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The Association between Human Leukocyte Antigen-DRB1 and Vitiligo

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Abstract

Human leukocyte antigen (HLA) as part of the immune system has a role in the disease process. Genetic factors play an important role in susceptibility to vitiligo. Our aim in this study is to assess the relationship between HLA-DRB1 alleles frequency in Iraqi patients with vitiligo compared with a healthy control group using the PCR-SSOP method. The patient group consisted of forty Iraqi Arab Muslims patients with vitiligo that consulted the dermatological department in Al-Kindy teaching hospital from September 2013 to June 2015 were assessed for HLA genotyping for HLA-DRB1. A control group consisted of thirty healthy volunteers among the staff of AL-Kindy

College of medicine that did not have vitiligo or family history of vitiligo. HLA genotyping for HLA-DRB1 was performed for each patient and for the control persons using PCR with sequence-specific-oligonucleotide primers. Results showed an increase in the frequency of HLA genotype DRB1* 07:0101 (P value= 0.026) and DRB1* 11:0101 (p-value = 0.016) in patients with vitiligo compared with healthy controls. In conclusions, our results suggest an association between HLA-DRB1* 07:0101 and DRB1* 11:0101 and susceptibility to vitiligo.

Keywords: vitiligo, genetic, PCR.

Introduction

Vitiligo is a common, multifactorial depigmenting disorder of the epidermis and hair follicles, manifesting clinically as white depigmented patches with natural margin or hyperpigmentation. It is the most common pigmentary disorder affecting 0.5–1 % of the world population. [1]

The disease pathogenesis is unknown. But it has been made clear that genetic and immunological factors play a significant role in its developing [2, 3].

Of these, autoimmune hypothesis remains most widely accepted because of frequent occurrence of other autoimmune disease in vitiligo cases, the presence of auto reactive T cells in the vitiliginous lesions and peripheral circulation, and the presence of circulating autoantibodies in the sera of patients. [3, 4, 5]

Clinically characterized by milky-white macules with fairly homogenous depigmentation and a well-defined border. Vitiligo is classified into generalized (vulgaris, acrofacial, and mixed), and localized (focal, segmental, and mucosal) types. [6]

Major histocompatibility complex (MHC) represents a gene region that the histocompatibility molecules responsible for antigen presentation to the immune system. In humans, MHC is located in the short arm of chromosome 6 and it is called HLA (human leukocyte antigens) system. The genes in the HLA system have been classified into three regions: Class I, II and III.

Class I comprises HLA – A, B and C loci which modify the classical histocompatibility molecules expressed on the surface of all nucleated cells.

Class II region is composed by HLA-DR, DQ and DP loci, and class III that includes other molecules such as tumor necrosis factor, proteins, C4, C2 and factor B of the complement system, heat shock protein and 21-hydroxylase enzymes. [7, 8]

Given the polymorphism of the HLA system, its association is highly variable, as a result depending on the genetic load, an individual may present a higher or lower risk of developing a certain disease. So the aim of this study is to investigate whether there is an association between HLA class II DRB1 and Vitiligo.

Patients and methods

The study consisted of forty Iraqi Arab Muslims patients who had vitiligo that consulted the dermatological department in Al-Kindy teaching hospital from September 2013 to September 2015 were assessed for HLA typing class II DRB1.

The study took place at the HLA typing research unit, AL-Kindy college of Medicine, Baghdad University. The second control group consisted of thirty healthy volunteers' age and sex matched among the staff of Al-Kindy College of medicine that did not have vitiligo or other autoimmune diseases and had negative family history for vitiligo.

The age of the patients ranged from 6-45 years with a mean age of 35.

The ethical committee of Al-Kindy College of medicine, Baghdad University approved the study; all samples were obtained with informed consent in accordance with the Al-Kindy Teaching hospital declaration.

HLA genotyping

Peripheral venous blood samples from patients and control groups were collected in ethylene diaminetetracetic acid containing tubes and then stored at -20° c until testing for class II HLA-DRB1 using the polymerase chain reaction (PCR). Sequence specific primer (SSOP) method. Genomic DNA was extracted using promega DNA extraction Kit (promega corporation, fitchbury, Wisconsin USA). All DNA was stored at -20° C until testing. Locus and allele-specific amplification

of genomic DNA was performed for DRB1. Amplification and Hybridization was performed using a panel of sequence-specific.

Oligonucleotide probes (SSOP) using HLA-DRB1 amplification and hybridization kits (SSO HLA type DRB1 plus and mastermix for HLA type DRB1 Amp plus kits-Innogenetics-Belgium) using automated method by Autolipa-48 Innogenetics-Belgium. The results were interpreted using LiRas version-5.0 software-Innogenetics-Belgium.

Statistical analysis:

The distribution of HLA alleles in the patient and control group was compared using chi-square for continuous variables using minitab version 15 software in each comparison, the odds ratio (OR) along with the 95 % confidence interval (95 % CI) was used. A P-value less than 0.05 were considered statistically significant.

Results

Patient group with vitiligo and control group were typed for identification of the HLA-DRB1 alleles using DNA-based methodology (PCR-SSOP). Allele's frequencies of HLA-DRB1 for vitiligo patients and control group are shown in Table 1.

There was an increased frequencies of HLA-DRB1* 07:0101 in patients with vitiligo than control group (P-value = 0.026, odd ratio = 3.285, CI= 1.151 – 9.378)

Also there was an increased frequencies of HLA-DRB1* 11:0101 in patients with vitiligo than control group (P-value = 0.016, odd ratio = 3.631, CI= 1.271 -1 0.370).

Discussion

Association of MHC alleles with a disease gains importance because of the antigen-presenting function of the MHC. The peptides presented by the MHC molecules have allele-specific motifs. The affinity of the peptide to be particular MHC molecule is determined by the amino-acid residues present in peptide-binding groove. Shared amino acids in the peptid-binding pockets have been demonstrated in autoimmune diseases [8].

In this study, a significant increase was found in frequency of HLA-DRB1* :07 and HLA-DRB1* 11 in patients with vitiligo than control group, that is in agreement with Singh et al [9], who reported an increase in HLA-DRB1*07:01, HLA-A*33:01 & HLA-B*44:03 in patients with vitiligo than the controls in North India and in Gujarat. Also, our results agreed with that reported increased frequency of DRB1*07:01 in vitiligo patients from Slovakia (Buc et al, 1996) [10] and in China (Ren et al, 2009) [11]. Also a study on Turkish Vitiligo showed increase frequency of DRB1*03, DRB1*04 & DRB1*07 (Tastan et al. [12]

Genetic models of vitiligo also show a positive association among DQB1*0303, DQ1*0503 & DRB1*0901 alleles with vitiligo susceptibility. [13]

A study found increased frequency of HLA-B7, B15, BW6, CW6, DRB4*0101 and a decrease frequency of HLA-A9, B5, DQ1, DRB3*010101 in Saudi patients with vitiligo. [14]

Other study in Omani patients with vitiligo found that the frequency of HLA-DR7 was significantly increased. [15]

The inheritance pattern of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. Vitiligo seems to be a complex hereditary disease governed by a set of recessive alleles situated at several unlinked autosomal loci which may be involved in the generation of oxidative stress, melanin synthesis, autoimmunity etc that could collectively confer the vitiligo phenotype. [16] The presence of circulating antimelanocyte and antikeratinocyte antibodies in the sera of Vitiligo patients, the selective destruction of melanocytes might be due to antibody reactivity directed to the antigens preferentially expressed on pigment cells or from an antibody response against antigens expressed on a variety of cell types that might selectively destroy melanocytes because they are intrinsically more sensitive to immune mediated injury than other cells [17] many studies have indicated a role for both cellular [18], And humoral immunity in the pathogenesis of vitiligo [19, 20].

Histological evidence further supports an autoimmune etiology. Vitiligo lesions have an infiltrate of inflammatory cells, particularly cytotoxic and helper T cells and macrophages, this infiltrate is most prominent in the perilesional skin just prior to the clinical appearance of vitiligo. More over vitiligo is epidemiologically associated with increased risk of several other autoimmune

diseases, both in patients & their relatives. Suggesting that these autoimmune diseases involve shared susceptibility genes. [21]

The major contribution of the study of HLA and vitiligo has been done to show the genetic aspects of the disease, further more this study has contributed a great deal to a better in sight in the pathogenesis of the disease.

The differences in the association of HLA antigens in Vitiligo in our study with other studies can be attributed to racial group variations, religion, sample size and methods used in the study. Discrepancy of our results as compared to other studies may be due to a racial factor and normal distribution of HLA antigens in different populations.

Conclusions

Vitiligo is associated with HLA-class II DRB1* 07:0101 and DRB1* 11:0101 which has a role in the etiopathogenesis of the disease.

Acknowledgments

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Table 1: Human leukocyte antigens (HLA-DRB1) alleles frequencies in patients with vitiligo and healthy control groups

HLA-DRB1* alleles	Patient No=40		Control No=30		Odd ratio 95% CI	Pvalue
	No.	%	No.	%		
02:0301	0	0	2	6.66	Na	Na
03:0101	3	7.5	4	13.33	0.527 0.108-2.555	0.426
03:0102	0	0	2	6.66	Na	Na
03:1701	0	0	4	13.33	Na	Na
03:1101	0	0	1	3.33	Na	Na
04:0201	3	7.5	0	0	Na	Na
04:2201	2	5	0	0	Na	Na
07:0101	20	50	7	23.33	3.285 1.151-9.378	0.026
08:0101	0	0	2	6.66	Na	Na
08:0201	0	0	2	6.66	Na	Na
08:0701	5	12.5	0	0	Na	Na
11:0101	21	52.5	7	23.33	3.631 1.271-10.370	0.016
11:0701	2	5	0	0	Na	Na
11:2201	1	2.5	0	0	Na	Na
12:0901	0	0	2	6.66	Na	Na
13:0501	0	0	2	6.66	Na	Na
13:1801	0	0	7	23.33	Na	Na
13:2201	7	17.5	0	0	Na	Na
14:0101	3	7.5	2	6.66	1.135 0.177-7.258	0.893
14:0201	0	0	8	26.66	Na	Na
15:0101	2	5	0	0	Na	Na
16:0101	1	2.5	0	0	Na	Na
Other	10	25	-	-	Na	Na

Na = not applicable

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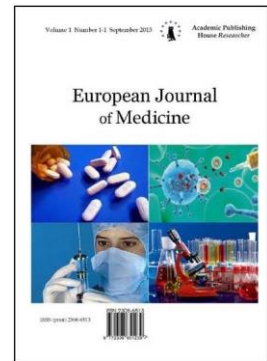
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The Power of Beliefs on Health Seeking Behaviour: Implication for Therapeutic Relationships for Cardiovascular Care

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Abstract

This study had as its aim, to examine health seeking behaviour among people suffering from cardiovascular disorders in the New Juaben Municipality in the Eastern Region of Ghana. Six hundred (600) participants, comprising male and female patients aged between 35-75 years and suffering from cardiovascular disorders for the past 12 months were selected used for the study. Cultural belief systems were found to significantly influence the health seeking behaviour of the people. Additionally, the perception of the aetiology of CVDs was socially constructed from a cultural point of view, which deviated from the orthodox views of disease causation. The finding of this study underscore the need for therapists and clinicians to adopt a culturally congruent approach to the provision of healthcare. This further calls for therapists to understand the cultural values and beliefs of the people in order to provide healthcare that would be acceptable to the people.

Keywords: cardiovascular disorders, traditional medicine, culture, belief systems, health seeking behaviour, utilisation, healthcare, biomedical, Ghanaians; diagnoses.

Introduction

Culture shapes the worldview in that it interprets experiences, determines and animates over 90% of an individual's daily activities (Kraft, 2000). The term culture in the African thought is very inclusive. It takes account the religious, social, psychological, linguistic, political, economic, and many other aspects of life. Helman (1994; p 48) defines culture as set of guidelines (both explicit and implicit) which individuals inherit as members of a particular society, and which tells them how to view the world, how to experience it emotionally, and how to behave in relation to other people, to supernatural forces or gods, and to the natural environment. Yet, very few studies have explored the cultural acceptability dimension, which, probably is as a result of difficulties in defining and measuring the concept of 'culture'.

In this study, culture is defined as 'customary beliefs and values that ethnic, religious and social groups transmit fairly unchanged from generation to generation' (Fernandez, & Fogli, 2006; Guiso et al., 2006) and is measured by a series of attitudes and beliefs held by individuals about the use of both orthodox and traditional medicine (TM). This definition is employed alongside a unique to test how cultural belief systems affect traditional medical utilisation in Ghana. Affordability, accessibility and availability have all been put forward as key reasons for utilisation

of healthcare in Ghana and elsewhere in Africa (Anyinam, 1987; World Health Organization, 2008) and accordingly quantified (for example, Hausmann-Muela, et al., 2000; Leonard & Zivin, 2005; Peltzer, 2009).

Many existing studies investigating the role of culture and healthcare utilisation are anthropological in nature and they tend to evaluate health-seeking behaviour within a social constructivist framework (Anyinam, 1987; Evans-Pritchard, 1937; Hielscher & Sommerfeld, 1985; Stoner, 1986; Tsey, 1997; Twumasi, 1979). Culture is central to anthropological approaches, because an individual's behaviour is influenced by preconceptions, including those related to illness (Winkelman, 2009; Wiredu & Nyame, 2001). Historical review by Rivers (1924) argues that we must first start with an understanding of how an individual perceives disease before we can understand observed actions: all health seeking behaviour is the result of a process involving identification of causation, followed by aetiology, diagnosis then prognosis. While biomedical diagnoses would consider disease to be caused by, or the result of, biological, physical or chemical abnormalities within the body (informed in large part but not exclusively by germ theory), anthropological understandings would deem illness to involve more than simply a biomedical explanation, placing the individual within a societal context.

Rivers again (1924) illustrates differences in interpretation by depicting a person falling from a tree. Modern medicine would characterise this as an accident, perhaps owing to a loose branch or carelessness whereas traditional explanations would blame a sorcerer or spirit for loosening a branch. In these two scenarios, given that the theory of causation differs, it naturally follows that the appropriate treatment, or response, is divergent. In one causal schema, often found in folk theories of causation in Africa and elsewhere, societal ills can also manifest within an individual, at which point social responses are crucial (Hevi, 1989). Thus, legitimacy of medicinal products is attributed to local communities, institutions, and symbolic values (van der Geest, Whyte, & Hardon, 1996).

Bierlich (1999) and Kirby (1997) demonstrate how Ghanaian traditional medical practitioners ascribe colours to medicines to distinguish their potency, type and use, and to label various stages of illness. At the start of an illness, the 'white' stage, individual self-help is the dominant action. If the situation worsens, the colour 'red' is assigned and society is expected to step in with prescribed and agreed upon interventions. Further, it is believed that some plants are inactive as medicines until prayers and libations to ancestors are carried out. Under such scenarios healers would be important sources of care for their perceived 'ability to cure' and complementary rituals. The idea that cultural beliefs linger and evolve only slowly, however, is documented. There is an argument by some researchers that when individuals emigrate, people hold ethnically-linked beliefs over their lifetime. Owusu-Daaku and Smith (2005) show that Ghanaian women who have moved to the UK uphold Ghanaian perspectives about health and illness while adapting to the British system.

Barimah and Teijlingen (2008) studied attitudes toward TM of Ghanaians living in Canada and found that 73% of respondents had not changed their views about TM as a result of emigration. There were no significant differences in results between individuals who had been abroad for a long and short period of time and individuals show strong acts of agency, whereby Ghanaians import TM back to Canada from their homeland in order that supplies do not run out. Ransford et al (2010) and Senah (1988) highlight the importance of cultural alternatives for Mexican immigrants as a result of belief and structural barriers to accessing formal health care in the United States. Sometimes, cultural alternatives were shown to be a coping strategy, but similarly individuals had considerable control over their health and explicitly chose TM.

However, cultural beliefs are not always the dominant force for explaining utilisation of health care facilities. Jenkins et al (1996), in a study, show no significant associations between traditional beliefs held by Vietnamese immigrants and access to modern preventive care. Additionally, Young and Garro (1994) examine medical choices made in two Mexican villages and found that, despite similar attitudes and beliefs toward traditional and folk medical knowledge, the village with better accessibility in the form of easier transport links and cheaper cost of care utilised physicians significantly more than the village with poor accessibility. Young and Garro calculate that only a fifth of traditional care users stated cultural preference as a key reason for utilisation.

Since traditional medicine has been with Ghanaians and mostly rural dwellers for generations and also for the fact that orthodox medicine is often in short supply, people's approach in times of suffering from any health condition is first towards traditional medicine. It is when this fails that they resort to chemist shops or medicine vendors and then the hospital as a last resort (Katung, 2001). In traditional medicine, divination (consulting the oracles), confession, ritual sacrifices, incantations and potions made from plant and animal parts are essential components of illness management (Sallah, 2007). These are aimed at restoring the patient to a harmonious relationship with his environment and/or counteract the effect of evil forces. In every instance where an illness is diagnosed to be due to ancestor spirit anger, there is usually an antisocial act of commission or omission by the person who must usually confess the misdemeanour, followed by ritual sacrifices to appease the offended supernatural agency before he can be expected to recover (Danquah, 2008; Badru, 2001).

