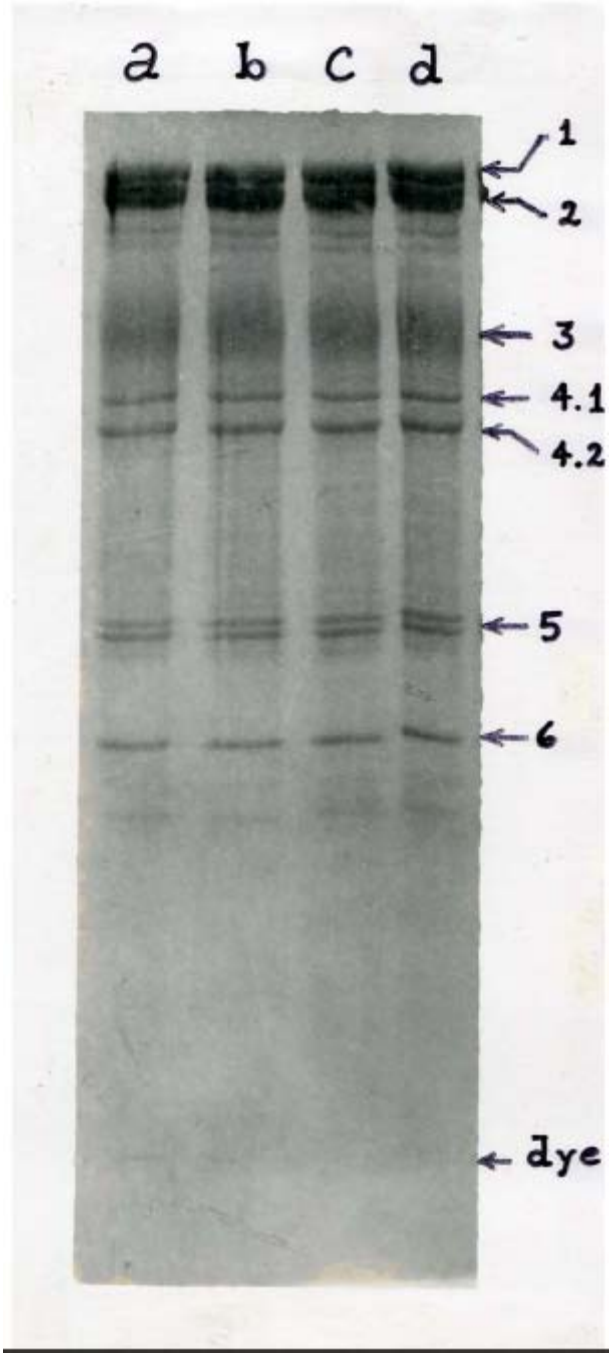

Figure 1 : Effects of iron and ascorbate on erythrocyte membrane

Incubation of erythrocyte ghosts were carried out for 4 h as described in the methods. All lanes contained equal amount of protein. Lanes (a, b, c, d):- erythrocyte ghosts incubated in the buffer alone (a) or with 0.2 mM $FeSO_4$ (b) or 0.5 mM ascorbate (c) or 0.2 mM $FeSO_4$ and 0.5 mM ascorbate (d). The bands in Lane (d) were numbered according to Fairbanks et al (1971).

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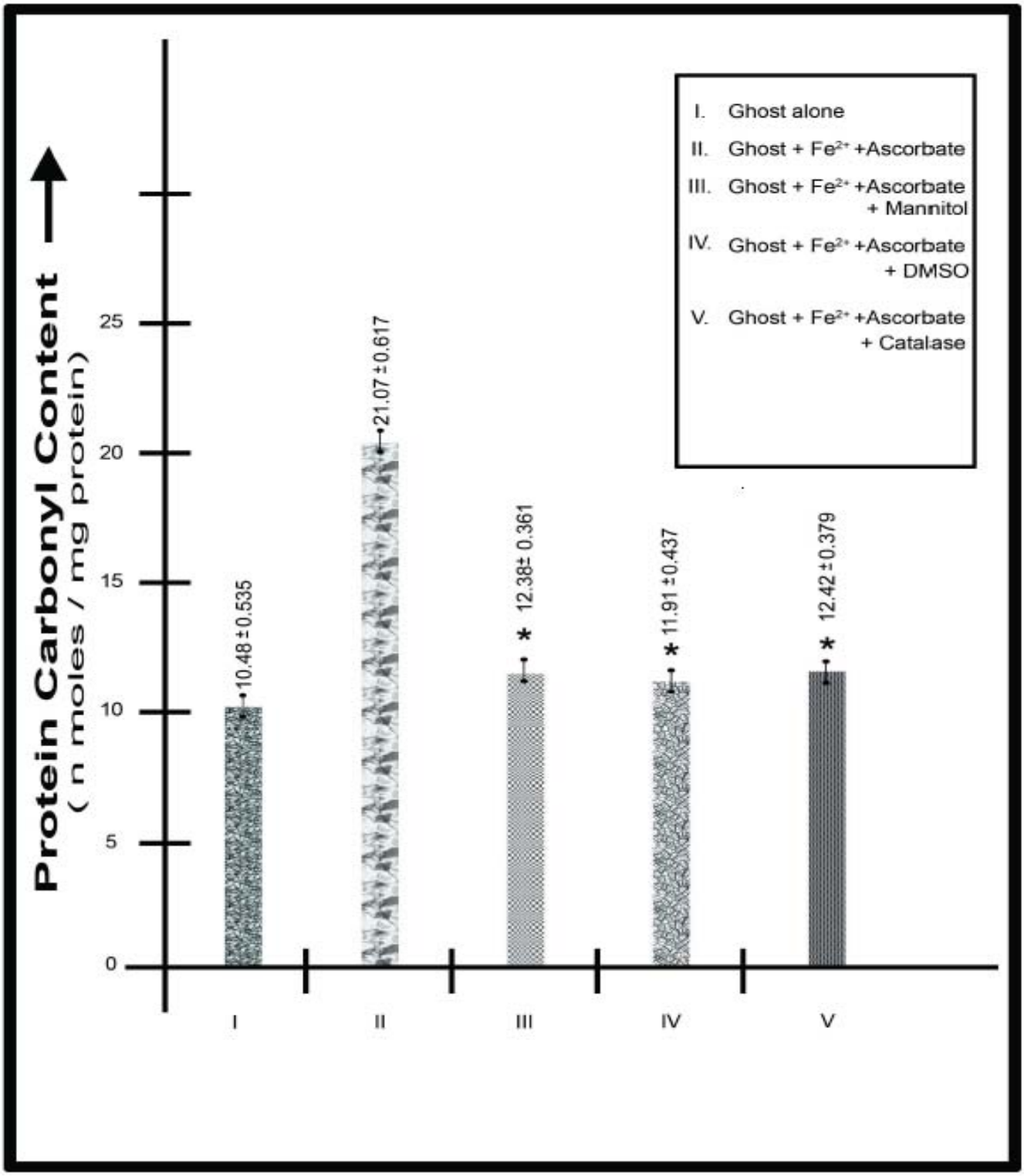

