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RESEARCH ARTICLE

Chromatographic and antiproliferative assessment of the aerial root of *Ficus thonningii* Blume (Moraceae)

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ABSTRACT

Ficus thonningii (Blume) has long history of use for variety of ailments. The hot aqueous extract of *Ficus thonningii* aerial root (FT) was obtained by infusion. The antiproliferative activity of FT was evaluated using *Sorghum bicolor* seed radicle over a period of 24 h to 96 h. The mean radicle length (mm), percentage inhibition and percentage growth were calculated. Chemical characterization of FT was done using chromatographic techniques. Thin layer chromatography revealed the presence of β-sitosterol. High performance liquid chromatography showed ten peaks with gallic acid, tannins, caffeic acid, rutin, ferulic acid and morin eluting at 3.530, 3.928, 4.668, 6.706, 7.669 and 18.844 minutes respectively. Compared with negative control, FT at 1 mg/ml to 32 mg/ml significantly ($p<0.0001$) inhibited *S. bicolor* seed radicle growth over 24 h-96 h. At 96h, FT dose-dependently inhibited *S. bicolor* seed growth, giving a percentage inhibition of 20.31%, 24.30%, 31.71%, 53.23%, 78.74%, 95.37% at 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 32 mg/ml, respectively. Methotrexate 50 µg/ml used as the positive control gave inhibition of 70.62% at 96h. The result revealed the potential of FT to inhibit rapid proliferating cells of *S. bicolor* seed radicle and by extension cancer cells.

Keywords: *Ficus thonningii*; Antiproliferative; β-sitosterol; Caffeic acid; Ferulic acid.

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INTRODUCTION

Cancer is a generic name describing a collection of related diseases which can affect any part of the body [1]. Cancer may be malignant or neoplastic, involving the rapid growth of abnormal cells beyond their normal location or boundaries, a process called metastases which constitutes the major cause of mortality from cancer. Globally, cancer is being ranked the second leading cause of mortality, being responsible for 8.8 million deaths

in year 2015. Nearly 1 in 6 deaths globally is due to cancer and approximately 70% of mortality from cancer occurs in countries with low or middle income capacity [2].

The use of phytomedicines in disease management dates to as old as the beginning of man's existence, as various plant materials have served as medicinal recipes even to the modern man. About 75-90% of the world's population use remedies from plants as their primary health care sources [3]. Plant and their metabolites have proved to be useful in treating and managing of diseases proven by their extensive application in herbal medicine practice [4].

F. thonningii (Blume) belonging to the family Moraceae, also called the common wild fig [4] is an evergreen, multi-stemmed, deciduous tree with a crown that is round or spreading, distributed mainly in the upland forests of subtropical and tropical Africa, having altitudes varying between 1,000-2,500 m [5]. It is also found in open grasslands, rocky and riverine areas and sometimes found in Savannah. It is draught resistant occurring naturally in Tanzania, DR Congo and South Africa [6]. The local names include Chediya in Hausa, India-laurel fig in French, Odan in Yoruba; Strangler fig, common wild fig, bark-cloth fig in English [6]. *Ficus thonningii* Blume is about 6-21 m high, the leaves are alternate or whorled mid dark green and sub-glossy [7]. The leaves have smooth margins, may be leathery and papery, elongated sometimes, glabrous with the apex obtuse or sometimes rounded with stipules about 12 mm long [6]. The fruits are borne singly or in pairs and are about 10 mm in diameter usually hairy and turn yellowish and rarely pink on ripening [7]. It has dense, spreading or rounded crown. Younger branches have hairy bark with caps that are stipular covering the growth tip while older stems and branches are smooth. It is lenticellate with aerial roots often hanging down the branches. The plant exudes abundant milky latex that turns pink. Figs fruit are borne in leaf axil or below the leave sometimes. It is native to Africa, possessing diverse economic and environmental uses across many communities in Africa [8].

Research has shown that *Ficus thonningii* extracts possess biological properties such as antioxidant [9, 10], antimicrobial [11], antihelmintic [12], antiprotozoal [13], antifungal [14], anti-psychotic [15] and anti-inflammatory [16].

Current treatment for cancer includes surgery, radiation therapy, chemotherapy, hormone therapy, among others, which are expensive and have many side effects, hence the growing need for new anticancer drugs that are more effective and less toxic. Cancer patients burdened with drug - induced toxicity are getting help from complementary and alternative medicines that are plant based [17].

Medicinal plants and natural products have significant roles in the prophylaxis and treatment of cancer through multiple therapeutic effects which include inhibition of cancer activating enzymes and hormones stimulation of DNA repair mechanism enhancing production of protective enzymes, antioxidant and immune boosting activities [18]. The bark of *Ficus thonningii* is useful in ethnomedicine for the treatment of dysentery, sore throat, cold, constipation, nose bleeding, wounds and the latex is useful for fever. The fibre and root are infused and taken orally to prevent abortion while the latex may also be instilled in the eye to treat cataract [6].

Ficus thonningii has been reported to contain alkaloids, flavonoids, terpenoids, tannins and saponins. It is also useful in the treatment of diabetes, arthritis, gastric discomfort, headache, asthmatic conditions, fever fungal infections and mental illness [11]. All parts of *F. thonningii* are medicinally useful, people prefer to use the leaves and bark which exudes latex, because latex has traditionally been associated with potency [19].

The aim of this study is to evaluate the phytochemical, chromatographic profile and antiproliferative effect of the aerial root of *F. thonningii* collected from Northern Nigeria. Antiproliferative effect was evaluated using rapidly growing *Sorghum bicolor* seed radicles.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise stated all chemicals and reagents used were of analytical grade and purchased from Sigma Aldrich (Germany).

Experimental plants

Ficus thonningii leaves and aerial roots were collected from Kwandere, Nasarawa state, Nigeria. The plants were identified and authenticated by a taxonomist at the herbarium of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria, where the voucher specimen was deposited.

Sorghum bicolor (Guinea corn) seeds were purchased from Karmo market, Abuja and identified and authenticated by a taxonomist of NIPRD Abuja. The seeds viability test was determined by placing them inside a beaker containing water, the seeds that floated were discarded while the totally submerged seeds were cleansed with methylated spirit and dried for usage [20, 21].

Plant preparation and extraction

The aerial roots of *F. thonningii* were air-dried at ambient temperature (28-30^{o}C) for two weeks. The dried plant material was pulverized. Then 50 g of the powdered sample was weighed and extracted by hot water (1000 ml) infusion in air tight container for 24 h. The resultant mixture was filtered with filter paper (Whatman No. 1) under gravity [22]. The filtrate was dried at 80^{o}C on a water bath to yield *F. thonningii* aerial root aqueous extract (FT) as brown residue.

Thin layer chromatography (TLC)

Thin layer chromatography was carried out on both the hexane and ethylacetate extracts using silica gel pre-coated glass plate. 2 g of the *Ficus thonningii* aerial root was extracted successfully with hexane and ethyl acetate at ambient temperature (28-30^{o}C) for 24 h. Micro syringe was used to uniformly apply 10 µl of the extracts on the TLC plate and allowed to dry, β-sitosterol reference standard (Sigma) was spotted alongside as control. The plates were developed in a chromatographic tank using mobile phase comprising hexane and ethyl acetate (5:1) for the hexane extract and hexane and ethyl acetate (3:1) for the ethyl acetate extract. The developed plates were air-dried and visualized under ultraviolet light at 366 nm and iodine vapour tank. The retardation factor (R_f) for each component was calculated using the following formula:

R_f = Distance moved by the solute / Distance moved by the solvent front.

High performance liquid chromatography analysis

The bioactive constituents of FT were analyzed by high performance liquid chromatography (HPLC) with UV diode array detector (UV-DAD). The HPLC consisted of Ultra-Fast LC-20AB equipped with SIL-20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, 5 µm VP-ODS C_{18} and dimensions (4.6 x 150 mm). The chromatographic conditions included mobile phase: 0.2% v/v formic acid and acetonitrile (20:80); mode: isocratic; flow rate 0.6 ml/min; injection volume 10 µl of 100 mg/ml solution of extract in water; detection UV 254 nm. The HPLC operating conditions were programmed to give solvent B: 20%. Column oven temperature was 40^{o}C. The total run time was 30 minutes. Flavonoids and phenolic acid standards such as apigenin, rutin, quercetin, caffeic acid, morin and ferulic acid were employed for the identification of the phytoconstituents of FT by comparing the retention time under similar experimental conditions [23].

Determination of growth inhibitory effects

The modified method of Okhale et al., 2017 [23] was used for this study. *Ficus thonningii* aerial root hot water extract FT (3200 mg) was dissolved in 100 ml of distilled water to obtain 32 mg/ml stock solution.

Various concentrations of FT were prepared (1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml and 32 mg/ml). Methotrexate, an anticancer drug, was made to a concentration of 50 µg/ml as positive control. Petri dishes were layered with cotton wool and filter paper. Twenty seeds (20) of *Sorghum bicolor* were placed in each of the Petri dishes. The control was treated with 15 ml of distilled water (negative control) and methotrexate (50 µg/ml) respectively. They were made in duplicates (for each concentration, control and methotrexate two (2) Petri dishes were used) for the samples. The test seeds were treated with different preparations of FT as the seed in each specific Petri dish received 15 ml of a particular concentration (the seed in one set of Petri dishes were treated with 1 mg/ml concentration, seeds in another set of Petri dishes received 2 mg/ml, another received 4 mg/ml, another received 8 mg/ml, the next received 16 mg/ml, followed by 32 mg/ml and 64 mg/ml, 1mg/ml concentration was prepared in the first set of Petri dishes seeds, another set Petri dishes received 2 mg/ml, another received 4 mg/ml, another received 8mg/ml, the next received 16 mg/ml, followed by 32 mg/ml respectively). The seeds were incubated in a dark cupboard and observed for further growth after 24, 48, 72 and 96 h. The mean radicle lengths (mm) of the seeds were measured after 24, 48, 72 and 96h. The percentage inhibition was calculated as:

$$\frac{\text{(Mean radicle length of Control - Mean radicle length treated)}}{\text{Mean radicle length Control}} \times 100$$

Percentage growth was calculated as 100 - percentage inhibition. Percentage inhibition and percentage growth at 24, 48, 72 and 96 h for seed treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 32 mg/ml of FT and the positive control methotrexate at 50 µg/ml are shown in Table 1.

Statistical analysis

The data obtained were expressed as mean ± standard error of mean and analyzed using GraphPad Prism (version 7.03).

RESULTS

Extraction of 50 g of *Ficus thonningii* aerial root powdered sample with hot water yielded 3.42g (6.84% w/w) of the dried extract (FT).

Thin layer chromatography (TLC)

The chromatogram of the hexane extract showed a total of 8 spots of which 5 were detected under ultraviolet light at 366 nm with R_f of 0.03 (pink), 0.07 (pink), 0.16 (pink), 0.25 (pink), 0.46 (white) and 3 of the spots were detected in iodine vapour with R_f of 0.29 (β-sitosterol), 0.51 and 0.77. The ethyl acetate extract showed 6 spots on TLC of which 5 were detected under ultraviolet light at 366 nm with R_f of 0.25 (pink), 0.38 (pink), 0.56 (pink), 0.68 (pink), 0.71 (white) and 1 of the spots was detected in iodine vapour with R_f of 0.45 (β-sitosterol).

High performance liquid chromatography analysis

From the HPLC chromatogram of FT ten peaks were detected as the bioactive constituents with retention time in minutes of 3.530, 3.928, 4.668, 6.706, 7.669, 8.727, 10.517, 12.475, 14.904 and 18.844. Compounds with retention time in minutes of 3.530, 3.928, 4.668, 6.706, 7.669 and 18.844 corresponded to gallic acid (33.95%), tannin (28.74%), caffeic acid (23%), rutin (5.25%), ferulic acid (3.18%) and morin respectively (Table 1 and Fig. 1).

Table 1. Chemical constituents of the hot aqueous extract of *Ficus thonningii* aerial root (FT) from HPLC analysis.

Peak number	Name	Ret. time (minute)	Peak area	Percentage composition*
1	Gallic acid	3.530	15543282	33.949887
2	Tannin	3.928	13158311	28.740595
3	Caffeic acid	4.668	10535024	23.007693
4	Rutin	6.706	2401990	5.2464653
5	Ferulic acid	7.669	1455720	3.1796071
6	ND	8.727	1572372	3.4344003
7	ND	10.517	822115	1.7956768
8	ND	12.457	235737	0.5149006
9	ND	14.904	38192	0.0834196
10	Morin	18.844	20272	0.0442784
Total			45783015	99.9969231

ND = Not Detected; * Percentage composition of each peak = Peak Area / Total Peak Area x 100

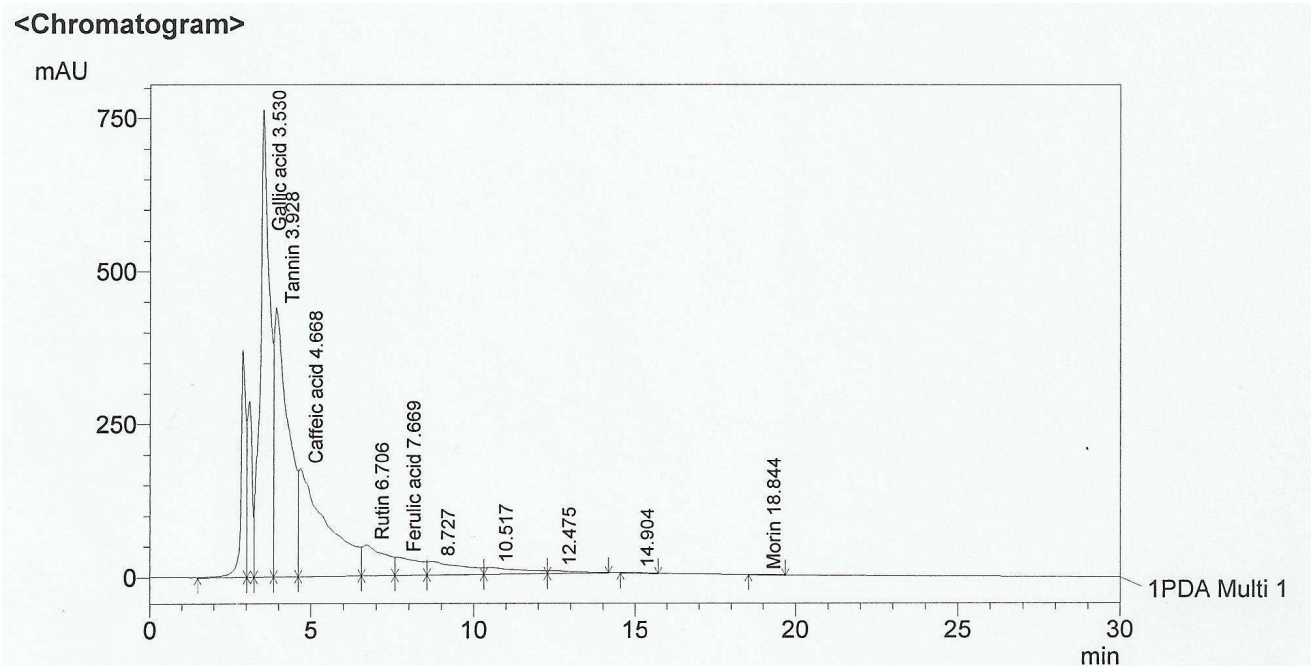

Figure 1. HPLC spectrum of hot aqueous extract of *Ficus thonningii* (FT) aerial root. Compounds with retention time in minutes of 3.530, 3.928, 4.668, 6.706, 7.669 and 18.844 corresponded to gallic acid (33.95%), tannin (28.74%), caffeic acid (23%), rutin (5.25%), ferulic acid (3.18%) and morin, respectively.

Growth inhibitory effect of FT on *Sorghum bicolor* seeds

There was appreciable and observable reduction in the radicle length of *Sorghum bicolor* seeds treated with various concentrations of the extract. The seed radical length increased over the incubation period of 24h-96h. There was an observable, progressive and rapid growth of the seed radical length in the negative control (distilled water). At 96h, the mean radicle length (mm) of the control seed was 96.10±3.49 while the mean radicle length of the seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml and 32 mg/ml were 76.58±1.37, 72.75±2.20, 65.63±3.52, 44.95±4.09, 20.43±1.49, 4.450±0.66, respectively (Fig. 2) corresponding to percentage inhibition of 20.32%, 24.30%, 31.71%, 53.23%, 78.74% and 95.37% showing that the growth inhibitory effect of FT was concentration-dependent. Radicle lengths were measured at 24h, 48h, 72h and 96h. The negative control used was distilled water while the positive control was methotrexate (50 µg/ml). Mean

radicle length, percentage inhibition and percentage growth at 24h, 48h, 72h and 96h for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml and 32 mg/ml of FT as well as negative control (H_2O) and positive control (methotrexate) 50 µg/ml as shown in Table 1.

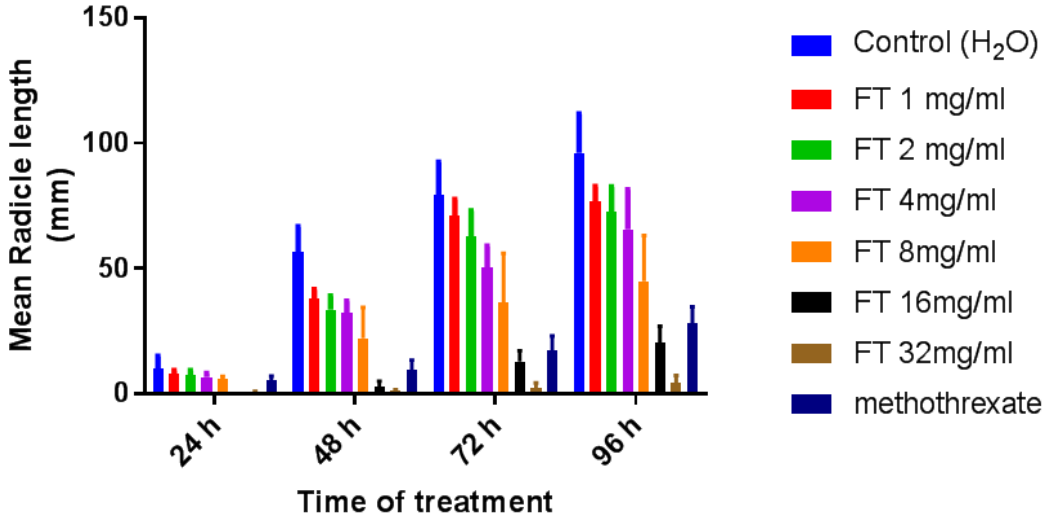

Figure 2. Growth inhibitory effects of *Ficus thonningii* aerial root aqueous extract (FT) on *Sorghum bicolor* seed radical.

Table 1. Mean radicle length, percentage inhibition and percentage growth for *Sorghum bicolor* seeds treated with FT.

Treatment	Mean radicle length (mm)				% Inhibition*				% Growth†			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Control (H_2O)	10.32±1.07	56.65±2.25	79.33±2.95	96.10±3.47	0	0	0	0	100	100	100	100
Methotrexate	5.600±0.35	9.775±0.84	17.25±1.33	28.23±1.48	45.74	82.74	78.26	70.62	54.26	17.26	21.74	29.38
FT (1 mg/ml)	7.925±0.34	37.9/3±0.84	71.15±1.43	76.58±1.37	23.21	33.05	10.31	20.31	76.79	66.95	89.69	79.69
FT (2 mg/ml)	7.500±0.43	33.70±1.19	62.90±2.27	72.75±2.20	27.33	40.51	20.71	24.30	72.67	59.49	79.29	75.70
FT (4 mg/ml)	6.750±0.31	32.43±1.04	50.38±1.90	65.63±3.52	34.59	42.75	36.49	31.71	65.41	57.25	63.51	68.29
FT (8 mg/ml)	6.025±0.25	22.35±2.72	36.28±4.44	44.95±4.09	41.62	60.55	54.27	53.23	58.38	39.45	45.73	46.77
FT (16 mg/ml)	1.000±0.00	3.050±0.48	12.86±1.00	20.43±1.49	90.31	94.62	83.79	78.74	9.690	5.380	16.21	21.26
FT (32 mg/ml)	0.950±0.05	1.350±0.11	2.425±0.44	4.450±0.66	90.79	97.62	96.94	95.37	9.210	2.380	3.060	4.630

*Percentage Inhibition = [(mean radicle length of control - mean radicle length of treated) / mean radicle length of control] x 100. †Percentage growth = 100 - percentage inhibition, n = 20. $p<0.0001$

DISCUSSION

One distinguishing feature between cancer cells and normal body cells is the ability of cancerous cells to proliferate without responding to cell feedback mechanism or apoptotic mechanisms that regulate cell death, which is replicated by meristematic stems of seeds and hence the choice of *Sorghum bicolor*. Under favourable environmental conditions meristematic cells of *Sorghum bicolor* seeds retain ability to proliferate similar to cancer cells [24]. The extractive value obtained for hot aqueous extract of *Ficus thonningii* aerial root (FT) was 6.84%. Thin layer chromatography of the hexane and ethyl acetate extracts of *Ficus thonningii* aerial root revealed the presence of β-sitosterol. HPLC chromatogram of FT revealed gallic acid, tannin, caffeic acid, rutin and morin. Gallic acid and its derivatives have been implicated as antimutagenic, anticarcinogenic, antiangiogenic, antimicrobial and anti-inflammatory agents [25]. Gallic acid is present in almost every part of plants such as roots, seeds, bark, wood and leaf and is also a known antioxidant [26]. Tannins are polyphenolic

compounds of high molecular weight found in roots, stems, barks and outer layers of plant tissues [27]. They possess antioxidant, antitumour and antibacterial activities [28]. Tannin had been reported to have anticancer properties as in maplexin A-1 in red maples [29], cuphiin DI [30], ellagitannins [31]. Corilagin, tannin, inhibits growth of ovarian cancer cell lines [32]; tannic acids also prevent the activation of PARP-1, thereby preventing doxorubicin-induced cell death [33]. Cancer can be linked to oxidative stress [34]. Caffeic acid (3,4-dihydro-cinnamic acid) is a polyphenolic acid possessing antioxidant and anticancer properties [35]. Plant phenolic such as morin, tannins, ferulic and caffeic acids serve as potent antioxidants [36]. Rutin, a polyphenol, is implicated in cytoprotection, antioxidant, anticarcinogenic cardioprotective and neuroprotective effects [37]. The bark of aerial roots of *Ficus elastica* (Moraceae) had growth inhibitory activity against the human A549 lung cancer cell line [38]. Aerial root of *Ficus microcarpa* had been reported to contain compounds with anticancer activities [39]. *Ficus thonningii* stem bark and aerial root are used as cancer remedy in Northern Nigeria [40]. This study provided preliminary scientific support for folkloric use of *Ficus thonningii* aerial root as anticancer agent.