Confession, that is admission of guilt, is crucial for therapeutic success. In other words, although the illness is attributed to ancestor spirit anger, the trigger for this is the sin against moral laws committed by the afflicted person (Calhoun, 1992; Jegede & Onoja, 1994; Sallah, 2007). It is only after rituals have been performed to appease the gods and ancestors that the individual could be restored to this or her normal health. For example, among the Akans of Ghana, there is the concept of "*funusoa*" where an individual who has angered the ancestral spirits has to carry a coffin with a dead body inside from one end of the town and paraded through the town to the other end before he or she could be restored to normal health status.

Method

Participants

A representative sample of six hundred (600) male and female adults aged 35-75 years who were currently living in the New Juaben Municipal Area in the Eastern Region of Ghana. These were patients who were suffering from cardiovascular disorders and were of sound mind and had insight into their conditions, but have not suffered any complications that might have impaired their cognitive functioning. This was done to sample participants who could adequately and appropriately express their beliefs, perceptions and opinions on the subject under study.

The study sought information on cultural values, belief systems and views of cardiovascular disorders that occur within the Ghanaian context. It was the belief of the researcher that information on these cultural values and belief systems would go a long way to build a strong understanding of the socio-cultural underpinnings that influence the health seeking behaviour of the people.

Respondents for the study had diverse demographic backgrounds which contribute to the enrichment of the primary data gathered. Female participants were dominant in the sample 296 (53%) compared to their male counterparts 264 (47%). Thirty five (6%) were unemployed, self-employed 300 (54%), employed 207 (37%) and students 18 (3%). Most respondents were Akans 294 (53%). The rest comprise Ewe 127(23%), Guan 16 (3%), Ga-Adangbe 83(15%), Gruma 12(2%), Mole-Dagbani 14(3%) and Grusi 14(3%). They were also of diverse religious affiliations, with the dominant group being Christians 451 (81%), African Traditional Religion practitioners 62 (11%) and Moslems 47 (8%). Description of the localities of the participants includes Urban 289 (52%), Semi-urban 234 (42%) and rural 37 (7%).

Measures

The Cultural Values and Belief Systems and Health Seeking Behaviour Questionnaire (CVBSHSB) is a 19-item scale that was used to assess participants' belief systems. This instrument was used to measure categories of beliefs involving 1) spiritual influences 2) physical influence, 3) behavioural influence and 4) perceptual influences. This questionnaire used a 7- point Likert Scale [with responses ranging from "Don't Know = 0 to Strongly Agree = 6]. The reliability statistics after piloting indicated a Cronbach's Alpha Based on Total Standardized Items is .879. Correlation Between Forms recorded a reliability of .664 while Spearman-Brown Coefficient indicated for both Equal Length and Unequal Length values of .798 and .798 respectively. Finally, Guttman Split-Half Coefficient recorded a reliability statistics of .792.

Procedure

Approval was obtained from the Department of Psychology, University of Ghana to carry out the study. Written consents were subsequently obtained from all participants before data collection was done. Questionnaires were then distributed to participants who were selected using a survey method of sample selection, and it took an average of one hour, fifteen minutes to complete each questionnaire. In the course of answering the questionnaires, participants were given enough time to break and get refreshed so that they could answer the questions as objectively as possible. In order to ensure the confidentiality and safety of responses obtained, completed questionnaires were scored and packed in sealed envelopes and safely paced in locked file cabinets.

Data Analysis

Preliminary analysis was done by testing the frequencies of demographic characteristics for the entire sample and the various study groups, analysis of the normal distribution of the variables, means, standard deviation, correlation among the key study variables and internal consistency reliability. The study tested for both normality and homogeneity.

Test for normality using skewness and kurtosis was within the acceptable range of ± 2 (Tabachnick & Fidell, 2007) for all the scales. All the scales used in this analysis also yielded acceptable results of Cronbach's alpha coefficients ranging from 0.72 to 0.88. The Cultural Values and Belief Scale was further subjected to principal component factor analysis after the suitability of data for factor analysis was assessed. Inspection of the correlation matrix revealed the presence of only four coefficients of .3 and above.

The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.724, meeting the commonly recommended value of 0.6 and above. The Bartlett's Test of Sphericity reached statistical significance, ($\chi^2 (21) = 75.481, p = 0.000$). Finally, the communalities were all above .3 further confirming that each item shared some common variance with other items. Given these overall indicators, factor analysis was deemed to be suitable with all the 19 items. Principal components analysis revealed the presence of one component with eigen value exceeding 1, explaining 39.12% of the variance. All the factors loaded onto one component.

Results

Hierarchical Multiple Regression analysis showed a significant positive relationship on whether cultural values and belief systems will have a moderating effect on the relationship between the development of cardiovascular disorders and health seeking behaviour. This allowed the interaction term to be included as part of the variables used in the analysis to examine the moderating effect. The study regressed 'health seeking behaviour' variable scores on the cultural values and belief systems variable scores, and the cultural values and belief systems; and cardiovascular disorders interaction term scores. A summary of the analysis is shown in Table 1.

Table 1: Summary of Hierarchical Multiple Regression of Variables

Variables	R-square	Changed R-square	Standardised Beta (β)	F
Independent Variables	.306**	.306**		121.621**
Cultural Values and Belief Systems			.213**	
All Variables	.332**	.026**		91.206**
Cultural Values and Belief Systems			.228**	
Cultural Values and Belief Systems of Cardiovascular Disorders interaction term			-.161**	

** $p < .01$

A significant model emerged ($F_{(3,551)} = 91.206, p < .01$) when the hierarchical multiple regression analysis (enter method) was conducted, (see Table 4.2). The R^2 was .332 indicating that the model as a whole explained 33.2% of the variance with the interaction term explaining additional 2.6% (Changed $R^2 = .026$) of the variance when cultural values and belief systems; and cardiovascular disorders were statistically controlled.

The contribution of the cultural values and belief systems; and cardiovascular disorders interaction term though small (Changed $R^2 = .026$) was statistically significant, Changed $F_{(1,551)} = 21.390, p < .01$. In other words, for the independent and moderator variable and their interaction term considered in the model, about 2.6% of the variances in 'health seeking behaviour' can be predicted by the cultural values and belief systems; and cardiovascular disorders interaction term.

The results also indicate that apart from the main effect of cardiovascular disorders ($\beta = -.375, p < .01$); the interaction term of cultural values and belief systems; and cardiovascular disorders variables ($\beta = -.161, p < .01$) was significantly and negatively related to 'health seeking behaviour'.

In addition, the significant model that emerged when the interaction term was introduced into the model ($F_{(3,551)} = 91.206, p < .01$) is an indication that cultural values and belief systems do have a significant moderating effect on the relationship between the cardiovascular disorders and health seeking behaviour. although the strength of relationship is small (Cohen, 2007), the interactive term is shown by the analysis as a significant predictor of 'health seeking behaviour', an indication that cultural values and belief systems do have a significant moderating effect on the relationship between cardiovascular disorders and health seeking behaviour.

Thus, cultural values and belief systems will have a moderating effect on the relationship between cardiovascular disorders and health seeking behaviour was supported by the results

Discussion

Cultural values and belief systems had a moderating effect on the relationship between cardiovascular disorders and health seeking behaviour. Health seeking behaviour of the people of New Juaben, thus hinges on the influences of culture comprising the social, cognitive, behavioural, material aspects of the individuals' lives. This finding showed that when a person suffers from cardiovascular disorders, cultural values and belief systems determine the kind of healthcare system her/she would prefer to use. This gives credence to the assertion by Dutta-Bergman (2004) that culture shapes health seeking behaviours and serves as the lenses for perceiving and interpreting experiences.

This finding is also consistent with a study by Pramukh and Palkumar (2006) that in the traditional system, cardiovascular disorders are attributed to certain deviant behaviours and for that matter they believe in the power of prayers and rituals that enable some herbs to heal their diseased conditions. Cultural beliefs affect the health of a people in many ways, where they use culturally specific explanatory models to think about, talk about, and direct care for health problems. The study found that the kind of health care to seek, whether self-care, home remedies, formal public health system and/or consultation with traditional healers and spiritualist are intricately linked with cultural beliefs (Nyamongo, 2002). This same belief system has led to different patterns of health-seeking and prevention, as well as mismatched provision of care (Hunt & Bhopal, 2004).

This finding could be due to the influence of the traditional system where belief systems that have been passed from generation to generation have permeated every aspect of the individuals' lives. There is a belief in a diabolical interference existent in almost all illnesses in Ghana. (Atindanbila, 2011; Onyina, 2002). This belief is deeply infused into belief systems and practices and thus, must have influenced respondents' attitude towards healthcare based on the cultural definition of the condition. Issues relating to culturally ingrained beliefs like disease causation and influences of evil spirits have not been discounted even in contemporary Ghanaian society. This lends some credence to a strong belief in a supernatural cosmology which influences people's health seeking behaviour for cardiovascular disorders.

In this study, belief in the aetiology of cardiovascular disorders had a direct relationship with the health seeking behaviour of respondents. Respondents who believed that their condition was caused by evil spirits and other supernatural powers sought treatment from traditional healers, prayer camps and other unorthodox sources for treatment consistent with the finding of other

studies (Walker, 2006). For those who held unto a biomedical cause of cardiovascular disorders, they sought healthcare from orthodox health care providers and pharmacies. In explaining these health seeking behaviours, a cue can be taken from the assertion by Yawney (2005) that there must be a proper understanding of the culture of a people. On the basis of that assertion, it is plausible that people believe that allopathic medicine can only explain cardiovascular disease conditions within the confines of the medical model at the expense of local cultural explanatory models.

Thus though medical science can describe illness clinically without recourse to cultural factors, it cannot explain or treat all illnesses successfully. Against the backdrop of such belief systems, it could be explained that people rely on culturally relevant explanatory models of their CVDs than from the orthodox. The condition is therefore seen from a cultural point of view than medical.

Important aspects of the socially constructed meaning of CVDs as a consequence of diabolical interference featured prominently in the study under the concepts of 'enemyship' and the '*sale of diseases*' which is referred to in the local Ghanaian parlance as "*nton yare*", which means a disease that has been literally sold to the victim by perceived enemies. The diabolical explanatory model for CVDs as found in this study generally reflects the social construction of the aetiology of diseases.

That is, people assign meanings to particular diseases and that subsequently determine where to seek help. Patronage of prayer camps, herbal centres therefore could be understood from such socially constructed viewpoint of CVDs. Respondents believed in the malevolent cause of cardiovascular disorders, and felt that the disease had been sold to them by their enemies, including witches. This belief presupposes that people's perception about diseases is not viewed as just simple biomedical disruptions, but rather due to an external diabolical agent. Despite the major inroads of education and urbanization, there is a strong belief that people can be bewitched by envious relatives who didn't want to see them prosper.

There are further beliefs held by participants that one could be bewitched to develop cardiovascular disorders as a result of litigation over a piece of land or some other properties left behind by a dead relative. The study found that a reasonable number of participants currently suffering from hypertension and on antihypertensive therapy from orthodox health systems still hold on to the belief that they had been bewitched and therefore sought help from the prayer camps and the *akomfo*. Those who were on antihypertensive medication claimed they have been advised by their Prophets, Prophetesses and relatives to combine prayers with medication and that would give them a complete cure. Other respondents who believed that their CVDs were caused by witchcraft were of the view that Western medicine could not cure them, and therefore sought healing from herbalists and prayer camps.

Related to this is the perception that diseases sold to victims by enemies can be transmitted from one generation to another as long as the causative agent of the disorder has not been dealt with through prayers and other rites in order to cure the victim, and that one cannot be completely cured without confronting the source of the disorder. For this reason, many collective rites like fasting and prayers, application of concoctions for ritual bathing, ingestion of herbal preparations and other practices such as getting the causative agent to confess and perform rituals to overturn the spell they cast on the victim. The aim of such practices is to stop the transmission of some diseases from generation to generation. This practice is in line with the assertion by Tsa-Tsala (2005), that the belief systems that disease is systematically acknowledged as having a supernatural or malevolent origin, the practice of sorcery and various evil spells are employed to cure the victim of the disorder.

This portrayal of sale of CVDs to victims by enemies is in line with the general belief regarding the causes of illness (Odejide, Oyewumi & Ohaeri, 2006; Makinde, 2005; Field, 2003). This fact might be partly responsible for the relatively high uptake for far less effective traditional care compared with orthodox health treatment. This is in line with the situation in Africa, where traditional healers and spiritualists still enjoy large patronage for health care (Odejide, Oyewumi & Ohaeri, 2006; Prince, 2002, Ebigbo and Tyodza, 2007), probably borne out of the perceived causative factors mentioned previously. On the other hand, with this perceived cause of CVDs orthodox hospital care has been erroneously, shown to be largely ineffective. Hence this could be a factor that discourages people from seeking early orthodox care. In general, the belief in aetiology

of CVDs as found in the study was a major determining factor of the health seeking behaviour of respondents.

Subjective beliefs about cardiovascular disorders was deduced to have influenced many patients to search for different kinds of healers. This was without regard to whether or not the practices of these healers were beneficial to them. These practices resulted in different health seeking behaviours, with patients going on a wild goose chase in search of what is responsible for their dilemmas (Jain, & Agrawal, 2005).

Some patients indicated titrating orthodox medications with traditional remedies concurrently or alternately according to their perceived symptoms. These behaviours stemmed from different beliefs about the aetiology of hypertension and stroke. While some believed in witchcraft as a cause, others believed too much blood in the body and genetics as a cause of cardiovascular disorders and that the occurrence of the disorder cannot be controlled. These findings are in consonance with recommendations by Benson (2006), whose conclusions to their study findings were that patients' perception of illness may be influenced by their subjective beliefs but that further studies were required to identify such contributory factors.

This study has found that belief in the cause of cardiovascular disorders as one of such contributory factors. The study found, further that the multiple use of medications for the same condition created more complications for patients, and a sizeable number of them ended up at hospitals, for further management. In this study, there was a belief in spiritual causation of cardiovascular disorders that resulted in the use of traditional medicine by study participants. This provides direct evidence for previously described notions (Amira & O kubadejo, 2007; Chuma, Thiede & Molyneux, 2006; Shafiq, Gupta, Kumari & Pandhi, 2003) that non-orthodox medicinal use are perceived to work in ways that orthodox medication may not.

This belief could be explained in terms of the view that non-orthodox medicine can work on supernatural causes (unlike orthodox medication) and may more likely lead to a complete cure. These traditional beliefs have continually increased the popularity of traditional medicine among the people who attributed the cause of hypertension and diabetes to a curse or witchcraft. Also, majority of the hypertensive patients were unaware of the symptomless nature of the disease (hypertension), which is also called the silent killer Olivera et al. (2005) and Babaei, Moeini, Sabouchi and Mohammadi (2008). These attributes of the disease and lack of in-depth understanding of cardiovascular disorders may be responsible for individual's negative attitude to treatment, high non-adherence and inadequate lifestyle adjustments, including over reliance on concoctions prescribed and given by traditional healers which have long term negative implications on their health. Consistent with the findings of Pearce (2007), these belief systems are based on cultural and social values, philosophies and expressions.

It is important to explore the reasons for which participants sought help from such quarters. Some of the reasons gleaned from the results included: perception of aetiology and efficacy of treatment, relational issues between healthcare providers and patients, proximity and acceptability of healthcare services, and availability of health services and involvement of family members.

The belief in the cause of cardiovascular disorders was a major factor that influenced the health seeking behaviour of respondents as explained by Boyle (2007). Findings of this study showed that respondents patronised either orthodox or traditional health care system based on their belief in the aetiology of the condition. This belief, as defined by cultural norms of supernatural causation could explain why some respondents were of the opinion that the orthodox health system was no place for them; and that seeking help from the traditional healer was the most probable option. In the same vein, those who believed in the biomedical explanation of the causes of their illnesses sought help mainly from orthodox health systems and saw these as the best places to receive treatment. With the perception of efficacy of treatment of particular health systems and the eventual health seeking behaviour notwithstanding, cases of multiple health care was not ruled out by respondents (Addo, Smeeth & Leon, 2007). This attitude of respondents presupposes that health seeking for orthodox and traditional systems are not strictly dichotomous and that there are some nuances like the length of the illness and the desire to get complete cure, which over ride allegiance to a particular health system and that. This further reveals that perception of health systems change in relation to the success or failure of health care delivered by a particular system.

Relational issues between health care providers and patients that influence the preference of healthcare systems among patients. For therapeutic success and eventual confidence in the health system, a good health care giver-patient relationship is of essence. These relational issues are in the form of patient abuses and communication systems (Ademuwagun, 1998; Iyalomhe, 2009). Some of the abuses, as gathered were in the form of insults, scornful looks, refusal to respond to patients' enquiries and humiliating patients in public through shouting at them. These attitudes of health staff and other instances of perceived neglect of patients and lack of proper care and concern for their welfare were perceived as factors that moved patients from these facilities. These attitudes were mainly found in the orthodox health facilities as opposed to the traditional systems that were very receptive and welcome patients with warm attitudes. These negative behaviours could stem from a variety of factors. The orthodox health facilities are more often than not inundated with hundreds of patients thus putting the health staff under a lot of pressure making them easily irritated. This is in sharp contrast to the non-orthodox health system where their source of income is hugely dependent on the patronage of their services.

