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CONTENTS

1. Prevalence of the Burnout Syndrome Among Medical Staff 4
Hogea L, Hogea B, Bredicean CA, Chiriac D

2. *Momordica Charantia* Restores Compromised Haematological Parameters in Streptozotocin-Induced Diabetic Rats 8
Omobola Aderibigbe Komolafe, David Adesanya Ofusori, Samson Ayodeji Odukoya, Olarinde Stephen Adewole

3. Quality of Life in Haemophilia Patients with Knee Haemarthrosis 13
Hogea B, Nussbaum L, Aungurence M, Hogea L

4. Body Mass Dependence of Reactive Oxygen Species Compartmental Distribution in the Rat Diabetic Kidneys 16
Vaduva A, Sturza A, Tiugan DA, Muntean D, Dema A

5. Bioprinting: Current Techniques and Applications 21
Hurmuz M, Bojin F, Miu C, Ionac M, Tatu F

6. Histochemical and Heamatological Study of the Effects of Ethanolic Leaf Extract C. Zambesicus on the Stomach in Diabetic Rats 26
DA Ofusori, OA Komolafe, OS Adewole

7. Usefulness of Real-Time Shear Wave Elastography in the Diagnosis of Malignant Thyroid Nodules in Patients with Autoimmune Thyroiditis 32
Oprea MC, Lazar F

8. Risk Factors Associated with Chronic Venous Insufficiency in Central India 37
Samita R Sorte, Sachin B Rathod

9. Emotional regulation of children diagnosed with Autistic Spectrum Disorder 43
Cristina Gianina Anghel, Liana Kobylinska, Mihaela Stancu, Iuliana Dobrescu

CUPRINS

1. Prevalența sindromului burnout in randul personalului medical 4
Hogea L, Hogea B, Bredicean CA, Chiriac D

2. *Momordica Charantia* restaureaza parametrii hematologici compromise la sobolanii cu diabet indus de streptozotocin 8
Omobola Aderibigbe Komolafe, David Adesanya Ofusori, Samson Ayodeji Odukoya, Olarinde Stephen Adewole

3. Calitatea vietii la pacientii hemofilici cu hemartroza la genunchi 13
Hogea B, Nussbaum L, Aungurence M, Hogea L

4. Masa corporala influenteaza distributia speciilor reactive de oxigen in diferite compartimente ale rinichiului de sobolan diabetic 16
Vaduva A, Sturza A, Tiugan DA, Muntean D, Dema A

5. Bioimprimarea: Tehnologii actuale si aplicatii 21
Hurmuz M, Bojin F, Miu C, Ionac M, Tatu F

6. Studii histochimice si hematologice asupra efectelor induse la nivelul stomacului sobolanilor diabetici de catre extractul etanolic din frunze de *C. Zambesicus* 26
DA Ofusori, OA Komolafe, OS Adewole

7. Utilitatea elastografiei shearwave in timp real in diagnosticul nodulilor tiroidieni maligni la pacientii cu tiroidita autoimuna ...32
Oprea MC, Lazar F

8. Factorii de risc asociati insuficientei venoase cornice in India Centrala 37
Samita R Sorte, Sachin B Rathod

9. Emotional regulation of children diagnosed with Autistic Spectrum Disorder 43
Cristina Gianina Anghel, Liana Kobylinska, Mihaela Stancu, Iuliana Dobrescu

PREVALENCE OF THE BURNOUT SYNDROME AMONG MEDICAL STAFF

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ABSTRACT
The burnout syndrome got the attention of both researchers and practitioners because of its insidious effects on an individual, organizational and health department level. It is considered a public health problem because of its increased frequency but mainly due to the negative consequences that it has regarding the health department. It hampers the health system, indirectly by affecting the health of the people that have burnout syndrome and indirectly by the consequences that it has over the quality of the medical caring.
The purpose of this thesis is to provide an analysis of the burnout syndrome inside a study group from the medical staff of Romania. Following this purpose, 100 medics have been selected from different departments from hospitals in Timisoara with ages between 30 and 55 years to whom a questionnaire for the measurement of the burnout syndrome has been applied. The results showed the fact that selected medics confront themselves with this syndrome, a syndrome that affects their personal performance, leading in time to exhaustion.
Keywords: burnout syndrome, stress, depersonalization syndrome

INTRODUCTION

Some specialists that dealt with the study of the burnout syndrome among the medical staff, got to the conclusion that the etiology of this system consists of the imperious need of the people in it to think that their lives have a purpose in this world and that all of the things they do are useful, valuable, important and even heroic [1].

Because of this, when the defeat, failing sensation appears, the burnout also appears [2]. But this thing is not generally true for everybody, it happens mostly to the persons who chose this line of work because they thought that they have a vocation for this sort of thing, and for the people that enter this work field realistically, but also very motivated, the risk for the syndrome to appear is much lower.

These observation and research have helped strengthen the ideas presented by Maslach in 1988 related to the differences between burnout and stress [3].

The medics from Romania are the most prone to develop burnout, because they are always at risk to get exhausted both emotional and physical. The accumulation of tiredness from the sleepless nights which the medic spends trying to minimalize the suffering and the sickness of a huge number of patients, the less and less free time that he needs to relax and recover his energy combined with the necessity to react promptly to the different solicitations lead to exhaustion, stress and finally to burnout.

Therefore, it is very important to identify the signs associated with the burnout syndrome so they can be differentiated from the signs of stress, and assuming that these signs exist, measurements regarding its development must be taken thus avoiding the critical faze with grave consequences on an individual and institutional level.

MATERIAL AND METHODS

100 medics employed in the emergency clinics from hospitals in Timisoara, aged between 30 and 55 years have taken part in this study. 40 of them are females and 60 of them are males.

A questionnaire build based on the theory of Maslach and Jackson, that measures the level of professional exhaustion from this line of work, was used for the collecting of data. The questionnaire includes 25 terms that are structured on 3 dimensions: emotional exhaustion (9items), depersonalization (6items) and the reduction of personal achievements (10items) [4, 5]. The Likert scale was use as

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a response method because it confers the advantage of a greater variety of answers.

RESULTS

The studies made in the ranks of medical staff from different European countries have shown that approximately a third of them experience the burnout phenomena [6-10].

The negative effects of these illness from which the medical personnel suffers are being felt by the individual, the institution as well as the patients. According to some studies, the medics, the residents, and the medical assistants who suffer from this syndrome are more prone to substance usage [11], depression [12], insomnia [13], or show some higher and higher rates of suicidal ideation [1].

Some studies made in 2001 and 2009 have confirmed the fact that the burnout syndrome affects the performance of hospitals by favoring the intentions to change jobs [2], the absence from work [3], the increase of demotivation or even by favoring the big amount of intentions to retire before term [14].

The medium presence (41%) of the burnout syndrome among medics owes itself to the fact that the medics must permanently deal with emotional stimuli from with their relation with their patients as well as with physical stimuli from the exhausting work that they must do in order to fill in for the lack of medical personnel. All of these are affecting the professional performance and lead to exhaustion and stress [Figure 1].

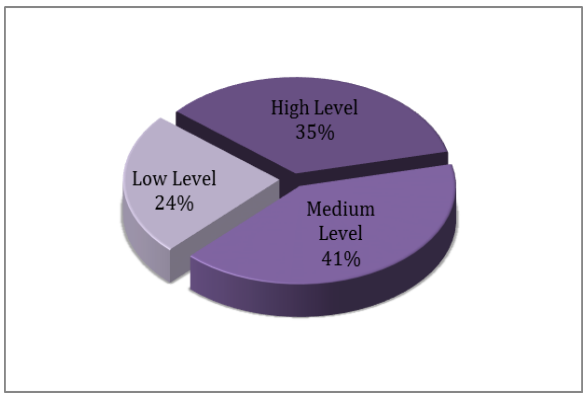

Fig. 1. The total score of burnout syndrome

The most important aspect of the burnout syndrome is the one regarding the emotional exhaustion and the results show that 44.4% from the total number have a high level of stress.

Emotional exhaustion represents the wasting of emotional energy caused by the excessive psychological requirements of the different tasks at the job.

The relations with the patients may be very demanding because they require empathy and emotional involvement and in time these have led to vulnerability towards stressors [Figure 2].

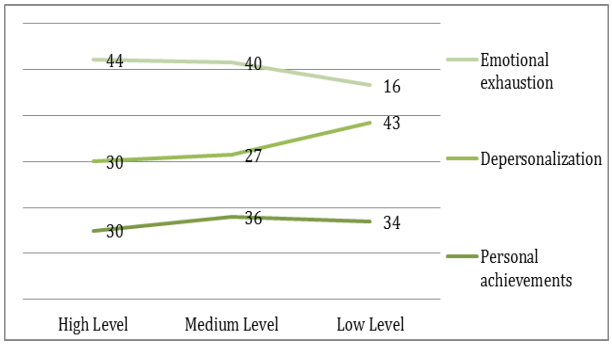

Fig. 2. Distribution of subjects on the three domains of burnout syndrome

Depersonalization is another dimension represented in the figure number 2. Depersonalization refers to the attempt of people to detach themselves from others by associating and treating them like objects. The aspects of depersonalization are reflected either by language, by the usage of labels to describe patients or their illnesses, either by negativity or a cynical attitude.

Voluntarily or not, each of us witnessed once in our lives, some of these treatments from medics and we judged them without thinking that they may suffer from the depersonalization syndrome. 29.8% of the subjects from this questionnaire present symptoms of a depersonalization syndrome [Figure 2].

The last component of the burnout syndrome is the reduction of personal achievements and it is characterized by the tendency of negatively evaluation of one's capacities, achievements or professional success (30.2%) [Figure 2].

These phenomena start faster at people who chose this line of work because they considered that they have a vocation towards it, and at the moment of the failure apparition, one thinks of himself as professional incompetent and incapable of reaching one's initial goal.

Emotional exhaustion makes its presence felt at people with a job experience of more than 5-10 years and respectively over 20 years.

The lack of experience and the lack of means of protection may lead in time towards emotional exhaustion, thus may be explained the higher score of people with less than 10 years of experience. Whereas people with more than 20 years of experience are experiencing this syndrome because they have accumulated a large number of losses that they didn't knew or couldn't express or manage [Figure 3].

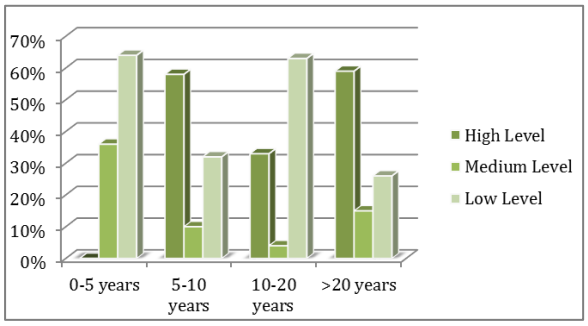

Fig. 3. Correlation between emotional exhaustion and professional experience

Regarding the case of the depersonalization syndrome, it can be observed in the figure bellow a directly proportional increase between itself and the acquired professional experience. If in the first years of experience only two out of ten people experienced the depersonalization phenomena, their number also rose with 12 out of 35 people to manifest this phenomenon [Figure 4].

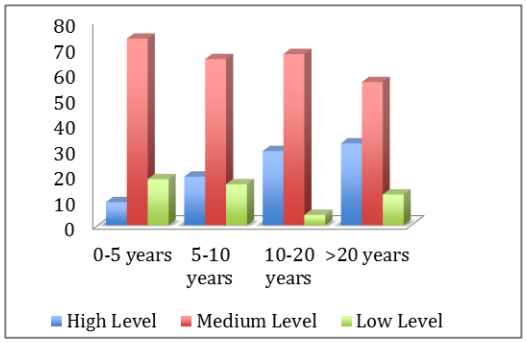

Fig. 4. Correlation between professional experience and depersonalization

Persons with more than 20 years of experience are more affected and they feel more the effects of the reduction of their personal achievements, declaring that they feel unsatisfied on a professional plan [Figure 5].

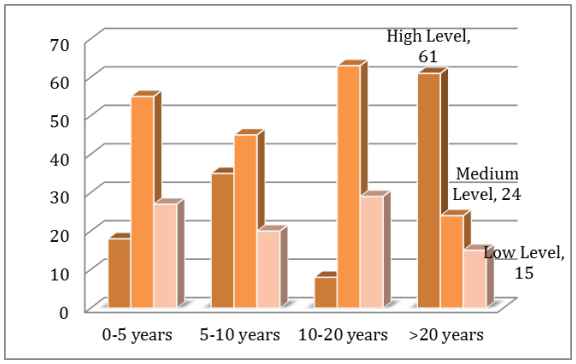

Fig. 5. Correlation between the reduction of personal achievements and professional experience

CONCLUSIONS

The analysis of the results based on the questionnaire created based on the theory of Maslach and Jackson, confirmed the worrying reasons regarding the presence of this syndrome among medics and it also helped to accomplish the research objective, the identification of a possible prevalence of the burnout syndrome among the medics of Timisoara. The results of this study have shown that the reduction of personal achievements and depersonalization are of a medium level, whilst the emotional exhaustion presents a high score.

A brief or prolonged work experience presents a factor that leads to an increased level of stress and implicitly to an increase of the possibility to develop the burnout syndrome.

Schaufeli and Enzman identified in their researches some affective signs on the medical personnel level like irritability, over-sensibility, low empathy towards patients or increased anger [15]. Simultaneously they also underlined typical cognitive signs as cynical perceptions or dehumanization towards patients, negativity/pessimism towards patients or their labeling in a derogatory way.

The behavior signs at an interpersonal level are violent outbursts, aggressiveness towards patients, interpersonal conflicts, the loss of interest or being indifferent towards the patients and the mechanical responses for the patients [15].

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PREVALENȚA SINDROMULUI BURNOUT ÎN RÂNDUL PERSONALULUI MEDICAL

REZUMAT

Sindromul burnout a captat interesul atat al cercatatorilor cat si al practicienilor din cauza efectelor insidiuase pe care le are la nivel individual, organizational si a sistemului de sanatate. Este considerat o problema de sanatate publica din cauza frecventei in crestere dar in principal din cauza consecintelor negative pe care le are asupra sistemului de sanatate. Ingreuneaza sistemul e sanatate in mod indirect prin afectarea sanatatii persoanelor cu sindrom burnout si indirect prin consecintele pe care le are asupra calitatii ingrijirii medicale.

Scopul acestei lucrări este de a oferi o analiza a sindromului burnout in randul unui esantion din personalul medical din Romania. In acest scop au fost selectati 100 de medici din diferite sectii din spitalele din Timisoara cu varste cuprinse intre 30-55 ani carora li s-a aplicat un chestionar de masurare a sindromului burnout. Rezultatele au relevat faptul ca medici selectati se confrunta cu acest sindrom, sindrom ce le afecteaza performanta profesionala, ducand in timp la extenuare.

Cuvinte cheie: sindromul burnout, stres, depersonalizare

MOMORDICA CHARANTIA RESTORES COMPROMISED HAEMATOLOGICAL PARAMETERS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

Background: The present study investigated the effects of *M. charantia* on haematological changes in streptozotocin-induced diabetic Wistar rats.

Method: Forty healthy adult Wistar rats of both sexes were randomly assigned into five groups A, B, C, D and E of eight rats each. Group A were the control (normal rats); B were the experimentally-induced diabetic rats; C were diabetic rats treated with methanolic extracts of *M. charantia* for two weeks (withdrawal group); D were diabetic rats treated with methanolic extracts of *M. charantia* for four weeks. E was diabetic rats treated with glimepiride for four weeks. Blood samples obtained by cardiac puncture were used for haematological studies.

Results: Results showed an improvement in most RBC indices in groups D and E as compared with untreated diabetic group. The groups treated with *Momordica charantia* and glimepiride enhanced WBC profiles as compared with the untreated diabetic group. Platelet count showed a significant ($p<0.05$) increase in groups D and E as compared with the untreated diabetic group. PDW and MPV were not significantly reduced in groups D and E as compared with the untreated diabetic group.

Conclusion: *M. charantia* restored some haematological indices in diabetic animals, and in a manner similar to glimepiride.

Keywords: Momordica charantia; Haematological; Streptozotocin; Diabetic Rats

INTRODUCTION

Diabetes mellitus (DM) is a serious metabolic disorder with micro- and macro-vascular complications that result in significant morbidity and mortality. Increasing proportion of the aging population, consumption of calorie rich diet, obesity and sedentary lifestyle have led to a tremendous increase in the number of diabetics worldwide [1]. Thus researches are always ongoing to increase and improve the understanding of pathogenesis of the disorder, its associated complications and develop better, safer and cheaper treatment. *Momordica charantia* (cucurbitaceae) is one of the popular herbs that grow in different regions of Nigeria. It is believed to have beneficial effects in prevention and treatment of many diseases in folkloric medicine, especially in the treatment of DM in individuals with non-insulin dependent diabetes. It has hypoglycemic properties as it significantly suppressed the rise in blood glucose concentrations in albino rats [2]. Bitter melon grows in tropical areas, including parts of the Amazon, east Africa, Asia, and the Caribbean, and is cultivated throughout South America as a food and medicine. In the Amazon, local people and indigenous tribes grow bitter melon in their gardens for food and medicine. They add the fruit and/or leaves to beans and soup for a bitter or sour flavor: Parboiling it with a dash of salt may remove some of the bitter taste. Medicinally, the plant has a long history of use by the indigenous people of the Amazon. A leaf tea is used for diabetes, to expel intestinal gas, to promote menstruation, and as an antiviral for measles, hepatitis, and feverish conditions. It is used topically for sores, wounds, and infections, internally and externally for worms and parasites [3].