Figure 2 : Protein carbonyl content of human erythrocyte ghost

Incubation of erythrocyte ghosts was carried out at 37°C for 2 h with or without addition of $FeSO_4$ (0.2 mM) and ascorbate (0.5 mM) in presence or absence of mannitol (20 mM), DMSO (20 mM) and catalase (50 µg/ml) and protein carbonyl content was measured as described in the methods.
Results presented were mean ± SEM of 10 observations (N=10) followed by Student's 't' test (paired).
Value of II was significantly higher than the value of I; where, IIp < 0.001.
*Values of III, IV and V, marked with asterisks (*) indicated statistically significant inhibition with respect to the value of II; where *p < 0.001.*

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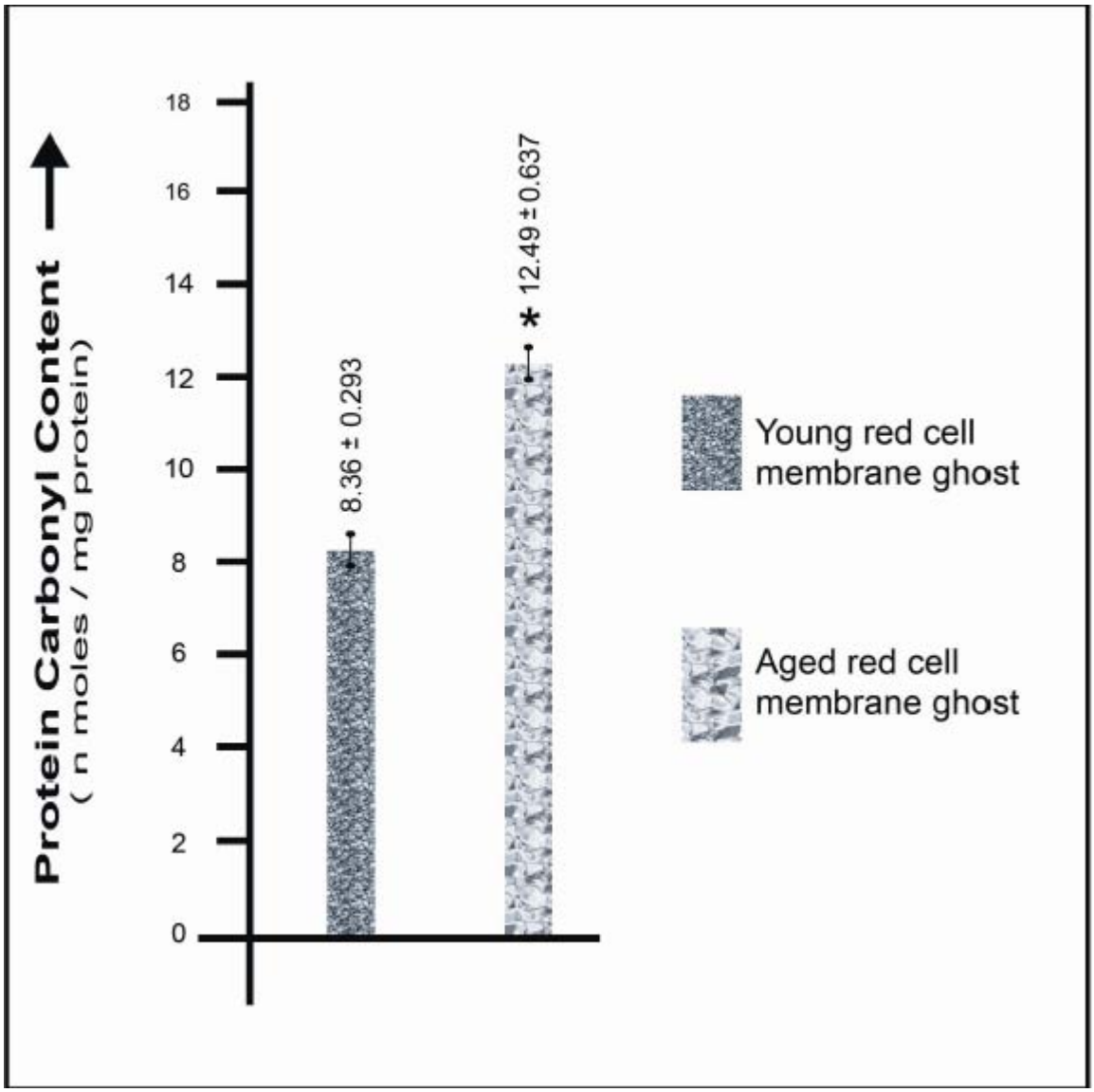