CONCLUSION

The hot aqueous aerial root extract of *Ficus thonningii* exhibited antiproliferative activity on the fast proliferating meristematic cells of *Sorghum bicolor* and hence can be said to be potential inhibitor of cancerous growth. This claim may be attributed to antioxidant rich secondary metabolites such as gallic acid, tannin, ferulic acid, rutin and morin which have been reported to possess anticancer potentials.

AUTHOR'S CONTRIBUTION

MOA wrote the initial draft of the manuscript; SEO designed and supervised the study; SFA assisted with the antiproliferative evaluation; UOE wrote the final draft of the manuscript and did the statistical analysis; SEO proof read, and edited the word. All authors were involved in the execution of the research plan. The final manuscript was read and approved by all authors.

REFERENCES

1. National Cancer Institute, 2015. https://www.cancer.gov/aboutcancer/understanding/what-is-cancer
2. World Health Organization Cancer Fact Sheet, 2017. http://www.who.int/mediacentre/factsheets/fs297/en/
3. Benzie IFF, Watchtel-Galor S. Herbal medicine: biomolecular and clinical aspects. CRC Press, 2nd edn, 2011.
4. Dangarembizi R, Erlwanger KH, Moyo D, Chivandi E. Phytochemistry, pharmacology and ethnomedical uses of *Ficus thonningii* Blume (Moraceae): a review. Afr J Tradit Complem Altern Med. 2013; 10(2): 203-212.
5. Agroforestry Tree Database, 2017. http://www.worldagroforestrycentre.org/sea/Products/AFDbases/AF/asp/speciesInfo.asp?SpID=866
6. Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S, 2009. Agroforestree Database: a tree reference and selection guide. http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp
7. Ndukwe IG, Bello AI, Habila JD, John C. Phytochemical and antimicrobial screening of the crude petroleum spirit and methanol extracts of the stem bark, leaves and roots of *Ficus thonningii* Blume. Afr J Biotechnol. 2007; 6(3): 2645-2649.
8. Balehegn M, Eik Lars O, Tesfay Y. Silvopastoral system based on *Ficus thonningii*: an adaptation to climate change in northern Ethiopia. Afr J Range Forage Sci. 2015; 32(3): 183-191.
9. Olas B, Wachowicz B, Nowak P, Stochmal A, Oleszek W, Glowacki A, Bald E. Comparative studies of the antioxidant effects of a naturally occurring resveratrol analogue trans 3, 3', 5, 5-tetrahydroxy-4-methoxystilbene and resveratrol-against oxidation and nitration of biomolecules in blood platelets. Cell Biol Toxicol. 2008; 24: 331-340.
10. Grassi D, Desideri D, Ferri C. Flavonoids antioxidants against atherosclerosis. Nutrients. 2010; 2: 889-902

11. Usman H, Abdulrahman FI, Usman A. Qualitative phytochemical screening and in vitro antimicrobial effects of methanol stem bark extract of *Ficus thonningii* Moraceae. Afr J Tradit Complem Altern Med. 2009; 6(3): 289-295.
12. Krief S, Hladik C, Mand HC. Ethnomedicinal and bioactive properties of plants ingested by wild chimpanzees in Uganda. J Ethnopharmacol. 2005; 101: 1-15
13. Jansen O, Angenot L, Tits M, Nicolas JP, De Mol P, Nikiema JB, Frederich M. Evaluation of 13 selected medicinal plants from Burkina Faso for their antiplasmodial properties. J Ethnophamacol. 2010; 13: 143-150.
14. Oyelana OA, Durugbo EU, Olukanni OD, Ayodele EA, Aikulola ZO, Adewole AI. Antimicrobial activity of *Ficus* leaf extracts on some fungal and bacterial pathogens of *Dioscorea rotundata* from Southwest Nigeria. J Biol Sci. 2011; 11: 359-366.
15. Maiha BB, Mohammed B, Magaji MG. Psychopharmacological potential of methanol leaf extract of *Ficus thonningii* (Blume) in mice. Nigerian J Pharmac Sci. 2013; 12(2): 30-34.
16. Otimenyin SO, Uguru MO, Atang BL. Antiinflammatory and analgesic activities of *Ficus thonningii* and *Pseudocedrela kotschyi* extracts. Nigerian J Pharmac Res. 2004; 3: 82-85.
17. Okhale SE, Tijani AY, Ezugwu BO. Assessment of antiproliferative potential of *Hexalobus crispiflorus* (Annonaceae). Brit Biotechnol J. 2016; 15(3): 1-7.
18. Macha MA, Krishn SR, Jahan R, Banerjee K, Batra SK, Jain M. Emerging potential of natural products for targeting mucins for therapy against inflammation and cancer. Cancer Treat Rev. 2015; 41(3): 277-288.
19. Dangarembizi R. 2012. Effects of crude leaf extracts of *Ficus thonningii* on growth, gastrointestinal morphometry and clinical biochemistry of suckling spraque dawley rats: A thesis submitted to the faculty of Health Sciences, University of the Witwatersrand, Johannesburg.
20. Chinedu E, Arome D, Ameh SF, Ameh GE. Evaluation of antiproliferative and cytostatic effect of *Citrus sinensis* (orange) fruit juice. Int J App Basic Med Res. 2014; 4(3): 20-22.
21. Ayinde BA, Agbakwuru U. Cytotoxic and growth inhibitory effects of the methanol extract *Struchium sparganophora* Ktze (Asteraceae) leaves. Pharmacogn Mag. 2010; 6(24): 293-297.
22. Fernandes AC, Cromarty DA, Albrecht C, van Rensburg CEJ. The antioxidant potential of *Sutherlandia frutescens*. J Ethnopharmacol. 2004; 95: 1-5.
23. Okhale SE, Nnachor AC, Bassey UE. Evaluation of HPLC-UV-DAD and antiproliferative characteristics of the leaf infusion of *Ximenia americana* Linn. MicroMed. 2017; 5(2): 45-52.
24. David A, Chinedu ES, Fidelis A, Amarachi A, Rose ME. Evaluation of anti-proliferative activities of aqueous leaf and root extracts of *Cymbopogon citratus.* J Pharmac Biol Sci. 2013; 1: 56-60.
25. Daglia M, Di Lorenzo A, Nabavi SF, Talas ZS, Nabavi SM. Polyphenols: well beyond the antioxidant capacity: gallic acid and related compounds as neuroprotective agents: you are what you eat! Curr Pharm Biotechnol. 2014; 15(4): 362-372.
26. Choubey S, Varughese LR, Kumar V, Beniwal V. Medicinal importance of gallic acid and its ester derivatives: a patent review. Pharmac Patent Anal. 2015; 4(4): 305-315.
27. Kar A. Pharmacognosy and pharmacobiotechnology. 2nd edn, New Age International Limited, New Delhi, India, 2007: 332-600.
28. Widsten P, Cruz CD, Fletcher GC, Pajak MA, McGhie TK. Tannins and extracts of fruit by-products: antibacterial activity and antioxidant capacity. J Agric Food Chem. 2014; 62: 11146-11156.
29. Gonzalex-Sarria A, Yuan T, Seeram NP. Cytotoxicity and structure activity relationship studies of maplexin A-1, gallotannins from red maple (*Acer rubrum*). Food Chem Toxicol. 2012; 50: 1369-1376.
30. Wang CC, Chen JG, Yang LL. Cuphiin D_1, the macrocyclic hydrolysable tannin induced apoptosis in HL-60 cell line. Cancer Lett. 2000; 149: 77-83.
31. Barrajon-Catalan E, Fernandez-Arroyo S, Saura D, Guillen E, Fernandez-Gutierrez A, Segura-Carretero A. *V. cistaceae* aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity and cytotoxic activity against human cancer cells. Food Chem Toxicol. 2010; 48: 2273-2282.
32. Jia L, Jin H, Zhou J, Chen L, Lu Y, Ming Y, Yu Y. A potential antitumor herbal medicine, corilagin, inhibits ovarian cancer cell growth blocking the TGF-β signalling pathways. BMC Complem Altern Med. 2013; 13: 33.

33. Tikoo K, Sane MS, Gupta C. Tannic acid ameliorates doxorubicin-induced cardiotoxicity and potentiates its anti-cancer activity: Potential roles of tannins in cancer chemotherapy. Toxicol Appl Pharmacol. 2011; 251: 191-200.
34. Jezierska-Drutel A, Rosenzweig SA, Neumann CA. Role of oxidative stress and the microenvironment in breast cancer development and progression. Adv Cancer Res. 2013; 119: 107-125.
35. Ryota S, Yutaka I, Isamu M, Ikuo K. Effect of antioxidant properties of caffeic acid with cyclodextrin using ground mixture method. Asian J Pharmac Sci. 2017. http://doi.org/10.1016/j.ajps.2017.08.006
36. Sajid M, Soottawat B, Aisha A, Asifa A. Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: a detailed review. Compreh Rev Food Sci Food Safety. 2014; 13(6): 1125-1140.
37. Javed H, Khan MM, Ahmed A, Vaibhav K, Ahmed ME, Khan Ashafag M, et al. Rutin prevents cognitive impairments by ameliorating oxidative stress and neuroinflammation in rat model of sporadic dementia of Alzheimer type. Neuroscie. 2012; 17: 340-352.
38. Mbosso EJT, Nguedia JCA, Meyer Fr, Lenta BN, Ngouela S, Lallemand B, et al. Ceramide, cerebroside and triterpenoid saponin from the bark of aerial roots of *Ficus elastica* (Moraceae). Phytochem. 2012; 83: 95-103.
39. Chan EWC, Tangah J, Inoue T, Kainuma M, Baba K, Oshiro N, et al. Botany, uses, chemistry and pharmacology of *Ficus microcarpa*: a short review. Sys Rev Pharm. 2017; 8(1): 103-111.
40. Ngulde SI, Sandabe UK, Hussaini IM. Ethnobotanical survey of anticancer plants in Askira/Uba local government area of Borno State, Nigeria. Afr J Pharm Pharmacol. 2015; 9(5): 123-130.

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RESEARCH ARTICLE

Predictors of residual disease after loop electrosurgical excision procedure

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ABSTRACT

The study aims to evaluate the importance of resection margins in the risk of residual disease (RD) and to investigate other factors that could potentially predict RD before patients engage in follow-up. Eighty-six women with a histologically confirmed diagnosis of cervical intraepithelial neoplasms (CIN) treated by loop electrosurgical excision procedure (LEEP), were included in this retrospective study, between January 2015 and May 2016. Age, smoking habit, menopause status, and LEEP margins were evaluated as possible predictors of RD. The mean age at diagnosis was 35.8 years (range 18-61). The mean follow-up period was 12 months. 11.6% of patients (09/86) were lost in follow-up. 64% of patients (55/86) had clear margins in the specimen and 34.8% of patients had positive surgical margins (30/86). In 1.2% of patients (01/86) the resection margins were uncertain. RD was demonstrated by positive Pap Smear and by colposcopy-guided biopsy in 26.7% of patients (23/86). We found significant differences in the frequency of RD depending on the status of margins: 65.2% of cases with positive margins vs. 24.5% of cases with negative margins ($p<0.0001$). Multivariate analysis showed that only high-grade squamous intraepithelial lesion (H-SIL) detection in cervical biopsy and status of the LEEP margins were significantly predictive of RD (OR 5.4, 95%CI 1.08-27.7, $p<0.05$ and OR 7.05, 95%CI 2.1-23.1, $p=0.001$; respectively). The combination of histological examination of resection margins plus H-SIL detection in cervical biopsy would help to classify LEEP-treated patients into categories of different risk levels of residual disease.

Keywords: Cervical intraepithelial neoplasia; Minimal residual disease; Conization; Papillomavirus infections.

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INTRODUCTION

Uterine cervical carcinoma is one of the most common cancers in the female population in Brazil and worldwide [1]. The infection caused by human papillomavirus (HPV) is involved in the pathogenesis of this type of cancer, which has as precursor lesions cervical intraepithelial neoplasms (CIN), particularly those of a high degree. There are several options in the treatment of this type of pre-invasive disease, specifically the high-grade squamous intraepithelial lesion (H-SIL) or recurring low-grade squamous intraepithelial lesion (L-SIL), which include among others: cold knife conization (CKC) and loop electrosurgical excision procedure (LEEP) [2].

LEEP was introduced in 1989 by Prendiville et al. to treat lesions that could be visualized by colposcopy [3]. The procedure involves excision of cervical tissue through the thermal effect of alternating electric current with high frequency with the use of a small loop. It is an outpatient procedure, safe, conducted under local anesthesia and has a lower bleeding rate when compared to CKC, and is therefore increasingly preferred [4, 5]. Moreover, it is related to lower obstetric adverse outcome rates, ensuring the preservation of women's fertility and proper evaluation of the resection margins, and can has healthy healing rates in up to 95% of cases [6, 7].

The literature is still controversial about the influence of margin compromise on the occurrence of residual disease (RD). However, most studies show that there is a positive relationship [6-9]. There is also divergence in the literature on what would be the predictors of RD, with some authors pointing to age [8-11] and others looking at persistent HPV infection [9-11] associated with a higher risk of persistent disease. On the other hand, studies are rare in which the LEEP is performed only by resident physicians, enabling the assessment of whether the experience and skill of the surgeon contribute to the increase of the compromised margin (CM) and RD prevalence.

Moreover, few studies have been conducted in Brazil on the prevalence of CM and RD after LEEP [12]. It is not well-defined in this country which factors are related to the higher risk of the development of RD and how prevalent CM and RD are in Brazilian women.

The objective of this study is to evaluate the prevalence of compromised margins and residual disease post LEEP and check the predictors of RD in a reference cervical pathology center.

MATERIALS AND METHODS

This is a retrospective analysis of 86 women with histologically confirmed CIN 1, 2 or 3 who were treated by LEEP in the Cervical Pathology Department of a Federal University Hospital in Brazil between January 2015 and May 2016. Approval was obtained from the institutional review board.

Conization was performed exclusively by gynecology residents using loop diathermy with a blend setting and a power output of 40 W. A 5-mm cautery ball with a power setting of 50 W was used to achieve hemostasis. Monsel solution was applied as needed. All specimens were marked for orientation with a delayed absorbable suture at the 12-o'clock position for pathology examination.

Standard follow-up in our department was a visit at 6 and 12 months after cervical conization with clinical examination, Pap smear (PS), colposcopy and eventual biopsy. However, if surgical margins were affected, the first check-up was performed at 3 months after conization. Cervical smears were stained using the Papanicolaou method and were evaluated following the 2014 Bethesda criteria [13]. All women with abnormal cytology and/or an abnormal transformation zone underwent a colposcopy directed biopsy. When the transformation zone was not visible or only partially visible or no colposcopic abnormality was identified, an endocervical curettage using a Kervokian curette was also performed.

Criteria for defining RD were based on positive surgical margins at conization and/or abnormal check-up at 6-12 months. The presence of RD was based on positive histology of colposcopy-directed biopsy or endocervical curettage. Histologic evidence of CIN of any grade was considered as RD. Women with two consecutive negative Pap smears and normal colposcopy were considered negative for RD.

Statistical analysis was performed using SPSS statistical software version 20.0 for Windows. Quantitative variables were compared using Student's t-test or Mann Whitney test. Categorical variables were compared using the chi-squared test or Fisher's exact test. Odds ratio and 95% confidence intervals (95% CI) were estimated by logistic regression analysis. Variables found to be significant by univariate analysis were examined by multivariate analysis using the Cox proportional hazards regression model. P values <0.05 were considered statistically significant for all statistical tests.

RESULTS

In the study period, 93 women were treated with LEEP for the first time, however, only 86 could be included, since 7 were excluded from the analysis because they did not attend any follow-up visit, or because they were diagnosed with invasive cervical carcinoma.

The mean age of patients was 35.8 ± 9.5 years (range 18-61). Among the patients, 15.1% were aged between 18 and 25 years, 75.6% between 25 and 50 years and 9.3% were older than 50. Regarding the number of pregnancies and childbirths, the majority had had between 1 and 3 (46.5%). Most women, more than 90%, were in the menace. Clinically, most patients presented negative serologies for syphilis, HIV and hepatitis. Previously with the LEEP, 81.4% had a biopsy revealing H-SIL. Table 1 presents the sociodemographic and clinical characteristics of the patients.

Table 1. Sociodemographic and clinical characteristics of the patients.

Variable	Category	n	%
Age	18-25	13	15.1
	25-50	65	75.6
	>50	8	9.3
Color	White	9	10.5
	Black	2	2.3
	Mestice	75	87.2
	Yellow	0	0
Parity	Nulliparous	15	17.4
	Multiparous	71	82.6
Menopause	No	79	91.9
	Yes	7	8.1
Cervical biopsy	Normal	2	2.3
	L-SIL	9	10.5
	H-SIL	70	81.4
	Unknown	5	5.8
Total		86	100

Abbreviations: L-SIL = low-grade squamous intraepithelial lesion; H-SIL = high-grade squamous intraepithelial lesion.

The LEEP histopathology was positive for H-SIL or higher in 58.1% of cases (50/86) and for L-SIL or lower in 41.9% of cases (36/86). After LEEP, a total of 64% patients (55/86) had clear margins in the operation specimens and the corresponding number of patients with positive surgical margins was 34.8% (30/86). In 1.2% of patients (01/86) the resection margins were uncertain. Of the overall group of patients with positive margins, the exocervical margin was apparent in 3.3% (01/30), the endocervical margin in 16.6% (05/30), and both margins in 80% cases (24/30) (Table 2).

Table 2. Anatomopathological characteristics of loop electrosurgical excision procedure specimens.

	n %	n RD %
Clear surgical margins	55 (64%)	8 (14.5%)
Positive surgical margins	30 (34.8%)	15 (50%)
Exocervical	01	01
Endocervical	05	05
Both	24	09
Uncertain surgical margins	01 (1.2%)	01 (100%)
Total	86 (100%)	

Abbreviations: RD = Residual Disease.

Table 3. Multivariate analysis of residual disease risk.

	OR	95% CI		p value
		L	H	
Positive surgicalmargins	7.05	2.1	23.1	0.001
H-SIL cervical biopsy	5.4	1.08	27.7	<0.05

Abbreviations: OR = Odds Ratio; CI = Confidence Interval; L = Lower; H = Higher; H-SIL = high-grade squamous intraepithelial lesion.

The total number of patients with residual disease (RD) was 26.7% (23/86). The mean age of patients was 38.2 ± 8.7 in the RD group and 34.6 ± 10.4 in non-recurrent cases. In our series, age is not predictive of RD (p=0.075). Significant differences in risk of RD depending on the involved margin were observed: 65.2% (15/23) of cases with positive margins vs. 24.5% (13/53) of cases with clear margins (p<0.0001). Patients with high-grade lesions in the cervical biopsy had a higher percentage of RD compared to low-grade lesions or lower (p<0.05).

In multivariate analysis, only previous cervical biopsy with H-SIL and positive cervical margins were significant predictive factors of residual disease (OR 5.4; 95% CI 1.08-27.7; p<0.05 and OR 7.05; 95% CI 2.1-23.1; p=0.001) (Table 3). Only cases with complete data (77 patients) were included in the analysis.

In the follow-up, 27.9% of cases (24/86) underwent surgery a second time in response to an abnormal smear test or colposcopy. Of these 24 reintervention cases, 14 patients were treated with LEEP and 10 with CKC.

DISCUSSION

In managing women with CIN, the goal is to prevent possible progression to invasive cancer while avoiding over-treatment of lesions that are likely to regress. In a previous study it was seen that the risk of cervical cancer is elevated for at least 20 years after the initial treatment of CIN [14], which underlines the importance of detecting factors that can predict, prior to follow-up, the eventual development of residual disease (RD). In our study, 26.7% of RD was observed.

The status of resection margins has been shown to be a predictor of RD, but the frequencies quoted in the literature are extremely variable, with values ranging from 11.9% to 53.4% [2, 8]. In this study, loop electrosurgical excision procedures were performed exclusively by residents and the frequency of positive margins after LEEP was 34.9%, which is comparable to the average for cases described in the literature. For the present research, the relationship between compromised margins and RD was statistically significant, according to the results found by Cejtin et al. [15], which corroborated findings of meta-analysis with more than 35 thousand women undergoing treatment for CIN [7].

Other predictive indicators of RD such as age, lesion severity and smoking have been described [16]. In our study, we found no significant difference with respect to age and smoking between patients with normal follow-up and those who had a RD. However, there was a statistically significant relationship between the lesion severity, represented by previous LEEP cervical biopsy with H-SIL, and RD.

This is a retrospective observational study that aims to provide an analysis of risk factors for RD after LEEP. There are certain limitations to be considered about our study. First, the retrospective nature of our case-series data unfortunately does not warrant a very high level of evidence. Secondly, the surprisingly large proportion of cases with positive or non-free margins suggests that the surgical technique was not optimal, which probably occurred because the procedure was performed exclusively by resident physicians. Moreover, although LEEP margins in pathology reports may be positive, the patient may in fact have no residual disease. This can be attributed to the thermal effect of the loop during surgery at the margins of the remaining cervix and to the use of diathermy for hemostasis, which both eradicate any remaining dysplastic cells.

Finally, after the LEEP it was not possible to perform, apart from Pap smear, colposcopy and cervical biopsy, a molecular method - PCR for detection of HPV in tissues, which may have generated a prevalence of RD underestimated in our analysis. Reliable data on the prevalence of HPV types is important for determining the types that should be included in a screening and follow-up program, since women with negative PS considered without RD, but infected with high-risk viral types should be more closely monitored because infection with a high oncogenic potential virus such as HPV 16 or 18 significantly increases the risk of cervical cancer in the future.