Various parts of *M. charantia* such as the seed, fruit and even the whole plant has been reported to have beneficial effects in prevention and treatment of many diseases in folkloric medicine, especially in the treatment of DM in individuals with non-insulin dependent diabetes [4,5]. It has hypoglycaemic properties as it significantly suppressed the rise in blood glucose concentrations in albino rats [4,2]. The first clinical study into the influence of the fresh juice of bittergourd on the management of DM was by Akhtar 1981[6]. These findings suggested the intervention would effectively treat all symptoms of diabetes including polyuria, polydipsia, and polyphagia. Sarkar et al. [7] and Miura et al. [8] indicated that the fresh bitter-gourd juice caused a significant reduction

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in plasma glucose concentration, and an improvement in the response to an oral glucose load. Bitter melon contains an array of biologically active plant chemicals including triterpenes, proteins and steroids. In addition, a protein found in bitter melon, momordin, has clinically demonstrated anticancerous activity against Hodgkin's lymphoma in animals. Other proteins in the plant, alpha- and beta-momorcharin and cucurbitacin B have been tested for possible anticancerous effects [9]. In numerous studies, at least three different groups of constituents found in all parts of bitter melon have clinically demonstrated hypoglycemic (blood sugar lowering) properties or other actions of potential benefit against diabetes mellitus [10]. These chemicals that lower blood sugar include a mixture of steroidal saponins known as charantins, insulin-like peptides, and alkaloids. The hypoglycemic effect is more pronounced in the fruit of bitter melon where these chemicals are found in greater abundance.

The present study investigated the restorative effects of *M. charantia* on haematological parameters in strepto-zotocin-induced diabetic Wistar rats.

MATERIALS AND METHODS

Animal care:

Forty healthy adult Wistar rats of both sexes, with average weight of 134.4 g were used for this experiment. The rats were bred in the animal holding of College of Health Sciences, Obafemi Awolowo University, Ile-Ife. They were maintained on standard rat pellet (Capsfeed, Ibadan, Nigeria) and water was provided *ad libitum*.

The animals were randomly assigned into five groups A, B, C, D and E of eight rats each.

- group A were the control (normal rats)
- group B were the experimentally-induced diabetic rats administered with 10% tween 80
- group C were the experimentally-induced diabetic rats treated with methanolic extracts of *Momordica charantia* dissolved in 10% tween 80 for two weeks (withdrawal group)
- group D were the experimentally-induced diabetic rats treated with methanolic extracts of *Momordica charantia* dissolved in 10% tween 80 for four weeks
- group E were the diabetic rats treated with a standard diabetic drug (2 mg/kg of glimepiride) dissolved in 10% tween 80 for four weeks

The animals received humane treatment as outlined in the "Care and Management of Laboratory Animals" published by the National Institute of Health [11].

Plant material:

Matured leaves of *Momordica charantia* (Cucurbitaceae) were collected during the raining season from suburban villages of Ile-Ife metropolis in Osun State of Nigeria. The leaves were taken to the Herbarium in the Department of Botany, Obafemi Awolowo University, Ile-Ife to confirm identification and a voucher specimen number (UHI 16510) was placed in the Herbarium.

Preparation of methanolic extract of *M. charantia*:

Leaves of *Mormodica charantia* (MC) were air dried and powdered in a warring blender. A 765 g of the powdered leaves were extracted in 1,950 mls of absolute methanol for 72 hours with intermittent shaking and filtered. The filtrate were concentrated *in vacuo* at 35^0C using a vacuum rotary evaporator (Bŭchi Rotavapor R110, Schweiz). The extract was partitioned between water and dichloromethane, the dichloromethane fraction (5.94%) was oven-dried at 37^oC and stored until it is ready to be used. The aqueous portion obtained was very little. Aliquot portions of the extract were weighed and dissolved in 10% tween 80 for use on each day of the experiment.

Induction of diabetes:

Diabetes mellitus was experimentally-induced in groups B, C, D and E by a single intraperitoneal injection of 65 mg/kg body weight of streptozotocin (Tocris Bioscience, UK) dissolved in 0.1M sodium citrate buffer (pH 6.3). Diabetes was confirmed in animals 48 hours after induction, by determining fasting blood glucose level using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany) consisting of a digital meter and the test strips using blood samples obtained from the tail vein of the rats. Animals in group A were given equal volume of citrate buffer used in dissolving streptozotocin intraperitoneally.

Administration of extract and anti-diabetic drug:

Methanolic extracts of the leaves of *M. charantia* (100 mg/kg) was dissolved in10% tween 80 and administered daily (orally) by gastric intubation to the rats in groups C and D for 2 and 4 weeks respectively. The standard antidiabetic drug (glimepiride, 2 mg/kg) was administered to group E rats for four weeks [12] while those in group B were left untreated.

Haematological parameters:

Blood samples were by cardiac puncture from the animals under slight chloroform anesthesia using syringes containing the 10%-EDTA. This procedure was carried out within 0.8-1.2 minutes to minimize stress. The blood was later transferred into EDTA K2 vacuum tube (GD040EK). Haematological parameters were performed using automated heamatology analyzer (Sysmex: KX-21N). The following heamatological parameters were determined: white blood cell (WBC), red blood cell (RBC), heamoglomin concentration (HGB), mean cell volume (MCV), Mean corpuscular heamoglobin (MCH), Mean heamoglobim concentration (MCHC), platelet (PLT), lymphocytes (LYM), neutrophil (NEUT), red blood cell distribution weight (RDW_SD), red blood cell distribution weight (RDW_CV), Platelet distribution weight (PDW), mean platelet volume (MPV), P_LCR.

Statistical analysis:

All values were presented as means ± SEM. Data were analyzed using one way analysis of variance (ANOVA) with Duncan multiple range test (DMRT) using Statistical Package for Social Science (SPSS 17).

RESULTS

WBC profile (WBC, LYMP & NEUT):

White Blood Cell Count

There was a significant (p>0.05) decrease in the WBC count in the diabetic group (group B, 6.60±0.20) when compared with the control (12.15±2.85). Administration of MC extract for only two weeks to the animals in group C (withdrawal group) reduced the WBC count to 3.70±0.90. The continuation of the extract administration for four weeks (group D), increased the WBC count to 7.95±1.25. The group administered with gimepiride (group E) showed unprecedented significant (p<0.05) increase in the WBC count when compared with all other animal groups (Table I).

Lymphocytes Count

There was no significant (p>0.05) difference in the lymphocyte count of the diabetic (79.35 ± 3.65) group when compared with the control group (61.95±3.25). The lymphocyte count of group C (withdrawal group) was reduced to 41.90±18.20 when compared with group D. However, the rats treated with extract for four weeks (group D) showed an increase in the lymphocyte count by 63.13% when compared with the group C. The lymphocyte count in group D (68.35±13.45) was not significantly different (p>0.05) when compared with the group treated with glimepiride (group E, 75.90±7.50) (Table I).

Neutrophil count

There was a significant (p<0.05) decrease in the neutrophil count of the diabetic rats (group B, 20.65±3.65) when compared with the control group (group A, 38.05±3.25). The animals in group C (withdrawal group) shows an increase in the neutrophil count (58.10±18.20) as compared with group D. Continuation of the MC extract administration for four weeks reduces the neutropil count by 45.52%. There was no significant (p>0.05) difference in the effect of glimepiride on neutropil count (24.10±7.50) when compared with group D (31.65±13.45) (Table I).

RBC Indices (RBC, HGB, HCT, MCV, MCH, MCHC, RDW_SD, RDW_CV):

Red Blood Cell (RBC) Count

There was no significant (p>0.05) increase in RBC count in the diabetic group (7.94±0.04) when compared with the control group (7.64±0.63). There was a slight decrease (p>0.05) in the RBC count of the animals administered with extracts for two weeks (withdrawal group C) when compared with group D rats that increased with 38.77%. The group administered with glimepiride (group E, 8.33±0.05) compared non significantly with group D (8.59±0.30) rats that were administered with extract for four weeks (Table I).

HGB

The HGB concentration increases significantly (p<0.05) in the diabetic group (group B) when compared with the control group (group A).With the administration of MC extract for four weeks (group D), HGB concentration increased to 15.60± 0.20 when compared with the control (13.05±0.75) rats (group A). Following withdrawal of the MC extract for two weeks (group C), the HGB concentration reduced to 10.60 ±2.00. The group administered with glimepiride (group E) shows no significant difference (p>0.05) with group D animals treated with MC extract for four weeks after the initial four weeks of STZ induction (Table I).

HCT

There was a significant (p<0.05) increase in HCT in the diabetic group (group B) when compared with the control (group A) as shown in Table 4.9. HCT count shows a significant (50.90 +_1.80 p<0.05) increase in the group treated with MC extract for four weeks (group D) when compared with the control group. Withdrawal of MC extract adminisration for two weeks (group C) lowered the HCT to 41.05±4.95 when compared with the diabetic group (group B, 55.40±1.60). There was no significant (p>0.05) difference between the group administered with glimepiride (group E) and the group that was treated with MC extract for four weeks after the induction of STZ (Table I).

MCV, MCH and MCHC

There was an increase in the MCV and MCH in the diabetic group (69.70±1.70 and 19.75±0.55 respectively) when compared with the control group (55.50±1.30, 17.10±0.40, respectively). However, MCHC shows a significant decrease (p<0.05, 28.35±0.05) in the diabetic group when compared with the control group (30.85 ±0.05). Administration of MC extract for four weeks (group D) further reduced the MCV and MCH count to 59.15 ±0.05 and 18.15 ±0.15 respectively while MCHC increased to 30.60 ±0.30. Withdrawal of the extract administration in (group C) rats significantly increased the MCV count but shows a decrease in the MCH and MCHC count.The group administered with glimepiride (group E) shows a non significant difference (p>0.05) when compared with the group treated with MC extract for four weeks (group D) (Table I).

RDW_SD, RDW_CV

The RDW_SD and RDW_CV shows an increase in the diabetic group (group B) when compared with the control group (Table 1). Administration of extract for four weeks further increased the RDW _SD but reduced the RDW _CV. Withdrawal of the extract administration in group C significantly (p<0.05) increased the RDW_SD to 48.80±1.50

when compared with the group D treated with extract for four weeks (37.85±0.95), while the RDW _CV was increased to 21.46±4.45 when compared with the group D treated with extract for four weeks (19.65±0.25). The group E treated with glimepiride presented a non significant difference ($p>0.05$) when compared with the group treated with MC extract for four weeks (group D) (Table I).

Platelet (PLT, PDW and MPV)

The PLT, PDW and MPV were increased in the diabetic rats (group B) (1033.00±73.00, 11.85±1-25 and 9.25±0.85 respectively) as compared with the control (group A) (770.50±32.50, 10.75 ±0.05 and 8.45 ±0.05 respectively). Administration of MC extract for four weeks reduced the PLT, PDW and MPV to 828.00 ±29.00, 10.95±0.65 and 8.55±0.35 respectively. Withdrawal of extract administration for two weeks further reduced the PLT and MPV to 544.00±371 and 8.00 ±2.40 respectively while PDW was increased to 12.60±5.10. Group E animals treated with glimepiride showed a decrease of PLT to 725.00 ± 23.00 and increased the PDW and MPV to 13.50 ± 0.40 and 9.55 ± 0.05, respectively (Table I).

Table I. The effect of M. Charantia on Heamatological Paramaters

	WBC $x10^3/\mu l$	LYM %	NEUT %	RBC $x10^6/\mu l$	HGB g/dl	HCT %	MCV Fl	MCH Pg	MCHC g/dl	RDW_SD fl	RDW_CV %	PLT $x10^3/\mu l$	PDW fl	MPV fl
Group A	12.15 ± 2.85^{a}	61.95 ± 3.25^{abc}	38.05 ± 3.25^{a}	7.64 ± 0.63^{b}	13.05 ± 0.75^{ab}	42.30 ± 2.50^{a}	55.50 ± 1.30^{a}	17.10 ± 0.40^{a}	30.85 ± 0.05^{b}	33.85 ± 0.85^{a}	17.15 ± 1.75^{a}	770.50 ± 32.50^{ab}	10.75 ± 0.05^{a}	8.45 ± 0.05^{a}
Group B	6.60 ± 0.20^{ab}	79.35 ± 3.65^{ab}	20.65 ± 3.65^{ab}	7.94 ± 0.04^{b}	15.70 ± 0.50^{b}	55.40 ± 1.60^{b}	69.70 ± 1.70^{c}	19.75 ± 0.55^{a}	28.35 ± 0.05^{ab}	45.00 ± 1.50^{bc}	18.10 ± 0.50^{a}	1033.00 ± 73.00^{b}	11.85 ± 1.25^{a}	9.25 ± 0.85^{a}
Group C	3.70 ± 0.90^{c}	41.90 ± 18.20^{a}	58.10 ± 18.20^{abc}	6.19 ± 0.38^{a}	10.60± 2.00^{a}	41.05 ± 4.95^{a}	66.05 ± 3.95^{bc}	17.00 ± 2.20^{a}	25.60 ± 1.80^{a}	48.80 ± 5.30^{c}	21.45 ± 4.45^{a}	544.00 ± 371.00^{a}	12.60 ± 5.10^{a}	8.00 ± 2.40^{a}
Group D	7.95 ± 1.25^{ab}	68.35 ± 13.45^{a}	31.65 ± 13.45^{a}	8.59 ± 0.30^{b}	15.60 ± 0.70^{b}	50.90 ± 1.80^{ab}	59.15 ± 0.05^{ab}	18.15 ± 0.15^{a}	30.60 ± 0.30^{b}	37.85 ± 0.95^{ab}	19.65 ± 0.25^{a}	828.00 ± 29.00^{ab}	10.95 ± 0.65^{a}	8.55 ± 0.35^{a}
Group E	17.50 ± 8.10^{a}	75.90 ± 7.50^{a}	24.10 ± 7.50^{ab}	8.33 ± 0.05^{b}	14.75 ± 0.05^{b}	48.80 ± 0.60^{ab}	58.55 ± 0.35^{ab}	17.70 ± 0.20^{a}	30.25 ± 0.45^{b}	34.85 ± 0.85^{a}	17.25 ± 0.65^{a}	725.00 ± 23.00^{ab}	13.50 ± 0.40^{a}	9.55 ± 0.05^{a}

Values are given as Mean ± SEM for each haematological parameters WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYM, NEUT, RDW_SD, RDW_CV, PDW and MPV respectively in each group. a, b, c, d, ab, bc, cd, abc within column signifies that means with different letters differs significantly at $p < 0.05$ while means with the same letters does not differ significantly at $p < 0.05$ (using one way ANOVA with Duncan multiple range test)

DISCUSSION

Reactive oxygen species has also been implicated in the mechanism of red cell damage [13]. In diabetes, the excess glucose present in the blood reacts with the haemoglobin to form glycosylated haemoglobin which leads to a decrease in total haemoglobin level [14]. The present result showed an increase in RBC indices in diabetic rats which when treated with *M. charantia* and glimepiride for four weeks significantly increase it except RBC, MCH and RDW-CV as compared with the control group. The reason for this observation calls for further investigation but the duration of this study may not be enough to have allowed pronounced pathogenesis required for full expression of anaemia. Withdrawal of the *M. charantia* decreased the RBC indices with the exception of MCV and RDW_SD. The reason for this occurrence is not yet known. It has also been reported that occurrence of anemia in DM is more common and is mostly due to an increase in non-enzymatic glycosylation of erythrocyte membrane glycoproteins, which correlates with hyperglycemia [15]. These increases in oxidation of glycoproteins induce an increase in the production of lipid peroxides causing hemolysis of erythrocytes [16].

The WBC and NEUT were significantly reduced in the diabetic animals except the lymphocyte count which was significantly increased. Treatment with *M. charantia* and glimepiride for four weeks increased the WBC and NEUT while the lymphocyte count was not affected significantly. Following the withdrawal of the *M. charantia*, the NEUT counts showed further increase when compared with the diabetic rats, the reason for the decrease in WBC and NEUT counts in diabetic rats may be due to reduction in the immunity in corroboration with other investigation [17]. The increase noticed in lymphocyte count in diabetic rats may be a strategy to cope with infection associated with diabetes mellitus [16].

Platelet count, PDW and MPV was significantly increased in the diabetic rats when compared with the control group. Increase in PLT and MPV has been reported to be implicated in diabetes [18]. This increase may be due to dehydration which often characterizes diabetic animals as result of polyuria [19]. Zuberi *et al* [20] also reported an increase in MPV to be due platelet hyperactivity. Treatment with *M. charantia* and glimepiride restored the Platelet count, PDW and MPV to normal.

This study has revealed that methanolic *M. charantia* could restore some haematological parameters in diabetic rats in a manner similar to glimepiride. Though the present study showed that *M. charantia* restored some compromised haematological parameters in DM, there is need for further study to fully understand the mechanisms of adverse haematological changes associated with diabetic rat models and the effects of useful therapies on these changes.

CONFLICT OF INTEREST

There was no conflict of interest among the authors and every necessary detail was agreed upon during the preparation of the work.

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QUALITY OF LIFE IN HAEMOPHILIA PATIENTS WITH KNEE HAEMARTHROSIS

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ABSTRACT
Purpose. The study brings to your attention a rare pathology whose treatment is expensive but with its help encouraging results may be obtained, with consecutive growth of the quality of life of hemophilic patients and their social reintegration.
Material and methods. The study included a number of 83 patients with hemarthrosis with ages between 6 and 40 years. Data collecting was made by using the EQ-5D quality of life questionnaire, HAL and the VAS visual scale.
Results. The quality of life questionnaires show a significant difference with improvements to the quality of life between the time period previous to the recovery and the after recovery time period (p=0.002).
Conclusions. Patients suffering from hemarthrosis tend to report that the ability to perform daily activities, social and physical activities is severely affected. Furthermore, the ones with the severe illness have the lowest quality of life score in all areas.
Keywords: haemophilia, quality of life, haemarthrose

INTRODUCTION

Haemarthrosis represent one of the hemorrhagic manifestations with a high incidence at hemophiliacs, considering that 3 out of 4 patients suffer from a hemarthrosis.

Hemarthrosis generally appears with the begging of walking, between the ages 1 and 5 and represents the first manifestation of the illness. After its apparition, after 10 years, as a unique hemorrhage it may indicate an attenuated form of illness.

The triggering factor is always traumatizing, and the affected articulations with the highest predilection are the knees, ankles and elbows. The articulations of the shoulders, hands, thighs and fingers are affected less often [1, 2, 3].

The hemarthorsis triggering is insidious, being preceded by sensations of intraarticular tension, accompanied by articular instability, after which the very live pain appears accompanied by the tumefaction and functional deficiency of the articulation. The evolution towards hemarthorsis may be rapid, in a few minutes or hours, according to the importance of the suffered traumatism [1, 4, 5].

