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Prevalence of Bovine Fasciolosis and its Economic Significane in and Around Assela, Ethiopia Shiferaw Mulugeta, Feyisa Begna, Ephrem Tsegaye

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Abstract – A cross-sectional study was carried out from November 2009 to march 2010 on bovine fasciolosis in and around Assela and at Assela municipal abattoir to assess its prevalence and economic importance. From total of 400 cattle examined coprlogically 45.25 % (181) were found positive for fasciolosis. The prevalence of bovine fasciolosis was higher in male cattle than females and also higher in younger cattle (> 3 yrs) than older ones (> 5 yrs). The prevalence of bovine fasciolosis in the study sites was significantly ($p<0.05$) affected by sex, age and months of the year, however, its prevalence was not significantly ($p>0.05$) affected by body conditions. Post mortem examination was done on a total of 183 cattle and 34.97% were found infected with Fasciola at Assela municipal Abattoi. F. hepatica was found to be the predominant facsiola species causing bovine fasciolsis in the study areas. The economic significance of bovine fasciolosis was also assessed from condemned liver and carcass weight loss. Thus based on the retail value of bovine liver and 1kg of beef the total annual economic loss from fasciolosis during the study time was estimated to be 698,700.6 Eth. Birr (51,909.4 USD).

Keywords : *Assela, Bovine, Economic significant, Fasciola, Prevalence.*

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Prevalence of Bovine Fasciolosis and its Economic Significance in and Around Assela, Ethiopia Shiferaw Mulugeta, Feyisa Begna, Ephrem Tsegaye

Ephrem Tsegaye[α], Feyisa Begna[Ω], Shiferaw Mulugeta[β]

Abstract - A cross-sectional study was carried out from November 2009 to march 2010 on bovine fasciolosis in and around Assela and at Assela municipal abattoir to assess its prevalence and economic importance. From total of 400 cattle examined coprlogically 45.25 % (181) were found positive for fasciolosis. The prevalence of bovine fasciolosis was higher in male cattle than females and also higher in younger cattle (> 3 yrs) than older ones (> 5 yrs). The prevalence of bovine fasciolosis in the study sites was significantly ($p<0.05$) affected by sex, age and months of the year, however, its prevalence was not significantly ($p>0.05$) affected by body conditions. Post mortem examination was done on a total of 183 cattle and 34.97% were found infected with Fasciola at Assela municipal Abattoi. F. hepatica was found to be the predominant facsiola species causing bovine fasciolsis in the study areas. The economic significance of bovine fasciolosis was also assessed from condemned liver and carcass weight loss. Thus based on the retail value of bovine liver and 1kg of beef the total annual economic loss from fasciolosis during the study time was estimated to be 698,700.6 Eth. Birr (51,909.4 USD).

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I. Introduction

Fasciolosis is an economically important parasitic disease, which caused by trematodes of the genus *Fasciola* that migrate in the hepatic parenchyma, and establish and develop in the bile ducts (30). *Fasciola* is commonly recognized as liver flukes and they are responsible for wide spread of morbidity and mortality in cattle characterized by weight loss, anemia and hypoproteinemia. The two most important species, *Fasciola hepatica* found in temperate area and in cooler areas of high altitude in the tropics and subtropics and *Fasciola gigantica*, which predominates in tropical area. *Fasciola hepatica* is found in area above 1800 m.a.s.l. In between these altitude limits, both species coexists where ecology is conductive for both snail hosts, and mixed infections prevailed (35). The snail of the genus *Lymnae natalensis* and *Lymnae truncatula* are known as intermediate host in life cycle of fasciolosis.Infection with *Lymnea truncatula* is usually associated with herds and flocks grazing wet marshy land. On the other hand, *Fasciola gigantica* is a fresh water snail and infection with this species is associated with livestock drinking from snails infected watering places as well as with grazing wetland, which may be seasonally in undated (22). Fasciolosis is an economically important disease of domestic livestock, in particular cattle and sheep and occasionally man Ahmed *et al*. (3). The disease is responsible for considerable economic losses in the cattle industry, mainly through mortality, liver condemnation, reduced production of meat, milk, and wool, and expenditures for anthelmintics (11) and (19). The world-wide losses in animal productivity due to fasciolosis were estimated at US $200 million per annum, to rural agricultural communities and commercial producers (8), with over 600 million animals infected Ramajo *et al*. (26). In developed counties, the incidence of *F. hepatica* can reach up to 77%. In tropical countries, fasciolosis is considered the single most important helminth infection of cattle, with reported prevalence of 30-90% Spithill *et al*. (28). The prevalence of fasciolosis in many parts of Africa has been determined mainly at slaughter. However estimation of economic loss due to fasciolosis at national or regional level is limited by lack of accurate estimation of the prevalence of disease Phiri *et al*. (23).

The presence of fasciolosis due to *F. hepatica* and *F. gigantica* in Ethiopia has long been known and its prevalence and economic significance has been reported by several workers (17), (16), (5), (35), (36), (29), Fufa *et al*. (15). Available published reports have indicated that bovine fasciolosis causes economic losses of roughly 350 million Birr per annum due to deceased productivity alone (5). More recently, (29) and Fufa *et al*. (15) have reported financial losses of 6300 USD and 4000 USD per annum, respectively due to liver condemnations at slaughter houses.

Assela is one of town in Ethiopia, located in the South-east of Addis Ababa where huge livestock populations are found. The major land cover is thus used for grazing which support on an average 27 livestock per hectare. Livestock are major agricultural resource in the area. Assela is one of the areas where the environmental conditions and altitude of the area is conducive for the occurrence of fasciolosis. However; little information is available about its prevances and its economic significance in the study area. Therefore, the

objectives of this study were to study the prevalence of bovine fasciolosis and assess direct (liver condemnation) and indirect (carcass weight) economic losses caused by fasciolosis in the study area.

II. Materials and Method

a) Description of the Study Area

Topography and climate: Assela is situated at $6^0 59^1$-$8^0 49$ N latitude and $38^0 41^1$-$40^0 44^1$ E longitude in central Ethiopia, 175 km south cast of Addis Ababa. The altitude of the area ranges from 1780-3100 m.a.s.l and characterized by mid subtropical temperature ranging from 5^0c-28^0c. The annual average rainfall is 1200 mm and mostly with clay type of soil and in rare case black soil. The area covers 23674.72 km square and topographically has highland escapement and lowland areas. The high land areas are found centrally and the low lands dominate the periphery of the area (4).

b) The study sites

Tiyo Woreda has 18 Peasant Aassociations (PA) and out of these only three representative PAs (*Dosha, Kobolcha area*, and *Kulumsa*) were selected by considering their similarity in production system, their differences in altitude and livestock populations.

c) Study population

During sampling of the study animals in the present study, sexes, ages and body conditions of all the sampled cattle from the selected three PAs of Tiyo woreda were recorded.

d) Sample size Determination

Since there was no previous study in Tiyo woreda to establish the prevalence and economic significance of bovine fasciolosis, the sample size was determined by taking the prevalence of 50% fasciolosis using the formula given by (31).

$$n = \frac{1.96^2 . P_{exp} (1\text{-}Pexp)}{d^2}$$

Where n = required sample size
P_{exp} = expected prevalence= 50%
d = desired absolute precision=5%
Hence, d = 0.05 and p= 0.5 (50%).

Accordingly 384 animals were supposed to be sampled but in order to increase the precession a total of 400 study animals were used.

e) Study Design and Sampling Method

A cross-sectional investigation of the prevalence of bovine fasciolosis in the three PAs of Tiyo woreda was carried out from November 2009 to March 2010. Simple random sampling technique was the sampling strategy used to collect all the necessary data from feacal samples and abattoir survey of the study animals.

f) Study Methodology

i. *Coprological Examination*

Faecal samples for parasitological examination were collected directly from the rectum of each animal and freshly defected faeces in to plastic bottles with gloved hand. The samples were clearly labeled with universal bottles preserved with 5% formalin and each sample was clearly labeled with animal's identification, date and place of collection. Samples were packed and dispatched in cool box to avoid development of eggs and hatching. In the laboratory, coprospic examination was performed to detect the presence of fasciola eggs using the standard sedimentation techniques (18).

ii. *Abattoir Survey*

Active abattoir survey was conducted based on cross sectional study during routine meat inspection on randomly selected cattle slaughtered at Assela municipal abattoir. A total of 183 cattle were examined during the study. During ante-mortum examination detail records about the species, breeds, sexes, origins and body conditions of the animals were performed. The origin of the animal and age whenever possible while body condition scoring based on (20). During post-morton inspection, each liver visually inspected, palpated and incised based on routine meat inspection by (14). All livers having *Fasciola* species condemned were registered and flukes were conducted for species identification.

iii. *Species Identification*

After making systematic incision on liver parenchyma, and bile ducts, flukes were collected in the universal bottle containing 10% formalin in preservative and examined to identify the involved species. *Fasciola gigantica* (20 - 75 mm x 3.12mm) resembles *Fasciola hepatica* (20 - 30mm x 10mm) but readily recognized by its larger size, the shoulders are not prominent and the body is more transparent. It is grayish- brown in color changed to grey when preserved (27).

iv. *Economic loss assessment* .

The total economic loss due to fasciolosis in cattle slaughtered from the summation of annual liver condemnation cost (direct loss) and cost due to carcass weight reduction (indirect loss) was assessed.

v. *Direct Economic loss*

Direct economic loss was resulted from condemnation of liver affected by fasciolosis. All livers affected with fasciolosis were totally condemned. The annual loss from liver condemnation was assessed by considering the overall annually slaughtered animal in the abattoir and retail market price of an average zebu liver. Annual slaughtered rate was estimated from retrospective abattoir records of the last three years, while retail market price of an average size zebu liver was determined from the information collected from butcheries in Assela Town. The information obtained

was subjected to mathematical computation using the formula set by (21).

ALC = CSR X LCX P
Where ALC = Annual loss from liver condemnation
CSR = Mean annual cattle slaughtered at Assela municipality abattoir
LC = Mean cost of one liver in Assela Town.
P=Prevalence rate of the disease at the study abattoir

vi. *Indirect Economic loss*

Indirect economic loss was associated with carcass weight reduction due to fasciolosis. A 10% carcass weight loss in cattle is due to fasciolosis. Average carcass weight of an Ethiopian Zebu was taken as 126 kg (20). The annual carcass weight loss due to bovine fasciolosis assessed using the following formula set by (21).

ACW = CSR XCL XBC X PX 126 Kg

Where ACW = Annual loss from carcass weight reduction.

CSR = Average No cattle slaughtered per annual at annual at the study abattoir.
CL = Carcass weight loss in individual cattle due to fasciolosis.
BC = An average price of 1kg beef at Assela town
P=Prevalence rate of fasciolosis at the study abattoir.
126 kg = Average carcass weight of Ethiopian Zebu.

vii. *Data Management Analysis*

All the data collected during the study period were stored in excel spreadsheet for statistical analysis and were analyzed using intercooled stata 7.0 for windows (2001) to determine prevalence and analyze the associations with risk factors.

III. Results

a) Coprological finding

From a total of 400 faecal samples examined from cattle during the study period, 181 (45.25%) samples were found positive for fasciolosis.

The prevalence of bovine fasciolosis between the two sexes in the current study (table 1) revealed 62.73% (female) and 11.67% (male) and differ significantly ($p<0.05$). From the result of the present study on the prevalence of fasciolosis between different age groups showed an inverse correlation (table 1) and also the prevalence of fasciolosis was statistically significant difference ($p <0.05$) between cattle of different age groups.

b) Fasciola species Identification

From the total of 183 slaughtered animals whose liver were inspected in the abattoir, 64 livers were found to be positive for liver fluke infection (table 2) and from these 42 livers (65.625%) harbored with *F. Hepatica,* 16 livers (25%) harbored with *F.gigantica* and the remaining 6 livers (9.375%) harbored mixed infection.

Table 3 of the present study shows that there was no a statistical significant difference ($p>0.05$) in the prevalence of fasciolsis in cattle with different body conditions. But the higher prevalence (75%) was observed with cattle whose body conditions were thin and the lowest prevalence (40%) was recorded for cattle whose body conditions were good.

The prevalence of Bovine *Fasciolosis* between the three different study sites in the present study (table 4) showed the presence of variability on the prevalence of Bovine *Fasciolosis* between them. The highest (54.13%) and the lowest (39.61%) prevalence Bovine *Fasciolosis* were observed Kulumsa and Dosha, respectively.