Figure 3 : Protein carbonyl content in young and aged red cell membrane ghosts

Values presented were mean ± SEM of 6 observations (N=6) followed by Student's 't' test (unpaired). ''indicated significant increase in protein carbonyl content in aged red cell membrane ghosts when compared with that of young red cell membrane ghost. *p < 0.001.*

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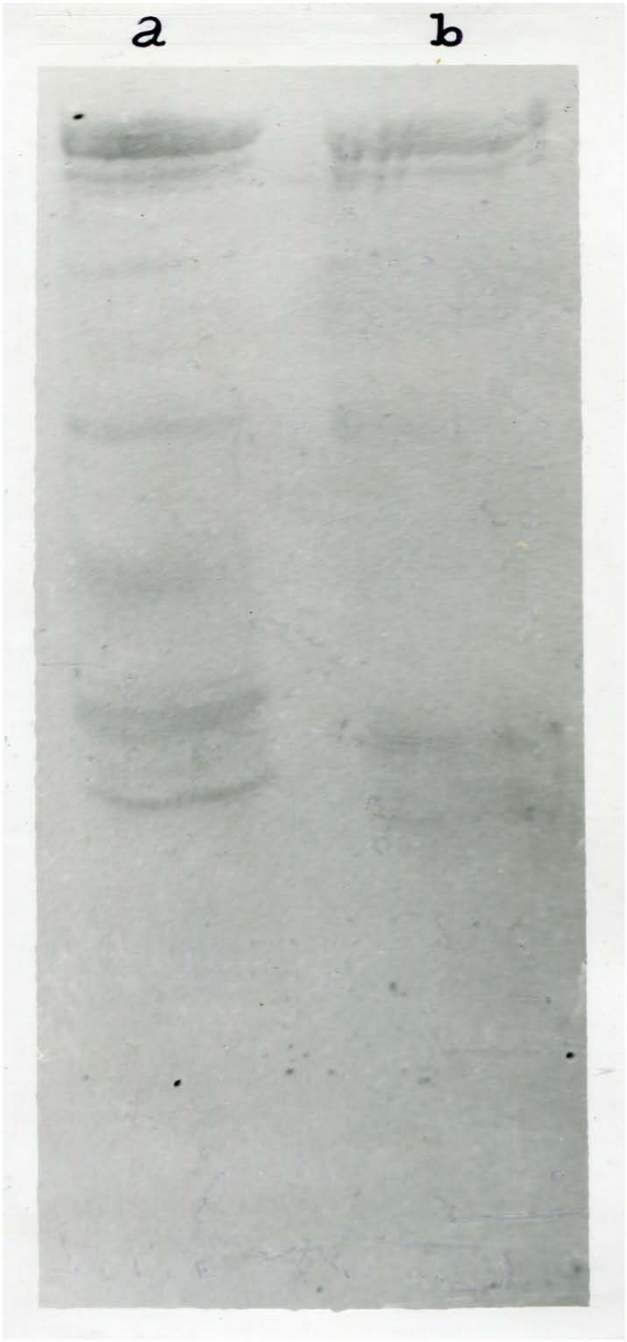

Figure 4 : Immunodetection of protein carbonyls in young and aged erythrocyte membrane

Young and aged erythrocyte membrane protein carbonyls were derivatized with DNPH followed by SDS-PAGE and transfer of protein bands to PVDF membrane and subsequent immunodetection by anti-DNP antibodies as described in methods.