The increased frequency of hemarthrosis to patients with a severe form of illness, the apparition and evolution of chronic hemophiliac arthropathy, which produces disability and modifies the body image may lead to a decrease in the quality of life of the hemophilia patients.

The purpose of the evaluations of the patient's quality of life consists of underlying the effect which different pathological events and therapeutic methods have on the patient's life taking into consideration aspects regarding the personal satisfaction, regarding social life. The evaluation of the treatment quality of a chronic affection implies the imperious reference to the quality of life of the patient who benefits from that treatment [6, 7].

MATERIAL AND METHOD

The present study was conducted on a group formed by 83 subjects diagnosed with severe hemophilia and who also presented hemarthrosis. The patients presented themselves for the comprehensive evaluation and for the locomotory recovery treatment. The patients had ages between 6 and 40 years.

Their quality of life has been evaluated with the help of the EQ-5D and HAL questionnaires, and the VAS scale. The EQ-5D questionnaire contains five questions, each measuring a health domain: mobility, self-care, usual activities, pain/discomfort, restlessness/depression. Based

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on the given answers a profile was calculated that indicates the affected level in each health domain as well as an average score of each domain. Furthermore, a health evaluation of the patients was done using the VAS visual scale (the biggest value is 100 and the smallest is 0).

With the help of the HAL questionnaire the patients completed their daily activities that they can participate to and the way the muscle-skeletal functioning influences it. Aspects related to the following domains were pursued: arm functions, leg functions, self-caring, home activities, leisure activities, using the public means of transport, others means of transport.

Global and domain centered scores were calculated for every questionnaire before and after the recovery process.

The statistical processing of data was made using the SPSS program that allowed the calculation of the frequency, mean, standard deviation, the appraisal of the p and r probability and the Pearson coefficient.

RESULTS

After you apply the EQ-5 d questionnaire, the proportion of patients with and without problems on the five areas before and after recovery was appreciated, it has thus been observed an increased proportion of patients with or without problems, namely the decrease in the number of patients with large problem after recovery. The average scores were assessed to the 83 patients of the group. Thus, before recovery, the average scores obtained varied between DS = 0.46 in the area of self-care and DS = 0.54 in the area of pain/discomfort. In the field of common activities, mobility and restlessness/depression scores do not vary greatly, being roughly equal, DS = 0.52 [Figure 1].

We may conclude that patients with hemarthrosis appreciate the quality of life as negative on all five areas: mobility, usual activities, pain, anxiety/depression and self-care [8, 9, 10].

The field of pain and discomfort measures the unpleasant physical sensations which a person felt and the degree of influence over their life. At the same time, it measures the person's control that it has on the pain and the ease with which he frees himself by the sensation of pain.

Being a subjective sensation, response to pain differs from person to person, depending on the degree of tolerance, acceptance, but it is certain that it has an impact on the quality of life [11, 12].

Studies have shown that pain and discomfort have a strong impact on quality of life, and the presence of pain influences the perception of other areas of the quality of life [13].

After the recovery intervention it was found a decrease in the average score across all fields, values vary between DS = 0.52 in the self-care domain and DS = 0.50 in the area of pain/discomfort. In the areas of common activities, mobility, and anxiety/depression the scores are DS = 0.51 [Figure 1].

The results found that the areas of self-care and pain are more affected in patients with a severe form of illness.

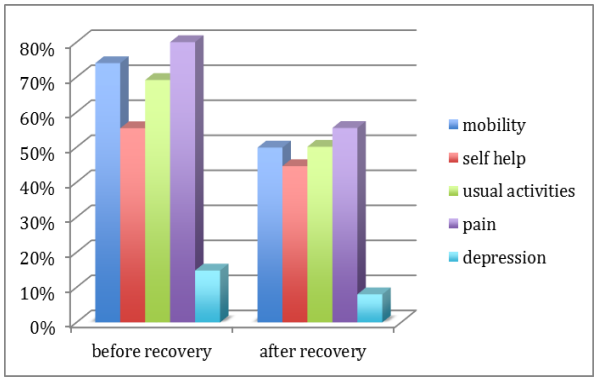

Fig. 1. The graphical representation of quality of the life before and after recovery

The VAS score appreciated depending on the severity, proved a significant increase after recovery in the case of patients with a severe form, the significant difference being p = 0,002.

The evaluation of the quality of life with the aid of the HAL list of activities has shown that patient had increased scores, the initial self-evaluation reveals the affecting of all functional abilities. The highest score was in the area of of daily living activity, DS = 24.1.

After the recovery, a significant decrease in score was reported from fields related to arms functions - p=0.003, posture - p=0.003, p=0.005- domestic activities.

The study carried out by Prof. Schramm *et al.* over the quality of life of patients with hemarthrosis from the countries where the prophylactic treatment is being provided compared to those who don't receive treatment have a better quality of life.

CONCLUSION

Patient assessment has revealed that in the absence of a replacement treatment, from a tender age the chronic suffering and impairment within a joint arises. The progresive affected joints will result in disability, handicap and a decrease of the quality of life. Most patients had their knee joint damaged , 96,6%, 46% had a damaged elbow, , 23.2% ankle 6.15%, the scapula-humerus joint and 1%, the coxo-femoral joint [14, 15].

Questionnaires measuring the quality of life show a significant difference with the improvement of the quality of life between the period prior to the recovery and after recovery (p = 0,002).

The implementation of a prophylaxis program and home treatment is imperative, starting at an early age in order to prevent the appearance of hemarthrosis and arthropathy with incapacitating potential [16].

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CALITATEA VIEȚII LA PACIENȚII HEMOFILICI CU HEMARTROZĂ LA GENUNCHI

REZUMAT

Scop. *Studiul aduce in atentie o patologie rara al carei tratament este costisitor, dar cu ajutorul caruia se pot obtine rezultate incurajatoare, cu cresterea consecutive a calitatii vietii pacientilor hemofilici si reintegrarea lor sociala.*

Material si metode*. Studiul a inclus un numar de 83 de pacienti cu hemartroza, cu vârsta cuprinsă între 6-40. Colectarea datelor s-a realizată folosind chestionarul de masurare a calitatii vietii EQ-5D, HAL si scala vizuala VAS.*

Rezultate. Chestionarele de masurare a calitatii vietii arata o diferenta semnificativa cu imbunatatirea calitatii vietii intre perioada anterioara recuprarii si dupa recuperare (p=0,002).

Concluzii*. Pacientii cu hemartoza tind să raporteze că le sunt afectate sever în capacitatea de a efectua activitatile de zi cu zi, activitățile lor fizice și sociale. Deasemenea, cei cu boala severa au cel mai scazut scor al calitatii vietii pe toate domeniile.*

Cuvinte cheie: hemofilie, calitatea vietii, hemartroza

BODY MASS DEPENDENCE OF REACTIVE OXYGEN SPECIES COMPARTMENTAL DISTRIBUTION IN THE RAT DIABETIC KIDNEYS

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ABSTRACT

Background: Oxidative stress is widely recognized as a major contributor to kidney damage in the setting of diabetes. We aimed to characterize reactive oxygen species (ROS) production in the different kidney compartments in rats.

Material and methods: Diabetes was induced in Wistar rats (n=12) by a single IP injection (50 mg/kg) of streptozotocin. After 2 months, the rats were separated in two groups, under and above 400g body weight, respectively. The kidneys were harvested and dihidroethidium (DHE) stained frozen sections were examined under an Olympus Fluoview FV1000 laser scanning confocal microscope. Fluorescence intensity was measured using a custom designed protocol in Icy bioimage analysis software.

Results: In animals weighing less than 400g, the average fluorescence intensity was over 900 AU for 2/6 rats (33%), while in the over 400g group all rats (6/6) had higher levels. Interestingly, in the > 400g group (at variance from the < 400g group) a major ROS production occurred in the vascular compartment (followed by the glomeruli) that displayed the highest DHE intensities. No changes were evident between the groups for the tubular ROS production.

Conclusion: Our results strongly suggest that an increased body mass influences the ROS generation, especially in blood vessels and glomeruli, an observation that clearly warrants further investigations with the appropriate data reporting.

Keywords: kidney ROS, confocal DHE, weight adjusted ROS levels, diabetic kidney disease

INTRODUCTION

Diabetic nephropathy (DN) is a distinct renal disease incurred by long time exposure to high glycaemic levels in diabetic patients. The spectrum of lesions is wide, as DN affects the glomeruli (mesangial expansion, podocyte damage, thickening of the basement membranes and eventually obliteration of the capillary tuft), the tubular and interstitial compartment (thickening of basement membranes, tubular atrophy, interstitial fibrosis), and the vascular structures, respectively (hyaline change) (17).

In the past decades, oxidative stress (OS) has emerged both as a central pathomechanism for tissue damage triggered by persistent high glucose levels in diabetic patients as well as potential a therapeutic target (7,12). OS is defined by the imbalance between the increase in reactive oxygen species (ROS) generation and the decrease in native antioxidant mechanisms (3). ROS comprise a family of oxygen metabolism-derived intermediates, superoxide being the foremost element, which can be further converted in hydrogen peroxide and hydroxyl radical. Superoxide is also responsible for the generation of peroxynitrite, which results from its reaction with nitric oxide(13).

In living cells, several sources for superoxide were described. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and mitochondria are the major sources for superoxide generation (15). Interestingly, Brodsky demonstrated that under hyperglycaemic stress, *in vitro*, endothelial cells supress nitric oxide production and superoxide is generated at higher rates, via the mitochondrial nitric oxide synthase (4).

Over the years, several fluorescent probes were developed to adequately monitor ROS production, like dihidroethidium (DHE) and mitochondrial targeted DHE – MitoSOX for superoxide, dichlorodihydrofluorescein-diacetate (DCF) for H202, dihydrorhodamine for peroxynitrite (9). DHE is a freely permeable fluorescent dye that can easily cross membranes and upon oxidation by

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ROS forms ethidine and 2-hydroxyethidium. It was demonstrated that out of these two, 2-hydroxyethidium is a specific product resulting only through superoxide oxidation of DHE; accordingly, the detection of the latter compound appears to be a superior marker for the superoxide quantification (20). Since the oxidized forms of DHE have overlapping emission spectra and, in order to separate the fluorescent signal, a specific setting for excitation was documented: the first of the two excitation peaks of the DHE seems to be specific for superoxide generated 2-hydroxyethidium (396nm) (16).

We aimed to quantify the levels of ROS generated in the diabetic kidneys, using the well-established model of streptozotocin (STZ)-induced experimental diabetes in rat (2). Our main outcome was to characterize the compartmentalization of ROS production in the different structures of the kidney: glomeruli, blood vessels and tubules, using the confocal microscopy technique in order to quantify DHE fluorescence.

MATERIAL AND METHODS

All experimental procedures used in this research were conducted according to the Romanian Law no.43/2014 and the 2010/63/EU Directive regarding protection of the animals used in scientific experiments, upon approval of the experimental protocol by the Committee for Research Ethics of the University of Medicine and Pharmacy "Victor Babes" Timisoara.

Diabetes induction and evolution

We used the previously described streptozotocin method to induce diabetes in adult male Wistar rats (n = 12). A single IP injection (50mg/kg) of STZ was administered and glycemia values of > 200mg/dL detected after 2 days in the blood sample harvested from the tail vein was considered a positive test for successful induction of diabetes. The animals were monitored over a period of 2 months, being housed in a controlled environment (22.5 +/- 2^{o}C, alternating 12h light/dark cycles, food and water *ad libitum*). On the day prior to organ harvesting, total food restriction was instated.

Organ harvesting and imaging

Kidneys were removed under general anaesthesia and immediately cryo-embedded in OCT (TissueTek). 20um thick sections were cut using a cryostat (SLEE MTC) and incubated for 30 minutes with DHE (Sigma-Aldrich, D7008). Control slides were created by incubating them with PBS instead of DHE. The resulting slides were coverslipped using Vectashield (Vector Labs) mounting medium and immediately examined on an Olympus Fluoview FV1000 laser scanning confocal microscope (UPLSAPO 20x objective (NA=0.75), 405nm laser excitation, barrier filter position at 500nm, barrier filter range 100nm and 4us/pixel sampling speed). Randomly selected images were acquired from the cortical area of every kidney (Fig. 1a).

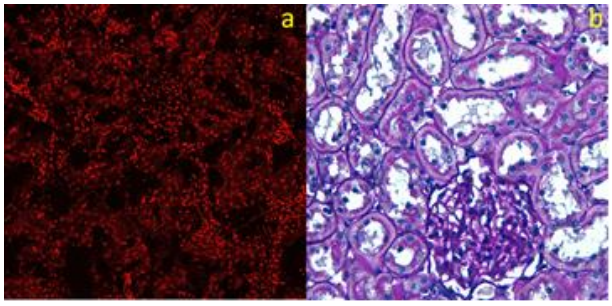

Fig. 1. a. Sample confocal image of DHE stained kidney. **b.** Sample PAS stained kidney section showing no histological changes.

Image analysis

The first step in image analysis was background correction to remove auto fluorescent signal. This was done in Olympus Fluoview F10-ASW 4.0 software, by subtraction of the unstained images from the stained ones. From every acquired image, sample areas representing glomeruli, tubulo-interstitial and vascular compartments were manually cropped. The images thus obtained were then fed through a custom designed batch processing algorithm in Icy bioimage analysis software (Fig.2). Briefly, nuclei were segmented as regions of interest (ROI) and ROI statistics were computed. We used the average fluorescence intensity values to determine the mean values for nuclear staining intensity in each compartment of the kidney cortex from every rat. Fluorescence intensity was expressed in arbitrary units (AU).

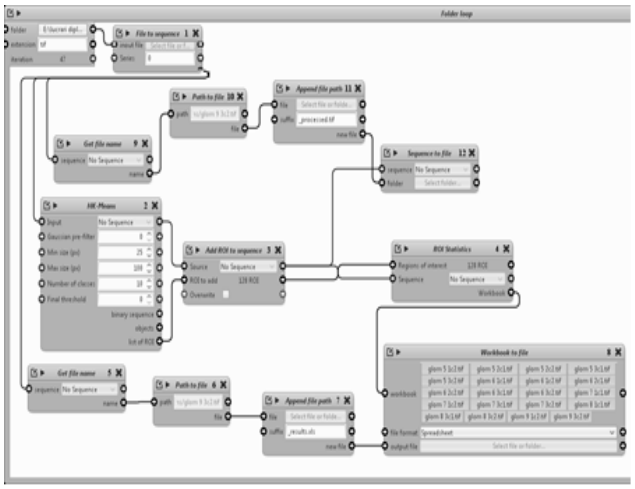

Fig. 2. Custom designed protocol for batch analysis of DHE stained kidney sections.

Statistical analysis included two-way ANOVA tests performed in GraphPad Prism. A *p* value less than 0.05 was considered significant. All values were expressed as means +/-SEM.

RESULTS

Diabetic state and body weight

All animals developed diabetes and the glycaemia levels at the end of the experiment were ≥ 285mg/dl. The average weight of the rats at the beginning of the experiment was 520 ± 28g. At the end of the experiment, 6 rats had weights up to 400g, while the remaining 6 were over 400g. Therefore, we decided to separate them in 2 groups, under and above 400g, respectively.

DHE staining

The average fluorescence intensity was over 900 AU for 2/6 (33%) in the under 400g group, while in the over 400g group all rats (6/6) had higher than 900 AU levels (Fig.3).

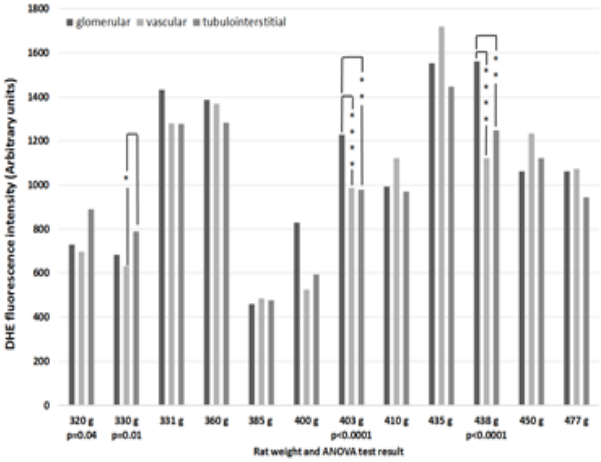

Fig. 3. ROS levels in different kidney compartments, expressed as average values per rat. ANOVA test results are shown underneath the rat's weight, while significant Tukey's multiple comparison test results are displayed over the bars.

The analysis of variance for the three kidney compartments (glomerular, vascular and tubulo-interstitial) showed significance in 2 out of 6 rats under 400g (p=0.01, respectively p=0.04), while for the over 400g group, 2 out of 6 rats presented higher significance values (p<0.001) (Fig.3) The ANOVA analysis was completed with Tukey's multiple comparison test for individual variances in the kidney compartments and it yielded significant differences between the vascular and tubulo-interstitial compartment in only 1 of the 6 rats under 400g group. In the over 400g group, 2 out of 6 rats presented very significant p values when comparing the glomerular with the vascular compartment, respectively glomerular versus tubulo-interstitial compartment.

Of note, in the <400g group, 4 out of 6 rats presented higher ROS levels in glomeruli than in blood vessels, while in the other group, vascular levels were higher than glomerular in 4 out 6 rats and higher than tubular level in 5 out six rats.

The two-way ANOVA analysis of the 2 groups (below and above 400g) showed very significant effect for the weight factor (p=0.006), while the kidney compartment factor had no effect overall (p=0.8123).

Weight adjusted ROS levels

We computed weight adjusted values by dividing the detected DHE fluorescence levels to the body mass of the animal. The resulting values showed similar ROS levels for the tubular compartment in both groups, while the glomerular and vascular DHE values were higher in the >400g group. We noticed that the vascular compartment had the most striking change, as it appear to produce the highest levels of ROS (followed by glomeruli and then by the tubules in the >400g group. Interestingly, in the <400g group, blood vessels displayed the lowest values for DHE fluorescence no significant difference between the two groups was recorded for the ROS generation in the tubulo-interstitial compartment.