The prevalence of bovine fasciolosis in each month of the study periods was also indicated in table 5. The table shows the presence of significant difference ($p<0.05$) in the prevalence of bovine fasciolosis between each month of the study periods. The highest (69.767%) and the lowest (32.18%) prevalence were observed in March and November, respectively.

c) Economic loss assessment

i. *Direct Economic loss*

Direct economic loss was resulted from liver condemnation as the result of fasciolosis. Generally all infected livers with fasciolosis are unfit for human consumption. The 64 fasciolosis infected livers of cattle were corresponding to an estimated total loss of about 805.71 ETB. In the study abattoir the average annual cattle slaughtered rate was estimated to be 3000 while mean retail price of bovine liver in Assela town as 36 ETB. Prevalence of fasciolosis in Assela municipality abattoir estimated as (34.97%). Therefore the estimated annual loss form organ condemnation is calculated according to the formula:

ALC = CSR X LC X P
= 3000 X 36 X ETB X 34.97%
= 3000 X 36 ETB X 0.3497
= 37,767.6 ETB

ii. *Indirect Economic loss*

Indirect economic loss was due to carcass weight reduction as result of *Fasciola* infection. In the study area the average price of 1kg beef was 50 ETB. The annual economic loss from carcass weight reduction due to bovine fasciolsis is calculated by using the formula: ACW

= CSR X CL X BC X P X 126kg
= 3000 X 10% X 50 ETB X 34.97% X 126kg
= 3000 X 0.1 X 50 ETB X 0.3497 X 126kg
= 660, 933 ETB

Therefore, the total annual economic loss due to bovine fasciolosis in the study abattoir is the summation of the losses from organ condemnation (direct loss) and carcass weight reduction (indirect loss) and thus a total of 698,700.6 ETB (51, 909.4056 USD).

NB: 1 USD was equivalent to 13.4600

IV. Discussions

Fascioliosis is an important parasitic disease of domestic ruminants caused by two liver fluke species: *Fasciola hepatica* and *F. gigantica* (Trematoda). *Fasciola hepatica* has a cosmopolitan distribution, mainly in temperate zones, while *F. gigantica* is found in tropical regions of Africa and Asia.

Bovine fasciolsi exists in almost all region of Ethiopia. However, the prevalence, epidemiology and *Fasciola* species involved vary with locality. This is mainly attributed to the variation in the climate and ecological condition such as altitude, rainfall, temperature and livestock management system (17), (5). The result of present study indicated that bovine fasciolsis relatively spread with moderate prevalence of 45.25% in the study area as compared to high prevalence of 86% in Keffa (5), 80% in and around Debre Berhan (9), 88.57% and 82.5% in western shoa (33). Moreover, (5), (34) and (13) have reported prevalence of 61%, 52% and 62.2% in Gonder, around Tan, and around Bahir Dar, respectively. The result of these workers are relatively higher than the present finding and this variation might be attributed to the difference in the infestation, level of study area and the present study were conducted during the dry period of the year when the infections rate of fasciolsis is expected to be low. The result of the present study is similar with the prevalence of bovine fasciolosis reported at Jimma. Sodo and Ziway abattoirs by (29), (1) and (2), respectively but higher than that of (10) from Dire Dawa municipality abattoir. This is probably due to the ecological and climatic difference between the two localities.

The results of the present study revealed that sex and age have significant effect on the prevalence of bovine fasciolosis. However, the work done by (25) and (9) concluded that sex has no impact on the infection rate and hence both male and female are equally susceptible and exposed to the disease. The significant effects of sex on the prevalence of bovine fasciolosis might be attributed to the management system with longer exposure of male outdoor when females are kept indoor at the beginning of lactation (6).

Different works reported similar finding with the present work and clearly justified that the decrease in infection rate (prevalence) as age increase is the result of acquired immunity which is manifested by humeral respond and tissue reaction in bovine liver due to previous challenge (21), and Dwinger *et al.* (12). They also reported that the increase resistance (low prevalence) as age increase is most likely related to the high level of tissue reaction seen in bovine liver, server fibrosis which impedes the passage of immature fluke, acquired resistance, thickening, stenosis and calcification of bile ducts, assumed unfavorable site for adult parasites and consequently fasten their explosion. Additionally the experimental result by (27) and Radostits *et al.* (24) confirmed the occurrence of higher infection rate in younger animals. Moreover, inverse co-relation of prevalence and age of cattle were also reported by (13), (25), (9) and (7).

Post mortem examination on the 64 *Fasciola* infected livers of the current result indicated that *F. hepatica* and *F. gigantica* were the main fasciola species in the study areas, however, *F. hepatica* were found to be the predominant facsiola species causing bovine fasciolsis in the study areas. Similar study conducted at Jimma and Zeway abattoir reported 63.3% and 60.3% of the liver harbored F. hepatica (29), (2), respectively. This is attributed mainly due to the variation in the climatic and ecological conditions such as altitude, rainfall, temperature and livestock management system (35). Moreover, Garber and Daynes reported that; in Ethiopia F. hepatica and F. gigantica infections occur in areas above 1800 m.a.s.l. and below 1200 m.a.s.l. respectively. The high prevalence rate of F. hepatica may be associated with the existence of favourable ecological biotops for L. truncatula. Relatively small proportion of cattle were found infected with F. gigantica alone or mixed infection with both spp. This may be explained by cattle coming for slaughter from highland and middle altitude zone flood prone areas, drainage ditches are favourable habitat to natalensis Urquhart *et al.* (32).

Infection rate of bovine fasciolsis was statistically analyzed on the base of body condition to study the impact of the disease in debilitating (emaciating) infected animals. The result of study indicated that infection rate has no significant difference ($p>0.05$) on the prevalence of fasciolsis weather the animal in thin, moderate or good body conditions. This body condition in cattle manifested when fasciolsis reaches at its chronic stage even though there is a difference in infection rate between thin (75%), moderate (49%) and good (40%) body conditions. The monthly/seasonal/ variation in the prevalence of fasciolosis has been studded for 5 dry months in the study area. It was difficult to indicate the effect of seasonal variation on the prevalence of bovine fasciolosis since the study period was too short without incorporating wet months of the season. An accurate description of seasonal occurrence requires long term epidemiological investigation over several years. In this study high infection rate (69.767%) was encountered in March while lower infection (32.18%) in November having statistical significant difference ($p<0.05$). However, (29) reported October was when the highest

prevalence rate was analyzed during, when the wet ecological conditions still prevailed. It has been described that the bionomic requirements for breeding of the Lymnaea snails and development of the intramolascan stages of the flukes often reach the optimum threshold during the wet months of the year. During the dry periods, breeding of the snails and development of the larval flukes slow down or stops completely and snails undergo a state of aestivation (35).

(21) emphasized on the statement that even if it is realized estimating the actual economic loss due to individual parasitic disease is difficult, this should not be medicate against an attempt to emphasize the cause of the disease. The direct economic loss incurred during this study as a result of condemnation of liver of cattle was estimated about 37, 767.6 ETB per annum and indirect economic loss due to carcass weight reduction was estimated about 660, 933 ETB per annum. Therefore, the total annual economic loss due to fasciolsis in the study abattoir is the summation of losses from organ condemnation and carcass weight reduction which is equal to 698,700.6 ETB. This finding is by far lower than the result reported by (29), (2) and (10) a total economic loss of about 55,080.00, 154, 188 and 215,000 Ethiopian birr per annum in cattle due to fasciolosis at Jimma, Ziway and Dire Dawa municipal slaughterhouses, respectively. This is probably due to the ecological and climatic difference between the two localities.

v. Conclusions

The present study revealed that although a moderate prevalence of bovine fasciolosis in the study sites recorded; the prevalence was significantly affected by sex, age and months of the year. Higher prevalence of bovine fasciolosis was recorded in male cattle than females and in younger cattle (> 3 yrs) than older (> 5 yrs) ones. *F. hepatica* was found to be the predominant. facsiola species causing bovine fasciolsis in the study

Finally the total annual economic losses due to bovine fasciolosis in the study abattoir from organ condemnation (direct loss) and carcass weight reduction (indirect loss) were high.

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Table 1 : Prevalence of bovine fasciolosis on sex basis.

Sex	No of sample examined	No of sample Positive	No of sample Negative	Prevalence (%)	χ^2	P-value
M	263	165	98	62.73	18.575	0.00
F	137	16	121	11.67		
Age						
<3	69	35	34	50.72	1.271	0.00
3-5	191	118	73	61.78		
>5	140	28	112	20		

Table 2 : Species of *Fasciola* identified during post mortem examination of slaughtered animals.

Species of fasciola	No. of lives condemned	Percentage (%)
F. hepatica	42	65.625
F. gigantic	16	25.00
Mixed	6	9.375
Total	64	100

Table 3 : Prevalence of fasciolsis in different body condition groups

Body condition	No of animals examined	No of positive cases	Prevalence (%)
Thin	8	6	75
Moderate	202	99	49
Good	190	76	40

$X^2 = 1.456$ $p = 0.483$

Table 4 : Prevalence of Bovine *Fasciolosis* by Study Sites

Study Sites	No of sample examined	No of sample positive	No of sample negative	Prevalence (%)
Dosha	154	61	93	39.61
Kombolha	113	48	65	42.47
Kulumsa	133	72	61	54.13

Table 5 : Monthly Prevalence of fasciolosis

Month	No. of animals examined	No. of positive sample	Prevalence (%)	χ^2	P- value
November	87	28	32.18	17.273	0.01
December	105	35	33.33		
January	83	42	50.60		
February	82	46	56.097		
March	43	30	69.767		

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Bioequivalence of of Two Oral Contraceptive Drugs Containing Ethinylestradiol and Drospirenone in Healthy Female Volunteers

By Eduardo Abib Junior, Luciana Fernandes Duarte, Joseane Montagner Pozzebon,Silvana Fidelis de Souza, Moisés Pirassol Vanunci

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Abstract – The bioavailability and bioequivalence of two different film coated tablets containing ethinylestradiol and drospirenone were investigated in 36 healthy female volunteers after oral single-dose administration. The study was performed according to a single-center, randomized, single-dose, 2-way cross-over design with a wash-out phase of 28 days. Blood samples for pharmacokinetic profiling were taken post-dose up to 72 h (ethinylestradiol) and 144 h (drospirenone). Ethinylestradiol and drospirenone plasma concentrations were determined with a validated LC-MS/MS method. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% I.C) for the ratio of AUC_{0-t} and C_{max} values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals of ethinylestradiol were 89.13% – 95.32%, and 88.13% – 96.38%, respectively. The 90% confidence intervals of drospirenone were 94.50% – 102.12%, and 95.11% – 111.11%, respectively. Since the 90% confidence intervals for C_{max} and AUC_{0-t} were within the 80 – 125% interval proposed by Food and Drug Administration, it was concluded that the two ethinylestradiol and drospirenone formulations are bioequivalent in their rate and extent of absorption.

GJMR-B Classification: NLMC Code: WD 200.5.G6

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Bioequivalence of of Two Oral Contraceptive Drugs Containing Ethinylestradiol and Drospirenone in Healthy Female Volunteers

Eduardo Abib Junior[α], Luciana Fernandes Duarte[Ω], Joseane Montagner Pozzebon[β], Silvana Fidelis de Souza[Ψ], Moisés Pirassol Vanunci[¥]

Abstract - The bioavailability and bioequivalence of two different film coated tablets containing ethinylestradiol and drospirenone were investigated in 36 healthy female volunteers after oral single-dose administration. The study was performed according to a single-center, randomized, single-dose, 2-way cross-over design with a wash-out phase of 28 days. Blood samples for pharmacokinetic profiling were taken post-dose up to 72 h (ethinylestradiol) and 144 h (drospirenone). Ethinylestradiol and drospirenone plasma concentrations were determined with a validated LC-MS/MS method. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% I.C) for the ratio of AUC_{0-t} and C_{max} values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals of ethinylestradiol were 89.13% – 95.32%, and 88.13% – 96.38%, respectively. The 90% confidence intervals of drospirenone were 94.50% – 102.12%, and 95.11% – 111.11%, respectively. Since the 90% confidence intervals for C_{max} and AUC_{0-t} were within the 80 – 125% interval proposed by Food and Drug Administration, it was concluded that the two ethinylestradiol and drospirenone formulations are bioequivalent in their rate and extent of absorption.

I. INTRODUCTION

Combination contraceptives are most effective means for contraception excluding sterilization. Contraceptives are hormonal agents; combination oral contraceptives contain both an estrogen (ethinylestradiol or mestranol) and a progestogen (many different progestogens are utilized throughout the world). Endogenous estrogens are largely responsible for the development and maintenance of the female reproductive system and secondary sexual characteristics. Estrogens act through binding to nuclear receptors in estrogen-responsive tissues. These will vary in proportion from tissue to tissue. Circulating estrogens modulate the pituitary secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), through a negative feedback mechanism. Drospirenone is a synthetic progestin and spironolactone analog with antimineralocorticoid activity. In animals and in vitro, drospirenone has antiandrogenic activity, but no glucocorticoid, antiglucocorticoid, estrogenic, or androgenic activity. Progestins counter estrogenic effects by decreasing the number of nuclear estradiol receptors and suppressing epithelial DNA synthesis in endometrial tissue. [1-5]

The primary estrogen used in oral contraceptives is ethinylestradiol. 17-Ethinylestradiol (EE), a synthetic estrogen developed in 1938, is an essential constituent of oral contraceptives, which have been widely prescribed since the 1970s. [6] In general, ethinylestradiol is used in combination to prevent pregnancy in women. [7,8] The mean bioavailability of EE is reported to be 45%. [9,10] Its metabolism occurs mainly in the liver and at least 10 metabolites of 17EE have been isolated from human urine, with the 2-hydroxy species being the major metabolites. [11,12]

Drospirenone is a novel synthetic progestogen with a pharmacological profile similar to that of natural progesterone. An analog of spironolactone, drospirenone has antimineralocorticoid and antiandrogenic activity. [13-17] It is almost completely metabolized: less than 1% of the administered dose is excreted in the urine as unchanged drug. The metabolites of drospirenone undergo both hepatic and renal elimination. Based on receptor-binding studies, the metabolites excreted in urine are devoid of pharmacologic activity. [18-20] The compound is part of certain birth control formulations. Combined with ethinyl estradiol in oral contraceptive formulations, drospirenone-containing contraceptives have similar efficacy and safety profiles to other low-dose oral contraceptives, but seem to offer improved tolerability with regard to weight gain, mood changes, acne and treatment of a severe form of the premenstrual syndrome called premenstrual dysphoric disorder . [21,22]

The aim of this study was to compare in healthy volunteers, the pharmacokinetics profiles and evaluate the bioequivalence of one test formulation containing 0.02 mg of ethinylestradiol and 3 mg of drospirenone, (test formulation). The test formulation was compared to one commercial formulation containing 0.02 mg of ethinylestradiol and 3 mg of drospirenone (reference formulation).