Therefore, the precise number of HPV-infected women is unknown in this study, and it is difficult to determine accurately, even with close follow-up with colposcopy/cytology. However, probably if the viral serotype research was performed in the patients, most would be found with persistent infection with HPV 16 and 18 as well as women from other developed countries, since a study with 97 women in the same region of Brazil where this study was performed demonstrated that approximately 40% had PCR positive for both serotypes [17].

In conclusion, we have shown that the most important prognostic markers for RD in patients with CIN treated with LEEP conization are affected surgical margins and H-SIL in cervical biopsy. Therefore, the combined evaluation of surgical margins status and cervical lesion severity could allow for the subdivision of patients treated with LEEP into categories of different risk levels of residual disease.

AUTHORS' CONTRIBUTION

RNC conceived the idea for and designed the study. ACH and AFS were involved in data collection and performed the research. ACH and TDF analyzed the data, conducted the literature search, study selection and prepared manuscript. RNC, AKG and CAJ revised the manuscript for final submission. The final manuscript was read and approved by all authors.

REFERENCES

1. Chen Y, Lu H, Wan X, Lv W, Xie X. Factors associated with positive margins in patients with cervical intraepithelial neoplasia grade 3 and postconization management. Int J Gynecol Obstet. 2009; 107(2): 107-110.
2. Bittencourt DD, Zanine RM, Sebastião AM, Taha NS, Speck NG, Ribalta JCL. Number of fragments, margin status and thermal artifacts of conized specimens from LLETZ surgery to treat cervical intraepithelial neoplasia. Sao Paulo Med J. 2012; 130(2): 92-96.
3. Prendiville W, Cullimore J, Norman S. Large loop excision of the transformation zone (LLETZ): a new method of management for women with cervical intraepithelial neoplasia. Br J Obstet Gynecol. 1989; 96: 1054-1060.
4. van Hamont D, van Ham MAPC, van der Zanden PHTH, Keijser KGG, Bulten J, Melchers WJG, et al. Long-term follow-up after large-loop excision of the transformation zone: evaluation of 22 years treatment of high-grade cervical intraepithelial neoplasia. Int J Gynecol Cancer. 2006; 16(2): 615-619.

5. Papoutsis D, Rodolakis A, Mesogitis S, Sotiropoulou M, Antsaklis A. Appropriate cone dimensions to achieve negative excision margins after large loop excision of transformation zone in the uterine cervix for cervical intraepithelial neoplasia. Gynecol Obstet Invest. 2013; 75(3): 163-168.
6. Serati M, Siesto G, Carollo S, Formenti G, Riva C, Cromi A, et al. Risk factors for cervical intraepithelial neoplasia recurrence after conization: a 10-year study. Eur J Obstet Gynecol Reprod Biol. 2012; 165(1): 86-90.
7. Ghaem-Maghami S, Sagi S, Majeed G, Soutter WP. Incomplete excision of cervical intraepithelial neoplasia and risk of treatment failure: a meta-analysis. Lancet Oncol. 2007; 8(11): 985-993.
8. Treacy A, Devaney D, Mulligan NJ, Boyd W, Keane JC. Can a more detailed evaluation of excision margins refine cytologic follow-up of women post-LLETZ for high-grade dysplasia? Int J Gynecol Pathol. 2010; 29(5): 479-482.
9. Lubrano A, Medina N, Benito V, Arencibia O, Falcón JM, Leon L, et al. Follow-up after LLETZ: a study of 682 cases of CIN 2–CIN 3 in a single institution. Eur J Obstet Gynecol Reprod Biol. 2012; 161(1): 71-74.
10. Baloglu A, Uysal D, Bezircioglu I, Bicer M, Inci A. Residual and recurrent disease rates following LEEP treatment in high-grade cervical intraepithelial lesions. Arch Gynecol Obstet. 2010; 282(1): 69-73.
11. Fu Y, Cheng X, Wang X, Xie X, Lü W, Chen C, et al. Residual disease and risk factors in patients with high-grade cervical intraepithelial neoplasia and positive margins after initial conization. Ther Clin Risk Manag. 2015; 2015(11): 851.
12. Figueira P, Reis B, Tacla M. Positive margins at loop electrosurgical excision procedure: are they a predictor of residual cervical intraepithelial neoplasia? Rev Bras Genitoscopia. 2008; 3(2): 41-45.
13. Nayar R, Wilbur DC. The Pap test and Bethesda 2014. Acta Cytologica. 2015; 59: 121-132.
14. Kalliala I, Anttila A, Pukkala E, Nieminen P. Risk of cervical and other cancers after treatment of cervical intraepithelial neoplasia: retrospective cohort study. BMJ. 2005; 331: 1183-1185.
15. Cejtin HE, Zimmerman L, Mathews M, Patel A. Predictors of persistent or recurrent disease after loop electrosurgical excision procedure. J Low Genit Tract Dis. 2017; 21(1): 59-63.
16. Liss J, Alston M, Krull MB, Mazzoni SE. Predictors of positive margins at time of loop electrosurgical excision procedure. J Low Genit Tract Dis. 2017; 21(1): 64-66.
17. Nunes JD, Vidal FC, Ferraro CT, Chein MB, Brito LM, Monteiro SC. Molecular detection of human papillomavirus in Brazilian women with cervical intrapithelial neoplasia in a northeast Brazilian city. Genet Mol Res. 2014; 13(4): 9077-9085.

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RESEARCH ARTICLE

Exposure to stress minimizes the zone of antimicrobial action: a phenotypic demonstration with six *Acinetobacter baumannii* strains

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ABSTRACT

Aim: To phenotypically study the role of domestic environmental stress in the emergence of antimicrobial resistance in *Acinetobacter baumannii*. **Materials and Methods:** Six strains of *A. baumannii* were initially subjected to AST and then were exposed to various stresses (temperature, pH and random combinations). Stressed cells were subcultured and then subjected for AST. The ZOIs before and after exposure to stress were compared. Statistical analysis was done using Student t-test at $p < 0.10$. **Results:** Exposure to stresses and combination of stresses resulted in substantial reduction in the ZOIs. Stress hardening was associated with further reduction in ZOIs. **Conclusion:** Exposure to domestic environmental stress imparted a significant and substantial reduction in the susceptibility of *A. baumannii* strains to antibiotics.

Keywords: *Acinetobacter baumannii*; Environmental stress; Antimicrobial resistance; Bacterial stress response; Stress-induced resistance.

INTRODUCTION

The advent of many potent antibiotics in the late 1960s rendered great hope in the effective management of infectious diseases while a few projected the utility of antibiotics to an extent of eradication [1, 2]. The upsurge in the global incidence of drug-resistant infections holds out a significant intimidation in the management of infectious diseases and to the attributed mortality and morbidity corresponding to all the levels of health care. Numerous initiatives have been directed by global health agencies to tackle this issue. Progressing prospectively across the timeline, we are no far from stumbling upon the 'end of antibiotic era' since the development of AMR has been entitled to an inevitable phenomenon [3-5]. ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus auerus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.) derive special attention of any infection preventionist (IP) due to their ability to persist refractory to antibiotics and the associated financial and morbidity burden [6].

Acinetobacter baumannii is an aerobic, Gram-negative, non-fermenting cocco-bacillus discovered in 1911 by a Dutch microbiologist, Martinus Willem Beigerinck. Early Acinetobacter outbreaks were reported in terminally ill patients and were effectively managed with sulfonamides and beta-lactams [2, 7, 8]. The isolation of multi-drug resistant (MDR) strains of *Acinetobacter baumannii* was reported in the early 1990s. Around the same time, the introduction of imipenem aided the effective treatment of MDR *Acinetobacter baumannii* infections. Unfortunately, resistance to imipenem vs reported in the same year due to the elaboration of OXA beta-lactamases making them extensively drug resistant (XDR) [9]. *Acinetobacter baumannii* have accumulated multiple drug resistance mechanisms which enable them to tolerate potent antimicrobials like carbapenems [10-12]. Surveillance carried out in 2010 revealed the incidence of isolation of MDR *A. baumannii* to be 74% and is currently the second most commonly isolated nosocomial pathogen [13, 14]. Currently, colistin is considered to be the drug of choice for the treatment of MDR and XDR *A. baumannii* infections [15]. However, the isolation of Pan-drug resistant (PDR) *A. baumannii* strains resistant to colistin has also been reported across the globe [16-18]. Owing to these challenges in effective management, it has been documented that isolation of *A. baumannii* from a hospitalized patient presents an approximate mortality of 30 % and is also associated with an escalation in morbidity, health-care costs and length of hospital stay [19-21].

In spite of the recent advancements in infection control and sterilization modalities, *A. baumannii* persist to be a liability in an intensive-care setup [12]. Environmental and human colonization, prolonged persistence on the surface of inanimate objects, the ability for spontaneous resistance development and biofilm formation are the exceptional properties of this organism [22-24]. Persistence of *A. baumannii* in the environment exposes them to multiple adverse factors that endanger their survival. These adverse factors elicit a strong protective stress response that enhances the probability of bacterial persistence. This stress response comprises of a definitive sequence of sub-cellular events resulting in altered gene expression and overall cellular physiology [25]. These protective stress responses are known to influence the action of antibiotics since antimicrobials are also considered to be growth-threatening factors [26-29]. Efforts to precisely decipher the interface of interaction between the action of domestic environmental stress factors, bacterial stress response and mechanisms of tolerating antimicrobial agents will be productive in understanding the molecular epidemiology of drug resistance development. Meanwhile, few scientists are also working on innovative alternative methods [30-32]. To our knowledge, the relation between AMR and stress due to food preservation has been studied in food-related pathogens such as *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes*. Previous works were directed to understand the effect of sublethal bacteriostatic stresses on antibiotic susceptibility [33-36] and few have worked on the relation between environmental factors and virulence [37]. But no work has been done to establish the influence of environmental conditions in harnessing AMR. *A. baumannii* has been chosen to be extensively studied owing to its long bacteria-environment interaction duration as a nosocomial pathogen which in turn predisposes it to adaptive responses in the form of higher frequencies of resistance-promoting genotypic and phenotypic alterations. As a preliminary step, in this study we phenotypically demonstrate the influence of exposure to domestic environmental stress on the antimicrobial susceptibility of *A. baumannii* by comparing the zones of inhibition (ZOI) before and after exposure to stress. Moreover, in this study, the model of demonstration was designed in such a way to possess a close resemblance to the actual scenario of contracting a health-care associated infection.

MATERIALS AND METHODS

The clinical isolates and the control strains were subjected to antimicrobial susceptibility testing by Kirby Bauer's disc diffusion method for nine commonly used drugs and their zones of inhibition were noted. Later they were exposed to various sub-lethal stresses, sub-cultured and again were subjected to antibiotic challenge. Owing to the non-availability of any evidence suggesting a standardized procedure to study the influence of stress on antimicrobial resistance, a novel scientifically coherent procedure was formulated and adopted throughout the study.

Ethical conduct

Ethical clearance for conducting the study was obtained from the Institutional Ethical Committee (IEC), Shridevi Institute of Medical Sciences and Research Hospital, Tumkur, Karnataka, India.

Bacterial strains

Five isolates (n=5) of *A. baumannii* grown from an array of samples isolated from the patients admitted to our hospital and other tertiary care centers in our locality were used in this study. The phenotypical and biochemical confirmation of the isolates was undertaken as per the recommendations of CLSI 2015 [38]. At first, the isolates were cultured in BHI broth (Himedia labs, Mumbai, India). Following this, the antibiotic susceptibility testing (AST) of the isolates was carried out for a few selected antibiotic agents that are commonly used for the treatment of *A. baumannii* infections by Kirby- Bauer disc diffusion method and were interpreted with reference to CLSI 2015 [38] guidelines. Standard strain *Acinetobacter baumannii* ATCC BAA 747 was included in the study for standardization and for comparison of variations in antibiotic susceptibility with other clinical isolates. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* 27592 were used as quality controls for interpretation of AST.

Antibiotic susceptibility testing

AST was carried out using Kirby- Bauer disc diffusion method. The inoculum was prepared by adjusting a 2 to 4-hour BHI broth to match the 0.5 McFarland turbidity standard, using saline. The dried surface of Muller- Hinton agar was inoculated by streaking a sterile swab dipped in the saline suspension over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 to 5 minutes, to allow for any excess surface moisture to be absorbed before applying the antibiotic impregnated disks. After 16 to 24 hours of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of an inverted perti plate. Susceptibility of the isolates for cefuroxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), amikacin (30 µg), norfloxacin (10 µg), imipenem (10 µg), meropenem (30 µg), ampicillin+sulbactam (10 µg + 10 µg), and piperacillin+tazobactam (1000 µg + 10 µg) were evaluated, recorded and interpreted according to CLSI 2015 [38] guidelines.

Phenotypical detection of enzyme production

All the isolates were screened for the production of enzymes such as ESBL, AmpC, and carbapenemase using disc diffusion method and were interpreted according to the manufacturer's instructions (Himedialabs, Mumbai, India). β-lactamase production was detected by comparing the ZOIs of drug and drug-inhibitor combination discs. Both the discs were applied aseptically to MHA plates inoculated with the *A. baumannii* in lawn pattern. The discs were placed at least 20 mm apart. The plates were incubated for 18 to 24 hours following which the ZOIs around drug and drug-inhibitor combination were measured and compared for interpretation. The results were interpreted as producer or non-producer.

Extended spectrum β- lactamase (ESBL) [39]

For ESBL detection, ceftazidime (30 µg) and ceftazidime-clavulanic acid (30 µg + 10 µg) combination discs were used. Any strain of *A. baumannii* which shows a difference in the zone diameter of ≥ 2 mm between the two discs was interpreted as ESBL producer.

AmpC cephalosporinase

Though not recommended by CLSI, AmpC production has been known to produce tolerance to extended spectrum cephalosporins and beta-lactam/beta-lactamase inhibitor combinations (40). *A. baumannii* was detected by comparing the ZOIs of cefoxitin (30 µg) and cefoxitin-cloxacillin (30 µg + 200 µg) combination. Any strain of *A. baumannii* which shows a difference in the zone diameter of ≥ 4 mm between the two discs was interpreted as AmpC producer.

Carbapenemase [41]

Carbapenemase production was detected by using two discs - imipenem (10 µg) and imipenem-EDTA (10 µg + 750 µg) which were placed not less than 20 mm apart. Any strain exhibiting a difference in ZOI ≥ 7 mm between the two discs was said to be an MBL producer.

Exposure to sublethal stress

Each of the five isolates and the control strain (*A. baumannii* ATCC BAA 747) were inoculated in BHI broth and were incubated for 24 hours under optimal conditions (37°C, pH 7.4). The overnight cultures were exposed to a variety of sub-lethal domestic environmental stresses such as sub-optimal temperature, super-optimal temperature, acidic pH, alkaline pH and random combinations of all for a specified duration of time.

Control

The temperature of 37°C and pH of 7.4 were considered to be the control conditions. All the obtained results were interpreted with reference to the ZOIs obtained by incubation at standard optimal conditions for 18 to 24 hours.

Temperature

All the strains were exposed to a range of temperature (5°C to 45°C) inclusive of sub-optimal and super-optimal temperatures.

Sub-optimal temperature
The overnight incubated broth of each bacterial strain was exposed to sub-optimal temperatures of 5°C, 20°C and 30°C for a duration of 24 hours.

Super-optimal temperature
BHI broth of each strain incubated for 24 hours was subjected to heat stress by exposing them to 40°C and 45°C for 2 to 4 hours in temperature controlled water bath.

pH

24-hour old inoculated BHI broths were acidified or alkalinized using acid or alkali to yield a range of pH from 3.0 to 10.0 and were incubated for 2 to 4 hours duration.

Acidic pH
Overnight incubated BHI tubes were acidified with diluted sulphuric acid (H_2SO_4) to attain final acidic pH of 3.0, 5.0 and 6.0. The tubes with supplemented acid were incubated at 37°C for 2 to 4 hours.

Alkaline pH

Alkalization was carried out by the addition of diluted potassium hydroxide (KOH) to render a final alkaline pH of 9.0 and 10.0 and the supplemented broth suspensions were incubated at 37°C for 2 to 4 hours.

Combination

All the strains were treated with random combinations of pH and temperature such as 5.0, 20°C; 5.0, 40°C; 9.0, 20°C; and 9.0, 40°C respectively. For this, 24-hour old broth cultures of each strain were adjusted to the specified pH by addition of acid/alkali and then stressed at a particular temperature for 2 to 4 hours. Moreover, a few additional test conditions were included. These additional conditions included exposure of cells which survived pH 3.0 to pH 1.0; pH 10.0 to pH 12.0; pH 3.0 to pH 12.0 and pH 10.0 to pH 1.0. The cells that survived the sub-lethal pH were cultured in BHI broth. 24-hour old broth cultures were then acidified or alkalinized to attain the specified pH and were incubated at 37°C for 2 to 4 hours.

Isolation of stressed bacterial cells

All the strains cultured in BHI broth were subjected to the action of various sub-lethal stresses for specified duration of time. Following exposure to stress, each strain was plated on an MHA plate which was further incubated for 18 to 24 hours at 37°C. From the colonies that appeared on the MHA plate, one or two were inoculated into another tube containing BHI broth and incubated for 2 to 4 hours. Growth in the broth was adjusted to 0.5 McFarland turbidity units by the addition of sterile physiological saline and then was subjected to antibiotic susceptibility testing as per the above-described procedure.

Statistical analysis

The ZOIs for various antibiotic agents at different test conditions were recorded on a spreadsheet. The mean and standard deviation of Zones of inhibition (mm) of all strains to a specific antibiotic were estimated. The differences in ZOIs between test conditions and standard condition for each antibiotic were compared and analyzed using MS Excel spreadsheet application. The significance of variation was assessed by one-tailed studentt-test. Since a novel, non-standardized methodology was adopted and due to the small sample size, a value of $p < 0.10$ was considered significant. At diverse test conditions, variation in ZOI was observed only with amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem. Hence, only the susceptibility for these five (n=5) antibiotics were further analyzed for statistical significance.

RESULTS

Antimicrobial susceptibility of unstressed *A. baumannii*

This study involved five (n=5) clinical isolates and one standard ATCC BAA 747 strain of *A. baumannii*. The data of five (n=5) clinical isolates has been furnished in Table 1. Initial AST was carried out by Kirby-Bauer disc diffusion method and the susceptibility pattern is shown in Table 2. All the five clinical isolates were resistant to a majority of antibiotics used in the study except for variable susceptibility to norfloxacin and amikacin. The diameter of the zone of inhibition for each drug was compared with the standard ATCC strain. Zone of inhibition (ZOI) was deemed to be the parameter for comparison and analysis. The diameter of zones around amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem for the clinical isolates were considerable and comparable. Hence, the clinical isolates, though exhibited resistance to most of the antibiotics, were included in the study. ATCC BAA 747 strain was susceptible to amikacin, norfloxacin, imipenem and meropenem; intermediately susceptible to piperacillin-tazobactam; and resistant to

cefuroxime, ceftazidime, cefepime, and ampicillin-sulbactam. ATCC BAA 747 was used as a substitute for susceptible strain.

Table 1. Clinical sites of isolation of *Acinetobacter baumannii.*

S No	Strain No	Age	Gender	Clinical Diagnosis	Sample	Source
1	ET400	60 yrs	M	VAP (Cardiac arrest)	Bronchial wash	ICU
2	ET401	65 yrs	F	VAP (COPD)	Tracheal aspirate	ICU
3	B2023	17 days	M	Neonatal sepsis	Blood	NICU
4	CT57	59 yrs	M	Catheter-associated UTI	Urine	ICU
5	CT58	68 yrs	F	Catheter-associated UTI	Urine	ICU

Table 2. Antibiogram of unstressed *Acinetobacter baumannii* isolates.

S. No.	Antibiotic	ET400	ET401	B2023	CT57	CT58	ATCC BAA 747
1.	Cefuroxime	R	R	R	R	R	R
2.	Ceftazidime	R	R	R	R	R	R
3.	Cefepime	R	R	R	R	R	R
4.	Amikacin	R	R	R	S	S	S
5.	Norfloxacin	I	R	I	S	S	S
6.	Amp-Sulb	R	R	R	R	R	R
7.	Pip-Tazo	R	R	R	R	R	I
8.	Imipenem	R	R	R	R	R	S
9.	Meropenem	R	R	R	R	R	S

Enzyme production

All the five (n=5) clinical isolates were screened for beta-lactamase (ESBL, AmpC cephalosporinase, and Carbapenemase) production. Among the five strains, only blood isolate (B2023) was found to be an AmpC cephalosporinase producer (Table 3).

Table 3. β-lactamase production among clinical isolates.

S. No.	Strain	ESBL	AmpC	Carbapenemase
1.	**ET400**	-	-	-
2.	**ET401**	-	-	-
3.	**B2023**	-	+	-
4.	**CT57**	-	-	-
5.	**CT58**	-	-	-

Influence of temperature stress on antibiotic susceptibility

Clinical isolates (n=5) and ATCC BAA 747 strain were exposed to a range of sub-optimal (5°C, 20°C, and 30°C) and super-optimal (40°C and 45°C) temperatures. Though the zones of inhibition mildly reduced

following exposure to sub-optimal temperatures of 20°C and 30°C, a significant reduction in ZOIs was observed on exposure to a super-optimal temperature of 45°C. A marked decrease in ZOI of ≥ 9mm for norfloxacin was observed when strains B2023 and CT57 were exposed to 45° C temperature. Effects of exposure to sub-optimal and super-optimal temperatures on the susceptibility for amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem were relatively significant. At 45°C, a majority of the bacterial strains exhibited resistance to all the antibiotics. UTI isolates (CT57 and CT58) were initially sensitive to amikacin and norfloxacin but exposure to 40°C and 45°C rendered them resistant. ATCC BAA 747 strain also showed a similar pattern of variation at different temperatures. Exposure to 5°C for 24 hours resulted in a mild reduction in the mean ZOI for a majority of the antibiotics but this variation in susceptibility was statistically insignificant ($p > 0.10$). Eventually, a significant reduction ($p < 0.10$) in the mean ZOI was recorded at 45°C for all antibiotics. The influence of other test temperatures on mean ZOI was statistically insignificant ($p > 0.10$) except for norfloxacin which exhibited significant variation in susceptibility at 20°C ($p=0.0693$) and at 40°C ($p=0.02908$). As a general trend, the mean ZOI decreased at sub-optimal and super-optimal temperatures. However, a significant reduction was observed only at 45°C (Figure 1, Table 4).