For this reason they are more likely to put up attitudes that would attract a lot of customers (Atindanbila, & Thompson, 2011). A converse factor was the perception of health staff that patients are ungrateful and that no amount of sacrifices made by hospital staff for them, will please them. Attitude towards healthcare providers is also an important factor that determines the level of patronage (Atindanbila, 2011). The perceived ungrateful attitude of patients has paradoxically turned hospital staff against the very patients they are supposed to care for (Omotosho, 2010). Adequate explanation of the situation may resolve these dilemmas of cyclical misunderstandings between healthcare providers and patients (Ademuwagun, 1998; Iyalomhe, 2009).

Health seeking behaviour is therefore not dependent only on affordability, accessibility or availability, but on other factors not captured by earlier researchers (Good, 1987; Omotosho, 2010). On the contrary, traditional healers interacted differently with their patients than providers who have been trained in Western medicine. They were significantly more patient-centred in several aspects: They focused more on psychosocial topics and on issues of daily life than on purely medical questions and in particular, they more often asked for the patient's opinion and frequently discussed their concept of illness (Niklaus, Sabine, Engelbert, Jozien & Wolf, 2010).

One might summarise these findings as stating that traditional healers followed a more 'biopsychosocial approach' in the sense that they actively sought common ground with patients. Patients do not appear intimidated but feel invited to ask more questions themselves. In short, one might say traditional healers try to approach their patients by talking about issues that matter in real life and by thoroughly exploring their beliefs. This attitude is reflected by patients' responses, who ask more questions themselves and provide less information in response to routine medical questions. Instead of a provider interrogating the patient about his/her symptoms, it is the patient, who uses his or her share in the consultation to interview the healer about the meaning of his/her symptoms, where they come from, and what can be done. These contrasting health care giver – patient relationships could stem from different working cultures where the orthodox system appear depersonalized and far removed from patients while the traditional system has more accepting attitude and encourage to air their problems, situation that is regarded as part of the therapeutic process.

Finally, the role of extended family members significantly influenced the health seeking behaviours of patients. Notably, this study found that typically family members lend support in areas of emotional and psychological care and "informational support" in terms of directions to the "best" places to seek treatment but support in terms of financial backing is not encouraged. At best family members discuss health maintenance and health enhancing behaviours and in a subtle manner avoid discussing issues that involve financial commitments with sick relatives. The involvement of extended family on where to seek help was more positively skewed towards the search for traditional medicine than orthodox medicine. For example, there was a general belief among some participants that hospitals do not have cure for such terminal conditions like CVDs and that if sufferers needed a complete cure, they could seek help from the traditional health system. Furthermore, participants hold a belief that the drugs used in the hospitals are poisonous which do more harm to the body than good.

This attitude to health reflects a contextualised understanding among the people that seeking help from the orthodox system directly involves paying huge sums of money for drugs that only harm that have the potential to further harm the body. This perception and financial constraints could, in a way explain the perpetuation of the use of traditional medicine. For this reason, extended family members without strong financial backing are likely to shy away from discussing health seeking with a sick relative in areas that involve financial commitments. The informational support given by the extended family members in support of the traditional health system could be due to the increasing advertisements on the mass media by the traditional healers who make very “wild” claims to have a completely curable antidote to every sickness, ranging from infectious diseases to non-communicable diseases such as cardiovascular disorders. The advertisements also go with the promises that patrons do not need to hold a lot of money to go there since payment for services is very meagre or could be deferred or paid in kind (Jonas, Franks, & Ingram, 1997).

These advertisements are tenaciously defended on daily basis with many of the traditional practitioners consistently buying airtime for this exercise, while at the same time these traditional healers try to run down the efficacy and the long term negative effects of medication prescribed by the orthodox health system (Haddad, 2005). These could be reasons why people are swayed by these actions due to a combination of factors like level of literacy, length of illness in addition to other socio-cultural factors mentioned in earlier pages. Based on these considerations, CVD patients were willing to devote as much money as possible to seek treatment from any source, provided they would be cured of their cardiovascular disorders.

Limitations

This study has some limitations that must be taken into consideration. First, the study was conducted in the New Juaben Municipal area, which though comprises people of diverse ethnic groups is highly dominated by Akans who make up more than fifty percent of participants for the study. Their views may not necessarily reflect that of other ethnic groups. Therefore, the findings of this study may not necessarily be generalizable to other ethnic groups who may have different cultural backgrounds that influence their health seeking behaviour.

Secondly, the scope of the study was limited to people suffering from cardiovascular disorders and excluded people with other conditions for which patients seek treatment. This is because cardiovascular disorders comprising hypertension, stroke and chronic cardiac failure as defined in this study are constantly among the first ten diseases in Ghana just like malaria, TB, HIV/AIDS. For which people seek health care (de Graft Aikins, 2006; Sarfo, Cudjoe, Fosu, & Schlatter, 2015). Unfortunately, CVDs are among the least studied disorders in Ghana. The researcher therefore decided to limit the scope of the research to cardiovascular disorders.

Furthermore, extending the scope to other categories of disorders could have compromised the effectiveness of the study since having many variables in a study that is being undertaken within a limited time could potentially lead to shoddy work done.

Conclusions

The study findings also indicate that a belief in the cause of cardiovascular disorders determined where an individual decides to seek treatment that is the approach to treatment options is determined by how the individual culturally defined the condition and the sociocultural definition of cardiovascular disorders. There was also a strong belief in the supernatural cause of cardiovascular disorders. These beliefs were based on the notion that evil spirits and envious relatives and co-tenants were capable of transmitting cardiovascular disorders to other people. The belief in the supernatural transmission of CVDs significantly influenced patronage of traditional medicine, a notion based on the perception of some diseases classified as “not-for-hospital” diseases and that orthodox medicine has no antidote to these ailments. These findings therefore supported the key objective of the study that cultural values and beliefs do influence health seeking behaviour for cardiovascular disorders.

Secondly, religiosity and spirituality were identified as major factors that influenced health seeking behaviour among participants. Religious affiliation and a sense of reliance on a Supreme Being who cared for the suffering of people was a source of encouragement that provided emotional and psychological healing to patients though some of them might be suffering from debilitating conditions. The concept of religious healing was a force that participants reckoned kept

them going even in very trying moments of their suffering from cardiovascular disorders. Both traditional Spiritual heads and Christian spiritual heal heads play very significant roles that should not be downplayed. They have constantly served as hope for those that need it.

Health professionals need to develop and inculcate into their practice the concept of cultural competence where healthcare providers would understand the culture of the patients' they take care of. Healthcare providers are encouraged to be sensitive to patients' concerns and to understand the conditions from the patients' point of view and incorporate these in their professional practice. There is the dire need for healthcare givers to pay close attention to the traditional aspect of healthcare provision, since patients seeking help interpret the condition from a cultural point of view. Currently this aspect of healthcare delivery is missing in the health system, due to the western oriented training given to health professional where patients are viewed from the clinical point of view without enough regard for the cultural values of the patients. Closely related to this is the barrier of communication channels between health care providers and their patients which prevent patients from adequately expressing themselves. There is therefore the need for the health facilities to provide cultural-congruent services where they would take the cultural backgrounds of their patients into consideration when they are treating them.

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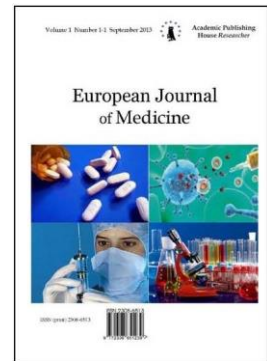
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UDC 61

Congenital Incus Anomaly: Any Role of HRCT As Single Diagnostic Tool?

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Abstract

We describe a rare case of isolated congenital incus anomaly without other otologic anomalies in this case report. We detected this condition in an adult who presented with long standing, non progressive unilateral reduced hearing. Isolated congenital incus anomaly was identified incidentally during her visit for other otological problem. We discussed regarding the case and the important role of HRCT imaging as a single tool for diagnosing the anomaly without surgical exploration.

Keywords: congenital, hearing loss, ossicle, ossicle anomaly, incus anomaly, HRCT.

Introduction

Ossicular anomalies with conductive deficit commonly associated with external auditory canal dysplasia. Stapes malformation is reported to be the most common ossicular anomaly. In this case report, we describe a rare case of isolated unilateral incus anomaly without other otologic manifestation which was found in a female patient who presented with long standing non progressive unilateral hearing loss.

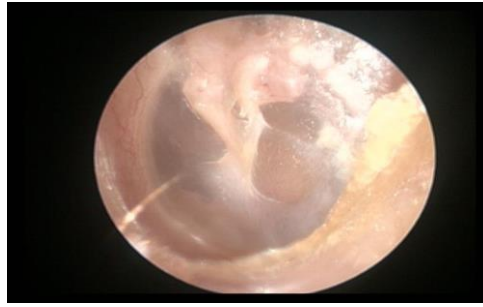


Figure 1: prominent handle of malleus, body and long process of incus

Case report

We report a case of 38 years old female presented with long standing and non progressive hearing loss of left ear over 26 years. She only noticed the hearing loss when she was 12 years old. There was no history of otitis media or trauma. There is no significant antenatal, perinatal and birth history.

Examination revealed normal bilateral pinna and ear canal. Otoscopic examination of left ear showed a retracted tympanic membrane with prominent lateral process of malleus, body and long process of incus (Figure 1). There was no clinical evidence of cholesteatoma seen. Rinne’s and Weber test consistent with left conductive hearing loss. Tympanometry test revealed a bilateral type A tympanogram (Figure 2) while her serial Pure Tone Audiogram revealed left conductive hearing loss with air bone gap of 30-50dB (Figure 3). The opposite ear has normal examination finding and normal hearing level.

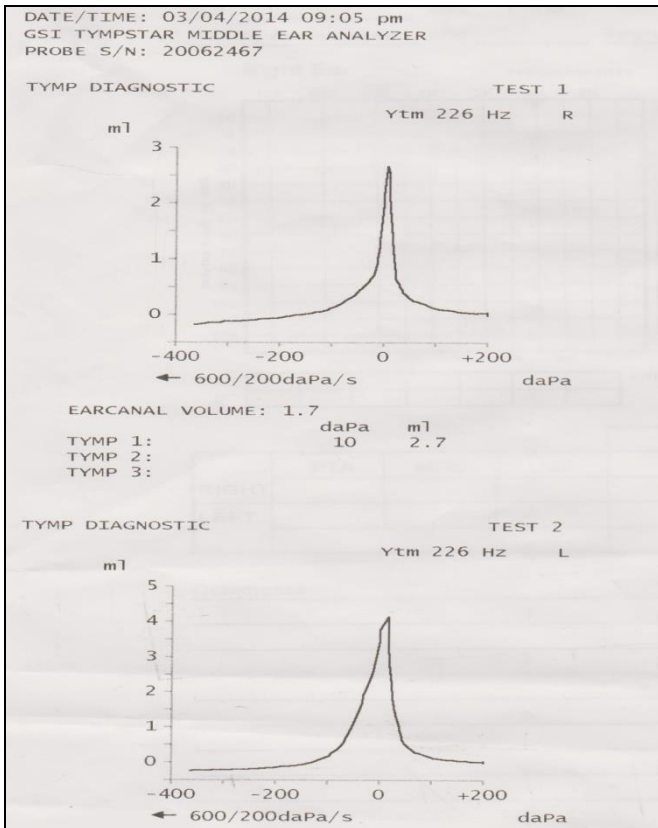


Figure 2: Tympanogram: Bilateral Type A

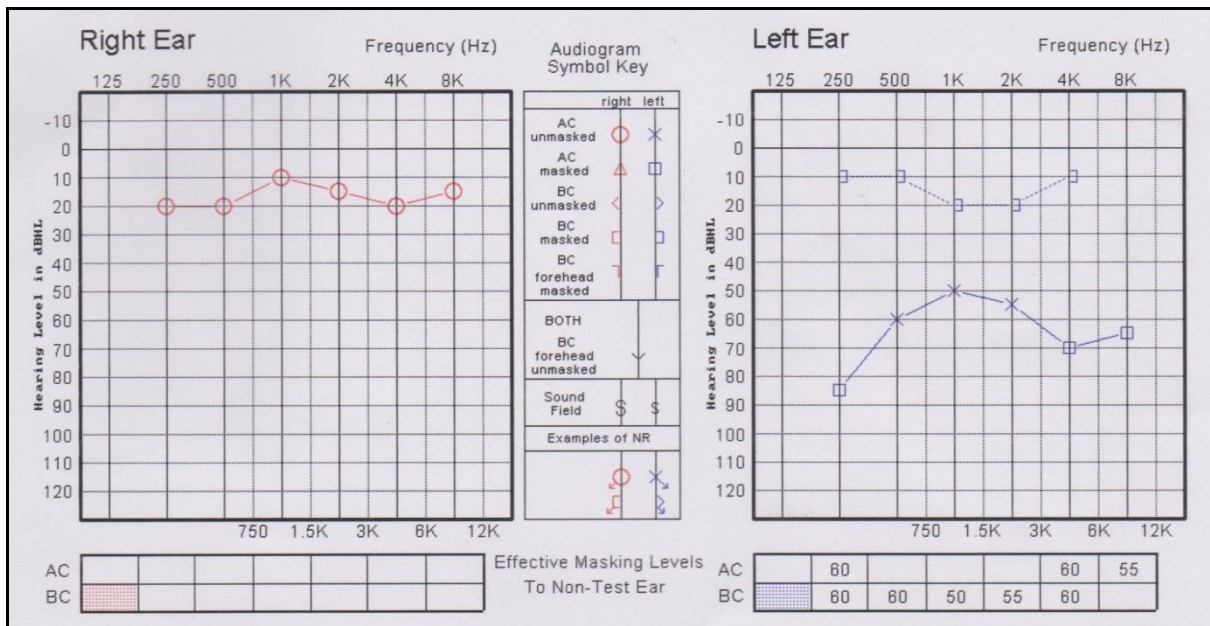


Figure 3: Pure Tone Audiogram showed left conductive hearing loss with air-bone gap (ABG) of 30-50dB

Further investigation was performed with High Resolution Computed Tomography (HRCT) of temporal which showed a dislocated left incudo-malleolar and incudo-stapedial joint with evidence of incus anomaly of left ear. Stapes and malleus appeared normal characteristically. There was no evidence of canal or middle ear cholesteatoma. The HRCT finding could explain her clinical symptoms, clinical finding and audiological examination result.

Hearing level was monitored in the past 6 years and it remained the same. She was offered surgical ossicles reconstruction with prosthesis, which has the possibility to have a good outcome. However, patient refused surgery and opted for conservative treatment.

Discussion

Congenital ossicular anomalies resulting in conductive deficit commonly associated with external auditory canal dysplasia while it is less common without any external ear anomalies. Stapes malformation is reported to be the most common ossicular anomaly. A unilateral absence of incus without other otologic manifestation is a rare entity [1]. Study by Swartz and Faerber in Medical College and Hospital of Pennsylvania, 4 out of 8 patients with isolated congenital ossicular deformity cases, the anomaly was bilateral. Stapes is the most common ossicle involved and was abnormal in all cases of isolated deformity. Numerous anomalies are possible and bilaterality is common [5]. The conductive hearing loss with absence of pinna and external auditory canal anomaly can also be caused by other conditions such as osteogenesis imperfecta, branchio-otorenal syndrome, orofacioidigital type II syndrome and Treachers Collins Syndrome [4].

A non-progressive and conductive hearing loss with a normal tympanic membrane, without history of trauma or middle ear infection, is highly suggestive of a congenital ossicular anomaly. In this case, her late presentation is likely due to her hearing disability being masked by good hearing of the opposite ear until it was detected incidentally. In ossicular disruption, PTA (Pure Tone Audiometry) usually demonstrates a large air bone gap and tympanometry may demonstrate an axis deviation curve (type Ad) [2, 3]. In our case her serial PTA corresponds well with the expected finding except her tympanometry showed normal type A bilaterally.