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II. Results

a) Demography and safety

Thirty one of the 36 enrolled subjects completed the study. Two subject dropped out after phase two for personal reasons. Three subjects dropped out before confinement for personal reasons and abnormal clinical laboratory investigations. Hence 31 completed cases for both treatments were available for analysis of ethinylestradiol and drospirenone plasma concentrations.The demographic characteristics of the study subjects are presented in **Table 1**, including age, height, weight and BMI. Ethinylestradiol and drospirenone were well tolerated at the administered dose. No serious adverse events occurred.

b) Pharmacokinetic and Statistical Analysis

The mean (± SD) plasma concentration-time profiles are presented in **Figure 01** (ethinylestradiol) and **Figure 02** (drospirenone) and the pharmacokinetic parameters of both substances are summarized in **Table 2** and **Table 3**.

The mean of Cmax of ethinylestradiol was 84.31 pg/mL in reference product and 77.76 pg/mL in test product. Both occurred 1.25 h after dose administration. Cmax of drospirenone was on average 56947.08 pg/mL in reference product and 58431.17 pg/mL in test product and occurred 1.25 h after dosing (reference) and 1 h (test). For ethinylestradiol, the geometric means of AUC0-∞, a measure of extent of absorption amount, were 854.86 pg.h/mL (reference) and 794.61 pg.h/mL (test). The geometric means of AUC0-∞ of drospirenone were 906099.00 pg.h/mL (reference) and 889520.52 pg.h/mL (test). The values of AUC0-t for ethinylestradiol were 807.17 pg.h/mL (reference) and 746.06 pg.h/mL (test). In the drospirenone evaluation the amounts of AUC0-t were 851151.59 pg.h/mL (reference) and 835564.88 pg.h/mL (test). No significant differences with respect to drug absorption were found. Elimination half-lives and elimination rate constants were well comparable between the different preparations.

The resulting 90% confidence intervals of the parameter ratios for for $AUC_{0-\infty}$, AUC_{0-t} and C_{max} as well as for differences in t_{max} are summarized in Table 4.

III. Discussion

Preventing unwanted pregnancy has been an important issue for women and their families all over the world for many hundreds of years. With the development of oral hormonal contraceptives, the so-called "Pill", in the early 1960s, women finally had access to a revolutionary method of contraception. [23,24]

Combined oral contraceptives are effective in normalizing irregular periods, reducing symptoms of premenstrual dysphoric disorder, improving acne, and allowing women to avoid having their period at inconvenient times. [25]

Combinations of drospirenone and estradiol, when compared with estradiol alone, were protective against endometrial hyperplasia. This combination was also effective in reducing menopausal symptoms, thereby elucidating improvements in health-related quality of life measures without significant adverse drug events.[26] Ethinylestradiol and drospirenone not only prevents pregnancy but also results in shorter, lighter periods, reduced cramps and a regular menstrual cycle. It also helps with some symptoms of premenstrual dysphoric disorder and helps control mild to moderate acne breakouts. [27,28]

When a new oral contraceptive formulation is developed, it is crucial to ensure optimum hormone exposure during concomitant therapy with other substances, while also guaranteeing the lowest dose to prevent pregnancy and avoid side effects. To enable testing that can deal with these concerns a highly sensitive analytical method with a low limit of quantification (LLOQ) is required to accurately measure oral contraceptives concentrations in human plasma samples.

Immunoassay methods have been the most sensitive analytical procedures available for the determination of estrogens in biological samples for many years. [29,30] These methods are sensitive, but are time consuming and prone to cross reactivity by steroids and their metabolites. Gas chromatographic coupled to mass spectrometric (GC-MS) methods typically employ some type of extraction, and one or multiple steps of derivatization.[31-34] Recently, liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection has been applied for the quantitative analysis of estrogens in environmental and biological samples.[35-41] LC-MS/MS is superior to immunoassay methods or GC/MS in terms of simplicity, sensitivity, selectivity and analytical throughput.

The LC–MS/MS method described here is specific due to the inherent selectivity of tandem mass spectrometry is in accordance with both Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA) requirements for pharmacokinetic studies. This method offers the advantage over those previously reported using LC–MS/MS [35,38,40,42,43], showing a low validated LOQ 1 pg mL^{-1} (ethinylestradiol) and LOQ 250 pg mL^{-1} (drospirenone).

The mean ratio of parameters C_{max} and AUC_{0-t} and 90% confidence intervals of correspondents were calculated to determine the bioequivalence. The point estimator and the 90% confidence intervals for the AUC_{0-t} ratio (test/reference: 92.17% [89.13% - 95.32%]) indicate high similarity of both formulations with respect to the extent of ethinylestradiol exposure. A high degree of similarity was also observed for C_{max} of ethinylestradiol, as the point estimator and the 90%

confidence interval for the C_{max} ratio are 92.16% (88.13% - 96.38%). Regarding the AUC_{0-t} ratio of drospirenone, the point estimator is 98.24% and the 90% confidence interval 94.50% - 102.12%. Furthermore, exchangeability of both formulations is also suggested by the point estimator and 90% confidence of C_{max} of this active agent (102.80% [95.11% - 111.11%]).

The AUC_{0-t} and AUC_{0-inf} are both recognized as an uncontaminated measurement of the extent of absorption. The present study showed that 90% CI of mean AUC_{0-t} and AUC_{0-inf} (after log-transformation of individual ratios) were included into the bioequivalence range (80-125%), consequently, the two formulations of ethinylestradiol and drospirenone are equivalent for the extend of absorption.

The statistical comparison of Cmax, AUC0-t and AUC0-inf clearly indicated no significant difference in the two formulations of ethinylestradiol and drospirenone. 90% confidence intervals for the mean ratio (T/R) of Cmax, AUC0-t and AUC0-inf were entirely is in accordance with both acceptance range the Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA). Based on the pharmacokinetic and statistical results of this study, we can conclude that ethinylestradiol and drospirenone (Test Formulation) is bioequivalent a formulation reference, and that then the test product can be considered interchangeable in medical practice.

IV. Methods

a) *Study subjects*

Thirty six healthy female volunteers were selected for the study. All volunteers were healthy as assessed by physical examination, gynecological examination, electrocardiogram (ECG), oncotic cytology (Papanicolaou) and the following laboratory tests: blood glucose, urea, creatinine, uric acid, alanine and aspartate aminotransferases (ALT and AST), gamma-gluthamil transferase (γ-GT), alkaline phosphatase, total billirubin, albumin and total protein, trygliceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, red blood cell counts, platelet counts and routine urinalysis. All subjects were negative for human immunodeficiency virus, and B (except for serological scar) and C hepatitis virus.

b) *Study procedures*

All subjects gave written informed consent and the study was conducted in accordance with the revised Declaration of Helsinki, the rules of Good Clinical Practice (ICH-GCP) and the Resolutions No. 196/96 and 251/97 of National Health Council – Health Ministry, Brazil. The clinical protocol was approved by the Research Ethics Committee of University of Campinas/Unicamp (São Paulo, Brazil) and the National Sanitary Surveillance Agency (ANVISA).

The study was a single dose, two-way randomized crossover design with a 28 days washout period between the doses. During each period, the volunteers were hospitalized at 7:00 p.m. They had the usual evening meal until 9:00 p.m., and an overnight fast (minimum of 10 hours).

The subjects were randomly assigned to one of the two treatment sequences. Each treatment consisted of a single dose of two tablets, corresponding to a dose of 0.04 mg ethinylestradiol and 6 mg drospirenone. The double of the daily dose was used, since administration of only 0.02 mg ethinylestradiol and 3 mg drospirenone tends to result in plasma concentrations that are too low for a rating of ethinylestradiol 72 h and of drospirenone 144 h after drug intake.

Both treatments were administered orally. Subjects have received 200 mL of water at room temperature with each administration. All volunteers were then fasted for 4 h following drug administration; afterwards a standard lunch was consumed. Standard snack and evening meal were provided 7-8 and 10-12 h after dosing, respectively. No other food was permitted during the confinement period. Liquid consumption was allowed *ad libitum* 2 h after drug administration. However, xanthine-containing drinks including tea, coffee, and cola were avoided.

Blood samples (06 mL) were collected by indwelling catheter into EDTA containing tubes before dosing and 15, 30, 45 min and also 1, 1.25, 1.5, 1.75, 2, 2.50, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144 h post-dosing for ethinylestradiol and drospirenone. The blood samples were centrifuged at 3.000 rpm for 10 min. at 4°C and the plasma decanted and storage at –20°C until assay for their ethinylestradiol and drospirenone content. All samples from a single volunteer were analyzed on the same day in order to avoid interassay variation. Arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and after drug administration at each full-hour sample collection.

c) *Chemicals and reagents*

Ethinylestradiol was purchased from United States Pharmacopea (lot number QOC162, Rockville, Maryland, USA). 17α-Ethinylestradiol-d4 was obtained from CDN Isotopes (lot number H352P54, Pointe-Claire, Quebec, Canada). Drospirenone was purchased from United States Pharmacopea (lot number F0G064, Rockville, Maryland, USA). Drospirenone-d4 was obtained from SynFine Research (lot number S-1211-081A4, Richmond Hill, Ontario, Canada). Acetonitrile, methanol, chlorobutane and hexane (HPLC grade). Ultrapure water was obtained from aMilli-Q system. Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant EDTA (BD Vacutainer®, BD, Franklin Lakes, NJ, USA). Blank pooled plasma was prepared and stored at −20 ◦C until needed.

d) Analytical method

Ethinylestradiol and Its internal standard 17α-ethinylestradiol-d4 were extracted from aliquot of human plasma by liquid-liquid extraction and derivatization. 1 chlorobutane is added to the samples. The organic phase is evaporated to dryness. The buffer solution of the derivatization and derivatization reagent are added to each sample. Samples is added to the hexane and the samples are centrifuged and vortexed adequately. The organic phase is evaporated to dryness. Samples reconstituted with reconstitution solution prepared with methanol and water type Milli-Q.

Drospirenone is extracted from aliquot of human plasma by solid phase extraction and derivatization procedure. To the plasma samples is added the internal standard working solution prepared in buffer solution. The samples are loaded on the top of activated cartridges and passed through the cartridges by gravity. The compound is eluted from the cartridge using methanol and evaporated to dryness. The methanol, catalyzing solution and the derivatization solution are added to each sample. The samples are mixed adequately and incubated for the derivatization step. The samples are evaporated to dryness and reconstituted with the reconstitution solution prepared with Milli-Q type water and acetonitrile.

e) Apparatus

The ethinylestradiol samples were injected into a Zorbax SB-C18, 4.6 x 50 mm, 3.5 µm column and a Applied Biosystems Sciex API 5000 tandem mass spectrometer. The mobile A phase was methanol–water (78:22, v/v), acetic acid glacial 0.2% (v/v), and the mobile phase B was a mixture of acetonitrile 100% and acetic acid glacial 0.2% (v/v) The chromatographic condition was a gradient mode performed at 35°C and at a flow rate of 1 mL/min. for pump n°. 1 and 0.5 mL/min. for pump n°. 2. The mass spectrometer was operated with + ESI and MRM using the optimized transitions 530.3 → 171.1 for the ethinylestradiol derivate and 534.4 → 171.1 for the 17α-ethinylestradiol-d4 derivative.