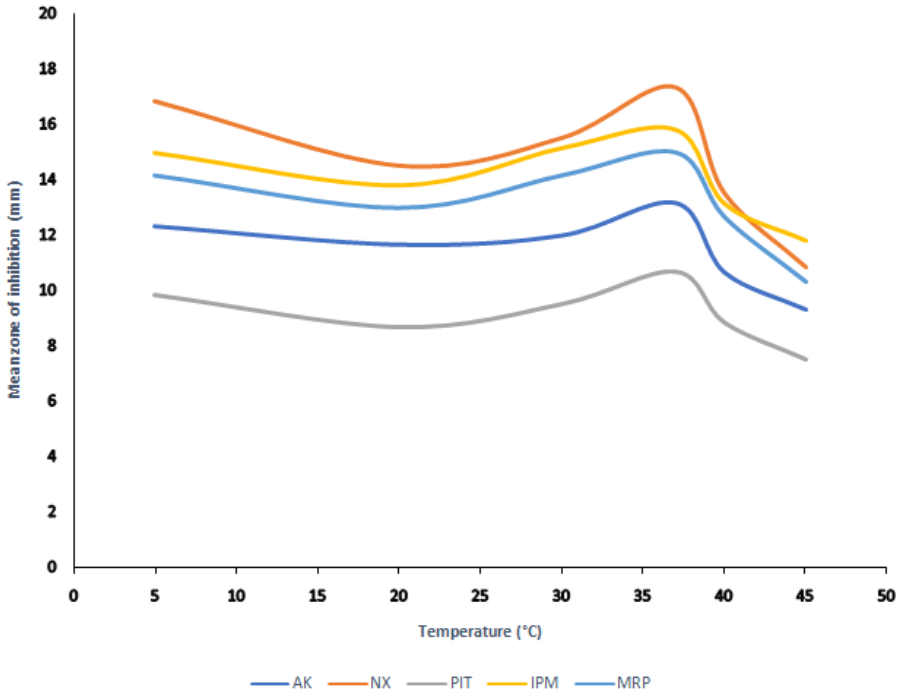


Figure 1. Influence of temperature on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is Temperature (°C) at pH 7.4 and along y-axis is the mean zone of inhibition (mm).

Influence of pH stress on antibiotic susceptibility

Overnight broth cultures of each *A. baumannii* strain was exposed to a collection of acidic (3.0, 5.0 and 6.0) and alkaline (9.0 and 10.0) pH for 2 hours and then were subjected to antibiotic challenge. On exposure to increasing pH from 3.0 to 7.4, there was a consistent increase in ZOI following which a steady fall in ZOI was recorded (Figure 2, Table 5) till an alkaline pH of 10.0 was attained. Least ZOI values were obtained at the extremes of acidic (3.0) and alkaline (10.0) pH. ATCC BAA 747 strain also followed the same trend of variation. The degree of reduction in ZOI was equivalent with all the bacterial strains. Exposure to acidic pH conferred greater resistance to the majority of the antibiotics when compared to alkaline pH (Table 5). A

significant fall in ZOI of ≥ 10 mm for norfloxacin was observed at pH 3.0 in UTI isolates (CT57 and CT58). ATCC strain did not survive an acidic pH of 3.0. At extremes of acidity and alkalinity, the ATCC strain showed a marked reduction in ZOI of ≥ 10 mm for amikacin and imipenem. A gross reduction in mean ZOI ≥ 4 mm was recorded for norfloxacin and carbapenems at extremes of acidic and alkaline pH. The reduction in mean ZOI for amikacin, piperacillin-tazobactam, and imipenem at pH 6.0 and 9.0 was statistically not significant. However, the mean ZOI for norfloxacin showed significant reduction at all levels of tested acidity and alkalinity.

Influence of combination of test conditions on antibiotic susceptibility

Bacterial cultures were subjected to stress in the form of a few combinations of pH and temperature; 5.0, 20°; 9.0, 20°C; 5.0, 40°C; and 9.0, 40°C respectively. The combinations of acidic pH with sub-optimal temperature and alkaline pH with super-optimal temperature induced a higher degree of resistance than other combinations (Table 6). It is noticeable that the combination of temperature and pH stresses caused a greater reduction in the ZOIs than the reduction caused due to the action of individual stresses themselves. For example, the ZOI of CT57 for amikacin at 20°C was 18 mm and at pH 5.0 was 14 mm while the zone markedly decreased to 12 mm when subjected to the combination of 5.0 pH and 20°C temperature. ATCC BAA 747 that was susceptible to carbapenems following exposure to all individual stresses, developed resistance when subjected to a combination of cold and acidic stress. But the ATCC strain did not withstand the combination of super-optimal temperature and acidity. A significant reduction ($p < 0.10$) in mean ZOI was observed at all combinations of temperature and pH (Table 6 and Figure 3). Hence, subjection to a random combination of stresses consistently enhanced resistance to all the antibiotic agents.

Table 4. Effect of temperature on susceptibility to various antibiotics.

Temperature (°C)		**37**	**5**	**20**	**30**	**40**	**45**
AK	Mean	13.17	12.33	11.67	12	10.67	9.33
	SD	7.91	6.98	6.25	6.66	5.2	3.88
	CI	±6.33	±5.58	±5.00	±5.33	±4.16	±3.10
	p- Value		0.39037	0.29069	0.34252	0.14579	0.03002
NX	Mean	17.33	16.83	14.5	15.5	13.5	10.83
	SD	5.09	5.42	3.94	4.04	3.83	3.97
	CI	±4.07	±4.34	±3.15	±3.23	±3.06	±3.18
	p- Value		0.41563	0.0693	0.1587	0.02908	0.00512
PIT	Mean	10.67	9.83	8.67	9.5	8.83	7.5
	SD	4.93	5.46	4.27	5.05	4.58	3.67
	CI	±3.94	±4.37	±3.42	±4.04	±3.66	±2.94
	p- Value		0.3613	0.15143	0.29744	0.18548	0.04412
IPM	Mean	15.83	15	13.83	15.17	13.17	11.83
	SD	8.18	8.44	7.05	7.52	7.17	6.55
	CI	±6.54	±6.75	±5.64	±6.02	±5.74	±5.24
	p- Value		0.40958	0.25951	0.41875	0.20222	0.09776
MRP	Mean	15	14.17	13	14.17	12.67	10.33
	SD	5.18	5.08	4.94	5.31	4.23	3.83
	CI	±4.14	±4.06	±3.95	±4.25	±3.38	±3.06
	p- Value		0.3521	0.18342	0.35817	0.11713	0.01532

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 5. Effect of pH on susceptibility to various antibiotics.

pH		7.4	3	5	6	9	10
AK	Mean	13.17	7.5	9.67	12	11.17	10.5
	SD	7.91	5.21	4.03	6.6	5.71	4.93
	CI	±6.33	±4.17	±3.22	±5.28	±4.14	±3.94
	p-Value		0.0222	0.0433	0.3412	0.2146	0.0222
NX	Mean	17.3	7.83	11.83	14.5	12.67	11.5
	SD	5.09	4.36	2.71	3.89	3.5	3.89
	CI	±4.07	±3.49	±2.17	±3.11	±2.80	±3.11
	p-Value		0.0015	0.0021	0.0673	0.0112	0.0072
PIT	Mean	10.7	5	8.17	9.17	8.17	7.67
	SD	4.93	2.45	3.49	4.07	4.36	4.08
	CI	±3.94	±1.96	±2.79	±3.26	±3.49	±3.26
	p-Value		0.0012	0.0695	0.2036	0.1091	0.0657
IPM	Mean	15.8	7.83	11.5	13.67	12.67	11.5
	SD	8.18	4.26	3.45	6.98	6.41	6.12
	CI	±6.54	±3.72	±2.76	±5.58	±5.13	±4.90
	p-Value		0.0029	0.0138	0.2409	0.1403	0.0719
MRP	Mean	15	8.17	12.17	12.83	12.33	11.33
	SD	5.18	4.49	3.66	4.45	4.41	4.72
	CI	±4.14	±3.92	±2.93	±3.56	±3.53	±3.78
	p-Value		0.0068	0.0581	0.1431	0.0994	0.0577

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 6. Effect of random combinations of temperature and pH on the susceptibility to various antibiotics.

Conditions (Temp, pH)		37, 7.4	20, 5.0	40, 5.0	20, 9.0	40, 9.0
AK	Mean	13.17	8.83	10	9.5	9.67
	SD	7.91	3.13	4.43	3.99	4.13
	CI	±6.33	±2.50	±3.54	±3.19	±3.30
	p- Value		0.00964	0.06991	0.03693	0.04619
NX	Mean	17.33	11	12.17	10.67	10.83
	SD	5.09	1.67	2.32	2.58	2.04
	CI	±4.07	±1.34	±1.86	±2.06	±1.63
	p- Value		0.00012	0.0014	0.00073	0.00028
PIT	Mean	10.67	7.17	7.83	7.33	7.33
	SD	4.93	2.86	4.49	3.27	3.27
	CI	±3.94	±2.29	±3.59	±2.62	±2.62
	p- Value		0.015	0.09124	0.02716	0.02716
IPM	Mean	15.83	10.5	11.33	11.67	11
	SD	8.18	1.76	5.24	5.82	5.18
	CI	±6.54	±1.41	±4.19	±4.66	±4.14
	p- Value		0.00035	0.04478	0.07005	0.03553
MRP	Mean	15	10.83	11.5	12	10.83
	SD	5.18	3.31	4.46	4.29	3.76
	CI	±4.14	±2.65	±3.57	±3.43	±3.01
	p- Value		0.0137	0.05633	0.07368	0.0211

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 7. Effect of stress hardening on susceptibility to various antibiotics.

Conditions (Pre-stressed pH, exposed pH)		37, 7.4	3.0, 1.0	10.0, 12.0	3.0, 12.0	10.0, 1.0
AK	Mean	13.17	6	4.67	7.67	7
	SD	7.91	5.37	3.93	5.47	6.03
	CI	±6.33	±4.30	±3.14	±4.38	±4.82
	p- Value		0.01107	0.0016	0.02838	0.02708
NX	Mean	17.33	6	7	7.67	6.67
	SD	5.09	4.9	5.66	4.27	5.61
	CI	±4.07	±3.92	±4.53	±3.42	±4.49
	p- Value		0.00119	0.00328	0.00132	0.00278
PIT	Mean	10.67	4	5.33	5	5
	SD	4.93	3.1	5.16	2.45	4.52
	CI	±3.94	±2.48	±4.13	±1.96	±3.62
	p- Value		0.00163	0.02622	0.00119	0.01381
IPM	Mean	15.83	5.83	6.83	7.67	8.33
	SD	8.18	4.67	7.31	4.5	7.66
	CI	±6.54	±3.74	±5.85	±3.60	±6.13
	p- Value		0.00167	0.01477	0.00338	0.01205
MRP	Mean	15	5.83	6.5	7.67	8.17
	SD	5.18	4.62	6.63	4.13	7.49
	CI	±4.14	±3.70	±5.30	±3.30	±5.99
	p- Value		0.00232	0.0128	0.00369	0.03792

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

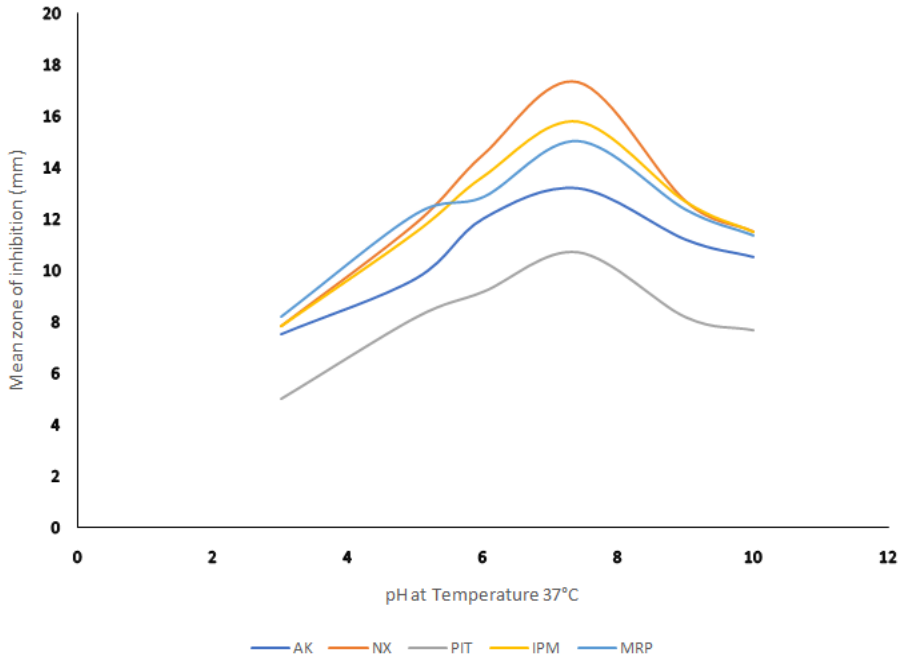


Figure 2. Influence of pH on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+ Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is pH at 37°C and along y-axis is the mean zone of inhibition (mm).

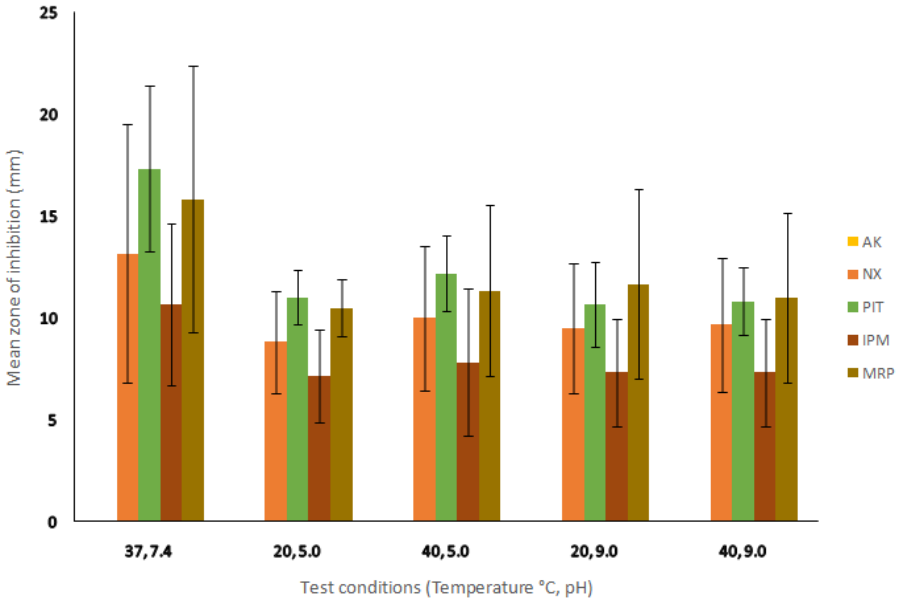


Figure 3. Influence of combined action of stresses on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is the test condition (temperature °C, pH) and along y-axis is the mean zone of inhibition (mm).

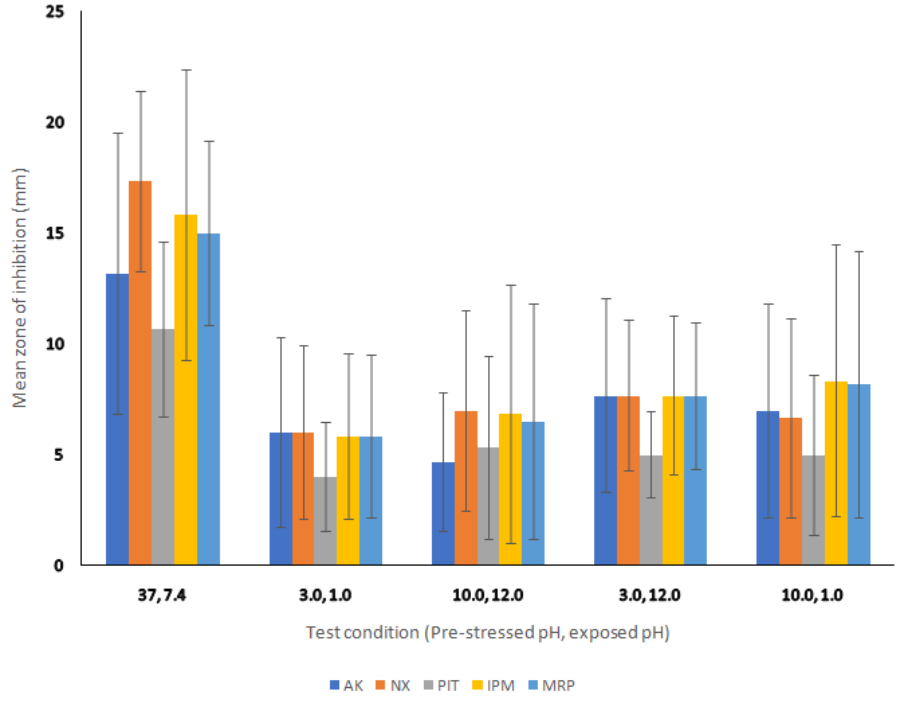


Figure 4. Effect of stress hardening on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is pH (sub-lethal pH, lethal pH) and along y-axis is the mean zone of inhibition (mm).

Few other combinations like exposure of pH-stressed cells to another stratified degree of acidity or alkalinity were also included in the study. The bacterial cells stressed at pH 3.0 and 10.0 were then exposed to pH 1.0 and 12.0 following which they were plated and then subjected for AST. It was observed that acid-stressed (pH 3.0) cells of all strains except for that of ET401 survived a higher acidic pH of 1.0 while all strains

survived alkaline pH 12.0. VAP isolates (ET400 and ET401) stressed at pH 10.0 did not survive pH 1.0. Likewise, UTI isolates stressed at pH 10.0 did not survive a higher alkaline pH 12.0. Notably, the post-stressed cells of strain B2023 tolerated all the designated higher levels of acidity and alkalinity. As a general rule, subjecting the post-stressed cells to a different degree of acidity or alkalinity resulted in a further curtailment of antibiotic susceptibility ($p < 0.10$) (Table 7 and Figure 4).

DISCUSSION

In the past two decades, *A. baumannii* has emerged out as a successful nosocomial pathogen exhibiting salient challenging features such as prolonged environmental persistence, spontaneous emergence of drug resistance and biofilm formation [20]. Especially, the spread of MDR *A. baumannii* worldwide poses a serious threat to the management of *A. baumannii* infections in health care setup. In the present study, five (n=5) clinical isolates of *A. baumannii* and one (n=1) standard ATCC BAA 747 strain were included. According to the recent definition [42], three isolates (ET400, ET401, and B2023) were extensively drug resistant (XDR) while the other two strains (CT57 and CT58) were multidrug resistant (MDR). Since all the clinical isolates were drug resistant, ATCC BAA 747 strain was used as a substitute to susceptible strain for interpretation of the results. The purpose of this study was to understand the influence of environmental stress-bacterial cell interaction on its susceptibility to antibiotic agents. Production of beta-lactamase enzymes is one of the chief mechanisms that confer a high degree of resistance to beta-lactam antibiotics. Of the collection of beta-lactamases, the roles of extended-spectrum β-lactamses, Metalloβ-lactamases (such as carbapenemases) and AmpC cephalosporinases are significant with special reference to *A. baumannii* [39-43]. We made an attempt to screen all the clinical isolates for β-lactamase production phenotype. Only one strain (B2023) was found to produce AmpC cephalosporinase while other strains were non-enzyme producers in spite of exhibiting resistance to all the β-lactam antibiotic agents. Hence, it can be inferred that the resistance exhibited by clinical isolates of *A. baumannii* is probably due to other mechanisms such as alterations in the expression of porin channels [43, 44] and induction of efflux pump expression [43]. A total of six (n=6) strains of *A. baumannii* were subjected to various forms of environmental stresses (sub-optimal and super-optimal temperature; acidic and alkaline pH; and few combinations of both) in vitro. The stationary phase of bacterial growth provides an advantage of enhancing the chances of environmental persistence [45]. As an effort to understand the survival strategies of bacteria, in our study, all the strains were exposed to sub-lethal domestic environmental stresses during their stationary phase of growth. Then, the post-stressed bacterial cells were subjected to antibiotic challenge.

All (n=6) *A. baumannii* strains were exposed to super-optimal and sub-optimal temperatures. On exposure of the bacterial cells to super-optimal temperatures of 40°C and 45°C, a consistent increase in the degree of resistance was observed. Remarkably at 45°C, a majority of the bacterial strains were resistant to all the test antibiotics. Similar observations were recorded by Faezi Ghasemi [34] where exposure of *Listeria monocytogenes* PTCC1297 strain to a high temperature of 45°C for 2 hours exhibited a marked 2 to 4 fold increase in the MIC of all the antibiotics tested. In contrary to this, McMahon et al. [33] reported that increased temperature stress enhances the susceptibility of food related pathogens like *S. aureus, E. coli* and *S. typhimurium* to antibiotics. Similar observations were reported by Bahram [46] that exposure of *Stenotrophomonsmaltophilia* to temperature stresses increased its susceptibility to aminoglycosides. It should be noticed that McMahon et al. and Bahram exposed the bacteria to environmental stress during lag phase or the phase of adaptation which leads to inhibition of bacterial proliferation and thus reduction in MIC. As discussed earlier, stationary phase of bacterial growth is considered to be the appropriate juncture to understand the instincts of bacterial persistence. Recent evidences suggest that exposure to heat stress induces the expression and synthesis of stress proteins especially sigma factors (SigB, SigX, and RpoE) which are regulated by σB, σX and σE respectively [45, 47-50]. These sigma factors have been shown to induce the expression of *mexCD-OprJ* multidrug efflux operon which is responsible for the reduction in susceptibility to a majority of the antibiotics [45]. Exposure to a sub-optimal temperature of 5°C did not significantly alter the

susceptibility of bacterial strains except for a mild reduction in the mean ZOI for all the antibiotics ($p > 0.10$). But a moderate reduction in susceptibility was noticed at 20°C and 30°C. According to McMahon et al. [33], exposure to cold stress did not alter the susceptibility of *E. coli* and *S. aureus* to a majority of antibiotics with a mild degree of increase in resistance to a few antibiotics while *S. typhimurium* showed an increased susceptibility. Al-Nabulsi [35] reported a two to four-fold increase in resistance (MIC) of *L. monocytogenes* following exposure to a low temperature of 10°C for 24 hours. In *E. coli* exposure to cold stress induced the expression of *RcsCDB* gene which in turn participates in protection against β-lactam agents and capsule synthesis [45, 51]. Holler [52] also demonstrated that 4°C had least stress effect while intermediate temperatures (10°C and 20°C) cause significant injury to the bacterial cell membrane of *Campylobacter coli* SP10 leading to adaptive changes in morphology and metabolism. However, the mechanism of enhanced susceptibility at low temperature has not yet been understood.