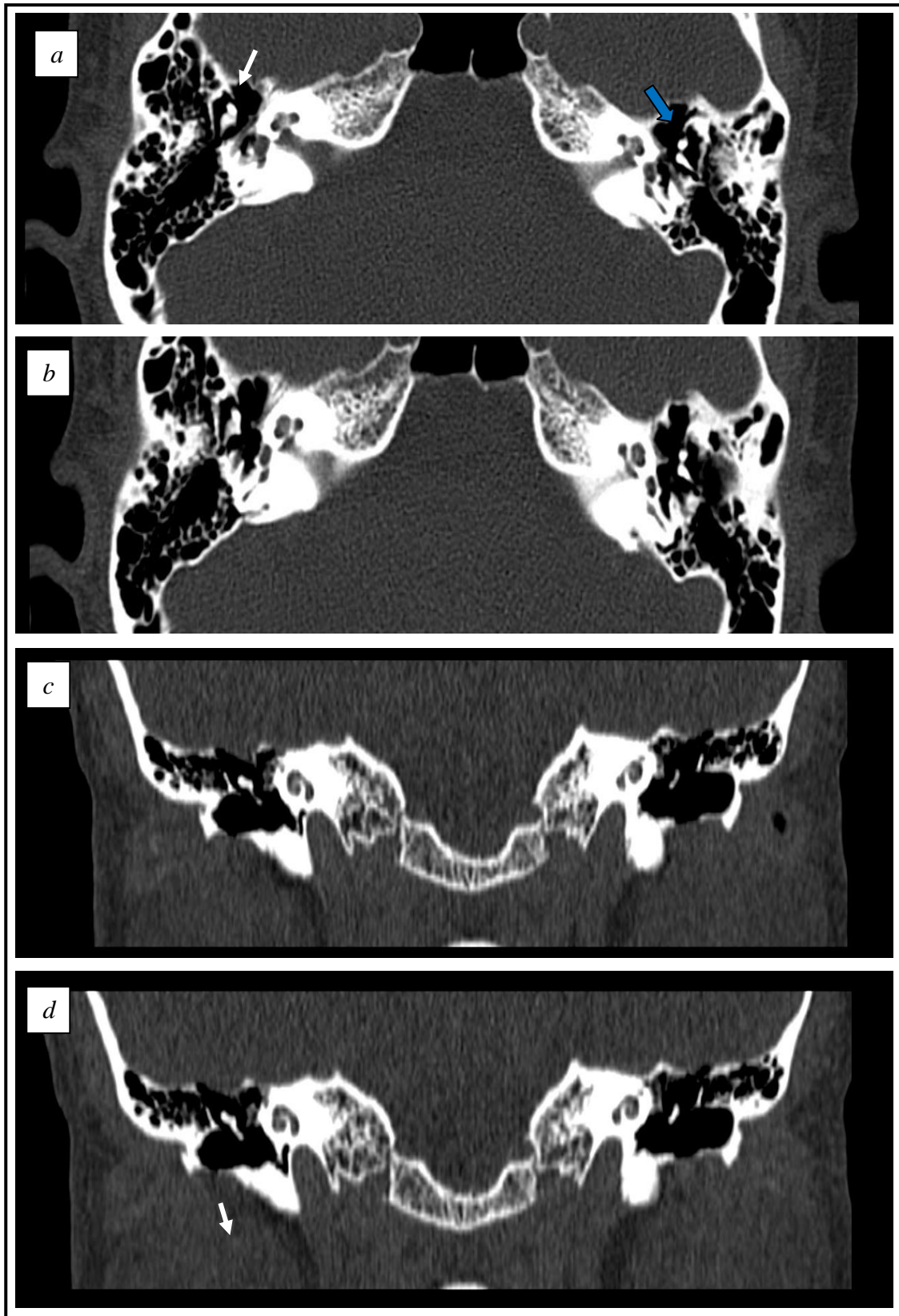


Figure 3: a and b are axial views of HRCT temporal of the patient. In a, 'ice cream' cone configuration (white arrow) is seen in right temporal represent normal head of malleus and incus while on the left temporal, there is separation of malleus and incus. The incus is seen dislocated and displaced (blue arrow). This finding can be compared with the normal incus in right temporal .c and d are coronal views of HRCT temporal. At this plane, right head of malleus and long process of incus seen in its normal anatomical position (arrow). Left long process of incus more prominent in this plane and head of malleus not visualized likely they are detached and long process of incus is displaced posteriorly.

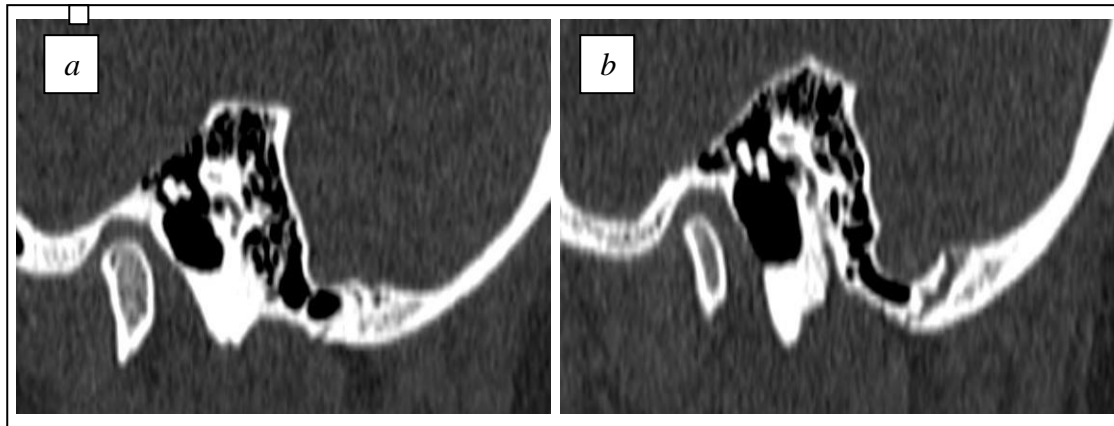


Figure 4: a : sagittal view of right temporal and b is sagittal view of left temporal, showing separation and dislocation of incus and malleus

Embryological development of middle ear is otherwise a complex process. The skeletal elements of the middle ear develop from the mesenchyme of the first two branchial arches. Although there are many hypothesis postulated differently, in general it was well accepted that middle ear develops primarily from first and second branchial arches and that the ossicles are derived from various contribution by Meckel's cartilage, Reichert's cartilage and the otic capsule. The malleus and incus are formed mainly by endochondral ossification. Their development starts as a condensation of concentric cells in the caudal extremity of Meckel's cartilage. This extends perpendicular to the main axis of Meckel's cartilage towards the otic capsule. This condensation then separates into two parallel components that remain connected dorsally but separate ventrally. The most caudally located will form the incus and the most rostral is the malleus [3]. In details, Meckel's cartilage was solely responsible for the head and neck of malleus and the body and short process of incus, while Reichert's cartilage was responsible for the handle of the malleus, the long process of incus, the crura and lateral stapedial footplate while the otic capsule was responsible for the medial stapedial footplate [4]. Therefore, the complexity of the development makes the concomitant anomalies are not uncommon.

Proper and timely diagnosis is essential to the management of these patients. A complete history, proper examination and hearing assessment could lead to the correct diagnosis. Imaging technique plays a vital role in detecting an ossicular anomaly. To date, High Resolution Computed Tomography (HRCT) is the most accurate diagnostic tool for identifying congenital ossicular anomaly pre-operatively. Often, preoperative HRCT of the temporal bone prior to exploratory tympanotomy provide guidance for otologist before revealing them intraoperatively. The major objectives of HRCT in such cases include the identification of the type of anomaly and determination of surgical correctibility. In this case, we highlight the role of HRCT as a single tool in diagnosing congenital ossicular anomaly. From HRCT, there was separation and dislocation of the incudomalleolar joint and incudostapedial joint. The incus appears abnormal. It was displaced posteriorly. The long and lenticular process of the incus was displaced inferiorly. (Figure 3, 4)

A thorough discussion on exploratory tympanotomy and ossicular reconstructive techniques is beyond the scope of this article. Exploratory tympanotomy and ossiculoplasty is helpful to confirm the diagnosis and produce a functional sound transformer mechanism to improve hearing in such cases. In **conclusion**, HRCT is a reliable diagnostic tool for ossicular anomaly and diagnosis could be obtained without surgery.

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UDC 61

Novel Computed Tomography-based Metric Reliably Estimates bone Strength, Offering Potentially Meaningful Enhancement in Clinical Fracture Risk Prediction

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Abstract

Osteoporosis with resultant fractures is a major global health problem with huge socio-economic implications for patients, families and healthcare services. Areal (2D bone mineral density (BMD) assessment is commonly used for predicting such fracture risk, but is unreliable, estimating only about 50% of bone strength. By contrast, computed tomography (CT) based techniques could provide improved metrics for estimating bone strength such as bone volume fraction (BVF; a 3D volumetric measure of mineralised bone), enabling cheap, safe and reliable strategies for clinical application, and to help divert resources to patients identified as most likely to benefit, meeting an unmet need.

Here we describe a novel method for measuring BVF at clinical-CT like low-resolution (550µm voxel size). Femoral heads (n=8) were micro-CT scanned *ex-vivo*. Micro-CT data were downgraded in resolution from 30µm to 550µm voxel size and BVF calculated at high and low resolution. Experimental mechanical testing was applied to measure *ex vivo* bone strength of samples. BVF measures collected at high-resolution showed high correlation (correlation coefficient $r^2=0.95$) with low-resolution data. Low-resolution BVF metrics showed high correlation ($r^2=0.96$) with calculated sample strength. These results demonstrate that measuring BVF at low resolution is feasible, which also predicts bone strength. Measures of BVF should be useful for

clinically estimating bone strength and fracture risk. The method needs to be validated using clinical CT scans.

Keywords: Osteoporosis, Fragility Fractures, Bone Mineral Density, Bone Volume Fraction, Computed Tomography.

Introduction

Osteoporosis is a systemic condition characterised by compromised bone strength due to abnormality in the amount and/or architecture of bone. The consequence of osteoporosis is a heightened risk of fragility (low trauma) fractures, the outcomes of which are serious morbidity and death (1). The disease is a grave public health concern and estimates suggest that ~200 million people are affected worldwide (2, 3). At present, osteoporosis and associated fracture risk are assessed clinically in terms of bone mineral density (BMD) assessment performed by dual energy x-ray absorptiometry (DEXA). BMD refers to the bone mass per unit area and is employed as an indirect indicator of fracture risk (1). Importantly, BMD does not reflect bone quality, which is an integration of several features influencing skeletal resistance to fractures.

Bone quality depends upon material and structural factors including but not limited to mineralisation, quality of collagen, rate of bone turnover, cortical geometry and trabecular micro-architecture (including trabecular number, thickness, orientation and connectivity) (4, 5). The ability of bone to resist fracture depends not only on the amount of bone but also the spatial distribution of bone mass and the intrinsic properties of the materials that constitute bone which come under the wide ranging concept of bone quality. Bone strength reflects the combination of bone mass and bone structure (6). BMD testing only measures the areal bone mass and does not capture any structural information because images are 2D. Thus BMD is not an adequate surrogate marker of bone strength; it only explains about 50 % of *ex vivo* strength (7).

Given the high socio-economic relevance of fragility fractures, there is a need for a clinical method that can detect specific traits of bone fragility more efficiently than the conventional DEXA-measured BMD. Development of such improved measures of bone strength can allow for identification and assessment of rapidly occurring skeletal changes seen with pathological or age-related deterioration in bone health. 3D imaging techniques including micro-computed tomography (micro-CT), are employed for assessing bone strength *ex-vivo* but are of limited clinical use at present owing to radiation hazards as well as technical issues, high costs and accessibility (8-10). Micro-CT enables *ex vivo* evaluation of various parameters such as volumetric bone mass (expressed as bone volume fraction 'BVF'), which has been identified as a strong determinant of bone strength ($r^2 > 0.8$) (11, 12).

Low-resolution clinical-CT is a non-invasive and accessible imaging technique available to most hospitals. 3D measures such as BVF derived from clinical-CT scans are potentially a useful option for estimating bone strength. Clinical-CT derived measures of skeletal morphology may be especially useful in cancer patients because these patients undergo CT scans for staging the disease and planning treatment, potentially offering low cost, easily accessible and safe technology for predicting bone strength (13).

Aims

To devise a method for measuring BVF of the femoral head trabecular bone at a low resolution (550 μ m voxel size; i.e. comparable to clinical-CT resolution), validate the method against high-resolution (30 μ m voxel size) micro-CT data and determine whether the BVF metrics calculated at low resolution relate to bone strength.

Materials and Methods

Femoral head samples (n=8) were collected post-operatively from patients giving informed consent undergoing hip arthroplasty for osteoarthritis at the Department of Orthopaedic Surgery, Imperial College Healthcare NHS Trust, UK and were obtained from the Imperial College Healthcare Tissue Bank (ICHTB; ref:13004 issued from sub-collection ref:MEDA). ICHTB is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London and approved by the National Research Ethics Service (NRES) to release human material for research (12/WA/0196).

Femoral heads were micro-CT scanned at the Natural History Museum, London, UK using a Nikon (Metris X-Tek) HMX-ST 225 CT system (Nikon Metrology, UK) (14). Specimens were fixed on a styrofoam base and placed onto a turntable inside the scanner for scanning at set parameters of 180kV and 165 μ A with a 0.1mm copper filter. A total of 6315 projections were collected at an angular interval of 0.057°. The resulting scan for each femoral head had 2000 serial cross-sectional views with a cubic voxel size of 30 μ m. Scans were reconstructed using CT Pro 2.2 (Metris X-Tek, UK) and converted to the DICOM (Digital Imaging and Communications in Medicine) file format using VG Studio Max 2.0 (Volume Graphics, Heidelberg, Germany). Data were collected on external hard drives (NTFS format) and analyses were performed on a high power workstation (HP Z800) at the Musculoskeletal Lab, Imperial College London, UK using the BoneJ plugin for bone image analysis in ImageJ software (Java image processing program) (15).

The micro-CT DICOM data files were imported into ImageJ and downgraded in resolution from 30 μ m to 550 μ m voxel size (Figure 1a, 1b). Spheres of trabecular bone were virtually sectioned from all the high and low-resolution 3D scans of the femoral heads using the sphere fitting algorithm in BoneJ. The largest sphere possible was collected about the centre of the femoral head. Frequency distribution plots of the voxel grey values were plotted using the 'histogram' function in ImageJ. Threshold grey values were calculated by finding the trough that separates bone and non-bone peaks (16, 17). Based on this value, a black and white binary image was generated using the 'threshold' function in ImageJ. Trabecular BVF was measured by counting voxels representing bone. The accuracy of lower-resolution metrics was validated against the high-resolution data by plotting scatter graph and calculating correlation coefficient (r^2).

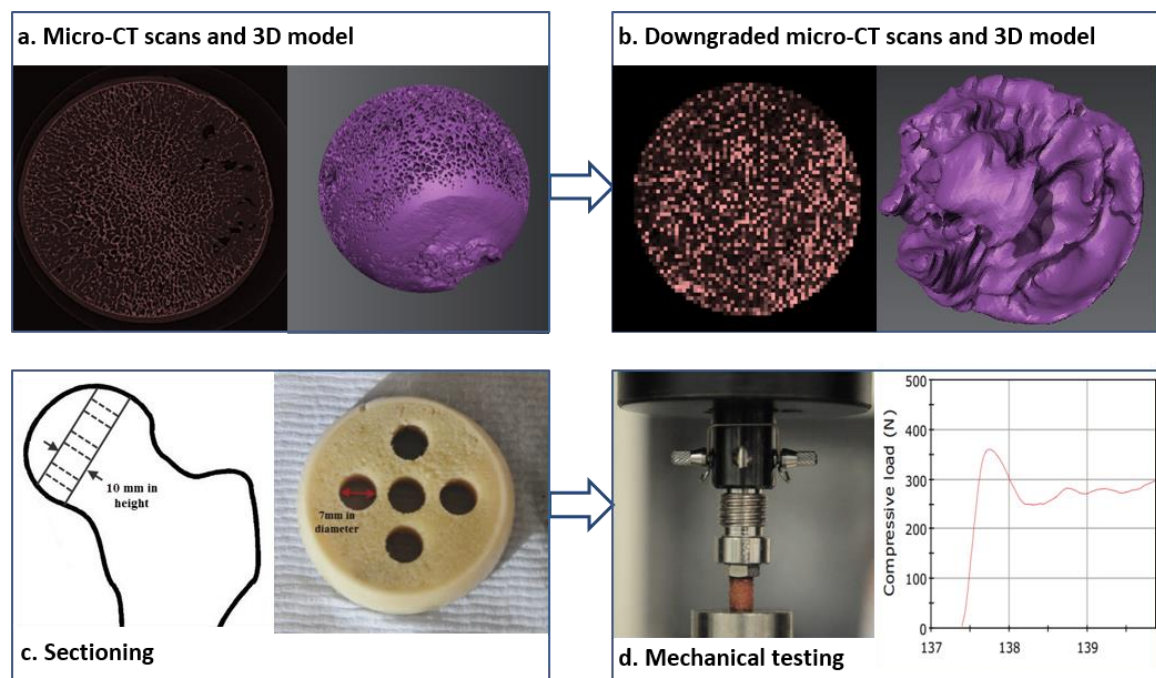


Figure 1 a. A slice of high resolution micro-CT scan and 3D model of femoral head built from it, BVF was measured from these high resolution scans; b. High resolution micro-CT scans were downgraded in resolution from 32 μ m to 550 μ m; 3D model of femoral head built from the low resolution scans, BVF was measured again at low resolution; c. Five cylindrical sub-samples (7mm in diameter, 10mm in height) were sectioned from the locations shown above; d. Two sub-samples were chosen randomly and compressively tested to failure for calculating mechanical strength, mechanical behaviour can be seen from load-displacement graph.