Lane (a):- aged erythrocyte membrane ghosts;
Lane (b):- young erythrocyte membrane ghosts

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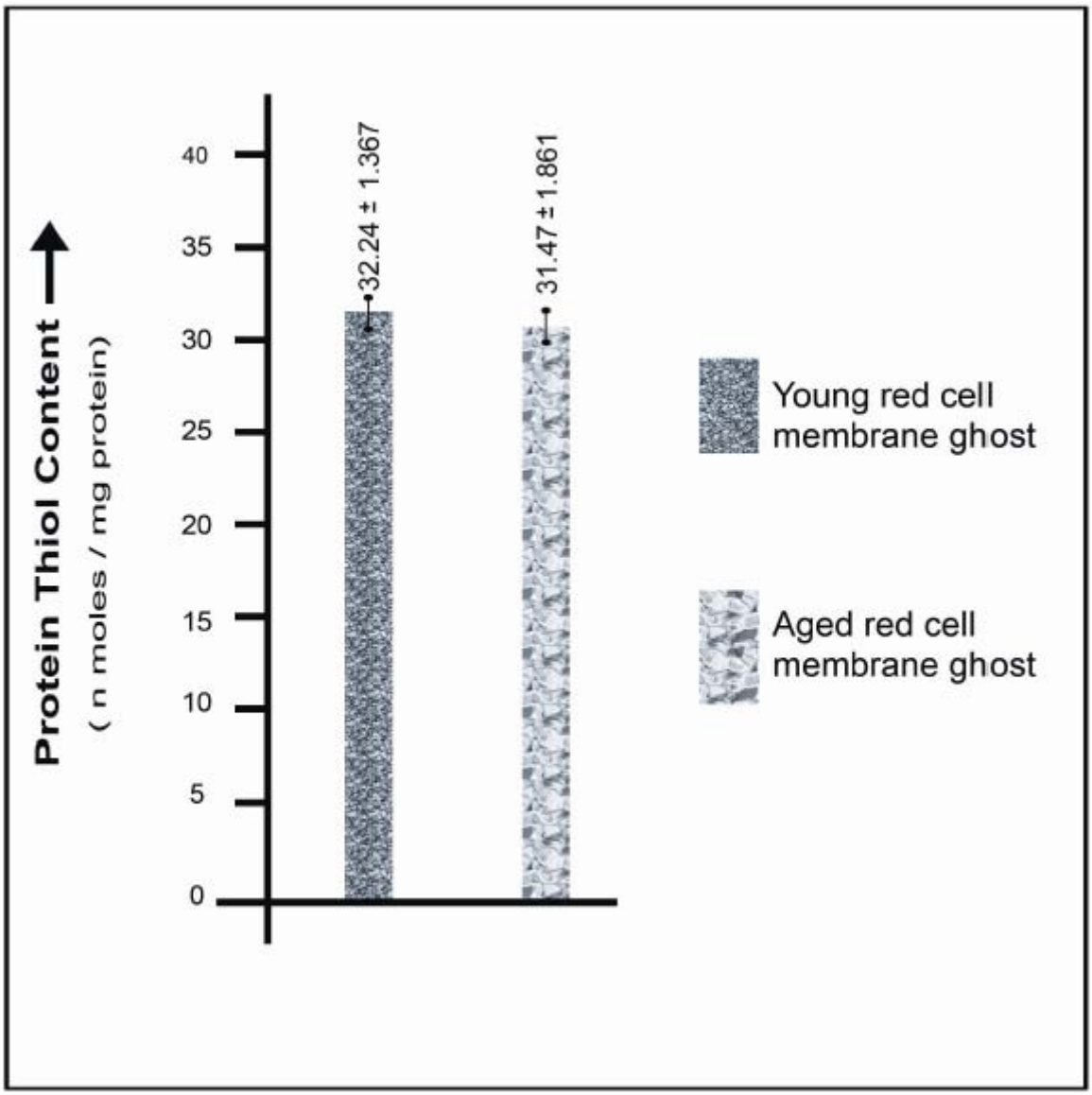

Figure 5 : Protein thiol content in young and aged red cell membrane ghost

Values presented were mean ± SEM of 6 observations (N=6) followed by Student's 't' test (unpaired). Values were not significantly different from each other.

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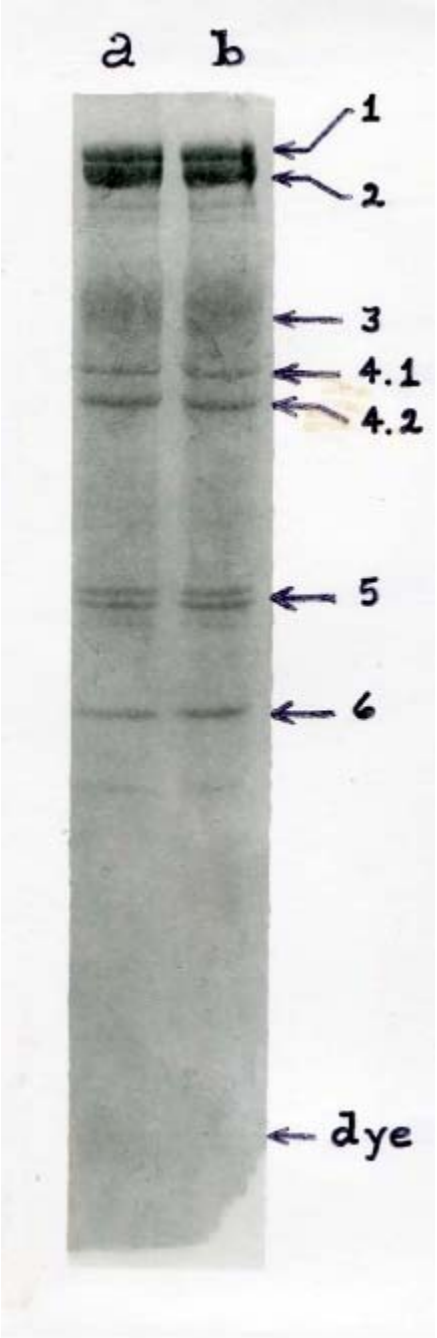

