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Prevalence and Sensitivity Patterns of Candidal Infections in Various Tertiary Care Health Subunits of Karachi

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Abstract

Mostly candida resides as an opportunistic organism on epithelial surfaces of human being. However, under auspicious conditions can cause infections including serious life threatening invasive candidiasis with subsequent mortality particularly in immune deficit and hospitalized patients having co-morbids. Limited data are published on the prevalence of candidiasis, based on the researches conducted at few tertiary care settings which are not representing the overall disease burden in our country, Pakistan. Therefore, this study was conducted to evaluate the frequency and sensitivity patterns of candidiasis in our community. **Methods:** Out of total 1020 specimens, 130 clinical samples were identified as candidal positive, obtained from March to May 2018. These samples were isolated from vagina, oropharynx, urine, tracheal aspirates, pus, blood, tips of the intubations, wounds and fluids of the body cavities. Identification of candida, its species and antifungal sensitivity screening was done by Kirby Bauer's disk diffusion method according to CLSI guide lines' (M - 44 A2 series, 2009). **Results:** A significant majority, 80 (61.5%) of candidal strains were isolated from females with female to male ratio 8:5 and most of these isolates were obtained from high vaginal swabs (43.75%). Four candidal species (*Candida albicans* 80%, *Candida tropicalis* 10%, *Candida glabrata* 9.2% *and Candida ciferrii* 0.8%) were isolated from all positive specimens. Maximum number of the positive samples 52 (40%) were obtained from ICU patients. Sensitivity test of candidal positive samples revealed that commonly used azole antifungal drugs, fluconazole and voriconazole were highly resistant, with respective 57.7% and 70.8% resistance. **Conclusion:** Candidiasis is highly prevalent in our clinical set up and more frequently infecting females in comparison to males as most of the positive isolates were retrieved from HVS (high vaginal swabs). Still, *C. albicans* was found to be the most preva-

lent specie isolated among all candida samples. Our study also demonstrated that the resistance of most commonly prescribed antifungals, azoles have shown a rapid rise. Therefore, it is recommended that before prescription of antifungal drugs the clinicians should routinely recommend culture and sensitivity testing of samples taken from candida infected individuals.

Keywords

Candida, Candidiasis, Antifungal Drugs, Fluconazole, Voriconazole, *C. albicans*, Candidal Infection, Sensitivity, Resistance

1. Introduction

In daily life, human beings are commonly exposed to numerous microorganisms including fungi, among which some behave as normal commensals while other could be pathogenic. According to CDC (Centers for Disease Control), three wide classes of fungi are of concern, these are; 1) opportunistic, 2) community acquired 3) hospital acquired [1]. Candida, an ascomycetous yeast is one of the common fungi responsible for infecting individuals across the globe [2]. It is one of the normal floras of our body surfaces, acts as opportunistic organism which dwells in 70% of human population. Under certain suitable and privileged circumstances candida can infect through its resident areas like oropharynx, vagina, vulva, urethra, ears externa and nails. It can also invade the affected distorted epithelial surfaces by forming hyphae and can become the source of systemic infection. It usually causes recurrent infections in spite of prolonged antifungal therapy [3] [4] [5] [6].

Global warming is continuously rising that declines the temperature gradient between human and fungi which becomes one of the reasons of rise in fungal infections around the world [7]. Pakistan is a land of varied geographical features with extremes of seasonal variations seen across the country [8]. These geographical factors, such as hot and humid weather, heavy rain falls during monsoons and floods are thought to increase the risk of acquiring fungal infections among our population. It is usually associated with people of extreme ages, weakened or altered immunity, prolonged hospitalization and organ transplantation. Prolonged catheterization, use of chemotherapeutics, immunosuppressants, corticosteroids (topical/oral), and chronically ill individuals on polypharmacy are also associated risk factors for candidiasis. Since 19th century, the incidence and prevalence rate of mycotic diseases is continuously rising in aforementioned high-risk population [9]. Candidiasis is now becoming a grave health related issue due to continuous rise of its high morbidity and mortality rates with passing time [9].

Routinely prescribed antifungal drugs for treating candidiasis included azoles, polyenes and echinocandins. Topographic variance has been observed in the

occurrence rate and antifungal sensitivity of various candidal species as evident from the data of different countries and clinical setups around the world [10]. Therefore, this study was conducted at four subunits of a tertiary care hospital located in different areas of Karachi to evaluate the prevalence of candidiasis along with species identification and their antifungal resistance patterns in our community.

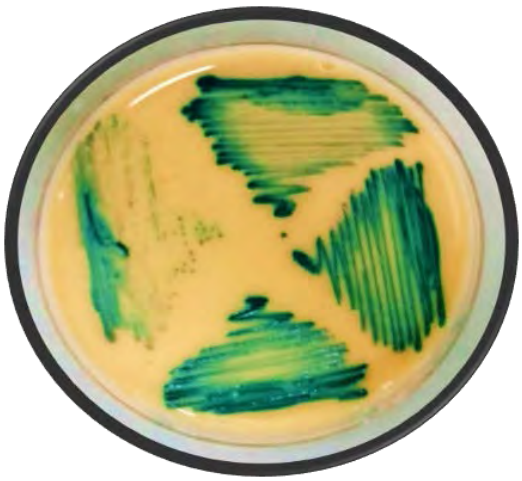
2. Materials and Methods

This prospective quasi experimental study was conducted at four subunits of a tertiary care, Ziauddin hospital located in different areas of Karachi, from March, 2018 to May, 2018. After approval from all competent committees including Ethical Review Committee of Ziauddin University, research work was initiated. After informed consent patients with signs and symptoms of fungal infections like white patchy and itchy skin or mucosal regions, additionally patients with their prolonged hospitalization and on prolonged antibiotics therapy presented with constant fatigue, recurrent infections of different regions of the body and whose laboratory test found candida positive, were included in this study. While those patients, didn't want to participate were excluded from the study. Their samples were taken from oropharynx (oral and throat swabs), blood, respiratory tract (sputum, tracheal secretions, nasopharyngeal aspirates, bronchoalveolar lavage), genitals (high vaginal swab), pus and urine for culture and sensitivity (C/S). All samples were processed in microbiology laboratory within 24 hours according to standard protocols. Patients' data was entered in electronic hospital software (LIS—Laboratory Information System).

2.1. Methodology

According to the standard procedures, yeast were identified by; BACTEC 9240™ for blood specimens, other specimens like vaginal swabs were gram stained and wet mounted. Germ tube test was also performed to identify hyphal yeast. Sabouraud dextrose agar media (SDA, Oxoid, UK) was prepared according to manufacturer's directions along with adding Chloramphenicol to inhibit bacterial growth on it. Candida was grown by streaking on media (SDA) as shown in **Picture 1**. After 48 hours of positive fungal growth, species identification was done on differential agar media, CHROME agar candida (Oxoid, UK) by incubating at 35˚C for 2 days which showed different colored grown colonies of various candidal species (**Picture 2**). Species identification was further confirmed by API 20 C AUX method. At 0.5 MacFarland's, turbidity culture sensitivity test was done by 'Kirby Bauer's method and CLSI guide lines' (M - 44 A2 series, 2009) [11]. For this purpose, Muller Hinton Agar Media (MHA) was prepared according to company instructions (Oxoid, UK), along with adding 2% glucose and 0.5% Methylene blue in it. Antifungal discs of fluconazole 10 mcg and voriconazole 1 mcg (HiMedia, India) were placed on these media. After two days of incubation, zones of inhibition (ZIH) around each disc were noted to estimate the sensitivity patterns of respective antifungal drugs [12] [13].

Picture 1. Candidal positive, white velvety colonies of a specimen grown on Sabouraud dextrose agar media.



Picture 2. Green colored colonies showing Candida albicans positive samples, grown on differential CHROMagar media.

2.2. Data Analysis

The sample size was calculated by Sealed Envelope calculator version 2012.

The calculated sample size was 64 but for this research 100 samples by consecutive sampling technique were taken according to the inclusion and exclusion criteria.

All collected data was entered and analyzed by using SPSS (Statistical Package for the Social Sciences) version 20 and transferred to Microsoft word 2016. Numerical data (zones of inhibition) was expressed by mean and standard deviations, while categorical data (sources of isolates, age groups, gender, clinical settings, species, antifungal sensitivity and resistance patterns of isolates) were stated in frequency and percentages and their associations were tested by applying Chi-square test. P-value < 0.05 considered significant.

3. Results

3.1. Demography and Sensitivity Pattern of Candidiasis

3.1.1. Number of Samples and Distribution of Candidiasis within Different Age Groups and Gender

From 1st March 2018 to 30th May 2018, 130 (12.75%) samples were found to be candida positive out of the total 1020. The age groups of patients reported in this study with candidal infections ranged from 2 months to 89 year with mean age of 51 ± 20.27 year. The most candidal infected age group was 51 to 60 year with an overall prevalence of 26.20% (Figure 1). Among males the most frequently candidal infected age group reported was ranged between 51 - 60 year of age whereas among females from 31 - 40 year with highly significant p value < 0.01 (Table 1).

3.1.2. Distribution of Clinical Settings and Basic Demography of Candidal Patients

As shown in Table 2, majority (40%) of the candida positive samples were isolated from ICU patients followed by patients admitted in wards and private rooms (36.2%). It was observed that females were predominantly 80 (61.54%) infected with candidiasis in comparison to the males (Figure 2). In females candida positive samples were frequently associated with candidal vaginitis 35 (43.75%) in comparison to males whose candidal positive samples were mostly related to candidal infected tracheal aspirates 14 (28%) with highly significant P value (<0.01). Overall in both gender, candidiasis was frequently retrieved from vagina (26.9%) sputum (19.23%) and urine (17.7%). Candidal specimens in both genders are displayed in Table 3.

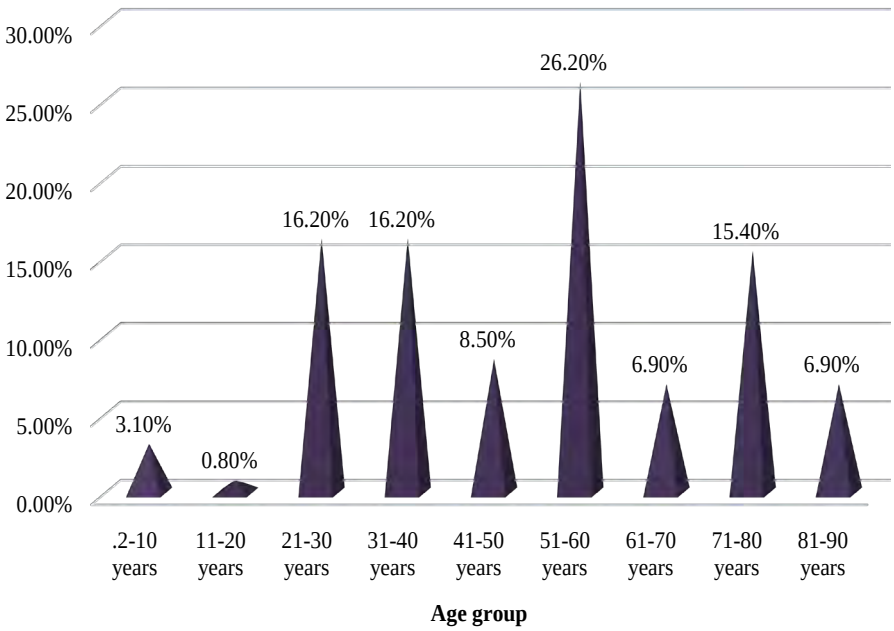


Figure 1. Frequency of candidal infections among various age groups.

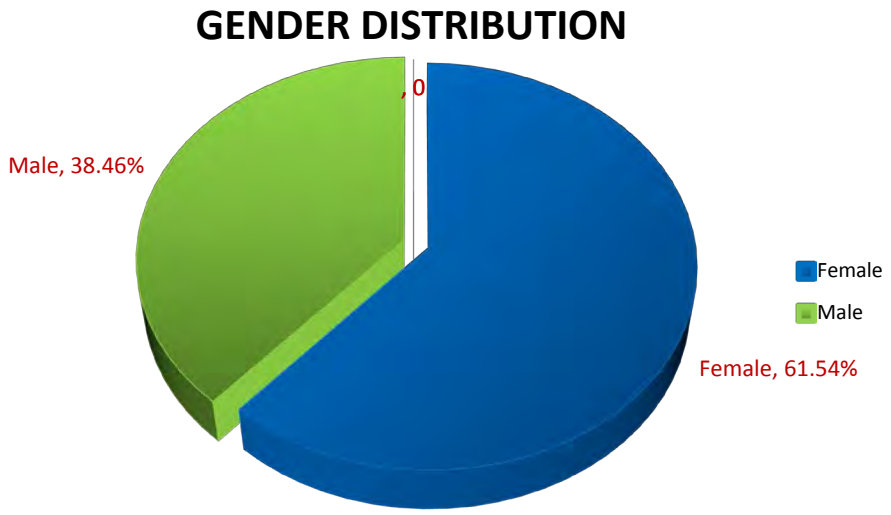


Figure 2. Frequency of gender distribution in isolated cases of candidiasis.

Table 1. Association of candidal patients' age group with their gender.

Age group	Gender		Total no. of gender in age groups	P - value
	Male 50 (38.48%)	Female 80 (61.5%)		
	n (%)	n (%)	n (%)	
0.2 - 10 years	3 (8)	0 (0)	3 (3.1)	< 0.01
11 - 20 years	1 (2)	0 (0)	1 (0.8)	
21 - 30 years	3 (6)	18 (22.5)	21 (16.2)	
31 - 40 years	2 (4)	19 (23.8)	21 (16.2)	
41 - 50 years	0 (0)	11 (13.8)	11 (8.5)	
51 - 60 years	16 (32)	18 (22.5)	34 (26.2)	
61 - 70 years	4 (8)	5 (6.2)	9 (6.9)	
71 - 80 years	14 (28)	6 (7.5)	20 (15.4)	
81 - 90 years	6 (12)	3 (3.8)	9 (6.9)	
Total	50 (100)	80 (100)	130 (100)	

Table 2. Overall frequency of number of candidal cases from different settings of Ziauddin group of hospitals in different regions of Karachi.

Clinical settings	Frequency (n)	Percent (%)	Total (%)
OPD	31	23.8	23.8
IPD - ICU/CCU/E.R/O.T	52	40.0	76.2
IPD - WARD/ADMIT/PVT ROOM	47	36.2	
Total	130	100	100

OPD—out patient department, IPD—in patient department, ICU—intensive care unit, CCU—critical care unit, ER—emergency department, OT—operation theatre, PVT ROOM—private room.

Table 3. Association of Gender with isolated candidal sources by Pearson Chi-Square.

Source of isolates	Gender				P - value
	Male (38.46%)		Female (61.54%)		
	n	%	n	%	
Ascitic fluid 1 (0.8%)	1	2	0	0	< 0.01
Blood 8 (6.2%)	5	10	3	3.75	
HVS* 35 (26.9%)	0	0	35	43.75	
N/G tube** 1 (0.8%)	0	0	1	1.25	
Peritoneal fluid 1 (0.8%)	1	2	0	0	
Pus 9 (6.9%)	9	18	0	0	
Sputum 25 (19.23%)	11	22	14	17.5	
Tip 6 (4.6%)	0	0	6	7.5	
Tracheal aspirate 17 (13.1%)	14	28	3	3.75	
Urine 23 (17.7%)	9	18	14	17.5	
Wound swab 4 (3.1%)	0	0	4	5	
Total 130 (100%)	50	100	80	100	

*HVS: High vaginal swab, **N/G: Nasogastric tube.

3.1.3. Frequency of Candidal Species

Among all candidal species, *C. albicans* was the most frequent specie isolated from 80% of the candida positive samples while among non albicans the isolated species were *C. tropicalis* 10%, *C. glabrata* 9.23% and *C. ciferrii* 0.8%. Overall 25.40% isolates showed co-infection with bacteria, as shown in Figure 3.

3.1.4. Sensitivity and Resistance Patterns of Clinical Isolates

Table 4 demonstrates the sensitivity and resistance patterns of identified candidal isolates against fluconazole and voriconazole. According to our results voriconazole was found to be more resistant (70.8%) in comparison to fluconazole showing resistance of 57.7% with overall resistance to both azoles was 64.25% (35.75% sensitivity). Though the cross resistance detected was (66) 50.7%.

3.2. Candidemia—A Life Threatening Invasive Blood Stream Infection

In present study out of 130 candidal positive isolates 8 specimens were obtained from blood as source of infection at a rate of 6.15%. Candidemia was observed to be 62.5% common in males in comparison to females (Table 5). Among species C. non albicans were the major offender for this blood stream infection involving *C. tropicalis* 87.5% and *C. ciferrii* 12.5% (Table 6). While mortality rate analyzed was 50% (Table 5). Among species 87.5 % of samples showed *C. tropicalis* predominance while overall sensitivity profile against both azoles showed 75% resistance, displayed in Table 7.

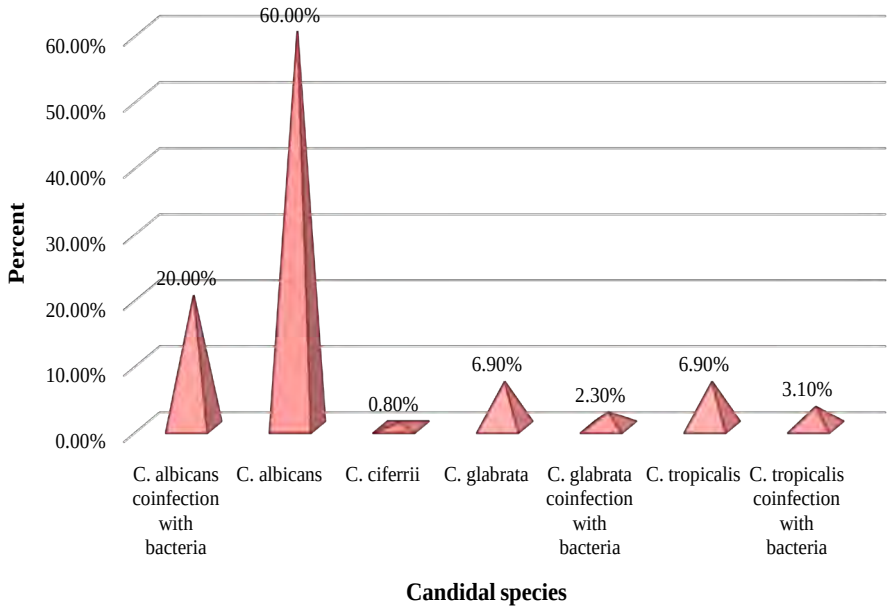


Figure 3. Bar chart showing frequency of Candidal species.

Table 4. Over all Sensitivity and resistance of clinical isolates to antifungal drugs.

Sensitivity pattern	Fluconazole		Voriconazole		Cross Resistance	
	n	%	n	%	n	%
Sensitive	55	42.3	38	29.2		
Resistant	75	57.7	92	70.8	66	50.7
Total	130	100	130	100		

Where, inhiboitry zone > 14 mm of fluconazole (25 mcg) and > 17 mm of voriconazole (1 mcg) were considered sensitive [11] [12] [13].

Table 5. Distribution of death rate with gender in candidemia patients.

Gender	Candidemia patients				P value
	Alive 4 (50%)		Expired 4 (50%)		
	n	%	n	%	
Male 5 (62.5%)	1	20	4	80	0.028*
Female 3 (37.5%)	3	100	0	0	

*p value is significant (<0.05).

Table 6. Distribution of candidal species responsible for candidemia.

Candidal species	Frequency (n)	Percent (%)
C. ciferrii	1	12.5
C. tropicalis	7	87.5
Total	8	100.0

Table 7. Antifungal sensitivity profile of candidemia isolates.

Sensitivity pattern	Fluconazole		Voriconazole	
	n	%	n	%
Sensitive	3	37.5	1	12.5
Resistant	5	62.5	7	87.5
Total	8	100	8	100

Where, inhiboitry zone > 14 mm of fluconazole (25 mcg) and >17 mm of voriconazole (1 mcg) were considered sensitive [11] [12] [13].

4. Discussion

Global rise in the occurrence of opportunistic fungal infections is exacerbating and has been quite challenging for the medical researchers to fix this issue with the development of new generation of antifungal agents. Since many of the currently available antifungal drugs have undesirable side effects, are ineffective against new or reemerging fungi and may lead to the rapid development of resistance. Furthermore, the improvement of new antifungal compounds is limited due to the eukaryotic architecture of fungal cell, which is very similar to the human cells, and has huge genome's plasticity [14] [15]. The knowledge of incidence and prevalence rate along with the resistance patterns of candidiasis in our country is lacking at national level which has a direct influence on treating these patients with the most appropriate antifungal drug [16]. Furthermore, antifungal susceptibility testing is not applied routinely as a standard protocol in most of the microbiological laboratories of Pakistan as the C/S testing is quite expensive, costs around 20 US $ (dollars) that cannot be managed by majority of our low socioeconomic population [5].

Candidal patients notified in our study were from 2 month to 89 year of ages. Most of these patients had weak immunity, comorbidity, long term antibiotic treatments and hospitalization. Globally candidiasis is known as the disease of people at extreme of ages, usually occurs at the ages of less than 1 and greater than 70 year, particularly, due to their compromised immunity [17]. However, a study conducted in a tertiary setting of Rawalpindi, Pakistan showed candidiasis commonest in the age group of 21 - 40 year [5]. In comparison, most of our study population was from 51 - 60 year age group with mean age of 51 ± 20.27 year (**Table 1**). Two other local studies showed its high frequency in people at 50 year and another study at >60 year of ages [18] [19].

According to **Figure 2**, higher frequency of candidal isolates belonged to females, being 61.54% with female to male ratio of 8:5. Our results are parallel to the findings of two studies conducted in other cities of Pakistan (Islamabad and Rawalpindi), showed candidaiasis predominantly in females (56% and 65.8%) [5] [17]. While contrary to our results, Bhattacharjee *et al.*, India, reported males (63.32%) more frequently infected with candidiasis than females (36.38%) [20].

In this study candidiasis was commonly isolated from vagina (26.9 %) followed by sputum (19.23%), urine (17.7%) and tracheal aspirates (13.1%) as

mentioned in **Table 3**. In comparison to our results a study conducted in Korea reported isolates commonly from urine 62.4% [21]. Candida is a common resident in areas of human body which are exposed to the environment therefore more prone to infect under advantageous circumstances as the patients' immunity decline, prolonged catheterization and pregnancy. Additionally, female reproductive hormones have receptors for candida due to which these organisms colonize and infect female genitalia (mainly vagina) [18] [22]. This is the reason why candidias was mainly isolated from females in our study.

In our study, majority of the candida positive samples were isolated from ICU patients with 40% frequency followed by patients admitted in wards and private rooms (36.2%) which is contradictory to the findings of Aslam *et al.*, Pakistan showed 52% of isolates, predominantly from Medical unit [5] and Noor *et al.*, Malaysia documented 25% from wards [23]. Previously it has been documented that Candidal strains are considered to be 80% responsible pathogenic opportunistic organism among hospital based fungal infections [20]. Protracted hospitalization is associated with increased risk of acquiring candida infections as these patients usually hospitalized for their co-morbids, had already poor health and weakened immune system [9].

In this study the principal pathogenic specie responsible for candidiasis in general are *C. albicans* 80% followed by *C. tropicalis* 10%, *C. glabrata* 9.23% and *C. ciferrii* 0.8% (**Table 3**). These findings are in accordance to a global surveillance study ARTEMIS conducted by Pfaller, expressed *C. albicans* as the main culprit among all other species with overall 57.7% prevalence internationally, after that *C. glabrata* 14.8% and *C. tropicalis* 9.4% were amongst the prevalent candida species [24]. Our results are also corresponding to a study of China by Park et al. showed *C. albicans* 64%, *C. tropicalis* 18.1% and *C. glabrata* 8.6% [16] [25]. While contrasting with the results of an Indian study which showed majority (63.3%) of the isolates were non albicans and the commonest isolated specie was *Candida tropicalis* (41.1%), followed by *Candida albicans* (36.7%) and *Candida glabrata* (10%) [26]. Considerable discrepancy has been observed in the frequency of Candidal species distribution among all candida positive cases across the world but the actual reason is still unknown [10].