The drospirenone samples were injected into a Betasil CN column and a Applied Biosystems Sciex API 5000 tandem mass spectrometer. The chromatographic separation was performed with a gradient, at room temperature and at a flow rate of 1.000 mL/min. The mobile phase A was a mixture of water–acetonitrile (65:35, v/v), formic acid 0.1% (v/v) and the mobile phase B was a mixture of acetonitrile–water (90:10, v/v), formic acid 0.1% (v/v). The mass spectrometer was operated with + ESI and MRM using the optimized transitions 500.3 → 421.2 for the drospirenone derivate and 504.3 → 425.2 for the drospirenone-d4 derivative.

f) Calibration

The calibration range of ethinylestradiol was 1.00-200.00 pg/mL. Calibration standards with 8 concentrations (1.00, 2.00, 4.00, 20.00, 40.00, 80.00, 160.00, 200.00 pg/mL) and quality control standards with 3 concentrations (3.02, 70.42, 150.90 pg/mL) were prepared in human EDTA plasma. The calibration range of drospirenone was 250.00-100000.00 pg/mL. Calibration standards with 8 concentrations (250.00, 500.00, 2500.00, 20000.00, 40000.00, 60000.00, 80000.00, 100000.00 pg/mL) and quality control standards with 3 concentrations (751.50, 30060.00, 70140.00 pg/mL) were prepared in human EDTA plasma.

g) Method validation

Quantitation was based on determination of relationship between ethinylestradiol and drospirenone peaks areas and I.S. peaks areas. Selectivity was evaluated by extracting plasma samples of plasma from different volunteers, including a lipemic and hemolysed plasma. Recoveries of ethinylestradiol and drospirenone at the three QC concentrations and I.S. were determined by comparing peak areas of spiked plasma samples with the peak area in solutions prepared with the same nominal concentration. For precision (as relative standard deviation, R.S.D.) and accuracy (as relative error,R.E.) studies, samples were prepared at three QC and were analysed in the same day (intraday precision and accuracy), and analysed in 3 consecutive days (inter-day precision and accuracy).

The calibration curves were processed and the correlation coefficient was equal to or greater than 0.9979 (ethinylestradiol) and 0.9947 (drospirenone). In ethinylestradiol the accuracy and precision of back-calculated calibration standard concentrations ranged from 89.94-99.21% and 1.35-6.81%, respectively. In drospirenone the accuracy and precision of back-calculated calibration standard concentrations ranged from 85.27-102.84% and 0.66-3.58%, respectively. In ethinylestradiol the intra-day accuracy and precision of the quality control samples ranged from 101.68-103.07% and 3.84-4.26%. In drospirenone the intra-day accuracy and precision of the quality control samples ranged from 95.55-99.12% and 2.20-3.70%. Similar accuracy and precision values were observed during the study sample analysis.

The stability of ethinylestradiol was also evaluated in plasma samples kept at -20 °C for 221 days and after being submitted to 2 freeze-thawing cycles (24 h each cycle). The stability of drospirenone was also evaluated in plasma samples kept at -20 °C for 93 days and after being submitted to 2 freeze-thawing cycles (24 h each cycle). All samples described above were compared to freshly prepared ethinylestradiol and drospirenone samples at the same concentration level. All sample analysis were carried out in a GLP-compliant manner and in accordance with the current Brazilian Regulatory Agency (ANVISA) requirements and the US Food and Drug Administration Bioanalytical method validation guidance.

h) Pharmacokinetics and Statistical analysis

The first-order terminal elimination rate constant (Ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot, using the software SAS® Institute (Version 9.1.3). Elimination half-life ($T_{1/2}$) was derived from this rate constant ($T_{1/2}$ = ln (2)/Ke). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the ethinylestradiol ($AUC_{0\text{-}72h}$) and drospirenone ($AUC_{0\text{-}144h}$) plasma concentration versus time curves from were calculated by applying the linear trapezoidal rule. In ethinylestradiol extrapolation of these areas to infinity ($AUC_{0\text{-}inf}$) was done by adding the value C72/Ke to the calculated $AUC_{0\text{-}72h}$ (where C72=plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 72 hours after dose). In drospirenone extrapolation of these areas to infinity ($AUC_{0\text{-}inf}$) was done by adding the value C144/Ke to the calculated $AUC_{0\text{-}144h}$ (where C144=plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 144 hours after dose).

The bioequivalence between both formulations was assessed by calculating individual C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$ and $C_{max}/AUC_{0\text{-}t}$ ratios (test/reference) together with their mean and 90% confidence intervals (CI) after log transformation of the data. The inclusion of the 90% CI for the ratio in the 80% to 125% range was analyzed by nonparametric (SAS® Institute Version 9.1.3) and parametric (ANOVA) methods.

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VI. Conflict of Interest

The authors declared no conflict of interest.

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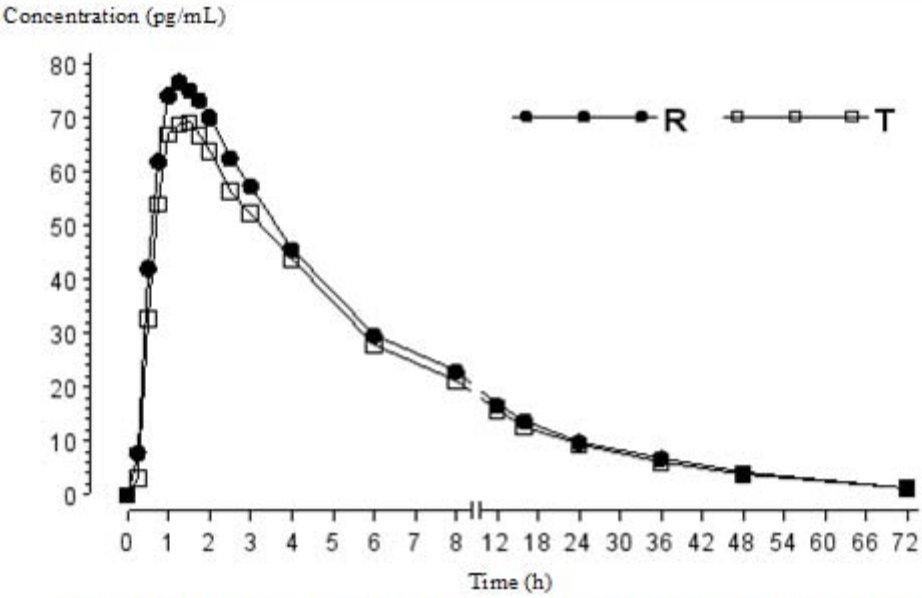

Figure 1 Mean plasma concentration-time profile of ethinylestradiol over the first 72 h after oral administration of the test and reference formulation.

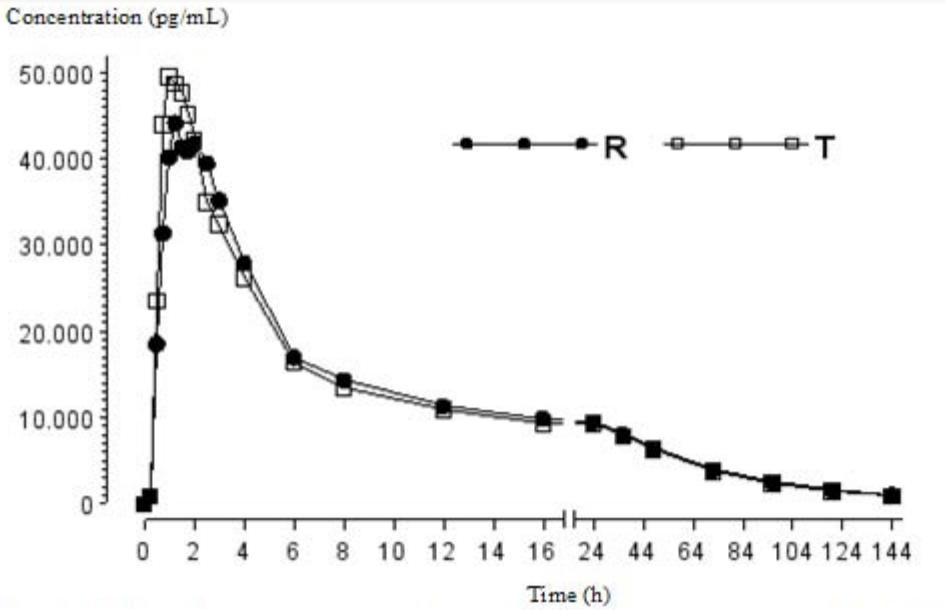

Figure 2 Mean plasma concentration-time profile of drospirenone over the first 144 h after oral administration of the test and reference formulation.

Table 1 Summary of demographic characteristics for the safety population for study (mean ± SD)

Category	Volunteers
n	36
Age (years)	36.04 ± 6.62
Height (cm)	1.60 ± 0.06
Weight (Kg)	64.90± 8.00
BMI (Kg/m²)	25.30± 2.39

Table 2 Mean pharmacokinetic parameters of ethinylestradiol and drospirenone of test and reference formulation

	ETHINYLESTRADIOL				DROSPIRENONE			
	TEST		REFERENCE		TEST		REFERENCE	
Parameter (unit)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)
AUC_{0-t} (pg.h/dL)	746.06	213.77	807.17	215.27	83.5564.88	199702.16	851151.59	203231.96
$AUC_{0-\infty}$ (pg.h/dL)	794.61	226.10	854.86	221.17	88952.0.52	218454.91	906099.00	226901.38
C_{max} (pg/dL)	77.76	25.87	84.31	26.32	58431.17	10841.48	56947.08	12172.15
T_{max} (median/amp) (h)	1.25	3.25	1.25	2.50	1.00	3.25	1.25	3.50
Kel (1/h)	0.04	0.01	0.04	0.00	0.02	0.00	0.02	0.00
T½ (median/amp) (h)	17.54	12.26	16.79	7.40	34.62	31.09	35.33	37.48

Table 3 Geometric mean pharmacokinetic parameters of ethinylestradiol and drospirenone of test and reference formulation

	ETHINYLESTRADIOL		DROSPIRENONE	
	TEST	REFERENCE	TEST	REFERENCE
Parameter (unit)	Geometric Mean	Geometric Mean	Geometric Mean	Geometric Mean
AUC_{0-t} (pg.h/dL)	718.43	779.42	810015.40	824536.02
$AUC_{0-\infty}$ (pg.h/dL)	765.74	827.38	860430.42	874937.12
C_{max} (pg/dL)	74.30	80.61	57205.81	55648.61

4 Ratios means and the 90% geometric confidence interval of test and reference formulation

	ETHINYLESTRADIOL					DROSPIRENONE				
Parameter	Ratio T/R (%)	Lower Limit (%)	Upper Limit (%)	Power (%)	Coefficient of Variation (%)	Ratio T/R (%)	Lower Limit (%)	Upper Limit (%)	Power (%)	Coefficient of Variation (%)
AUC_{0-t}	92.17	89.13	95.32	99.99	7.79	98.24	94.50	102.12	99.99	8.99
$AUC_{0-\infty}$	92.55	89.52	95.68	99.99	7.72	98.34	94.78	102.04	99.99	8.56
C_{max}	92.16	88.13	96.38	99.98	10.39	102.80	95.11	111.11	99.42	18.15
T_{max} (dif) (h)	0.25	0.00	0.50			-0.25	-0.50	0.25		

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Erythrocyte Membrane Lipid Alteration in Type 2 Diabetic Subjects

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Abstract – The lipid components present in biological membranes including erythrocyte membrane are significant in regulation of the membrane fluidity as well as transport across the membranes. The conflicting results of either increase or decrease or no change have been reported in erythrocyte membrane fluidity in type 2 diabetic subjects. The diabetes induced dyslipidemia possibly lead to an alteration in erythrocyte membrane lipid composition. Hence a study was undertaken to assess erythrocyte membrane lipid alterations in type 2 diabetic subjects. The blood samples from randomly selected type 2 diabetic subjects, attending Medical OPD of Basaveshwara Medical College Hospital, Chitradurga, were collected with heparin as an anticoagulant. Erythrocyte membrane total cholesterol (mTC), Erythrocyte membrane phospholipids (mPL), Erythrocyte membrane phosphatidyl choline, Erythrocyte membrane sphingomyelin and Erythrocyte membrane phospholipids/ Erythrocyte membrane cholesterol ratio were studied. The results suggests a significant rise in erythrocyte membrane cholesterol (mTC, N=1.25±0.31, T2DM= 1.54± 0.14, $p<0.001$), erythrocyte membrane phospholipid contents (mPL, N=6.99±1.84, T2DM=23.44±10.31, $p<0.001$), Erythrocyte membrane sphingomyelin, (N=9.28± 1.37, T2DM=19.32± 2.37), Erythrocyte membrane phosphatidyl choline (N=27.08± 1.58, T2DM=26.09±3.04, $p<0.001$), as well as phospholipid/cholesterol ratio (mPL/mTC, N=6.08±2.65, T2DM=11.76±5.31, $p<0.001$.) in type 2 diabetic subjects was observed. Though, there is a significant alteration seen between normal and diabetic groups, but there was no significant change was observed between male and female subjects of both normal as well as diabetic groups.

Keywords : membrane cholessterol, membrane phosplipids, membrane Phospholipids/ cholesterol ratio.