Antibiotic susceptibility of all the strains of *A. baumannii* reduced consistently at both acidic and alkaline pH. Susceptibility was proportional to pH as the pH was raised from 3.0 to 7.4. When the pH was further raised from 7.4 to 10.0, a fall in susceptibility was noted. In other words, both acidity and alkalinity reduced the susceptibility of *A. baumannii* strains to antibiotics. Exposure to acidity conferred a higher degree of resistance to antibiotics than alkalinity. However, at the extremes of acidic (3.0) and alkaline pH (10.0), all the *A. baumannii* strains were resistant to all the antibiotics. Al-Nabulsi [53] also reported that exposure to acidic and alkaline environment increased the resistance of *Cronobacter sakazakii* for antibiotics such as ampicillin, amoxicillin, kanamycin, neomycin, etc. McMahon reports a marked rise in MIC for *S.aureus, S. typhimurium* and *E.coli* following subjection to acid stress (pH 5.0). Another study by Hernando [54] demonstrated a fall in antibiotic susceptibility of *L. monocytogenes* consequential to citric acid exposure. Similar findings were recorded by Al-Nabulsi [35] on exposing three strains of *L. monocytogenes* to acidic stress. Controversial to the findings of our study, Faezi-Ghasemi [34] reported an increase in susceptibility of *L. monocytogenes* to beta-lactam agents, aminoglycosides, and rifampicin on exposure to acidic pH of 5.0. Faezi-Ghasemi exposed the bacteria to acidic pH in the log phase of bacterial growth during which active proliferation occurs and the bacteria are more vulnerable to any form of stress [55] while in this study bacteria were subjected to any form of stress during their stationary phase. Acidic and alkaline environmental pH enhance the expression of sigma factor, SigB [56, 57] and CpxRA TCS [58, 59] which in turn modulate the preferential synthesis of outer membrane proteins (OmpF and OmpC) [45, 60] and alter the cell membrane fluidity by modifying the membrane lipid composition [45, 57]. These changes in the membrane composition and permeability possess a direct influence on the influx and efflux of antibiotics across the cell membrane and thus on the antibiotic susceptibility of the bacterial cell itself.

Combined action of temperature and pH stress resulted in the development of a higher degree of resistance to antibiotics. Hot and cold temperatures; and acidic and alkaline pH initiate a sequence of specific or non-specific pathways that bring about alterations in the genotype and phenotype of a stressed bacterial cell that enable its survival. Most of the environmental stresses like osmotic pressure, acidity, alkalinity and cell wall-active antibiotic agents pose a direct impact on the bacterial cell envelope [45]. When a collective spectrum of envelope stresses acts simultaneously, the probability of induction of cross-resistance to other stresses is maximum [33, 35, 45]. Especially, in an intensive care setup, a myriad of stresses operate simultaneously on the bacteria. This enhances the chances of bacterial persistence in the environment with a significant reduction in antibiotic susceptibility. Al-Mahin [61] constructed a genetically modified strain of *Lactococcus lactis* NZ9000 that expressed an *E. coli dnaK* stress protein on exposure to various stresses. They reported that the genetically modified strain of *L. lacti* remained resilient to all the stresses. This higher degree of tolerance was attributed to the product of *dnaK* expression, hsp70, a heat shock protein. Hence, it can be inferred that induction of heat shock proteins or molecular chaperones non-specifically stimulate multiple pathways which enable a high degree of tolerance to various stresses. Other stress proteins such as PhoPQ, ParRS, BraRS and AlgU are responsible for induction of broad-spectrum cross-resistance to various cell-wall damaging factors, antibiotics, detergents and unfavorable temperature [45, 62-67]. In this study, we also tried to evaluate the relation between stress-hardening and antibiotic susceptibility. Our preliminary workup to determine the sub-

lethal levels of pH revealed that an acidic pH 1.0 and an alkaline pH 12.0 to be lethal to all the bacterial strains employed in the study. However, following exposure to sub-lethal levels of acidity and alkalinity, a majority of the strains survived lethal surges of acidity and alkalinity. Enhanced tolerance to higher levels of acidity and alkalinity were associated with a substantial reduction in the susceptibility of *A. baumannii* to various antibiotic agents. This proves that the genotypic and proteomic adaptive changes in the bacterial cell that are responsible for stress hardening also initiate mechanistic consequences that impart a significant measure of cross resistance to other stresses.

The control *Acinetobacter baumannii* ATCC BAA 747 strain followed a trend of variations similar to the other clinical strains following exposure to different test conditions which is supported by Al-Nabulsi. He reported that *Listeria monocytogenes* ATCC 7644 showed a pattern of variation similar to other two isolates from food products [35]. However, in our study, the tolerance of ATCC strain to various test conditions was much reduced. Clinical isolates remained viable after following exposure to a wide range of stresses compared to the ATCC strain. This is because the clinical isolates were multi-drug resistant and resistance to antibiotics, in turn, confers a certain degree of tolerance to environmental stress [33, 68]. From this it can be understood, that both carbapenem susceptible *Acinetobacter baumannii* (CSAB) and carbapenem resistant *Acinetobacter baumannii* (CRAB) project similar distinctions in antibiotic susceptibility following the exposure to various stresses.

McMahon et al. [33] reported that increase in MIC following exposure to stress was not associated with any change in the diameter of the zone of inhibition (ZOI). They accredited the increase in MIC to the occurrence of hyper-resistant mutants. But in this study, an obvious and significant reduction in the zones of inhibition (ZOIs) was noted following the exposure to stresses.

It has been understood that exposure to stress results in the emergence of a phenotypically heterogeneous population of bacterial cells that possess a higher degree of adaptability or resilience. Directed mutation or stress adaptation is a consequence of hypermutability, amplification of resistance genes, stress-induced inter- and intra-bacterial genetic transfer and recombination, and defects in DNA repair [33, 68-73]. These amendments in the bacterial genotype that enable environmental persistence of the bacteria also possess a direct influence on the bacterial susceptibility to antibiotic agents [72, 73]. In the current study, bacterial strains were subcultured following exposure to stress to ensure that the acquired resistant phenotype is not a transient phenomenon. The scientific reliability and acceptability of this work were ensured by exposing the *A. baumannii* strains to stresses in their stationary phase of growth and by calibrating the methodology to closely mimic the realistic picture of acquiring a nosocomial infection. For further evaluation of this hypothesis, demonstration of stress gene expression, stress protein elaboration, and expression of genetic determinants of antibiotic resistance by employing nucleic acid amplification techniques and advanced proteomic studies would be suggested. A major drawback of this study is the limited sample size. Hence, it would be recommended to include a considerable number of bacterial strains exhibiting heterologous patterns of antibiotic susceptibility. Structuring, designing and developing novel chemotherapeutic agents that will inhibit the action of stress proteins or the adaptive changes secondary to the production of stress proteins would serve a great deal in mitigating the emergence and spread of antimicrobial resistance.

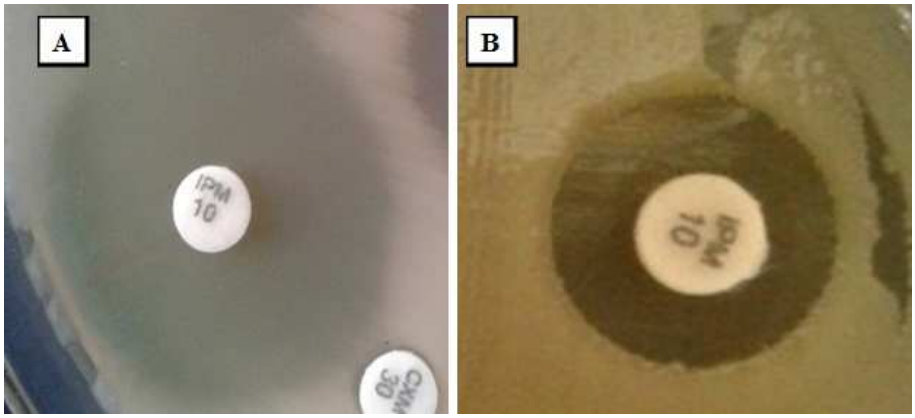
CONCLUSIONS

Acinetobacter baumannii has emerged to be a notorious superbug. This study was determined to elucidate the effect of interaction between domestic environmental stress and bacterial existence on bacterial susceptibility to antibiotics. There was a steady reduction in the antibiotic susceptibility of *A. baumannii* with exposure to various stresses. Post-stressed cells of carbapenem resistant *A. baumannii* (CRAB) strains (all five clinical isolates) and those of carbapenem sensitive *A. baumannii* (CSAB) strain (ATCC BAA 747) did not show any significant difference in susceptibility to antibiotics. Therefore, an intense and prolonged interaction between bacterial cell and environmental stress fosters the development of antimicrobial resistance. Thus, the

symbiotic interaction between environmental stresses and bacterial stress responses in the emergence of antimicrobial resistance is established.

IMAGES

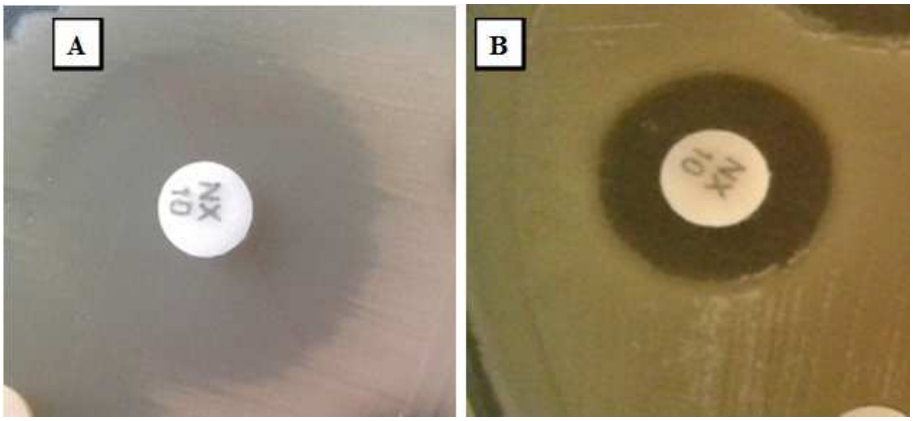
Image 1. Depiction of variations in the susceptibility of ATCC BAA 747 strain to imipenem following exposure to various stresses.



A. ZOI formed around imipenem disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around imipenem disc in ATCC BAA 747 after exposure to a pH of 5.0 at 20°C.

The ZOI of imipenem before exposure to stress was 32 mm. A least ZOI of 12 mm was obtained when ATCC BAA 747 was exposed to pH of 5.0 at 20°C.

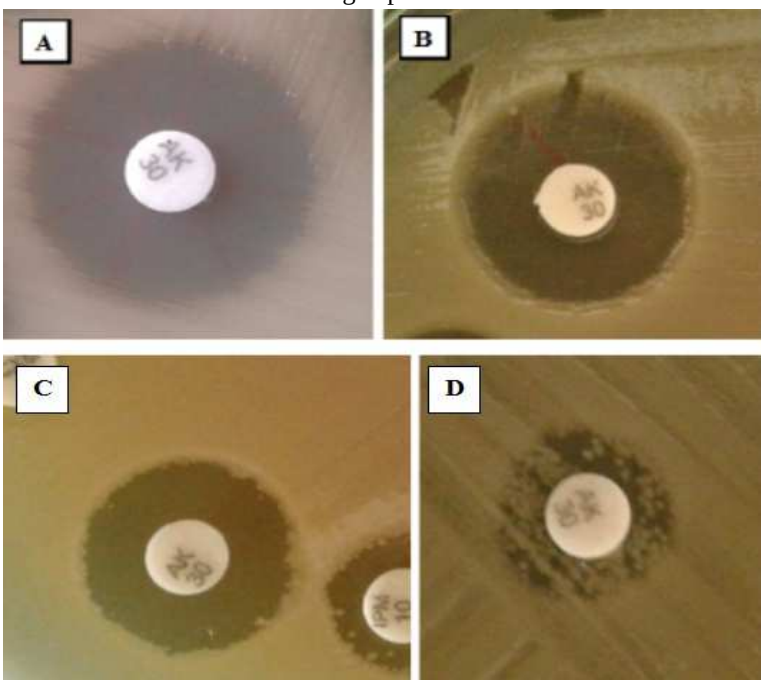
Image 2. Depiction of variations in the susceptibility of ATCC BAA 747 strain to norfloxacin following exposure to various stresses.



A. ZOI formed around norfloxacin disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around norfloxacin disc in ATCC BAA 747 after exposure to a pH of 9 at 40°C.

Before exposure to stress a ZOI of 24 mm was recorded. It is noted that the ZOI was significantly reduced to 12 mm after exposure to a pH of 9 at 40°C.

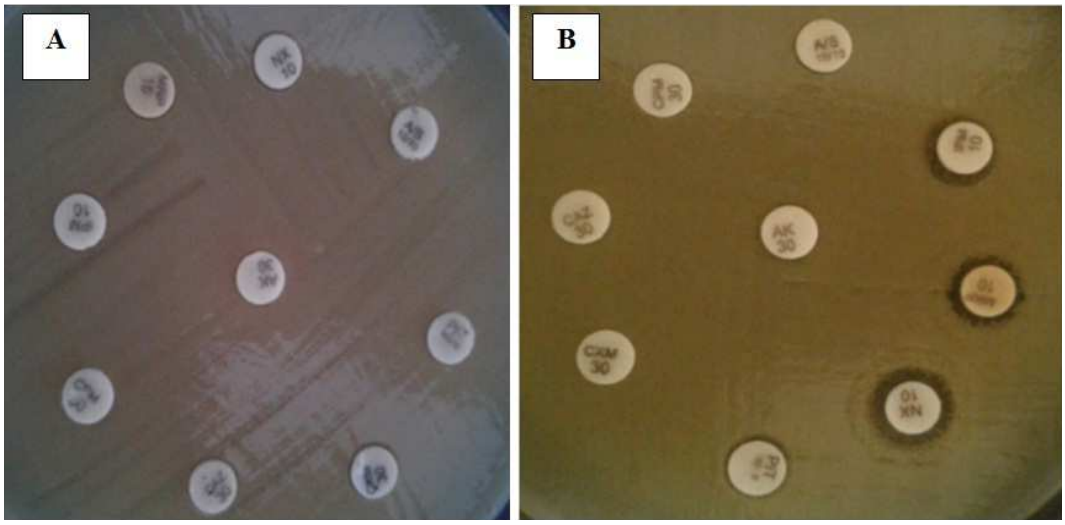
Image 3. Depiction of variations in the susceptibility of ATCC BAA 747 strain to amikacin following exposure to various stresses.



A. ZOI formed around amikacin disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to 20°C.
C. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to pH 10.0.
D. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to pH 9.0 at 40°C.

The ZOI for amikacin recorded before exposure to stress was 22 mm. It was reduced to 18 mm following the exposure to a temperature of 20°C. A further reduction in ZOI to 15 mm was observed when exposed to a pH of 10.0. A least ZOI of 12 mm was recorded after exposure to pH 9.0 at 40°C. Also note multiple hyper-resistant clones of *A. baumannii* growing up to to the margins of the disc (3D). It was initially thought to be a contaminant but was later confirmed to be *A. baumannii*.

Image 4. Absence of Zone of inhibition (ZOI).



A. Acid stressed ET401 after exposure to a pH 12.0.
B. B2023 following exposure to 45°C.

A significant fall in susceptibility to antimicrobial agents was observed when acid stresses ET401 was exposed to a lethal alkaline pH of 12.0 and when B2023 was heat stressed at a super-optimal temperature of 45°C. In these two test conditions, the strains exhibited absolute resistance to all the tested antibiotics. When acid stressed ET401 was further exposed to a pH 12.0, the cells did not only survive this lethal level of alkalinity but also were absolutely resistant to antibiotic action. After exposure to 45°C, a very negligible ZOI was noted. However, the bacterial cells were able to tolerate all the antibiotics just producing a very minimal ZOI for a few antibiotics.

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AUTHORS' CONTRIBUTION

AE conceptualized the study. AE and GSVK analyzed, interpreted data and wrote the manuscript. AE and TSK carried out bench work, generated data. The final manuscript was read and approved by all authors.

REFERENCES

1. Larson E. Community factors in development of antibiotic resistance. Annu Rev Pub Health. 2007; 28: 435-447.
2. Gonzalez Villoria AM, Valverde Garduno V. Antibiotic-resistant *Acinetobacter baumannii* increasing success remains a challenge as a nosocomial pathogen. J Pathogen. 2016: 7318075.
3. Celenza G, Pellegrini C, Caccamo M, Segatore B, Amicosante G, Perilli M. Spread of bla (CTX-M-type) and bla(PER-2) beta-lactamasegenes in clinicalisolates from Bolivian hospitals. J Antimicrob Chemother. 2006; 57: 975-978.
4. Goossens H. European status of resistance in nosocomial infections. Chemotherapy. 2005; 51: 177-181.
5. Leeb M. Antibiotics: a shot in the arm. Nature. 2004; 431: 892-893.
6. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis. 2008; 197: 1079-1081.
7. Ward NR, Wolfe RL, Justice CA, Olson BH. The identification of gram-negative, nonfermentative bacteria from water: problems and alternative approaches to identification. Adv Appl Microbiol. 1986; 31: 293-365.
8. Hrenovic J, Durn G, Goic-Barisic I, Kovacic A. Occurrence of an environmental *Acinetobacter baumannii* strain similar to clinical isolate in Paleosol from Croatia. Appl Environ Microbiol. 2014; 89: 2860-2866.
9. Lopez-Hernandez S, Alarcon T, Lopez-Brea M. Carbapenem resistance mediated by beta-lactamases in clinical isolates of *Acinetobacter baumannii* in Spain. Eur J Clin Microbiol Infect Dis. 1998; 17: 282-285.
10. Towner KJ. *Acinetobacter*: an old friend, but a new enemy. J Hosp Infect. 2009; 73: 355-363.
11. Livermore D-M. The threat from the pink corner. Ann Med. 2003; 35: 226-234.
12. Gaynes R, Edwards JR, the National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis. 2005; 41: 848-854.
13. Garza-Gonzalez E, Llaca-Dıaz JM, Bosques-Padilla FJ, Gonzalez GM. Prevalence of multidrug-resistant bacteria at a tertiary-care teaching hospital in Mexico: special focus on *Acinetobacter baumannii*. Chemotherapy. 2010; 56: 275-279.
14. Morfín-Otero R, Alcántar-Curiel MD, Rocha MJ, Alpuche-Aranda CM, Santos-Preciado JI, Gayosso-Vázquez C, et al. *Acinetobacter baumannii* infections in a tertiary care hospital in Mexico over the past 13 years. Chemotherapy. 2013; 59: 57-65.
15. Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis. 2006; 6: 589-601.

16. Gales AC, Jones RN, Sader HS. Global assessment of the antimicrobial activity of polymyxin B against 54731 clinical isolates of Gram-negative bacilli: report from the SENTRY Antimicrobial Surveillance Programme (2001-2004). Clin Microbiol Infect. 2006; 12: 315-321.
17. Hernan RC, Karina B, Gabriela G, Marcela N, Carlos V, Angela F. Selection of colistin-resistant *Acinetobacter baumannii* isolates in post- neurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagn Microbiol Infect Dis. 2009; 65: 188-191.
18. Park YK, Choi JY, Jung SI, Park KH, Lee H, Jung DS, et al. Two distinct clones of carbapenem-resistant *Acinetobacter baumannii* isolates from Korean hospitals. Diagn Microbiol Infect Dis. 2009; 64: 389-395.
19. Wilson SJ, Knipe CJ, Zieger MJ, Gabehart KM, Goodman JE, Volk HM, Sood R. Direct costs of multidrug-resistant *Acinetobacter baumannii* in the burn unit of a public teaching hospital. Am J Infect Control. 2004; 32: 342-344.
20. Bergogne BE, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical and epidemiological features. Clin Microbiol Rev. 1996; 9: 148-165.
21. Lee NY, Lee HC, Ko NY, Chang CM, Shih HI, Wu CJ, Ko WC. Clinical and economic impact of multidrug resistance in nosocomial *Acinetobacter baumannii* bacteremia. Infect Control Hosp Epidemiol. 2007; 28: 713-719.
22. Hota B. Contamination, disinfection, and cross-colonization: are hospital surfaces reservoirs for nosocomial infection? Clin Infect Dis. 2004; 39: 1182-1189.
23. Villegas MV, Hartstein AI. *Acinetobacter* outbreaks, 1977-2000. Infect Control Hosp Epidemiol. 2003; 24: 284-295.
24. Getchell-White SI, Donowitz LG, Gröschel DH. The inanimate environment of an intensive care unit as a potential source of nosocomial bacteria: evidence for long survival of *Acinetobacter calcoaceticus*. Infect Control Hosp Epidemiol. 1989; 10: 402-407.
25. Poole K. Bacterial stress responses as determinants of antimicrobial resistance. J Anitmicrob Chemother. 2012; 196: 1-21.
26. Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother. 1991; 35: 1824-1828.
27. Morita Y, Sobel ML, Poole K. Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa:* involvement of the antibiotic-inducible PA5471 gene product. J Bacteriol. 2006; 188: 1847-1855.
28. Cirz RT, Romesberg FE. Controlling mutation: intervening in evolution as a therapeutic strategy. Crit Rev Biochem Mol Biol. 2007; 42: 341-354.
29. Dorr T, Lewis K, Vulic M. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet. 2009; 5: e1000760.
30. Ebinesh A, Kailash TV. Looking into antibiotic failure: deemed a threat, indeed not. Int J Appl Res Stud. 2016; 5.
31. Ebinesh A, Kailash TV. Bacteriophage-mediated micro biome manipulation: a novel venture in fostering infant gut health. Int J Med Biotechol Genetics. 2016; 4: 34-39.
32. Ebinesh A, Kailash TV. Horizon of new hope towards a robust infantile gut: advent of bacteriophages in tuning gut microbiome. Arch Clin Microbiol. 2016; 7: 5.
33. McMahon MAS, Xu J, Moore JE, Blair IS, McDowell DA. Environmental stress and antibiotic resistance in food related pathogens. App Environ Microbiol. 2007; 73: 211-217.
34. FaeziGhasemi M, Gazemi S. Effects of sub-lethal environmental stresses on cell-survival and antibacterial susceptibility of *Listeria monocytogenes* PTCC1297. Zahedan J Res Med Sci. 2015; 17: 1-6.
35. Al-Nabulsi AA, Osaili TM, Shaker RR, Olaimat AN, Jaradat ZW, Zain Elabedeen NA, Holley RA. Effects of osmotic pressure, acid or cold stresses on antibiotic susceptibility of *Listeria monocytogenes.* Food Microbiol. 2015; 46: 154-160.
36. Ganjian H, Nikokar I, Tieshayar A, Mostafaei A, Amirmozafari N, Kiani S. Effect of salt stress and antimicrobial drug resistance and protein profile of *Staphylococcus aureus.* Jundishapur J Microbiol. 2012; 5: 328-331.
37. Doughari JH, Ndakidemi PA, Human IS, Benade S. Effect of oxidative stress on viability and virulence of environmental *Acinetobacter hemolyticus* isolates. Sci Res Essays. 2012; 7: 504-510.
38. Performance standards for antimicrobial disc susceptibility tests, M100-S25. CLSI, Vol. 35 No. 3, Jan 2015.
39. Litake GM, Ghole VS, Niphadkar KB, Joshi SG. Phenotypic ESBL detection in *Acinetobacter baumannii*: a real challenge. Am J Infect Dis. 2015; 11: 49-53.