Azoles are one of the most commonly prescribed antifungal drugs for both systemic and topical fungal infections and are administered by oral and topical routes .These are frequently over the counter (OTC) available drugs, mainly fluconazole in pharmacies since 19th century and their constant rising resistance is becoming a big threat globally [27] [28] [29]. According to a global surveillance study, conducted from the year 2001 to 2007 resistance to fluconazole reported 13.8% and voriconazole 6% [10]. While this current study displayed overall resistance of 64.25% for both of antifungals, fluconazole and voriconazole while individual resistance for fluconazole was 57.7% and for voriconazole was 70.8% (**Table 4**), in contrast a study conducted in Rawalpindi, Pakistan showed overall resistance of both antifungals, 46% whereas, respective resistance to fluconazole

and voriconazole was 85% and 4% [30]. This shows a drastic variation in resistance patterns of widely prescribed, azoles in both cities of Pakistan, Karachi and Rawalpindi. The emergence of resistance to voriconazole is alarming as it is used as an alternative to fluconazole and itraconazole which may represent its irrational use more than fluconazole in our clinical settings. In our study cross resistance to both azoles observed was 66 (50.7%) among all isolates. While according to the previous studies conducted in various countries including Pakistan, Brazil and India, cross resistance between these antifungal drugs reported in a range of 34%, to 74% [18] [30] [31] [32], which is due to their analogous chemical structure [33].

Candidemia

Candidal infection in blood is a big life threat for the affected individuals. In this present study it is reported 6.15% which is resembling to the report of CDC (first global report published on candidemia) In this study, candidemia was found to be fairly prevalent in male gender 62.5%. The overall mortality rate observed was 50%, while in earlier studies death rate was found to be approximately 26% to 52% [34] [35] [36] [37]. According to reports of ARTEMIS and SENTRY, globally 38% to 70% candidemia occurs due to C. albicans among all species since centuries but now this rate is shifting towards non albicans [38] which is also obvious from our study, showing Non albicans 100% responsible for candidemia. These non albicans species in this study are *C. tropicalis* 87.5% as the main culprit of candidemia followed by *C. ciferrii* 12.5%. A study conducted in same country showed *C. tropicalis* 33% responsible specie for candidemia [39]. Unexpectedly, in our study a rare specie, *C. ciferrii* was isolated in a patient with blood stream infection. As documented, it is an infrequent candidal specie, known for its superficial pathogenicity in the field of microbiology [40]. This is in contrast to our study displaying C. ciferrii causing blood stream infection, which illustrates that any superficial candidal specie could invade systemically when person get immunocompromised which is also true for our study sample. Although our sample size for candidemia was small but these results verified the results of previous studies to consider *C. tropicalis* as the main culprit in cases of candidemia in our clinical set up. Our results are also in agreement with the previous researches showing non-candida albicans as the main source of invasive candidiasis [41].

In our study, for candidemia both antifungals were highly resistant but fluconazole showed more (37.5%) sensitivity in comparison to voriconazole (12.5%) (**Table 5**), among which *C. tropicalis* was 71.4% resistant to fluconazole and 100% to voriconazole. The global study ARTEMIS conducted from 1997 to 2003 showed resistance of *C. tropicalis* against fluconazole from 3% to 6.6% [15], which is quite low as compared to our results. Surprisingly our results were contradictory to our local data conducted in 2013, according to which the most resistant specie was *C. krusei* 100%, followed by and *C. glabrata* 15% [39].

5. Conclusions

Candidiasis is highly prevalent in our clinical set up and more frequently invading females in comparison to males as most of the positive isolates were retrieved from HVS (high vaginal swabs), while *C. albicans* was still found to be the most prevalent isolated specie among all positive candidal samples. We didn't find the shift towards non albicans except in candidemia cases where absolutely non albicans were the main offenders. Amazingly a rare candidal specie *C. ciferrii*, known for superficial candidiasis was reported in our study as a cause of systemic (nosocomial) blood infection.

Our study also demonstrated that the resistance of the most commonly prescribed antifungals, azoles have shown a rapid rise. Therefore, it is recommended that before prescription of antifungal drugs the clinicians should routinely recommend culture and sensitivity testing of samples taken from candida infected individuals. This will help in prescription of more effective and appropriate antifungal drugs which will lower the development of their resistance in our clinical set up. Subsequently the effective antifungal therapy will reduce the repeated use of antifungal drugs, with decreased frequency of adverse effects, improvement in over health and cut down the cost of antifungal therapy on the pockets of the candidal infected patients. Consequently, there is an intense urge to introduce novel antifungals with better efficacy, less side effects, and should be affordable to the patients for prolonged therapy. Additionally, preventive measures should be taken to decrease the incidence of nosocomial candidiasis.

Limitations of the Study

In this study we tested the sensitivity patterns of only two commonly prescribed antifungals due to limited budget. Additionally, the sampling was conducted only in one major city of Pakistan, Karachi and could not include fungal infected patients of other regions, also because of the limited budget, and lack of access to their clinical set ups.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Effect of Specific Nutrients on Ovulation, Oocytes Development, Gene Expression and Coupling Success in Mice

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Abstract

Introduction: The roles of genetic, epigenetic, metabolic and other environmental factors such as nutrition and stress, are becoming evident for a successful and healthy pregnancy. This raises the possibility and question, if and how, we improve the probability of pregnancy and of a healthy fetus? The present study examined the role of metabolic, antioxidant and minerals and the results suggest that these factors may positively influence the oocyte quality and the pregnancy rate. Methods: CD1 female mice aged 15 and 5 weeks were divided into four groups of ten each and treated by intragastric gavage daily for 3 weeks. G1: Vehicle; G2: Carnitines (L-carnitine 0.4 mg and acetyl-L-carnitine 0.12 mg/mouse); G3: Microelements (Zinc 4 ng, Copper 0.8 ng, Iron 7 ng/mouse); G4: G3+G2. At the end of the treatment period superovulation was induced and oocytes were collected to assess their quantity and quality. Further, *in vitro* fertilization (IVF) experiments were performed to assess the preimplantation embryo development. The birth success rate was also analyzed in old and young female. The mice were *in vivo* fertilized. qRT-PCR were performed to analyze a possible modulation in key genes of the reproductive process. Results: The number of oocytes was significantly higher in groups 2 and 4 compared to the control group. The oocyte number in group 3 was not affected. The level of degraded oocytes was 29.1% and 19.3% (group 2 and 4) versus 34.3% (control). Concomitantly, the numbers of embryos arriving to successful birth were also increased in G4, both in the old and young group of mice. Preliminary analysis of genes affected evidenced that AMH was up regulated in the ovary and KITL in the uterus in group 2. Conclusion: Results showed that L-carnitine, acetyl-L-carnitine and micronutrients were able to improve both oocytes quality and success rate of pregnancy. Further studies are planned to further examine ways to improve pregnancy and fetal health.

Keywords

Pregnancy, L-Carnitine, Acetyl-L-Carnitine, Micronutrients, Oocytes, Birth, Fetal Health, Embryos, Key Genes, Fertilization, Reproductive

1. Introduction

Genes and cellular metabolism are intrinsically linked to fertility and successful pregnancy. It is estimated that 10 to 15 percent of couples have trouble getting pregnant or getting to a successful delivery, in the USA according to the Centers for Disease Control and Prevention (CDC). The infertility results due to issues with the female about one-third of the time and due to male infertility factors about one-third of the time. In the rest, the cause is either unknown or a combination of male and female factors. As stated by the National Center for Health Statistics (NCHS), the fertility rates in the US have declined over the last few decades and many couples have trouble conceiving. The reasons for the declines are not completely understood but are thought to be related to a number of factors such as nutrition, pollution, stress, and general lifestyle issues. Women who experience problems achieving a recognized conception also have elevated rates of early unrecognized pregnancy loss [1] and elevated rates of clinical spontaneous abortion [2]. Indeed, the recent review by Gray and Becker [2] suggests that delayed conception and the early pregnancy loss may share a common etiology, possibly through events or exposures prior to or during implantation and embryogenesis. The decline in fertility could in part be explained by a decrease in the ovum quality, as well as the physiological processes involved in female fertility. At birth, there are approximately one million eggs; and by the time of puberty, only about 300,000 remain. Of these only about 300 to 400 will be ovulated during a woman's reproductive lifetime, the question of how to improve egg quality is more recent. There is growing consensus that egg quality declines with age. This could be connected to the accumulation of cellular damage with time, a reduction in cellular metabolic processes, which in turn leads to a reduction in energy production and in increase in cellular oxidative stress, which can trigger abnormal physiological processes especially immune and inflammation processes.

1.1. Role of Cellular and Mitochondrial Metabolism in Fertility

The majority of cellular energy is produced in the mitochondria, which are specialized organelles found in the cytoplasm of the cells. The impairment of cytoplasmic and in particular the mitochondrial function would lead to a drastic reduction in cellular energy and an increased oxidative stress. The escape of reactive oxygen species (ROS) from the mitochondria could trigger a number of processes such as inflammation that in turn play a key role in initiating pathology. Damage or deficiency in appropriate metabolic cofactors and substrates to

mitochondria results in aberrant functionality of the respiratory chain and oxidative phosphorylation resulting in reduced energy and increased cytotoxicity. Free radical production also induces inflammatory processes due to the misfolding of proteins and other mechanisms. The use of antioxidant substances able to reduce or quench ROS may thus counteract cellular damage. Research into mitochondriotropic agents such as carnitines, coenzyme Q10 and nicotinamide have shown potential to improve various factors associated with body energy, hormonal regulation, uterus weight as well as possible stabilization of cellular membranes and the genome [3]. The role of these compounds in epigenetics and effects upon genomic stability per se is also becoming important in fertility [4]. L-Carnitine, which is essential in fatty acid metabolism, has been shown to prevent mitochondrial damage induced in the rat choroid plexus by medium chain fatty acids [5] or by mitochondrial toxins [6].

1.2. The Emerging Role of Nutrigenomics, Metabolomics and Oxidative Stress in Fertility

Many recent studies have shown that some biochemical and genetic alterations occur in the oocyte and take part in the natural history of female infertility [7] [8] [9]. Any dysfunction in the genes and therefore in the related protein, for example an enzyme protecting against reactive oxygen species (ROS) called superoxide dismutase I (SOD) is known to lead to female folliculogenesis defects and cause a failure in maintaining pregnancy.

Studies suggest that oxidative stress is associated with decreased female fertility in animals and in-vitro models; epidemiological evidences strongly suggest that exposure to oxidative stress can influence the timing and maintenance of a viable pregnancy (e.g. preeclampsia).

The identification and evaluation of appropriate genetic, metabolic and biochemical markers correlating with early steps of disease onset and progression can be used as a key in preventing this type of dysfunction.

1.3. Carnitines and Female Fertility

Carnitine is a natural product synthesized in mammals from the essential amino acids lysine and methionine or obtained from dietary sources (**Figure 1**).

It is essential in fatty acid metabolism and studies are showing its role in the epigenetic modulation of cellular functions. Carnitine is necessary for cellular energy production and its dysfunction results in various diseases. These are related to abnormal mitochondrial function and thus reduced energy production and increased oxidative stress. Growing evidence supports the hypothesis of the control of oxidative stress by metabolism. Carnitine plays an antioxidant role controlling ROS pathway, showing specific preventive action against lipid peroxidation and membrane damage. These represent some molecular events that culminate in the initiation of a disease process. Studies suggest that carnitine reduces oocyte cytoskeletal damage and embryo apoptosis [10], furthermore it may also stabilize the hypothalamic pituitary gonadal axis [6]-[11]. Carnitines

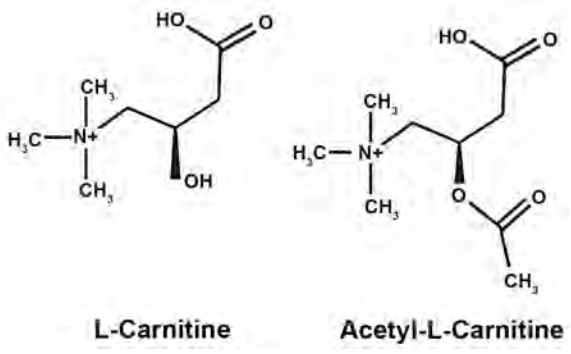

Figure 1. Chemical structures of L-carnitine and acetyl-L-carnitine.

play a central role in fatty acid beta oxidation, that is involved in the acquisition of oocyte competence during which specific enzymes are expressed [12]. Studies show a correlation between the levels of CPT2 expression and embryo developmental competence [13]. Indeed, the inhibition of beta-oxidation during oocyte maturation or zygote division impairs subsequent blastocyst development. In contrast, L-carnitine supplementation during oocyte maturation significantly increases beta-oxidation and improves developmental competence [14]. In addition, its acetylated form, acetyl-L-carnitine (ALC), protects cells exposed to neurotoxins and mitochondrial inhibitors [6]. Thus, together with antioxidant compounds like Q10 a protective action may be hypothesized on ovum and other tissues vital for successful fertility.

However, the quality of the ovum and the state of the uterus is also very important for a successful pregnancy. There is growing consensus that egg quality declines with age and in conditions of abnormal energy balance, such as in polycystic ovary syndrome (PCOS). Indeed, what remain to be elucidated is the critical period between ovulation and implantation. During this period the successfully fertilized egg has to rely exclusively on its own energy reserves to start growing form the zygote to the blastocysts stage. The energy during this period comes from the mitochondria of the oocyte; if this energy runs out the egg stops dividing and implantation is not achieved. This would result in pregnancy loss. In the 70% of PCOS affected women (coupled or not with insulin resistance) inhibition of ovulation, impaired of maturation of viable egg, and reduced egg quality and implantation rate, can occur. It is possible that improving the energy balance before the ovulation process would improve the success of pregnancy. Further the oocyte quality could be improved by substances such as carnitines and antioxidant compounds, increasing the chances of a successful pregnancy. All these factors are becoming important in the treatment of female infertility.

2. Material and Methods

2.1. Ethics Statement

All animal procedures were conducted under the approval of the Italian National

Institute of Health. Animal care was conformed to the European Council Directive 86/609/EEC and all experiments including animals were approved by the review board of the Italian National Institute of Health (Istituto Superiore di Sanita', ISS) and authorized by the Italian Ministry of Health. Animals were sacrificed by cervical dislocation. All efforts were made to minimize suffering.

2.2. The Experiments Were Conducted in Three Steps

- First step: Female 8 weeks old CD1 mice were divided into four groups of ten each and treated daily for 3 weeks by intragastrical gavage. G1: Vehicle; G2: Carnitines (L-carnitine 0.4 mg and acetyl-L-carnitine 0.12 mg/mouse); G3: microelements (Zinc 4 ng, Copper 0.8 ng, Iron 7 ng/mouse); G4: G3+G2. The treatment dose for the mice was calculated approximately based on the recommended healthy dietary allowances for human consumption. After treatment superovulation was induced, oocyte collected to assess quality and quantity. Moreover, *in vitro* fertilization (IVF) experiments were performed to evaluate the preimplantation embryos development [15]. Details for superovulation, oocyte collection and IVF are given below (**Figure 2**).
- Second step: The ovary of the mice were surgically explanted and the RNA extracted, and expression of AMH, FSHB, CEPBP, LHR, and CDX2 genes was tested, being them related to ovary aging and minor production of oocytes (CEPBP, LHR and CDX2), to ovarian reserve and stimulation of oocytes production (AMH and FSHB). RNA was extracted and gene expression evaluated by Reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR as described below.
- Third step: Female 15 and 5 weeks old CD1 mice were divided into four groups of ten each and treated daily for 3 weeks by intragastric gavage. G1: Vehicle; G2: Carnitines (L-carnitine 0.4 mg and acetyl-L-carnitine 0.12 mg/mouse); G3: microelements (Zinc 4 ng, Copper 0.8 ng, Iron 7 ng/mouse); G4: G3+G2. To assess the successful rate of birth in old and young female, mice were *in vivo* fertilized, as described below.

Female 15 and 5 weeks old CD1 mice each group N =10

	3 WEEKS OF TREATMENT (intragastric administration)	NUTRIENTS (per mouse)
1	CTRL	Vehicle
2	Carnitines	L-Carnitine 0.4 mg Acetyl-L-Carnitine 0.12 mg
3	Zinc, Copper, Iron	Zinc 4 ng Copper 0.8 ng Iron 7 ng
4	2 + 3 (Carnitines + Zinc, Copper, Iron)	

Figure 2. Methods.

2.3. Collection of Organs, Spermatozoa and Oocytes

Germ free CD1 female and male mice were purchased from Charles River (Calco, Italy). Viable somatic cell-free spermatozoa were obtained by puncturing cauda epididymis with a needle and collected after a 30 - 60 min "swim-up" selection step in FM medium [16] to perform IVF experiments. Oocytes for IVF experiment were obtained by surgical resection of ovaries as well as preimplantation embryos, obtained by natural breeding [16]. Surgically uterus and ovaries obtained were also stored in Trizol reagent (Invitrogen) for subsequent RNA extraction.

2.4. RNA Extraction

Organs from mice were lysed in Trizol reagent (Invitrogen) and total RNA was extracted according to manufacturer's protocol, without further modification, except for the final step including two EtOH 75% washes rather than one.

2.5. Reverse Transcriptase PCR (RT-PCR) and Quantitative Real-Time PCR

2 μg of total RNA extracted was reverse transcribed into cDNA using the iScript Advanced cDNA Synthesis kit for RT-qPCR (Bio-Rad). 200 ng of cDNA were qPCR amplified with specific primer pairs listed below:

AMH: For 5'-gtgagaggagaggggaacac-3', Rev: 5'-gttctccagtctcccctagc-3';
CDX2: For 5'-ctgtcccttccctcgtcttt-3', Rev: 5'-aactgtgttcggatcccctt-3'
CEBPB: For 5'-tgcggggttgttgatgtttt-3', Rev: 5'-tgctcgaaacggaaaaggtt-3';
FSHb: For 5'-tcgtctgccttttagagcca-3', Rev: 5'-ttcctcagccagcttcatca-3'
LHR: For 5'-acccggtgcttttacaaacc-3', Rev: 5'-cgtcgtcccattgaatgcat-3'
GAPDH: For 5'-accacagtccatgccatcac-3', Rev: 5-tccaccaccctgttgctgta-3'

Quantitative real time PCR (qPCR) was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's instructions in a 7500 Fast Real-Time PCR System (Applied Biosystems) under following conditions: one cycle of 95˚C for 30 s, 40 cycles of 98˚C for 15 s, 60˚C for 1 min and the instrument default settings for the Melt-Curve analysis. The annealing of the primers was at 60˚C. RNA levels were normalized to the level of GAPDH and calculated as delta-delta threshold cycle (ΔΔCT) [17]. All PCR experiments were performed in triplicate and the results were analyzed with qPCR 7500 Software Download v. 2.0.6.

2.6. Treatment and Induction of Superovulation in Mice

At the end of the relevant treatments, in the female 8 weeks old CD1 mice belonging to all the four groups, superovulation was induced by intraperitoneal injection (i.p.) of 5 IU of pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet) followed by 5 IU of human chorionic gonadotropin (hCG) (Corulon, Intervet) after 48 hours. Mice of young group were sacrificed and oocytes collected to assess quantity for each group and to perform *in vitro* fertilization.

2.7. *In Vitro* Fertilization

12 - 13 hours after hCG injection, female 8 weeks old CD1 mice were sacrificed; oviducts were removed and squeezed in 1 ml of FM medium. Aliquots of (1 – 2) × 10^6 spermatozoa were withdrawn and added to the egg-containing dishes. Dishes containing both sperm cells and eggs were incubated for 6 hours at 37˚C with 5.3% CO_2. Non-degraded eggs were then transferred to dishes containing 1 ml of fresh M-16 medium (Sigma) supplemented with BSA (4 mg/ml), overlaid with mineral oil and further cultured for 7 hours at 37˚C with 5.3% CO_2. After 24 hr fresh medium was replaced. Embryos were allowed to grow for 5 days up to the blastocyst stage and, on the basis of morphology, the number of degraded and normal developing embryos was assessed under an inverted photomicroscope stage by stage.

2.8. *In Vivo* Fertilization

12 - 13 hours after hCG injection, female 12 and 5 weeks old CD1 mice were mated with males of the same strain. The presence of a vaginal plug on the following morning indicated successful mating. Developmental progression was followed up till the naturally birth of pups.

2.9. Statistical Analysis

All the data were descriptively analyzed and presented as n, mean and standard deviation in **Table 1** and as mean in **Table 2**. The figures were created using the data included in the tables. The p-values were derived from the t-test comparing each group with the control one.

3. Results

3.1. Carnitine Treatment Improve Quality and Quantity of Oocyte

The results of the study that were in part already published [14] and show that the mean number of oocytes/mouse was higherin the groups 2 (carnitines) and 4 (microelements plus carnitines): 32.5 ($p = 0.273$) and 31.3 ($p = 0 .378$) respectively, versus control group: 24.5 (**Figure 3** and **Table 1**).

The number of oocytes in group 3 was not significantly affected (27.2). Furthermore, the number of degraded oocytes in these groups was positively regulated: 27.8% ($p = 0.163$) and 19.3% ($p = 0.004$) (group 2 and 4 respectively) versus 34.3% (control group) (**Figure 4** and **Table 1**).

This is an important result that showed a significant effect of microelements and carnitines to increase oocytes number and also to maintain their good quality. It is interesting to note, that, as shown in the same figure in the microelements only (Group 3) a higher percentage of degraded oocytes (35.3%) was observed and this was markedly decreased by the addition of the carnitines (19.3%), suggesting the positive effect of the combination of both classes of compounds. These observations suggest that the carnitine group seems to give some benefits in both genesis and ovulation process.

Table 1. Summary of relevant results.

Parameter (u.m.)	Control	Group 2	Group 3	Group 4
Oocytes				
N	10	9	5	5
Mean	24.5	32.5	27.2	31.3
SD	13.8	16.8	10.7	13.1
p-value*		0.273	0.712	0.378
CD1 mouse oocyte percentage degraded				
N	10	10	10	10
Mean	34.3	27.8	35.3	19.3
SD**	10	10	10	10
p-value*		0.163	0.826	0.004
2-cells stage embryos				
N	10	10	10	10
Mean	79.2	35.1	30.9	45.2
SD**	30	10	10	20
p-value*		>0.001	>0.001	0.008
Blastocystis stage embryos vs. 2-cells stage				
N	10	10	10	10
Mean	59.6	86.6	16.0	18.0
SD	15	20	8	8
p-value*		0.003	>0.001	>0.001
Pregnancy Rate Old mice				
N	10	10	10	10
Mean	70	80	30	60
SD**	4	4	NA	4
Pregnancy Rate Young mice				
N	10	10	10	10
Mean	90	100	80	100
SD**	4	4	NA	4

*p-values were derived from t-test on each group vs. control group; **estimated.

3.2. Carnitine Treatment Positively Affect Preimplantation Development

The results of IVF experiments performed to establish the magnitude of fertilized eggs and developed embryos, summarized in (**Figure 5** and **Table 1**), showed that despite the treated groups had a minor percentage of 2-cells embryos versus control group, the major part of them reach the blastocyst stage in Group 2 (86.6% versus 59.6%) (**Figure 6** and **Table 1**).

Table 2. Summary of CEBP, LHR and CDX2 gene expression by group.