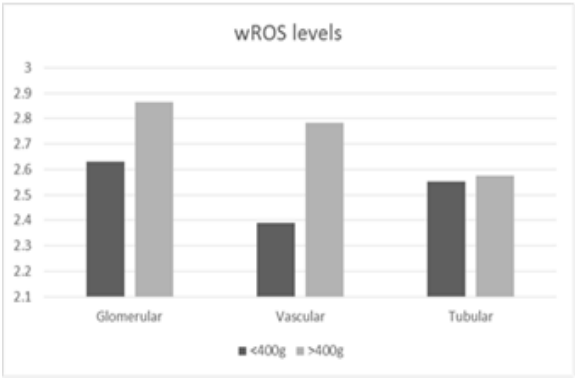

Fig. 4. Weight adjusted ROS levels in the different kidney compartments

Optical microscopy

We detected no chronic changes in the kidney parenchyma: absent glomerular nodular sclerosis, no thickening of the glomerular and tubular basement membranes, while the interstitium appeared to be within normal limits, with no added inflammatory changes (Fig.1b).

DISCUSSION

The present study was purported to characterize the compartmental distribution of ROS levels in the kidneys of diabetic rats, using confocal microscopy to quantify florescence intensity of DHE, a marker for superoxide.

Our major finding is that in STZ-induced diabetes in Wistar rats, the weight of the animals at the end of the experiment influenced the ROS levels detected in the diabetic kidneys. In the presence of diabetes, all rats became leaner over the two months period of diabetes follow-up and this weight loss is in concordance with trends previously described in literature (2). Interestingly, in our hands, the rats with final body weight >400g had overall

higher ROS values in all compartments in comparison to the <400g group. The compartmental distribution of ROS was also different between the groups. We identified that the vascular compartment presented a lower contribution to the overall ROS levels in the kidneys of the <400g group.

A fairly recent Cochrane meta-analysis, which took into consideration studies on the impact of administering antioxidants to chronic kidney disease patients (8), suggested that this therapy might slow the progression towards end-stage renal disease in patients not undergoing dialysis. As medication dosage is generally calculated in concordance to body mass, we normalized the ROS levels to the body weight of an individual animal (wROS). The compartmental distribution analysis on this new parameter showed that there was little difference at tubular level between the two groups, whereas in vascular and glomerular compartments higher levels of superoxide occurred in the >400g group. Of note, the vascular compartment, displayed the highest values of DHE fluorescence in the "fat" rats and the lowest in the "lean" rats.

Quigley et al. have shown that in the setting of obesity, the increased OS is a factor that elicits kidney injury (14). Interestingly, in obesity, the lesions in the kidney covered a different spectrum as compared to diabetic kidney disease, glomerulomegaly and focal and segmental glomerulosclerosis being the major components (5,10,17). It was documented that weight loss improves kidney function by decreasing total proteinuria and albuminuria (1,11). Moreover, higher ROS levels have been proven to modify the glomerular permeability (18). Our weight normalized parameter - wROS is in line with the previous observations and appears to indicate that weight reduction in diabetes is associated with improved levels of superoxide in vascular and glomerular compartments of the kidney. In this respect, it would be tempting to speculate that in the setting of type II obese diabetics, weight loss could also elicit a compartment dependent-mitigation of OS.

Interestingly, a recent study published by Dugan et al. (6), shows that in the setting of diabetes, the ROS levels in the kidney are actually decreased, contrary to previous literature findings. Corroborating these findings with our results, we conclude that body weight plays a definite role in increasing ROS levels in the diabetic rats, and this increase particularly occurs in the vascular compartments, regardless their location (interstitial or intra-glomerular – inside the capillary tuft).

Of note, the histological examination of the formalin fixed, paraffin embedded kidney samples showed no inflammatory cell infiltrates in the interstitium. It has been postulated that the infiltrating immune cells increase the local cellular ROS generation (19). In this setting, we conclude that in our experiment, the changes in ROS levels occurred, most probably, in response to the chronic hyperglycaemia.

CONCLUSIONS

In the STZ-induced diabetes in rats, ROS levels largely varied across the different compartments of the kidney. These levels showed a body mass - dependence, especially in glomeruli and blood vessels. In studies dealing with oxidative stress, we propose the normalization of ROS values to body weight in order to provide a more accurate interpretation of the results.

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MASA CORPORALĂ INFLUENȚEAZĂ DISTRIBUȚIA SPECIILOR REACTIVE DE OXIGEN ÎN DIFERITE COMPARTIMENTE ALE RINICHIULUI DE ȘOBOLAN DIABETIC

REZUMAT

Introducere: Stresul oxidativ este larg acceptat ca fiind un important factor implicat in lezarea rinichiului in diabet. Noi am dorit sa cuantificam productia de specii reactive de oxigen (ROS) in diferitele compartimente ale rinichiului de sobolan.

Material si metode: Diabetul zaharat a fost indus in sobolani Wistar (n=12) printr-o singura injectie intraperitoneala (50 mg/kg) de streptozotocin. Dupa 2 luni, sobolanii au fost separati in doua grupuri, cu greutate sub, respectiv peste 400g. Rinichii au fost recoltati, iar sectiunile facute la gheata au fost incubate cu dihidroethidium (DHE) si examinate cu un microscop confocal Olympus Fluoview FV1000. Intensitatea fluorescentei a fost masurata folosind un protocol personalizat dezvoltat in programul de analiza de imagini Icy.

Rezultate: La animalele cu greutate mai mica de 400g, 2/6 (33%) din sobolani au prezentat nivele medii de intensitate mai mari de 900 AU, in timp ce in grupul celor cu greutate peste 400g, toti au prezentat nivele mai mari. In mod interesant, in grupul celor de peste 400g (spre deosebire de cei <400g), s-a identificat o productie mult crescuta de ROS la nivel vascular (urmat de cel glomerular), ce au prezentat cele mai mari intensitati ale DHE. Nu am identificat diferente intre nivelele ROS la nivel tubular.

Concluzie: Rezultatele noastre sugereaza cu tarie faptul ca o masa corporala crescuta influenteaza productia renala de ROS in diabet, in special in vase si glomeruli, observatie ce necesita studii suplimentare.

Cuvinte cheie: specii reactive de oxigen in rinichi, DHE confocal, nivele ROS ajustate la greutate, rinichi diabetic

BIOPRINTING: CURRENT TECHNIQUES AND APPLICATIONS

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ABSTRACT
Bioprinting is an evolving technology that uses 3d printing in order to create complex macro and micro structures capable of replicating and enhancing the healing capacity of the native body. Through the printing process different materials and cells can be mixed in order to obtain artificial tissues and organs which in the future can represent an alternative to tissue transplant or implants. We have reviewed the main techniques of cell bioprinting options and presented the advantages and drawbacks of each method.
Key words: biofabrication, 3d printing, tissue engineering

INTRODUCTION

Tissue engineering is a combination of different scientific branches that combines engineer's principles with medical knowledge in order to repair damaged tissues, to restores specific functions of an organ or to replace it entirely [1-3]. The classical method implies the combination of different cells types with a scaffold to obtain an implantable construct. The overall result assures mechanical features similar to the native tissue, promote cell adhesions and proliferation [4]. Although in most of the cases the macro structures resembles to the anatomical shape most of the times the internal architecture lacks the complexity of natural tissues or organs. Common techniques for fabricating tissue engineering scaffolds such as gas foaming, solvent-casting, fiber bonding, phase separation, particulate leaching, and freeze drying provide macroscale scaffold but provide limited accuracy in cell placement, cell density, enable proper cell connectivity. The micro and nano structure of a design is equally important as the large scale. All these inconveniences have determined the scientists to search for other solutions.

Bioprinting has been intensely studied after the successful transformation in the early 2000s of a traditional ink jet printer when cells replaced the traditional ink from the cartridges [5]. In the recent years many publications have documented research in the 3d printing field (Fig.1). Bioprinting can be defined as an "innovative technology that allows for the generation of organized 3D tissue constructs via a layer-by-layer deposition process that combines cells and biomaterials in an ordered and predetermined way" [6]. One of the major advantages of 3D bioprinting is that patient-specific information can be directly incorporated into the biofabrication process in order to generate anatomically-correct shapes [7].

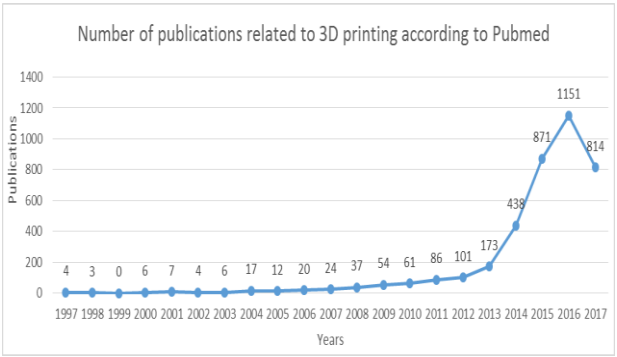

Fig. 1. Number of publications related to 3D printing according to Pubmed

BIOPRINTING TECHNIQUES

There are many bioprinting techniques available. We have tried to classify them and present there advantages and disadvantages in order to better understand the current status of knowledge. During the bioprinting process care

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must be taken in order to assure cell viability and cell function after the printing process has ended.

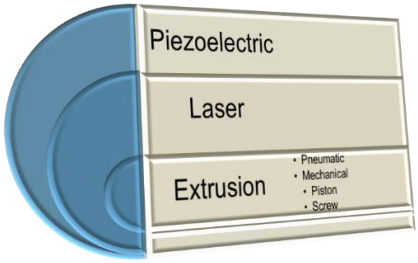

Fig. 2. Classification of bioprinting techniques

INKJET Bioprinting

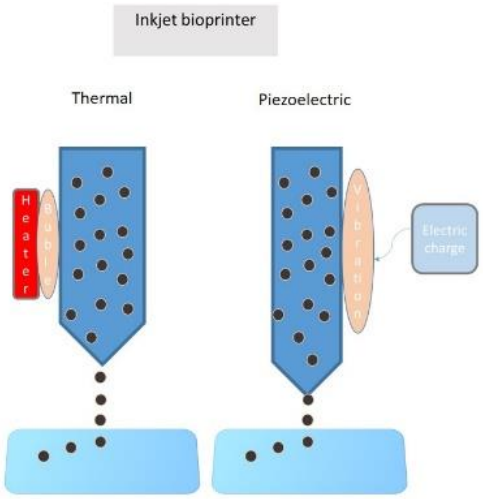

Fig. 3. Schematic representation of inkjet bioprinting

The bioprinting process with ink consists of adding cells to different biomaterials and through a nozzle discarded in layers to form the final construct. The printing process controls very accurate the amount of substances released and so very sophisticated forms can be created [8,9]. One of the reasons for the extensive us of this technique is the speed of the printing, the non-contact procedure, the resolution obtain and the reduced cost [10,11]. The increased speed poses though some problems regarding the material that can be used. The polymers used need to transform form liquid to solid, to gelatinate, very quickly, in order to be able to maintain the shape of printing [12]. During the gelatination phase chemical crosslinking is used and it can be physical, chemical or through ultraviolet methods obtained.

The thermal inkjet system contains the ink reservoir and a heating plate. The raising of the heat in the metal plate generates bubbles which will determine the drop of the substances from the reservoir [13-15]. The piezoelectric type of printer uses crystal that under an electric charge generate a vibration that will determine the ink to be discarded from the nozzle [16].

The heating process presumably affects the quality and viability of the cells [15]. However in 2014 Lorber et al proved using adult retinal ganglion cells and glia rat cells in a piezoelectric inkjet that the process has no side effects on cell characteristics [17].

Boland et al transformed a normal inkjet printer into bioprinter and managed to print multiple layers of cells and polymers [18]. Other groups of researchers have managed to adapt multiple printing heads to one printer combining several types of cells and polymers thus obtaining complex structures [19-21]. Watanabe et al, Cambell et al, Roda et al printed growth factors, proteins and biological cells using inkjet machines [22-24].

In order to be able to be printed the viscosity of the substances used needs to be below 0,1 Pa s^{-1} [25]. The size of the droplet can vary from 1 to 300 picoliters and the amount of droplets per second can be up to 10000 [8].

LASER bioprinting

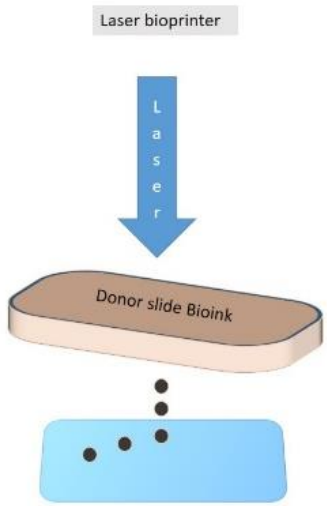

Fig. 4. Schematic representation of laser bioprinting

Laser technology for bioprinting was used by Odde et al in 1999 for the first time [26]. Laser-based direct writing (LDW) is one of the techniques on which a laser pulse determines the movement of the cells, each at a time, from the reservoir to a collector base. The laser pulse provokes the generation of a bubble which forces the exit of the cells from the donor slide to the collector slide. The LDW techniques can be divided supplementary in laser-induced forward transfer (LIFT) and matrix-assisted pulsed laser evaporation direct writing (MAPLE DW). All those laser procedures allow an accurate distribution of cells and so Lothar et al, Wang et al and Riggs et al managed to print skin cell(fibroblasts), human mesenchymal stem cells and

biopolymers [27-29]. The viability of skin cells after printing was 98% while for mesenchymal stem cells was 90%. Nathan et al. studied different types of cells (dermal, neural, pulmonary, cancer) from different sources (humans, rats, mice, bovine) and concluded that laser printing is an efficient and consistent technique in observation collagen formation, advancement of cancer and growth of neural cell [30].

The technique has no limitations regarding the viscosity of the gels unlike inkjet printing. So using high viscosity bioink can also be employed [31,32]. The precision of the bioprinting method has permitted Gaebel et al. to incorporate human umbilical vein endothelial cells on a polyester urethane cardiac patch and proved cell efficiency after several weeks [33]. By printing mammalian cells some authors have proved that stacks of cells of 50 microns can be obtained [34]. Guillotin et al. proved also that high density microscale construct can be printed with the laser practice [32].

Like every method employed the laser technology has its own side effects. The heat and pulse generated by the laser can alter the functionality and the outcome of the construct [32,35]. Laser printing techniques provide the most accurate and reliable cell placement but is limited in producing height for the scaffolds.

Extrusion bioprinting

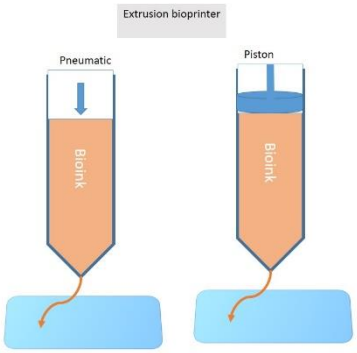

Fig. 5. Schematic representation of extrusion bioprinting

Extrusion bioprinting uses force, pneumatic or mechanic, to liberate the bioink from a dispenser. The piston mechanism allows a better control over the amount of liquid been dispensed. The bioink used for this process needs to have minimal resistance under flow. After dispense of the ink the composite is crosslinked by light thus allowing for 3d constructs with a continuous deposition of ink.

In a 2005 study Yan et al. printed artificial liver tissue using hepatocytes and adipose derived cells [36]. Sun and Lee proved that cell viability is directly correlated by the concentration of the ink, the pressure of the piston and nozzle geometry [37]. In latest studies researchers have reached angiogenesis by incorporating endothelial cells into a fibrin network filled with support cells [38]. Billiet et al. in a recent study ended up with an artificial liver made up out of hepatocytes and gelatin methacrylamide hydrogel and with a more than 95% cell viability after the printing process [39]. Horvath et al. realized a very accurate blood-air barrier in a printed lung tissue [40].

Pati et al. printed human adipose derived mesenchymal stem cells onto a decellularized matrix components of adipose tissue, being able to create various shape of structures and moreover proved that bioprinted cells have more important adipogenic differentiation capacity [41].

The extrusion printing process has great speed and the hardware required in not as expensive as in other methods. Through this techniques a wide variety of bioinks, including cell aggregates, cell-laden hydrogels, micro-carriers and decellularized matrix components can be utilized [42-47].

Although is a very convenient method, easy to use and adaptable, the extrusion process has some disadvantages also. The minimum resolution is over 100 microns and so cells can be grossly printed thus not allowing very fine deposition of cells. Hydrogel cannot be used due to gelatination and solidification process.

FUTURE DIRECTIONS

We are facing a rapid development of tissue engineering techniques and processes and 3d printing is among them. The new research has led to creation of tissue grafts and whole organs. The scientist managed to replace parts of intestines, to replace bladder or to help drug delivery [48-50].

There are still things that need to be sorted out such as quality and resolution of cell deposition, obtain both nano structure and macro structure that would resemble the native tissue, improve the speed of creation of structures. The current material used for scaffolds such as collagen, gelatin, hyaluronic acid, alginate should be improved and be accompanied be new ones which respect some rules like: biocompatibility, promote cell differentiation and cell multiplication.

The new lines to be investigated could include *in situ* printing to allow quicker and more reliable healing and rehabilitation outcomes.

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BIOIMPRIMAREA: TEHNOLOGII ACTUALE ȘI APLICAȚII

REZUMAT

Bioprintarea este o ramura tehonologica in plina dezvoltare care utilizeaza imprimarea tridimensionala pentru a crea structure complexe micro si macroscopice cu rol in replicarea si sporirea capacitatilor de vindecare ale organismului. Prin procesul de printare diferite material si celule sunt amestecate in vederea obtinerii tesuturilor si organelor artificiale care in viitor vor reprezenta o alternativa la transplantul tisular sau la implanturile existente. Ne-am aplecat atentia asupra tehnicilor principale de bioimprimare celulara prezentandu-le avantajele si dezavantajele.

Cuvinte cheie: inginerie tisulara, imprimare 3d, bioimprimare

HISTOCHEMICAL AND HEAMATOLOGICAL STUDY OF THE EFFECTS OF ETHANOLIC LEAF EXTRACT C. ZAMBESICUS ON THE STOMACH IN DIABETIC RATS

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ABSTRACT

The aim of this study was to verify the histochemical and haematological effects of *C. zambesicus* on the stomach in STZ-induced wistar rats.