40. Kumar E, Usha K, Chaudhury A, Ramana BV, Gopal DV. Detection of AmpC β-lactamases production in *Acinetobacter* species by inhibitor (disk) based & modified three dimensional (enzyme extraction) methods. Indian J Med Res. 2014; 140: 688-690.
41. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol. 2002; 40: 3798-3801.
42. Manchanda V, Sanchaita S, Singh NP. Multidrugresistant *Acinetobacter*. J Glob Infect Dis. 2010; 2: 291-304.
43. Bonomo RA, Szabo D. Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin Infect Dis. 2006; 43(suppl 2): S49-56.
44. Quale J, Bratu S, Landman D, Heddurshetti R. Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. Clin Infect Dis. 2003; 37: 214-220.
45. Patra P, Klumpp S. Population dynamics of bacterial persistence. PLoS One. 2013; 8: e62814.
46. Bahram RA, Magee JT, Jackson SK. Effect of temperature on aminoglycoside binding sites in *Stenotrophomonas maltophilia*. J Antimicrob Chemother. 1997; 39: 19-24.
47. Helmann JD. The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol. 2002; 46: 47-110.
48. Cao M, Helmann JD. The *Bacillus subtilis* extracytoplasmic-function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J Bacteriol. 2004; 186: 1136-1146.
49. Bandow JE, Brotz H, Hecker M. *Bacillus subtilis* tolerance of moderate concentrations of rifampin involves the σ^B-dependent general and multiple stress response. J Bacteriol. 2002; 184: 459-467.
50. Ades SE. Control of the alternative sigma factor σ^E in *Escherichia coli*. Curr Opin Microbiol. 2004; 7: 157-162.
51. Erickson KD, Detweiler CS. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. Mol Microbiol. 2006; 62: 883-894.
52. Höller C, Witthuhn D, Janzen-Blunck B. Effect of low temperature on growth, structure and metabolism of *Campylobacter coli* SP10. Appl Environ Microbiol. 1998; 64: 581-587.
53. Al-Nabulsi AA, Osaili TM, Elabedeen NA, Jaradat ZW, Shaker RR, Kheirallah KA, et al. Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. Int J Food Microbiol. 2011; 146: 137-143.
54. Alonso-Hernando A, Capita R, Prieto M, Alonso-Calleja C. Comparison of antibiotic resistance patterns in *Listeria monocytogenes* and *Salmonella enterica* strains pre-exposed and exposed to poultry decontaminats. Food Control. 2009; 20: 1108-1111.
55. Gilbert P, Collier PJ, Brown MR. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy and stringent response. Antimicrob Agents Chemother. 1990; 34: 1865-1868.
56. Bischoff M, Berger-Bachi B. Teicoplanin stress-selected mutations increasing σ^B activity in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2001; 45: 1714-1720.
57. Morikawa K, Maruyama A, Inose Y, Higashide M, Hayashi H, Ohta T. Overexpression of sigma factor, σ^B, urges *Staphylococcus aureus* to thicken the cell wall and to resist β-lactams. Biochem Biophys Res Commun. 2001; 288: 385-389.
58. Dorel C, Lejeune P, Rodrigue A. The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? Res Microbiol. 2006; 157: 306-314.
59. Raffa RG, Raivio TL. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol Microbiol. 2002; 45: 1599-1611.
60. Batchelor E, Walthers D, Kenney LJ, Goulian M. The *Escherichia coli* CpxA-CpxR envelope stress response system regulates expression of the porins OmpF and OmpC. J Bacteriol. 2005; 187: 5723-5731.
61. Al-Mahin A, Sugimoto S, Higashi C. Improvement of multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli dnaK*. Appl Environ Microbiol. 2010; 76: 4277-4285.
62. Kato A, Groisman EA. The PhoQ/PhoP regulatory network of *Salmonella enterica*. Adv Exp Med Biol. 2008; 631: 7-21.

63. Barrow K, Kwon DH. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa.* Antimicrob Agents Chemother. 2009; 53: 5150-5154.
64. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, et al. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2011; 55: 5761-5769.
65. Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother. 2010; 54: 3372-3382.
66. Muller C, Plesiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and b-lactams in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2010; 55: 1211-1221.
67. Fraud S, Campigotto AJ, Chen Z, Poole K. The MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in chlorhexidine resistance and induction by membrane damaging agents dependent upon the AlgU stress-response sigma factor. Antimicrob Agents Chemother. 2008; 52: 4478-4482.
68. Velkov VV. How environmental factors regulate mutagenesis and gene transfer in microorganisms. J Biosci. 1999; 24: 529-559.
69. Macia MD, Blanquer D, Togores B, Sauleda J, Perez JL, Oliver A. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob Agents Chemother. 2005; 49: 3382-3386.
70. Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother. 2000; 44: 1771-1777.
71. Rowan NJ. Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. Trends Food Sci Technol. 1999; 10: 261-270.
72. Ebinesh A. Conspiracy of domestic microenvironment, bacterial stress response and directed mutagenesis towards antimicrobial resistance: lessons for health care. J Infect Dis Med Microbiol. 2017; 1: 1-3.
73. Harms A, Maisonneuve E, Gerdes A. Mechanisms of bacterial persistence during stress and antibiotic exposure. Science. 2016; 354(6318): aaf4268.
74. Ebinesh A. Bacterial stress response and cross resistance to antibiotics in the light of natural selection. J Infect Dis Immune Ther. 2016; 1: 1.

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RESEARCH ARTICLE

Orthodontic anomalies in mixed dentition

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ABSTRACT

Aim: To estimate the frequency of orthodontic anomalies in the period of mixed dentition in children, and to highlight the importance of prompt treatment in order to prevent more serious disorders of the child oral health.

Materials and methods: For accomplishing the goal we've conducted systematic and control check-ups on children at the age of 9; 95 children from urban areas, and 68 from rural areas.

Results: Of 95 analyzed children from urban areas with ethnic Macedonian population, 81 have an orthodontic anomaly. Nearly half of them i.e. 39 have mobile appliances. The most common anomaly are protrusion of teeth standing at 28.3%, a deep bite with 21% and crowding with 17.2%. Of 68 analyzed children from rural areas of ethnic Albanians, 54 have an orthodontic anomaly, while only 3 children wear mobile appliances. The most common anomalies are both crowding and maxillary protrusion of teeth with an equal 27.7%. If we compare the results: the occurrence of orthodontic anomalies is slightly higher in the Macedonian population.

Conclusion: Orthodontic anomalies are diagnosed during regular systematic dental check-ups for children aged 7-13. The period of mixed dentition, which is characterized by an intense growth of the jaws, is ideal for orthodontic treatment. The parents have opportunities to inform themselves of the orthodontic anomaly of their children and promptly visit an orthodontist. With properly conducted activities on behalf of the preventive teams, we can severely decrease the percentage of children with orthodontic irregularities.

Keywords: Orthodontic anomalies; Mixed dentition; Orthodontic therapy; Pedodoncy.

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INTRODUCTION

The proper growth of jaws and the positioning of primary and permanent teeth is part of the oral health in

children. All functions of the oral cavity such as chewing, swallowing and talking, play a crucial role in the development of the jaws and the overall facial skeleton, as well as the correct aesthetical appearance. Properly positioned teeth and a nice smile contribute to a good social status of the individual, whereas poorly positioned teeth and jaws have a negative effect [1, 2].

Orthodontic anomalies, malocclusions, are both disorders of the growth and development of the jaws and teeth, with morphological and functional imbalance of the dentofacial system. Malocclusions don't just have an impact on teeth positioning, but also on the functions of the orofacial apparatus, and they also generate problems with the temporomandibular joint, thus increasing the predisposition of dental traumas, paradontal disorders and cavities [3-5]. Because malocclusion is not only a problem of the dental union, but rather of the whole craniofacial complex, all of this has a psychosocial reflection and impacts on the development of the each person.

The age from 6 to 12 is the period of mixed dentition. This stadium begins with the sprout of the first permanent molars, and sometimes of the lower central incisives. The correct intercuspidation is achieved when the mesiobuccal tuber of the first upper molar lies in the buccal cavity of the first lower molar [1]. The upper central incisives overlap the lower both horizontally and vertically for 2 mm. Every normal child forms their own model of normal occlusion [1, 6].

There is significant number of etiological factors that contribute to many orthodontic anomalies. For an easier interpretation of the complex effects and their causes, they can be divided into general factors (genetics, disorders, malnutrition, endocrinal dysfunctions, congenital anomalies) and local factors (disorders, traumas, outer pressure, improper functions, bad habits, hyperdontia, hypodontia, macrodontia, microdontia, improper positioning of the teeth, labial frenulum, early extraction of teeth, persistence of primary teeth, insignificant treatment of teeth and improper orthodontic treatment) [1].

In dental literature, we have a wide range of classifications regarding malocclusions, though the most detailed classification was made by Markovic, based on morphological characteristics and it encompasses: irregularities on individual teeth, irregularities in the dental pattern, irregularities in the aspect ratio of the jaws in a sagittal, transversal and vertical ways [1]. All irregularities in the orofacial region should promptly be diagnosed and treated. In the period of mixed dentition, the orthodontic treatment assures the correction of many small anomalies [6]. The prompt diagnosis and early treatment successfully prevents the occurrence and progression of malocclusions, which is the main goal of the orthodontic preventive and interceptive, in which pedodontics are actively involved. Kingsley and his colleagues, as one of the first orthodontists in the USA, only focused on equalizing the dental pattern and correction of the facial proportions, and they barely focused on the details of occlusions due to the rarity of good oral health in that time [7].

According to Meshtrovik, the ideal age of the first orthodontist visit is 7, which correlates to the sprouting of the first permanent molars and incisives. He also states that in some cases orthodontic therapy may begin at the age of 9-10, with the beginning of the first invasive change of the primary molars and the sprout of premolars [3]. Proffit WR, et al, believe that orthodontic therapy have to be started at the age of 6 and to be ended by puberty of the child [8].

There have been a large number of epidemiological experiments and surveys in the world, in order to create national strategies for preventive measures and early treatment. Also, in our country, the National Strategy for Prevention and Oral Diseases in children from 0 to 14 year of age, started to be implemented in 2007 [9].

Angle (1907) is the first to highlight the huge number of various dental anomalies [1, 8]. Chievaro highlighted the irregularities in the bite of 29% of children aged 3-6 [10]. Kraus highlighted it in 52% of children living in Prague, whereas Tielmann and Thatz highlighted an occurrence of 76% in the population of Munich [1, 8, 10]. Markovic and his colleagues also highlighted the large percentage of malocclusions in milk dentition, with 52% [1].

Many have pointed out the increase in the frequency of anomalies in mixed and permanent dentition. Bikar and Tabori have confirmed 60,5% of the dental anomalies are present in children aged 8-14 [10], and Mileusnic registered 77,1%, 30 years later [1]. The first epidemiological experiments on the territory of

Skopje, in children aged 3-14, were conducted by Serafimova and her colleagues, with 2032 case studies and a confirmed frequency of malocclusions in 68% of the participants. The next territorial experiment was conducted by Bojadziev and his colleagues, who confirmed an occurrence of 59%, of which 72,4% were first class, 25,5% were distoocclusion and 2,1% were mesiocclusion [11].

The large occurrence of anomalies points out the need of education and guidance to register these anomalies, as well as other specialties. Maximal prevention of early extraction of milk teeth and other non-genetic causes of anomalies is crucial, as well as promoting the importance of good oral hygiene and educating the population about a healthy diet and regular check-ups.

It is extremely important to know the occurrence of orthodontic anomalies, in order to plan the professional potential and infrastructure of dentists, as well as to manage the resources.

The goal of this project is to register the occurrence and the type of orthodontic anomalies in children with mixed dentition, to highlight the importance of orthodontic irregularities as well as their meaning and their consequences, why the period of mixed dentition is vital and why the timely orthodontic treatment is necessary.

MATERIALS AND METHODS

In order to achieve our goal, we have conducted a dental check-up of the oral cavities of 95 children of both sexes at the age of 9, all being students of primary school ,,Vlado Tasevski", a school from an urban area with children of ethnic Macedonian descent, and 68 children from primary school ,,Naim Frasheri" together with primary school ,,Ibe Palikukja", schools from rural areas with ethnic Albanian students.

Permission for the study was obtained from the kindergarten and school authorities, who sought and obtained consent from the parents of the children concerned. Ethical approval was obtained from the Ministry of Health. World Health Organization [12] caries diagnostic criteria were followed. The DMFT, decayed, missed, or filled surfaces for permanent teeth and DMFT, decayed, missed, or filled surfaces for primary teeth were used to evaluate children dental caries experience. It was decided to use cluster sampling because it was more economical and achievable within constraints of resources and finance. All classes (fourth grades) in these schools were included in study. Recently in the Republic of Macedonia, the compulsory primary education is prolonged for one more year. The starting time for primary education has been lowered with the introduction of the nine-year primary education, which starts from the age of 6 and lasts till the age of 15.

We conducted check-ups on both groups of children, in the 4^{th} grades. During the checkups, apart from the oral hygiene we also noted all irregularities and anomalies in the positioning of the teeth and the occlusion: crowding, protrusion of teeth, progeny, crossbite, deckbiss, open bite, deep bite and we also noted other anomalies (hypodontia, hiperdontia, diastema mediana, cheilognathopalatoshisis). The classification of malocclusions was done from the aspect of children and preventive dentistry, the way a pedodontics looks at orthodontic anomalies. In the cases where more anomalies were noted on the same child, we highlighted the anomaly that was most notable.

The children from urban areas attended all check-ups with their parents, who were introduced to the state of the oral cavity of their children, the occasional occurrence of an orthodontic anomaly and the need of orthodontic therapy. Simultaneously, they received advice about dental irregularities, the importance of orthodontic therapy and the results of its manifestation.

The children from rural areas attended the check-ups usually with an elder sibling, a grandfather or a grandmother, a neighbor or school teachers. The information about the children in need of orthodontic treatment was received by various people and was also advised to visit an orthodontist.

After holding conversations with parents and other responsible individuals who accompanied the children, we received various information. Some parents were unaware of their child's orthodontic anomaly and the need of an orthodontic apparatus, some even didn't know how to visit an orthodontist, and a small number of parents were completely uninterested in the preservation of the teeth or a visit to the orthodontist. Only a small percentage of parents in the Macedonian population seriously considered the advice and their children had already visited an orthodontist on multiple occasions.

We included participants at the age of 9, because mixed dentition occurs during this period, as well as with existence of primary and permanent teeth. In the participants of this age we noticed the effect of the 3-year-long education of parents and children; to estimate the health of the oral cavity, as well as to confirm the number of patients with orthodontic anomalies, as well as the occurrence of various types of malocclusions. We received information from some parents regarding conducted.

RESULTS

Statistical data that was collected were from primary school children in the Skopje Region of the Republic of Macedonia. All children were 9 year old.

For each child following data after the conducted preventive activities and advising of the parents and other company was recorded: sex (male or female), ethnic group, area (urban or rural). In Table 1, the distribution of individuals from the urban area, Macedonian ethnic group, and the mean DMFT of permanent teeth in studied sample are given.

In Table 2, the distribution of individuals from the rural area, Albanian ethnic group, and the mean DMFT of permanent teeth in six classes is given.

In Table 3, the distribution of individuals from the urban area, Macedonian ethnic group, and the mean DMFT of primary teeth in four classes from primary school Vlado Tasevski are given.

Table 1. DMFT on permanent teeth in participants from urban areas (Macedonian children), students of primary school Vlado Tasevski.

Grade	Decayed teeth			Extracted teeth			Filled teeth			Mean DMFT		
	M	F	T	M	F	T	M	F	T	M	F	T
IVa	15	17	32	8	1	9	0	0	0	2	14	16	0.68	0.88	0.76
IVb	14	12	26	11	10	21	0	0	0	3	8	11	1.00	1.48	1.24
IV c	10	11	21	5	7	12	0	0	0	1	8	9	0.64	1.36	1.00
IVd	8	8	16	2	8	10	0	0	0	1	5	6	0.37	1.63	1.00
Total	47	48	95	26	26	52	0	0	0	7	35	42	0.68	1.32	1.00

Mean DMFT=1.00

Table 2. DMFT on permanent teeth in participants from rural areas (Albanian children): students from primary school Naim Frasheri and primary school Ibe Palikukja.

Grade	Decayed teeth			Extracted teeth			Filled teeth			Mean DMFT		
	M	F	T	M	F	T	M	F	T	M	F	T
IVa- Bu.	8	5	13	5	5	10	1	1	2	1	2	3	0.87	1.60	1.15
IVb- Bu.	9	6	15	7	7	14	3	0	3	5	0	5	1.66	1.16	1.46
IV -Cha	5	3	8	6	5	11	0	1	1	2	0	2	1.60	2.00	1.75
IV -Ar.	4	6	10	4	7	11	0	2	2	0	0	0	1.00	1.50	1.30
IV -La.	13	5	18	11	6	17	1	1	2	0	2	2	0.92	1.80	1.16
IV -Pa	3	1	4	7	2	9	0	4	4	0	0	0	2.33	6.00	3.25
Total	42	26	68	40	32	72	5	9	14	8	4	12	1.26	1.73	1.44

Legend: Bu.- v.Bukovikj, Cha-v.Chajlane, Ar.-v.Arnakia, La.-v.Laskarci, Pa-v.Panichari. Mean DMFT=1.44

Table 3. DMFT of primary teeth in participants from urban areas, Macedonian ethnic group, students from primary school Vlado Tasevski.

Grade	Decayed teeth			Extracted teeth			Filled teeth			Mean DMFT		
	M	F	T	M	F	T	M	F	T	M	F	T
IVa	15	17	32	30	35	65	12	6	18	17	19	36	3.92	3.52	3.72
IVb	14	12	26	22	14	36	12	9	21	12	9	21	3.28	2.66	3.00
IV c	10	11	21	19	18	37	4	5	9	6	18	24	2.90	3.72	3.33
IVd	8	8	16	21	20	41	7	1	8	2	8	10	3.75	3.62	3.69
Total.	47	48	95	92	87	179	35	21	56	37	54	91	3.48	3.37	3.43

Mean DMFT=3.43

Table 4. Decayed, Missed, or Filled teeth DMFT of primary teeth in participants from rural areas, Albanian ethnic group, students from primary school Naim Frasheri and primary school Ibe Palikukja.

Grade	Decayed teeth			Extracted teeth			Filled teeth			Mean DMFT		
	M	F	T	M	F	T	M	F	T	M	F	T
IVa- Bu.	8	5	13	45	36	81	16	13	29	0	3	3	7.62	10.46	8.69
IVb- Bu.	9	6	15	32	27	59	41	9	50	2	1	3	8.33	6.16	7.46
IV -Cha	5	3	8	18	10	28	25	5	30	0	0	0	8.60	5.00	7.25
IV -Ar.	4	6	10	16	20	36	14	12	26	0	0	0	7.50	5.33	6.20
IV -La.	13	5	18	39	14	53	34	14	48	2	0	2	5.76	5.60	5.72
IV -Pa	3	1	4	5	0	5	14	8	22	0	0	0	6.33	8.00	6.75
Total	42	26	68	155	107	262	144	61	205	4	4	8	7.21	6.61	6.98

Legend: Bu.- v.Bukovikj, Cha-v.Chajlane, Ar.-v.Arnakia, La.-v.Laskarci, Pa-v.Panichari. Mean DMFT=6.98

The mean value of DMFT index for the whole sample is 1.18 for the permanent teeth and mean DMFT for the primary teeth was 4.91 (mean DMFT=4.91).

After the conducted preventive activities and advising of the parents and other companions of the children, we have reached the following conclusions.

In the children from urban areas (Macedonian children): good oral hygiene was present in the majority of the children, 85.3% had orthodontic anomalies, and 48.1% of the children had previously visited an orthodontist.

In the children from rural areas we noticed a lack of oral hygiene in most children, especially in the retrocanine region. In the rural area 79.4% of the children had orthodontic anomalies, and only 5.6% of them were mobile appliances.

In Table 5 we can see the participants from urban areas (Macedonian ethnic group of children).

Properly positioned teeth were present in only 14 children (14.7%), while 81 children had anomalies (85.3%), of which 39 (48.1%) wear mobile appliances.

In Table 6 we can see the occurence of malocclusions according to the sexes and the type in participants from urban areas (Macedonian children).