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Erythrocyte Membrane Lipid Alteration in Type 2 Diabetic Subjects

G. Rudrappa[α], Basavaraj Aski[Ω], Kashinath R.T.[β]

Abstract - The lipid components present in biological membranes including erythrocyte membrane are significant in regulation of the membrane fluidity as well as transport across the membranes. The conflicting results of either increase or decrease or no change have been reported in erythrocyte membrane fluidity in type 2 diabetic subjects. The diabetes induced dyslipidemia possibly lead to an alteration in erythrocyte membrane lipid composition. Hence a study was undertaken to assess erythrocyte membrane lipid alterations in type 2 diabetic subjects. The blood samples from randomly selected type 2 diabetic subjects, attending Medical OPD of Basaveshwara Medical College Hospital, Chitradurga, were collected with heparin as an anticoagulant. Erythrocyte membrane total cholesterol (mTC), Erythrocyte membrane phospholipids (mPL), Erythrocyte membrane phosphatidyl choline, Erythrocyte membrane sphingomyelin and Erythrocyte membrane phospholipids/ Erythrocyte membrane cholesterol ratio were studied. The results suggests a significant rise in erythrocyte membrane cholesterol (mTC, N=1.25±0.31, T2DM= 1.54± 0.14, p<0.001), erythrocyte membrane phospholipid contents (mPL, N=6.99±1.84, T2DM=23.44±10.31, p<0.001), Erythrocyte membrane sphingomyelin, (N=9.28± 1.37, T2DM=19.32± 2.37), Erythrocyte membrane phosphatidyl choline (N=27.08± 1.58, T2DM=26.09±3.04, p<0.001), as well as phospholipid/cholesterol ratio (mPL/mTC, N=6.08±2.65, T2DM=11.76±5.31, p<0.001.) in type 2 diabetic subjects was observed. Though, there is a significant alteration seen between normal and diabetic groups, but there was no significant change was observed between male and female subjects of both normal as well as diabetic groups.

Keywords : membrane cholesterol, membrane phospholipids, membrane Phospholipids / cholesterol ratio.

I. Introduction

Diabetes Mellitus (Type 2) is a metabolic syndrome characterized by chronic hyper glycemia and disturbances of carbohydrate, protein and lipid metabolism due to underlying insulin lack or subnormal functioning of insulin. In diabetic patients, a reduction of erythrocyte deformability and an increase in whole blood viscosity were correlated with microangiopathy (1). The membrane surrounding the erythrocytes forms a boundary between the interior of the cell and the plasma surrounding it, and severs as a barrier to help maintaining the interior of the red cell. It must be insoluble in aqueous solutions, approximately one half of the mass of the human erythrocyte membrane consists of lipid, largely arrange as a bilayer (4). Membrane lipids are either phospholipids or neutral lipids, mostly unesterified cholesterol. Membrane phospholipids are asymmetrically arranged in to a lipid bilayer two molecules thick. Cholesterol is intercalated between the phospholipid molecules. The relative amounts of phospholipids and cholesterol are responsible for the fluid properties of the erythrocyte membrane (19). It is also responsible for the biconcave shape and basic structural integrity of the erythrocyte.

The changes of erythrocyte membrane properties induced by high levels of free fatty acids or aldehydes which are produced in membrane during peroxidative processes, may be responsible for long-term complications in a number of diseases, such as diabetes mellitus (2). It has been also suggested that the ability of red blood cells to change their shape is decreased in diabetic patients. Such an impairment of the red blood cells deformability might be another contributing factor to the reduction of blood flow in the capillaries (7).

The differences of results in the literature led us to further investigations of the lipid composition of erythrocyte membrane in type2 diabetes.

II. Materials and Methods

The diabetic type 2 subjects (male and female) in the age group of 30-60 years attending Medical OPD of Basaveshwara Medical College Hospital and Research Center, Chitradurga, were randomly selected. The normal subjects (male and female) were randomly picked among medical students, house surgeons and employees of the college as well as Hospital, who were in the age group of 30-60 years. The normal subjects include 30 males and 06 females; whereas diabetic subjects include 59 males and 31 females.

Blood samples (6-7ml) from the selected normal subjects and type 2 diabetic subjects were collected, in the fasting state, with heparin as an anticoagulant by obtaining informed consent. Plasma was separated by centrifugation at 3500 rpm, for 10 minutes. Erythrocytes were washed three times with an aliquot of 5 ml normal saline and were preserved for further use.

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III. Preparation of Erythrocyte Membrane

To 1 ml of 50% saturated erythrocyte suspension 4 ml distilled water were added and the mixture was stirred vigorously with a clean glass rod to lyse the erythrocytes. This was centrifuged at 3500 rpm for 5 minutes. Supernatant was discarded. The sedimented membranes were washed 3 times with 3ml aliquots of normal saline. The washed erythrocyte membranes were employed for membrane lipid phosphorous profile studies.

One part of washed membranes was homogenized with 9 parts of chloroform: methanol (1:1 v/v) mixture for 8 minutes using Potter- Elvejham tissue homogenizer. The extracts were used for the estimation of membrane total cholesterol (mTC) (12) , Membrane total lipids (mTL) (8), and membrane total phospholipids (mPL) (17). The phospholipid profile-membrane phosphatidyl choline (PC), membrane sphingomyelin (SM) was estimated in the chloroform: methanol extract, using quantitative thin layer chromatography procedure (10). Another part of washed erythrocyte membranes were homogenized with 4 parts of normal saline for 10 minutes and the extracts were employed for membrane free and total phosphorous estimation (10).

IV. Results

In the present study, a total number of 126 subjects were employed which includes 36 normal subjects and 90 diabetic subjects. The normal subjects were consisted of 30 male subjects and 06 female subjects. Further the diabetic group consisted of 59 male diabetic subjects and 31 female diabetic subjects. The results of the present study are narrated in table 1 and 2.

Table 1 gives, membrane total lipids (mTL), membrane total cholesterol (mTC), membrane total phospholipids (mPL) and calculated ratios of mPL/mTC in erythrocytes of normal subjects (group 1), in erythrocytes of diabetic subjects (group 2), in erythrocytes of normal male subjects (group 3), in erythrocytes of diabetic male subjects (group 4), in erythrocytes of normal female subjects (group 5), and in erythrocytes of diabetic female subjects (group 6). As seen from the table 1 there is a significant elevation observed in mTC level ($p < 0.001$) , in mPL levels ($p < 0.001$) as well as in mPL/mTC ratio ($p < 0.001$) in group 2 as compared to group1, in group 4 as compared to group 3 and in group 6 as compared to group 5. However no significant alterations observed in mTL levels between group 2 as compared to group 1, group 4 as compared to group 3 and in group 6 as compared to group 5. It is also evident from the table that there is no significant alterations observed in mTL levels, mTC levels, mPL levels and mPL/mTC ratio between group 4 and group 6, showing that diabetes mellitus induced alterations are common in diabetic male and diabetic female subjects

Table 2 depicts membrane levels of lipid phosphorous profile - free phosphorous, bound phosphorous, total phosphorous as well as membrane phosphatidyl choline (PC) and membrane sphingomyelin (SM) in erythrocytes of normal subjects

Table-1 : Table showing membrane total lipids (mTL), membrane total cholesterol (mTC), membrane total phospholipids (mPL) and calculated ratio of membrane phospholipids/membrane cholesterol in normal erythrocytes (male/female) as well as in diabetic erythrocytes (male/female).

	Erythrocyte membrane total lipids mg/cc (mTL)	Erythrocyte membrane total cholesterol mg/cc (mTC)	Erythrocyte membrane total phospholipids mg/cc (mPL)	Erythrocyte membrane total phospholipids/ cholesterol ratio (mPL/mTC)
Erythrocyte of normal subjects (n = 36)Group-1	5.02 ± 1.62	1.25 ± 0.31	6.99 ± 1.84	6.08 ± 2.65
Erythrocyte of diabetic subjects (n = 90)Group-2	5.35 ± 1.53	1.54*** ± 0.14	10.31*** ± 3.44	11.76*** ± 5.31
Erythrocytes of normal male subjects (n=30) Group-3	4.88 ± 0.89	1.25 ± 0.32	7.04 ± 1.86	6.14 ± 2.7
Erythrocytes of diabetic male subjects (n=59) Group-4	5.35 ± 1.53	1.52*** ± 0.14	15.52*** ± 7.47	11.34*** ± 5.28
Erythrocytes of normal female subjects (n=06) Group-5	5.12 ± 1.01	1.26 ± 0.32	7.04 ± 1.86	6.14 ± 2.7
Erythrocytes of diabetic female subjects (n=31) Group-6	5.38 ± 1.38	1.48*** ± 0.12	16.03 *** ± 6.85	12.84 *** ± 5.30

Note: 1.The number in parenthesis shows the number of samples.
2. Values are expressed as their Mean ± SD.
3. p- value * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

(group 1), in erythrocytes diabetic subjects (group 2), in erythrocytes of normal male subjects (group 3), in erythrocytes of diabetic male subjects (group 4), in erythrocytes of normal female subjects (group 5), and in erythrocytes of diabetic female subjects (group 6). It is evident from the table that there is a significant elevation observed in membrane free phosphorous ($p<0.001$), membrane bound phosphorous ($p<0.001$), membrane total phosphorous ($p<0.001$), and membrane sphingomyelin ($p<0.001$) in group 2 as compared to group 1, in group 4 as compared to group 3, and in group 6 as compared to group 5. However, there is less striking change observed in membrane phosphatidyl choline in group 2 as compared to group1 ($p<0.01$), and in group 6 as compared to group 5. Further it is evident from the table 2 that there is a significant change observed in membrane bound phosphorous ($p<0.001$) and in membrane total phosphorous levels ($p<0.001$) levels in group 4 as compared to group 6, however there is no significant alteration observed in membrane free phosphorous, membrane SM levels and membrane PC levels between these two groups.

V. Discussion

Diabetes Mellitus (DM) is a chronic metabolic disorder which is widespread and is associated with substantial morbidity and mortality. Type 2 DM is a chronic disease characterized by hyperglycemia and dyslipidemia due to underlying insulin resistance (11). Apart from hyperglycemia and glucosuria in diabetes mellitus, lipid alteration has been observed by many workers (22, 25, 28). The Dyslipidemia in diabetes mellitus is related to the type of diabetes as well as to the level of glycemic control achieved in these subjects.

Table 2 : Table showing the membrane levels of membrane lipid phosphorous (Free, bound and total), PC as well as SM in erythrocytes of normal male/female and diabetic male/diabetic female subjects.

	Membrane free Phosphorous mg/dl	Membrane Bound Phosphorous mg/dl	Membrane Total Phosphorous mg/dl	Membrane Phosphatidyl Choline (PC) mg/dl	Membrane Sphingomyelin (SM) mg/dl
Erythrocyte of normal subjects(n=36) Group-1	55.94 ± 9.29	24.45 ± 6.07	80.10 ± 8.82	27.08 ± 1.58	9.28 ± 1.37
Erythrocyte of diabetic subjects (n= 90) Group-2	120.70*** ± 48.10	96.40*** ± 24.14	215.12*** ± 57.98	26.09** ± 3.04	19.32*** ± 2.37
Erythrocytes of normal male subjects (n=30) Group-3	54.12 ± 3.38	26.12 ± 4.80	76.80 ± 5.45	26.12 ± 0.96	9.38 ± 1.12
Erythrocytes of diabetic male subjects (n=59) Group-4	126.70*** ± 13.28	98.40*** ± 16.60	240.30*** ± 21.18	29.78*** ± 3.26	21.32*** ± 1.28
Erythrocytes of normal female subjects (n=06) Group-5	56.28 ± 4.38	23.38 ± 2.26	83.10 ± 6.68	28.32 ± 1.10	9.80 ± 1.60
Erythrocytes of diabetic female subjects (n=31) Group-6	128.20*** ± 8.60	90.30*** ± 12.11	224.10*** ± 18.80	29.32 ± 4.10	20.80*** ± 0.98

Note: 1. The number in parenthesis shows the number of samples.
2. Values are expressed as their Mean ± SD.
3. p- value * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

It is known that there exists a symmetrical bilayer distribution of lipids in biological membrane including erythrocyte membrane. Normally amine rich lipids are on the innerline of cytoplasmic side of the membrane where as choline rich sphingolipids are on outer surface. It has been shown by previous workers that erythrocyte membrane fluidity as well as deformability may be related to membrane lipid composition (5, 14,16).

In the present study a significant elevation as been observed in mTL, mTC, mPL as well as mPL/mTC ratio in diabetic erythrocytes as compared to normal erythrocytes (group 2 X group 1), (group 4 X group 3), and (group 6 X group 5). This is in agreement with the reports of Bryzewska et. al. (6), and other workers (9, 15, 18, 27).

Such an alteration in erythrocyte membrane lipid composition can induce changes in biochemical and biophysical properties of erythrocyte membrane (21), including activities of various membranes bound enzymes and proteins (24).

The erythrocyte membrane principally consisting of different phospholipid and cholesterol. Altered membrane cholesterol, as well as membrane phospholipids, including mPL/mTC ratio was observed in type 2 diabetic erythrocytes in the present study. Though there is an increase in both erythrocyte membrane cholesterol ($p<0.001$) and in erythrocyte membrane phospholipids ($p<0.001$), the increase in phospholipids in diabetic erythrocyte seem to be higher as there is a significant elevation seen in mPL/mTC ratio ($p<0.001$), (ref.table 1).

The membrane lipids specifically phospholipids play a significant role in maintenance of cell shape, cell permeability as well as movement of various compounds across the membrane. This is true in case of erythrocyte membranes also. It is well established that the phospholipid distribution across the erythrocyte membrane bilayer is asymmetrical (26). The changes in erythrocyte membrane lipid and phospholipid composition observed in the present study (ref. table 2) may induce changes in the physico-chemical properties of erythrocyte membrane as well as in fluidity / rigidity (20).