Forty adult wistar rats were divided into four groups n=10. Group A serves as negative control animals receiving equivalent amount of citrate buffer, group B animals were induced with 65mg/kg of STZ, group C were pretreated with *C. zambesicus* leaf extract two weeks before STZ induction and the treatment continued throughout the study while group D was administered with *C. zambesicus* leaf extract four weeks after STZ induction. At the expiration of the study, the animals were sacrificed by cervical dislocation. The blood samples were collected for haematological analysis while the stomachs were excised and fixed in 10% buffered formaline for the demonstration of connective tissue fibres (collagen and elastic) using Verhoeff's vanGieson staining and histochemical analysis for detection of glycogen deposit using PAS staining with diastase control.

The result showed that the ethanolic leaf extract of *C. zambesicus* was able to prevent hyperglycemia and also restore connective tissue fibres, glycogen deposit and haematological parameters to near normal in adult wistar rats.

We therefore concluded that *C. zambesicus* exhibited antidiabetic potentials and thus recommend it for further studies aimed at corroborating these findings in the quest to finding a safer alternative product in the management gastric disorders resulting from diabetes.

Key words: *C. zambesicus,* Diabetes, Stomach, Histochemistry, Haematology, Connective tissue fibers

INTRODUCTION

Diabetes is the failure of the body to metabolize blood sugar properly; it is now progressing very rapidly among the Americans and other developed countries [1]. In developing nations, diabetes is gradually becoming a deadly disease as they switch to westernized lifestyles [1]. Gastric tissue compromise has been implicated in diabetes [2]. At least 75% of diabetic patients have significant gastrointestinal symptoms [3,4]. It therefore becomes very important to study gastric complications resulting from diabetes and also try to unearth the possible remedy to these pathologic conditions. Some of the solutions of diabetic-mediated complications come with the treatment of diabetes itself while in some cases; diabetes becomes alleviated living behind the complications such as gastric disorders [5]. Most of the gastrointestinal manifestations in patients have been attributed to disordered motor function [2]. Streptozotocin (STZ) induction of diabetes is an experimental model widely used to study glycemic and lipidemic changes in plasma [6]. In mammalian cells, the mechanism of action of STZ that results in cell death has not been fully identified, but is thought to be a result of DNA and chromosomal damage brought forth by mechanisms involving free radical generation during STZ metabolism [1].

The extract of *C. zambesicus* has been documented to produce a significant reduction in blood glucose level after a single dose of the extract (150mg/kg) and in prolonged treatment for 7 days [7]. Also Ofusori *et al.,* [8] has reported an improvement in gastric emptying following the administration of *C. zambesicus* in adult wistar rats. Ngadjui *et al*., [9] has successfully identified some very important compounds in *C. zambesicus* which include abiatane, diterpenoids, quinines, triterpenoids, flavonoid, Labdane, clerodane and trachylobane.

In recent times, traditional therapy has been employed by different researchers with a view to finding lasting and safer solutions not only to diabetes but also the complications that come along with it. It is in this view we set

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out to assessing the histochemical and heamatological effects of *C. zambesicus* in the prevention and restoration of gastric integrity in diabetic rats.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ) was purchased from Tocris Bioscience, UK.. tween 80 was purchased from sigma chemical company, St Louis, Missouri. USA.

Animal Care

Forty adult male albino rats of the Wistar strain were procured and acclimatized for two weeks at the Animal Holdings of the College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria before the commencement of the research work. Animals were fed with standard rat feed (Capfeeds, Ibadan) and given water liberally.

All the animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, USA [10].

Preparation of Plant Extract

The leaves of *C. zambesius* Müll. Arg. (Euphorbiaceae) were collected and authenticated at the Botany Department, Obafemi Awolowo University where specimen sample with number (UHI 16511) was deposited. The fresh leaves of the plant were air dried and grinded using squeezing and crushing machine (Daiki Rika Kogyo Co-ltd, Japan). Thereafter, the ethanolic extract was obtained. The extract obtained was partitioned between dichloromethane and water. The dichloromethane fraction was dissolved in 10% tween 80 and administered orally at a dose of 200 mg/kg as the plant extract.

Induction of experimental diabetes

All the animals were fasted for 16-h, but still allowed free access to water throughout. At the end of the fasting period – taken as 0h, initial glycemia of all animals were determined and recorded. The animals in groups B, C and D were induced with streptozotocin (65mg/kg body weight) dissolved in 0.1M sodium citrate buffer (pH 4.5). These animals were stabilized for four weeks after which the leaf extract in aqueous solution was administered orally through gavages at a concentration of 200 mg/kg body weight/rat/day to group D for another 4 weeks. Animals in group C were pretreated with *C. zambesicus* therapy 2 weeks prior to induction of diabetes.

Experimental Design

The animals were divided into four groups as follows, with ten animals in each group.

Group A: Control rats administered intraperitoneally with 0.1 M sodium citrate buffer (pH 4.5)

Group B: Diabetic rats administered orally with 10% tween 80 for 4 weeks after the initial four weeks of diabetic induction.

Group C: Diabetic rats but in which *C. zambesicus* leaf extract (200 mg/kg body weight/day/rat) in 10% tween 80 therapy started 2 weeks prior to induction and continued throughout the period the experiment lasted (8 weeks).

Group D: Diabetic rats administered orally with *C. zambesicus leaf* extract (200 mg/kg body weight/day/rat) in 10% tween 80 for 4 weeks after the initial four weeks of diabetic induction.

Animal sacrifice

Twenty-four hours after the last administration, all animals were sacrificed by cervical dislocation.

Elastic and collagen fibers analysis

Stomach from each of the animals were excised and fixed in 10% Neutral buffered formalin and Verhoeff van Gieson stain was used for demonstration of collagen and elastic fibres.

Histochemical procedure

A periodic Acid Schiff (PAS) with diastase control method by Gomori (1946) was used to demonstrate Glycogen. This included fixation in 10% Neutral buffered formalin; thereafter, the specimen were deparaffinized and hydrated to water, treated with periodic acid, aqueous for 5 minutes, washed in running water for 5 min, treated with Schiff reagent for 10 min, transfered through sulfite solutions three changes for 1.5-2 minutes each, washed in running water for 5 min, counterstained, dehydrated, cleared and mounted with DPX for permanent storage.

Haematological procedure

Haematological Analysis was performed using automated heamatology analyzer (Sysmex KX-21N, USA). Blood samples were drawn using syringes containing the 10%-EDTA from the heart of the animals placed under slight chloroform anesthesia. Average period of 1min was adopted in this procedure to reduce stress. Blood samples were thereafter transferred into EDTA K2 vacuum tube (GD040EK). The following parameters were analysed: white blood cell (WBC), red blood cell (RBC), heamoglomin concentration (HGB), mean cell volume (MCV), Mean corpuscular heamoglobin (MCH), Mean heamoglobim concentration (MCHC), platelet (PLT), lymphocytes (LYM), neutrophil (NEUT), red blood cell distribution weight (RDW_SD), red blood cell distribution weight (RDW_CV), Platelet distribution weight (PDW), mean platelet volume (MPV).

Photomicrography

All the prepared slides were examined and archived at different magnification under a LEICA research microscope (LEICA DM750, Switzerland) with a digital camera attached (LEICA ICC50).

Statistical analysis

Data were expressed as Mean ± Standard Error of Mean (S.E.M). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 17.0 (SPSS, Cary, NC, USA) with Duncan's Multiple Range Test (DMRT) option. A value of $p<0.05$ was considered to indicate a significant difference between groups.

RESULTS

Blood Glucose Level (BGL)

The result from this study showed that there was a significant increase in the BGL in the untreated diabetic group when compared with the negative control (Fig. 1). Group C that was pretreated with *C. zambesicus* prevented the elevation of BGL while the group treated with *C. zambesicus* for 4 weeks reduced BGL to near normal as shown in Figure 1.

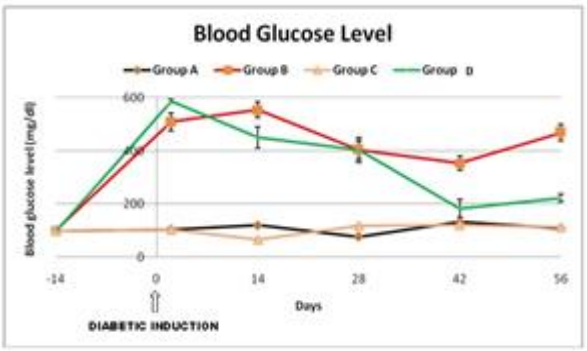

Fig. 1. Showing the antihyperglycemic effect of *C. Zambesicus* in diabetic rats.

Collagen and Elastic Fibers

There was general reduction in the elastic and collagen fibers in the untreated diabetic group when compared with the control animals. With early commencement of extract administration two weeks prior to STZ induction, there were evidences of increased concentration of elastic fibers in the muscularis externa as well as mucosa and increased concentration of collagen fibers in the submucosa and mucosa when compared with the untreated diabetic group and control (Figure 2). Administration of the extract for four weeks after four weeks of diabetic stabilization (group D) also showed an increase in the concentration of elastic fibers in the muscularis externa and mucosa as well as increased concentration of collagen fibers in the submucosa and mucosa when compared with histological sections of the untreated diabetic group (Figure 2). The histological outline is comparable with the groups pretreated with 200mg/kg of *C. Zambesicus*.

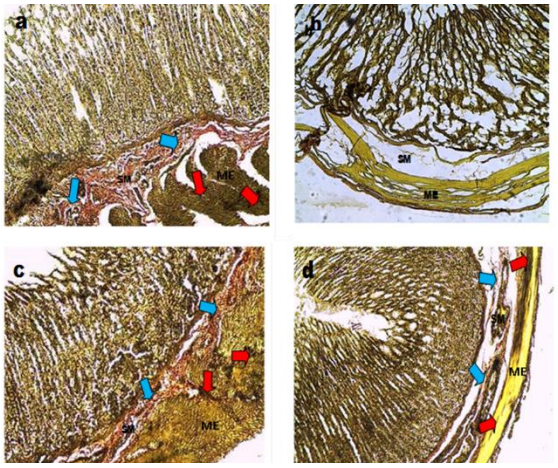

Fig. 2. Photomicrograph of the transverse section of the stomach of **(a)** Control group. Note the high deposits of collagen fibers in the submucosa (SM) and elastic fibers in the muscularis externa (ME). **(b)** Untreated diabetic group. Note the depletion of collagen fibers in the submucosa (SM) and elastic fibers in the muscularis externa (ME). BLUE ARROW-Collagen fibers, RED ARROW-Elastic fibers **(C)** Note the preservation of collagen fibers in the submucosa (SM) and elastic fibers in the muscularis externa (ME) **(d)** Note the few strands of collagen fibers in the submucosa (SM). BLUE ARROW-Collagen fibers, RED ARROW-Elastic fibers VVG x100.

Histochemical Analysis

The examination of the stomach for PAS staining in all animal groups suggest that the stomach of untreated diabetic group showed an increase in the glycogen deposit when compared with the control animals (Figures 3 & 4). With early commencement of extract administration two weeks prior to STZ induction, there was an evidence of reduction in glycogen deposit as marked by decreased PAS positive staining characterized by purple granules when compared with the untreated diabetic group and control (Figures 4 & 5). Administration of the extract for four weeks after four weeks of diabetic stabilization (Figure 6) also showed a reduction in glycogen deposit in a similar fashion with the group with early commencement of extract administration (Figures 5 & 6).

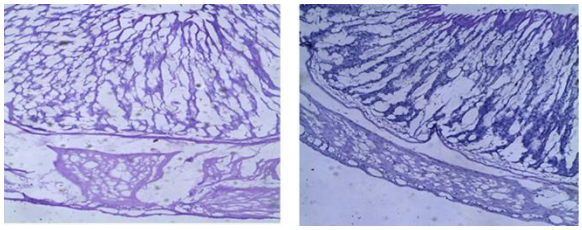

Fig. 3. Photomicrograph of the transverse section of the stomach of Negative control animals showing **(a)** Diastase control. **(b)** Normal staining. PAS x100

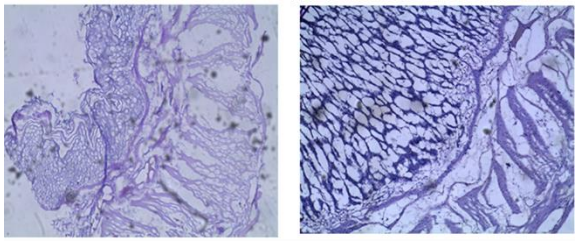

Fig. 4. Photomicrograph of the transverse section of the stomach of untreated diabetic animals showing **(a)** Diastase control. Note the depletion of glycogen **(b)** Normal staining. Note the accumulation of glycogen deposits as evident by the high staining intensity. PAS x100

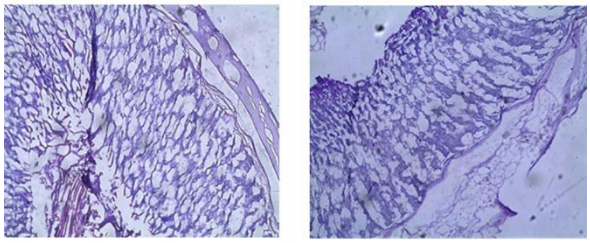

Fig. 5. Photomicrograph of the transverse section of the stomach of animals pretreated with *C. zambesicus* before STZ induction **(a)** Diastase control. **(b)** Normal staining. PAS x100

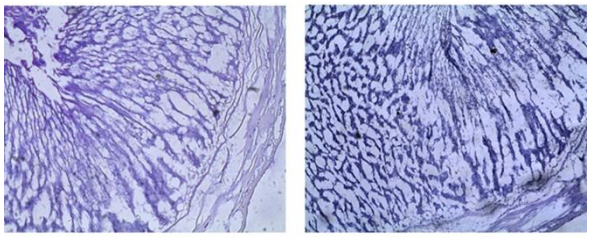

Fig. 6. Photomicrograph of the transverse section of the stomach of animals treated with *C. zambesicus* four weeks after STZ induction **(a)** Diastase control. **(b)** Normal staining. PAS x100

Heamatological Analysis

White blood cell profiles

WBC

The WBC in the untreated diabetic animals (group B) was significantly lowered by 44.31% when compared with the control group (group A). Early commencement of extract administration two weeks prior to STZ induction significantly ($p<0.05$) raised the WBC by 43.50% (Table I).

Treatment of diabetic animals with extract for four weeks (group D) significantly ($p<0.05$) raised the WBC count by 96.35% when compared with untreated diabetic group (group B).

Table I. Effects of *C. Zambesicus* on the Heamatological Parameters in STZ induced Diabetic rats

Groups	Red Blood Cell Indices								White Blood Cell Profile			Platelet Profile		
	RBC $x10^6/\mu l$	HGB g/dl	HCT %	MCV fl	MCH pg	MCHC g/dl	RDW_SD fl	RDW_CV %	WBC $x10^3/\mu l$	LYM %	NEUT %	PLT $x10^3/\mu l$	PDW fl	MPV fl
Group A	7.75 ± 0.71^{abc}	13.15 ± 0.95^{ab}	42.70 ± 3.10^{a}	55.20 ± 1.10^{a}	16.95 ± 0.35^{a}	30.80 ± 0.00^{de}	33.70 ± 0.60^{ab}	17.30 ± 1.60^{ab}	12.30 ± 3.00^{a}	61.65 ± 0.85^{a}	38.35 ± 0.85^{a}	728.00 ± 72.00^{ab}	10.85 ± 0.05^{a}	8.45 ± 0.05^{a}
Group B	7.88 ± 0.03^{bc}	15.75 ± 0.65^{c}	54.40 ± 1.90^{cd}	69.05 ± 2.15^{b}	19.95 ± 0.75^{c}	28.99 ± 0.11^{b}	44.80 ± 2.00^{c}	18.00 ± 0.20^{b}	6.85 ± 0.55^{a}	79.60 ± 3.10^{a}	20.40 ± 3.10^{a}	964.00 ± 48.00^{b}	12.10 ± 1.60^{a}	9.45 ± 1.05^{ab}
Group C	9.43 ± 0.32^{d}	16.55 ± 0.55^{c}	57.10 ± 2.50^{d}	60.50± 0.60^{a}	17.55 ± 0.05^{ab}	29.00 ± 0.30^{b}	37.95 ± 2.95^{b}	18.85 ± 1.75^{b}	17.65 ± 7.45^{a}	76.90 ± 2.60^{a}	23.10 ± 2.60^{a}	661.50 ± 237.50^{a}	11.30 ± 0.70^{a}	8.65 ± 0.05^{a}
Group D	8.79 ± 0.20^{cd}	15.50 ± 0.20^{c}	52.80 ± 0.60^{bcd}	60.05 ± 0.75^{a}	17.60 ± 0.20^{ab}	29.35 ± 0.05^{bc}	37.50 ± 0.50^{b}	18.90 ± 0.80^{b}	13.45 ± 1.75^{a}	85.45 ± 0.15^{a}	14.55 ± 0.15^{a}	865.50 ± 61.50^{ab}	11.05 ± 0.05^{a}	8.70 ± 0.20^{a}

Values are given as mean ± SEM for each haematological parameters in each group. a, b, c, d, ab, bc, cd, abc, bcd within column signifies that means with different letters differs significantly at $p < 0.05$ while means with the same letters does not differ significantly at $p < 0.05$ (using one way ANOVA with Duncan multiple range test).

LYM and NEUT

The LYM in the untreated diabetic animals (group B) was significantly ($p<0.05$) increased by 29.12% while the NEUT was lowered by 46.81% when compared with the control group. Early commencement of extract administration two weeks prior to STZ induction slightly ($p>0.05$) lowered the LYM count by 3.39% while the NEUT count was slightly ($p>0.05$) raised by 13.24% (Table I).

Treatment of diabetic animals with extract for four weeks (group D), raised ($p>0.05$) the LYM count by 7.35% and reduced the NEUT count by 28.68% when compared with untreated diabetic group (group B).

Red Blood Cell Indices

RBC, HGB and HCT

RBC, HGB and HCT counts were significantly ($p<0.05$) increased in untreated diabetic group (group B) when

compared with the control (Table 16). These values were increased by 19.67%, 5.08% and 4.96% respectively with early commencement of extract treatment two weeks prior to STZ-induction. Treatment of diabetic animals with extract for four weeks after four weeks of diabetic stabilization (group D) presented 11.55% increament in RBC as well as 1.59% and 2.94% decreament in HGB and HCT respeactively as compared with the untreated diabetic group (group B).