Five cylindrical sub-samples (10mm in height and 7mm in diameter) were drilled from each femoral head (Figure 1c). Two sub-samples from the five were selected randomly and mechanically tested until fracture under uniaxial compressive loading (Figure 1d) using an Instron 5565 mechanical test machine (Instron Engineering Corporation, USA) at the Department of Mechanical Engineering, Imperial College London, UK. The maximum load (the peak at mechanical behaviour

graph shown in Figure 1d, which corresponded with fracture) divided by specimen cross-section, was used to calculate the bone strength of each cylindrical specimen (18).

Results

BVF data collected at high resolution (30 μ m voxel size) were correlated with BVF data at low resolution (550 μ m voxel size). A high positive correlation ($r^2=0.95$) was seen between BVF measures at high and low resolutions (Figure 2).

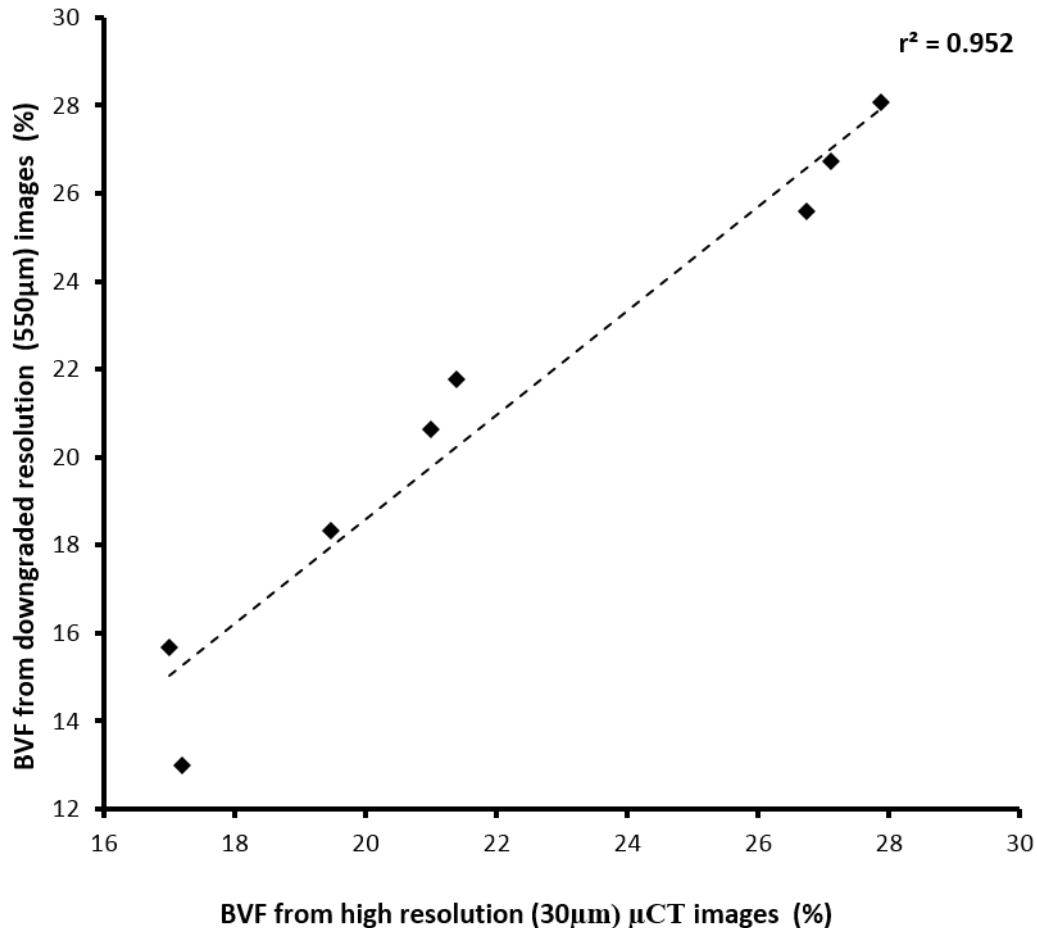


Figure 2. Bone volume fraction (BVF) measured from downgraded resolution images correlated with BVF measured from high-resolution μ CT images

The low-resolution BVF metrics were correlated with the means of the compressive bone strengths calculated *ex vivo* for the two cylindrical specimens from each femoral head. A high positive correlation ($r^2=0.96$) was observed between low-resolution BVF measures and *ex-vivo* bone strength (Figure 3).

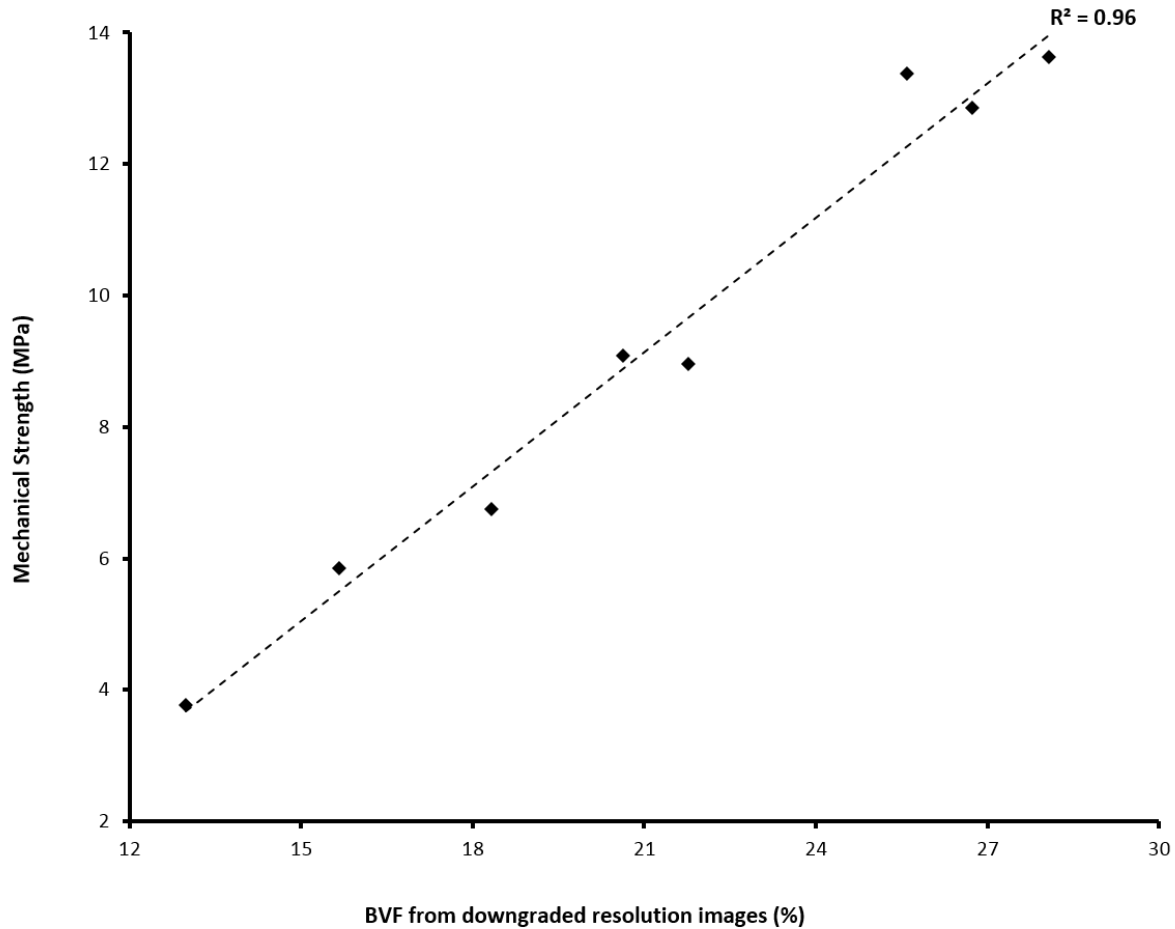


Figure 3. Mechanical strength correlated with bone volume fraction (BVF) measured from downgraded resolution (550 μ m) images

Discussion

BMD measurement using DEXA is the current mainstay for assessing osteoporosis and associated fragility fracture risk (1). However, the 2D DEXA technique only allows for areal measurement of bone mass and does not take into account the contribution of other characteristics such as volumetric bone mass and micro-architecture to bone strength (6). 3D techniques such as high-resolution micro-CT have been used to calculate BVF *ex vivo* but are clinically inapplicable currently due to high radiation exposure (9). Therefore this study explored the potential of using low-resolution clinical-CT for estimating bone strength as most oncological patients receive CT scans clinically for staging tumours and planning interventions.

The study has demonstrated that femoral head trabecular BVF can be calculated (> 95% correlation with BVF measured from high-resolution micro-CT images) from CT images downgraded in resolution comparable to clinical-CT scans of the pelvic region. BVF metrics from these low-resolution scans were also shown to have a 96% correlation with bone strength assessed *ex vivo*. These findings are concordant with results reported by Nazarian et al. who studied biopsy specimens of trabecular bone from spine and/or femur of patients with metastatic prostate, breast, lung, ovarian, or colon cancer (n=41) and non-cancer cadaveric samples (n=96). Specimens were imaged using micro-CT and mechanically tested by uniaxial compression. Measured BVF was shown to account for 84% of bone strength variations in all trabecular bone specimens irrespective of the skeletal site or pathology (12).

The results of the present study show that it is feasible to measure BVF at large voxel size (550 μ m), which is the resolution offered by modern clinical CT scanners. The calculated low resolution measures of BVF are also highly predictive of bone strength assessed *ex vivo* (96%

correlation) whereas BMD has previously been shown to have only a 50% correlation to bone strength (19).

A limitation of the study is the use of low-resolution data downgraded from high-resolution micro-CT data as a simulation of low resolution clinical-CT data. The micro-CT data do not contain the same noise and artefacts that are present in clinical-CT data. Further work using actual clinical CT data for determining BVF and its comparisons with BMD values from DEXA and 'Finite Element Analysis' measures of mechanical behaviour of bone is required to provide more conclusive results (20). Future investigations showing that such clinical CT-based measurements predict fractures better than the current methods may help establish the potential future use of BVF as an easy access, non-invasive and cost-effective method for clinical bone health assessment.

Conclusion

BVF may readily serve as an effective metric for predicting bone strength in cancer patients who routinely undergo CT scans of the pelvis as part of their cancer management regime and changes in bone strength over time can be tracked using BVF measures derived from those CT scans. Timely and accurate identification of high risk patients such as those with diminishing BVF can allow clinicians to modify treatment and/or prescribe bone sparing agents (21). Clinical trials of diet and/or exercise intervention for improving bone health can potentially utilise BVF instead of BMD (22, 23). With advancements in CT technology leading to fall in radiation dosage, this novel metric of bone strength could find widespread clinical use in the future for diagnosing osteoporosis in the general population, predicting fracture risk and monitoring treatment outcomes.

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The Effect of EEG Biofeedback Therapy on Motor Abilities of Children with Attention Deficit Hyperactivity Disorder

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Abstract

Background. Currently, EEG biofeedback (Neurofeedback) is used in the rehabilitation of children with brain damage with the symptoms of attention deficit disorder, hyperactivity and impulsivity. After treatment improvements were observed not only in the control of attention and impulsivity but also in voluntary and involuntary movements. The aim of the prospective clinical study was to measure the impact of EEG biofeedback on motor abilities of children with ADHD (Attention Deficit Hyperactivity Disorder) and compare the effectiveness of EEG biofeedback with classical rehabilitation. It was assumed that in children with ADHD in combination with central motor disorders EEG biofeedback therapy will strengthen not only the control of impulsivity and attention but also motor skills.

Material. The observed group consisted of 60 (N = 60) children with mild central motor disorders with ADHD. They were randomly assigned to either the EEG biofeedback group (N = 30, mean age 8.9 years) or the classical rehabilitation group (N = 30, mean age 8.5 years).

Methods. Both groups received thirty 30-45 minute sessions of training, at a frequency of 2-3 times a week. Pre-post assessment included testing of motor skills with PANESS test (Physical and Neurological Examination for Subtle Signs) for both groups and the EEG biofeedback group were assessed also for changes in impulse and attention control using CPT (Continuous Performance Test) test AX version and changes observed by parents using TLC Subjective Assessment (The Learning Curve, 2004).

Results. Achieved overall score of EEG biofeedback group was lower after therapy (Mdn = 24.00) than before therapy (Mdn = 55.00), $T = 0.00$, $p < 0.01$, $Z = -4.78$, $r = -0.62$. Values of significance (Asymp.Sig. 2-tailed = 0.000) and effect size (effect size $r = -0.62$) indicate a statistical and factual significant positive effect of EEG biofeedback to improve overall motor skills (lower score is better).

Conclusion. EEG biofeedback therapy in children with ADHD improved control of attention, impulsivity and also improved motor skills. There were no significant differences in improvement of performance of timed movements between groups. Also, parents of children who received the EEG biofeedback therapy observed positive changes in behavior, learning and motor skills.

Improvement in motor skills was significantly higher in the EEG Biofeedback group than in the classical rehabilitation group.

Keywords: Effect of EEG, Biofeedback Therapy, Motor Abilities, children.

Introduction

The incidences of mild cerebral dysfunction with motor and coordination disorders and cognitive and emotional deficits in children within the population are high. The American Academy of Pediatrics states that motor skills disorder also known as developmental coordination disorder is usually diagnosed only when motor skills problems significantly interfere with academic achievement or activities of daily living. Motor skills disorder involves a developmental delay of movement and posture that leaves children with coordination substantially below that of others of their age and intelligence level (American Academy of Pediatrics, 2010). These children seem clumsy and awkward and they have problems being accepted in the activities of their classmates. By adolescence, most children with motor skills disorder not only perform poorly in physical education classes, but may also have a poor physical self-image and perform below expectations academically. Therefore, new methods are being sought which will, together with classical rehabilitation, contribute to minimize the handicap of these children. One of these methods is EEG biofeedback. EEG biofeedback is a method, which specifically helps in the rehabilitation of neurological and psychological disorders. Its clinical application includes the treatment of attention deficit disorders, epilepsy, learning disorders, obsessive compulsive disorders and alcohol addiction. Meanwhile it has assumed a role in achieving optimal performance in optimizing musical and interpretative skills of students (Egner, a iní, 2003), in sports performance (Landers, et al., 1991) (Vernon, 2005), optimizing microsurgical skills in eye surgery (Ros, et al., 2009) and in NASA research aimed at minimizing pilot errors (Prinzel, et al., 2002).

Our facility is dedicated to the rehabilitation of children with different diagnoses. We are using a variety of rehabilitation equipment and devices. Also we are providing EEG biofeedback for attention, impulsivity and hyperactivity disorders. Children are sent to our facility by pediatrics when they have problems with behavior in school or their academic achievements are poor. Our results in this field are conforming to many studies that have examined the impact of EEG biofeedback on the improvement of attention and hyperactivity in children. Moreover we have also received positive feedback from the side of the parents. Parents reported not only about improvements in attention and hyperactivity but also in motor skills as well. We made measurements of motor skills and attention and hyperactivity before and after EEG biofeedback in order to confirm this. For motor skills we have used PANESS test (Physical and Neurological Examination for Subtle Signs – author M. Denckla, (1985)) and for attention and hyperactivity CPT (Continuous Performance Test) test AX version. Both tests have very good test re-test reliability and are very easy to perform.

The aim of the work was to measure the effect of EEG biofeedback therapy on motor, cognitive and emotional deficits of children with brain injury of different levels.

Material and methods

Material

60 children of both sexes were included into the prospective clinical study. They were sent to our rehabilitation facility by pediatric neurologists having motor disorders in combination with a diagnosis of ADHD-HI (predominantly hyperactive-impulsive) and ADHD-PI (predominantly inattentive) (Ramsay, 2007). The whole set was divided by randomized selection into two groups: EEG Biofeedback group (N = 30, mean age 8.90 years and the range 7-12, SD=1.539) and a group receiving classical rehabilitation (N = 30, mean age 8.50 years and range 7-11, SD=1.306). Before starting the treatments we obtained written consent from the legal guardians of children for them to participate in the study as well as division into groups. In EEG biofeedback group there were 25 boys and 5 girls. In classical rehabilitation group there were 19 boys and 11 girls. 20 boys and 2 girls were diagnosed with ADHD-HI in EEG biofeedback group and 13 boys and 7 girls in group with classical rehabilitation. ADHD-PI diagnosis had 5 boys and 3 girls in EEG biofeedback group and 6 boys and 4 girls in group with classical rehabilitation.

Inclusion criteria

The clinical study included children having diagnoses according to ICD-10 - other abnormalities of gait and mobility (R26.8), other lack of coordination (R27.8), abnormal posture (R29.3), cerebral palsy (G80.0) light forms, attention-deficit hyperactivity disorders (F90.0), ADHD according to DSM III. respectively DSM IV. and aged from 7 to 12 years.

Exclusion criteria

Children with a positive history of head trauma, a history of epilepsy, metabolic syndrome, a severe form of cerebral palsy, medical therapy (based on the assessment of a pediatric neurologist or neurologist), children with average IQ undersized by WISC III. set by psychologist (Category B and C).

The study was conducted in accordance with ethical principles based on the Declaration of Helsinki (1964).