Parameters	Control	Group 2	Group 3	Group 4
Stimulation of oocytes production AMH	1	2.278		
Stimulation of oocytes production FSHB	1	1.264		
Gene Analysis CEBPB	1	0.586	0.7804	0.7006
Gene Analysis LHR	1	0.7766	1.6521	0.0156
Gene Analysis CDX2	1	0.8876	0.5211	0.4408

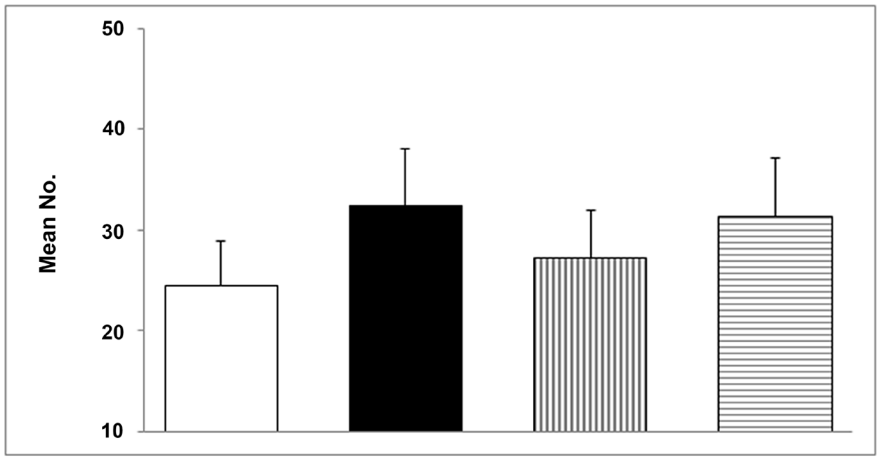

Figure 3. Mean of oocytes.

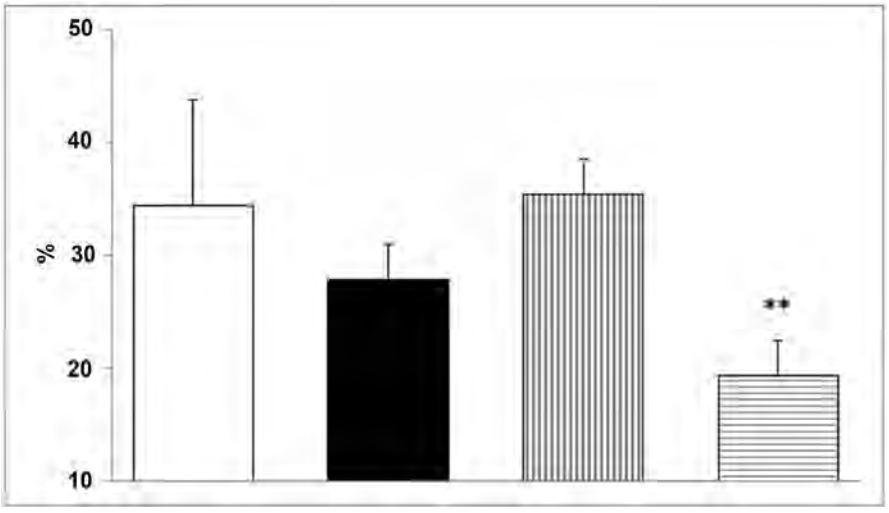

Figure 4. CD1 mouse oocyte percentage degraded.

These results show that carnitines have an important effect on embryo development on 8 weeks mice.

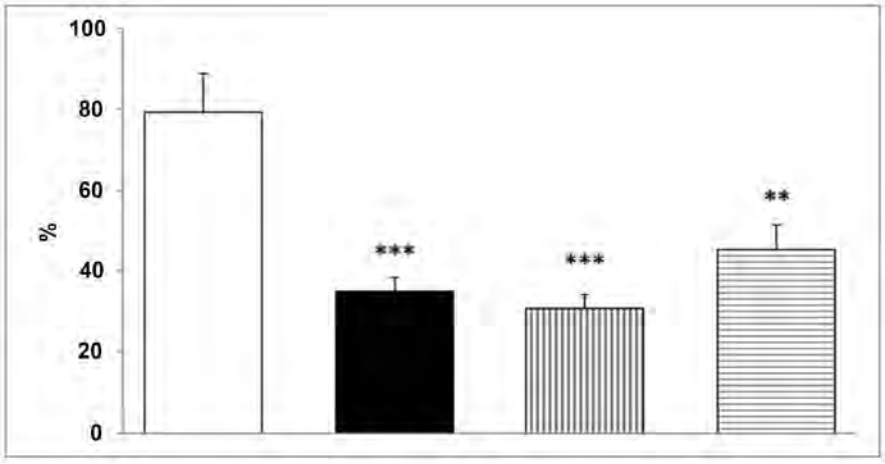

Figure 5. % 2-cells stage embryos.

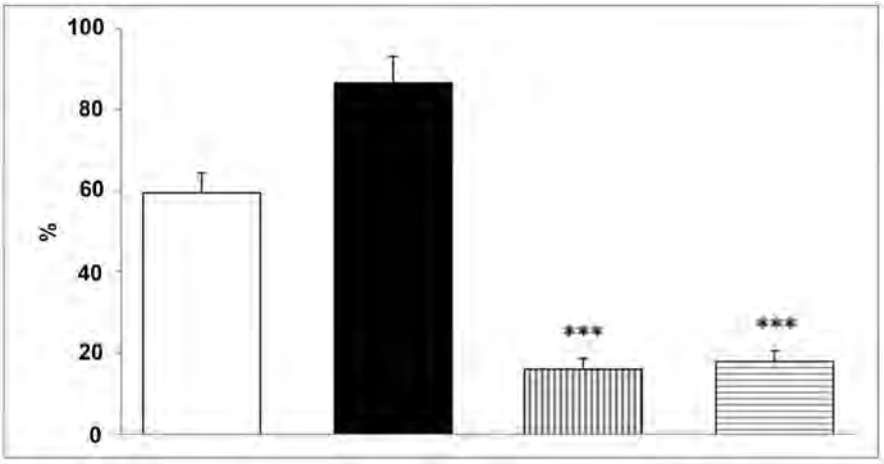

Figure 6. % Blastocysts stage embryos vs. 2-cells stage.

3.3. Gene Expression Analysis after Carnitine Treatment

After treatment, we decided to explore the possible effect of the different supplementations on the gene expression in ovary. Particularly we focused on CEPBP, LHR and CDX2 whose expression is related to ovary aging and minor production of oocytes, AMH and FSHB, influencing the ovarian reserve and stimulation of oocytes production.

Preliminary analysis of genes affected in these processes showed some interesting trends. The expression levels of AMH and FSHB was upregulated in group 2, the expression of AMH was more than 2 times higher than the control group, FSHB was slightly over expressed (Figure 7 and Table 2). In group 3 and 4 no significant effect on AMH or FSHB expression was evidenced.

The different behavior of CEBP, LHR and CDX2 gene expression was observed in the four groups; in particular, CEBP expression was down regulated 0.6 times compared to control in group 2, the down regulation was still evident, even if to minor extend, in group 3, about 0.8 times the control, and group 4, about 0.7 times of the control (Figure 8 and Table 2).

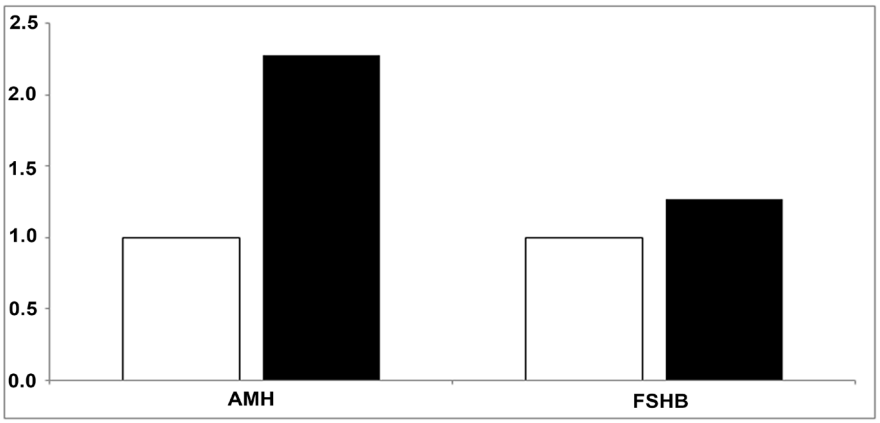

Figure 7. Stimulation of oocytes production.

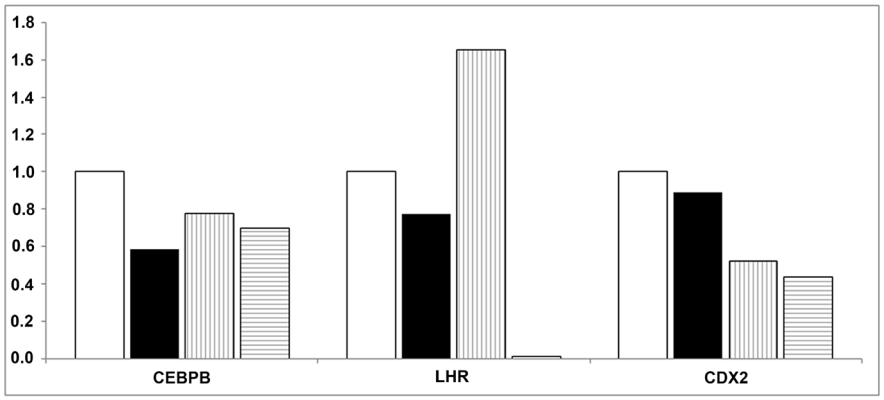

Figure 8. Gene analyses.

LHR expression shows a peculiar pattern; it was slightly down regulated in group 2 (0.8 times compared to the control), completely switched off in group 4, and upregulated in group 3 (1.6 times the control). The expression of CDX2 was down regulated in all the three groups, to a minor extent in group 2, about 0.9 times the control, and to a maximum extent in group 4, where it was reduced to about 0.4 times that of the control.

3.4. *In Vivo* Evaluation of Pregnancy Rate

We verified if the above described genes expression regulation had some effect on the pregnancy rate in young and old mice (Figure 9 and Table 1).

The Group 2 old mice showed a significantly higher rate of pregnancy compared to control (80% vs 70%), Group 2 and group 4 young mice show a comparable higher rate of pregnancy compared to control (100% compared to 90%). Group 3 of both young and old mice showed a lower rate of pregnancy compared to control group, in particular 30% vs 70% in old mice.

4. Discussion

We already published [14] our results on the effect of supplementation of carnitine with or without microelements on the fertility of 8 weeks old female mice.

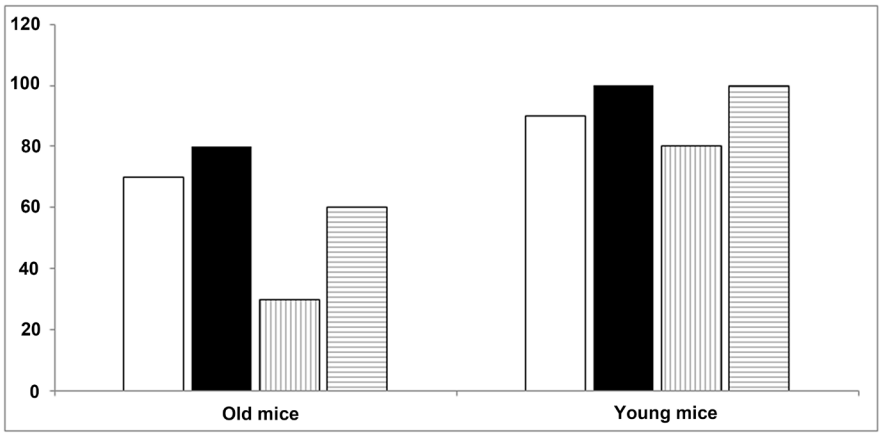

Figure 9. % Pregnant.

In particular, we targeted the oocytes, evaluating the effect of supplementation on their quantity and quality, which resulted significantly improved in the groups assuming carnitines alone ad carnitine plus microelements. These results, prompted us to investigate both the causes and the consequences of the improvement in terms of quality and quantity of oocytes. In order to start to elucidate the molecular mechanism underling the oocytes status, we performed a preliminary evaluation of the expression of genes that are known to be in some way related to the female fertility. In particular: AMH (anti mullerian hormone) is produced by pre-antral follicles and is a biomarker of granulosa cell mass, and thus indirectly, of ovarian reserve [18]; FSHB (Follicle stimulating hormone Beta subunit) is part of FSH, hormone inducing the oocytes production; the expression of these two genes is thus directly related to an improvement of ovarian reserve and stimulation of oocytes production. CEBPB (CCAAT/enhancer binding protein beta) encodes a transcription factor important for the transcription of genes involved in immune and inflammatory responses, among other processes; LHR (Luteinizing hormone receptor) is the receptor of Luteinizing hormone (LH) by gonadotropic cells in the anterior pituitary gland and triggers ovulation and development of the corpus luteum [19]; CDX2 is a member of the caudal-related homeobox gene family, is related to placenta development and important in early embryogenesis [20] [21].

The expression of these last three genes is correlated to ovary aging and minor production of oocytes. Taking into account that this was a preliminary evaluation of the expression of the above mentioned genes, requiring following investigation, it seems quite clear a general positive effect of carnitine supplementation, mainly alone, in the direction of up regulation of the genes that favorite the fertility (AMH, FSH, FSHB), and down regulation of the genes that impair it, as also related with aging (CEBPB, LHR, CDX2).

Finally, we wanted to verify if all the results have correspondence with *in vivo* main indicator of fertility, *i.e.* the pregnancy rate. With the purpose to maximize the eventual effect of different supplementations, and explore a possible age ef-

fect we administered the different supplementation protocols to two groups of mice differing in age young mice 5 weeks old, and old mice 8 weeks old. As expected, the global pregnancy rate was higher in all the four supplementation groups for young mice than old, but, among the groups of same age, again the groups receiving carnitine, alone or with macronutrients, showed a pregnancy rate significantly higher. Taking all together these results, we accumulated a strong evidence that the supplementation of carnitine has a beneficial effect on the female fertility, with an improvement of number and quality of oocytes, which is probably due or at least strongly connected with the up regulation of genes improving fertility and down regulation of genes which impair it, and with a final outcome in an increase of pregnancy rate.

An explanation could be that L-carnitine, which is essential in fatty acid metabolism, has been shown to prevent mitochondrial damage induced in the rat choroid plexus by medium chain fatty acids or by mitochondrial toxins. Key factors in the process of conception are the production of the ovum, the quality of the ovum and the state of the uterus. There is growing consensus that egg quality declines with age and in conditions of abnormal energy balance such as in polycystic ovary syndrome (PCOS). There is a critical period between ovulation and implantation when the egg has to rely exclusively on its own energy reserves contained within the zona pellucida. This energy comes from the fixed number of mitochondria present at moment of ovulation. If energy runs out the egg stops dividing and implantation is not achieved. In PCOS insulin resistance inhibits ovulation, impairs maturation of viable eggs, reduces egg quality and implantation. Restoring energy balance and providing adequate energy stores to the egg prior to ovulation would improve success of pregnancy. Further the oocyte genome quality could be improved by substances such as carnitines which improve genomic stability and therefore reduce chances of aneuploidy.

5. Conclusion

Environmental and stressful conditions can cause damage to the reproductive organs, eggs and general physiological processes of the body involved in the reproductive processes. The normal gene response may also be affected by metabolic dysfunctions. The metabolic compounds, such as carnitines, antioxidants and micronutrients may play an important role in fertility by effects on energy and free radical formation during cellular metabolic processes. Further improved mitochondrial function could provide adequate energy for successful conception as well as the period up to implantation in the uterus. Targeting ovum, tubal and uterus function may provide new approaches to improve fertility diseases however further experimental studies are needed in this field.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Intensity Modulated Radiotherapy (IMRT) in the Planning of Hypofractionated Treatment in Head and Neck Tumors

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Abstract

Head and neck cancer (HNCC) is the sixth most common cancer with an incidence of approximately 600,000 cases per year and 300,000 annual deaths worldwide[1y2]. In Cuba, cancer of the larynx is the fourth cause in incidence, being the fourth cause of cancer death in men. Radiotherapy constitutes an important modality in the control of these tumors and the Intensity Modulated Radiotherapy (IMRT) is a new advance in this field. With it, it is possible to improve dose distribution, decreasing the dose in adjacent healthy tissues and escalating dose in tumor. In this work we present 33 patients of National Institute of Oncology and Radiobiology in Cuba (INOR), in whom the IMRT was used as a treatment technique with a hypofractionation of the dose. Their response was observed at the end of the treatment and one month later. 56% (19) of the patients had a complete response to treatment at the primary site of the tumor and neck. 10% (3) had no response, progressed. 11 of the patients had no response at the lymph node site at the end of treatment, 8 of these 11 had complete remission one month after radiotherapy ended.

Keywords

IMRT, Hypofractionation, Response

1. Introduction

Head and neck cancer (HNCC) is the sixth most common cancer with an incidence of approximately 600,000 cases per year and 300,000 deaths per year worldwide [1] [2].

It preferentially affects male patients older than 50 years and its main risk

factors are smoking and excessive consumption of alcohol. However, an epidemiological transition is currently observed due to an increased incidence of head and neck cancer associated with human Papilloma virus infection. The most common histological type is Squamous, covering more than 90% of cases [1] [2]. In Cuba, laryngeal cancer is the fourth cause in incidence [3].

At the time of diagnosis, only 32% were in localized stage, 47% with regional commitment and 15% with remote commitment. The 5-year survival rate reaches 83% in localized tumors, 61% if there is regional commitment and 37% if there is distance commitment [4].

The treatment contemplates different alternatives, with the main surgery and radiotherapy associated or not with chemotherapy and/or biological therapies, depending on the tumor stage and the functional reserve of the patient [5].

Radiation therapy offers possible benefits by allowing the patient to heal, the preservation of organ functionality, and cost-effectiveness [6].

The evolution of the radiant methods from the conventional radiotherapy of the beginnings looks for to reduce to the minimum, the exhibition of healthy weaves neighbors to the tumor and to maximize the dose in the tumor weave.

The Intensity Modulated Radiotherapy (IMRT) is a technology consisting of the supply of tele-therapy beams with non-uniform fluency in the cross-section of the beam; calculated by computer programs with algorithms known as inverse planning; who seek to obtain the highly conformed distribution of radiation doses. In addition, it allows the treatment of various targets with different doses, at the same time radiation is minimized to critical structures not compromised with the disease [7] [8].

The hypofractionated radiotherapy uses fewer fractions with more doses for each of them. The typical dose values per fraction are in the range of 2.2 to 2.75 Gy, unlike conventional therapy with 2Gy fractions. The IMRT thus allows to decrease the time of treatment and also allows can obtain similar tumor response than conventional fractionation in tumors of the head and neck [9].

The waiting list of patients to start radiant treatment and what this implies for the patient, family member and society in general means that with a high level of evidence we incorporate them into these treatment regimens, optimizing more and more existing resources.

2. Materials and Methods

A prospective, observational study was carried out in which 33 patients diagnosed in the National Oncology and Radiobiology Institute (INOR) with loco-regional squamous cell carcinoma in pharynx, larynx and oral cavity (except nasopharynx, paranasal sinous, and stage I-II glottic carcinoma) were included in the period between 2014-2017. Prior surgical excision (except for biopsy purposes) is an exclusion criterion.

2.1. Inclusion Criteria

1) Tumor classified as stage I-IV located in oropharynx, hypopharynx, larynx

(not glottic stage I-II), or oral cavity according to the TNM classification of Malignant Tumors.

2) Histopathological diagnosis of invasive squamous cell carcinoma at the primary site.

3) Age > 18 years.

4) Informed consent according to the Helsinki declaration and local regulations.

5) The patient must be a candidate for external beam radical radiotherapy, and must be expected to complete the treatment.

6) World Health Organization (WHO) performance status of 0 - 2.

2.2. Exclusion Criteria

1) Distant metastases.

2) The patient should not be in a state or have major co-morbidity that could be expected to influence the outcome of treatment, or interfere with the assessment of treatment outcome at follow-up, or (apart from the present disease) considerably reduce the life expectancy.

3) Patients who test positive for human immunodeficiency virus (HIV).

4) Prior surgical excision (except biopsy).

5) Planned (elective) surgery.

6) The existence of synchronous multiple malignancies (not leukoplakia) or previous history of cancer.

7) The patient must not be pregnant.

8) Socio-demographic or other factors that make it unlikely that the patient will be available for follow up of long term treatment outcome.

They received hypofractionated treatment with a daily dose of 2.75 Gy and 55 Gy as the total dose in the high-risk white tumor volume (CTV-Hi) while the low-risk tumor target volume (CTV-Lo) received a daily dose of 2.2 Gy. And 44 Gy as the total dose. These doses were delivered with the Modulated Intensity Technique (IMRT), performed in the radiotherapy planning system, Elekta Xio.

Patients were fitted with an Orfit type 5-point immobilization mask for simulation and a computerized axial tomography before planning. The equipment used for the treatments was an Elekta Synergy device, with a coupled conical tomographic image system (CBCT). The CBCT was used to carry out a weekly verification of the positioning of the system.

3. Results

The most frequent age group of patients included in the study was between 55 - 64 years. Being the masculine sex the most frequent for a 94% and 79% with toxic habits (Table 1).

As the Figure 1 shows, the most frequent site of the primary tumor was the oropharynx (78%) and 93% of this patients were in advanced stage of the disease at diagnosis time, III and IVA.

Table 1. Demographic characteristic.

Sex	Male	31
	Female	2
Ages	35 - 44 ages	4
	45 - 54 ages	7
	55 - 64 ages	16
	+65 ages	6
Toxic Habits	Current Smoker	25
	Alcohol user	22
	Smoker + Alcohol	21
	Non Toxic Habits	7

Source: Clinical History.

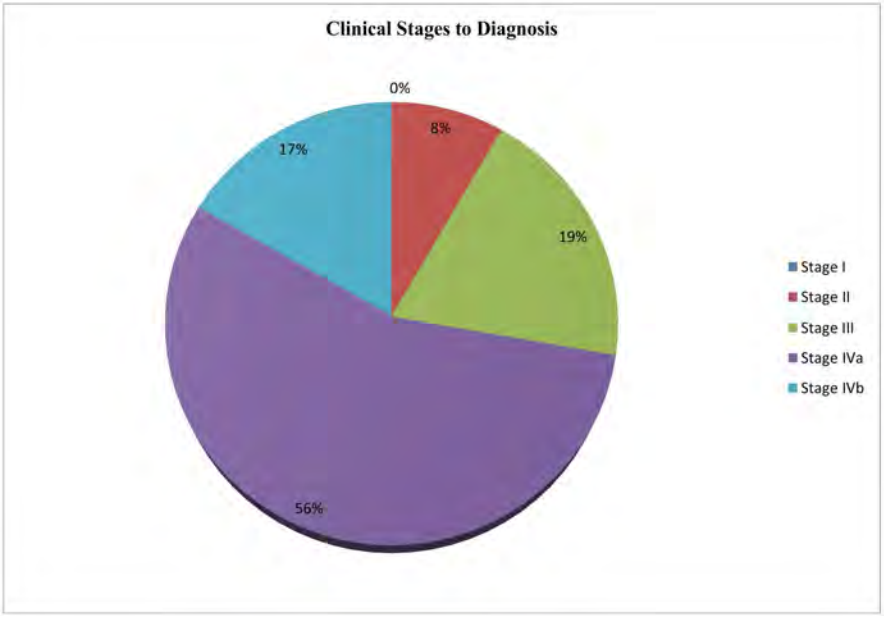

Figure 1. Clinical stages to diagnosis. Source: Clinical history.

100% of the patients were treated with concurrent Radio-Chemotherapy. In case of radiotherapy, with IMRT technique and hypo fractionated schedule.

The intensity modulated radiotherapy used in the plan of these patients allowed a differentiation of the doses in the different target volumes to be treated. CTV-Hi was scheduled a dose of 2.75 Gy per day and 55 Gy as a total dose with an equivalent dose administered in fractions of 2 Gy (EQD2) of 73.6 Gy.

For its part, the CTV-Lo included the less risky node chains received doses of 2.2 Gy daily with a total dose of 44 Gy. The intention of the planning included to diminish the dose in the healthy organs or tissues of risk in comparison with the traditional treatments in the department taught with other techniques. The thirty-three patients included in the study received their full treatment at the

planned time without interruptions between (26 - 28 days). When analyzing the response that they had to the treatment, the response of the tumor in the primary site and in the lymph node site of the neck was taken into account separately. Both were clinically defined by the Radio-Oncologist and Head and Neck Surgeon.