Figure 6 : SDS-PAGE pattern of young and aged erythrocyte membrane ghosts

Preparation of young and aged erythrocyte membrane ghosts and SDS-PAGE were conducted as described in the methods. Both the lanes contained equal amounts of proteins and membranes were not incubated. Lane (a):-young erythrocyte ghosts; Lane (b):-aged erythrocyte ghosts. The bands in Lane (b) were numbered according to Fairbanks et al (1971).

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FNAC as a Diagnostic Tool in the Evaluation of Lymphadenopathy-A Tertiary Hospital Experience

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Abstract- Introduction: Fine Needle Aspiration cytology (FNAC) is a rapid diagnostic technique for the initial evaluation of palpable swellings. Enlarged lymph nodes are a common lesion subjected to FNAC. The cytomorphological features observed on cytology correlate very well with the histological appearances. Besides initial diagnosis of lymphoma, it also helps in diagnosis of residual disease, recurrence, progression of low grade to high grade lymphoma and also the detection of metastasis as lymph nodes are a common site of metastasis of different cancers.

Materials and Methods: The present study comprises of 396 patients who presented with lymphadenopathy in the department of pathology, Giansagar medical college and hospital, Ramnagar, Patiala, Punjab from April 2008 to March 2011 (3 years). All the patients were subjected to FNAC using a 10 ml syringe and 22 gauge needles. 70 cases were subjected to lymph node biopsy. The cytological results were compared with the histological findings wherever possible.

Keywords: lymphoma, lymph nodes.

GJMR-C Classification : NLMC Code: WI 141

FNACASADIAGNOSTICTOOLINTHEEVALUATIONOFLYMPHADENOPATHYATERTIARYHOSPITALEXPERIENCE

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FNAC as a Diagnostic Tool in the Evaluation of Lymphadenopathy-A Tertiary Hospital Experience

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Abstract- Introduction: Fine Needle Aspiration cytology (FNAC) is a rapid diagnostic technique for the initial evaluation of palpable swellings. Enlarged lymph nodes are a common lesion subjected to FNAC. The cytomorphological features observed on cytology correlate very well with the histological appearances. Besides initial diagnosis of lymphoma, it also helps in diagnosis of residual disease, recurrence, progression of low grade to high grade lymphoma and also the detection of metastasis as lymph nodes are a common site of metastasis of different cancers.

Materials and Methods: The present study comprises of 396 patients who presented with lymphadenopathy in the department of pathology, Giansagar medical college and hospital, Ramnagar, Patiala, Punjab from April 2008 to March 2011 (3 years). All the patients were subjected to FNAC using a 10 ml syringe and 22 gauge needles. 70 cases were subjected to lymph node biopsy. The cytological results were compared with the histological findings wherever possible.

Observations & Results: Cervical lymph nodes were the commonest ones that were sampled (216) followed by axillary (115) and inguinal lymph nodes (65). FNAC diagnosis was divided into malignant and non malignant lesions. Metastatic cancer was the commonest malignancy observed whereas reactive hyperplasia was the commonest non malignant condition followed by granulomatous pathology. The cytological diagnosis correlated well with the histopathological diagnosis with the best correlation found in metastatic cancer (100%)

Conclusion: FNAC of lymph nodes proved to be a very useful tool in the diagnosis and early evaluation of enlarged lymph nodes. It is useful in diagnosis of both neoplastic as well as non neoplastic conditions.

Keywords: lymphoma, lymph nodes.

I. Introduction

Fine needle aspiration cytology (FNAC) is a simple and rapid diagnostic technique. It is an accepted procedure to diagnose lesions in the thoracic and abdominal cavities as well as superficial lumps in the body [1]. Since this technique lends itself to outpatient diagnosis, it is eminently suited for use in peripheral medical centers [2].

Lymphadenopathy is one of the commonest clinical presentations in the outdoor patients. Etiology can vary from an inflammatory process to a malignant condition [1,3]. FNAC is a safe accurate, repeatable, sensitive, specific and a cost-effective procedure in the diagnosis of lymphadenopathy [1,3,4]. In the past, the assessment of lymphadenopathy was made indirectly from the clinicopathological parameters or biopsy, but with the resurgence of FNAC as a diagnostic tool, procedure of biopsy is mostly avoided [3,5]. The cytomorphological features obtained in needle aspiration frequently correlate very well with histological appearance and in some cases has qualities of a micro biopsy [1].

FNAC can be more useful than open biopsy in cases where there is multiple lymphadenopathy. On open biopsy, the surgeon can excise only those lymph nodes accessible through the incision. Severe inflammation around the lymph nodes can hinder the identification of tissues and excised specimen may not be representative. With FNAC, nearly all the palpable nodes can be aspirated, thus providing better sampling and a higher diagnostic yield [3,5,6].