Methods

Research was conducted during the period of September 2008 to May 2011 at the Rehabilitation Centre Harmony, Kudlakova 2, Bratislava. Both groups received thirty 30-45 minutes sessions of training, at a frequency of 2-3 times a week.

EEG biofeedback group

We used the following training protocols with the children who have undergone EEG biofeedback rehabilitation. Protocols have been proven in controlled clinical studies (Monastra, et al., 2005).

PROTOCOL 1: "SMR up/Theta down"

Patients are learning to strengthen control of impulsivity by learning to increase the production of SMR (12-15 Hz, respectively from the zone of enhanced SMR 12-19 Hz) to C3 or C4 while at the same time suppressing theta rhythms (4-7 Hz or 4-8 Hz). Auditory (tones) and visual feedback (control of simple video game) is provided according to the patient's success in suppressing theta or increasing SMR (below the threshold theta or above the threshold SMR). This protocol was also used in the first controlled common study of the effectiveness of the EEG biofeedback for ADHD (Rossiter, et al., 1995).

PROTOCOL 2: "SMR up/Beta2 down"

In this protocol, patients with ADHD, predominantly hyperactive-impulsive type, were trained to increase SMR (12-15 Hz) while inhibiting beta2 (22-30 Hz). This protocol was administrated for 15 minutes in patients with combined type of ADHD. In the second half of the session was increased beta1 and suppressed theta activity on C3. This type of SMR training was tested in controlled study (Fuchs, et al., 2003).

PROTOCOL 3: "Theta down/Beta1 up"

In this training procedure, patients learn to increase the production of beta1 activity (16-20 Hz), whereas the theta activity (4-8 Hz) is inhibited. Fuchs et al. (2003) used a variation of this protocol in patients with ADHD predominantly inattentive type while training inhibition of theta and beta strengthening on C3. If the in-training found increased aggression or hyperactivity in the range of 13 to 35 training protocol was assessed as "hyper stimulated" and indicated a SMR training with the strengthening of 13-15 Hz and suppression of 2-7 Hz. This protocol has been studied in published works by Linden, Habib, Radojevic, (1996); Monastra, Monastra, & George, (2002); Rossiter & LaVaque (1995).

Group with classical rehabilitation

Children received kinesiotherapy, which included relaxation exercises, strengthening exercises, exercises for developing pattern movement quality, proprioceptive stimulation of soles of the legs.

Pre-post assessments of motor skills of the children in both groups were assessed with revised Physical And Neurological Examination for Soft Signs by Martha Denckla (1985) – **PANESS**. This test is very easy to use - examiner needs only stopwatch and assessment form and it takes only about 30 minutes. Test variables are: lateral preference, gaits, balance, motor persistence, coordination, overflow, dysrhythmia, and timed movements (repetitive and patterned).

Pre-post assessment of EEG Biofeedback group was enhanced with following tests:

1. Attention and impulsivity – Continuous Performance Test AX version (AX-CPT) provides data on attention deficit disorder and impulsivity control (Rosvold, et al., 1956).
2. The TLC Subjective Assessment (The Learning Curve, 2004) - parents subjective assessment of the problems of the child. The test contains 144 items.
3. The TLC Objective Assessment (The Learning Curve, 2004) - the process of objective assessment of EEG.

PANESS test

Lateral preference.

Lateral preference (hand, foot, and eye) was assessed by asking the child to demonstrate various lateralized tasks:

- the hand (show me how you: comb your hair, brush your teeth, cut with scissors, throw a ball, hit a ball with a bat, hit a ball with a racket, use a hammer, use a screwdriver, use a saw, flip a coin, and open a door with a key,
- the foot (show me how you: kick a soccer ball and stamp out a fire),
- the eye (show me how you: look through the lens of a camera).

Gaits

Children were asked to walk 10 steps over the line on toe, heels, sides of feet and tandem walk forward and backward (toe to heel).

Balance

Children were asked to hop on one foot (maximum 50 times) and stand on one foot for 20 seconds.

Attention and impulsivity – Continuous Performance Test AX version (AX-CPT)

The original CPT was developed in 1956 by Rosvold with his colleagues and although the original X-CPT had adequate classification accuracy, the classification accuracy improved with the more difficult AX-CPT version. We used AX version in our study. Children had to hit the keyboard when the letter X appeared but only if the X was immediately preceded by the letter A. Achieved points were converted to respective age (years) and compared (higher achieved age by output test is better).

The TLC Subjective Assessment

The test contains 144 items and assesses psychosocial characteristics of the child using a seven-point scale. Parents filled the questionnaire before and after EEG biofeedback rehabilitation. Questionnaire provides: suggestions for training protocols, subjective assessment of the problems of the child and impact of the therapy from the perspective of parent or guardian.

The TLC Objective Assessment

Because it is simple and relatively unassuming (30-45 minutes), the process of objective assessment can be repeated as often as needed. Using this assessment tool we can obtain important information for initial diagnosis and planning of the training. In our research we used software BioExplorer with the device "Pendant EEG" supplied by Australian "Pocket Neurobics" for data collection and the training EEG biofeedback. Pendant EEG is connected to a PC via USB or wireless over a short distance (3m), with dual-channel wiring electrodes.

Statistical analysis

We tested data for normality with Kolmogorov-Smirnov test and variation with Leven test. According to the results of these tests we used the Student pair t-test for parametric statistic and Wilcoxon signed rank test for non-parametric statistic. The same was applied for correlation – Pearson's test for parametric and Spearman's test for non-parametric statistic. Finally we calculated effect size.

Theory

In recent years EEG biofeedback training has been applied to an increasing number of psychological, neurological, and psychosomatic conditions. Sensorimotor (SMR) and Beta

neurofeedback (one of the training forms focused on immediate enhancement intermediate frequency amplitude) achieved very good results in treatment of not only epilepsy, but also in attention deficit hyperactivity disorder, specific learning disorders and several other conditions associated with ADHD, for example bruxism, tics, mood swings. Subjectively SMR approaches the alpha rhythm, but it is more centered on the body feeling - awareness of the body in a particular environment and readiness, which interact with the environment. High levels of SMR are a characteristic of motion talented and trained people. In the absence of SMR problems may occur with body sensations (low pain threshold, less control of the body functions). The obvious indicator of the minimum SMR is sleeping without physical exhaustion. SMR is described as a key frequency for effective performance of the mind and body (Faber, 2001). SMR training stimulates self-regulation and control functions (particularly motor control, sensors and affectivity, which stabilizes and facilitates perceptual-cognitive skills) and beta training executive functions (Tyl, et al., 2002). Head injury, multiple sclerosis, autism, chronic fatigue syndrome and the premenstrual syndrome, are only a few examples of the still growing list of conditions reported by clinicians to be partly or fully remediated by SMR-beta neurofeedback training.

The apparent diversity of disorders impacted by SMR-beta neurofeedback training suggests a commonality of mechanisms for these conditions, a fact that should be addressed by any theory that attempts to identify the therapeutic mechanism of SMR-beta neurofeedback. Sterman (1982) proposed that SMR neurofeedback may restore regulated function of thalamocortical mechanisms associated with arousal. In particular, abnormal sensorimotor arousal or excitability may interfere with higher cognitive functions in a resource-limited competitive model (Sterman, 1996). Abarbanel (1995) formulated a similar model of self-regulation in which attentional processing were modulated by thalamocortical and limbic circuitry. In his model long-term potentiation was responsible for any functional permanence associated with training. Both models presume SMR-beta neurofeedback impacts functions that modulate arousal (Sterman, 1982); (Abarbanel, 1995). Both models readily address the symptomatology and possible mechanisms of ADHD and epilepsy. The primary symptoms of ADHD, inattention, impulsivity, or hyperactivity, are associated with decreased arousal in frontal cortex and subcortical regions (Zametkin, et al., 1990); (Mann, et al., 1992). The cortical hyperexcitability associated with epilepsy may reflect an arousal dysfunction, possibly due to a loss of integrity in the thalamic gating mechanism (Sterman, 1982). In addition to motor or vocal tics, sufferers of Tourette's Syndrome often exhibit somnambulism, night terrors, and other disorders of arousal (Barabas, 1984). Attentional processes in particular appear to be uniquely sensitive to problems of arousal, and thus they serve to be a good measure of effectiveness in restoring such functions.

A pioneering collaboration between two laboratories from the University of London has provided the evidence of neuroplastic changes occurring directly after natural brainwave training. Researchers from Goldsmiths and the Institute of Neurology have demonstrated that half an hour of voluntary control of brain rhythms is sufficient to induce a lasting shift in cortical excitability and intracortical function. These after-effects are comparable in magnitude to those observed following interventions with artificial forms of brain stimulation involving magnetic or electrical pulses (Ros, et al., 2010).

Results

Laterality

In the group of children with EEG biofeedback (EEG group) were 29 right-handers (97%) and 1 mixed (3%). In the group of children with classical rehabilitation (control group) were 27 right-handers (90%), 2 left-handers (7%) and 1 mixed (3%).

Gaits and station

Results in gaits and station were better in EEG group then in the control group. Children in EEG group made fewer errors and involuntary movements after EEG biofeedback training as children in the control group. EEG biofeedback group achieved significantly lower score ($M=16.17$, $SD=6.46$) after therapy than before therapy ($M=28.90$, $SD=8.72$), $t(29)=9.81$, $p<0.05$, $r=0.88$. Significance value (Sig. 2-tailed=0.000) and effect size ($r=0.88$) indicates a high positive effect of EEG biofeedback in reduction of errors, involuntary movements and increased motor persistence. Control group achieved score after therapy was higher ($M=23.83$, $SD=5.97$) than before therapy ($M=21.27$, $SD=5.89$), $t(29)=-2.13$, $p<0.05$, $r=0.37$. Children in control group did not show any improvement after rehabilitation.

TABLE 1: GAITS AND STATION

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	28.9	1.592	29.5	8.719	11	60
	after	30	16.17	1.178	16	6.455	3	30
Control group	before therapy	30	21.27	1.075	21	5.889	9	32
	after	30	23.83	1.089	23.5	5.966	12	36

Dysrhythmia

Results in Dysrhythmia were better in EEG group then in the control group. EEG biofeedback group achieved a score after therapy that was significantly lower (Mdn=1.00, SD=1.80) than the score before therapy (Mdn=2.50, SD=2.97), $T=38.00$, $p<0.05$, $Z=-3.23$, $r=-0.42$. Significance value (Sig. 2-tailed=0.001) and effect size ($r=-0.42$) indicates a positive effect of EEG biofeedback in reduction of symptoms of dysrhythmia. Control group achieved a score after therapy that was lower (Mdn=1.50, SD=3.19) than the score before therapy (Mdn=2.00, SD=2.39), $T=163.50$, $p>0.05$, $Z=-0.78$, $r=-0.10$. The result of classical rehabilitation in reduction of symptoms of dysrhythmia is statistically not significant.

TABLE 2: DYSRHYTHMIA

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	3.53	0.542	2.5	2.968	0	10
	after	30	1.7	0.329	1	1.803	0	5
Control group	before therapy	30	2.87	0.436	2	2.389	0	9
	after	30	2.6	0.583	1.5	3.191	0	12

Overflow movements

Results in overflow movements were better in EEG group then in control group. EEG biofeedback group achieved score after therapy was significantly lower (Mdn=6.00) than the score before therapy (Mdn=12.50), $T=13.50$, $p<0.05$, $Z=-4.51$, $r=-0.58$. Significance value (Sig. 2-tailed=0.000) and effect size ($r=-0.58$) indicates a positive effect of EEG biofeedback in reduction of overflow. Control group achieved score after therapy was lower (Mdn=15.50, SD=6.10) than the score before therapy (Mdn=16.00, SD=5.39), $T=167.50$, $p>0.05$, $Z=-4.52$, $r=-0.07$. The result of classical rehabilitation in reduction of overflow movements is statistically not significant.

TABLE 3: OVERFLOW MOVEMENTS

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	13.53	1.194	12.5	6.538	2	29
	after	30	6.33	0.667	6	3.651	0	17
Control group	before therapy	30	16.03	0.983	16	5.385	3	28
	after	30	16.57	1.114	15.5	6.101	7	32

Repetitive movements

Results in repetitive movements were better in EEG group then in the control group. EEG biofeedback group achieved an after therapy score that was significantly lower (Mdn=3.00) than the before therapy score (Mdn=7.00), $T=20.50$, $p<0.05$, $Z=-4.16$, $r=-0.54$. Significance value (Sig. 2-tailed=0.000) and effect size ($r=-0.58$) indicates a positive effect of EEG biofeedback in reduction of errors in repetitive movements. The control group achieved an after therapy score that

was higher (Mdn=7.50, SD=4.35) than before therapy score (Mdn=7.00, SD=3.17), $T=126.50$, $p>0.05$, $Z= -1.75$, $r=-0.22$. The result of classical rehabilitation in reduction of errors in repetitive movements is statistically not significant.

TABLE 4: REPETITIVE MOVEMENTS

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	8.23	1.079	7	5.911	0	26
	after	30	3.1	0.427	3	2.339	0	10
Control group	before therapy	30	6.73	0.579	7	3.172	1	13
	after	30	8.03	0.794	7.5	4.351	1	18

Patterned movements

Results in patterned movements were better in EEG group than in the control group. EEG biofeedback group achieved an after therapy score that was significantly lower (Mdn=5.00, SD=4.50) than the before therapy score (Mdn=19.50, SD=13.52), $T=6.00$, $p<0.01$, $Z=-4.66$, $r=-0.60$. Significance value (Sig. 2-tailed=0.000) and effect size ($r=-0.60$) indicates a positive effect of EEG biofeedback in reduction of errors in patterned movements. The Control group achieved an after therapy score that was higher (Mdn=13.50, SD=4.81) than the before therapy score (Mdn=13.00, SD=4.51), $T=175.50$, $p>0.05$, $Z= -0.325$, $r=-0.22$. The result of classical rehabilitation in reduction of errors in patterned movements is statistically not significant.

TABLE 5: PATTERNED MOVEMENTS

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	21.43	2.469	19.5	13.521	6	67
	after	30	6.6	0.822	5	4.5	0	17
Control group	before therapy	30	13.9	0.824	13	4.513	8	31
	after	30	13.97	0.878	13.5	4.81	7	28

Overall time of timed movements

Overall time comprises the time of repetitive and patterned movements and tongue wagging. EEG biofeedback group achieved time after therapy was significantly lower (Mdn=88.95, SD=18.26) than before therapy (Mdn=101.60, SD=25.84), $T=36.00$, $p<0.01$, $Z=-4.04$, $r=-0.52$. Significance value (Sig. 2-tailed=0.000) and effect size ($r=-0.52$) indicates a positive effect of EEG biofeedback on decreasing of achieved time. The control group achieved time after therapy was significantly lower (Mdn=107.85, SD=21.93) than before therapy (Mdn=137.06, SD=23.57), $T=16.00$, $p<0.05$, $Z= -0.325$, $r=-0.22$ indicates a positive effect of classical rehabilitation on decreasing of achieved time.

TABLE 6: OVERALL TIME OF TIMED MOVEMENTS

		N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
EEG group	before therapy	30	111.78	4.72	101.6	25.84	81.1	188.9
	after	30	95.02	3.33	88.95	18.26	74.8	145
Control group	before therapy	30	132.79	4.3	137.06	23.57	84.8	189.7
	after	30	110.58	4	107.85	21.93	76.9	157.7

Attention and impulsivity – Continuous Performance Test AX version (AX-CP)

Continuous performance test AX version was only performed on children from the EEG biofeedback group. Achieved age of attention was significantly higher after therapy (Mdn = 11.00) than before therapy (Mdn = 9.00), $T = 0.00$, $p < 0.01$, $Z = -4.349$, $r = -0.56$. Values of significance (Asymp.Sig. 2-tailed = 0.000) and effect size (effect size $r = -0.56$) indicate positive effect of EEG biofeedback on improvement of attention.

Achieved age of impulsivity was significantly higher after therapy (Mdn = 9.00) than before therapy (Mdn = 7.00), $T = 0.00$, $p < 0.01$, $Z = -4.417$, $r = -0.57$. Values of significance (Asymp.Sig. 2-tailed = 0.000) and effect size (effect size $r = -0.57$) indicate positive effect of EEG biofeedback on improvement of impulsivity control.

TABLE 7: CONTINUOUS PERFORMANCE TEST AX VERSION

	N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
Before - attention	30	9.23	0.278	9.00	1.524	6	13
After - attention	30	10.97	0.341	11.00	1.866	6	13
Before impulsivity	30	7.73	0.310	7.00	1.701	6	11
After - impulsivity	30	9.57	0.361	9.00	1.977	6	13

The TLC Subjective Assessment (The Learning Curve. 2004)

The subjective assessment was only performed on children from EEG Biofeedback group. Achieved score was significantly lower after (Mdn = 344.00) than before therapy (Mdn = 386.50), $T = 2.00$, $Z = -4.74$, $p < 0.05$, $r = -0.61$. Value of significance and effect size (effect size $r = -0.57$) indicate positive effect of EEG biofeedback on improvement from the point of view of their parents.

TABLE 8: PARENTS SUBJECTIVE ASSESSMENT

	N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.
Input	30	415.83	12.168	386.50	66.647	319	552
Output	30	350.63	13.188	344.00	72.234	212	503

Overall score of PANESS test

We have assessed achieved scores of all subtests before and after EEG biofeedback therapy and kinesiotherapy except laterality.