According to the sex we have an equal ratio of occurrence of orthodontic anomalies, with 49.5% in males to 50.5% in females. The deep bite is twice as present in males as it is in females. According to the types of anomalies, in all children the most present is protrusion of teeth with 28.3%, with a deep bite coming in second with 21% and crowding coming in third with 17.2%.

In Table 7 we can see the participants from rural areas (Albanian children).

Table 5. Orthodontic anomalies in participants from urban areas (Macedonian ethnic group of children), students of primary school Vlado Tasevski.

Grade				Participants with proper teeth			Participants with orthodontic anomalies			Participants who wear braces			Participants who don't wear braces		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
IVa	15	17	32	1	1	2	14	16	30	9	7	16	5	9	14
IVb	14	12	26	4	0	4	11	11	22	4	7	11	6	5	11
IVc	10	11	21	3	3	6	7	8	15	3	6	9	4	2	6
IVd	8	8	16	0	2	2	8	6	14	0	3	3	8	3	11
Total.	47	48	95	8	6	14 /14.7%	40	41	81 /85.3%	16	23	39 /48.1%	23	19	42 /51.9%

Legend: m-male, f-female, t-total number.

Table 6. The occurrence of malocclusions in accordance to their type and the sex of the participants from urban areas (Macedonian children).

Types of malocclusions	Participants with orthodontic anomalies from urban areas					
	M	%	F	%	Total	%
Crowding	7	8.6	7	8.6	14	17.2
Spacing	2	2.5	2	2.5	4	5.0
Protrusion of teeth	10	12.3	13	16.0	23	28.3
Deck biss	2	2.5	3	3.7	5	6.2
Progenio	0	0	0	0	0	0
Crossbite	2	2.5	5	6.1	7	8.6
Open bite	4	5.0	2	2.5	6	7.5
Deep bite	11	13.6	6	7.4	17	21.0
Other anomalies	2	2.5	3	3.7	5	6.2
Total	40	49.5	41	50.5	81	100

Table 7. Orthodontic anomalies in participants from rural areas (Albanian children): students from primary school Naim Frasheri and primary school Ibe Palikukja.

Grade				Participants with proper teeth			Participants with orthodontic anomalies			Participants who wear braces			Participants who don't wear braces		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
IVa- Bu.	. 8	5	13	1	0	1	7	5	12	0	0	0	7	5	12
IVb- Bu.	9	6	15	2	0	2	7	6	13	1	1	2	6	5	11
IV -Cha	5	3	8	1	0	1	4	3	7	1	0	1	3	3	6
IV -Ar.	4	6	10	1	3	4	3	3	6	0	0	0	3	3	6
IV -La.	13	5	18	4	0	4	9	5	14	0	0	0	9	5	14
IV -Pa	3	1	4	1	1	2	2	0	2	0	0	0	2	0	2
Total	42	26	68	10	4	14 / 20.6%	32	22	54 /79.4%	2	1	3 /5.6%	30	21	51 /94.4%

Legend: Bu.- v.Bukovikj, Cha-v.Chajlane, Ar.-v.Arnakia, La.-v.Laskarci, Pa.-v.Panichari, m-male, f-female, t-total number.

Only 14 children have properly positioned teeth (20.6%). 54 children have an orthodontic anomaly (79.4%), of which only 3 children (5.6%) wear braces.

In Table 8 we can see the occurrence of malocclusions according to their type and the sex of participants from rural areas (Albanian ethnic group of children).

Table 8. The occurrence of malocclusions according to their type and the sex of participants from rural areas (Albanian ethnic group of children).

Types of malocclusions	Participants with orthodontic anomalies from rural areas					
	M	%	F	%	Total	%
Crowding	6	11.0	9	16.7	15	27.7
Spacing	0	0	2	3.7	2	3.7
Protrusion of teeth	11	20.3	4	7.4	15	27.7
Deck biss	2	3.7	1	1.9	3	5.6
Progenio	0	0	0	0	0	0
Crossbite	3	5.6	1	1.9	4	7.4
Open bite	5	9.3	4	7.4	9	16.7
Deep bite	4	7.4	1	1.9	5	9.3
Other anomalies	1	1.9	0	0	1	1.9
Total	32	59.2	22	40.8	54	100

According to the sexes the occurrence of orthodontic anomalies is 59.2% in males to 40.8% in females. According to the type of the present anomalies, the most frequent ones are crowding and protrusion of teeth with an equal 27.7%. According to the sexes, protrusion of teeth is three times more present in males than in females.

The anomalies in participants from the Macedonian children are present in a much lesser percentage as opposed to the Albanian ethnic group of children where they are more prevalent.

DISCUSSION

Etiological factors that cause various orthodontic anomalies can be divided into general and local. The general etiological factors are: genetics, general sicknesses, malnutrition, endocrinal dysfunctions and congenital anomalies. Local etiological factors are: local sicknesses, traumas, external pressure, improper functions, bad habits, hyperdontia, hypodontia, macrodontia, microdontia, improper positioning of milk teeth, improper treatment of teeth and improper orthodontic treatment, dental trauma, periodontal sicknesses and cavities [1, 3, 4, 13].

Pedodontics is in regular contact with children and that allows them to be the first ones to recognize orthodontic anomalies and to take the necessary precautions for their prevention and treatment [13].

There is a lack of data on dental caries experience in the literature with regard to the Skopje region from the Republic of Macedonia. Ambarkova et al. in 2013 conducted cross-sectional study among 15-year old children from Vardar region. The mean DMFT was 4.97, with standard deviation of 3.5 and 95% confidence interval (CI) of 4.36-5.59 [14]. Dental caries experience vs seen to be moderate (mean DMFT=3.55) among 15-year old students from Strumica city and its surrounding [15]. Also, dental caries experience was seen to be high (mean DMFT=3.47) among 12-year old children in Eastern Region of the Republic of Macedonia [16].
Dental caries experience was seen to be high (mean DMFT=6.01) among 5 year old children from the two municipalities Berovo and Pechcevo in the Eastern region of the Republic of Macedonia [17].

The data from the systematic check-ups shows that we have a high percentage of children who face orthodontic anomalies in both ethnic groups.

From the conversation with the parents and the status of the oral cavity we can infer that there are several factors that contributed to this large number of orthodontic abnormalities, some of them are: improper nutrition and bad dietary habits in children, mainly the consumption of processed food (white bread, cakes, chocolate, sweets, crisps) which led to a decrease in the chewing function of the teeth. Second is the loss of space for the permanent teeth due to untreated decayed primary teeth. It is also worth mentioning that these anomalies are also caused by other bad habits in individual children (sucking of fingers, the lower lip or other objects). And of course, a vital role is played by the negligence on the parents' behalf [18].

We can compare our data with other countries in the region or the world.

There is a study in Split, Croatia, conducted on 1600 children at the age of 7-14. Orthodontic anomalies are present in 52.87% of children of which primary compressions number take up 15.81%, secondary compressions 15.81%, protrusion of teeth takes up 13.53% and crossbite takes up 5.99%, open bite takes up 5.75%, progeny has 5.87%, and the least frequent anomaly is cheilognatopalatoshisys with 0.60% [18].

There is a study in Lithuania conducted within 1681 children at the age of 7-15 that has shown that 84.7% of participants have different types of orthodontic anomalies which correlates with our study [19].

According to the study conducted by Anne-Marie Rauten, with the goal of preventing the need of late orthodontic treatment, in Romania they conducted a study of children aged 6-9 in dental offices where it was revealed that there is a frequent early loss of milk teeth due to untreated cavities. And as a direct result of the above, there is less space for the sprout of permanent teeth, again resulting in orthodontic anomalies i.e. 10.13% of children aged 6 and 24.35% of children aged 9 are in need of early orthodontic treatment [6].

Nicholas Karaiskos conducted a study in 395 Canadian children aged 6-9 that had cavities on their milk teeth which resulted in early extraction. He came to the realization that 28% of the participating children had a chance to develop an orthodontic anomaly in the near future [20]. Morris Al concluded that only 5% of the population has orthodontic anomalies that can be regarded as a handicap [21].

In the participants from the primary school Vlado Tasevski we can see that half of the children that have orthodontic anomalies have not received any orthodontic treatment. On the other hand, the number of children that wear mobile appliances in the rural area is negligible.

When we posed the question as to why children who have orthodontic anomalies don't wear appliances, the parents gave us the following answers: some of the children just refused to wear braces, some of the parents believe their children are in no need of orthodontic therapy and that the anomaly will be resolved by itself, and some believe that it's better to wear fixed braces, thus missing the period when the anomaly can be resolved with mobile ones. In some cases the children were accompanied by someone else, so contact with the parents couldn't always be established.

All preliminary anomalies can be detected by pedodontics in the earliest stages of their occurrence, and working together with the dentists they can team up to prevent these orthodontic anomalies from progressing further, says German Ramirez-Yanez [22].

Some authors recommend that all children will neutralize their bad habits, if their primary teeth receive appropriate dental treatment when after caries cavities formation, if we control sprouting and intercuspidation of the first permanent molar, and finally if we monitor occlusion and the positioning of the teeth over the mix dentition period.

A cross-sectional survey was conducted among 2335 children aged 3-5 years from kindergartens in Shanghai, China by Zhou X, et al. [23]. Several occlusal parameters were clinically assessed, including second deciduous molar terminal plane, canine relationship, degree of overjet and overbite, anterior and posterior crossbite, and the presence or absence of physiologic spaces and crowding. All parents of subjects were asked to fill in the oral health knowledge questionnaires. The prevalence of malocclusion in primary dentition in Shanghai was 83.9%, and no significant differences were found in genders. Data showed that the prevalence of deep overbite (63.7%) was the highest in children with malocclusion, followed by deep overjet (33.9%), midline deviation (26.6%), anterior crossbite (8.0%) and anterior crowding (6.5%). They concluded that the

need for preventive orthodontic therapy is extremely desired and oral health education about malocclusion should be strengthened [23].

A large number of orthodontists regard the mixed dentition period as suitable for orthodontic therapy because in this period we can seize the growth potential in children. The goal of early orthodontic treatment is to eliminate and model irregularities and deviations from the normal skeletal development and disorders in the functional matrix.

The early orthodontic treatment guarantees a complete or partial correction of many initial discrepancies or at least reduction of their growth capacity, with the goal to positive impact growth, function, aesthetics and the psychological state of the children [24-27].

The benefits of early orthodontic treatments are:

- correction of bad habits
- reduction or elimination of abnormal swallowing and speech problems
- reduced risk of trauma on protruded frontal teeth
- an opportunity to assure proper growth of the jaws
- an opportunity to assure proper growth of permanent teeth in their right position, and assuring they have enough space
- reduced need of extraction of permanent teeth
- reduced or eliminated need of maxillofacial surgery
- an opportunity to minimize the need of further more expensive treatment
- increased confidence in children.

CONCLUSION

The period of mixed dentition is one of the most important moments for children to receive prompt orthodontic treatment and to prevent a large number of anomalies as well as to reduce the intensity of the anomaly, while at the same time reducing the number of children with anomalities. Pedodontics and dentists who work with children should focus on:

- knowing the difference between normal occlusion and malocclusion in milk mixed and permanent dentition
- discovering abnormalities, sprouts and the replacement of milk teeth with permanent teeth
- recognizing the predispository factors and to suggest the elimination of bad habits that cause orthodontic abnormalities
- taking precautionary measures to control the formation of cavities: proper brushing and preservation of milk teeth, to make proper space for the permanent teeth
- sending the children to an orthodontist who later confirms the anomaly and suggests when the treatment should begin.

Other than the pedodontics and orthodontists, a key role in the timely orthodontic treatment is also played by the parents. That's why they should be completely informed of their child's anomaly. The parents should receive information regarding the benefits of timely orthodontic treatment as well as the repercussions of late or undeceived treatment.

AUTHORS' CONTRIBUTIONS

Concept and design: GT. Acquisition, analysis and interpretation of data: GT, VA, BD, NTS. Drafting the article: GT. Collection of literature: OKI, KS. Revising it critically for important intellectual content: VA. Approved final version of the manuscript: VA. The final manuscript was read and approved by all authors.

REFERENCES

1. Markovik M, Antolič II. Orthodontics Ortodoncija. Ortodontska sekcija Srbije Beograd, 1982.
2. Mandall NA, McCord JF, Blinkhorn AS, Worthington HV, O'Brien KD. Perceived aesthetic impact of malocclusion and oral selfperceptions in 14-15-year-old Asian and Caucasian children in greater Manchester. Eur J Orthop. 2000; 22: 175-183.
3. Magdalenić-Meštrović M. Ortodontske anomalije i mogućnosti liječenja [Orthodontic malocclusion and treatment planning]. Medicus. 2010; 19(1): 75-90.
4. Ooshima T, Sugiyama K, Sobue S. Oligodontia in the primary dentition with permanent successors: report of case. ASDC J Dent Child. 1988; 55(1): 75-77.
5. Bajraktarova B. Trajna denticija, embriologija i anatomohistomorfologija [Permanent dentition, embryology and anatomy histomorphology]. SkenPoint, Skopje, 2000.
6. Rauten AM, Georgescu C, Popescu MR, Maglaviceanu CF, Popescu D, Gheorghe D, et al. Orthodontic treatment needs in mixed dentition - for children of 6 and 9 years old. Romanian J Oral Rehabilit. 2016; 8(1) :28-39.
7. Kingsley NW. A treatise on oral deformities as a branch of mechanical surgery. Gyan Books Pvt. Ltd. (Delhi, India), 1880.
8. Profitt WR, Fields HW, Ackerman JL, Sinclair PM, Thomas PM, Tulloch JFC. Contemporary orthodontic. The C.V. Mosby Company, 5th edn., 2012.
9. The National Strategy for Prevention of Oral Diseases for children of 0-14 year of the Republic of Macedonia. Ministry of Health, Department for Dental Health Care, Skopje, 2007. Revised 2010.
10. Bikar I. Osnovi ortopedije vilica [Orthodontic fundamentals]. Stomatoloska sekcija SLD, Beograd, 1962.
11. Dzipunova B: Morfoloski karakteristiki na malokluzija vtora klasa prvo oddelenie [Morphologic characteristics in Class II Division 1 maloocclusion]. Master thesis, Faculty of Dentistry, Skopje, 2000.
12. Oral Health Surveys. Basic Methods 5th edn. World Health Organization, Geneva, 2013.
13. Rajić Z, Kostić A, Jelinek E. Pediatric and preventive dentistry [Dječja i preventivna stomatologija]. Zagreb, Jugoslavenska medicinska naklada, 1985.
14. Ambarkova V, Jankulovska M, Arian D, Glavina D, Soleva A. Dental caries experience among secondary school children in the Vardar Region of the Republic of Macedonia. Oral Health Dental Manag. 2014; 13(3): 805-810.
15. Ambarkova V, Panova O. Dental caries experience among 15-years old children in the southeast region of the Republic of Macedonia. Oral Health Dental Manag. 2015; 14(6): 353-360.
16. Ambarkova V, Ivanova V. Dental caries experience among primary school children in the eastern region of the Republic of Macedonia. Oral Health Dental Manag. 2014; 13(1): 514-520.
17. Ambarkova V, Apostolova D, Gothe RM. Dental caries experience among 5 year age children from two municipalities Berovo and Pechcevo in the eastern region of the Republic of Macedonia. J Dent App. 2014; 1(4): 61-67.
18. Radica-Soric V. Analiza malokluzija izabranog uzorka školske djece [Malocclusion analysis of the selected sample of school children]. Acta Stom Croat. 1982; 16(3): 183-188.
19. Šidlauskas A, Lopatienė K. The prevalence of malocclusion among 7-15-year-old Lithuanian schoolchildren. Medicina (Kaunas). 2009; 45(2): 147-152.
20. Karaiskos N, Wiltshire WA, Odlum O, Hassard TH. Preventive and interceptive orthodontic treatment needs of an inner-city group of 6- and 9-year-old Canadian children. JCDA. 2005; 71(9): 649-649e.
21. Morris AL. Seriously handicapping orthodontic conditions. Washington, DC: National Academy of Sciences, 1977.
22. Ramirez-Yanez G. Early treatment of malocclusions. Prevention and interception in primary dentition. J Ramirez Press Calle O No.11E-71 Quinta Oriental Cucuta, Colombia, 2009.
23. Zhou X, Zhang Y, Wang Y, Zhang H, Chen L, Liu Y. Prevalence of malocclusion in 3- to 5-year-old children in Shanghai, China. Int J Environ Res Public Health. 2017; 14(3): pii: E328.
24. Keski-Nisula K, Hernesniemi R, Heiskanen M, Keski-Nisula L, Varrelae Vaasa J. Orthodontic intervention in the early mixed dentition: A prospective, controlled study on the effects of the eruption guidance appliance. Am J Orthod Dentofacial Orthop. 2008; 133(2): 254-260.

25. Baume LJ. Uniform methods for the epidemiologic assessment of malocclusion. Am J Orthod. 1974; 66: 251-272.
26. Corrucini RS. Anthropological aspects of orofacial and occlusal variations and anomalies. In: Kelly MA, Larsen CS. Advances in Dental Anthropology. New York: Wiley-Liss, 1991.
27. Seehra J, Newton JT, Dibiase AT. Interceptive orthodontic treatment in bullied adolescents and its impact on self-esteem and oral-health-related quality of life. Eur J Orthod. 2013; 35(5): 615-621.

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RESEARCH ARTICLE

Effect of age on chemical element contents in female thyroid investigated by some nuclear analytical methods

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ABSTRACT

A prevalence of thyroid dysfunction is higher in the elderly as compared to the younger population. An excess or deficiency of chemical element contents in thyroid may play an important role in goitro- and carcinogenesis of gland. The variation with age of the mass fraction of twenty chemical elements (Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn) in intact (normal) thyroid of 33 females (mean age 54.5 years, range 3.5-87) was investigated by energy dispersive X-ray fluorescent analysis and instrumental neutron activation analysis with high resolution spectrometry of short- and long-lived radionuclides. This work revealed that there is an increase in Br, Ca, Co, Fe, Rb, Sb, Se, and Zn mass fraction, as well as a decrease in Cl mass fraction in the normal thyroid of female during a lifespan. Therefore, a goitrogenic and carcinogenic effect of inadequate Br, Ca, Co, Fe, Rb, Sb, Se, and Zn level in the thyroid of old females with increasing age may be assumed.

Keywords: Thyroid; Chemical elements; Age-related changes; X-ray fluorescent analysis; Neutron activation analysis.

INTRODUCTION

The endocrine organs, including the thyroid gland, undergo important functional changes during aging and a prevalence of thyroid dysfunction is higher in the elderly as compared to the younger population [1, 2]. Advancing age is known to influence the formation of adenomatous goiter and thyroid cancer [3]. The prevalence of thyroid nodules is increased in the elderly, reaching a frequency of nearly 50% by the age of 65 [4]. Both prevalence and aggressiveness of thyroid cancer increase with age [2]. Women are affected by thyroid nodule and cancer two to five times more often than men [2-5].

Aging is characterized by progressive impairment of body functions caused by the accumulation of molecular damage in DNA, proteins and lipids, is also characterized by an increase in intracellular oxidative stress due to the progressive decrease of the intracellular reactive oxygen species (ROS) scavenging [6, 7]. Oxidative damage to cellular macromolecules which induce age-related diseases, including cancer, can also arise through overproduction of ROS and faulty antioxidant and/or DNA repair mechanisms [8].

Overproduction of ROS is associated with stress, inflammation, radiation, and some other factors, including overload of certain chemical elements, in both blood and certain tissues, or deficiency of other chemical elements with antioxidant properties [9-15]. The imbalance in the composition of chemical elements in cells, tissues and organs may cause different types of pathology. The importance of appropriate levels of many chemical elements is indisputable, due to their beneficial roles when present in specific concentration ranges, while on the other hand they can cause toxic effects with excessively high or low concentrations [12].

In our previous studies [16-24] the high mass fraction of iodine and some other chemical element were observed in intact human thyroid gland when compared with their levels in non-thyroid soft tissues of the human body. However, the age-dependence of chemical element mass fraction in thyroid of adult and, particularly, elderly females is still need to be evaluated. One valuable way to elucidate the situation is to compare the mass fractions of chemical elements in young adult (the control group) with those in older adult and geriatric thyroid. The findings of the excess or deficiency of chemical element contents in thyroid of adult and elderly females may indicate their roles in a higher prevalence of thyroid dysfunction in the elderly population.

The reliable data on chemical element mass fractions in normal geriatric thyroid is apparently extremely limited. There are multiple studies reporting chemical element content in human thyroid, using chemical techniques and instrumental methods [25-42]. However, majority of the analytical methods currently used and validated for the determination of major and trace elements in thyroid and other human organs are based on techniques requiring sample digestion. The most frequently used digestion procedures are the traditional dry ashing and high-pressure wet digestion that cause destruction of organic matter of the sample. Sample digestion is a critical step in elemental analysis and due to the risk of contamination and analytes loss, a digestion step contributes to the systematic uncontrolled analysis errors [43-45]. Moreover, only a few of the previous studies employed quality control using certified/standard reference materials (CRM/SRM) for determination of the chemical element mass fractions. Therefore, sample-nondestructive technique like energy dispersive X-ray fluorescent analysis (EDXRF) as well as instrumental neutron activation analysis with high resolution spectrometry of short- and long-lived radionuclides (INAA-SLR and INAA-LLR, respectively) combined with a quality assurance using CRM/SRM is good alternatives for multi-element determination in the samples of thyroid parenchyma.

There were three aims in this study. The primary purpose of the study was to determine reliable values for chemical element mass fractions in the normal (intact) thyroid of subjects ranging from children to elderly females using EDXRF, INAA-SLR, and INAA-LLR. The second aim was to compare the chemical mass fractions determined in thyroid gland of age group 2 (adults and elderly persons aged 41 to 87 years), with those of group 1 (from 3.5 to 40 years).The final aim was to find the correlations between age and chemical element contents.