The results in table 2 show a significant raise in erythrocyte membrane free phosphorous ($p<0.001$), bound phosphorous ($p<0.001$), total phosphorous ($p<0.001$), mPL levels ($p<0.001$) as well as membrane SM levels ($p<0.001$), in erythrocytes of diabetic subjects (group 2) as compared to erythrocytes of normal subjects (group 1). The observed results with respect to membrane phosphorous profile may suggest a reciprocation for alteration in erythrocyte membrane phospholipids levels as well as an alteration in erythrocyte membrane phospholipid profile in diabetic subjects.

The results observed in the present studies in erythrocytes of diabetic female subjects as well as in erythrocytes of diabetic male subjects with respect to erythrocyte membrane phospholipid profile, mTC levels, mPL levels, and mPL/mTC ratios are in (both male/ female) agreement with the reports of Juhan Vagus et. al. (20).

It may be concluded from the present studies, the lipid changes in erythrocyte membrane in type 2 diabetic subjects may lead to changes in membrane fluidity resulting in possible membrane functional alteration, including transport across the membranes. However, present study does not show any significant alterations in erythrocyte membrane levels of mTL, mTC, mPL, and mPL/mTC in erythrocytes diabetic male subjects as compared to erythrocytes of diabetic female subjects indicating that the changes observed in erythrocyte membrane of diabetic population as compared to erythrocyte membrane of normal population is common to both sexes.

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Effect of Thiopropanol on Glucose Utilization in Alloxan Diabetic Rat Liver

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Abstract – Cellular thiol-disulfide ratio can be altered by exogenously added, readily absorbable thiols or disulfides. Many sulphydryl enzymes including glycolytic kinases are known to be affected by changes in thiol-disulfide balance. It is known that in diabetes mellitus the tissue total thiol concentration is reduced thereby creating disturbances in various metabolic pathways, especially the pathways of carbohydrate metabolism. Few studies have suggested that the alterations in carbohydrate metabolism can be directly attributed to modifications in tissue thiol-disulfide balance. Certain low molecular weight thiols are known to influence glucose utilization in adipocytes probably by replenishing cellular NADP levels hence favoring utility of glucose through HMP pathway. A study was undertaken to assess the effect of Thiopropanol(3-mercapto-1- propanol), a low molecular weight thiol, on glucose utilization in isolated alloxan diabetic liver slices. The results indicate that the thiopropanol at the dosage employed in the present study influences glucose utilization, lactate production, pyruvate production, glucose-6-phosphate dehydrogenase as well as hexokinase activities in isolated alloxan diabetic liver slices, probably by favoring glucose utilization through glycolysis as well as through HMP pathway.

Keywords : *low molecular weight thiol, 3mercapto-1-propanol, thiol-disulfide balance, glucose utilization, diabetes mellitus.*

GJMR-C Classification: *NLMC Code:QU 83, WD 200.5.G6,QU 131, QU 85*

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Effect of Thiopropanol on Glucose Utilization in Alloxan Diabetic Rat Liver

Vickram[α], Divya D[Ω], Veena G Raiker[β], Kashinath R T[Ψ]

Abstract - Cellular thiol-disulfide ratio can be altered by exogenously added, readily absorbable thiols or disulfides. Many sulphydryl enzymes including glycolytic kinases are known to be affected by changes in thiol-disulfide balance. It is known that in diabetes mellitus the tissue total thiol concentration is reduced thereby creating disturbances in various metabolic pathways, especially the pathways of carbohydrate metabolism. Few studies have suggested that the alterations in carbohydrate metabolism can be directly attributed to modifications in tissue thiol-disulfide balance. Certain low molecular weight thiols are known to influence glucose utilization in adipocytes probably by replenishing cellular NADP levels hence favoring utility of glucose through HMP pathway. A study was undertaken to assess the effect of Thiopropanol(3-mercapto-1- propanol), a low molecular weight thiol, on glucose utilization in isolated alloxan diabetic liver slices. The results indicate that the thiopropanol at the dosage employed in the present study influences glucose utilization, lactate production, pyruvate production, glucose-6-phosphate dehydrogenase as well as hexokinase activities in isolated alloxan diabetic liver slices, probably by favoring glucose utilization through glycolysis as well as through HMP pathway.

Keywords : low molecular weight thiol, 3mercapto-1-propanol, thiol-disulfide balance, glucose utilization, diabetes mellitus.

I. Introduction

In principle, any enzyme or protein having an accessible thiol essential for its activity is capable of yielding itself to cellular changes in thiol-disulfide ratio thus making such enzymes or proteins for easy modulation [1]. This cellular thiol - disulfide balance can be altered by treating animals or isolated tissue with readily absorbable thiols or disulfides [1,2,3]. It is known that many enzymes particularly glycolytic kinases are sulphydryl enzymes and are affected by changes in thiol-disulfide balance [1,4-7]. In diabetes mellitus the tissue total-thiol concentration is reduced [8] there by creating disturbances in various metabolic pathways especially the pathways of carbohydrate metabolism. There are few studies that suggests that changes in carbohydrate metabolism can be directly attributed to modifications in tissue thiol-disulfide balance [9,10,11,12]. Certain low molecular weight thiols are known to influence glucose utilization in adipocytes [13,14] which is thought to be probably through replenishing cellular NADP levels hence favoring utilization of glucose through HMP pathway . Hence a study was undertaken to assess the effect of thiopropanol (3-mercapto 1-propanol), a low molecular weight thiol, on the glucose utilization in isolated alloxan diabetic liver slices.

II. Materials & Methods

All the chemicals employed were of analar grade. Alloxan was obtained from Loba chemicals. 3-mercapto 1-propanol (Thiopropanol) (TP) was procured from Sigma-Aldrich chemicals Pvt. Ltd. USA. Male albino rats weighing 150-250 g were selected randomly from the stock colony of animal house of Basaveshwara Medical College & Hospital, Chitradurga, were employed in the present study. The chosen rats were housed in plastic well aerated cages at normal atmospheric temperature (25 ± 5 °C) and normal 12- hour light/dark cycle. The rats were maintained on standard stock diet (Amruth Rat Feed, supplied by Pranav Agro Industries, Pune, India). The feed and the tap water were accessible to the animals ad libitum.

a) Induction of Diabetes Mellitus :

A single intraperitoneal injection of freshly prepared aqueous Alloxan monohydrate (150 mg per kg body weight) [15,16] was given to 12 hours fasted rats . The onset of diabetes was monitored 48 hours after alloxan treatment by using standard Urine Glucose Strips(from Qualigens).The rats whose urine showing positive for glucose for 3 consecutive days were labeled diabetic and were used in the present work.

b) Experimental Design :

The rats were divided into two groups.

Normal group – consisting of 6 male albino rats maintained on stock lab diet and tap water *ad libitum.*

Diabetic group – consisting of 6 male albino alloxan diabetic rats maintained on stock lab diet and tap water *ad libitum.*

The rats of both the groups were anesthetized and sacrificed after 30 days. They were immediately dissected, the liver tissue was procured,

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washed and refrigerated with cold PBS(Phosphate buffer saline , pH 7.4) at 0-2 ^{0}C till further use.

The isolated livers of both normal as well as alloxan diabetic rats were cut into small slices of 0.5 g each and were employed in the present studies.

c) Glucose Utilization Studies and Lactate Assay :

The glucose [17], lactic acid [18] as well as the glycogen[19] contents of both pre and post incubated liver samples were estimated .Glucose utilization by the isolated normal liver slices, control alloxan diabetic liver slices(control) and TP-exposed-alloxan-diabetic liver slices were studied.

Procedure :

The zero minute contents of Glucose and lactic acid were estimated as follows. To 0.5g of normal liver tissue slice or control alloxan diabetic liver slice or TP-exposed- alloxan- diabetic liver slice(Conc. 5mg thiopropanol/0.5g liver tissue slice) 1ml of freshly prepared buffered glucose solution (0.1g % glucose in phosphate buffer, pH 7.4) was added and immediately 3.5ml of 10% TCA(trichloro acetic acid) was added and allowed to stand at room temperature for 15 minutes for protein precipitation. The contents were thoroughly homogenized using Potter Elvehjam Homogenizer and centrifuged at 3000rpm for 5minutes. The supernatant obtained was employed for both Glucose and Lactic acid estimations. Like wise, for the 60 minutes (post incubation) levels of glucose and lactic acid, 0.5g normal liver slice or control alloxan diabetic liver slice or TP-exposed-alloxan- diabetic liver slice was added with 1ml buffered glucose solution and the tubes were incubated at 37^{0}C in a thermostatically regulated water bath for 60 minutes. Then processed to get the protein free supernatant as described above. The glucose formed by the liver glycogen breakdown during this period was also taken into account by estimating glycogen content in the beginning (at zero minute) and at the end of incubation period(at 60 minutes). This glycogen-glucose value was taken into consideration during glucose utilization calculations.

Glucose utilization was calculated as follows:

Glucose utilization/hr/g liver tissue ={zero min. glucose + (zero min. glycogen-60 min. glycogen). - 60 min.glucose}

Lactate Production was calculated by subtracting zero min lactate from 60 minutes lactate.

d) Enzyme Assays :

Glucose - 6 - phosphatedehydrogenase (G6PD) {EC:1.1.1.49} and Hexokinase (HK){EC:2.7.11} activities were estimated in isolated normal liver slices , in control alloxan diabetic liver slices as well as in TP-exposed-alloxan-diabetic liver slices(5mg thiopropanol/0.5g liver tissue).

Procedure : 0.5g of normal liver slice or control alloxan diabetic liver slice or TP-exposed-alloxan-diabetic liver slice was taken in a test tube containing 1ml of phosphate buffer (pH 7.4) and the contents were incubated for 60 minutes at 37 ^{0}C in a thermostatically regulated water bath. At the end of the incubation period , the tubes were removed from the water bath and 3.5ml of Phosphate buffer, (pH7.4) was added to all the tubes. Then contents were homogenized and centrifuged for 5 min at 3000rpm. The supernatant was employed for the estimation of G6PD [20,21,22] and HK[23].

e) Pyruvate Assay :

The pyruvate content in isolated normal liver slices , in control alloxan diabetic liver slices as well as in TP - exposed – alloxan - diabetic liver slices (5mg thiopropanol/0.5g liver tissue) was estimated using Dinitro phenyl hydrazine (DNPH) [24] reaction. The same supernatant which was used for the enzyme assays as described above was employed for pyruvate estimation also.

Procedure :

Four test tubes were taken and marked as B(reagent blank), S(standard), T(test), C(test control). Then 0.2 ml of buffered substrate(L-Alanine [200mMol'L] , Oxo-2 – Glutarate [2mMol/L] prepared in Phosphate buffer, pH 7.4) was taken in all 4 test tubes. The tubes were kept at 37^{0}C in a thermostatically controlled water bath for 5 minutes. Then 0.02 ml of glass distilled water, 0.02 ml standard pyruvate solution (2mMol/L) and 0.02ml supernatant were added into tubes B, S and T respectively and the contents were mixed well. All the tubes were incubated for 30 min. at 37^{0}C in a water bath. At the end of the incubation, 0.2ml of DNPH (1mMol/L) was added to all the tubes. Then 0.02 ml of supernatant was added to the tube 'C' and all the tubes were allowed to stand at room temperature for 20minutes. Later 2ml of 0.4N NaOH was finally added into all the tubes, the contents were mixed and the tubes were allowed to stand for 5 min. at room temperature. The optical density (OD) was read at 540nm in Spectrophotometer against glass distilled water. The test-control OD gives the pyruvate content in the beginning ie, at zero minute and the test OD gives the pyruvate content at the end of 30minutes. Pyruvate produced was calculated by subtracting T from C.

f) Statistical Evaluation :

The statistical analysis of the data obtained was done using Microsoft Office Excel worksheet and the P (probability) value was calculated by Student't' test.

g) Ethical Consideration :

The animal experiments were conducted as per the norms of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi and IAEC (Institutional Animal Ethical Committee) of Basaveshwara Medical College, Chitradurga.

III. Results

Table - 1 gives the glucose utilization per hour, lactate production per hour, pyruvate production per hour, as well as G6PD and HK activity in isolated normal liver slices, control alloxan diabetic liver slices and alloxan diabetic liver slices exposed to thiopropanol. It is evident from the table that glucose utilized per hour, lactate produced per hour, pyruvate produced per hour, G6PD activity as well as HK activity in control alloxan diabetic liver is significantly lowered($p<0.001$) as compared to normal liver values. Where as the same parameters are significantly increased ($p<0.001$) in TP-exposed-alloxan diabetic liver slices as compared to control alloxan diabetic liver slices.

Graph 1, 2 and 3 gives the comparative results of glucose utilization, pyruvate production, lactate production, HK activity as well as G6PD activity in isolated normal liver slices, control alloxan diabetic liver slices and in TP-exposed-alloxan diabetic liver slices. It is evident from these graphs that these parameters are significantly lowered in control alloxan diabetic liver slices as compared to normal liver slices while the same parameters are statistically improved upon exposure of alloxan diabetic liver slices to thiopropanol(5mg/0.5g liver).