MCV, MCH and MCHC

The MCV and MCH count was significantly ($p<0.05$) increased in untreated diabetic group (group B) while MCHC was significantly decreased ($p<0.05$) when compared with the control (Table I). MCV and MCH values were decreased by 12.38% and 12.03% respectively with early commencement of extract treatment two weeks prior to STZ induction while MCHC was increased by 0.03% when compared with the untreated diabetic group. Treatment of diabetic animals with extract for four weeks after four weeks of diabetic stabilization also presented a 11.55% and 11.78% decreament in MCV and MCH respectively while MCHC presented a decement as compared with the untreated diabetic group (group B).

RDW_SD and RDW_CV

The RDW_SD and RDW_CV count was significantly ($p<0.05$) increased in untreated diabetic group (group B) when compared with the control (Table I). RDW_SD value was decreased by 15.29% while RDW_CV was increased by 4.72% with early commencement of extract treatment two weeks prior to STZ induction. Treatment of diabetic animals with extract for four weeks after four weeks of diabetic stabilization also presented a 16.29% decreament in RDW_SD and 5.00% increament in RDW_CV as compared with the untreated diabetic group (Group B).

Platelet profile

PLT

The PLT count was significantly ($p<0.05$) increased in untreated diabetic group (group B) when compared with the control (Table I). This was reduced by 31.38% with early commencement of extract treatment two weeks prior to STZ induction. Treatment of diabetic animals with extract for four weeks after four weeks of diabetic stabilization also presented 11.55% decreament in PLT as compared with the untreated diabetic group (group B).

PDW and MPV

The PDW and MPV count was increased in untreated diabetic group (group B) when compared with the control (Table I). These values were reduced by 19.67% and 5.08% respectively with early commencement of extract treatment two weeks prior to STZ induction. Treatment of diabetic animals with extract for four weeks after four weeks of diabetic stabilization also presented a reduction in PDW and MPV as compared with the untreated diabetic group (group B) (Table I).

DISCUSSION

Our finding showed that *C. zambesicus* reduced the BGL significantly and also prevented the elevation in the group pretreated before diabetic induction. This observation is in inline with previously documentations [11,12] This further established that *C. zambesicus,* if properly adopted in our diet can serve as preventive measure for hyperglycemia.

Positive staining indicating the presence of collagen and elastic tissue fibers were observed in varying degrees of intensity in the stomach of the negative control. These fibers were drastically depleted in the untreated diabetic group. The tunica mucosa is composed of epithelium, a lamina propria (of collagen, elastic and reticular fibers) and a muscularis mucosae. The submucosa contains collagen fibers, fat and the submucosal nerve plexuses [13]. The tunica muscularis externa has two coats: an inner circular and an outer longitudinal layer. The reduction in the staining intensity of collagen fibers in the submucosa of untreated diabetic animals is indicative of reduction in collagen fibers. This condition will subsequently lead to a reduction in the tensile strength which is required to support the mechanical activity of the stomach. This may have been responsible for the poor gastric emptying earlier noticed by Ofusori *et al.,* [8]. A reduction in the elastic tissue fibers may compromise the churning process in the stomach. The elastic fibers are responsible for the stretching and recoiling property needed for the proper churning of the ingesta. Pretreatment and treatment with *C. zambesicus* moderately restored the connective tissue fibers to near normal in a similar fashion as glimepiride.

The histochemical results showed an increase in glycogen deposits in the entire gastric outline of untreated diabetic rats. Gastric pathology is a common complication in DM. Glycogen deposit is a reference point for probable compromise in carbohydrate metabolism in alloxan induced diabetes thus resulting in hyperglycaemia [14]. It is therefore presumed that depletion of insulin secretion, hyperglycaemia and altered metabolism of lipids, carbohydrates and proteins, in addition to damaged β cells of pancreas are factors which contributed to the increase in glycogen deposit in the untreated diabetic group. In this study, the restoration of the glycogen deposits towards normal levels when treated with *C. zambesicus* presented similar pattern with that of glimepiride treated group, thus unearthing the gastroprotective effect of *C. zambesicus*.

Some WBC profiles (WBC and NEUT) were significantly reduced in the diabetic animals except the lymphocyte count which was increased. Preatment with *C. zambesicus* two weeks prio to STZ induction and treatment with glimepiride for four weeks provide better remediation vis-à-vis groups

treated with *C. zambesicus* for four weeks after four weeks of diabetes stabilization. The lymphocyte count was only moderately affected. This suggests that pretreatment with *C. zambesicus* could reasonably boost the immunity response in diabetes as evident in this study. The increament of lymphocytes in the diabetic state may be an adaptive means of coping with series of infections that are diabetic related [15]. Lymphocytes are the main constituents of the immune system which is a defense against the attack of pathogenic micro-organisms such as viruses, bacteria, fungi and protista [16].

Platelet count, PDW and MPV were significantly increased in the untreated diabetic rats when compared with the control group. Previous study has shown that increase in PLT and MPV are evident in diabetic state 17. This increase may be connected with dehydration implicated in diabetes as a result of polyuria [18]. Evidence from previous study has also shown that platelet hyperactivity could lead to an increase in MPV [17]. Pretreatment and treatment with *C. zambesicus* restored the platelet count, PDW and MPV to near normal in a similar fashion as glimepiride.

In conclusion, this study has shown that *C. zambesicus* is capable of restoring connective tissue fibres, glycogen deposit and also boost the immunity in the treated groups. We therefore, advocate for further studies to corroborate this study.

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USEFULNESS OF REAL-TIME SHEAR WAVE ELASTOGRAPHY IN THE DIAGNOSIS OF MALIGNANT THYROID NODULES IN PATIENTS WITH AUTOIMMUNE THYROIDITIS

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ABSTRACT

Thyroid disorders are common and both low and high iodine intake are responsible for a wide range of health consequences. Thyroid nodules and autoimmune thyroiditis are also associated with changes in iodine intake. Diagnosis of both diffuse thyroid disease and thyroid nodules is often challenging. Elastography, a new imaging modality, has recently emerged and has been shown to be useful in the assessment of thyroid nodules, with good inter- and intra-observer reproducibility and accuracy in detecting thyroid malignant disease.

We have analysed 19 thyroid nodules (13 benign, 6 malignant) assessed by means of conventional and Doppler ultrasound, as well as by shear wave elastography. The ultrasound and shear wave elastography studies were performed during a single procedure on the same equipment. At conventional ultrasound, we found only one feature associated with malignancy (transonic areas) was statistical significant. The colour-flow Doppler correctly detected blood flow patterns correlated with benign and malignant disease. SWE produced statistically significant results for all the four parameters analysed (mean and maximum elasticities, standard deviation in the elasticity index and the ratio between elasticities inside and outside the nodule). However, in our group, the elasticity scores did not help us discriminate between benign and malignant lesions.

SWE proves to be a helpful diagnostic tool, but further studies are needed to confirm its potential.

Keywords: shear wave elastography, thyroid nodule, thyroid cancer, autoimmune thyroiditis

INTRODUCTION

Thyroid disorders are common, particularly in areas of iodine deficiency, with high prevalence of goitre, reaching up to 80% in areas of severe iodine deficiency [1]. The health consequences of iodine deficiency also include infant mortality, as well as altered growth and development due to impaired thyroid hormone production [2] Conversely, high iodine intake may lead to autoimmune thyroid disorders, with estimated incidences of hypothyroidism and hyperthyroidism reaching 350/100,000/year and 80/100,000/year, respectively, in women, while the figures are much lower in men (80/100,000/year and 8/100,000/year) [3]. Changes in iodine intake from low to high elicit effects on thyroid function, resulting in increased prevalence of thyroiditis [4]. Thyroid dysfunctions are associated with levels of thyroid-stimulating hormone (TSH) so that high TSH levels are seen in hypothyroidism and low levels in hyperthyroidism. Low serum TSH in iodine-deficient areas leads to formation of thyroid nodules, which is a common pathology (50%-60% of healthy subjects) [5]. These nodules are either unable to synthesize hormones or, inversely, have the capacity to autonomously synthesize and release thyroid hormones independently of the TSH level. Finally, some nodules are seen in the setting of simple or multinodular goitre. Elevated serum TSH in iodine-replete areas, on the other hand, results in increased hypothyroidism prevalence due to thyroid hypofunction secondary to autoimmune thyroiditis, and inhibition of thyroid hormone synthesis and release caused by excessive iodine [4]. Patients with chronic autoimmune thyroiditis are exposed to an increased risk of overt hypothyroidism with increasing serum TSH levels above 2 mIU/L, and in the presence of both anti-thyroid microsomal and anti-thyroglobulin antibodies [6].

Diagnosis of both diffuse thyroid disease and thyroid nodules is often challenging and includes history, clinical examination, laboratory tests, fine-needle aspiration biopsy (FNAB), conventional ultrasound (US) and other imaging techniques. Assessment of various thyroid hormones and antibodies may guide further investigation in patients with

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nodular and/or diffuse thyroid disease [7]. Conventional US allows for the identification of both pathologies, while FNAB is recommended for nodules larger than 1 cm with suspicious features at ultrasound examination [8].

Elastography, a new imaging modality, has recently emerged and has been shown to be useful in the assessment of thyroid nodules, with good inter- and intra-observer reproducibility and accuracy in detecting thyroid malignant disease [9,10]. Various elastographic techniques currently used in clinical practice are able to assess tissue elasticity using a compression force to measure the degree of tissue displacement [11]. Tissue stiffness is associated with the risk of malignancy [12], although thyroid tissue is also stiffer in patients with thyroiditis. However, data reported so far suggest elastography may become a useful tool in discriminating benign from malignant nodules in the presence of autoimmune thyroid disorders [13,14].

We have analysed 19 thyroid nodules by means of conventional and Doppler ultrasound, as well as by shear wave elastography (SWE) in order to assess the diagnostic ability of the latter technique to discriminate between benign and malignant nodules in the presence of autoimmune thyroiditis.

MATERIALS AND METHODS

We conducted this preliminary retrospective study in order to evaluate the ability of SWE to correctly diagnose benign and malignant thyroid nodules in patients with autoimmune thyroiditis. We have thus extracted only those nodules found in patients with autoimmune thyroiditis as confirmed by histopathological diagnosis.

The study was conducted from October 2015 to February 2016 in the Endocrinology Department of the Victor Babes University of Medicine and Pharmacy Timisoara (UMFVBT) and the 2nd Surgery Clinic within the Pius Brinzeu County Clinical Emergency Hospital Timisoara (SCJUT). Out of the 43 thyroid nodules assessed, we extracted the 19 thyroid nodules found in 11 patients with autoimmune thyroiditis, all women, mean age 58.36 years (min. 39 years, max. 76 years). The nodules were split in two subgroups, i.e. benign (n=13) and malignant (n=16). All nodules were surgically removed in the 2nd Surgery Clinic of SCJUT, and blinded histopathologic diagnosis served both as inclusion criterion and reference standard. All patients gave their informed consent prior to inclusion in the study.

We have performed our ultrasound and shear wave elastography studies during a single procedure on the same equipment, an Aixplorer™ ultrasound system (SuperSonic Imagine, Aix-en-Provence, France), using a SC5-1 linear transducer. First, we have assessed the sonographic features of the nodules by conventional ultrasound, namely nodule number, sizes and volume, aspect of margins, echogenicity, homogeneity, halo sign, microcalcifications and transonic areas inside nodules. Afterwards, the blood flow in the nodule and the surrounding area was evaluated by means of colour-flow Doppler and classified into intranodular, perinodular or absent blood flow.

Lastly, we performed real-time SWE, with the elastographic and B-mode images simultaneously shown on two separate screens. Shear wave elastography relies on an acoustic radiation force that creates quasiplane shear waves, with the shear source being moved at supersonic speed [15]. During the procedure, we have recorded the quantitative parameters displayed by the SWE system: mean elasticity index of the nodule (SWE-mean), maximum elasticity index of the nodule (SWE-max), standard deviation in the elasticity index (SWE-SD), and the ratio between elasticity of the nodule and that of the normal surrounding thyroid tissue (SWE-ratio), all expressed in kilo-Pascals (kPa).

Statistical Analysis

We used the Statistical Package for the Social Sciences (SPSS) programme (version 17.0 for Windows, SPSS Inc., Chicago, Illinois, USA) to conduct statistical analysis. The 19 nodules were assigned to benign (13 nodules, 68.4%) or malignant (6 nodules, 31.6%) subgroups. Student's t-test was used to compare data and p-values <0.05 were considered statistically significant.

RESULTS

All patients included underwent either lobectomy or total thyroidectomy in the 2nd Surgery Clinic of SCJUT. The blinded histopathologic diagnosis was performed according to the World Health Organization guidelines [16], revealing 6 papillary carcinomas, out of which 1 was classical variant, 1 microcarcinoma and 4 - follicular variant. Among the 13 benign nodules, 2 were follicular and 1 oxyphilic adenomas, 2 hyperplasic nodules, and 8 cystic colloid nodules.

All findings at conventional and Doppler ultrasound, as well as shear wave elastography described below were compared with the histopathologic diagnosis to assess the accuracy of imaging techniques used in this study.

Conventional ultrasound is routinely used to assess thyroid nodules, being able to identify suspicious nodules that need further evaluation by means of FNAB and/or histology, as recommended by guidelines [5, 17, 18]. Several features of thyroid nodules are considered to be predictive for malignancy, such as irregular margins, hypoechogenicity, inhomogeneity, absence of hallo sign, presence of microcalcifications and transonic areas, while US features of thyroiditis can vary according to the disease phase and severity, usually presenting as an array of hypoechoic nodules and echogenic fibrous bands [13].

In our group, we had 10 patients with solitary nodules, one with 3 nodules and other 3 with 2 nodules. The average nodule volume was 2.2550 ml in the benign

subgroup and 3.0017 ml in the malignant nodules subgroup. Measurements of length, width, thickness and maximum diameter showed no statistical differences between the benign and malignant nodules. Among the US features associated with malignancy, the presence of transonic areas was the only parameter to reach statistical significance, at p = 0.033. In our series, only 7 nodules presented with irregular margins, most of which (n = 5) were diagnosed as benign conditions. Because most of the nodules (n = 16) in our group were hypoechoic, including all malignant nodules, this feature was not useful in discriminating between benign and malignant lesions. Similarly, more than half of the nodules (n = 12, out of which 5 were malignant) had a non-homogenous appearance. The halo was absent in 12 nodules (2 malignant) in our series, while 17, including all malignant, nodules, did not had microcalcifications.

Doppler ultrasound is reliably detecting the blood flow in thyroid nodules, with marked intranodular hypervascularity being a distinctive pattern of malignancy, and perinodular blood flow more often seen in benign thyroid nodules, while avascular thyroid nodules are almost always benign. In our group, 4 of the 6 malignant nodules had intravascular blood flow, 2 had perivascular blood flow, while all avascular nodules (n = 5) were benign. These results proved to be statistically significant, at a p-value of 0.043.

Shear wave elastography, a recent imaging technique demonstrating good diagnostic potential for thyroid pathologies, enables the quantitative assessment of tissue elasticity by measuring the shear wave propagation speed, owing to the high sensitivity of shear modulus (i.e., elasticity coefficient for shear force) to physiological and pathological changes in tissues under investigation.

The transducer covered with US gel was placed on patient's skin without applying tissue compression. With SWE, images are acquired within seconds and then a region of interest is placed on the frozen image to perform the measurements.

We have recorded four quantitative parameters (SWE-mean, SWE-max, SWE-SD and SWE-ratio), the mean values of which are shown in Table 1 for both subgroups, with statistically significant (p-value <0.05) higher values recorded in the malignant nodules subgroup, consistent with reports in literature. Nevertheless, elastographic findings in diffuse thyroid diseases, such as autoimmune thyroiditis, indicate higher tissue stiffness as well, correlated with the severity of disease [19]. Despite this, we found that tissue stiffness in malignant nodules in patients with concurrent autoimmune thyroiditis is significantly higher for all SWE parameters, with great differences between the highest values recorded in the two subgroups, as follows: SWE mean 59.1 kPa in a malignant nodule and 33.2 kPa in a benign one; SWE max 96.06 kPa/46.5 kPa; SWE SD 14.4 kPa/4.9 kPa; SWE ratio 2.03 kPa and 1.53 kPa, respectively.

Table I. Mean values of SWE parameters in the two subgroups

Parameter		Mean value	Standard Deviation	p-value
SWE mean	benign	19.33 kPa	9.09	0.005
	malignant	28.41 kPa	15.52	
SWE max	benign	36.11 kPa	10.71	0.010
	malignant	56.65 kPa	27.43	
SWE SD	benign	5.92 kPa	3.18	0.020
	malignant	9.42 kPa	5.31	
SWE ratio	benign	0.93 kPa	0.38	0.010
	malignant	1.63 kPa	0.56	

Tissue elasticity can also be assessed qualitatively, the elastographic image showing a range of colours, from blue for maximum tissue elasticity to dark red for marked stiffness. We have assigned scores from 1 to 5 according to Ueno scale [20], where score 1 is assigned to normal tissue elasticity and score 5 to stiffness in the entire nodule and the surrounding area. In our study, the results were not statistically significant, as almost all nodules had elasticity scores of 2, with only one elasticity score of 3 recorded on a malignant nodule.

The US and SWE images of a benign and a malignant nodule in our study group are shown in Figures 1 and 2, revealing differences in appearance and particularly in the recorded SWE parameters.