19 (56%) of the patients had complete response (CR) to treatment at the primary site of the tumor and neck at the end of treatment. 3 (10%) had not response, progressed. At the same time 11 (33%) had a complete response at the site of the primary tumor but not at the lymph node; however, 8 of these 11 had complete remission one month after radiotherapy ended. In all, 27 of the 33 patients had CR after one month of treatment at the primary and regional tumor site (**Figure 2**).

A neck dissection was performed on patients who did not respond or with partial response of the lymph node site. As an interesting fact, it was proved that the results of the biopsies were negative to the tumor.

To date, 25 (83%) of the patients are controlled for their disease and 5 have died (17%) (Three who progressed, one due to metastatic disease and the other due to an accident). Three patients are in tumor relapse at the primary site.

4. Discussion

Many authors agree that radiotherapy with IMRT techniques for head and neck cancer is an excellent curative therapeutic tool.

The control rates of our patients are similar to those of Chao et al, who in 2003, presented their experience in a study conducted at the University of Washington in 126 patients with epidermoid carcinomas of the head and neck and intensity modulated radiotherapy technique (IMRT). They reported a loco-regional control rate of 85% at two years [10].

Similarly Dawson et al, in 2000, observed 58 patients with primary head and neck tumors treated with IMRT too and followed for a mean of 27 months. They reported a local control rate of 79% and 12 patients relapsed at 2 years [11].

Butler et al reported also, a study with 20 patients with primary head and neck tumors, showing 19 patients with a complete response to IMRT therapy [12].

Meta-analyses and collective data showed that loco-regional control may be improved at high level of evidence by concomitant chemo-radiotherapy, reason why 100% of our patients were treated with concurrent treatments [13].

At the same time, Di Nicola et al. showed in their study that both radiotherapy regimens (Conventional and hypofractionated) achieve similar results in terms of local control and complications [14].

The use of hypofractionated treatments in radiotherapy centers in low-income countries has become an attractive method to improve the probability of tumor control and maximize service productivity. Roy S *et al.* showed that hypofractionated radiotherapy administered to patients with squamous cell carcinoma of the head and neck using IMRT techniques results in a LCR similar to the

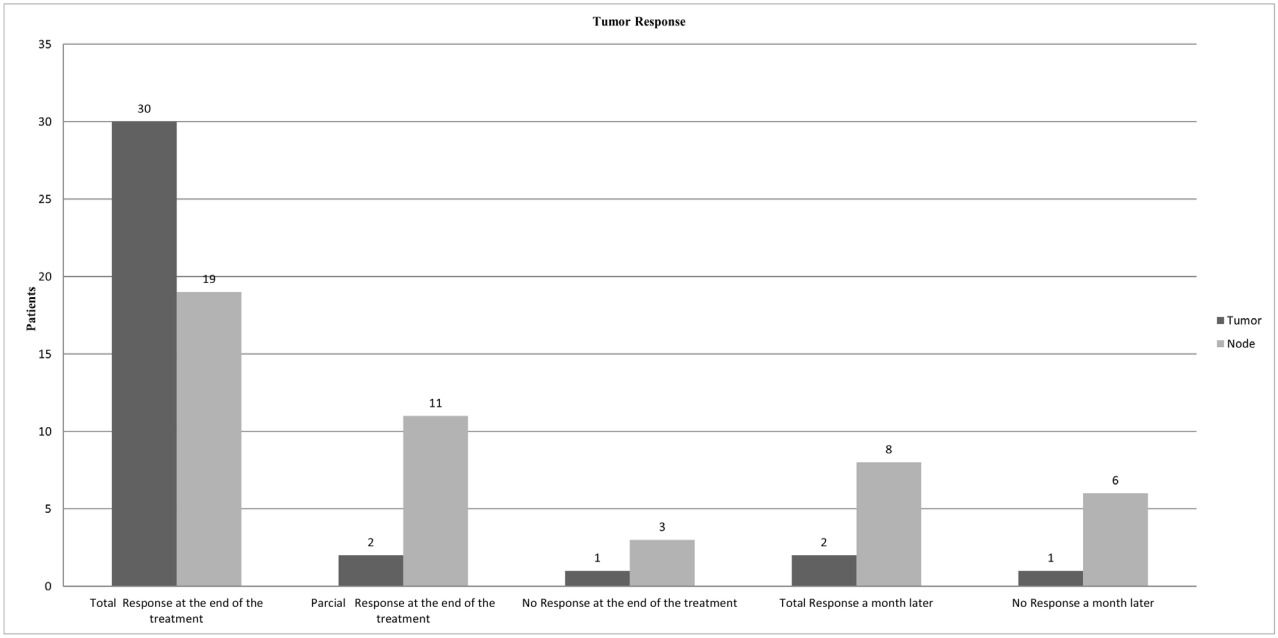

Figure 2. Tumor Response. Source: Clinical history.

conventionally fractionated program [15]. A similar result was observed in our center. The shorter treatment times thus attained resulted in the shortening of the patient's waiting list of patients (Hypofractionation, 4 weeks vs. Normofractionation, 7 weeks).

In the meta-analysis of radiation therapy for head and neck carcinomas (MARCH), which included 15 phase III trials and 6515 patients, there was a global survival benefit of 3.4% at 5 years for altered fractionation versus conventional fractionation. However, there is still the possibility of not obtaining an answer as it happened in three of our six IVB staged patients who progressed despite the treatment. This result coincides with what was proposed by this collaboration group, where they express that the majority of patients with insane failure-regional were in the groups of stage III - IV with bulky node state in the initial presentation. This implies that patients with a large nodal load are likely to be less likely to benefit from hypofractionated treatment. This finding is well corroborated with the meta-analysis that demonstrated the effect of altered fractionation, being significantly more pronounced in the primary tumor than in the lymph node disease. Occurring the same with 3 out of four pts of the IVB state of our series [16].

Other authors such as: Sanghera *et al.* analyzed 81 patients with squamous cell cancer of the larynx, oropharynx, oral cavity and hypopharynx (International Union against Cancer Stages II-IV), who received hypofractionated radiotherapy with a dose of 55 Gy in 20 fractions with concurrent chemotherapy. The 2-year local control rate was 75.4%. The 2-year Global Survival rate was 71.6% and the 2-year Disease-free Survival rate was 68.6% [17].

A multi-institutional intensity modified hypofraction radiotherapy trial for early-stage oropharyngeal cancer showed that hypofractionated radiotherapy

without chemotherapy for early oropharyngeal cancer is feasible, achieving high rates of tumor control [18].

The Phase I dose increase trial without concurrent chemotherapy indicated that 2.36 Gy per fraction for a total of 70.8 Gy was the maximum tolerable dose delivered to the gross tumor volume while using an integrated simultaneous boost for head and neck cancers. With a median follow-up of 17 months, the LRC rate was almost similar for the hypofractionated group compared to a conventional group (76% vs. 80%). When analyzed by subgroups, the benefit of hypofractionation was probably more pronounced in patients with tumors of the oral cavity and cancer of the oropharynx. The differences in response observed were consistent with that experience [19].

Also, Weissberg *et al.* compared the conventional fractionation (60 Gy to 70 Gy in 6 - 7 weeks) with the hypofractionated (40 Gy to 48 Gy in 10/12 fractions at 400 cGy/fr) No significant differences were found in tumor control in 64 patients with surgically unresectable Squamous Cell carcinoma in stages III and IV [20].

Saikat Das *et al.* conducted a study in 36 patients with locally advanced and inoperable head and neck cancer, who received doses of 40 Gy equivalents to 49.8 Gy in conventional fractionation and concluded that hypofractionated schemes are a viable option for these patients [21].

5. Conclusion

Epidemiologically Head and neck tumors in Cuba have a behavior similar to that reported in the literature. The use of radiotherapy techniques such as IMRT, allows us to concentrate and increase the dose at the tumor site and to protect the healthy organs as much as possible. Thus, different targets are used at the same time and hypofraction schemes are used with the same results as conventional treatments, as reported in the literature it's very advantageous in our environment due to the limited availability of radiotherapy equipment and the advanced diagnosis of these patients.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Culture and Sensitivity Patterns of Various Antibiotics Used for the Treatment of Pediatric Infectious Diarrhea in Children under 5 Years of Age: A Tertiary Care Experience from Karachi

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Abstract

Background and objective: Infectious diarrhea is one of the most common infections and a significant cause of morbidity and mortality in children under 5 years of age. Frequent and irrational use of antibiotics has resulted in increased bacterial resistance. The aim of our study was to determine the culture and sensitivity patterns of antibiotics used for the treatment of diarrhea in children less than 5 years of age in a tertiary care hospital of Karachi, Pakistan. Methodology: This cross sectional study was conducted for a period of six months in the children of ages ranging between 6 months and 5 years. Stool samples were obtained from the patients presented with signs and symptoms of diarrhea in OPD or being referred to microbiology department for stool C/S (culture and sensitivity). Data were analyzed on SPSS version 19.0. Results: A total number of 325 stool samples were collected, out of which 152 samples were positive for pathogens. The most common pathogen isolated was *E. coli* 92 (60%), followed by *Klebsiella* 56 (37%) and *Salmonella* 4 (3%). All 152 isolates were highly resistant to majority of the standard antibiotics. Conclusion: Infectious diarrhea is highly prevalent among children under 5 years of age. *E. coli* was found to be the most frequent pathogen isolated in stool samples of the patients presenting with diarrhea and was highly resistant to many of the commonly used standard antibiotics in our clinical set up. Male children were predominantly affected by infectious diarrhea as compared to female children. Therefore, injudicious use of antibiotics should be stopped as well as should not be prescribed empirically for the treatment of all cases of pediatric diarrhea.

Keywords

Infectious Diarrhea, Antibiotic Resistance, Antibiotic Sensitivity, Stool Culture

1. Introduction

Universally, diarrhea is one of the most common infectious diseases in humans and is one of the major causes of deaths in children. The most vulnerable age group affected by diarrhea is children less than 5 years of age [1] stands as the second leading cause of death among this age group. WHO defined diarrhea as the passage of three or more loose stools in the last 24 hours [2]. According to WHO updated factsheet (2017), approximately 1.7 billion cases of diarrhea were reported among children, out of which 525,000 children died per year worldwide [3]. In the developed world like Germany, every sixth child under the age of 5 years is affected by diarrhea, once or twice a year [4]. Whereas in under developed world; Africa and South East Asia, mortalities due to diarrhea occur in about 78% of children. In our neighboring country, India, approximately 14.6% of children less than 5 years of age group suffered from diarrheal diseases [5].

On the contrary, the scenario related to pediatric diarrheal diseases in our country, Pakistan is grave evident from various local data, out of every 10, one child died before reaching the age of 5 years [6]. In the same year another study revealed that the total population of children less than 5 years of age is 16.34%, out of which 11.76% suffered from diarrhea [7]. According to Pakistan Demographic and Health Survey conducted in 2013, 53,000 children died due to diarrhea per year [8].

The fundamental principle of treating bacterial diarrhea includes the use of ORS (oral rehydrating solution) along with zinc supplements which helps in reducing volume and duration of the stools [9]. Nevertheless, bacterial diarrhea in children is treated with antibiotics which include ampicillin, cefixime, ceftriaxone, ceftazidime, amikacin, gentamicin, tetracycline, nalidixic acid, ciprofloxacin, co-trimoxazole, etc. [10] [11]. Nowadays, increased antimicrobials' resistance has been reported worldwide. The consequences of increased resistance of antibiotics can cause the prolonged length of the ailment, increased cost of the treatment, manifestations of complications and even death can occur in some children [12].

In Pakistan over-the-counter availability, self-medication as well as irrational and repeated use of antibiotics for the treatment of diarrhea has been observed practiced in our clinical setups. Subsequently due to the overuse of antibiotics, pathogens have attained a high level of resistance and have also resulted in the alteration of the normal gut flora; which could become a risk for future serious infections in children [13] [14]. However, only few studies have been documented in Pakistan regarding the evaluation of the resistance patterns of com-

monly used antibiotics for diarrhea. Keeping in view all stated facts related to pediatric diarrhea in our community there is an intense urge to conduct more research work with a specific set of goals in order to assess where we are standing in the era of antibiotics' resistance. The aim of this study was to determine the culture and sensitivity patterns of diarrhea in children less than five years of age group in our clinical set up.

2. Materials and Methods

This cross-sectional study was conducted in pediatric unit of a tertiary care hospital of Karachi from December 2017 till May 2018, on children of 6 month to 5 years of age. After approval from institutional ethical review committee (ERC) written and informed consents were taken from the patient's parent or guardian. Patient's parents or guardians were instructed to collect at least 5 grams of feces in a given sterilized stool culture bottle. The children included in the study were selected according to the WHO criteria for acute diarrhea *i.e.* who had at least four liquid stools during the last 24 hours with the signs and symptoms of dehydration. While the children suffering from malabsorption syndrome, systemic infection, severe acute malnutrition, significant medical abnormalities or those who had recently received antibiotic for their current infection were excluded from the study.

According to inclusion criteria, in our study period stool samples were collected from 325 children, out of which 152 samples showed positive bacterial isolates. The stool samples were examined within 4 hours of collection for the presence of bacteria. The samples were grossly and microscopically examined for the color, consistency, presence of cellular elements (RBCs, WBCs, and pus cells), protozoa, eggs and cysts of parasites etc. Fecal specimens for culture were inoculated with the help of swab on *SS* agar (*Salmonella Shigella* agar), MacConkeys agar and selenite enrichment broth. The samples were incubated aerobically overnight at 37 degrees Celsius. Next day subculture from selenite F broth on *Salmonella shigella* agar (SS agar) were also performed. After 24 hours the cultures were re-examined and reported for the presence of organisms *i.e. E. coli, klebsiella* and *salmonella*. Serology and biochemical identification were carried out for the confirmation of pathogenic strains, by using; Simon citrate agar, SIM medium agar, Urea agar and TSI agar. All the isolated pathogenic strains were tested for susceptibility to a panel of 16 antibiotics with the Kirby-Bauer disc-diffusion method.

Results were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2018). According to these guidelines, all the standard antibiotics were tested on 152 stool samples showing positive growths for *E. coli*, *Klebsiella*, and *Salmonella.* After testing these antibiotics the sensitivity and resistance patterns of the bacterial isolates obtained from stool samples were assessed on the basis of zone of inhibitions of respective antibiotics. For the calculation of the zones of inhibition of antibiotics, the diameters of the respective an-

tibiotic's disc plus the surrounding clear area till the edges of the clear zone (showing no bacterial growth) were measured in millimeters (mm)" with the help of a graduated ruler.

Statistical Analysis

For data entry and analysis, SPSS version 19.0 was used. Mean and standard deviations were used to express numerical data (Zone of inhibitions and age without grouping), while frequency and percentages were used for age groups, gender, organisms isolated, sensitivity and resistance patterns of antibiotics etc. To find the association between various pediatric age groups, gender and organisms isolated, chi-square test was used. Comparison of average zone of inhibitions of antibiotics with organisms was done by analysis of variance (ANOVA). A p-value of < 0.05 was considered as statistically significant.

3. Results

Most (80%) of our study population belonged to low socioeconomic background. During our study period, out of 325 stool samples, 152 samples found to be positive. Majority of the stool samples, 92 (60%) showed *E. coli,* followed by *Klebsiella* in 56 (37%), While only 4 (3%) samples were positive for *Salmonella* as presented in Table 1 and Figure 1. The frequency of isolates detected in both genders and in various age subgroups of children less than 5 years age are mentioned in table I, which shows that the frequency of various organisms in both genders and in various age subgroups is different but statistically non-significant ($p = 0.286$) and ($p = 0.253$) respectively.

Our results displayed that all 152 isolates were highly resistant to majority of standard antibiotics. Overall, the highest resistance of isolates was shown by ampicillin. Whereas, the antibiotics which has shown least resistance are imipenem and amikacin as given in Table 2.

As shown in Figure 2 and Table 3, the sensitivity pattern of various antibiotics analyzed for individual organism showed that *E. coli* was resistant to majority of commonly used antibiotics, highest resistance was shown for ampicillin (93.5%), followed by ceftazidime (80%) and cefotaxime (80%), whereas most sensitive antibiotic for *E. coli* were found to be amikacin (97.8%) and imipenem (97.8%). For *klebsiella* most resistant antibiotics was ampicillin (92.9%) and then Amoxicillin-clavulanic acid (82.6%),while most effective antibiotics were imipenem (96.4%), amikacin (92.9%) and gentamycin (92.9%). While *Salmonella* was isolated only in 4 samples and maximum sensitivity, (100%) was observed for amikacin, gentamycin, ciprofloxacin and levofloxacin.

4. Discussion

Annually, 1.5 million children lose their lives on account of diarrhea out of the total 10 million deaths in the pediatric age group [3] and it is also regarded as one of the leading causes of morbidity and mortality in children less than 5 years of age throughout the world [15].

Table 1. Basic demographic features of children under 5 years of age with diarrhea.

Total stool samples N		Organisms isolated in samples n = 152			
		E. coli n (%)	*Klebsiella* n (%)	*Salmonella typhi* n (%)	
325		92 (60%)	56 (37%)	4 (3%)	
Gender					**P-value**
Male	87	56 (60.9%)	30 (53.6%)	1 (25.0%)	0.286
Female	65	36 (39.1%)	26 (46.4%)	3 (75.0%)	
Age (months)					
6 - 12	50	32 (34.7 %)	16 (28.57%)	2 (50%)	0.253
13 - 24	53	35 (38.04 %)	17 (30.35%)	1 (25%)	
25 - 36	25	16 (17.39%)	9 (16.07 %)	-	
37 - 48	12	6 (6.52%)	6 (10.71 %)	-	
49 - 60	12	3 (3.26 %)	8 (14.28 %)	1 (25%)	

*P value significance; level: ($P < 0.05$).

Table 2. Over all antibiotics sensitivity and resistance patterns of bacterial specimens isolated from the stool samples of children with diarrhea, total N = 152.

Antibiotics	M.I.C. (Minimum inhibitory concentration) (µg)	Z.O.I (mm) Mean ± std. deviation	Sensitivity pattern n (%)	
			S	R
Ampicillin	10	12.01 ± 1.82	10 (6.5%)	142 (92.8%)
Amoxicillin	20	11.97 ± 3.41	22 (14.4%)	130 (85.0%)
Piperacillin/Tazobactam	100/10	21.67 ± 2.70	134 (87.6%)	18 (11.8%)
Cefotaxime	30	21.90 ± 3.58	38 (24.8%)	114 (74.5%)
Ceftriaxone	30	20.42 ± 4.20	50 (32.7%)	102 (66.7%)
Cefuroxime	30	14.17 ± 4.00	44 (28.8 %)	106 (69.3%)
Ceftazidime	30	17.09 ± 3.61	40 (26.1%)	112 (73.2%)
Cefoperazone/Sulbactam	75/10	19.48 ± 4.66	108 (70.6%)	44 (28.8%)
Nalidixic acid	30	15.12 ± 4.62	60 (39.2%)	92 (60.1%)
Ciprofloxacin	5	18.93 ± 4.82	94 (61.4%)	58 (37.9%)
Levofloxacin	5	15.69 ± 3.83	92 (60.1%)	60 (39.2%)
Aztreonam	30	17.71 ± 3.85	54 (35.3%)	98 (64.1%)
Imipenim	10	23.66 ± 1.77	146 (95.4%)	6 (3.9%)
Amikacin	30	18.75 ± 1.81	146 (95.4%)	6 (3.9 %)
Gentamicin	10	16.50 ± 1.81	142 (92.8%)	10 (6.5%)
Trimethoprim/ Sulfamethoxazole	12.5/23.75	11.13 ± 4.92	54 (35.3%)	98 (64.1%)

Z.O.I = Zone of inhibition (diameter in mm), R = Resistance, S = Sensitive.

Table 3. Sensitivity pattern of drug with organisms (n = 152).

Drugs	Organism (n = 152)					
	E. coli (n = 92)		*Klebsiella* (n = 56)		*Salmonella typhi* (n = 4)	
	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	Resistant n (%)
Amikacin	90 (97.8)	2 (2.2)	52 (92.9)	4 (7.1)	4 (100)	-
Amoxicillin-clavulanic acid	16 (17.4)	76 (82.6)	6 (10.7)	50 (89.3)	-	-
Gentamycin	86 (93.5)	6 (6.5)	52 (92.9)	4 (7.1)	4 (100)	-
Cefuroxime	32 (34.8)	60 (65.2)	10 (17.9)	46 (82.1)	2 (50.0)	2 (50.0)
Ceftazidime	12 (13.0)	80 (87.0)	28 (50.0)	28 (50.0)	-	-
Aztreonam	28 (30.4)	64 (69.6)	26 (46.4)	30 (53.6)	-	-
Ampicillin	6 (6.5)	86 (93.5)	4 (7.1)	52 (92.9)	-	-
Cefotaxime	12 (13.0)	80 (87.0)	26 (46.4)	30 (53.6)	-	-
Ciprofloxacin	54 (58.7)	38 (41.3)	36 (64.3)	20 (35.7)	4 (100)	-
Nalidixic acid	24 (26.1)	68 (73.9)	36 (64.3)	20 (35.7)	-	-
Levofloxacin	50 (54.3)	42 (45.7)	38 (67.9)	18 (32.1)	4 (100)	-
Ceftriaxone	34 (37.0)	58 (63.0)	14 (25.0)	42 (75.0)	2 (50.0)	2 (50.0)
Imipenem	90 (97.8)	2 (2.2)	54 (96.4)	2 (3.6)	2 (50.0)	2 (50.0)
Sulfamethoxazole Trimethoprim	32 (34.8)	60 (65.2)	20 (35.7)	36 (64.3)	2 (50.0)	2 (50.0)
Cefoperazone/ Sulbactam	58 (63.0)	34 (37.0)	46 (82.1)	10 (17.9)	4 (100)	-
Piperacillin/ Tazobactam	78 (84.9)	14 (15.1)	52 (92.9)	4 (7.1)	4 (100)	-

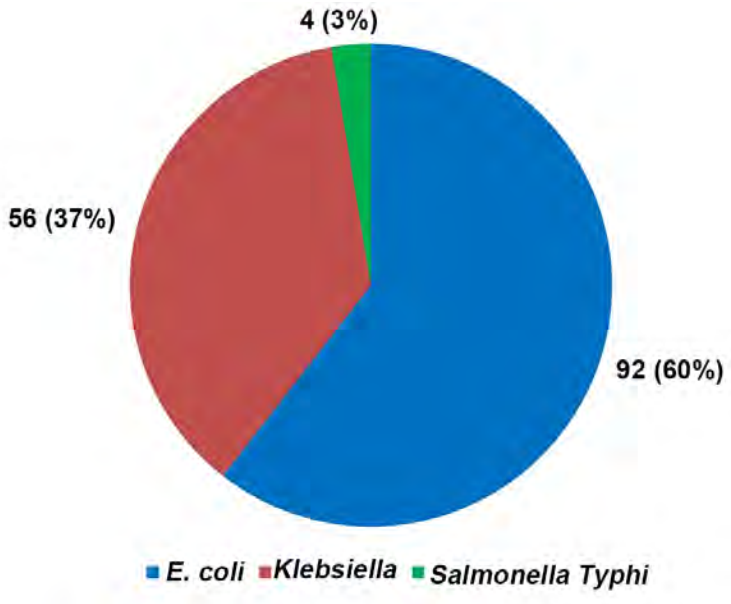

Figure 1. Distribution of organisms.