The advent of Human immunodeficiency virus (HIV) infection makes FNA particularly attractive for surgeons as it involves lesser risk to the performer than open biopsies. Acquired immunodeficiency syndrome (AIDS) related lymphadenopathy has definite patterns like florid reactive hyperplasia, folliculolysis, explosive follicular hyperplasia, lymphocytic depletion. Though FNAC cannot clearly demarcate all these lesions, it has definite identifiable reactive patterns described and is useful in detecting specific infective etiologies [7,8].

FNAC remains the first line investigation in cases of lymphadenopathy. Besides initial diagnosis of lymphoma, it helps in diagnosis of residual disease, recurrence, progression of low grade to high grade lymphoma and also the detection of metastasis as lymph nodes are a common site of metastasis of different cancers [3].

FNAC is also a very useful tool for the diagnosis of various infectious diseases particularly tuberculosis as acid fast staining can be easily done on the aspirates. This is very important in view of high prevalence of tuberculosis in our country, atypical presentation of tuberculosis and due to the fact that AFB are mostly seen in purulent aspirate smears which don't show any granulomas and can be dismissed as acute

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suppurative lymphadenitis in the absence of Ziehl-Nellsen staining [2,4,10].

FNAC sometimes does pose problems in diagnosing lymphomas [2,11]. Diagnosis and particularly their subclassification is still controversial and cytological diagnosis of lymphoma is usually followed by biopsy [3,12]. The diagnosis of lymphoma on FNAC can further be improved if combined with various specialized techniques i.e. ultrasound (USG) and computed tomography (CT) guidance, cytochemistry, immunophenotyping, molecular diagnosis and flow cytometry[3,13].

II. Materials and Methods

The present study comprises of 396 patients who presented with lymphadenopathy in the department of pathology, Giansagar medical college and hospital, Ramnagar, Patiala, Punjab from April 2008 to March 2011 (3 years).

All the patients were subjected to FNAC using a 10 ml syringe and 22 gauge needles. Some of the slides were air dried and the rest were put in 95% ethanol and stained with May Grunwald Giemsa (MGG) stain and Papanicolaou (PAP) stain respectively. Additional stains i.e. Ziehl-Neelsen (ZN) stain, Periodic acid Schiff (PAS) were performed wherever necessary.

The detailed history of the patient i.e. age, sex and duration of involvement and other investigations performed, were recorded. The patients were followed up. 70 cases were subjected to lymph node biopsy. The cytological results were compared with the histological findings wherever possible.

III. Observations and Results

- A total of 396 patients were included in the study.
- The age of the patients ranged from 4 to 88 years. Male to female ratio was 1.3:1.
- Cervical lymph nodes were the commonest ones that were sampled (216) followed by axillary (115) and inguinal lymph nodes (65).

FNAC diagnosis was divided into malignant and non malignant lesions. (Fig 1, 2)

Figures

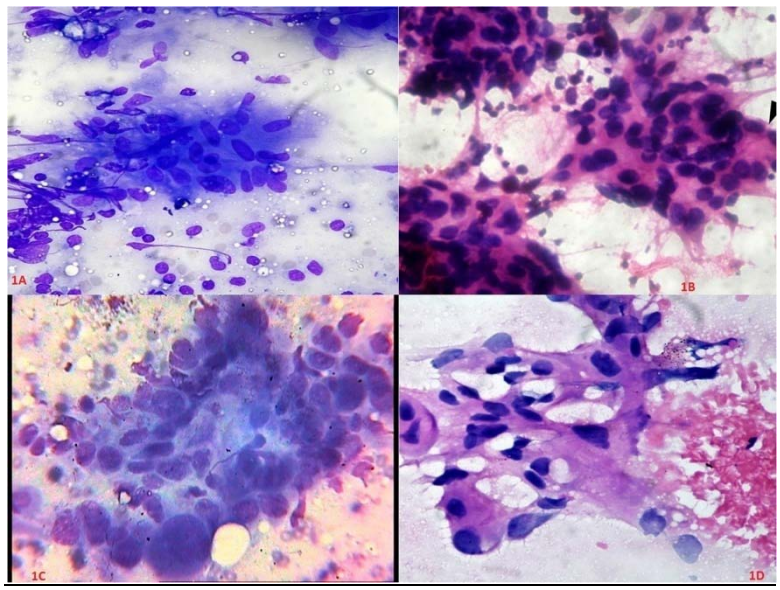

Figure 1

Fig 1 A : Granulomatous lesion – A cluster of epitheloid cells and few lymphocytes (MGG x 400)

Fig 1 B : Metastatic Adenocarcinoma – Neoplastic cells with delicate cytoplasm and pleomorphic, hyperchromatic nucleus arranged in cell clusters and glandular pattern (Papx 400)

Fig 1 C : Metastatic Squamous cell carcinoma – A cluster of neoplastic squamous cells with highly pleomorphic cells having ample basophilic cytoplasm (MGG x 400)

Fig 1 D : Metastatic Squamous cell carcinoma – A cluster of neoplastic squamous cells with highly pleomorphic cells having ample eosinophilic cytoplasm (Pap x 400)