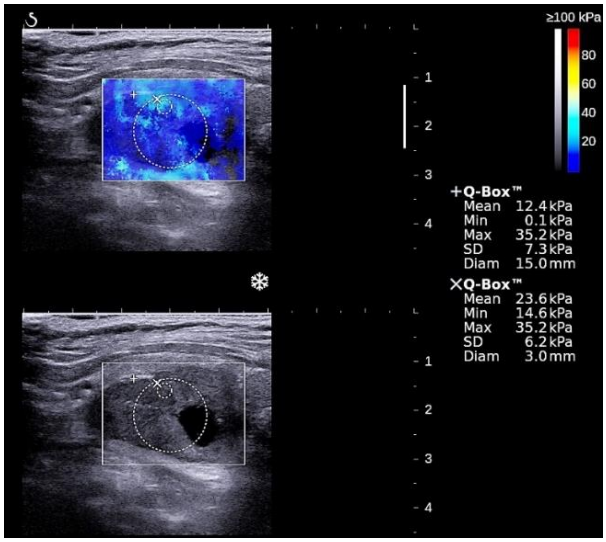

Fig. 1. SWE and US images of a benign nodule**.** A 53-year old woman with a follicular adenoma and chronic autoimmune thyroiditis, ES 2 on SWE. US shows a homogeneous hypoechoic nodule, with regular margins, no microcalcifications, absence of halo sign and cystic content.

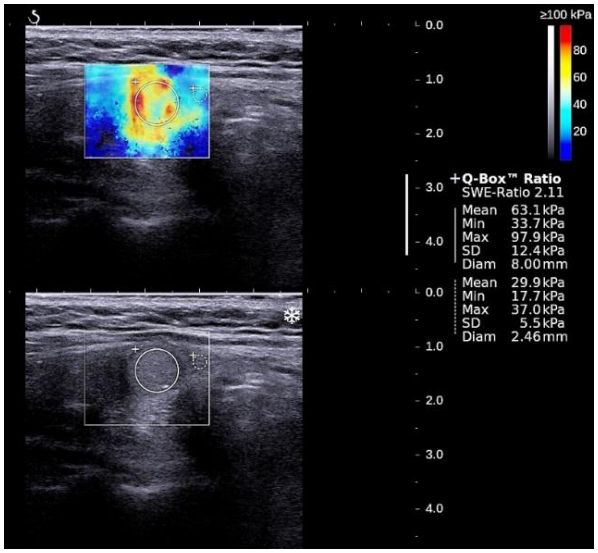

Fig. 2. SWE and US images of a malignant nodule. A 57-year-old woman with papillary carcinoma, follicular variant, and chronic autoimmune thyroiditis, ES 3 on SWE. US shows a nonhomogeneous hypoechoic nodule, taller than wide, with an incomplete hypoechoic halo and an area of microcalcification.

DISCUSSIONS

Conventional ultrasound of thyroid nodules is useful in identifying suspicious masses, but further investigation is needed to confirm the diagnosis, particularly in patients with concurrent autoimmune thyroiditis, as many of the features considered to be predictive of malignancy are also seen in a large number of benign nodules. On the other hand, Bonavita *et al*. [21] identified four US patterns associated with benign nodules: spongiform configuration, cyst with colloid clot, giraffe pattern, and diffuse hyperechogenicity. The US findings in the setting of thyroiditis typically shows enlarged thyroid gland with one of the following patterns: several diffuse small hypoechoic nodules interspersed with echogenic bands; round hyperechoic areas surrounded by linear hypoechoic areas; or a hyperechoic nodule [19].**Error! Bookmark not defined.** Therefore, the hypoechoic pattern in our group was not useful in discriminating between between benign and malignant lesions, as hypoechogenicity is not only a distinctive US feature for malignancy, but is also characteristic to thyroiditis as a result of marked inflammatory process following infiltration of thyroid with lymphocytes and plasma cells [22,23]. The non-homogenous appearance is also a common finding in nodular or adenomatous goiters and a typical aspect of thyroid parenchyma seen in patients with thyroiditis [21,24]. Absence of a hypoechoic rim around the nodule is due to lymphocytic infiltration, development of fibrous connective tissue and compression of thyroid parenchyma [25], and this may explain the absence of halo sign in both cancerous and benign nodules in our group. It is therefore not surprising that a halo was absent in 12 nodules (2 malignant) in our series. Similarly, microcalcifications, which are considered a hallmark of thyroid malignancy, are also found in autoimmune thyroiditis and follicular adenomas [26]. In our study, we only found two nodules with microcalcifications.

Marked intranodular blood flow identified at colour-flow Doppler is considered a distinctive pattern of malignancy, although it is also present in more than half of benign solid thyroid nodules [27]. Perinodular blood flow is more often seen in benign thyroid nodules, but is not uncommon in malignant thyroid conditions [28], while avascular thyroid nodules are almost always benign. The patterns of blood flow seen in our study allowed for the fair discrimination between benign and malignant nodules.

SWE has emerged as an imaging technique with great potential in the diagnosis of thyroid diseases and provides both qualitative and quantitative data regarding tissue elasticity. Various studies in literature [9,29-31] have proved the value of SWE in diagnosing thyroid malignancies, while others [13,14,24] have shown it can be useful in detecting thyroid cancer even in the setting of autoimmune thyroiditis, which is also characterized by an increased tissue stiffness. Though, the higher stiffness due to autoimmune thyroiditis seems not to tackle the ability of SWE to differentiate between benign and malignant conditions, as values of SWE parameters in autoimmune thyroiditis appear to be closer to those recorded in benign thyroid lesions. Szczepanek-Parulska *et al*.[32], for instance, compared the stiffness of benign thyroid nodules in patients with and without autoimmune thyroiditis and found no significant differences between groups. Nodules in our study were all found in patients with autoimmune thyroiditis, so that a comparison with benign lesions in absence of thyroiditis is not possible. However, we have shown that malignant nodules exhibit significantly higher stiffness and that concurrent thyroiditis is not affecting the results. SWE allowed for the correct diagnosis of nodules and, more important, was able to detect all malignancies. Conversely, US produced significant results for only one parameter, while colour-flow Doppler added in the characterisation of blood flow within nodules and the surrounding region, confirming the presence of vascularity inside malignant nodules. A limitation of our study is the small number of nodules included. Because of this, we could not establish an optimal cut-off, nor sensitivities and specificities.

CONCLUSIONS

Shear wave elastography is consistently proving its diagnostic value in thyroid disorders, being able to identify malignancies with high accuracy. Being a recent technique, studies are still needed to confirm its precision. For now, it is being used as an adjunct to conventional ultrasound, aiding in patient guidance to FNAB or surgery. If its value and diagnostic potential will be confirmed in the future, SWE will

help reduce the number of unnecessary FNAB procedures and surgeries, to the best benefit and care of patient.

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RISK FACTORS ASSOCIATED WITH CHRONIC VENOUS INSUFFICIENCY IN CENTRAL INDIA

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ABSTRACT

Chronic venous insufficiency is not only an individual problem but also a national problem as it had increased economic burden due to recurrence, treatment, disability and decreasing quality of life. Incidence of chronic venous insufficiency varies in different populations ranges from 10% to 65% which may be due to varied risk factors affecting diverse population. We want to find risk factor in central India rural population who lack awareness of this condition. A case control study was done, on 57 cases and 29 age-matched healthy controls. Various risk factors were studied – sex, age, height, weight, parity, history of prolong standing, prolong sitting, any family history, constipation, OCP, Post thrombotic and any prior invasive surgery of vein, smoking, cancer, polycythemia, hypertension and diabetes. Factors like prolong standing, prolong sitting, family history, prior invasive venous surgery, post thrombotic was higher in cases than controls which was found to be statistically significant ($P<0.05$).
Keywords: varicose veins, risk factors, chronic venous insufficiency

INTRODUCTION

Venous disorders of the legs occur frequently which range in severity from minor asymptomatic incompetence of venous valves to chronic leg ulceration. Varicose veins are a common manifestation of venous incompetence in the lower limb, and they appear as dilated, elongated, tortuous, pouched, thickened, inelastic and friable veins which have permanently lost its valvular efficiency. Incompetence of the deep, superficial and/or perforating veins leads to raised venous pressure in the lower leg, which can result in skin changes such as hyperpigmentation and indurations with eventual ulceration. These changes in the skin and subcutaneous tissues of the lower leg are often referred to clinically as chronic venous insufficiency (CVI) (13).

Incidence of varicose veins and their complication is not only an individual, but also a national problem of great importance especially in western countries due to its high prevalence, cost of investigations and treatment, and loss of working days (1,20). Varicose veins are present in 25% to 33% of female and 10% to 20% of male adults. (2,3,8.9,14,25,28,38,39,42,44) Prevalence estimates vary widely by geographic location, with the highest reported rates in Western countries. . Data from the Brazilian Security System show that CVI is the 14th most-frequently quoted disease leading to temporary work absenteeism and the 32nd most frequent reason for permanent disability and public financial assistance.(11) The annual cost of venous ulcers has been estimated to be £400 to 600 million for the United Kingdom (6,16) and >$1billion for the United States (15). The total direct and indirect cost of CVI to society is likely to be $1 billion US dollars in each of 3 European countries (the United kingdom, France, and Germany) (21, 22). The reported ranges in prevalence estimations presumably reflect differences in the population distribution of risk factors, accuracy in application of diagnostic criteria, the quality and availability of medical diagnostic resources.

Wardha is District in Maharashtra which lies in central India. The district has 6,309 sq km and population of 1,300,774 out of which 67.46 % live in rural areas according to the 2011 Census. The percentage of population living in rural Wardha is far greater than in the state as a whole. If not treated then, nearly 50% of patients of superficial venous insufficiency will eventually experience chronic venous insufficiency which is characterized by swelling of lower extremity, pigmentation, hemorrhage, eczema and ulceration (43). Chronic venous insufficiency (CVI) can be regarded as the common clinical endpoint of various venous diseases.

Till now, no study has been conducted to see the severity of the problem. People are not aware of the hazards of prolong standing. Chronic venous insufficiency, like a glacier, moves slowly but forcefully over time. It has enormous clinical and socioeconomic consequences,

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because the severity and chronicity of the CVI associated signs and symptoms can profoundly interfere with the patients' quality of life and work capacity on the one side, and the financial resources of the health care system on the other(12,31,32,41).

Scarcity of information regarding health and safety of rural population provides strong rationale for rural health professionals to initiate research and educational programs focusing on health promotion/safety needs of rural population. Our study aims to find the risk factors for chronic venous insufficiency in central India.

METHOD AND MATERIAL

A case control study was done. Clearance from Institutional review board was obtained. Informed written consent was taken from the patient. 57 patients were enrolled for the study. Patients attending Acharya Vinoba Bhave Rural Hospital for management of Chronic Venous insufficiency were subjected to standard questionnaire. The following data was collected: age, height, weight, parity, history of prolong standing, prolong sitting, any family history, constipation, OCP, Post thrombotic and any prior invasive surgery of vein, , smoking, cancer, polycythemia, hypertension and diabetes.

Clinical examination of leg was done in following heads -Venous dilatation, edema, Skin pigmentation and venous ulceration. A classification and grading of chronic venous disease was done according to the American Venous Forum is **CEAP** classification. It was followed by duplex scanning examination with colour Doppler unit. All tests were performed in morning hours between 0900Hrs to 1200Hrs. The diagnosis of varicose veins was done by combination of clinical and duplex ultrasonographic examination. All patients in the study had normal arterial examination on duplex scanning. Inclusion criteria were varicose veins of grade C3-C6. Exclusion criteria were Occlusive arterial diseases of leg, obliterative arteriosclerosis, atherosclerosis of leg veins; varicose veins grade C1, C2 .Twenty nine normal, healthy age matched volunteer were selected as controls. They had no sign or symptom of venous or arterial diseases in any limb. Colour Doppler findings were normal.

Statistical analysis

All parameters in the study were assumed nonparametric as the control group has less than 30 patients and nonparametric tests was used for statistical analyses. Comparison between groups was tested by Mann-Whitney U at 95% confidence interval (CI; α = 0, 05 level). All analyses were performed using SPSS 16.0

RESULTS

Table I and II shows Classification of chronic venous disease.

Table I. Classification of chronic venous disease (Porter and Moneta 1995) Mark Definition

C	Clinical signs (grade 0-6), supplemented by(S) for symptomatic and (A) for asymptomatic presentation
E	Etiologic Classification (Congenital, Primary, Secondary)
A	Anatomic distribution (Superficial, Deep, or Perforator, alone or in combination)
P	Pathophysiologic dysfunction (reflux or obstruction, alone or in combination)

Table II. CEAP Clinical classification of chronic lower extremity venous disease (Porter and Moneta 1995)

Class	Clinical signs
C0	No visible or palpable signs of venous disease
C1	Superficial spider veins, Telangiectasias, reticular veins, malleolar flare
C2	Simple varicose veins only
C3	Ankle edema of venous origin (not foot edema)
C4	Skin changes ascribed to venous disease (pigmentation, venous eczema, Lipodermatosclerosis)
C5	Skin changes (as defined above) in conjunction with healed ulceration
C6	Skin changes (as defined above) in conjunction with active ulceration

Table III shows clinical data of cases and control group. Mean age of the cases and control was 43.9 $\pm$ 12.2 yrs and 42.94 $\pm$ 13.6yrs respectively. There was no statistical difference between ages of two groups. The sex ratio (M: F) in cases and control was 4.7:1 and 1.9:1. BMI, Height, OCP (in females), smokers, hypertensive, diabetics and Constipation history in cases was greater than control group but not statistically significant (P>0.05). Other factors like Prolong Standing, Prolong Sitting, Family History, Prior surgery, Post thrombotic was higher in cases than controls which was found statistically significant (P<0.05).

Table III. Demographic and clinical data of cases and control

Sr no	Parameters	Cases	Controls	P Value
1	Number of patients n	57	29	-
2	Age (years; mean ± SD)	43.9 ±12	42.94 ± 13.60	0.56
3	Sex ratio (Male: Females)	47/10	19/10	-
4	BMI (kg/m2,mean ± SD)	21.31 ± 3.608	20.96 ± 4.5	0.75
5	Height (m, mean ± SD)	1.68±0.09	1.65 ± 0.06	0.66
6	Parity (birth no >1) (%)	10 (100%)	10 (100%)	-
7	**Prolong Standing n, (%)**	**42 (73.68%)**	**6 (20.68%)**	**0.01**
8	**Prolong Sitting n, (%)**	**10 (17.54%)**	**2(6.89%)**	**0.01**
9	**Family History (%)**	**28 (49.12%)**	**4 (13.79%)**	**0.05**
10	OCP (in females)	3 (30%)	2 (20%)	0.2
11	Constipation	8 (14.03%)	6 (20.68%)	0.59
12	**Prior surgery**	**10 (17.54%)**	**1 (3.44%)**	**0.05**
13	**Post thrombotic**	**7 (12.28%)**	**0 (0%)**	**0.01**
14	Smoking history	18	15	0.82
15	Hypertension	27	15	0.67
16	Diabetes	15	9	0.74

Table IV shows age and Gender wise distribution of cases and Table V shows distribution of cases according to occupation. CEAP classification of chronic venous insufficiency was shown in Table VI.

Table IV. Age wise and Gender wise distribution of cases

Age Group (yrs)	Male	Female	Total
11-20	1(1.75%)	1(1.75%)	2(3.51%)
21-30	7(12.2%)	1(1.75%)	8(14.04%)
31-40	10(17.54%)	1(1.75%)	11(19.30%)
41-50	13(22.81%)	5(8.77%)	18(31.58%)
>50	16(28.07%)	2(3.51%)	18(31.58%)
Total	47(82.46%)	10(17.54%)	57(100%)

Table V. Distribution of cases according to occupation

Occupation	No.of patients	Percentage (%)
Prolonged standing	42	73.68421
Prolonged sitting	10	17.54386
Weight pullers	5	8.77193
Total	57	100.00

Table VI. CEAP Classification of patients

SR NO	CEAP	Cases		Control	
		Males	Females	Males	Females
1	C0	-	-	-	-
2	C1	-	-	-	-
3	C2	-	-	-	-
4	C3	10 (21.27%)	3 (30%)	-	-
5	C4	12 (25.53%)	3 (30%)	-	-
6	C5	13(27.65%)	2 (20%)	-	-
7	C6	12 (25.53%)	2 (20%)		

DISCUSSION

Incidence of chronic venous insufficiency varies in different populations ranges from 10% to 65% which may be due to varied risk factors affecting diverse population. We had tried to find risk factor in central India rural population who lack awareness of this condition.

The sex ratio in the cases was male: female 4.7:1. We may interpret that chronic venous insufficiency is more

common in males as compared to female in rural Wardha population of central India. It might be due to; firstly males are occupied more in occupation requiring prolonged standing. Secondly women bother less about the medical problem and do not seek medical advice till the condition becomes severe and unbearable. Being a rural area female consume less oral contraceptive pills and so are at less risk of deep venous thrombosis and Chronic venous insufficiency. These findings are in accordance with Nobl (27) 1910 and Nicholson (26) 1927 who found male predominance. Stanhope J M (36) 1975 showed modest prevalence in men and very low prevalence in women. Most authors (Briscoe G 1918, Gibson 1937, Hooker 1914, Leconte 1922, Lyons 1922, Villaret 1921, Winsor 1946) found lower values for women as cited by Ochsner A (29) (1951). Callman M J (7) cited following table showing female predominance in the prevalence of varicose veins and the male: female ratio was between 1: 0.6 to 1: 4.0 depending upon the definition used.

Reference	Year	Sex Ratio M:F
Nobl	1910	1:0.6
Nicholson	1927	1:0.6
Widmer	1978	1:1.0
Brand et al	1988	1:1.3
De Takats	1929	1:1.5
Dodd and Cockett	1956	1:1.5
Prior et al	1970	1:1.7
Lartson and smith	1943	1:2.0
Pratt	1950	1:2.0
Dodd	1964	1:2.0
Drury	1965	1:2.0
Lake et al	1942	1:2.0
Babek et al	1962	1:2.1
Arnoldi	1958	1:2.1
Lofgren	1966	1:2.1
Logan and Cushion	1958	1:2.5
Logan and Brooke	1957	1:2.6
Harding Le Riche et al	1962	1:2.7
Abramson	1981	1:2.8
Danish National Morbidity Survey	1954	1:2.9
Haakenaasen	1963	1:3.0
Dick	1966	1:3.5
Weddell	1969	1:3.5
Payne	1936	1:4.0
Phillips	1963	1:4.0
US National Health Survey	1938	1:4.0

According to Callman M J (7) (1994), use of a wide variety of sampling techniques, definition of varicose veins (VV) and regardless of whether they were looking at incidence, point or period prevalence, various studies have shown a female predominance with a female: male ratio up to 4: 1. Two factors may account at least in part for the wide variation in results. First, the studies that gave a very high female predominance were almost exclusively questionnaire based and this method tends to be less accurate Dale JJ 1983 (**10)**, Weddell JM 1969 (42). Second, the data from Bernlsens (5) 1927 and the Basle study by Widmer LK (44) 1978 among others, suggest that the female: male ratio increases with age. The varying ages of the samples in different studies may have contributed to the wide range of results.