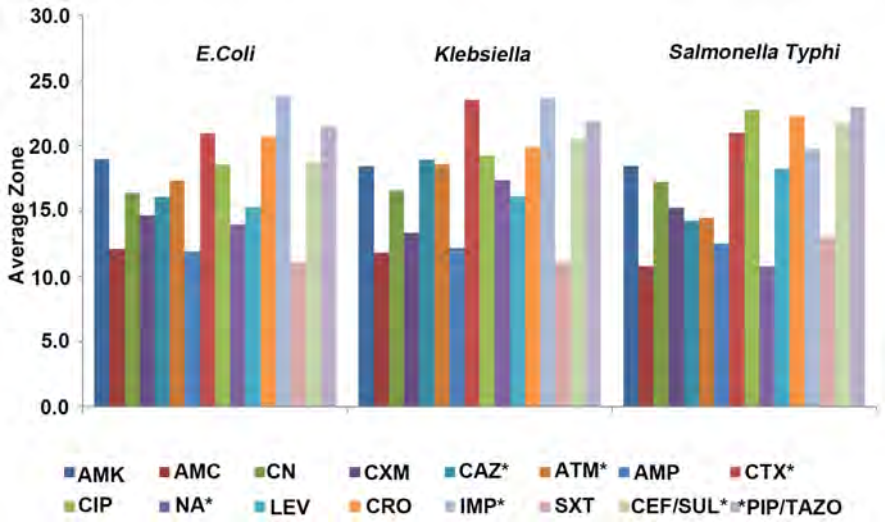

Figure 2. Average Zone of inhibitions of different antibiotics with organisms. AMK; Amikacin, AMC; Amoxicillin-clavulanic acid, CN; Gentamycin, CXM; Cefuroxime, CAZ; Ceftazidime, ATM; Aztreonam, AMP; ampicillin, CTX; cefotaxime, CIP; Ciprofloxacin, NA; Nalidixic acid, LEV; Levofloxacin, CRO; Ceftriaxone, IMP; Imipenem, SXT; Trimethoprim-Sulfamethoxazole, CEF/SUL; Cefoperazone/Sulbactam, PIP/TAZO; Piperacillin/Tazobactam [*shows significant P values].

Pakistan is one of the fifteen developing countries, which has shown the highest burden of diarrheal diseases [16]. Our study conducted at pediatric unit of a tertiary care hospital of Karachi displayed that most (80%) of the cases of pediatric diarrhea were presented from low socioeconomic class. According to our study, 152 (46.77%) stool samples were found to be positive for bacterial pathogens out of 325 total samples as shown in table I, which revealed that infectious diarrhea is highly prevalent in our clinical set up. One of the previous studies conducted from the year 2002 till 2006 on children <5 years of age living in squatter settlements of central Karachi, displayed that the burden of infectious diarrhea varied from 29% to 37% [17]. In 2016, WHO statistics of Pakistan stated the prevalence of diarrhea in children is 23% [18]. This is clearly indicating that disease burden of diarrhea is rising in our population with time. Presumably the reason behind rising pattern and higher burden of diarrheal disease among pediatric age group in our country is that the major chunk of our study population comes from the low socioeconomic areas which are directly linked to poor quality of drinking water and hygiene especially hand washing, improper disposal of waste material, lack of health education of mothers, immunization and nutritional status not maintained according to the age. This is quite evident from a report published in 2017 in the most leading and widely read English newspaper Dawn, which highlighted that Pakistan is the seventh worst country in terms of access to basic sanitation, as its 42 percent of the population remains without access to basic sanitation [19]. On the basis of National Nutritional Survey conducted in different parts of the world the frequency of diarrhea in children less than 5 years of age was found to be variable, ranging from 22.3% in

Ethiopia, 22.1% in Iraq, 21.3% in Egypt and 12% in Tanzania. In 2010, the study performed in Fatemieh children hospital, Central Iran, 514 (57.6%) children were diagnosed with infectious gastroenteritis [18].

According to our results more bacterial isolates were obtained from stool samples of male children *i.e.* 57% as compared to female children in which 43%, of samples were positive (**Table 1**) but this difference was non-significant (p = 0.286) as shown in table I. Whereas, the study published in the journal of Global Health Action in 2014, revealed that there was no gender predominance related to the frequency of diarrhea among children less than 5 years of age [20]. Another study conducted in central Iran in 2010, also showed insignificant difference in the frequency of infectious diarrhea between male and female children (p = 0.22) [21].

In our study, *E. coli* was the most common pathogen isolated in 92 (60 %) stool samples out of the total 152, followed by the second common organism *Klebsiella* 56 (37%) and then *Salmonella* 4 (3%) as shown in **Figure 1**. *E. coli* is the facultative anaerobic gram negative bacillus which belongs to the family of *Enterobacteriaceae.* It is transmitted through fecal-oral route by using contaminated water and food. It was also detected in the milk given to the infants and young children through feeding bottles which were mishandled by the uneducated mothers especially seen in the children coming from low socioeconomic background [22]. Similarly, *E. coli* was detected to be the frequent cause of acute infectious diarrhea in the developing countries, Sudan and India [10] [23]. On the contrary recent studies conducted in Turkey and Spain, displayed the highest number of *Campylobacter* and *Salmonella* species isolated from stool samples of children having diarrhea [24] [25]. Likewise, in the region of South America, Ecuador, *Shigella* species and *Campylobacter jejuni* were found to be prevalent, whereas in China, *Shigella* species were found to be the most common agents for pediatric infectious diarrhea [26] [27].

Our current study showed that the majority of cases of *E. coli* and *klebsiella* were found in 13 - 24 months of age, subsequently the number of cases decreased in 49 - 60 and 37 - 48 months of age respectively as shown in table I. Similar results were presented by Amir Saeed *et al.* in 2015, which stated, *E. coli* is the most common causative agent of diarrhea in children under five year of age in Khartoum, Sudan [10]. Another study published in Tanzania 2014, the epidemics of diarrhea is significantly higher in the age groups of 18 - 23 months [28].

Various microorganisms are resistant to multiple antibiotics and hence named as multidrug resistant organisms. In our study the organism which showed the resistance to majority (more than 60%) of the antibiotics is *E. coli*. The study published in the American Journal of Tropical Medicine and Hygiene stated that *Escherichia coli (E. coli)* is the most frequently occurring organism in children under 5 years of age and is almost entirely resistant to many antibiotics [29]. As mentioned in **Figure 2** and **Table 3**, our current study revealed that *E. coli* is re-

sistant to majority of the commonly prescribed antibiotics, appropriate for pediatric age group, including among penicillins (amoxiclav, ampicillin, amoxicillin), cephalosporins (cefotaxime, ceftriaxone cefuroxime, ceftazidime), while gentamicin, aztreonam, and trimethoprim-sulfmethoxazole were also resistant. Likewise, *Klebsiella* has also showed resistance against commonly prescribed antibiotics, amoxicillin, cefuroxime, cefotaxime, ceftriaxone, ampicillin and trimethoprim-sulfmethoxazole. Although in our study, *Salmonella* was isolated in 4 samples only out of the total 152 but like other two organisms *Salmonella* has also shown higher resistance for cephalosporins (cefuroxime, ceftriaxone) and trimethoprim-sulfmethoxazole. Surprisingly in comparison to both of the organisms, *Salmonella* has showed higher resistance to imipenem as well, which is not prescribed routinely and is reserved for infections due to resistant *Pseudomonas Aeruginosa* [30]. Interestingly the intra group comparison of sensitivity of *E. coli, Klebsiella and Salmonella* displayed that among all antibiotics, the ZOIs of ceftazidime, aztreonam, ceftriaxone, nalidixic acid, imipenem and cefoperazone/sulbactam were highly significant for all the three organisms as shown in **Figure 2**, probably showing the clear difference between ZOIs of sensitive and resistant antibiotics mentioned above for all of the three organisms. Similarly, a recent study published in Feb. 2018, Italy, precisely exposed *E. coli* strains resistant to ampicillin, cotrimoxazole, chloramphenicol, ceftriaxone, and ceftazidime. In the same study, *Klebsiella* species were found resistant to ampicillin, cefotaxime, cefuroxime, co-amoxiclav, mezlocillin, chloramphenicol, gentamicin, and ceftazidime , whereas *Salmonella* strains were resistant to ampicillin, cephalotin, ceftriaxone, gentamicin, amikacin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline [31]. Despite that Italy is among developed countries and our country, Pakistan is a developing country but the scenario of antibiotics resistance is same in both of the countries, possibly this is due to empirical and extensive use of antibiotics in clinical set-ups of both countries. Our results were also parallel to studies reported in other countries like China, which also approximately demonstrates the comparative outcomes [32].

In our study most sensitive antibiotic for *E. coli* and *Klebsiella* were shown to be aminoglycosides (amikacin and gentamycin) as well as imipenem, while *Salmonella* showed highest sensitivity (100%) to both aminoglycosides and fluoroquinolones (ciprofloxacin and levofloxacin) but was resistant to imipenem (50%) as shown in **Figure 2** and **Table 3**. However, the study conducted in India 2016, showed the highest sensitive (100%) antibiotics for *E. coli* and *Klebsiella* are ciprofloxacin, norfloxacin and gentamicin [33]. Whereas, the data as observed in Ethiopia 2017 reveals, *Salmonella* is highly sensitive to ceftriaxone and norfloxacin but resistant to amoxicillin [34].

In the light of above discussion it is evident that currently majority of commonly prescribed antibiotics, suitable for pediatric use in the treatment of bacterial diarrhea have been resistant. The reason is that globally the empirical use of antibiotics for different clinical situations as well as for all cases of pediatric

diarrhea has been increased. Furthermore in our country, over-the-counter availability of various antibiotics has led people to self-medications. Subsequently this has increased resistance of highly useful antibiotics in our population, hence limited antibiotics are left for the treatment of serious infections in pediatric age group as majority of the sensitive drugs, such as aminoglycosides, fluoroquinolones and imipenem cannot be used in children due to systemic toxic effects.

5. Conclusion

Infectious diarrhea is highly prevalent among children of less than 5 years of age, affecting more commonly male children in our clinical setup. *E. coli* is responsible for majority of the cases and was found to be highly resistant to many of the standard antibiotics used currently for the treatment of infectious diarrhea in children. Although in comparison, our study displayed that imipenem, fluoroquinolones and gentamycin have better sensitivity profiles but owing to their limited use in pediatric age group, could not be recommended in pediatric diarrhea leaving less treatment options for this age group. This situation is quite grave and can lead to even greater number of morbidity and mortality in this age group on account of diarrhea only.

6. Recommendations

In order to minimize the resistance, injudicious use of antibiotics should be stopped as well as should not be prescribed empirically for the treatment of all cases of pediatric diarrhea. The decision of giving antibiotics should be based on patient's detailed history, clinical examination and according to the culture and sensitivity reports. Subsequently, this will help in reducing the cost and economic burden due to the overuse of antibiotics on our underprivileged population and will also improve the overall health of children.

The increasing resistance of antibiotics in children requires serious measures and implementations at national and international levels which emphasizes the need to explore other simple, supportive, effective and alternative treatment for resolving the burden of diarrheal diseases in children less than 5 years of age. Furthermore, the use of some antibiotics should be restricted to serious systemic and life-threatening infections in pediatric age group.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Paediatric Surgical Outreach to Papua New Guinea: Initial Experience

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Abstract

This is a report of our early experience in establishing a Paediatric surgical outreach program to Papua New Guinea (PNG) to build capacity and improve care. A Paediatric surgical outreach mission was initiated about 4 years ago in collaboration with Singhealth and PNG along with the multidisciplinary team of health care professionals from Cardiology, Cardiothoracic Surgery, Orthopaedics, Urology, Plastics and Anaesthesia. On each mission trip of 4 - 7 days duration, Singhealth doctors supervise or perform surgeries for complex cases, conduct patient consultations and give lectures to impart knowledge and transfer skills to the local health care community in PNG. In addition, a 6 - 12 months training program in Singapore for PNG doctors has been started. For complex cases which cannot be managed in PNG, there is provision for transfer of patients to Singapore under KKROK fund for further treatment. So far 4 mission trips have been conducted in past 4 years for paediatric surgical unit in Port Moresby General Hospital of PNG focussing on teaching and training of local Paediatric surgeons. Based on evaluation of the Singhealth medical team, there is severe shortage of medical manpower, surgical skill and specialised expertise. Due to lack of intensive care facility, adequate medications and proper medical equipment in the hospital, there are many conditions being left untreated causing high morbidity and mortality among infants and neonates. Such humanitarian work inspires Singhealth towards its global health mission of promoting health equity in responding to the medical needs of PNG through training and leveraging on strength through partnership. In addition, our doctors gain invaluable learning from the exposure, hone their skill as they treat a diversity of cases and are innovative in their treatment options by working in an environment with limited resources.

Keywords

Paediatric Surgery Outreach, Papua New Guinae

1. Introduction

Papua New Guinea (PNG) is a young nation that became independent in 1975 [1]. It is located in Oceania and is composed of the eastern half of the island of Guinea. With a population of nearly 8 million, and more than 800 native languages, cultures and traditions, its main economic activity is farming and harvesting natural resources. The nation is divided into four regions; namely the New Guinea Islands region, Northern region (Momase), Highlands region and Southern region (Papua). It constitutes a total of 22 provinces. The main referral and teaching Hospital (Port Moresby General Hospital) is located in the southern region.

Healthcare in PNG is primarily provided by the government. The country spends about 4.5% of its gross domestic product on health [2]. A significant portion of this comes from international donations. The doctor to patient ratio is 1:17,068. Apart from there being an absolute shortage of physicians, there is also an issue of uneven distribution of healthcare resources [3] [4] [5]. Nearly 85% of the population lives in rural areas, but only 50% of the doctors work outside the capital Port Moresby [6].

2. Paediatric Surgery at PNG

General paediatric surgical service were delivered by the general surgeons or by overseas paediatric surgeons during ad hoc visits for many years, until the first Papua New Guinea paediatric surgeon was trained and graduated from the Papua New Guinea Medical School in 2002. Most of surgical training developments have been supported by AusAid as well as Australian surgeons [7]. Currently there are only 4 surgeons in PNG including 2 in PMGH, doing Paediatric Surgery for a population of 8 million. There is no formal curriculum for the Paediatric surgery training program yet, but for general surgery training, there are well structured criteria similar to a British Training program. After completion of general surgical training for 4 years, they are encouraged to do subspeciality training under the higher postgraduate diploma program [8] [9]. Besides doing the specialist training, the surgeon also has to share the emergency workload of adult general surgery.

The Paediatric surgery service is offered at PMGH, Mount Hagen General hospital for the highland regions and Kevieng General hopsital in the new Guinea islands region, where cases are referred from district/peripheral health centres. At the peripheries where there is no paediatric surgeon, all the children's surgery services are delivered by general surgeons including colostomy for anorectal malformation and hirschsprung's disease.

Port Moresby General Hospital (PMGH) is the main specialist medical centre in the country, located in the capital city. It is also the teaching hospital for University of Papua New Guinea's School of Medicine and Health Sciences. The Port Morsby General Hospital has 700 bed capacity of which the paediatric surgical division is given only 10 beds. The main operating theatre composed of

only 4 operating theatres, where 10 surgical teams squeeze themselves to operate on these 5 days of a week. The number of paediatric surgical procedure can range from 150 - 200 cases per year. They have only 7 beds in ICU which is also shared by adult surgical cases. There is no neonatal intensive care unit.

3. Initiation of the Outreach Program

With the limited capacity and needs in mind, an initiative was launched in the year 2014. The main aim of this initiative was to create an outreach program whereby paediatric surgeons from KK Hospital would share their skills to build capacity and improve care in paediatric surgery in PNG.

The initiative was collaboration between Singhealth International Collaboration Office (ICO) and Port Moresby General Hospital (PMGH). The initiative had 3 key elements:

1) Planned outreach surgical trips where surgeons would undertake training visits to PMGH.

2) Exchange fellowship for trainee Paediatric surgeons at KK Hospital.

3) Provision for transfer and management of complex cases under the Regional Outreach Kids Program.

4. Program Structure

The program was structured to be a series of visits by a multidisciplinary team from KKH involving Paediatric surgeons, anaesthetists and nurses. Each visit was between 4 - 7 days long. The team mostly consisted of two paediatric surgeons, an anaesthetist accompanying the other adult surgical subspecialty teams from other hospitals under Singhealth. The team stayed close to the airport for operational and safety reasons. They would ferry between the accommodation and PMGH by special transport arrangement. For each trip, the PMGH team would have three main modes of interaction:

1) Operable cases: the team worked up and scheduled complex cases with good training and educational value. The KKH team usually operated at least 2 sessions with immediate post-operative management done at PMGH under the guidance of the anaesthetist. In all cases, a surgeon from PMGH would either assist or perform under guidance of the KKH team during surgery. This allowed the KKH team to share specific surgical skills and techniques with the host team.

2) Case management discussions: this would involve grand ward rounds, and discussions on case management of cases admitted to the wards. These discussions gave a different perspective to the PMGH teams thus enriching their management strategy for similar cases.

3) Patient consultations: This involved outpatient consultations for challenging cases brought in by the PMGH team. These sessions were mainly educational and allowed teaching of the local healthcare community as well.

As of June 2018, 4 trips have been completed successfully. Each trip we have managed mostly difficult and complex cases including complex hypospadias,

extensive lymphangiomas, Hirschprung's disease, anorectal malformations, cloacal anomalies, diaphragmatic hernia, infected cystic airway malformations, parasitic twin etc. Most of cases (90%) did well and recovered fully.

5. Challenges and Opportunities

The outreach program identified many opportunities to contribute to skills development in PNG. As the young nation strives to improve the care for its people, such initiatives can play an important role in helping aspiring trainees in acquiring skill sets that would go a long way in addressing the needs of their communities. The team at PMGH was enthusiastic and eager to maximize their learning. Apart from core surgical capacity development, there is also room for improvement in the support system for the paediatric surgeons.

The program has not been without a fair share of challenges. These can be grouped as being related to the pathology, operational issues and specific areas of need. In a way these also represent opportunities for organizations to help in capacity building.

1) Complex pathology: Usually cases selected for management are complex and difficult cases like Hirschprung's disease, hypospadias, anorectal malformation, cloacal anomaly or complex thoracic lesions with failed previous operations. Because they are technically challenging, most of them need paediatric anaesthetist support and close post-operative monitoring in intensive care.

2) Operational issues: The most important issue is that of limited facilities. The operating room size, equipment and layout were challenging, as was the availability of instruments for paediatric surgery, as opposed to general adult surgical instruments. Radiological and pathology support was also noted to be very limited—primarily because of resource constraints at PMGH.

For operated cases, there is minimal ICU support despite best efforts of the nursing department. There is scope for development of neonatal and children's ICU facilities.

Another aspect is the limitation of skilled manpower. There is a definite need to increase the number of nurses and doctors with a defined formal training program. Once a critical mass of well-trained healthcare staff is available, a multidisciplinary approach would be required for holistic care of children presenting with surgical conditions.

HIV prevalence rates continue to be high in PNG. As such, preoperative screening is not available and needs to be instituted to minimize the risks to the surgeons and nurses.

One unique challenge faced by the surgical teams at PMGH is the lack of follow up and communication with the patient. This is because mostly parents of the children are farmers who wish to resume their daily routines as soon as possible. Besides, the threshold to seek medical care is very high. These factors, coupled with long travel times from the faraway provinces and tough terrain, significantly reduce the number of children returning for scheduled follow ups.

Foreigners wanting to work in PNG would also need to deal with some restrictions on movement due to security concerns. Any new initiatives would need to address this issue of personnel safety carefully.

3) Specific clinical areas of need:

The absence of a neonatal ICU (NICU) is a very big challenge for managing premature babies of less than 2000 grams. This also then prevents paediatric surgeons from attempting more complex procedures in such children. Complex neonatal surgical cases done include tracheo-esophageal fistula/esophageal atresia (type 3), bowel atresia, gastroschisis and anorectal anomalies requiring colostomy, of some had survived undersstressfull conditions but other did not make it all due to lack of NICU. A good NICU is extremely important to ensure good post-operative care for premature babies who often present with congenital anomalies requiring surgical intervention. The neonatal surgical cases have very high morbidity and mortality rate in perioperative period due to lack of adequate facility and high infection rate.

6. Insights Gained during the Trips

Working outside the comfort zone of one's daily practice is not easy. At PMGH, we realized that working with minimal facilities and limited resources forces one to adapt and improvise and innovate based on the needs that arise. Hence they have recorded case of neonatal surgical case, survivor cases such as gastroschisis, tracheo-eosophageal fistula and all anorectal anomallies requiring colostomy. This is a very meaningful insight that we gained from our trips. Besides this, we appreciated the need to plan properly, manage time realistically and anticipate unexpected developments along the way. To perform in the best possible way, we relied on team work and close coordination within the team, and also with our gracious hosts.

So despite the fact that there were resource constraints, the trips so far have been unanimously perceived as being very insightful and gratifying.

7. Outcomes and Future Plans

By the end of the fourth trip, the surgical team at PMGH was able to manage some of the index paediatric surgical cases much more confidently. This was achieved by hands-on surgical co-management of cases during the trips. Further, a 6 - 12 months fellowship for trainee paediatric surgeons from PMGH has also been instituted.

Looking forward, there are plans to train other professionals as well. These include other key members involved in the management of paediatric surgical cases, namely paediatric anaesthetists, nurses and neonatologists. There is also a realization for need to train personnel in proper administration of total parenteral nutrition. With a strong team in place, the outcomes are likely to be better.

Apart from manpower training, there are also considerations for building in-

frastructure in terms of a separate paediatric surgical unit. An improvement in pathology and radiology support is also required. All these would allow a focused delivery of paediatric surgical service to the people of PNG.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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In-Vitro Comparison of Antifungal Activity of Herbs (Darehald and Pomegranate) with Azoles

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Abstract

Aim: Candida, an opportunistic organism is one of the commonest causes of hospital acquired infections among fungi. Currently available antifungal drugs have numerous adverse effects and drug-drug interactions (DDIs) along with increase in resistance over the time. Therefore, it is highly emergent to consider alternative treatments for candidal infections, having fewer adverse effects and is cost-effective. The current *in-vitro* study is undertaken to assess and compare the antifungal effects of the herbs, *Berberis aristata* (*B. aristata*, *Darehald*/*Darhald*) and *Punica granatum* (*P. granatum*, *Pomegranate*) with fluconazole and voriconazole, based on culture and sensitivity of candidal isolates. **Materials and Methods:** Ethanolic extracts of herbs (*Berberis aristata and Punica granatum*) and concentrations were formulated as per standard procedure. 130 samples were obtained for the study from in and out patients reported in clinical subsets of Ziauddin Hospital, Karachi from March to May, 2018. Samples were collected and grown according to the standard procedures like, wet mount test and gram's staining. Species were identified by CHROM agar candida and API 20 C AUX methods. Sensitivity tests were performed by Kirby Bauer's disc diffusion method according to CLSI guide lines M-44 A2, 2009. Data analysis was done by one-way ANOVA to compare the antifungal activities of drugs and herbs. **Results:** Mean inhibitory zones of herbs, *B. aristata* and *P. granatum* were highly significant against clinical candidal isolates with respective p-values of 0.00 and 0.02. Both of the herbs, *B. aristata* and *P. granatum* were found to be more sensitive, 98.5% and 97.7% respectively in comparison to fluconazole showing 42.3% and voriconazole showing 29.2% sensitivity against candidal isolates. The most resistant candidal specie was *C. tropicalis* that showed resistance against both fluconazole and voriconazole, contrary to that, this

specie was highly sensitive to both of the herbs, showing sensitivity of 100% respective for *Darehald* and *Pomegranate*. **Conclusion:** In comparison to azoles: Culture sensitivity of both herbs (*B. aristata and P. granatum*) displayed more sensitivity against candidal isolates of patients having non-invasive and invasive candidiasis. These herbs can be considered as substitute or alternative antifungal agents to the conventional antifungal therapy, particularly in cases of treating candidemia patients, which is a life threating condition.

Keywords

Candida, Fungi, Azoles, Antifungal Sensitivity, Antifungals, *Berberis aristata*, *Punica granatum*, Pomegranate, Darhald, Darehald

1. Introduction

The use of herbs in folk medicine has been practiced by humans since ancient times. The traditional herbal therapy is considered as a "shotgun approach" because of multiple constituents of a herb's extract, having many therapeutic mechanisms of actions which result in greater therapeutic activity against various diseases [1]. It is estimated by WHO that around 80% of world's population rely on natural herbal treatments for their primary cure, mainly in developing world [1] [2]. In the field of ethnobotanic studies, unpurified herbal extracts considered to be safer and more potent as they contain multiple constituents having synergistic activity to achieve required therapeutic effects along with reduced toxicity in comparison to its isolated buffered ingredients [3].