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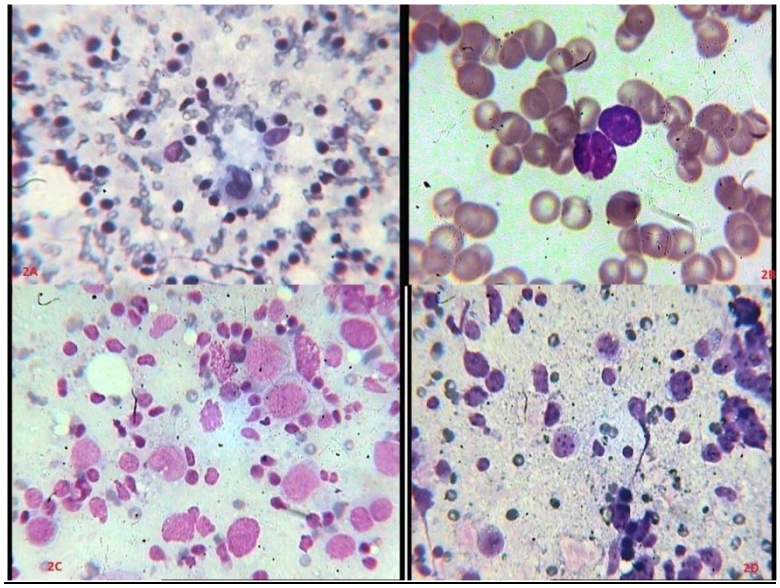

Figure 2

Fig 2 A : Hodgkin's Lymphoma: A Reed Sternberg cells (RS cell) present in a background of mixed Inflammatory cells (MGGx400)

Fig 2 B : Follicular Lymphoma (Blood film): An atypical lymphoid cell with abnormal chromatin and cleaved nucleus (Leishmann stainx1000). The blood film was examined due to the presence of increased lymphoid cells in the FNAC with atypical features.

Fig 2 C : Diffuse large B cell lymphoma: Large cells with increased N:C ratio, multiple nucleoli present against a dirty background. (MGGx400). On IHC, these cells were CD19,20,22 positive.

Fig 2 D : Anaplastic large cell lymphoma: Large cells with abundant cytoplasm, pleomorphic, irregular nuclei and multiple nucleoli. (MGGx400). On IHC, these cells were CD 30 positive.

Table I : shows the distribution of malignant lesions

Hodgkins lymphoma (HL)	11	
Non Hodgkins lymphoma (NHL)	25	
NHL with Leukemic infiltration	02	
Metastatic	51	
Total	89	

Table II : shows the distribution of non-malignant lesions

Reactive lymphadenopathy	185	
Acute suppurative lesion	41	
Granulomatous with necrosis	51	
Granulomatous without necrosis	24	
Total	301	

6 cases were excluded from the study as the material was insufficient for diagnosis.

Biopsy of the lymph node was done in 70 cases. FNAC diagnosis of these was as follows.

HL: 11

NHL: 27

Metastatic: 20

Granulomatous lesion: 12

Histopathological diagnoses of the above cases were as follows.

1) All the cases of HL on FNAC were confirmed by biopsy. 7 were mixed cellularity and 4 were nodular sclerosis. These cases were confirmed by immunophenotyping. They were positive for CD 15 and CD 30. However 1 case of HL was diagnosed as granulomatous lesion on FNAC due to intense granulomatous response. Also 2 cases of HL were diagnosed as NHL on FNAC but turned out to be HL on histopathology. Therefore, FNAC showed a sensitivity of 78.6% in diagnosing HL whereas the

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specificity was 100% considering histopathology as the gold standard.

2) 25 out of 27 cases diagnosed as NHL on FNAC were confirmed by biopsy. 2 cases turned out to be lymphocyte predominant HL which were confirmed by immunophenotyping. Thus the sensitivity of FNAC in diagnosing NHL was 100%where as specificity was 95.7%. 25 cases of NHL were further sub typed as follows on immunophenotyping.
   - Follicular lymphoma
   - Small lymphocytic lymphoma
   - Diffuse large B cell lymphoma
   - Burkitt's lymphoma
   - Anaplastic large cell lymphoma
3) All the metastatic lesions diagnosed by FNAC were confirmed on histopathology. Their distribution was as follows:
   - Squamous cell carcinoma:11
   - Adenocarcinoma:07
   - Small cell carcinoma: 1
   - Undifferentiated carcinoma: 1

Therefore, FNAC showed a high sensitivity and specificity of 100% in diagnosing metastatic lesions.

4) Out of 12 granulomatous lesions diagnosed on FNAC, 11 showed positive correlation on histopathology, 1 was diagnosed to be a case of HL with granulomatous reaction. This case was missed on FNAC due to intense granulomatous reaction which obscured the presence of Reed Sternberg (RS) cells. Thus the sensitivity of FNAC was 100% in these cases. Specificity was found to be 97.67%.
5) All 58 malignant cases diagnosed on FNAC, which were subjected to biopsy were confirmed on histopathology. Thus the sensitivity and specificity of FNAC in diagnosing metastasis was found to be 100% each.

IV. Discussion

FNAC is a simple, safe, cost effective, quick as well as an efficient diagnostic procedure with relatively no contraindications and no side effects. It forms an important tool for diagnosing various causes of lymphadenopathy [1,2,4].