TABLE 9: OVERALL SCORE OF PANESS TEST

	N	Mean	Std. Error	Median	Std. Deviation	Min.	Max.	
EEG group	before training	30	58.57	3.675	55	20.128	26	108
	after	30	25.87	1.894	24	10.371	4	49
Control group	before training	30	41.9	1.371	42.5	7.508	20	59
	after	30	45.83	2.077	42.5	11.378	29	72

Overall scores were better in EEG group then in the control group. EEG biofeedback group achieved significantly lower scores after neurofeedback therapy (Mdn=24.00) than before therapy (Mdn=55.00), $T=0.00$, $p < 0.01$, $Z = -4.78$, $r = -0.62$. Significance value (Sig. 2-tailed=0.000) and effect size ($r = -0.62$) indicates a positive effect of EEG biofeedback in overall improvement in motor skills. Control group achieved scores before and after kinesiotherapy that were the same

(Mdn=42.50, SD=4.35), $T=144.50$, $p>0.05$, $Z= -1.58$, $r=-0.20$. There were no improvements in followed parameters.

Discussion

The aim of the prospective clinical study was to measure the impact of EEG biofeedback on motor abilities of children with ADHD (Attention Deficit Hyperactivity Disorder) and compare the effectiveness of EEG biofeedback with classical rehabilitation. EEG biofeedback therapy in children with ADHD improved control of attention, impulsivity and also improved motor skills. Improvement in motor skills was significantly higher in the EEG Biofeedback group than in the classical rehabilitation group.

Up to 52 % of children with ADHD are characterized as children with impaired motor coordination (Barkley, et al., 1990a) (Barkley, 1990c), (Hartsough, et al., 1985), (Szatmari, et al., 1989b), which particularly relates to tasks requiring fine motor movements. In healthy children and adults inhibition and facilitation mechanisms are in balance, used to control and regulate the voluntary and involuntary movements, but also emotions and other manifestations. If these mechanisms do not work properly, voluntary and automatic motoric will deteriorate and, moreover, subtle or pronounced uncontrollable movements, hyperkinesia and overflow movements appear (Samson, 2011).

Larson (2007) defined the overflow movement as co-movement of body that is not needed to perform the task. As typically children mature, they manifest less imprecise movements (Largo, et al., 2003). Among overflow movements, the most studied are mirror movements (also referred to as synkinesis). The presence of mirror overflow movements in adolescents and adults with disorders of both the motor cortex and the corpus callosum suggests that the ability to perform unilateral fine motor movements is dependent upon intact interhemispheric and corticospinal connections (Knyazeva, et al., 1997); (Meyer, et al., 1998); (Nass, 1985)). Using transcranial magnetic stimulation (TMS) investigators have demonstrated that transcallosal inhibition is absent in children under 6 years of age and that it gradually matures to adult levels by early adolescence (Garvey, et al., 2003) (Heinen, et al., 1998). Thus, when intra and inter-cortical inhibitory and excitatory systems are immature, overflow movements in children are at their peak; as these cortical systems mature, overflow movements are more difficult to elicit. The persistence of overflow into late childhood and adolescence, often seen in children with ADHD (Morris, et al., 2001); (Mostofsky, et al., 2003) and other developmental disabilities suggests a neurodevelopmental lag in systems supporting the inhibition of overflow. Choreiform movements are characterized by involuntary random, jerking motions, most often in the extremities (Delgado, et al., 2003), and often described as “dance-like” movements. Choreiform movements suggest lapses in postural control and implicate immaturity of the postural system. They can affect execution of motor tasks, contributing to dysgraphia and fatigue during writing (Denckla, 1997). Wolff and Hurwitz (1973) found choreiform movements to be more prevalent in children who were reported to be inattentive, disorganized and immature, positing that the presence of this subtle sign may implicate “minimal brain dysfunction.”

Overflow movements were recorded by all tests. A summary of all overflow movements pre and post treatment gave us information concerning their reduction. When comparing groups of children treated with EEG biofeedback and children treated with conventional rehabilitation, we observed significant differences in favor of EEG biofeedback.

Pattern movements

Mostofsky (2003) used the functional magnetic resonance imaging (fMRI) to determine differences in the brain activation during finger sequences in children with ADHD and children with normal development. The group consisted of 11 children with ADHD and 11 children with normal development. All children have a right-handed laterality. Groups showed no significant difference when measuring the speed of finger sequence but contralateral primary motor cortex (Brodmann Area 4) and right parietal cortex, showed significantly less activity throughout the fMRI during finger sequences with left and right hand in children with ADHD. This finding shows that children with ADHD have anomalous cortical development system needed to implement the pattern movement. This finding correlates with our results where in subtest pattern movement of PANESS was the sum of all errors high.

Gaits and station

Hammond (2005) presents the results of successful treatment of balance disorders in 4 patients. Two patients aged 32 and 50 years after a light head injury and two patients after suffering a stroke aged 45 and 46 years had trouble keeping their balance. In case reports were observed improvement in motor skills in standing, standing with closed eyes and tandem walking after EEG biofeedback. Similar results were also found in our study, except that it was a) a larger group of patients, b) significantly younger age set, c) set with a different diagnosis, d) set with mild disabilities, e) set with disabilities both hemispheres.

Dysrhythmia

Dysrhythmia is an abnormality in an otherwise normal pattern of movements; it can be seen as an improper rhythm or timing of the movement. Dysmetria is the failure to focus the trajectory of an intentional movement (extremity coordination) and whereas an intention tremor, produced by goal-directed motor movements, involves increased rhythmic oscillation at a right angle to the line of movement as the target is approached (Larson, et al., 2007). In our study, we evaluated dysrhythmia separately. Achieved scores of children after EEG biofeedback was significantly lower after treatment (Mdn = 1.00) than before treatment (Mdn = 2.50).

Timed movements

Speed of repetitive movements (toe tap, hand pat and finger tap) and patterned movements (heel-toe, hand pronate/supinate and finger sequences) we interpreted either as a sign of delayed development or manifestation of impaired development in the context of the causes that led to the formation of cerebral palsy (damage during pregnancy, hypoxia during labor, postpartum damage etc.). Wolff (1985) reported age-related improvement in the speed of repetitive and patterned movement tasks executed with hand, foot and fingers. Examining the same timed repetitive movements, Denckla (1973); (1974)) found that speed of performance improves with age and begins to plateau between ages 8–10 years. Largo et al. (2003) also reported age-related improvement performance of repetitive and patterned hand and finger movements; however, their findings suggested that speed of hand movements does not plateau until puberty, and speed of sequenced finger movements continues to improve beyond 18 years of age. In our study we didn't observe any major differences in timed movements between the group with EEG biofeedback and the group with classical rehabilitation. Both groups performed better after therapies. However, we have noticed that in EEG group in some cases the speed of performance dropped but on the other hand also errors dropped. It is obvious that these children paid more attention to performing the tasks without any errors than to increase the speed of performance.

Attention and impulsivity

The positive effect of EEG biofeedback to influence attention, impulsivity and hyperactivity has been studied over the years by a number of authors. Arns (2009) reported a meta-analysis of fifth level, comparing studies published in scientific journals or as part of a dissertation. (To meet the criteria for fifth level classification it is necessary to show that the treatment is in terms of statistics better than placebo therapy, reliable medical therapy or other treatments regarded as positive in at least two independent studies.) There was included prospective controlled studies ((Rossiter, et al., 1995); (Monastra, et al., 2002); (Fuchs, et al., 2003); (Heinrich, et al., 2004); (Levesque, et al., 2006); (Bakhshayesh, 2007); (Drechsler, et al., 2007); (Gevensleben, et al., 2009), (Holtman, et al., 2006)) and studies using pre-post design (Kropotov, et al., 2005); (Xiong, et al., 2005); (Strehl, et al., 2006); (Leins, et al., 2007). Both groups of studies confirmed statistically significant effect size (ES) of EEG biofeedback on impulsivity and attention and middle ES on hyperactivity. Average ES for attention was 0.8097 and for impulsivity 0.6862. These results are identical with our findings because ES for attention was ($r = -0.56$) and for impulsivity ($r = -0.57$) with statistical significance ($p < 0.01$) for both parameters in our group of children with EEG biofeedback.

Overall score of PANESS test

We know so far of case reports that describe the efficiency of the motor skills in children but with severe motor deficits (Hammond, 2007); (Ayers, 2004); (Bachers, 2004). Empirical experience (achievement of walk alone of a 5 year old child after EEG biofeedback, which walked with compensation aids) led us to investigate the effect of EEG biofeedback on motor function. Due to the fact that we wanted to verify the quality level we have chosen as the material children with mild degrees of central motor disorders and also cognitive deficits. The results achieved in

individual subtests of Paness test we have summarized in the total score, which reflects the overall effect of EEG biofeedback on child's motor skills. Final score of EEG biofeedback group was lower at the end of therapy (Mdn = 24.00) than in the beginning of therapy (Mdn = 55.00). The values of significance (Asymp.Sig. 2-tailed = 0,000) and the effect size ($r = -0.62$) indicate a very good factual and statistical significant positive effect of EEG biofeedback on overall improvement. Group with classical rehabilitation achieved scores was the same after therapy (Mdn = 42.50) as before therapy (Mdn = 42.50) - there were no improvement.

Parent's subjective assessment

Core light brain dysfunction (attention deficit disorder and hyperactive syndrome) is diagnosed by behavioral markers. Bragdon (2006) reports diagnosis by the American Diagnostic and Statistical Manual of Mental Disorders (DSM IV), which is the result of numerous epidemiological and clinical studies and gives for determination diagnosis an exact list of characters. Diagnosis of ADHD according to DSM IV is based on historical data, withdraw from a parent or guardian, or teacher. Subjective assessment of the child's behavior before EEG biofeedback therapy and after 30 sessions we evaluated on the basis of an 144 item questionnaire. Individual items of the questionnaire surveyed in detail cognitive, behavioral and motor aspects of the child from the parent's perspective. From their perspective there has been an improvement after the end of EEG treatment in the cognitive and behavioral aspects as well as motor skills of the child.

Conclusions

Rehabilitation of children with central motor disorders is one of the fundamental measures in their comprehensive care. Kinesiotherapy, however, is not the only system in the framework of the rehabilitation. A holistic approach is necessary, and the right knowledge about behavioral disorders combined with motor deficit can significantly affect the success of rehabilitation. The aim of the work was to determine whether the use of EEG biofeedback will strengthen the control of the impulsivity and the attention and at the same time will have an effect on motor skills. The results of the study demonstrated:

1. strengthening control of attention and impulsivity in a group of children between 7 and 12 years with a central motor disorders in combination with ADD and ADHD,
2. improvement of motor skills in postural functions (walking, standing and balance), voluntary movements (repetitive movements and pattern), the involuntary movements (reducing errors and overflow movements) and speed of movement
3. in the group of classical rehabilitation, improvement was observed only in the timed movement,
4. from child's parent perspective there was positive changes in behavioral and motor disorders after EEG biofeedback therapy,
5. EEG biofeedback is a method that clearly makes the process of rehabilitation more efficient, contributes to the patient care, and improves his health and quality of life.

This is the first study that uses mutual comparison of neurofeedback with classical rehabilitation, and clearly demonstrated the effectiveness of neurofeedback. This result can be interpreted as a result of the creation of new neural connections and demonstration of the plasticity of the nervous system. Its result is a significant improvement in motor and coordination abilities of the patient as well as significant improvement in impulsivity and attention.

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Effect of Zinc Oxide Nanoparticles on *Candida albicans* of Human Saliva (*in vitro* study)

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Abstract

The potential use of zinc oxide and other metal oxide nanoparticles in biomedicine are gaining interest in the scientific and medical communities, largely due to the physical and chemical properties of these nanoparticles, therefore there is an urgent need to develop new classes of antimicrobial agents, and recent studies demonstrate that hold a considerable promises. *Candida albicans* were isolated from saliva of forty eight volunteers of both sexes their age range between 18-22 years and then purified and diagnosed according to morphological characteristic and biochemical tests. Different concentrations of ZnO NPs were prepared from the stock solution; all the experiments were conducted *in vitro*. Disk diffusion method was used to study the sensitivity of *Candida albicans* to different concentrations of zinc oxide nanoparticles in comparison to effect of de-ionized water. *Candida albicans* were sensitive to all cocentrations (0.01, 0.05, 0.1, 0.5, 1, 3 and 5.8 mg/ml) of the zinc oxide nanoparticles solution in comparison to de-ionized water, revealing a highly significant difference in all concentrations. This study revealed that zinc oxide nanoparticles were effective against *Candida albicans*.

Keywords: *Candida albicans*, ZnO NPs, Human saliva.

Introduction

Nanotechnology is referred to the emerging technology involving fabrication or application of nanosized structures or materials [1]. Nanoparticles are commonly defined as particles with the size of at least one dimension ranging from 1 to 100 nm, which serve as a bridge between bulk materials and atoms/molecules [2]. Zinc oxide nanoparticles [ZnO NPs], is an inorganic white powder insoluble in water [3]. Zinc oxide nanoparticles have been shown to be useful antibacterial and antifungal agents when used as a surface coating on materials and textiles [4]. ZnO nanorod arrays diminished the growth of *Candida albicans* with stable action for two months [5]. *Candida albicans* is a commensal fungal species commonly colonizing human mucosal surfaces [6]. *Candida* infections can be seen in various parts of the body, including the skin, oral cavity, gastrointestinal tract and vagina. In the oral cavity *Candida* infection is sometimes visible as thrush, white/yellowish cream like patches on the oral mucosa and the tongue [7]. The incidence of these organisms appears to increase with the age. These fungi can cause characteristic infections that may be quite serious and

even life threatening [8]. Some studies have shown a significant association between *C. albicans* and dental caries in children and young adults [9]. The findings from *in vitro* and animal studies attributing a role for *C. albicans* in caries development and/or progression were solidified by data from a clinical study where the occurrence of caries in children was positively correlated with the frequency of oral candidal carriage [10]. ZnO NPs have shown to have a photo-catalytic effect, which is related their effectiveness as inhibitor of bacteria and fungi [11].

Material and methods

Collection of Saliva Samples

Collection of stimulated salivary samples were taken from forty eight apparently healthy college students from Baghdad University/Collage of Dentistry; their age range between 18-22 years of both sexes. The collection of stimulated salivary samples was performed under standard condition following instruction cited by Tenovou and Lagerlof [12]. Each individual was asked to chew a piece of Arabic gum (0.5-0.7 g) for 1 min, then remove all saliva by expectoration, after that chewing was continued for ten minutes with the same piece of gum and saliva collected in a sterile screw capped bottle. After disappearance of salivary foam, 0.1 ml of saliva is transferred to 0.9 ml of sterile phosphate buffer saline of pH 7.0-7.2 for microbiological analysis.

Preparation of Culture Media

Sabouraud Dextrose Agar [SDA]

The medium which is selective one for cultivation and isolation of *Candida albicans* was prepared and sterilized according to manufacturer's leftlet; 65 g-were suspended in 1000 distilled water. Sterilization was done by autoclaving at 121°C at 15 pounds per square inch for 15 minutes, left to cool to 45-50°C and then chloramphenicol antibiotic [2 g for each 1000 ml of media] was added and poured into petri dishes, left to solidify then put them in incubator at 37°C for 24 hours then stored in refrigerator until being used.

Brain Heart Infusion Broth [BHI]

Preparation of the media was according to the manufacturer's instruction which involved the suspension of 37 g in one liter of de-ionized water. After being completely dissolved, the pH adjusted to 7.2. The media was sterilized in autoclave at 121°C at 15 pounds per square inch for 15 min. then left to cool down to room temperature and thereafter kept in the refrigerator until use.

Mueller Hinton Agar [MHA]

These were prepared according to manufacturer's instruction which involved the suspension of 38 g in 1 L of de-ionized water, after being completely dissolved with boiling, it was sterilized in autoclave, then left to cool at 45-50°C, poured and left to solidify then put them in incubator at 37°C for 24 hrs then stored in refrigerator until being used.

Isolation of *C. albicans*

After mixing of saliva as mentioned previously, ten- folds dilution was performed, from dilution (10^{-1} , 10^{-2}) of salivary samples then 0.1 ml was taken and spread on sabouraud dextrose agar [SDA], and the plates were incubated aerobically for 48 hr at 37°C [13].

Identification of *C. albicans*

A. Colony morphology

Colonies of *C. albicans* appeared smooth creamy in color with a yeast odor and typically medium size 1.5-2 mm diameter which later developed into high convex, off-white larger colonies after about 2 days [14].

B. Gram stain

The same procedure as described for *Streptococcus mutans* was used, *C. albicans* appeared as Gram- positive small oval or budding yeast cell.

C. Germ tubes formation

Very small inoculums from isolated colonies were suspended in 0.5 ml of normal human serum. The inoculated tubes were incubated at 37°C for 3 hr. After incubation, a drop of yeast suspension was placed on a clean microscopic slide covered with a cover slip and examined under low power magnification for presence of germ tube. Production of germ tubes is characteristic of *C. albicans* [15].