Globally, the frequency of fungal infections is increasing just like bacterial infections [4] [5]. Among fungal infections, candidiasis is one of the common opportunistic organisms related to yeast family [6]. It is responsible for 70% of all hospital acquired mycological infections [7] [8]. In the field of medicine, till date synthetic drugs are considered as conventional empirical therapy for the treatment of fungal infections. About 18 antifungal drugs were synthesized and approved by authorities from 1980 to 2002 in which majority (83%) of the drugs were related to the azoles class [9]. For candidiasis, the preference of antifungal therapy depends up on type, nature and extent of the lesion along with patient's generalized health and immune status [10]. Besides, numerous drug-drug interactions, the adverse effects of antifungal drugs are hepatotoxicity, nephrotoxicity, headache, altered taste sensation, anemia, GI upsets, hair loss etc. [11] [12]. Nowadays, the rising resistance to antifungals and cross resistance among its azole class of drugs is a big threat which shows variation topographically [13] [14]. These ground realities are sufficient for the motivation of researchers to consider and evaluate new antifungal drugs or alternative agents for the treatment of candidal infections, showing promising results with better

efficacy, lower resistance, fewer adverse effects and affordability of the patients [15].

Therefore, this current study was undertaken to evaluate and compare the anticandidal effects of herbs *Punica granatum* (*Pomegranate*) *peels* and *Berberis aristata* (*Darehald*) *root bark* with commonly used azoles (fluconazole and voriconazole) antifungal drugs. The aforementioned herbs are commonly grown and available in regions of Asia [16] [17]. They are used in folk medicine for many therapeutic purposes, *Darehald* (*Darhald*) has been used as an antimicrobial, antipyretic, antioxidant, antihypertensive, anti-osteoporotic and anti-arrhythmic herb [18] [19]. Its root and stem barks extracts have potent antipyretic, antimalarial and antimicrobial effects [20] [21]. In the past, *pomegranate* was popularly called as a "fruit of dead" by Greek mythologists [22]. It is an extensively studied plant in the field of pharmacotherapeutics, having antimicrobial, neuroprotective and immune modulating properties, also being used in treating diabetes mellitus, infantile brain ischemia, weight gain, Alzheimer's disease and arthritis [20] [23]. Regarding toxicity of herbs, Berberis was studied as safe drug with no mortality and hepatoprotective effects in therapeutic doses of even 2000 mg [24] [25] [26]. *P. granatum* (*Punica granatum*) was also reported to be safe with no noxious effects at therapeutic doses < 2000 mg daily along with hepatoprotective and nephroprotective effects [23] [27]. It is known as "superfood" by the mythologists because of its apparent non-toxic, curative and probable nutritious values [28].

2. Materials and Methods

Study settings:

Total 1020 samples were collected from in and out-patients of different wards, private rooms, operation theaters, emergency, coronary and intensive units of Dr. Ziauddin group of hospitals (Clifton, Kemari, Ziauddin Memorial and North Nazimabad Ziauddin Hospitals Karachi). All samples were transported within 24 hours to microbiology laboratory of North Nazimabad hospital where the bench work was performed.

Data Analysis:

The numerical data (mean zone of inhibitions and ± standard deviations) of herbs (*B. aristata* and *P. granatum*) and azoles (fluconazole and voriconazole) were observed and compared by applying one-way analysis of variance (ANOVA) test through a statistical software programanalysis, SPSS 21. 95% confidence interval was taken with p-value less than 0.05 was considered to be significant. While sensitivity and resistance pattern of these variables (herbs and azoles) was evaluated by percentage.

2.1. Preparation of Crude Extracts

All herbs were bought from a registered herb dealer, cleaned, dried and stored at room temperature. These herbs were authenticated and placed in herbarium of

Pharmacognosy department of Jinnah Sind Medical University (JSMU). They were grinded in electric grinder [29] [30]. The powder of each herb was placed in a glass container to mix and soak with the absolute (100%) ethanol at 3:10 ratio for 20 days. These extracts were filtered by using Whatmann No. 1 filter paper (**Figure 1(a)** and **Figure 1(b)**) [4] and further extracted by a rotary evaporator (BUCHI, Switzerland) (**Figure 1(c)**) [31]. These crude extracts were placed open in ventilated laboratory to let them dry for 7 to 8 days and stored in airtight bottles. [32] [33]. The minimum inhibitory concentrations (MICs) of *Berberis aristata* (25 mcg) and *Punica granatum* (20 mcg) extracts were made with per ml of 5% Tween 20 [31].

2.2. Culture Assays Procedure for Yeast Sensitivity Testing

After approval from the institutional ethical committee, and written informed consent, the 130 (out of 1020) samples of the patients from suspected infected urine, pus, wound, vagina, tracheal aspirates and body fluids (ascitic, peritoneal and pleural fluids), nasogastric tube, incubate tips and blood was taken. Those suspected samples with positive sign and symptoms of infection (e.g. itchy subcutaneous regions of mouth etc., white velvety lesions and history of chronic fatigue, fever and recurrent infections) were identified candidal positive by the following procedure which was processed according to standard microbiological technique. The isolates of Candidal species from clinical samples of vagina, tracheal aspirate, pus, wound, body cavity fluids, sputum, urine, tips and blood identified by Wet mount (for vaginal swabs) (**Figure 2(a)**), Gram's stain (**Figure 2(b)**) and Germ tube test (for sputum specimens) (**Figure 2(c)**) and BACTEC 9240 system (Automated Blood Culture Systems, for candidemia samples) according to microbiological standard procedure. These isolates further identified on the species level by using CHRO Magar Candida (Oxoid Ltd., UK) (**Figure 2(d)**), and API 20 C AUX test [4] [34]. For screening purpose, simple discs soaked in 200 µL of each extract for 37 hours. Modified Kirby Bauer's disc diffusion method (**Figure 3**) at 0.5 McFarland turbidity was applied as per CLSI M-44 A2series guidelines, 2009 [35] [36] [37]. Muller Hilton Agar (MHA, Oxoid Ltd. UK) was prepared by adding 2% glucose and 0.5 µg/ml methylene blue in it. Fluconazole (SD 232, 25 mcg/disc) and voriconazole (SD 277, 1 mcg/disc, Hi-Media, India) discs were placed on MHA and incubated at 35˚C for 37 hours [17] [38]. The inhibitory zones of samples > 14 mm for fluconazole (25 mcg) and >17 mm for voriconazole (1 mcg) were considered sensitive, as per standard by CLSI M 444-S3 guidelines, 2009 [39] [40] [41]. To the best of our knowledge the cut off values for herbs' (*B. aristata* and *P. granatum*) inhibitory zones (ZIH) were not mentioned in any of the previous studies and thus a pilot study was conducted on 20 candidal samples in order to calculate the cut offs for the inhibitory zones of *B. aristata* and *P. granatum* which were found to be greater than 12 and 8 mm respectively. All the tests were repeated and confirmed three times [4].

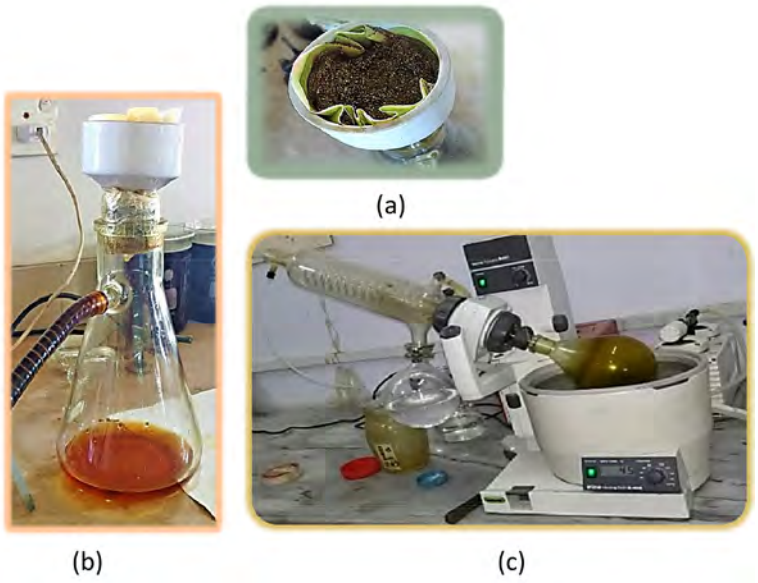

Figure 1. (a): Filtration of soaked pomegranate ethanolic extract by Whatmann number 1, filter paper. (b): Residual pomegranate coarse powder after filtration of its soaked ethanolic extract. (c): Final herb's (darehald/darhald) extraction by vacuum evaporator.

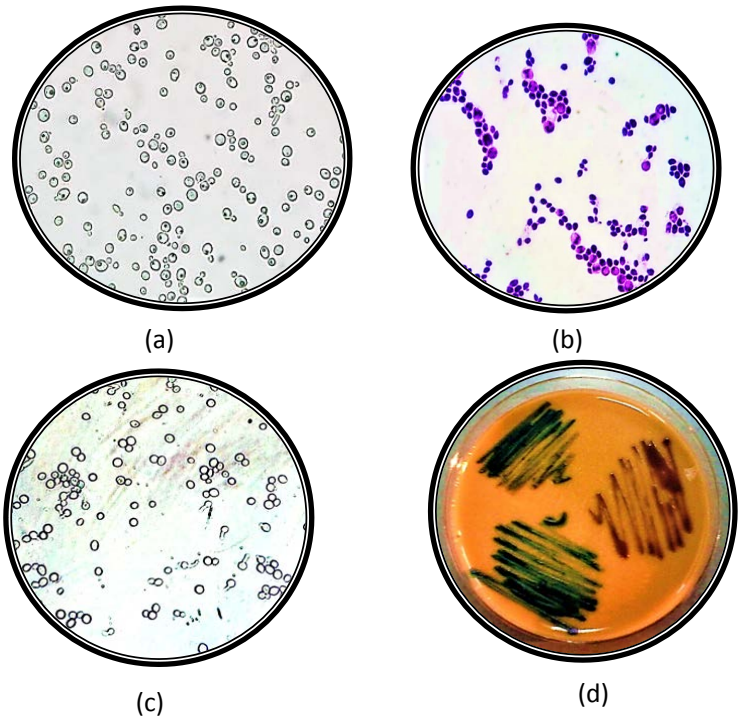

Figure 2. (a): Microscopic view of a candida positive wet mounted slide of a high vaginal swab specimen. (b): Microscopic view of a clinical specimen showing candidal positive on Gram stain. (c): Microscopic view of a clinical specimen showing candida positive germ tube test. (d): Candidal specie identification of different candidal positive specimens on CHROMEagar media showing colonies of *C. albicans* (green color) and *C. glabrata* (pink/mergenda color).

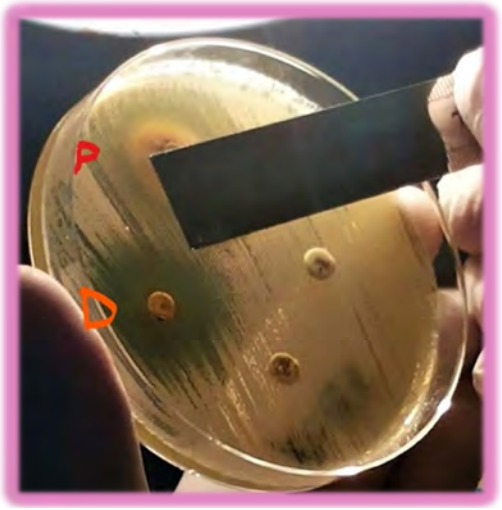

Figure 3. Measuring of inhibitory zones around both herbs (*B. aristata* and *P. granatum*) in azole (fluconazole and voriconazole) resistant strain.

3. Results

In this current study two herbs, *darhald* and *pomegranate* were evaluated for *in-vitro* antifungal activity against candidal isolates to assess their effectiveness in our clinical set ups. The inhibitory zones of azoles (fluconazole and voriconazole), *Berberis aristata* (*B. aristata*, *darhald/darehald*) and *Punica granatum* (*P. granatum*, *pomegranate*) peels' extracts against candidal strains measured in millimeters (mm). The highest mean zone of inhibition (ZIH) calculated on candidal samples was 22.27 ± 4.9 mm of *B. aristata* with statistically significant p-value < 0.05 while the least ZIH was 14.29 ± 12.86 of fluconazole with p-value > 0.05, shown in Table 1. Though according to culture sensitivity pattern of clinical candidal isolates, *B. aristata* was the most sensitive antifungal agent 98.5% and the least sensitive agent was voriconazole 29.2%, displaying in Table 2.

The candidal species identified from clinical specimens were *Candida albicans* (*C. albicans*, 104), *Candida tropicalis* (13), *Candida glabrata* (12) and *Candida ciferrii* (1). While the sensitivity of antifungal agents identified for these various candidal species was; *B. aristata* - *C. albicans* 103 (99.03%), NCACS 25 (98.16%), *P. granatum* - *C. albicans* 101 (95.5%), NCACS 26 (100%), fluconazole - *C. albicans* 43 (37.85%), NCACS 16 (62.01%) and voriconazole - *C. albicans* 31 (30.15%), NCACS 13 (51%) as showing in Figure 4.

These results displayed *C. tropicalis* as the least sensitive among identified species, showing sensitivity of 30.55% (69.45% resistant) to fluconazole and 5.55% sensitive (94.45% resistant) to voriconazole (Figure 4).

4. Discussion

Nowadays herbs are gaining popularity across the globe and are a focus of interest in the field medicine and ethnobotany. Evidence based researches are playing

Table 1. Mean zone of inhibitions of herbs and azoles on candidal isolates.

S. No.	Drugs/Herbs (MIC in mcg)	Zone of inhibitions (mm)	P-value
		Mean ± Std. Deviation	
1	Fluconazole (25)	14.29 ± 12.86	0.434
2	Voriconazole (1)	16.90 ± 13.92	0.648
3	*Berberis aristata* (25)	22.27 ± 4.93	0.000*
4	*Punica granatum* (20)	17.48 ± 4.03	0.020*

P-value calculated by one-way ANOVA followed by Post Hoc Tukey test. *P-value is significant (<0.05).

Table 2. Sensitivity pattern of azole antifungal drugs and herbs against clinical candidal isolates.

	Fluconazole N (%)	Voriconazole N (%)	*B. aristata* N (%)	*P. granatum* N (%)
Sensitive	55 (42.3)	38 (29.2)	125 (98.5)	124 (97.7)
Resistant	75 (57.7)	92 (70.8)	5 (3.8)	6 (4.6)

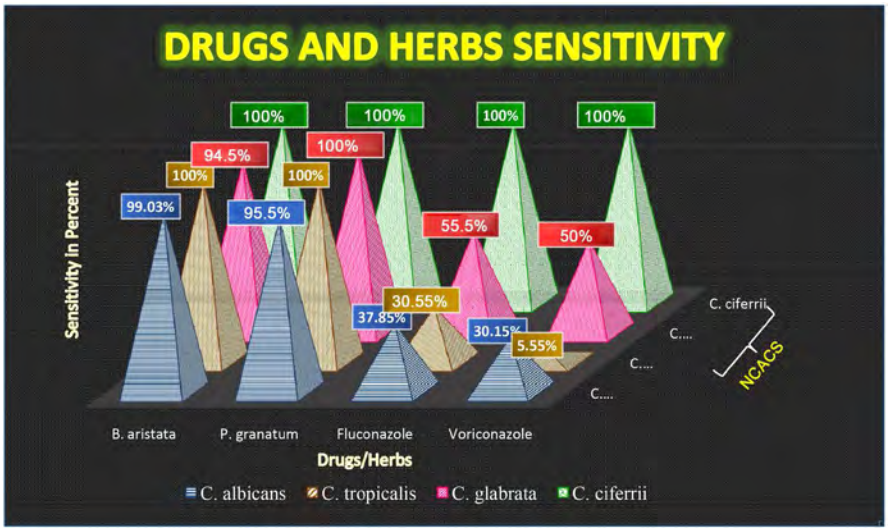

Figure 4. Antifungal sensitivity of azoles and herbs among candida albicans and Non-candida albicans (NCACS).

a crucial role in the exploration and development of new therapeutics [42] The limitations of recently available antifungal drugs, their rise in resistance along with recurrence in fungal (candidal) infections have emphasized the researchers to explore novel antifungal therapies with unique mode of actions against pathogens as well as have few adverse effects, inexpensive and easily available [9]. Since their discovery, azoles (fluconazole and voriconazole) are most widely prescribed antifungal drugs around the world and are fungicidal for aspergillus but are fungistatic for candida with subsequent rise in its resistance over the time [43]. Hence in the current study, two herbs were compared with two most commonly used antifungal drugs of class azoles, fluconazole and voriconazole on the basis of their zones of inhibition for various candidal strains isolated in

order to know their effectiveness in our community.

In our study, the most sensitive agent on *in-vitro* culture sensitivity of candidal clinical specimens was *B. aristata* with mean ZIH of 22.27 ± 4.93 mm (**Table 1** and **Table 2**). Similarly, a study conducted on *B. aristata* displayed mean ZIH 20.1 ± 0.15 mm on candida samples [44]. It is well documented that antifungal activity of *B. aristata* is mainly due to its alkaloid constituent known as "*Berberine*" that inhibits fungal cell wall, ergosterol biosynthesis and mitochondrial functions. Interestingly it has been evaluated innocuous to human being cells [45]. According to our results shown in **Table 1**, the second highest mean inhibitory zone (17.48 ± 4.03 mm) against candidal strains was shown by *P. granatum*. This is in accordance to an Indian study in which *pomegranate* displayed 13 ± 1.225 mm of ZIH on candida samples [46]. The antifungal activity of *pomegranate*'s peels (*P. granatum*) is due to its alkaloid content, tannins containing punicalagin, granatins A and B, gallagyldilacton, casuarinin, pedunculagin, tellimagrandin I and corilagin. These constituents cause fungal cell wall alteration, inhibition of candidal cells' habitation, dwelling and growth on host [47] [48]. In our study the sensitivity of both herbs, *B. aristata* and *P. granatum* for candidal isolates were shown to be 98.5% and 97.7% receptively which was greater than both azoles (**Table 2**). To the best of our knowledge our study is innovative as we reported and compared for the first time the sensitivity pattern of the herbs for candidal samples as in the previous studies only mean zone of inhibitions were mentioned, which already have been discussed above. While the mean inhibitory zones of azoles noticed in this current study were 14.29 ± 12.86 mm for fluconazole and 16.90 ± 13.92 mm for voriconazole with 42.3% sensitivity to fluconazole (57.7% resistance), and sensitivity to voriconazole was 29.2% (70.8% resistance). These results are comparable with global surveillance data by Pfaller, 2010 reported sensitivity of 90.2% (6.2% resistant) to fluconazole and 95% (3% resistance) to voriconazole [48]. In the current research resistance pattern of fluconazole among species noted was, *C. tropicalis* 69.45%, *C. glabrata* 11.1% and *C. albicans* 62.15% while for voriconazole the resistance was shown for *C. tropicalis* 94.45%, *C. albicans* 69.85%, *C. glabrata* 50% (**Figure 1**). Our results clearly displayed that among all isolated candidal species the most resistant specie was *C. tropicalis* with respective 69.45% and 94.45% resistance against both fluconazole and voriconazole. Contrasting results were reported by Pfaller where the highest resistance to fluconazole was observed for *C. glabrata*, 15.7% followed by *C. tropicalis* 4.1% and *C. albicans* 1.4% while for voriconazole the resistance shown was *C. glabrata* 10%, *C. tropicalis* 5.4% and *C. albicans* 1.2% [49]. A study conducted in Iran also displayed contradictory results to our study; the most resistant specie to fluconazole was *C. glabrata* 79.1% though similar to our study the most resistant specie was *C. tropicalis* showing 41.6% resistance against voriconazole [50]. We hoped that the results of this current study may benefit clinicians to recommend appropriate antifungal drugs according to the culture and sensitivity patterns of the candidal isolates prevalent in our community. This current study also highlighted the role of commonly available herbs as

alternative treatment for candidal infection even for those showing high level of resistance to azoles.

5. Conclusions

Punica granatum and *berberis aristata* exhibited superior antifungal activity on all cultured samples of candida in comparison to two widely prescribed azoles, fluconazole and voriconazole in our population. To the best of our knowledge the current study is the first one to report sensitivity patterns of the *B. Aristata* and *P. granatum* on candidal isolates. *C. tropicalis* was greatly resistant to both of the azoles but in contrast, our studied herbs, *B. aristata* and *P. granatum* showed remarkable sensitivity for all candidal species including even those species showing high resistance to both of the commonly prescribed azoles, fluconazole and voriconazole.

Furthermore, both herbs are economical, easily available and documented to have better safety profiles with health boosting properties in general. While conventional antifungal drugs have lots of side effects including drug-drug interactions and are not cost-effective. Hence it is highly recommendable that both herbs, *B. aristata* and *P. granatum* can be considered as alternative drugs for the treatment of candidiasis in near future after subsequent clinical trials and further verification of our results.

6. Limitations of the Study

We conducted our study in few tertiary care health centers of only in one city (Karachi) of our country and could not include other cities and rural areas due to a limited budget.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Herpes Zoster in Diverse Situations: A Review

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Abstract

Herpes zoster occurred when the suppressive ability of immune system failed to prevent the reactivation of initial varicella-zoster virus infection. Its frequency is higher among immune compromised individuals. Herpes zoster presents with characteristic painful grouped vesicles on erythematous background along the dermatome area and could be complicated by post-herpetic neuralgia. The current review examined the risk factors and discussed herpes zoster in different situations, treatment and concluded by discussing the future research trend of herpes zoster.

Keywords

Herpes Zoster, Post Herpetic Neuralgia, Immunocompromised, Malignancy, HIV

1. Introduction

Herpes zoster (HZ) typically occurs when suppressed varicella-zoster virus (VZV) in the neuronal cell bodies is reactivated after resolution of the initial occurrence of chicken pox. Usually, after the occurrence of chicken pox, immune system eliminates the VZV and suppresses its reactivation, but sometimes, the suppressive ability of the immune system fails leading to the reactivation of the VZV [1]. Although the concept of reactivation of suppressed VZV has been established since the 1950's [2], the exact causes of the suppression and subsequent reactivation of VZV leading to HZ remain unclear. Herpes zoster is also called shingles and frequently occurs in immune-compromised individuals as a result of aging, psychological stress and infections such as human immunodeficiency virus (HIV) [1]. Herpes zoster is clinically characterized by painful grouped vesicles on erythematous rash along the dermatome area with clinical symptoms which could include headache, photophobia, malaise, localized abnormal skin

sensations and rarely fever [3]. Herpes zoster can also lead to post-herpetic neuralgia mostly in elderly individuals [4] [5]. Although, the occurrence of HZ in children and young adults is rare, HZ has been reported among sub-Saharan Africa young adults where it has been considered as one of the most influential predictors of HIV [1] [6].

2. Epidemiology

Varicella-zoster virus causes two different diseases *i.e.* the primary infection and reactivated disease known as HZ. The primary infection*:* usually present as a contagious but benign disease called chickenpox among vulnerable children aged between 2 - 7 years [7]. The mode of transmission is via droplet inhalation into the respiratory system [7]. After about 10 - 23 days of incubation period, small pus filled-vesicles break out on the face or upper trunk which get busted and become covered by skin with intense itching. Individuals who recover from chicken pox become subsequently immune to this disease; however, they are not free of the VZV as the viral DNA remains in a latent state in the dorsal root ganglia [8].

The reactivated disease: immunosuppression may occur following immunosuppressive illness or medical treatments or naturally through aging in individuals previously infected with chickenpox [9]. In this context, the VZV may become reactivated and migrate along the afferent sensory nerves to the skin and eye where they replicate and cause characteristic lesions [10]. This subsequent reactivation of latent VZV in dorsal root ganglia results in a localized cutaneous eruption called "herpes zoster."