In our study, there was a slight male preponderance with male:female ratio of 1.3:1. This is in accordance with various other studies with similar findings [1,4,5].

Lymph node enlargement can occur in a diverse age group from very early age to elderly. The youngest patient in our study was 4 years of age where as the oldest was 88 years. These figures compare to the findings in other studies [1,4,5,7,9].

The commonest lymph nodes sampled were cervical, followed by axillary and inguinal. These findings are also consistent with those observed in other studies [1,2,3,5,7].

The causes of lymphadenopathy are diverse and range from innocuous reactive hyperplasia, suppurative pathologies and granulomatous lesions to dreaded malignancies i.e. lymphomas and metastatic carcinomas. Out of a total of 396 patients in our study, 89 were diagnosed as malignant and 301 as non malignant on FNAC. 6 patients were excluded from the study as the material was insufficient and the patient was lost for follow up. These values are also in accordance with results of other studies [1,3,4,7,8,13]. The predominance of infective conditions in the etiology correlates well with the fact that in our country, the infectious diseases outnumber the malignant ones and also because the malignancies tend to be examined at a later age in contrast to the western countries where malignancies are reported earlier.

Majority of the cases were reactive in nature (47.5%). These results correlate well with other studies [1,3,9].

Granulomatous pathology accounted for 75 cases (19.2%). 51 were with necrosis and 24 without necrosis. The incidence of AFB positivity was more in cases with necrosis (35/51) in contrast to those without necrosis (04/24). This is due to the reason that abundant necrosis is seen in individuals with immune-compromised status which leads to greater AFB positivity whereas in patients with a good immune status, there is formation of more granulomas, less necrosis and less AFB positivity.

Among the malignancies, metastatic carcinomas constitute the predominant group constituting a total of 51 cases (13.07%). All the cases which were subjected to histopathology (20) showed positive correlation. The majority of the metastatic carcinomas were Squamous cell carcinoma which was in accordance with various other studies [1,3,12,13,14,15].

Lymphomas on FNAC constituted 38 of the total cases (9.74%) which correlated well with other studies [1,2,11]. 11 were diagnosed as HL and 27 as NHL on FNA. 02/27 diagnosed as NHL also showed leukemic infiltration in the peripheral blood and were diagnosed as follicular lymphoma on histopathology. All the cases of HL on FNAC were confirmed by biopsy. 7 were mixed cellularity and 4 were nodular sclerosis. These cases were confirmed by immunophenotyping. They were positive for CD 15 and CD 30. However 1 case of HL was diagnosed as granulomatous lesion on FNAC due to intense granulomatous response. Also 2 cases of HL were diagnosed as NHL on FNAC but turned out to be HL on histopathology. Therefore, FNAC showed a sensitivity of 78.6% in diagnosing HL whereas the specificity was 100% considering histopathology as the gold standard.

25 out of 27 cases diagnosed as NHL on FNAC were confirmed by biopsy. 2 cases turned out to be lymphocyte predominant HL which were confirmed by immunophenotyping. Thus the sensitivity of FNAC in

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diagnosing NHL was 100%where as specificity was 95.7%.

V. Conclusion

FNAC of lymph nodes proved to be a very useful tool in the diagnosis and early evaluation of enlarged lymph nodes. It is useful in the diagnosis of both neoplastic and non neoplastic conditions with good sensitivity and specificity. In many cases, it reduces the need for a surgical procedure to be performed on the patient.

- FNAC showed a sensitivity of 78.6% in diagnosing HL whereas the specificity was 100% considering histopathology as the gold standard.
- The sensitivity of FNAC in diagnosing NHL was 100%where as specificity was 95.7%.
- FNAC showed a high sensitivity and specificity of 100% in diagnosing metastatic lesions.
- The sensitivity of FNAC in diagnosing granulomatous lesions was 100%. Specificity was found to be 97.67%.
- The sensitivity and specificity of FNAC in diagnosing metastasis was found to be 100% each.

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- Font type of all text should be Swis 721 Lt BT.
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- Author Name in Font Size of 11 with one column as of Title.
- Abstract Font size of 9 Bold, "Abstract" word in Italic Bold.
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- Two Column with Equal Column with of 3.38 and Gaping of .2
- First Character must be three lines Drop capped.
- Paragraph before Spacing of 1 pt and After of 0 pt.
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- Large Images must be in One Column
- Numbering of First Main Headings (Heading 1) must be in Roman Letters, Capital Letter, and Font Size of 10.
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You can use your own standard format also.
Author Guidelines:

1. General,

2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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Complete support for both authors and co-author is provided.

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Research articles: These are handled with small investigation and applications

Research letters: The letters are small and concise comments on previously published matters.

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(b) A brief Summary, "Abstract" (less than 150 words) containing the major results and conclusions.

(c) Up to ten keywords, that precisely identifies the paper's subject, purpose, and focus.

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(e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition; sources of information must be given and numerical methods must be specified by reference, unless non-standard.

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(g) Discussion should cover the implications and consequences, not just recapitulating the results; conclusions should be summarizing.

(h) Brief Acknowledgements.

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References

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27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

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30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

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Key points to remember:

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- Please note the criterion for grading the final paper by peer-reviewers.

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- To the point depiction of the research
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- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

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Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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