In our study, 18 (31.58%) cases were from the age group 41 to 50yrs and 18 (31.58%) cases were in the age group of >51. Mean age of the cases was 43.9 yrs $\pm$ 12.2 yrs. The present study shows that chronic venous insufficiency is more common in more than 40 yrs of age in population of rural Wardha. Our finding are in accordance with Mekky S et al (25) in 1969, Beaglehole R (4) in 1986, Staffa R (35) 2002 and Widmer LK (44) 1978 who found that varicose veins increase with increasing age. Data from other studies (Abramson JH (12) 1978, Coon W (8) W1973, US Department, Hirai M, Lorenzi G) Show similar trends, although the absolute percentages varied, because different definition of varicose veins and population samples was used as cited by Callman M J in 1994.

The cause for increase varicosity with increase age can be explained on the basis of Structural alterations of the venous wall. There is reduction of smooth muscle fibers of the venous wall and qualitative and quantitative alterations in the wall of the varicose vein. Fibers are deformed with reduced collagen and disorderly disposed, with an excess of 'proteoglycans.' In addition, there is an increase in all the activities of lysosomal enzymes (hyaluronidases, glucosaminidases, and phosphatases) Sciannameo (33) 1993. According to Silveira (34) 1993, varicose saphenous veins presents significant structural modifications in its wall, occurring, in addition to a greater intimal thickening, deep modifications in the structure of the tunica media, with interposition of elastic fibers to smooth muscle clusters, consequently altering the resistance of the damaged venous wall. The loss of elasticity in the venous wall leads to age-related increases of venous diameter in the venous wall and may predispose for varicose veins (17).

BMI, Height, OCP (in females), history of constipation, hypertension, diabetics and smoking, in cases was greater than control group but not statistically significant ($p>0.05$). Other factors like prolong standing, prolong sitting, family history, prior venous surgery, post thrombotic was higher in cases than controls which was found statistically significant ($P<0.05$).

In our study, out of 57 patients, 46 were occupied in occupation requiring prolonged standing. This study

elucidates that, patients whose occupation involves prolonged standing, are at greater risk for developing chronic venous insufficiency. These findings are in line with Lake (19) et al 1942 and Kontosic (23) 2000 study showing role of posture in causing varicose veins. According to Stanhope J M[100] in 1975 sitting habits may be important in the etiology of varicose veins. According to Malhotra S L (24) 1972 and Beaglehole (3) 1975 and Weddell J M (35) 1969 posture does not appear to contribute to the causation of varicose veins. Weddell J M[5] postulated men who were involved in heavy lifting work did have a significant influence. Following table shows the association between occupation and varicose veins, as cited by Callman M J (7).

Reference	Year	Occupation	Cause
POSITIVE ASSOCIATION			
Mekky et al	1969	Cotton workers	Standing
Weddell	1969	Community samples	Heavy lifting
Abramson	1981	Community samples	Standing
Lorenzi et al	1986	Metal workers	Standing
NO ASSOCIATION			
Guberan et al	1973	Store employee	Standing
Maffei	1986	Out patients	Standing
Weddell	1969	Community sample	Standing

These finding can be explained as venous pressure in the healthy human leg is determined by the rate of arterial inflow and venous outflow. In supine position, venous pressure in foot vein is 10 mmHg and about 90 mm Hg approximately in standing position which is equal to the pressure of a column of blood extending vertically from that vein to the level of the heart Christian Kügler. With walking, lower extremity venous pressure is reduced from approximately 100 mm Hg (depending on height) to a mean of 22 mm Hg within 7 to 12 steps (44).

The regular contraction and relaxation of the deep leg muscles is largely responsible for the upward blood flow. Prolonged standing, on the other hand, is not a natural habit in man, and it certainly hampers the blood flow in the lower half of the body A. M. Stewart (37). The biological basis for the standing hypothesis is, the muscle pump is less utilized in people who stand continuously during work. The blood is not pumped towards heart, the venous pressure remains constantly raised. The veins became locally dilated distal to a valve and that there is media hypertrophy, which later progresses to atrophy and sacculation (King (18) 1950). Disorganization of the circular muscle in the media occurs, and that the collagen contents decreases and the water content increases (Prerovsky et al. (20) 1962).

Veins lose their elasticity and its diameter increases to accommodate the increased venous volume. Due to which the venous valve does not approximate and become functionless in long run. They can no longer prevent the back flow of the blood in the leg. Once the venous valves are incompetent, then even walking may actually increase venous pressure in the lower extremities because of a reversal in blood flow. Increase in leg veins blood volume and increase in the hydrostatic pressure produce high pressure zone in deep veins. This high pressure then damages the perforator valves and leaks into the superficial veins. Impeded blood flow leads to stasis in veins of the lower extremities. Stasis in the venous system is a key mechanism in venous vascular disease. Stasis increases the risk for coagulation and thrombus formation. (Finn Tuchsen (40) 2000).

People occupied in occupation which requires prolonged standing should be advised to walking at work and alternate with other positions such as sitting, preferably with the legs in an elevated position. They should do "vajrasan" which helps to make calf muscle stronger and use compression stockings as a preventive measure.

CONCLUSION

Our case control study of small sample size found the following risk factors were associated with chronic venous insufficiency: male sex, increasing age, prolong standing, prolong sitting, family history, prior venous surgery and post thrombotic conditions. Further studies with larger sample size and cohort study may show significance of parameter which we found to be insignificant in our study like parity, OCP and constipation.

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Emotional regulation of children diagnosed with Autistic Spectrum Disorder

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Abstract: Emotional regulation refers to the evaluation and appropriate modulation of emotional responses according to the social context or for achieving a goal. Emotional regulation is a spontaneous process which starts from an early age and is influenced by family interactions, observational learning and modeling and represents one of the pillars of social skills. A deficiency in ER could explain the emotional and behavioral problems in ASD, as well as the impaired social functioning of these individuals. This article tries to point out the particularities of ER in ASD and underline the need for further research in this area, as well as for the development of therapeutic programs focusing on improving ER.

Abstract: Reglarea emotionala se refera la evaluarea si modularea adecvata a raspunsurilor emotionale in functie de contextul social sau pentru atingerea unui scop. Reglarea emotionala este un proces spontan care incepe de la varsta mica si este influentat de interactiunile familiale, invatarea observationala si sta la baza interactiunii sociale. Un deficit al reglarii emotionale ar putea explica problemele emotionale si comportamentale din TSA, precum si deficitul in interactiunea sociala. Articolul de fata incearca sa sublinieze particularitatile reglarii emotionale in TSA si importanta continuarii studiilor in acest domeniu, precum si dezvoltarea unor programe de terapie bazate pe imbunatatirea RE.

Key-words: emotional regulation, autism, alexithymia, facial processing

Key-words: reglare emotionala, autism, alexitimie, procesare faciala

INTRODUCTION

The Autistic Spectrum Disorder (ASD) diagnosis criteria include marked difficulties in social interaction, using non-verbal behaviors and the presence of repetitive and stereotype behaviors [1]. Children with ASD have frequently been described as having difficulties in all emotional aspects; deficiencies in understanding and expression of emotions, in decoding facial expressions, and appropriate management of emotions. [2] There is currently a growing interest in emotional regulation (RE) literature in TSA due to behavioral consequences that affect quality of life and require appropriate therapeutic measures [3].

Emotional regulation (ER) refers to the complex and dynamic processes involved in evaluating and appropriately modulating emotional responses according to the social context or for achieving a goal [4]. ER is a multifactorial process with biological, cognitive, behavioral and observational components [2]. In ASD, emotional regulation could explain some of the behavioral and emotional issues that affect functioning in all areas of child activity.

Studies have shown that there is a lack of emotional regulation in children with ASD, which leads to increased use of psychiatric services [5]. 74% of children with ASD have difficulty of ER compared to only 18% in children with typical development and these deficits occur early and could be observed starting with the age of 12 months [6]. Emotional dysregulation is associated with major difficulties especially in school environment, and often children with ASD fail to graduate school or experience frequent class changes due to atypical RE strategies [7]. At the age of adolescence these difficulties are associated with depression, anxiety and behavioral problems. Depression has recently been recognized as an important contributor to suicidal thoughts experienced by adults diagnosed with ADS [2].

Currently, efforts are made for trying to conceptualize poor ER specific to ASD. Because these deficits are not found in the diagnostic criteria, there is a growing tendency to diagnose an associated condition in TSA that encompasses consecutive behavioral manifestations caused by the deficit of ER.

Thus, emotional dysregulation in ASD could no longer be considered as a mark of ADS. In support of this statement come the literature that shows that emotion dysregulation is not always present in ASD [3].

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In addition, there is evidence of a possible biological vulnerability that affects ER in ASD, including the variability of cardiac frequency and neuronal activity. [3]

Neural mechanisms involved in ER

Studies show that in ASD there is an up-regulative failure in the brain regions involved in adaptive RE strategies such as cognitive re-evaluation that involves changing cognition about a situation. ER activates areas of prefrontal cortex (ventrolateral, medial and dorsolateral) involved in cognitive control and subcortical areas involved in arousal and motivation (Critchley et al, 2007). The prefrontal cortex modulates the activity of the limbic regions that alter the experiential, behavioral and neurobiological aspects of emotional responses. Connectivity abnormalities between these areas have been described in ASD, some studies showing that there is a hypo-connectivity, while others have described cerebral hyper-connectivity [3][8]. It is currently believed that in ASD there is a hyper-connectivity of brain areas that are close to each other and a decrease in connectivity in long brain circuits that ensure large-scale integration [9]

But there are other brain structures involved in modulation of ER from ASD. Recent neuro-imaging studies have shown the insulae involvement in experimenting and anticipating the negative consequences and striated cortex in the rewards component of RE and the abnormal impairment or functioning of these brain areas in ASD. [5]

Behavioral Strategies for ER

Children with typical development use adaptive strategies for ER: goal-oriented behaviors, social support, cognitive re-evaluation. Cognitive revaluation involves reassigning the meaning of a stimulus to changing the trajectory of the emotional response [2]. Adaptive strategies are context-dependent and selectively applied depending on the situation. In contrast, maladaptive tends to be universally applied. Compared to TD, ASDs use maladaptive strategies due to cognitive inflexibility, deficit in perspective taking, and difficulty in modulating behaviors [5].

Individuals with ASD use multiple maladaptive strategies that are associated with a wide range of adverse consequences such as impairment of social functioning and the more frequent occurrence of symptoms of anxiety and depression [3]. Because they fail to properly manage their emotions, they often act impulsively to emotional stimuli through crises of anger, self-harm and aggression, and these behaviors are interpreted as deliberate and defiant. Pre-school children with ASD often use simpler tactics such as self-soothing or proximity-seeking, and do not resort to complex strategies involving cognitive and attention-based processes such as attention diversion or substitutive play. [10]

Difficulties in affection sharing , limited understanding of emotional messages, reduced eye-to-eye communication, and immature theory-of-mind abilities, characteristic of preschoolers with ASD, further complicate their ability to regulate social stress. [10]

Maladaptive strategies used in ASD: ¼ of restrictive and stereotype behaviors appear in response to emotional triggering as a ER trial; this strategy can be effective in the short term but maladaptive over time. Studies that examined ER strategies and their effectiveness in ASD for 3 different emotions (anger, anxiety, and amusement) showed that those with ASD experienced more negative emotions and less fun. Expression of anger and anxiety through tantrum crises, accompanied by low levels of positive emotions, most affects the daily functioning and well-being of children with ASD [12]. Other maladaptive behaviors used are avoidance, suppression of emotions, self-talk, remaining focused on the stressor which are often ineffective in lowering negative activation [2,11]. Studies have shown that in ASD there is a trial-error approach because children with ASD aged 3-10 years use a wider range of strategies than TD [13].

In ASD, the ER adaptive strategies are used less and repetitive behaviors occur more frequently. These behaviors can be used to control the environment and deal with overwhelming emotions, but could not explain the presence of behavioral speculations in general. However, they can be effective in helping the child with ASD to cope with undesirable emotions and could be a way of accepted by the RE when they are not potentially dangerous for the child or others and the cognitive modalities of ER could not be accessed. Behavioral stereotypes may, however, also reflect a state of distress, so caution is recommended in interpreting them in the conducted studies [5].

Among the possible reasons why preschoolers with TSA have difficulty with RE, there may be an increased frequency of anxiety and irritability, abnormal brain response, difficulty in understanding the mental status of others [2].

Negative emotions and Anxiety in ASD

Impaired ER has been associated with several disorders including anxiety and mood disorders. Another possible explanation for the ER deficiency in ASD is the presence of these associated conditions that may be responsible for these emotional difficulties. On the other hand, the DE deficiency can also be intrinsic to the underlying pervasive disorder and then it becomes difficult to identify the source of consecutive behavioral problems [3].

The tripartite model of anxiety and depression claims that general distress is a common factor but that the physiological hyper-arousal is specific to anxiety and anhedony is specific to depression. In ASD we encounter increased levels of irritability since small ages and hyper-arousal physiology in response to emotional distress. These findings explain the excess presence of ASD anxiety and the efficacy of arousal management therapy. [5]

Increased levels of anxiety, anger, and negative reactions such as tantrum and aggression crises are common in children and adolescents with ASD and are a risk factor for the development of other psychiatric disorders. Moreover, increased negative emotional affects social functioning leading to difficulties in interpreting clues social and consequent inappropriate behavior in these situations [3]. In order to objectively determine the symptoms of anxiety in children, we used measurements of the physiological response in response to stress and especially the autonomic nervous system response: heart rate, electrodermal activity and body temperature. In the studies performed, children with ASD had an increased response to electrodermal activity (dampened EDA response to faces as compared to objects (Kushki et al, 2013).

Recognizing emotions in facial expressions

Developing ER skills at young ages is a spontaneous process, due to family interactions, observational learning, modeling, and social reference. Social deficiencies in the ASD and especially the reduced preference for human faces and the difficulties in recognizing emotions interfere with the learning process through observation and social experience. [14]

Neuroimaging and behavioral evidences indicate to the fact that people with ASD show important anomalies in the facial processing. These differences are often explained as a result of a innate deficiency of neural circuits or as a secondary consequence of low social interest levels.

Literature reviews that analyze typical and atypical facial processing show that this represents a developmental ability which is mainly mediated by early experiences. People with ASD may experience cerebral defficiencies which hinder their ability to atribute statuses to faces and expressions, therefore narrowing down the number of visual stimuli and limiting the development of specialized neural areas for facial processing. Recognizing facial emotions represents the base of emotion regulation because these offer us information regarding the mental and emotional state of others. The difficulties in facial emotion recognition are heavily associated with ASD, although there are contradictions of study results.

Eye-tracking and electrophysiology studies show that from early childhood people with ASD experience a diminishing interest for faces and a lack of social motivation. [15] These features are maintained by avoiding eye contact which can occur as early as 6 months. [16] At the age of 15 months, children with ASD focus more on the mouth region of the face, which negatively impacts face expressions recognition and causes a loss of social stimuli. Therefore, there is established an early deficient base of ER development and social interaction.

Studies that analyzed the deficit of emotion recognition in individuals with ASD show a great variability with results between 5-70%. These differences made the authors conclude that there can't be said that emotion recognition deficit is a universal trait of ASD phenotype. [17]

A 43 transversal studies meta-analysis of facial emotion recognition (FER) in people with ASD have shown that a common difficulty is especially indentifying fear and anger and other subtle emotions expressions. These studies say that a lack of facial emotion recognition in different social contexts contribute to the social communication deficit. FER is one of the main problems in ASD and represent a main target in therapeutic intervention. Improvement of FER through therapy leads to an improvement in social interaction. [18]

It is still unclear if FER deficit is applicable for the whole emotional range or is limited to a division of basic emotions, or just for complex or subtle emotions. The processes responsible for FER variability are not yet well known. [19]

Furthermore, children with ASD also have difficulties in adequately expressing emotions. Even if they have a rich emotional experience, often times their emotional expression is inadequate to the context or being described as "plain".

Alexithymia has been proposed as a factor which can explain the FER difficulties and inadequate emotion expression, met in certain children with ASD. The main characteristics of alexithymia consist in difficulties in identifying and describing emotions, which affects the ability of understanding the emotional state of oneself and expressing an emotional adequate response in a situation. It is associated with social-affective deficits as well as lack of empathy. Studies have proven that alexithymia is more frequently met in children with ASD compared to typical children. Moreover, the facial expressions of those with high levels of alexithymia are much more limited. Therefore, the overlap of alexthymia and neurodevelopmental disorders could explain difficulties in identifying and expressing emotions. [20]

ASD individuals also experience a deficiency in the theory of mind (ToM). TOM refers to the ability to determine the internal status of others, their thoughts, beliefs and motivation of the persons we came in contact with, all these with the purpose of foreseeing their behavior. Studies have shown that from a very early age, children react depending on the emotions of others. Once a child understands that facial expression reveals something about the internal status of a person, this knowledge represents the pillar of "mind-reading" development, which reflects in the ability to attribute a mental status to other people. ToM underlines the importance of emotional information and therefore the attention for facial expressions is being stimulated. Baron-Cohen et al, 1986) A deficiency in ToM development contributes to the difficulties regarding emotional expression and regulation [21]

Although emotional regulation (ER) is not included in ASD criteria, it is frequently observed in this pathology. ER

studies are still in an early stage, but it is important that the mechanisms of ER deficiency in ASD be elucidated. Emotional and behavioral problems in ASD have been associated with a low level of independence and a severe deficit in self-care as an adult. [3] Studies observing the behavior in emotional contexts are rare in ASD and the authors pointed out the necessity of further research in ER behaviors in these children for pinpointing the social stressing moments. The development of therapeutic programs focusing on stimulation of using adaptive strategies are bound to increase the coping in stressful situations while also optimizing long term results for young people with ASD.

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