The global annual incidence rate of HZ ranges from 1.2 - 3.4 cases per 1000 healthy individuals [11] [12]. Since increasing age is a crucial risk factor for the development of HZ; there is an increase of the annual incidence to 3.9 - 11.8 per 1000 individuals among those older than 65 years [11] and exceeds 10 cases per 1000 persons among individuals older than 75 years of age. The lifetime risk of herpes HZ is estimated to be 10 to 20 percent [12].

Several risk factors for herpes zoster have been identified. These include altered cell-mediated immunity, human immunodeficiency virus infection, neoplastic diseases, patients on immunosuppressive drugs and organ-transplant recipients. A study has shown an incidence of 29.4 cases of HZ per 1000 individuals who were HIV-seropositive, compared to 2 cases per 1000 individuals among HIV-seronegative [13]. In the context of HIV infection, HZ may occur in asymptomatic HIV-positive individuals. Therefore, HZ might be an indication for HIV serology testing in patients with high risk of HIV infection due to its ability to cause early alteration in cell-mediated immunity and there are also several studies showing that individuals with HZ are more likely to have HIV [14] [15].

3. Clinical Manifestations of Herpes Zoster

The prodrome of HZ could include a report of a headache, photophobia, ma-

laise, and rarely fever. Most patients present with localized abnormal skin sensations, ranging from itching or tingling to severe pain, which precedes the typical skin lesions by 1 - 5 days. The pain in HZ is of variable severity, and virtually all patients with acute HZ complain of pain. Following these, the patient developed erythematous macule and papular rash which progresses to clusters of clear vesicles which continue to form for about 3 - 5 days and evolve through stages of pustulation, ulceration, and crusting [**Figure 1**, **Figure 2**]. The cutaneous eruption is usually unilateral and does not cross the midline. Healing occurs over a period of 2 - 4 weeks and often results in scarring and permanent changes in pigmentation of the affected area [**Figures 3-5**]. The involvement of multiple noncontiguous dermatomes almost never occurs in immune-competent patients, although in 20% of cases, lesions overlap adjacent dermatomes. Presence of few skin lesions outside the main or contiguous dermatomes is not rare in immune-competent patients [3].

4. Diagnosis of Herpes Zoster

The diagnosis of HZ is essentially clinical when the presentation is typical. However, the manifestations may be bizarre or unusual particularly in immune-compromised individuals, and laboratory confirmation may be necessary. Available confirmatory investigations may include viral culture, polymerase-chain-reaction, and direct immunofluorescence assay. The lability of VZV makes it relatively hard to recover the virus from swabs of cutaneous lesions. Direct immunofluorescence assay is a more sensitive laboratory technique and has the additional advantages of a lower cost [16]. Like culture, the direct immunofluorescence assay can also distinguish herpes simplex virus infections from VZV infections. Polymerase-chain-reaction techniques are also useful for detecting VZV DNA in fluid and tissues [17].

Figure 1. Vesiculation, ulceration and crusting of Acute Herpes Zoster.

Figure 2. Hutchinson's sign, pustulation, crusting and swelling of in herpes zoster ophthalmicus in HIV patient

Figure 3. Cervical Post herpertic scarring and dyspigmentation in patient with Leucopenia.

5. Prognosis of Herpes Zoster

Herpes zoster rarely causes fatalities in immune-competent individuals; however, HZ can be life-threatening in individuals with compromised immunity. Ocular complications occur in about 50% to 90% of the HZ cases, resulting in either

Figure 4. ThoracicPost herpertic scarring and dyspigmentation in patient with Leucopenia

Figure 5. Necrotic Herpes zoster in 75 year old woman with Chronic Lymphocytic Leukaemia.

temporary or permanently decreased visual acuity or total blindness if untreated [18] [19]. Other complications of HZ include sensory loss, peripheral nerve palsies, bacterial super-infections, and disseminated HZ [20].

6. Postherpetic Neuralgia and Other Complications

Post-herpetic neuralgia is defined as pain that persists more than 30 days after the onset of rash or after cutaneous healing. It is the most dreaded complication

in immune-competent individuals because both the incidence and duration of post-herpetic neuralgia are directly associated with the individuals' age [12] [21]. The reported incidence of post-herpetic neuralgia ranges from 8% - 70% and incidence increases with advancing age [12]. In addition to neuropathic pain, other sensory abnormalities within the affected dermatome could include allodynia, in which non-noxious stimuli is perceived as painful. Pain could continue for months and at times for years. Other complications of herpes zoster in immune-competent individuals include cranial and peripheral-nerve palsies, myelitis, encephalitis, and a syndrome of delayed contralateral hemiparesis [17].

Disseminated Herpes Zoster: Before the introduction of antiviral drugs, cutaneous spreading of VZV was reported in 6% - 26% of immune-compromised individuals [22] with the disseminated form of the disease restricted to the skin; however, there is visceral involvement like hepatitis, pneumonitis or encephalitis in about 10% - 50% of these patients. Even after the introduction of intravenous acyclovir therapy, the mortality rate from HZ with visceral dissemination reduced to about 5% - 15%, with most deaths caused by pneumonitis [22].

Although acute retinal necrosis caused by VZV rarely occurs in immune-competent individuals, recent reports have focused more on ocular disease in immune-compromised individuals particularly HIV-infected persons [23]. Visual changes start weeks or months after HZ have resolved and funduscopic examination shows characteristic granular, yellowish, non-hemorrhagic lesions. Usually, in HIV-infected patients, the lesions rapidly extend and coalesce, respond poorly to antiviral therapy, and almost inevitably cause blindness in the involved eye. Retinitis is less aggressive in immune-competent persons and can often be arrested with antiviral therapy than in immune-compromised persons [3].

7. Herpes Zoster in HIV

Human immunodeficiency virus (HIV) is a recognized risk factor for HIV. Other well documented immunosuppressive conditions include cancers and chronic medical conditions [24] [25] [26]. Numerous evidences have shown that the prevalence of HIV infection is very high among persons with highly threatening HZ [14] [27]. Other studies have also shown that HZ could be the first sign of undiagnosed HIV infection because of its role in cell-mediated immunity [14] [15].

The incidence of HZ in HIV infected individuals has been reported by several researchers to be between 8.4% - 26.7% [6] [13] [28], while in HIV seronegative patients are between 1% - 2% [5] [28]. The association between HZ and duration of HIV infection is still not clear. However, some literature has described HZ as the first indicator [29] and an important predictor of more rapid progression of HIV to acquired immunodeficiency syndrome (AIDS) [30], while others differ on this [31].

In the context of HIV infection, relapse of HZ is assumed to be an indication

of the more advanced stage of HIV infection. Cutaneous lesions in the majority of the cases of HZ have classical course, some have dispersed infection with more destructive and necrotic lesions. Systemic complications especially meningitis and encephalitis may also occur with dispersed or persistent eruptions [32]. While acute retinal necrosis caused by VZV occasionally occurs in immune-competent patients, it is frequent in HIV and a case of jaw osteonecrosis caused by HZ in HIV has also been reported [23] [33].

7.1. Herpes Zoster in HIV-Infected Adults on Combined Antiretroviral Therapy

Herpes zoster is mainly a disease of aged and immune-compromised individuals, likely due to impaired cell-mediated immunity [1]. Before the availability of combined antiretroviral therapy, the incidence of HZ in HIV infected individuals was about 10 to 30 times higher when compared to HIV-seronegative individuals [6] [13] [28] [29] with low CD4 cell counts being the most potent predictor of HZ [34].

Since the introduction of combined antiretroviral therapy (cART), studies opinion on prevalence of HZ varies. Several studies of both adults and children suggest that the incidence of HZ has not changed, whereas other studies have shown a significant decrease [34] [35] [36]. Also, numerous literature have reported a rise in the incidence of HZ during the first months following cART initiation, suggesting that HZ may be a feature of the immune reconstitution inflammatory syndrome (IRIS) [36].

7.2. Immune Reconstitution Herpes Zoster

Immune reconstitution inflammatory syndrome (IRIS) is an inflammatory reaction that ensues as a result of immune reconstitution after the start of highly active antiretroviral therapy (HAART) in HIV seropositive patients [37], giving way to deteriorating opportunistic infection like herpes zoster which is believed to be the most common manifestation of IRIS in spite of an increase in CD4+ cell count and a decrease in HIV viral load [38] [39]. HIV patients with CD4+ cell counts between 50 and 200 cells/mm^3 and those on HAART is believed to have the highest risk for HZ occurrence [40]. The IRIS can be classified into unmasking IRIS and paradoxical IRIS. Unmasking IRIS occurs when opportunistic diseases which are not present at the start of ART begin to manifest as a result of ART-induced immune reconstitution. However, paradoxical IRIS occurs when patients who are already on medication for an opportunistic disease, and in whom immune reconstitution after initiation of ART provokes the clinical worsening of that disease during the initial treatment [41]. The use of HAART in HIV management has led to a reduction in mortality rate; however, about 25% - 35% of patients on HAART have been reported to develop IRIS [42].

The period between onset of disease and commencement of HAART is so va-

riable that controversy exists when trying to define the interval which the disease must manifest for it to be termed IRIS. The duration between the start of HAART and the onset of an opportunistic disease has been reported to be greater than 90 days [38]. However, another report concluded that there was no difference in the risk of HZ occurrence before and after 90 days from the start of HAART [43]. Conversely, other authors believe that onset of IRIS-induced HZ tend to increase approximately after four weeks [44].

Although HZ can occur at any CD4+ count in HIV-seropositive individuals, the occurrence is highest with CD4+ counts of < 200 cell/mm^3. The occurrence of IRIS after the initiation of HAART is rare in patients with CD4+ cell counts of >350 cell/mm^3 [45]. On the other hand, individuals with a baseline CD4+ cell percentage of < 10% has been reported to have a three-times increase in the risk of IRIS occurrence [37] [46].

Furthermore, the diagnostic value of CD8+ cells remains controversial. The number of circulating CD8+ cells does not necessarily show either their numbers in infected, inflamed tissues, or their functional competence [47]. Thus, CD8+ cells cannot be used as a laboratory marker for differentiation of HIV-IRIS from an opportunistic infection in immune-compromised patient. In spite of these reports, findings of some studies have established the significance of CD8+ cells in the pathogenesis of IRIS, but not its importance as a diagnostic tool. The percentage of CD8+ cells at baseline and the magnitude of their increase one month after initiation of antiretroviral therapy have shown a strong association with an increased risk for herpes zoster [48].

HAART has become a vital therapy in the management of HIV. Globally, numerous cases of IRIS are reported annually. However, many controversies still exist. While some literature has documented case definitions of IRIS [42], lack of agreement about the definition of IRIS makes differentiation of IRIS from recurrence or relapse of an infection a challenge. Also, a few risk factors, including CD4+ T-cell count, HIV-1 RNA level, and HAART regimen, have been reported. However, these are too complex to simplify as definite risk factors [42].

8. Herpes Zoster Ophthalmicus

Herpes zoster ophthalmicus (HZO) occurs due to reactivation of latent VZV in the ophthalmic division of the trigeminal nerve. Although HZO could damage the eye and surrounding structures through secondary perineural and intraneural inflammation of sensory nerves, it does not certainly affect the structures of the eye [49].

HZO accounts for about 10% - 25% of all cases of HZ and about 50% of persons diagnosed with HZO will develop complications [12]. While HZO usually produces a characteristic dermatomal rash, few of patients may have only ophthalmic findings, limited mainly to the cornea.

8.1. Extra-Ocular Manifestations of Herpes Zoster Ophthalmicus

HZO usually begins with an influenza-like illness with fatigue, malaise, and

low-grade fever for about one week before the appearance of rash over the forehead [50] with approximately 60% having varying degrees of dermatomal pain in the course of the ophthalmic nerve [51]. Later, erythematous macules, papules, vesicles containing serous fluid and pustules appear along the involved dermatome. These lesions rupture, crust heals over several weeks [52]. The risk of HZO is higher in immune-compromised individuals compared to immune-competent individuals [53]; with generalized vesicular rash and more serious visual complications occurring more in immune-compromised individuals [54].

8.2. Ocular Manifestations of Herpes Zoster Ophthalmicus

The affectation of the nose tip, also known as Hutchinson's sign [**Figure 2**] is believed to be a clinical predictor of ocular involvement. Although patients with Hutchinson's sign have double the occurrence of ocular manifestations, only one-third of patients without Hutchinson's sign develop ocular involvement [18]. Ocular manifestations in persons with HZO include the following:

a) Blepharitis and Conjunctivitis

The eyelids are frequently involved in HZO. Patients may develop blepharitis and present with ptosis secondary to edema and inflammation. Many of them usually have vesicular lesions on the eyelids which resolve with minimal scarring. Conjunctivitis is also a common manifestation of HZO wherein the conjunctiva appears injected, edematous, often with petechial hemorrhages [55] [56].

b) Corneal Disease

Corneal involvement occurs in almost 65% of HZO and can lead to varying degree of vision loss, pain, and light sensitivity [54]. Punctate epithelial keratitis, the first corneal finding [56] may occur between one or two days after the initial skin rash and may either resolve or progress to dendrite formation which appears at four to six days or even several weeks after [57]. In approximately 25% - 30% of persons with HZO, both punctate and dendritic lesions could cause anterior stromal corneal infiltrates which is characterized by multiple fine granular infiltrates in the anterior corneal stroma below the epithelial layer [57] [58]. Neurotrophic keratitis is the end consequence of decreased corneal sensation from HZO-mediated destruction, including susceptibility to mechanical trauma, decreased lacrimation, and delayed epithelial healing [54].

c) Uveitis

Anterior uveitis frequently occurs in HZO and may be isolated or associated with keratitis. It causes a mild and transient inflammation with a mild rise in intraocular pressure. It could lead to atrophy of the iris, irregular pupil, glaucoma and cataract formation [19].

d) Retinal Necrosis

HZV is usually thought to be the causative agent in most cases of acute retinal necrosis and progressive outer retinal necrosis syndromes. Both conditions commonly cause retinal detachment. Acute retinal necrosisis characterized by peripheral patches and rapidly coalescing retinal necrosis, occlusive vasculitis,

and vitreous inflammation. However, progressive outer retinal necrosis is a more severe viral retinitis observed in immune-compromised individuals when compared with acute retinal necrosis. The severity of progressive outer retinal necrosis has been related to in ability immune incompetent individuals to mount a clear inflammatory response, leading to a rapid involvement of the macula. [19].

9. Herpes Zoster in Malignancy

It has been well established that immune suppression is the link between HZ and malignancy [59]; however, the outcomes of studies that investigated the relationship between HZ and malignancy have been conflicting. [60]-[65]. Some previous studies observed no link between HZ and occurrence of cancer [62] [63] [65]. One of the studies reported that HZ diagnosis did not increase the overall risk of cancer and the prevalence of cancer among patients with HZ was similar to the expected rate in the healthy population. However, HZ was reported to have increased the risk of multiple myeloma [65].

Conversely, studies that investigated the association between hematological malignancies such as multiple myeloma, non-Hodgkin's lymphoma, leukemia and the occurrence of HZ showed that there is a link between HZ and haematological malignancy [60] [61]. The factors associated with increase risk of cancer in patients with HZ include; age above 65 years and female gender. A certain prospective study that followed up patients with HZ showed that in the first year after HZ diagnosis, no difference was observed in the occurrence of cancer, suggesting that HZ may not be a sign of undiagnosed malignancy. Therefore, the study indicated that patients with HZ needed not to be screened for underlying malignancy [64].

Finally, a study concluded that there is an increased risk of malignancy development after HZ diagnosis, and this risk affects both gender and all age groups older than 18 years equally. The authors reported that the risk was greatest in the first six months after HZ and continued for about five years. They also concluded that though malignancy screening cannot be recommended because of the modest increase in risk and the lack of specificity regarding malignancy type, they suggested that clinicians should be observant for malignancy in HZ patients [66].

10. Herpes Zoster in Children

In children, HZ is a rare disease but could occur in children at any time after varicella infection or varicella vaccination. The HZ is less frequent after varicella vaccination compared to the higher frequency following natural progression from varicella infection. The prevalence of reactivated varicella is lower in children compared to adults. The incidence of HZ in children below 14 years is about 0.45 per 1000 persons, but the occurrence could rise to about 4.5 per 1000 persons in adults above 75 years [67] [68] [69]. Generally, the course HZ is relatively mild in children with a mean duration of 1 - 3 weeks. Although lesional pru-

ritus and pain may be present, the occurrence of postherpetic neuralgia or other common complications of HZ in adults is negligible [68] [69].

The presence of HZ in children is a marker of an underlying malignancy, particularly acute lymphatic leukemia. However, the increase in the prevalence of malignancy has not been shown in children with HZ, but about 3% of children with HZ had malignancies [70]. Increase incidence of HZ in immune-competent healthy children has been attributed to primary VZV infection *in-utero*, or in infancy when immunity was not fully developed. Immunologic findings associated with observed suppressed early life immunity include a low level of lymphocytes, cytokines, natural killer (NK) cells and virus-specific immunoglobulins, which may lead to an inability of preserving the dormancy of the VZV causing the early occurrence of HZ in children. To a lesser extent, vaccination with live attenuated varicella virus may also contribute [71]. The diagnosis of HZ in children is clinical but could be confirmed by viral culture [72].

11. Therapy for Herpes Zoster

The goals of effective HZ therapy include rapid healing, limitation of severity, limitation of the duration of acute/chronic pain, and reduction of complications. Furthermore, in immune-compromised persons, an additional therapeutic objective is to reduce the risk of spreading of VZV.

11.1. Symptomatic Treatment

The skin lesion of HZ must be clean and dry to reduce the risk of superimposed bacterial infection. A sterile, non-occlusive and non-adherent dressing should be placed over the involved dermatome to prevent the lesions from contact with clothing. Clinicians should not underestimate neuralgic pain. Sympathetic-nerve blockade can provide rapid, temporary relief of severe pain [73]. For mild to moderate pain, scheduled short-acting narcotic analgesics could be prescribed, but for persistent pain, long-acting and controlled-release opioids (oral or transdermal) are preferred. Some studies of the pathogenesis of postherpetic neuralgia suggest that early reduction of acute pain may prevent central mechanisms of chronic pain initiation, thereby reducing the risk of post-herpetic neuralgia [74].

11.2. General Antiviral Therapy

In the United States, acyclovir, a prodrug of acyclovir, valacyclovir, and famciclovir are drugs that are approved for the treatment of HZ. The three drugs are very safe and well tolerated, and there are no contraindications to their use, although dosage adjustment is required in patients with renal insufficiency. However, none of these drugs are currently approved for use in pregnant women by the Food and Drug Administration, and there is no role for topical antiviral therapy in HZ management [13] [75].

A previous study using 800 mg of acyclovir five times daily reduced the extent

of viral shedding, stopped the rapid formation of new lesions, hastened to heal, and reduced acute pain severity [75]. However, the roles of acyclovir in the reduction of frequency and duration of post-herpetic neuralgia have been inconsistent [76]. Valacyclovir given as 1000 mg every 8 hours produced about 3 - 5 times higher serum levels of acyclovir levels than oral acyclovir therapy and resulted in equal degrees of cutaneous healing compared to acyclovir therapy [77]. In another study, famciclovir a pro-drug of penciclovir given 500 mg every 8 hours significantly reduced the extent of viral shedding, prevent and limits the formation of the new lesion and hasten to heal when compared with controls on placebo [78].

Valacyclovir and famciclovir have been shown to be both therapeutically equal in cutaneous healing and pain resolution [79]. The higher pharmacokinetic profiles and simpler dosing regimens of valacyclovir and famciclovir make them choice medication in the treatment of HZ.

11.3. Therapy for Herpes Zoster Ophthalmicus

Without antiviral treatment, approximately 50% of these individuals will have ocular complications such as keratopathy, episcleritis, iritis, or stromal keratitis, some of which are sight-threatening [50]. Oral antiviral therapy has been reported to also reduce the frequency of late ocular complications from about 50% to 20% [77].

11.4. Therapy for Herpes Zoster in HIV-Seropositive Patients

Although HZ seen in HIV-seropositive patients and immune-competent individuals is typically similar after adjustment for age, distinct features such as frequent recurrences and atypical lesions are well described [80]. Acyclovir is proven to be useful for HZ in HIV-infected patients [81] and cases of disease caused by acyclovir-resistant VZV in patients with advanced acquired immunodeficiency syndrome (AIDS) are rare [82]. Though famciclovir and valacyclovir have not been assessed systematically; anecdotal experience suggests that they are likely to be efficacious [82]. Also, because of the risk of relapsing infection in these patients, VZV disease should be treated until all lesions have resolved completely [3].

11.5. Corticosteroids Therapy

Previous clinical trials evaluating the role of corticosteroids in combination with acyclovir showed that individuals on corticosteroids had a moderate hastening in the rate of cutaneous healing and reduction of acute pain [83]. However, the use of corticosteroids without concomitant antiviral therapy is not recommended and should be avoided in patients at risk for corticosteroid-induced toxicity such as diabetes mellitus [3]. Also, combination therapy resulted in an improved quality of life, as measured by reductions in the use of analgesics, the time to uninterrupted sleep, and the time to resumption of usual activities [83]. Howev-

er, none of the studies established the influence of corticosteroids on the incidence or duration of post-herpetic neuralgia. Combination therapy using valacyclovir or famciclovir with corticosteroids is assumed to be equally effective, but it has not been examined in clinical trials [3].

11.6. Therapy for Post-Herpetic Neuralgia

Treatment of post-herpetic neuralgia is complex since it usually requires a multifaceted approach [84] [85]. Although clinical trials have shown that opioids, tricyclic antidepressants, and gabapentin reduce the severity or duration of post-herpetic neuralgia, either as single agents or in combination [86], their adverse effects may, however, be additive, particularly in elderly individuals. Also, the topical use of lidocaine patches or capsaicin cream can provide relief for some individuals [84] [85]. A study has also shown that intrathecal injection of methylprednisolone acetate once every week for four weeks significantly reduced pain in individuals with intractable post-herpetic neuralgia [87]. However, because some patients still experience significant pain and intolerable side effects even after the use of the aforementioned therapies, further studies revealed that the use of topical peppermint oil (or menthol) [88], geranium oil [89] and adenosine monophosphate (AMP) [90] yielded great success rate with lessened pain and little or no side effect.

In the study conducted using topical peppermint oil (or menthol), it was reported that a 76-year-old woman who had tried numerous drug treatments such as tricyclic antidepressants amitriptyline and dosulepin; anticonvulsants carbamazepine and gabapentin for about three years had experienced no relief. She had also use intravenous lidocaine and capsaicin cream but has also been unhelpful. Surprisingly, the patient was successfully treated with topical peppermint oil, and after two months of follow-up, she has had only a little side effect with continuing analgesia [88]. Another study which assessed neuropathic pain relief and the timing of onset of the relief with the use of topical geranium oil showed that when compared to topical capsaicin which relieves neuropathic pain gradually over two weeks, topical use of geranium oil relieves pain in minutes and it's well tolerated with minimal side effect [89]. In the study using AMP for the treatment and prevention of post-herpetic neuralgia, it was documented that intramuscular injection of gel-sustained AMP thrice weekly for four weeks reduces pain, decreases desquamation time and virus shedding, and promoted faster skin healing with no side effect and toxicity during and after treatment. No pain or lesion reoccurrence was seen 3 - 18 months post-treatment [90].

12. Future Research Aspect

The most threatening future of HZ burden is the rising cost, driven primarily by demographic factors of increasing and aging population [91]. The pandemic of HIV/AIDS in sub-Sahara of Africa and other immunosuppression related dis-

eases are clinical related factors driving the burden of HZ. The availability of biologics and other immunosuppressant therapies in clinical use are also playing their roles. Varicella and HZ vaccines are safe and effective preventive tools that have made their inroads into the reducing the global burden of HZ. Vaccine reduces incident zoster in both immunocompetent and immunocompromised [92] [93]. Some live attenuated vaccine is bedeviled by chance of reactivation. Similarly, vaccine uptake is low especially among the blacks where it is available and not freely available for use in many African nations. Research should be directed towards ensuring wide availability of safe and efficient vaccines to reduce burden of HZ in every groups.