IV. Discussion

Alloxan is known to induce diabetes by selectively damaging beta-cells of pancreas[15] thereby affecting insulin production and insulin release. This decreased or non-availability of insulin results in lowered glucose uptake and utilization by alloxan diabetic liver slices. The decreased glucose utilization in control alloxan diabetic liver as compared to normal liver observed in the present study may be due to decreased insulin levels in alloxan diabetic rats. There are few earlier studies regarding influence of thiols on glucose utilization [25-29] suggesting that thiols stimulate utilization of glucose through pentose cycle as well as favor incorporation of glucose- carbon into fatty acids which are more similar to insulin action. Many enzymes of glycolytic pathway, including hexokinase, phosphofructokinase and pyruvate kinase are thiol enzymes[1,4-7] and are expected to be altered by cellular thiol concentrations. The data of the present study given in table 1 as well as in graphs 1, 2 and 3 are in agreement with this hypothesis that thiopropanol added to the alloxan diabetic liver slices (conc. 5mg/0.5g) might have improved the cellular thiol levels hence keeping the enzymes in their thiol nature thus favoring their activities resulting in increased glucose utilization as evidenced by increased lactate and pyruvate production as well as raise in HK activity in TP-exposed-alloxan diabetic liver slices as compared to control alloxan diabetic liver slices. The raise in lactate production as well as HK activity in TP-exposed-alloxan diabetic liver slices, observed in the present study agrees with our previous report [30].Further it is known that certain low molecular weight thiols mimics the actions of insulin probably by acting as substrates for NADPH oxidase (NOX) system [31] thus, may show certain actions of insulin, hence may favor glucose utilization. This action of low molecular weight thiols through NOX system may increase the cellular NADP levels and may facilitate glucose utilization through HMP pathway. Our results shown in table-1 as well as in graphs 1, 2 and 3 agrees with this as there is an increase in glucose utilization with a parallel raise in the G6PD activities in TP-exposed-alloxan –diabetic rat liver slices(test) as compared to control alloxan diabetic rat liver slices

It may be concluded from the present study that thiopropanol at the concentration of 5mg/0.5 g liver tissue slice increases glucose utilization by the alloxan diabetic liver slices probably by favoring glucose-utilization through glycolysis as well as HMP pathway.

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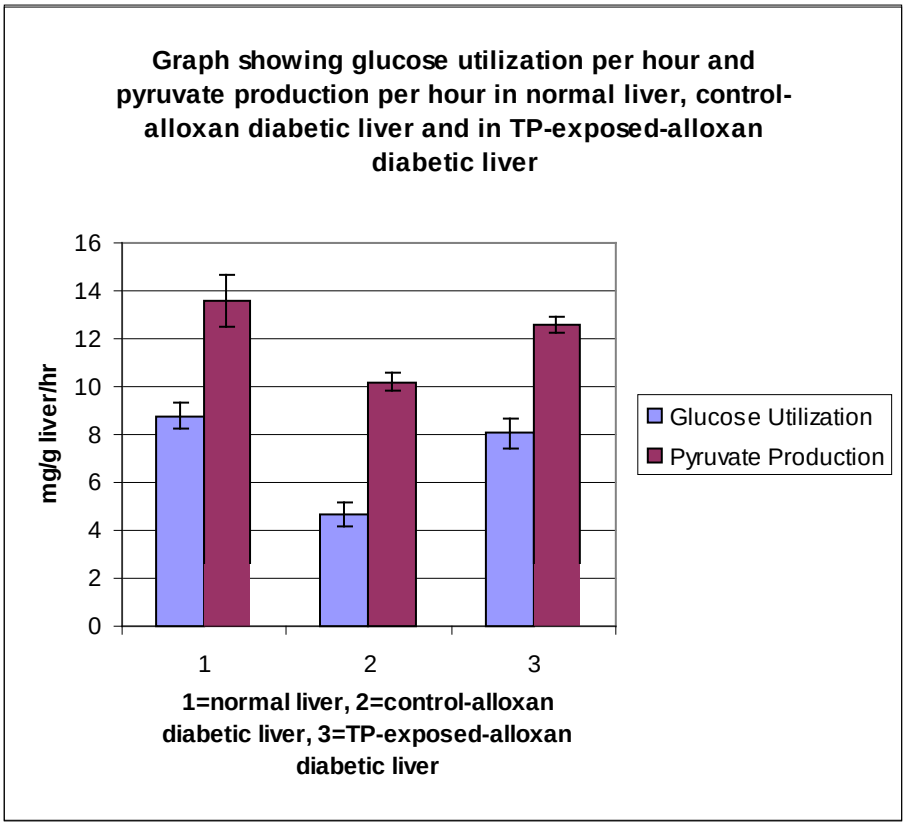
Table 1 : Table showing the glucose utilization per hour, lactate production per hour, pyruvate production per hour as well as G6PD & HK activity in isolated normal liver slices, alloxan diabetic liver slices & in alloxan diabetic liver slices exposed to thiopropanol.

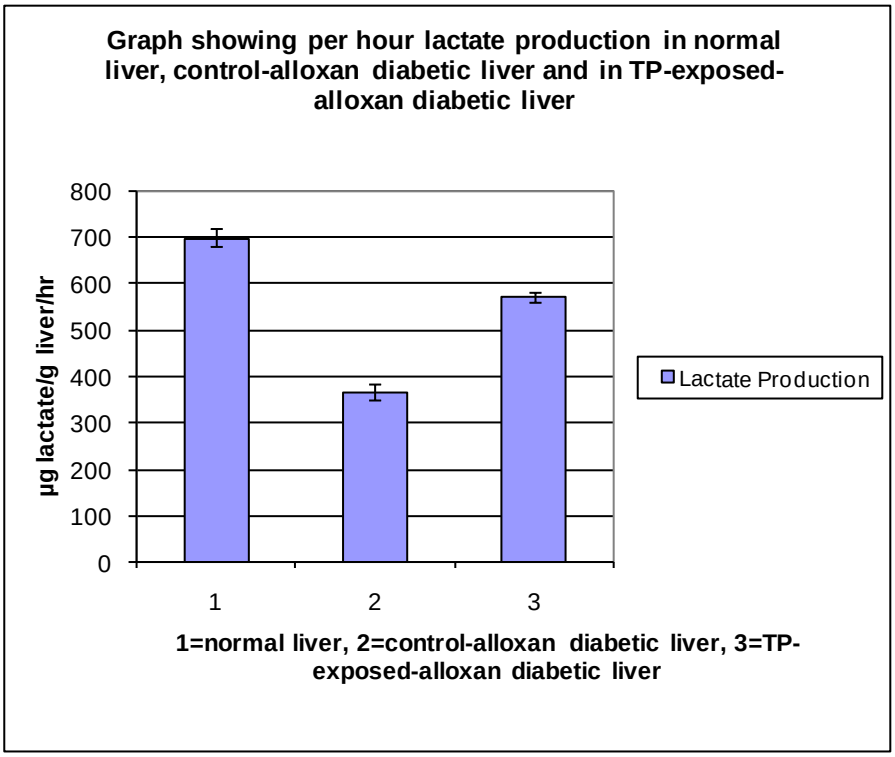
Groups	Glucose Utilization mg/g/hr	Lactate production µg/g/hr	Pyruvate Production mg/g/hr	G6PD Units	Hexokinase Units
Normal Liver (6)	8.78 ± 0.56	698.91 ± 18.48	13.59 ± 0.36	77.17 ± 1.44	170.04 ± 2.13
Control Alloxan Diabetic Liver (6)	4.64*** ± 0.50	366.20 *** ± 15.35	10.19*** ± 0.49	16.08*** ± 1.44	88.15*** ± 2.86
TP-Exposed-Alloxan Diabetic Liver (6)	8.05*** ± 0.64	571.53 *** ± 10.80	12.61*** ± 0.98	41.80*** ± 1.44	127.47*** ± 1.51

Note: 1. Number in parenthesis indicate the number of liver specimen
2. The values are expressed as their mean ± SD
3. Statistical evaluation-probability level* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
4. G6PD 1 unit = amount of NADPH produced/minute/g liver tissue
5. HK 1 unit = 1mµMol phosphate transferred /hr/mg liver tissue

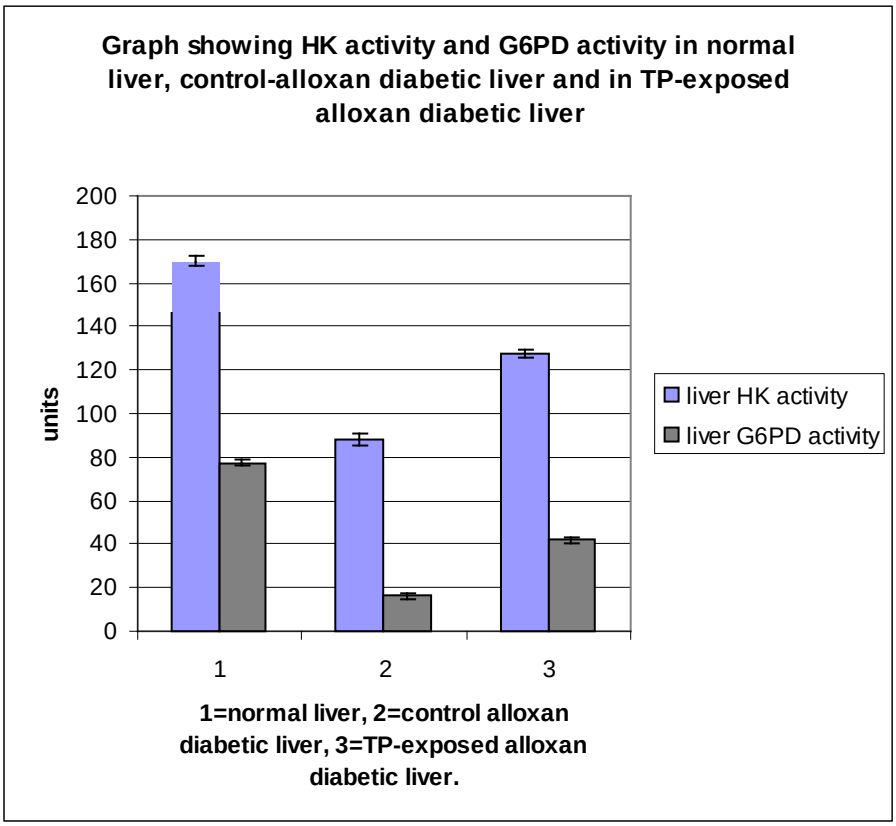
Graph-1



Graph-2



Graph-3



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Effect of Diallyl Disulphide on Renal Glycated Proteins and Plasma Sialic Acid Levels in Alloxan Diabetic Rats

By Vijay V, Vickram, Veena G Raiker, Kashinath R T

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Abstract – Diabetes mellitus (DM) induced hyperglycation of cellular and membrane proteins may result in altered ionic nature as well as an alteration in 3 dimensional structures of these molecules, thus resulting in a possible functional variation. Sialic acid (SA), a constituent of glomerular basement membrane (GBM) is a newly established potent indicator for the development of macro and microvascular complications in DM and its elevated levels are observed in DM patients with microalbuminuria and clinical proteinuria. Probably this elevation in SA may be due to increased SA release from the renal GBM due to hyperglycation. This biochemical alteration is the main initiating factor for the pathophysiology of diabetic complication, nephropathy. Diallyl disulphide (DADS), the principle compound of garlic oil, is well known for its anti-diabetic properties. Hence a study was undertaken to assess the anti-glycation properties of DADS and its usefulness in prevention of de-sialation of GBM, in alloxan diabetic kidneys, thereby to establish any beneficial effects of DADS in prevention of renal complications in DM. The current study showed a significant decrease ($p<0.001$) in kidney glycated proteins and plasma SA levels in DADS treated diabetic rats as compared to diabetic control rats. Hence it can be concluded that DADS helps in preventing glycation of renal proteins and de-sialation of GBM which may be useful in prevention of diabetic nephropathy.

Keywords : *Diallyl disulphide, protein glycation, sialic acid, Diabetic nephropathy.*

GJMR-C Classification : *NLMC Code: QU 55, WD 200.5.G6, QU 55.5*

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Abstract - Diabetes mellitus (DM) induced hyperglycation of cellular and membrane proteins may result in altered ionic nature as well as an alteration in 3 dimensional structures of these molecules, thus resulting in a possible functional variation. Sialic acid (SA), a constituent of glomerular basement membrane (GBM) is a newly established potent indicator for the development of macro and microvascular complications in DM and its elevated levels are observed in DM patients with microalbuminuria and clinical proteinuria. Probably this elevation in SA may be due to increased SA release from the renal GBM due to hyperglycation. This biochemical alteration is the main initiating factor for the pathophysiology of diabetic complication, nephropathy. Diallyl disulphide (DADS), the principle compound of garlic oil, is well known for its anti-diabetic properties. Hence a study was undertaken to assess the anti-glycation properties of DADS and its usefulness in prevention of de-sialation of GBM, in alloxan diabetic kidneys, thereby to establish any beneficial effects of DADS in prevention of renal complications in DM. The current study showed a significant decrease ($p<0.001$) in kidney glycated proteins and plasma SA levels in DADS treated diabetic rats as compared to diabetic control rats. Hence it can be concluded that DADS helps in preventing glycation of renal proteins and de-sialation of GBM which may be useful in prevention of diabetic nephropathy.