D. Identification of *C. albicans* by Rapid Yeast Plus System

Rapid Yeast Plus System has several reaction cavities molded into the periphery of plastic disposable tray. Reaction cavities contain dehydrated reactions and the tray allows the simultaneous inoculation of each cavity with a predetermined amount of inoculum [16].

Purification and Maintenance of *C. albicans*

A single colony from *C. albicans* was transferred to 10 ml sterile brain heart infusion [BHI] broth and then incubated for 24 hours aerobically at 37°C. The purity of isolates was checked by re-inoculation of 0.1 ml of culture broth BHI on SDA. The plates were incubated aerobically for 48 hr at 37°C, then one colony from each isolates was transferred to 10 ml of sterile BHI broth and then incubated for 24 hrs aerobically at 37°C [17].

Activation of *C. albicans*

Inoculums of *C. albicans* were activated by the addition of 0.1ml of pure broth culture to 10 ml of BHI broth followed by incubation for 18 hr at 37°C [18].

Characterization of Zinc Oxide nanoparticles

Zinc oxide nanoparticles provided from ministry of sciences and technology, with the concentration 5.8 mg/ml for stock solution and the particles size >50 nm papered by sol gel method. We make different concentration from the stock solution by using dilution low ($N_1V_1 = N_2V_2$). To confirm the activity of zinc oxide nanoparticles solution we make the

UV-Vis spectra of ZnO NPs shown in Figure 1. The absorption peak of the prepared ZnO NPs was found at around 400-500nm.

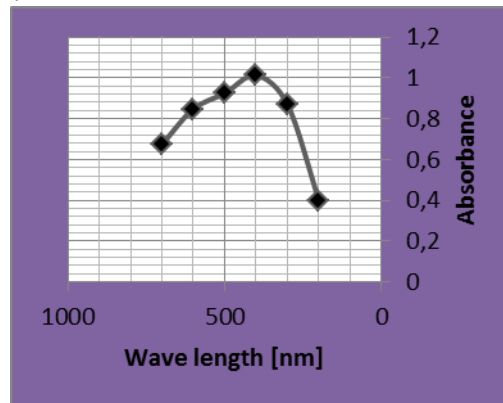


Figure 1. UV-Vis spectra of the ZnO NPs

Determining the Sensitivity of *C. albicans* to Different Concentrations of ZnO NPs and de-ionized water

Fungal inoculums used was prepared by adding a few pure colonies of *C. albicans* to 10 ml of sterile brain heart infusion broth (pH 7.0), then incubated aerobically for 18 hrs at 37°C. The disk diffusion method was used as antifungal susceptibility test. Disposable plates containing Muller-Hinton agar inoculated were applied to study the antibacterial effects of different concentrations of zinc oxide nanoparticles (0.01, 0.05, 0.1, 0.5, 1, 3, and 5.8 mg/ml) compared with de-ionized water as negative control on Mueller Hinton Agar (MHA) media. These experiments were conducted on 48 isolates of *C. albicans*.

The sensitivity of *Streptococcus mutans* and *C. albicans* to ZnO NPs

1. A volume of 25 ml of MHA (pH 7.0) was poured into sterile Petri dishes then left at room temperature for 24 hours.
2. To each plate 0.1 ml of activated *C. albicans* inoculum was spread, left at room temperature for 20 mins.
3. Eight filter papers (wattman no.1) of equal size (7 mm in diameter) were prepared; each filter was impregnated with 40 μ l of ZnO NPs with different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.8 mg/ml) and de-ionized water respectively, and put in each agar plate.
4. Plates were left at room temperature for 1 hour then incubated aerobically for *C. albicans* 24 hour at 37°C. Zone of inhibitions which appears as a clear zone of inhibition around disk were measured across the diameter of each filter paper by using a ruler, no inhibition zone indicated a complete resistance of *C. albicans* to the agents.

Results

Identification of *C. albicans*

Colony morphology

Colony of *C. albicans* appeared smooth, creamy in color with yeast odor and typically medium sized (1.5-2 mm) diameter within 2 days, they develop into high convex, off- white large colonies (Figure 2).

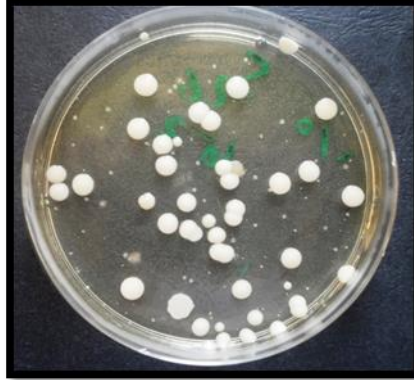


Figure 2. *C. albicans* colonies on SDA

Microscopic examination

The slide was examined under light microscope; the rounded or oval yeast cells were Gram-positive (Figure 3).

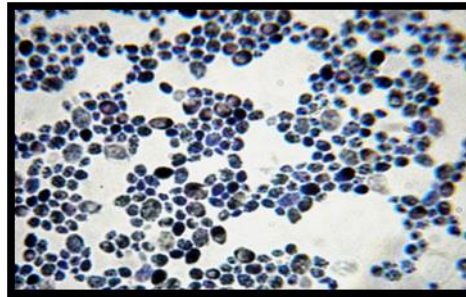


Figure 3. Gram's stain of *C. albicans*

Germ Tube Formation

All isolates of *C. albicans* under light microscope (100×magnification) show the presence of germ tubes which is a characteristic feature of *C. albicans* (Fig. 4).



Figure 4. Different shapes of germ tube of *C. albicans*

RapID Yeast Plus System for identification of *C. albicans*:

The RapID Yeast Plus System Differential Chart illustrates the expected results for *C. albicans*. Table 3-8 show the results after incubation at 30°C in incubator for 4 hours.

Table 1: The Results of RapID Yeast Plus System for Identification of *C. albicans*

Test	Abbreviations	Positive result of <i>C.albicans</i>
Glucose	GLU	+
Maltose	MAL	+
Sucrose	SUC	—
Trehalose	TRE	—
Raffinose	RAF	—
Fatty acid ester	LIP	—
ρ -Nitrophenyle-N-acetyle- β ,D-galactosaminide	NAGA	+
ρ -Nitrophenyl- α , D-glucoside	α GLU	+
ρ -Nitrophenyl- β , D-glucoside	β GLU	—
ρ -Nitrophenyl- β , D-galactoside	ONPG	—
ρ -Nitrophenyl- α ,D-galactoside	α GAL	—
ρ -Nitrophenyl- β , D-fucoside	FUC β	—
ρ -Nitrophenyl phosphate	PHS	V
ρ -Nitrophenyl phosphorylcholine	PCHO	—
Urea	URE	—
Proline- β -naphthylamide	PRO	+
Histidine β - naphthylamide	HiST	V
Leucyl-glycine naphthylamide	LGY	V

+, positive; -, negative; V, variable

Sensitivity of *C. albicans* to Different Concentrations of ZnO NPs Solution and De-ionized Water

The diameter of inhibition zones for zinc oxide nanoparticles solution (clear zone of no growth for *C. albicans* around each filter paper) as found to be increased as the concentration of the solution increased. The stock solution of ZnO NPs which equal 5.8 mg/ml showed higher zone of inhibition compared to other concentrations. De-ionized water showed no zone of inhibition (Figure 5).

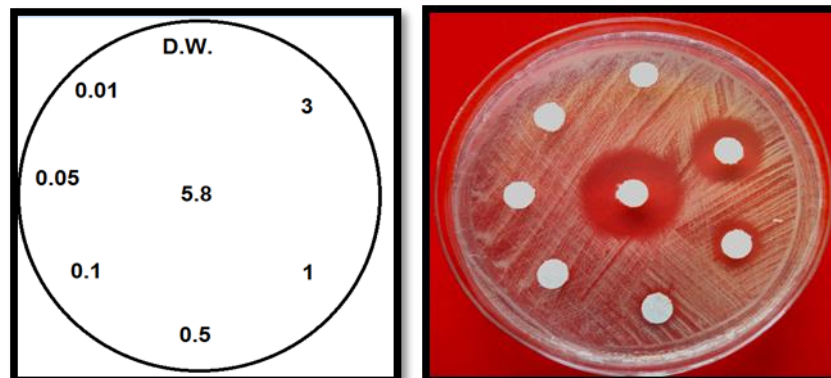


Figure 5. Sensitivity of *C. albicans* to different concentrations of ZnO NPs

Statistical Analysis Tests for Inhibition Zone of ZnO NPs on *C. albicans*

All data for the inhibition zone for all groups concentrations of ZnO NPs (except 5.8) is not normally distributed (sig. < 0.05) by Kolmogorov-Smirnov test for test of normality (Table 2). Therefore the tests used are non-parametric.

Table 2. Test of Normality

Kolmogorov-Smirnov Test		
I.Zones	Groups	Sig.
	0.01	.000
	0.05	.000
	0.1	.001
	0.5	.005
	1	.000
	3	.000
	5.8	.020

Descriptive statistics for inhibition zones are used to examine the differences among different concentrations of ZnO NPs (0.01, 0.05, 0.1, 0.5, 1, 3, 5.8 mg/ml) with de-ionized water to make eight groups respectively in (Table 3) each group consist of 48 tests for the same concentration and the mean of inhibition zones measured in mm which included the diameter of filter paper in measurements, So when there is no inhibition zone we measure the diameter of filter paper only which is equal to 7 mm and refer to it in Table 4.

Table 3: Descriptive statistics of inhibition zones of ZnO NPs and de-ionized water on *C. albicans*.

Groups	No.	Median	Mean	SD	Interquartile range
0.01 mg/ml	48	8.00	8.00	0.97	2.00
0.05 mg/ml	48	9.00	8.88	1.48	3.00
0.1 mg/ml	48	10.00	10.25	1.87	4.00
0.5 mg/ml	48	12.00	12.19	2.16	4.00
1 mg/ml	48	14.00	14.69	2.59	3.00
3 mg/ml	48	17.00	19.08	3.65	4.75
5.8 mg/ml	48	25.00	25.69	3.54	4.75
D.W	48	7.00	7.00	0.00	0.00

The Kruskal-Wallis H and Mann-Whitney U non-parametric statistical tests test are used to analyze concentrations of ZnO NPs groups: Results of Kruskal-Wallis test showed highly significant differences among all groups $p < 0.01$ (Table 4).

Table 4: Zones of inhibition of *C. albicans* to different concentrations of ZnO NPs

	Groups	N	Mean Rank	Kruskal-Wallis H Test
I.Zones	1	48	88.53	Chi-Square = 334.877 df= 7 P < 0.01 HS
	2	48	116.74	
	3	48	161.91	
	4	48	210.44	
	5	48	257.63	
	6	48	309.09	
	7	48	354.67	
	8	48	41.00	

Further analysis using a Mann-Whitney U Test was done to determine which of the eight groups of ZnO NPs was different from the other groups. The results of Mann-Whitney U test with each other groups showed highly significant $P < 0.01$ between all the groups (Table 5).

Table 5: Mann-Whitney U test of inhibition zones of *C. albicans*

Group s	Medi an	Mean Rank	U Value	Z Value	P Value	Sig.
0.01	8.00	39.67	728.00	3.22	< 0.01	HS
0.05	9.00	57.33				
0.01	8.00	32.41	379.50	5.80	< 0.01	HS
0.1	10.00	64.59				
0.01	8.00	25.36	41.50	8.22	< 0.01	HS
0.5	12.00	71.64				
0.01	8.00	24.59	4.50	8.49	< 0.01	HS
1	14.00	72.41				
0.01	8.00	24.50	0.00	8.53	< 0.01	HS
3	17.00	72.50				
0.01	8.00	24.50	0.00	8.51	< 0.01	HS
5.8	25.00	72.50				
0.05	9.00	38.23	659.00	3.67	< 0.01	HS
0.1	10.00	58.77				
0.05	9.00	29.32	231.50	6.85	< 0.01	HS
0.5	12.00	67.68				
0.05	9.00	24.85	17.00	8.38	< 0.01	HS
1	14.00	72.15				
0.05	9.00	24.50	0.00	8.51	< 0.01	HS
3	17.00	72.50				
0.05	9.00	24.50	0.00	8.50	< 0.01	HS
5.8	25.00	72.50				
0.1	10.00	37.48	623.00	3.93	< 0.01	HS
0.5	12.00	59.52				
0.1	10.00	27.56	147.00	7.44	< 0.01	HS
1	14.00	69.44				
0.1	10.00	24.50	0.00	8.49	< 0.01	HS
3	17.00	72.50				
0.1	10.00	24.50	0.00	8.47	< 0.01	HS
5.8	25.00	72.50				
0.5	12.00	35.79	542.00	4.52	< 0.01	HS
1	14.00	61.21				
0.5	12.00	25.80	62.50	8.03	< 0.01	HS
3	17.00	71.20				
0.5	12.00	24.51	0.50	8.46	< 0.01	HS
5.8	25.00	72.49				
1	14.00	32.07	363.50	5.81	< 0.01	HS
3	17.00	64.93				
1	14.00	24.85	17.00	8.35	< 0.01	HS
5.8	25.00	72.15				
3	17.00	29.97	262.50	6.56	< 0.01	HS
5.8	25.00	67.03				

The results of Mann-Whitney U test for the de-ionized water with other groups showed highly significant differences between all groups (0.01, 0.05, 0.1, 0.5, 1, 3 and 5.8 mg/ml) $P < 0.01$ (Table 6).

Table 6: Mann-Whitney U test of inhibition zones of *C. albicans*

Groups	Median	Mean Rank	U Value	Z Value	P Value	Sig.
0.01	8.00	64.50	384.00	6.76	< 0.01	HS
D.W	7.00	32.50				
0.05	9.00	65.00	360.00	6.88	< 0.01	HS
D.W	7.00	32.00				
0.1	10.00	71.50	48.00	8.76	< 0.01	HS
D.W	7.00	25.50				
0.5	12.00	72.50	0.00	9.04	< 0.01	HS
D.W	7.00	24.50				
1	14.00	72.50	0.00	9.05	< 0.01	HS
D.W	7.00	24.50				
3	17.00	72.50	0.00	9.06	< 0.01	HS
D.W	7.00	24.50				
5.8	25.00	72.50	0.00	9.04	< 0.01	HS
D.W	7.00	24.50				

Discussion

The results of present study showed the significantly antifungal activity against *C. albicans* using the ZnO NPs as low as 0.01 mg/ml. As the concentration of ZnO NPs increased from 0.01 to 5.8 mg/ml the efficacy of ZnO NPs treatment was enhanced. The fungicidal activity of ZnO NPs was due to destroying cell membrane integrity [19]. The results of the quantitative antifungal assessment by disk diffusion method are reported in Table 3 from which it is observed that the size of the inhibition zone (the antifungal activity) was found to depend strongly on the concentration of ZnO NPs, and these results agree with Eman *et al* in 2013 [20] who showed the fungicidal effect of ZnO NPs is concentration dependent and also indicate that the mechanism of the fungicidal action of ZnO NPs involves disrupting the membrane. These results agree with those obtained by Shi *et al* in 2010 and Lipovsky *et al* 2011 [21, 22], who recorded the ability of ZnO NPs to affect the viability of the pathogenic yeast, *C. albicans*, as well as a concentration-dependent effect, but does not agree with present results in that the minimal fungicidal concentration of ZnO NPs was found to be 0.1 mg/ml. This concentration caused an inhibition of over 95% in the growth of *C. albicans*. While the current results observed that the minimal fungicidal concentration of ZnO NPs was found to be 0.01 mg/ml. This may be due to the incubation time which was 5 days in study of Eman *et al* 2013 [20] while in our study it was 24 hours only, or may be due to *C. albicans* that is isolated from the skin in study of Eman *et al* 2013 [20] which differ from *C. albicans* isolate that is isolated from human saliva in that study. The current study found that the least inhibition zone of *C. albicans* was 8 mm in concentration 0.01 mg/ml of ZnO NPs but this result in contrast to the results of Jehad *et al* 2012 [23] who showed that the best inhibition zone of *C. albicans* was 18 mm in concentration 10 µg/ml which is equal to 0.01 mg/ml the least conc. That is used in this study. This may be attributed to the antibacterial technique that used in both study as Jehad *et al* 2012 [23] used Agar Diffusion Technique in which hollows of 10 millimeters diameter wells were cut from the agar, and 0.1 ml of each of the tested solutions were poured into the wells. While in this study the disk diffusion method was used in which the filter paper of 7 mm in diameter was impregnated in 40 µl only the difference in amount of ZnO NPs may cause the difference in inhibition zone diameter for the same concentration.

The study of [21] mentioned that, for almost all fungi, the central core of the cell wall is a branched β -1, 3, 1, 6 glucan that is linked to chitin via β -1, 4 linkages. The binding of the oxides particles on the fungal cell surface through electrostatic interactions could be a possible mechanism.

Conclusion

1. Zinc oxide nanoparticles have inhibition effect in different concentrations on *Candida albicans*, starting from the concentration 0.05 mg/ml.

2. Sensitivity of *Candida albicans*, to ZnO NPs increases with the increase of concentration of ZnO NPs solution in comparison to de-ionized water.

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