The Advisory Committee on Immunization Practices has suggested VZV Oka strain vaccine for universal childhood vaccination. This vaccine is also believed to induce immune enhancement in older seropositive individuals by increasing cytotoxic-lymphocyte responses specific for VZV [94]. However, further studies are required to be done to determine if the vaccine-induced immune enhancement could reduce the frequency or severity of HZ in elderly individuals.

13. Conclusion

Herpes zoster develops when there is a reactivation of suppressed VZV with increased incidence in older adults and immune-compromised individuals. The diagnosis of HZ is essentially clinical when the presentation is typical. However, laboratory confirmation such as viral culture, polymerase-chain-reaction, and direct immunofluorescence assay may be necessary in atypical cases. Although HZ is not usually fatal in immune-competent individuals, it could be life-threatening in individuals with compromised immunity. Apart from symptomatic treatment of HZ, acyclovir, valacyclovir, and famciclovir are very useful drugs that have been approved for HZ treatment. These drugs are very safe and well tolerated. Both valacyclovir and famciclovir are therapeutically equal, and because of their higher pharmacokinetic profiles and simpler dosing regimens, they are both preferred to acyclovir for the treatment of HZ. The possibility of the patients experiencing severe pain should never be underestimated or overlooked. Although, no single therapy has proven to be effective for post-herpetic neuralgia, however, strong analgesics will often be needed.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Occupational Performance of Women with Chronic Pelvic Pain and the Potential of Coping as an Unassisted Intervention

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Abstract

Objective: To assess the satisfaction and occupational performance of women with chronic pelvic pain and to discuss unassisted intervention strategies by the occupational therapist for the care of these patients. Methods: A case-control study was conducted on 75 women with chronic pelvic pain and 75 apparently healthy women. The study was approved by the Research Ethics Committee of the institution and all subjects gave written informed consent to participate. Pain intensity was determined using a visual analogue scale and each patient was submitted to psychometric assessment using the Patient Health Questionnaire, the Self-Reporting Questionnaire of Psychiatric Screening and the Tampa Scale of Kinesiophofobia in their Brazilian version. The main outcomes regarding occupational performance and satisfaction were evaluated using the Canadian Measure of Occupational Performance. Results: The performance and satisfaction scores of women with chronic pelvic pain were significantly lower than those of healthy women. The presence of pain and kinesophobia was directly and independently correlated with low performance and satisfaction scores regardless of ethnicity, marital status, schooling, or psychometric scores. Conclusion: women with chronic pelvic pain present significant impairment of satisfaction and occupational performance. Coping is a potential unassisted intervention strategy to be applied to this population by occupational therapists.

Keywords

Coping, Occupational Therapy, Performance, Resilience, Satisfaction

1. Introduction

Chronic pain is a problem affecting a considerable number of persons all over

the world, with women being usually more affected than men [1]. Among the conditions involving persistent pain among women, chronic pelvic pain (CPP) is one of the most frequent conditions [2]. The estimated worldwide prevalence of this condition is approximately 4% [3], although it appears to be higher in Brazil and in all other developing countries [4] [5]. Since this condition primarily affects women of reproductive age and since most women are active during this phase of life, it is plausible to hypothesize that CPP, in addition to interfering in a negative manner with the personal, marital and social life of women [6] [7], may involve a significant impairment of their occupational performance, *i.e.*, their ability to perform routines, roles and tasks in response to their external and internal environment.

Occupational therapy, mainly over the last decades, has focused on physical, psychosocial and environmental factors aggravated by pain, especially in the self-care, leisure and work spheres. Since the 1980 decade, programs of occupational training have proved to be effective in the counseling and rehabilitation of women with chronic pain [8]. And, within a multidisciplinary context, the Intion (https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1581). Twiddy *et al.* have reported that a multidisciplinary approach is effective in significantly improving the occupational performance measure of the CPP population [9]. The authors cited areas for improvement such as intimate relationships, establishing a routine, personal care and general activity. Nevertheless, to our knowledge, no objective or detailed data are available in the literature about the occupational performance of women with CPP, nor is there an objective detailed description of possible unassisted techniques to be practiced by these professionals.

Thus, the objective of the present study was to assess the satisfaction and occupational performance of women with CPP and to present coping strategies on the basis of a narrative review (comprehensive review) of unassisted interventions by occupational therapists as part of the multi-professional care for these patients.

2. Methods

A case-control study was conducted on 75 women of reproductive age older than 18 years from February 2014 to January 2015. The subjects were seen at the Chronic Pelvic Pain Center of the University Hospital, Ribeirão Preto Medical School, University of São Paulo, a regional reference center. Seventy-five apparently healthy women selected from those accompanying the patients were used as controls. No type of pairing was performed.

2.1. Ethics

All participants were individually informed about the objectives, relevance and methodology of the study. The principles of reliability of the data obtained, maintenance of autonomy of the participants, protected personal identification

and beneficence of the purposes were respected and the subjects gave written informed consent to participate. The study was approved by the Ethics Committee of the institution (protocol n˚ 9091/2012).

2.2. Eligibility

Women of reproductive age, with no history of neoplasias or chronic diseases such as diabetes and hypertension, or of recent acute pain events, and receiving no treatment modality for their current condition were considered to be eligible for the study. All participants answered the questionnaires before any therapeutic measure was instituted. For the disease group of the study we considered women with noncyclic pain of six or more months' duration localized to the anatomic pelvis, or anterior abdominal wall below the umbilicus, and of sufficient severity to cause functional disability or to require medical care. Women with isolated dyspareunia or dysmenorrhea were not included. The control group of healthy women was recruited from subjects with the same sociodemographic characteristics as the patients, exclusively attending a primary health care unit of the Unified Health System of the country.

2.3. Variables and Data Measurement

An initial interview was held with each woman in a private room, during which all procedures were first explained. We then applied a semistructured questionnaire, including investigation about age, parity, marital relationship, schooling, professional activity, religion, race, abdominal surgery, age at menarche, body mass index, time and intensity of pain measured with a visual analogue scale. We also performed psychometric assessment using the Brazilian version of the Patient Health Questionnaire (PHQ) [10], of the Self-Reporting Questionnaire of Psychiatric Screening (SRQ) [11], and of the Tampa Scale of Kinesiophofobia (TSK) [12].

The main outcomes of occupational performance and satisfaction were assessed with the Canadian Occupational Performance Measure (COPM) [13], a validated instrument of broad applicability [14] consisting of three stages:

1) description of daily activities and occupations. In this stage the reported activities were assigned to the following categories and subcategories:

a) self-care: personal care (hygiene, eating habits, clothing), mobility (transfers, mobility inside and outside the home), independence outside the home (transportation, shopping, finances);

b) productivity: job (looking for and/or keeping employment, voluntary activities), household chores (cleaning, washing clothes, preparation of meals), duties or school activities (duties in addition to a job and household chores);

c) leisure: quiet recreation (hobbies, reading, handcraft, TV, computer), active recreation (sports, excursions, trips), and socializing (visits, telephone calls, parties, letter writing);

2) scoring and hierarchization of the importance of the activities: the partici-

pant scored the relevance of each activity on a 100 mm visual scale (ranging from "of no importance" to "extremely important"). According to the order of relevance, the subject was asked to choose three to five activities with higher scores;

3) classification of performance and satisfaction: for each chosen activity the participant quantitated her performance in it and her satisfaction with it also using a 100 mm visual scale (ranging from "unable to do" to "able to do extremely well" for performance, and "not satisfied" to extremely satisfied" for satisfaction). The final result of this evaluation considered the mean value of the sum of scores for performance and the sum of scores for satisfaction divided by the number of activities assessed.

2.4. Data Bank

The data obtained were recorded on a form specifically prepared for this purpose and transferred to the electronic data bank, managed in such a way as to guarantee confidentiality of the information and preservation of patient identity.

2.5. Statistical Analysis

Sample size was calculated in order to compare two means (two-sample, two-sided equality) by an online calculator (http://www.powerandsamplesize.com/). Based on a pilot sample of healthy subjects (n = 30), the parameters used were power of 90%, type I error rate of 5%, mean of performance/satisfaction of 9.6, standard deviation of 3.75, sampling ratio of 1, and estimated significant difference of at least 2.0 points below the mean for the healthy group, *i.e.*, a disease group mean of at least 7.6. Exploratory data analysis was carried out based on the means and standard deviations and the frequency distribution. The Fisher exact test was used to compare the frequencies of each quantitative variable of the study between groups and the Student t-test was used to compare the quantitative variables. The distribution of the data was checked using normalized graphs. Linear regression analysis was carried out to determine the effect of covariables in relation to the variables of interest only for the group of patient with CPP. The fit of the model was determined by residue analysis. All analyses were carried out with the aid of the SAS software, version 9.3, with the level of significance set at 5%.

2.6. Comprehensive Review

Studies in English reporting the interventions of occupational therapists in adult subjects with non-oncologic chronic pain were considered eligible, regardless of sex or class of the population studied. Studies that used assistance technologies or that did not present or describe the technique used were not considered to be eligible. Review articles and guidelines were read in full but were not included in the analysis. All reference lists were explored in order to expand the search for information.

The search was carried out in the Pubmed and Scopus databases using the following key words: ["occupational therapy" or "pacing" or "relax*" or "goal*" or "cognit*" or "exerc*"] and ["chronic pain" or "persistent pain"]. In this step, we investigated whether the title and abstract contained the above key words. The search covered the period of the last 25 years (1994-2018). All articles selected were obtained in full and were evaluated independently by two authors (RVB and OBPN) and classified by the Jaddad criteria of quality [15]. Eventual doubts were resolved in a consensus meeting. A total of 264 articles were detected; 171 of them were not considered because they did not specify the professional involved in the intervention and 23 that were bibliographic reviews and/or citations were also excluded. Thus, 70 articles were selected and read in full. Of these, 66 were discarded, 2 of them because they were guidelines, 17 because they were bibliographic reviews and 47 because they involved a multidisciplinary team (these last articles were discarded because they did not describe the type of intervention used by occupational therapy within the multidisciplinary approach). Only four articles were considered eligible [16] [17] [18] [19], as listed in Table 1.

3. Results

The characterization of the series is presented in Table 2.

Women with CPP had significantly lower performance and satisfaction scores than healthy women regarding all categories presented, *i.e.*, self-care, productivity and leisure (Table 3). A significantly higher proportion of women with CPP attributed more importance to functional mobility than healthy ones (18.7% versus 12.0%, respectively) and to household chores (38.7% versus 17.3%, respectively). On the other hand, sick women attributed lower importance to school activities or duties other than work or domestic activities than controls (48.0% versus 77.3%, respectively) (Table 4).

A direct correlation was detected between low satisfaction scores and the presence of CPP, regardless of ethnicity, marital status, schooling, or PHQ and

Table 1. Information contained in comprehensive review articles.

Authors	Country	Journal	Year	Intervention	Follow-up	Instruments	Score
Krischak et al.	Germany	J Shoulder Elbow	2013	Functional restoration	8 weeks	Visual analogue Scale (VAS) EuroQol questionnaire (EQ-5D)	5
Murphy et al.	USA	Am J Occup Ther	2010	Pacing	10 weeks	Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) Brief Fatigue Inventory (BFI) Six Minute Walk Test Timed Up and Go Test Actiwatch-Score	4
Park and Sonty	USA	J Pain	2010	Coping	1 interview	Treatment Outcomes in Pain Survey (TOPS) Numerical rating scale (NRS)	1
Persson *et al.*	Sweden	Scand J Occup Ther	2011	Coping	1 interview	Interview with narrative structure	2

Note: Score classified by Jaddad criteria of quality.

Table 2. Characterization of the casuistic series.

	Healthy (n = 75)	CPP (n = 75)	p
Age (mean ± sd)	35.5 ± 4.3	34.6 ± 7.1	0.7320
Stable union (n, %)	46 (61.3)	54 (72.0)	0.1659
Schooling (n, %)			<0.0001
<8y	7 (10.0)	19 (25.3)	
8 to 11y	10 (13.3)	24 (32.4)	
12 to 15y	20 (26.7)	23 (31.1)	
16 + y	38 (50.0)	9 (12.2)	
Paid work activity (n, %)	15 (20.0)	18 (24.0)	0.5543
Religion (n, %)			0.0256
Catholicism	39 (51.7)	29 (39.1)	
Atheism	7 (10.0)	2 (2.8)	
Others	29 (38.3)	44 (58.7)	
Self-declared color (n, %)			0.2504
White	63 (85.2)	69 (91.8)	
Black	6 (8.2)	12 (17.6)	
Menarche (mean ± sd)	13.0	12.5	0.3247
Parity (median, range)	1 (0 - 4)	1 (0 - 6)	0.3211
Abdominal surgery (n, %)	9 (12.2)	56 (75.3)	<0.0001
Dysmenorrhea (n, %)	0 (0.0)	39 (52.0)	<0.0001
Dyspareunia (n, %)	0 (0.0)	32 (42.5)	<0.0001
PHQ-4 (mean ± sd)	2.8 ± 2.4	5.6 ± 3.9	<0.0001
PHQ-4 ≥ 5 (n, %)	15 (25.0)	40 (54.8)	0.0004
SRQ (mean ± sd)	4.5 ± 2.8	8.4 ± 4.9	<0.0001
SRQ ≥ 8 (n, %)	12 (20.0)	40 (54.8)	<0.0001
TSK (mean ± sd)	-	44.8 ± 9.1	—
Duration of pain in months(mean ± sd)	-	99.8 ± 59.8	—
Pain intensity in millimeters (mean ± sd)	-	69.6 ± 22.1	—

Note: CPP: chronic pelvic pain; Intensity of pain evaluated by visual analogue scale; sd: standard deviation.

SRQ scores. We also identified a consistent, independent and indirect correlation between kinesiophobia scores and leisure satisfaction (t statistic = −2.5 and p = 0.04), as well as with total scores of satisfaction (t statistic = −3.1 and p < 0.01). Similarly, we observed an independent and indirect correlation between performance scores and presence of CPP for all categories. However, kinesiophobia was not correlated to performance, despite being important for some categories of satisfaction (**Table 5**).

Our comprehensive review identified three objective intervention alternatives, all of them finally culminating with a coping process: the practice of exercise for functional reestablishment [16], pacing [17], and coping itself [18] [19]. Krischak *et al.*'s paper [16] prospectively reported the effect of a supervised

Table 3. Performance and satisfaction score measured with the COPM scale.

	Healthy	CPP	p
Performance			
Self-care	9.8 ± 0.6	6.3 ± 2.8	<0.0001
Productivity	9.6 ± 0.6	5.4 ± 3.0	<0.0001
Leisure	9.6 ± 0.9	4.8 ± 3.1	<0.0001
Total score	9.8 ± 1.2	5.4 ± 2.4	<0.0001
Satisfaction			
Self-care	9.7 ± 0.76	4.7 ± 3.1	<0.0001
Productivity	9.6 ± 0.8	4.2 ± 3.0	<0.0001
Leisure	9.5 ± 1.0	4.1 ± 3.1	<0.0001
Total score	9.6 ± 0.7	4.4 ± 2.4	<0.0001

Note: COPM: Canadian Occupational Performance Measure; CPP: chronic pelvic pain. Analysis performed by the Wilcoxon (Kruskal-Wallis) test, and confirmed by linear regression, considering marital status, paid work activity, schooling, religion, abdominal surgery, and age.

Table 4. List of activates to which the participants attributed higher importance.

	Healthy (n = 75)	CPP (n = 75)	p
Self-care, n (%)	69 (92.0)	61 (81.3)	0.055
Personal care	65 (86.7)	54 (72.0)	0.058
Functional mobility	9 (12.0)	14 (18.7)	0.00013
Independence outside the home	9 (12.0)	6 (8.0)	0.199
Productivity, n (%)	68 (90.7)	64 (85.3)	0.315
Job	30 (40.0)	26 (34.7)	0.7297
Household chores	13 (17.3)	29 (38.7)	0.0001
Duties or school activities	58 (77.3)	36 (48.0)	0.0001
Leisure n (%)	63 (84.0)	69 (92.0)	0.132
Quiet recreation	21 (28.0)	29 (38.7)	0.2252
Active recreation	31 (41.3)	35 (46.7)	0.6219
Socializing	40 (53.3)	44 (58.7)	0.6219

Note: CPP: chronic pelvic pain.

occupational physical therapy protocol versus written home exercise guidelines on 43 patients with unilateral atraumatic rotator cuff tears, randomly allocated to the intervention groups. They observed that clinical improvement occurred in approximately two-thirds of the patients, regardless of the intervention. However, they also observed an improvement in the quality of life scores among those patients who did the intervention at home without supervision. Murphy *et al.*'s paper [17] reported a study with 32 subjects with osteoarthritis of the knee or hip who were randomized to two pacing interventions. One group received

Table 5. Linear regression model used to determine the effect of the covariables on occupational satisfaction and performance of women with chronic pelvic pain.

	Marital status	Job	Color	PHQ	SRQ	TSK	CPP
Satisfaction							
Self-care	[0.500], 0.615	[−0.140], 0.886	[−0.780], 0.436	[−0.290], 0.770	[−0.560], 0.579	[−1.950], 0.053	[−8.070], <0.0001
Productivity	[0.270], 0.788	[1.330], 0.185	[0.980], 0.328	[−0.500], 0.619	[0.530], 0.594	[−1.880], 0.062	[−9.620], <0.0001
Leisure	[1.800], 0.074	[2.060], 0.042	[−0.400] 0.690	[1.170], 0.245	[−0.370], 0.711	[−2.510], 0.013	[−9.020], <0.0001
Total score	[0.140], 0.890	[1.930], 0.055	[0.540], 0.593	[−0.040], 0.971	[−0.930], 0.354	[−3.070], 0.003	[−11.310], <0.0001
Performance		[0.540], 0.593	[0.540], 0.593				
Self-care	[0.840], 0.402	[0.390], 0.696	[−0.880], 0.381	[−0.350], 0.730	[0.730], 0.466	[−1,790], 0.077	[−6.200], <0.0001
Productivity	[−0.190], 0.847	[0.540], 0.588	[0.550], 0.585	[−0.560], 0.576	[−1.600], 0.112	[−1.120], 0.266	[−7.360], <0.0001
Leisurer	[1.600], 0.112	[0.080], 0.935	[−1.190], 0.238	[−1.170], 0.245	[1.640], 0.103	[−0.320], 0.752	[−8.810], <0.0001
Total score	[−0.340], 0.738	[0.750], 0.453	[−0.180], 0.854	[−0.210], 0.837	[−0.760], 0.450	[−1.790], 0.076	[−9.000], <0.0001

Note: The model was adjusted by residue analysis, considering 5% as significant. T statistic is given in brackets and p follows; PHQ: Patient Health Questionnaire; SRQ: Self-Reporting Questionnaire of Psychiatric Screening; TSK: Tampa Scale of Kinesiophofobia.

general instructions on activity pacing such as pre-planning activities, alternating activity with rest before exacerbation of symptoms, and the other group received personalized guidance based on detailed data on the relationship between developed activity and symptoms. They observed that the second group showed considerable improvement of fatigue. However, there was no significant impact on the control of clinical pain in any of the groups. Park and Sonty's study [18] retrospectively assessed 106 subjects with non-cancer chronic pain. They observed that there was a direct correlation between positive emotions and coping efficacy considering the interference of pain in the subject's social activities. Persson *et al.*'s study [19], in turn, selected 12 subjects with chronic pain and performed a qualitative study based on a narrative interview. They noted that when coping occupations were pleasurable and appreciated by the subjects, the pain was commonly shifted out of focus.

4. Discussion

The present study shows that performance and satisfaction scores for activities judged to be important are considerably lower among women with CPP than among healthy women, even when weighted according to the influence of other variables. Although never before reported for this population of women, this significant occupational impairment was expected to be similar to that previously

observed among other subjects with signs and symptoms of persistent pain [20] [21] [22] [23]. Many reasons may be behind this fact. The first and most obvious reason is the limitation of daily activity due to pain itself and the underlying disease. However, another element with a potential role in determining this limitation is coping [24] [25]. The fact that women with CPP attribute less importance to the self-care category unfortunately is not explained by our study. However, the higher prevalence of stigmatization and poor self-esteem is usual among chronic pain patients [26]. Furthermore, the scores of general and psychological symptoms observed in our series reflected by PHQ-4 and SRQ scores may be an indirect evidence of this. In addition, it is known that the presence of symptoms of depression, anxiety, catastrophization, low self-esteem, negative emotions, poor adaptation, social isolation, loss of identity [27] [28] and, probably, genetic inheritance [29] may compromise the ability of the patient to feel able to deal with the situation, that is, to control or adapt to the symptoms. Anyway, our study has some limitations that are inherent to the methodological design. Although plausible, it is impossible to define a causality association between CPP and occupational performance/satisfaction. Ideally, a prospective study would bring more valuable information about the actual impact of the clinical condition in determining low levels of satisfaction and performance. Nevertheless, the cost and time spent with this design would not be feasible without the existence of prior information such as that provided by our study.

As identified in our comprehensive review, the coping ability has a beneficial effect on the quality of life of patients with chronic pain, although it is not necessarily related to the improvement of clinical pain. There is no uniform conceptualization of coping. It is a complex process defined as thoughts and actions on which the persons focus their efforts in order to manage pain on a daily basis [30]. Although some authors state that coping is a confuse concept [31], a considerable diversity of strategies, instruments and questionnaires have been extensively described in the pertinent literature [32]. This complexity is due, at least in part, to the fact that coping strategies are multidimensional, involving cognitive, affective, behavioral and physiological human functioning. In general, coping consists of a response to stress and may be both voluntary or involuntary. The first response is a search for social support or the anticipation and prevention of stressors, while the second is also called defense. Within this involuntary group it is also possible to define at least a dichotomization of the characteristics: a set of "mature" or "adaptive" elements such as fever, tachycardia, coughing, muscle contracture, sublimation, mood change, and a set of "pathological", "immature" or "maladaptative" elements that seem to be more damaging, particularly when seen as elements external to the subject, such as fantasies, self-aggression, isolation, phobia, somatization, and rumination [33]. Maladaptive coping may also paradoxically culminate with reverberation of the perception of symptoms and a sensation of impotence in the face of the problem [34]. Unfortunately, the chemical processes and the neuronal pathways involved in

these defense and coping processes have not been identified. Furthermore, coping is directly associated with resilience [35]. And women with chronic pain, age close to 40 years, 9 to 12 years of schooling, and inserted in the labor market constitute a profile of patients classified as potentially "non resilient" [36]. This profile is very similar to that of our population of women with chronic pelvic pain.

Another element identified in our comprehensive review was pacing which is also a coping strategy widely recommended for patients with persistent pain within a multidisciplinary context. However, a lack of conceptual clarity implies restriction of the application of the method and limits the execution of rigorous studies [37]. The definition that seems most adequate to us is the following "*Pacing is an active self-management strategy whereby individuals learn to balance time spent on activity and rest for the purpose of achieving increased function and participation in meaningful activities*" [38]. This article cites important and highly illustrative cases of how the technique can be applied in practice. The establishment of goals and tasks to be carried out with intervals of rest or leisure between them can help the patients to program their daily activities and to carry them out in a more effective manner and with greater well being and quality of life. Some strategies are frequently applied spontaneously by the patients themselves, taking into account their life context. However, some of them only become effective if set up and/or accompanied by professional help. In any case, it is not possible to separate coping and resilience from the experiences of an individual and from his family, social, cultural, educational and religious relationships [39].

In the last years, neuroscience has been increasingly demonstrating the overlapping and in dissociation of physical pain and social pain. The social medium can increase the vulnerability of an individual to pain and also compromise his resilience in the face of the problem. Although social support is important to promote effective adaptation to pain, the social intelligence and resilience of an individual are of fundamental importance [40]. Unfortunately, we identified only a few papers whose key professional was an occupational therapist. We believe that these professionals can direct the preparation of a protocol of objective activity. The professional can acts as a mediator or moderator and helps the subjects to identify stressful events early, consequently helping with the construction of active strategies for the prevention and elimination or minimization of damage.

5. Conclusion

Women with CPP exhibit significant impairment of satisfaction and occupational performance in all categories considered. Coping is an important strategy that can be proposed and supervised by occupational therapists within a multidisciplinary context dealing with this population. However, the resilience of the subject is fundamental for the success of the strategy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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