Keywords : *Diallyl disulphide, protein glycation, sialic acid, Diabetic nephropathy.*

I. Introduction

Non enzymatic glycosylation of cellular and membrane proteins will be normally proportional to available free glucose in the tissues (1). It can be expected that a consistent hyperglycemia in diabetic subject may induce hyperglycation of tissue proteins, membrane proteins or even membrane lipids. This probably may result in altered ionic nature as well as 3 dimensional structures of these molecules, thus resulting in a possible functional alteration. It has been observed by earlier workers that glycation of collagen of glomerular basement membrane (GBM) alters its structure and function, including changes in net charge (2-4).

Sialic acids (SA), a class of 9 membered ketoses, a common terminal sugar unit of the oligosaccharide of glycoproteins and glycolipids, which are components of GBM proteins. SA plays important role in maintaining negative charge of the renal glomerular basement membrane, one of the main regulators of glomerular permeability (5,6). It may be speculated that vascular permeability is regulated by SA moieties. This SA apparently enter the circulation by either shedding or cell lysis and are of considerable interest because of their potential diagnostic value in various conditions (7-9) including microangiopathies, observed in DM (10).

Garlic (Allium sativum) is one of the most commonly studied medicinal plant worldwide, for its antihyperglycaemic and antihyperlipidemic properties. Diallyl disulphide (DADS), the principle sulphur compound of steam-distilled garlic oil (11,12) is probably responsible for the anti-diabetic, anti-hyperlipidemic, anti-atherogenic as well as anti-carcinogenic actions of garlic (13-17).

Hence a study was undertaken to study the effects of DADS on renal protein glycation and its usefulness in prevention of de-sialation of GBM in alloxan diabetic rats.

II. Materials and Methods

Alloxan and Diallyl disulphide (DADS) were procured from Sigma Chemical Company. Sialic acid (analytical grade) was obtained by the courtesy of Biochemistry Department, VM Medical College, Sholapur, India. All other chemicals employed were of analytical grade. Male albino rats, weighing 200-250g randomly selected from Central Animal House, BMCH, Chitradurga, India, were used for the present investigation. The animals were maintained on a standard rat feed from Amrut rat feeds, Bangalore, supplied by Pranav Agro Industries, Pune, India. The experiments were conducted according to the norms approved by Ministry of Social Justice and empowerment, Government of India, and Institutional Animal Ethics Committee (IAEC) guidelines. The animals were fasted overnight and Diabetes was induced by a single intraperitoneal injection of freshly prepared alloxan (150mg/kg body wt.) (18), in sterile normal saline. The animals were considered diabetic if their blood glucose were above 250mg/dl and urine showed consistent glucosuria. The treatment was started on 5th day after alloxan injection and was considered as first

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day of treatment. The rats were divided into three groups comprising six rats in each group as follows:

Group I : Normal rats – which were fed on 30 ml of normal saline per kg body weight, through gastric intubation, daily for 90 days.

Group II : Diabetic Control rats - which were fed on normal saline 30ml / kg body weight, through gastric intubation, daily for 90 days.

Group III : Diallyl disulphide (DADS) treated Diabetic rats – which were fed on DADS (100mg/ kg body weight) prepared in normal saline, given as 30ml / kg body weight suspension, through gastric intubation, daily for 90 days.

On completion of the stipulated period the rats were anaesthetised and were sacrificed. Blood was collected in heparinised tubes. Kidneys were dissected and their net weight was noted. Immediately the kidneys were processed as follows. One part of kidney was homogenised with 9 parts of cold Phosphate buffer (pH 7.4) and the extract was used for total proteins (19) and carbohydrate content of these protein [Glycated protein] (20). The free sugar content of phosphate buffer extract was estimated by Folin Wu method (19) and the value obtained was deducted from the total carbohydrate content of phosphate buffer protein to calculate glycated protein content. A part of whole blood was centrifuged at 3500 rpm for 6-8mins and the plasma was used for estimation of glucose (19) and sialic acid levels (21). Part of renal tissue was collected in buffered formalin and was processed for histopathological studies.

III. Results

Results obtained in the present study are elaborated in Table 1.

IV. Discussion

The most severe chronic complication of DM is nephropathy, which involves a definite alteration in GBM thickness as well as GBM composition. There are several reports indicating alterations in GBM in diabetes which is thought due to hyperglycation of GBM proteins (4) and possibly including renal lipids also. The results obtained in present study which is narrated in table 1 clearly indicates that there is a significant increase in protein glycation ($p < 0.001$) in alloxan diabetic rats as compared to normal rats. This hyperglycation of renal proteins including GBM proteins may lead to altered functioning of these proteins which may result in changed GBM functions leading to the renal complications.

Sialic acid contributes to the maintenance of the negative charge of the renal glomerular basement membrane (22). It is well established that vascular endothelium carries a high level of SA (23), and the vascular damage leads to its release into the circulation. It has been reported that serum SA levels are increased in diabetic patients with albuminuria (24) and further, several authors found the increased urinary concentration of SA in diabetic patients with microangiopathy (25). In addition, decrease in SA content in glomerulus is observed in human diabetes and also in alloxan diabetic rats (26). A relationship between serum SA levels and microvascular complications has been observed before, in diabetic patients with microalbuminuria and clinical proteinuria (24).

Protein hyperglycation, a common phenomenon observed in DM, is responsible for glycation of tissue and membrane proteins, resulting in misorientation and malfunction of these proteins. This glycation of membrane proteins do alter the 3 dimensional structure (4) as well as ionic nature of protein resulting in the altered orientation of its domains. Such an alteration in the GBM proteins may lead to exposure of the sialated portions of the membrane proteins, hence making them easily accessible for the action of sialidase enzyme. This may lead to removal of SA which in part may account for the increased plasma SA levels which is in agreement with the results given in table I. The observed elevated SA levels in alloxan diabetic rats is in agreement with earlier studies in diabetic nephropathy (24,27,28). A parallel raise in plasma SA levels along with increased glycation of renal proteins (ref. table I) indicates, the raised SA observed in alloxan diabetic rats might have araised from de-sialation of GBM proteins which may be due to glycation induced 3 dimensional alteration in GBM proteins. This is in agreement with the Studies of Alvaro C *et al* (29) and Lisette CF *et al* (30). This de-sialation of GBM proteins may alter their ionic nature, a decrease in negative charges leading to percolation of albumin which probably results in microalbuminuria, a predisposing factor observed prior to frank nephropathy.

A significant decrease in plasma SA levels ($p<0.001$) as well as a significant decrease in renal protein glycation ($p<0.001$) in DADS treated alloxan diabetic rats (group II rats) decisively indicates that DADS got a significant role in inhibiting protein glycation in alloxan diabetic rats. DADS, a disulphide, may be involved in sulphydryl exchange reactions with proteins or enzymes (31) similar to any other disulphide as follows:

$$R1\text{-}S\text{-}S\text{-}R1 + R2\text{–}SH \text{ ----} > R1\text{-}S\text{-}S\text{-}R2 + R1\text{-}SH$$

Sialidase being a sulphydryl enzyme (32), might have undergone similar sulphydryl exchange reaction with DADS, that might have lowered the activity of sialidase enzyme, thereby retaining SA residues on GBM proteins, hence maintaining its shape as well as their negative ionic nature, thus preventing the percolation of albumin. This hypothesis is further supported by decreased plasma SA levels in the DADS treated diabetic rats, observed in present study (ref. table I) and supported by histopathological studies (ref.

figure 1,2,3), suggesting a usefulness of DADS in prevention of renal complication in alloxan diabetic rats.

Hence it can be concluded that DADS, a disulphide may decrease GBM protein glycation and prevents de-sialation of GBM, thus retaining the normalcy of GBM proteins resulting in delaying or decreasing diabetes induced renal changes. Thereby DADS may be useful in prevention of renal complication in alloxan diabetic rats. Further studies with respect to other animal models may certainly prove the therapeutic application of DADS in prevention of development of diabetic nephropathy.

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Table 1 : shows the plasma glucose levels, plasma sialic acid levels along with renal glycated proteins of normal rats (group I), alloxan diabetic control rats (group II) and DADS treated alloxan diabetic rats (group III) :

	Plasma glucose mg/dl	Renal Total Proteins mg/g	Renal Glycated Protein %	Plasma Sialic acid mg/dl
Group I (n=6)	112.26 ± 19.6	145 ± 32.01	5.8 ± 1.7	66.12 ± 9.40
Group II (n=6)	623.66*** ± 102.08	120* ± 24.5	22.5*** ± 5.0	100.32*** ± 15.12
Group III (n=6)	565.00 ± 135.01	110 ± 22.36	13.1*** ± 3.0	84.82** ± 10.24

X

Note: 1. Number in parentheses indicate the number of animals in each group.
2. The values are expressed as their mean ± SD
3. Significance level * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

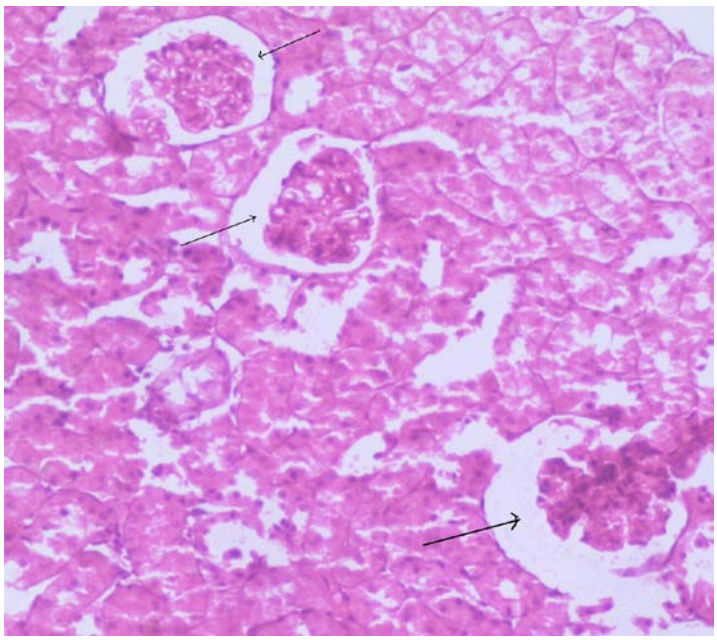

Fig. 1 : Normal rat kidney — slide showing normal glomeruli indicated by arrows. (H&E X10).

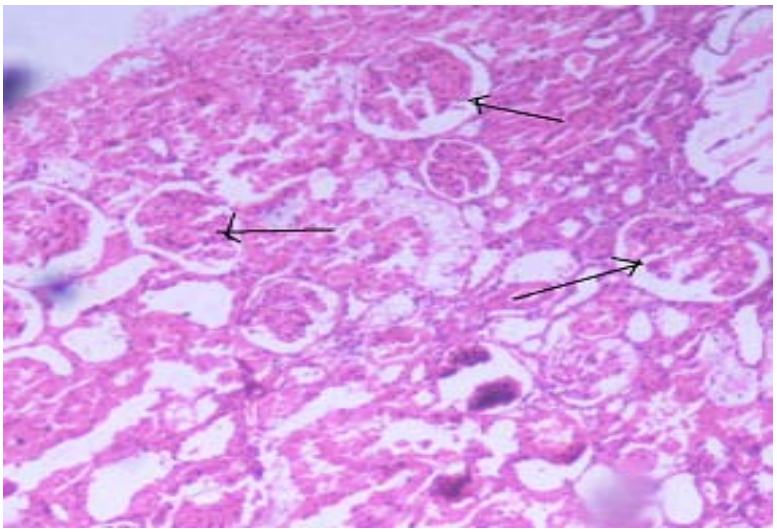

Fig. 2 : Alloxan diabetic rat kidney -- slide showing oedematous glomeruli indicated by arrows along with increased cellular infiltration and glycogen granules. (H&E X10).

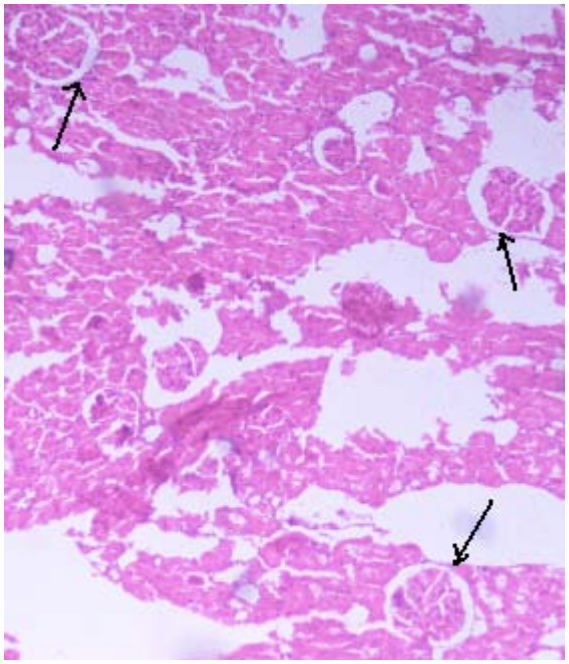

Fig. 3 : DADS treated alloxan diabetic rat kidney – slide showing normal glomerulus indicated by arrows. (H&E X10).

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Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
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Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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