All studies were approved by the Ethical Committee of the Medical Radiological Research Centre. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

MATERIALS AND METHODS

Samples

Samples of the human thyroid were obtained from randomly selected autopsy specimens of 33 females (European-Caucasian) aged 3.5 to 87years within 48 hours after a sudden death.All the deceased were citizens of Obninsk and had undergone routine autopsy at the Forensic Medicine Department of City Hospital, Obninsk. Subjects were divided into two age groups, group 1 with 3.5-40 years (30.9±3.1 years, M±SEM, n=11) and group 2 with 41–87 years (66.3±2.7 years, M±SEM, n=22). These groups were selected to reflect the condition of thyroid tissue in the children, teenagers, young adults and first period of adult life (group 1) and in the

second period of adult life as well as in old age (group 2). The available clinical data were reviewed for each subject. None of the subjects had a history of an intersex condition, endocrine disorder, or other chronic disease that could affect the normal development of the thyroid. None of the subjects were receiving medications or used any supplements known to affect thyroid chemical element contents. The typical causes of sudden death of most of these subjects included trauma or suicide and also acute illness (cardiac insufficiency, stroke, embolism of pulmonary artery, alcohol poisoning).

Sample preparation

All right lobes of thyroid glands were divided into two portions using a titanium scalpel [46]. One tissue portion was reviewed by an anatomical pathologist while the other was used for the chemical element content determination. A histological examination was used to control the age norm conformity as well as the unavailability of microadenomatosis and latent cancer.

After the samples intended for chemical element analysis were weighed, they were transferred to -20°C and stored until the day of transportation in the Medical Radiological Research Center, Obninsk, where all samples were freeze-dried and homogenized [47-49].

For EDXRF the pounded sample weighing about 8 mg was applied to the piece of Scotch tape serving as an adhesive fixing backing [50, 51]. To determine the contents of the elements by comparison with a known standard, aliquots of commercial, chemically pure compounds were used [52]. The microliter standards prepared from aliquots of commercially available pure compounds were placed on disks made of thin, ash-free filter papers fixed on the Scotch tape pieces and dried in a vacuum.

The sample weighing about 100 mg was used for chemical element measurement by INAA-SLR. The samples for INAA-SLR were sealed separately in thin polyethylene films washed beforehand with acetone and rectified alcohol. The sealed samples were placed in labeled polyethylene ampoules. Biological synthetic standards (BSS) prepared from phenol-formaldehyde resins were used as standards [52]. In addition to BSS, aliquots of commercially available pure compounds were also used.

The sample weighing about 50 mg was used for trace element measurement by INAA-LLR. The samples for INAA-LLR were wrapped separately in a high-purity aluminum foil washed with rectified alcohol beforehand and placed in a nitric acid-washed quartz ampoule.BSS were used as standards [52].

Certified Reference Materials

Ten subsamples of the Certified Reference Materials (CRM) IAEA H-4 (animal muscle) and IAEA HH-1 (human hair) were analyzed to estimate the precision and accuracy of results obtained by EDXRF, INAA-SLR, and INAA-LLR. In each method the CRMs subsamples were prepared and analyzed in the same way as the samples of thyroid tissue.

Instrumentation and methods

The facility for EDXRF included an annular ^{109}Cd source with an activity of 2.56 GBq, Si(Li) detector and portable multichannel analyzer combined with a PC(NUC 8100, Hungary). Its resolution was 270 eV at the 5.9 keV line of ^{55}Fe-source. The duration of the Br, Cu, Fe, Rb, Sr, and Zn measurements was 60 min. The intensity of K_α-line of Br, Cu, Fe, Rb, Sr, and Zn for samples and standards was estimated on calculation basis of the total area of the corresponding photopeak in the spectra. The trace element content was calculated by the relative way of comparing between intensities of K_α-lines for samples and standards. More details of the facility and method of analysis were presented in our previous publication [50, 51].

A horizontal channel equipped with the pneumatic rabbit system of the WWR-c research nuclear reactor (Karpov Institute of Physical Chemistry, Obninsk Branch) was used for INAA-SLR. The neutron flux in the channel was 1.7×10^{13}n cm^{-2} s^{-1}. Ampoules with thyroid tissue samples, SSB, intralaboratory-made standards,

and certified reference material were put into polyethylene rabbits and then irradiated separately for 180 s. Copper foils were used to assess neutron flux. The measurement of each sample was made twice, 1 and 120 min after irradiation. The duration of the first and second measurements was 10 and 20 min, respectively. Spectrometric measurements were performed using a coaxial 98-cm^3 Ge (Li) detector and a spectrometric unit (NUC 8100, Hungary), including a PC-coupled multichannel analyzer. Resolution of the spectrometric unit was 2.9-keV at the ^{60}Co 1,332-keV line. Details of used nuclear reactions, radionuclides, and gamma-energies were reported in our earlier publications concerning the INAA chemical element contents in human scalp hair [53].

A vertical channel of the WWR-c research nuclear reactor (Karpov Institute of Physical Chemistry, Obninsk Branch) was applied to determine the content of trace elements by INAA-LLR. The quartz ampoule with thyroid samples, standards, and certified reference material was soldered, positioned in a transport aluminum container and exposed to a 24-hour neutron irradiation in a vertical channel with a neutron flux of $1.3 \cdot 10^{13}$ $n \cdot cm^{-2} \cdot s^{-1}$. Ten days after irradiation samples were reweighed and repacked. The samples were measured for period from 10 to 30 days after irradiation. The duration of measurements was from 20 min to 10 hours subject to pulse counting rate. The gamma spectrometer included the 100 cm^3 Ge(Li) detector and a spectrometric unit (NUC 8100, Hungary), including a PC-coupled multichannel analyzer. The spectrometer provided a resolution of 1.9 keV on the ^{60}Co 1332 keV line. Details of used nuclear reactions, radionuclides, and gamma-energies were presented in our earlier publications concerning the INAA chemical element contents in human prostate and scalp hair [53, 54].

Computer programs and statistic

A dedicated computer program for INAA mode optimization was used [55]. All thyroid samples were prepared in duplicate, and mean values of chemical element contents were used in final calculation. Using Microsoft Office Excel, a summary of the statistics, including, arithmetic mean, standard deviation, standard error of mean, minimum and maximum values, median, percentiles with 0.025 and 0.975 levels was calculated for chemical element contents. The difference in the results between two age groups was evaluated by the parametric Student's t-test and non-parametric Wilcoxon-Mann-Whitney U-test. For the construction of "age - chemical element mass fraction" diagrams (including lines of trend with age) and the estimation of the Pearson correlation coefficient between age and chemical element mass fraction the Microsoft Office Excel programs were also used. To identify the trend of the age dependency of chemical element contents, we applied approximation methods using exponential, linear, polynomial, logarithmic and power function. The maximum of corresponding values of R^2 parameter, reflecting the accuracy of approximation, was used for the selection of function.

RESULTS

Table 1 indicates our data for twenty chemical elements in ten sub-samples of CRM IAEA H-4 (animal muscle) and IAEA HH-1 (human hair) in comparison with the certified values of this material.

The comparison of our results for the Br, Fe, Rb, and Zn mass fractions (mg/kg, dry mass basis) in the normal thyroid of female obtained by both EDXRF and INAA methods is shown in Table 2.

Table 3 represents certain statistical parameters (arithmetic mean, standard deviation, standard error of mean, minimal and maximal values, median, percentiles with 0.025 and 0.975 levels) of the Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fractions in intact (normal) thyroid of females.

The comparison of our results with published data for the Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn contents in the human thyroid is shown in Table 4.

To estimate the effect of age on the chemical element contents we examined two age groups, described above (Table 5). In addition, the Pearson correlation coefficient between age and chemical element mass fraction was calculated (Table 6). Figure 1 shows the individual data sets for the Br, Ca, Co, Fe, Rb, Sb, Se, and Zn mass fraction in all samples of thyroid, and also lines of trend with age. Since the age dependency of these element contents was best described by a polynomial function, this approximation was reflected in Figure 1.

Table 1. EDXRF, INAA-SLR and INAA-LLR data of chemical element contents in certified reference material IAEA H-4 (animal muscle) and IAEA HH-1 (human hair) compared to certified values (mg/kg, dry mass basis).

Element	IAEA H-4 animal muscle	This work results	IAEA HH-1 human hair	This work results
Ag	-	0.033±0.008	0.19±0.06[b]	0.18±0.05
Br	4.1±1.1[a]	5.0±09	4.2±2.1[b]	3.9±1.6
Ca	188±58[b]	238±59	522±160[a]	525±42
Cl	1890±130[b]	1950±230	2265±478[a]	2210±340
Co	0.0027±0.0010[b]	0.0034±0.0008	5.97±0.42[a]	5.4±1.1
Cr	0.06±0.04[b]	0.071±0.010	0.27±0.16[b]	≤0.3
Cu	4.0±1.0[a]	3.9±1.1	10.2±3.2[a]	-
Fe	49.1±6.5[a]	47.0±1.0	23.7±3.1[a]	25.1±4.3
Hg	0.014±0.005[b]	0.015±0.004	1.70±0.09[a]	1.54±0.14
I	0.08±0.10[b]	<1.0	20.3±8.9[b]	19.1±6.2
K	15840±1440[a]	16200±3800	9.2±5.2[b]	10.7±4.0
Mg	1050±140[a]	1100±190	62.0±9.6[b]	64.7±18.6
Mn	0.52±0.08[a]	0.55±0.11	0.85±0.25[a]	0.93±0.16
Na	2060±330[a]	2190±140	12.6±4.8[b]	14.0±2.7
Rb	18.7±3.5[a]	22±4	0.94±0.09[b]	0.89±0.17
Sb	0.0056±0.0031[b]	0.0061±0.0021	0.031±0.010[b]	0.033±0.009
Sc	0.0059±0.0034[b]	0.0015±0.0009	-	-
Se	0.28±0.08[a]	0.281±0.014	0.35±0.02[a]	0.37±0.08
Sr	-	<1	0.82±0.16[b]	1.24±0.57
Zn	86.3±11.5[a]	91±2	174±9[a]	173±17

M – arithmetic mean, SD – standard deviation, a – certified values, b – information values.

Table 2. Comparison of the mean values (M±SEM) of the chemical element mass fractions (mg/kg, dry mass basis) in the normal thyroid of female obtained by both EDXRF and INAA methods.

Element	EDXRF (1)	INAA (2)	Δ=[(M1 – M2)/M1] ·100%
Br	20.4±2.6	22.4±3.2	-9.8
Fe	223±21	232±22	-4.0
Rb	6.64±0.48	6.16±0.48	7.2
Zn	89.0±8.4	85.7±7.4	3.7

M – arithmetic mean, SEM – standard error of mean.

Table 3. Some statistical parameters of Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, J, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fraction (mg/kg, dry mass basis) in the normal thyroid of female.

Gender	El	M	SD	SEM	Min	Max	Median	P 0.025	P 0.975
Females n=33	Ag	0.0140	0.0093	0.0020	0.0012	0.0331	0.0130	0.0021	0.0321
	Br	20.6	14.3	2.7	3.10	54.1	16.3	4.86	52.2
	Ca	1663	970	198	461	3640	1170	670	3600
	Cl	3317	1480	290	1200	6000	3375	1386	5906
	Co	0.0505	0.0322	0.0064	0.0170	0.140	0.0405	0.0183	0.130
	Cr	0.573	0.246	0.049	0.290	1.22	0.488	0.303	1.11
	Cu	4.18	1.72	0.43	0.50	6.50	4.05	1.18	6.50
	Fe	228	105	21	74.0	512	191	87.2	422
	Hg	0.0329	0.0246	0.0051	0.0065	0.100	0.0263	0.0079	0.100
	I	1956	1199	219	114	5061	1562	309	4662
	K	5395	3245	723	1740	13700	4835	2120	13230
	Mg	212	97	24	66.0	364	215	67.5	356
	Mn	1.50	0.84	0.22	0.550	4.18	1.37	0.603	3.41
	Na	6421	1721	320	3800	10450	6700	4122	9924
	Rb	6.40	2.33	0.46	1.66	12.8	6.38	2.87	10.8
	Sb	0.116	0.063	0.012	0.0115	0.248	0.108	0.0183	0.247
	Sc	0.0042	0.0040	0.0012	0.0002	0.0143	0.0032	0.0003	0.0124
	Se	2.22	1.19	0.23	0.439	5.32	2.07	0.773	4.85
	Sr	4.67	3.11	0.78	0.65	10.9	4.40	0.82	10.8
	Zn	87.4	38.7	7.58	7.10	166	83.5	23.0	156

El – element, M – arithmetic mean, SD – standard deviation, SEM – standard error of mean, Min – minimum value, Max – maximum value, P 0.025 – percentile with 0.025 level, P 0.975 – percentile with 0.975 level.

Table 4. Median, minimum and maximum value of means Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, J, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn contents in the normal thyroid according to data from the literature in comparison with our results (mg/kg, dry mass basis).

El	Published data [Reference]			This work
	Median of means (n)*	Minimum of means M or M±SD, (n)**	Maximum of means M or M±SD, (n)**	M±SD
Ag	0.25 (12)	0.000784 (16) [25]	1.20±1.24 (105) [26]	0.014±0.009
Br	18.1 (11)	5.12 (44) [25]	284±44 (14) [27]	21±14
Ca	1600 (17)	840±240 (10) [28]	3800±320 (29) [28]	1663±970
Cl	6800 (5)	804±80 (4) [29]	8000 (-) [30]	3317±1480
Co	0.336 (17)	0.026±0.031 (46) [31]	70.4±40.8 (14) [27]	0.051±0.032
Cr	0.69 (17)	0.105 (18) [32]	24.8±2.4 (4) [29]	0.57±0.25
Cu	6.1 (57)	1.42 (120) [33]	220±22 (10) [29]	4.2±1.7
Fe	252 (21)	56 (120) [33]	2444±700 (14) [27]	228±105
Hg	0.08 (13)	0.0008±0.0002 (10) [28]	396±40 (4) [29]	0.033±0.025
I	1888 (95)	159±8 (23) [34]	5772±2708 (50) [35]	1956±1199
K	4400 (17)	46.4±4.8 (4) [29]	6090 (17) [36]	5395±3245
Mg	390 (16)	3.5 (-) [37]	840±400 (14) [38]	212±97

El	Published data [Reference]			This work
	Median of means (n)*	Minimum of means M or M±SD, (n)**	Maximum of means M or M±SD, (n)**	M±SD
Mn	1.82 (36)	0.44±11 (12) [39]	69.2±7.2 (4) [29]	1.50±0.84
Na	8000 (9)	438 (-) [40]	10000±5000 (11) [38]	6421±1721
Rb	12.3 (9)	≤0.85 (29) [28]	294±191 (14) [27]	6.40±2.33
Sb	0.105 (10)	0.040±0.003 (-) [40]	4.0 (-) [41]	0.116±0.063
Sc	0.009 (4)	0.0018±0.0003 (17) [42]	0.0135±0.0045 (10) [28]	0.0042±0.0040
Se	2.61 (17)	0.95±0.08 (29) [28]	756±680 (14) [27]	2.22±1.19
Sr	0.73 (9)	0.55±0.26 (21) [32]	46.8±4.8 (4) [29]	4.7±3.1
Zn	118 (51)	32 (120) [33]	820±204 (14) [27]	87.4±38.7

El – element, M –arithmetic mean, SD – standard deviation, (n)* – number of all references, (n)** – number of samples.

Table 5. Differences between mean values (M±SEM) of Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, J, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fraction (mg/kg, dry mass basis) in the normal female thyroid of two age groups (AG).

Element	Female thyroid tissue				Ratio
	AG1 3.5-40 years n=11	AG2 41-87 years n=22	t-test $p\leq$	U-test p	AG2 to AG1
Ag	0.0143±0.0032	0.0138±0.0027	0.909	>0.05	0.97
Br	11.8±1.7	25.8±3.7	**0.0028**	**≤0.01**	2.19
Ca	1052±65	2029±276	**0.0034**	**≤0.01**	1.93
Cl	4109±544	2965±318	0.0947	>0.05	0.72
Co	0.0328±0.0042	0.0644±0.0096	**0.0076**	**≤0.01**	1.96
Cr	0.567±0.065	0.578±0.073	0.913	>0.05	1.02
Cu	4.01±0.60	4.45±0.61	0.616	>0.05	1.11
Fe	172±23	271±28	**0.0126**	**≤0.01**	1.58
Hg	0.0275±0.0046	0.0370±0.0084	0.333	>0.05	1.35
I	1876±346	2002±288	0.782	>0.05	1.07
K	5379±1101	5408±1013	0.984	>0.05	1.01
Mg	212±39	212±31	0.994	>0.05	1.00
Mn	1.43±0.13	1.57±0.46	0.772	>0.05	1.10
Na	5969±458	6025±414	0.300	>0.05	1.01
Rb	5.13±0.56	7.33±0.58	**0.0115**	**≤0.01**	1.43
Sb	0.0880±0.0096	0.136±0.019	**0.0344**	**≤0.01**	1.55
Sc	0.0026±0.0017	0.0045±0.0014	0.438	>0.05	1.73
Se	1.86±0.27	2.48±0.34	0.169	>0.05	1.33
Sr	5.29±1.12	3.63±0.86	0.262	>0.05	0.69
Zn	62.7±9.8	105.5±8.5	**0.0033**	**≤0.01**	1.68

M – arithmetic mean, SEM – standard error of mean, *t*-test - Student's *t*-test, U-test - Wilcoxon-Mann-Whitney *U*-test, Statistically significant values are in bold.

Table 6. Correlations between age (years) and chemical element mass fractions (mg/kg, dry mass basis) in the normal thyroid of female (*r* – coefficient of correlation).

Element	Ag	Br	Ca	Cl	Co	Cr	Cu	Fe	Hg	I
r	0.09	0.26	0.37[a]	-0.43[a]	0.57[b]	0.20	0.18	0.27	0.04	0.08
Element	**K**	**Mg**	**Mn**	**Na**	**Rb**	**Sb**	**Sc**	**Se**	**Sr**	**Zn**
r	-0.05	-0.20	-0.04	0.18	0.48[a]	0.47[a]	0.29	0.47[a]	-0.10	0.67[c]

Statistically significant values: [a]$p \leq 0.05$, [b]$p \leq 0.01$, [c]$p \leq 0.001$.

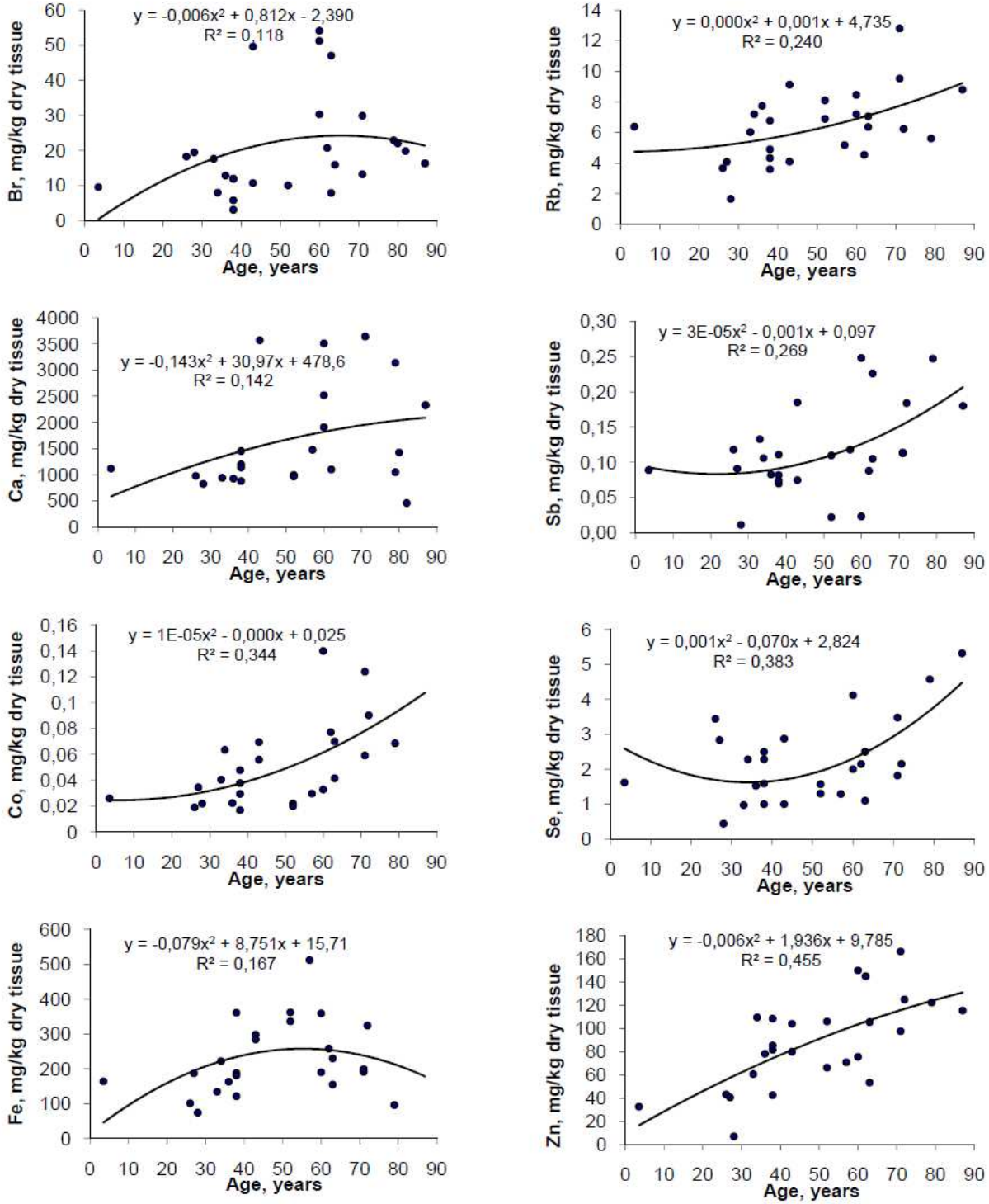

Figure 1. Data sets of individual Br, Ca, Co, Fe, Rb, Sb, Se, and Zn mass fraction values in the normal thyroid of females and their trend lines.

DISCUSSION

Precision and accuracy of results

A good agreement of our results for the Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fractions with the certified values of CRM IAEA H-4 and CRM IAEA HH-1 (Table 1) as well as the similarity of the means of the Br, Fe, Rb, and Zn mass fractions in the normal thyroid of female determined by both EDXRF and INAA methods (Table 2) demonstrates an acceptable precision and accuracy of the results obtained in the study and presented in Tables 3-6 and Figure 1.

Comparison with published data

The obtained means for Br, Ca, Cl, Cr, Cu, Fe, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, and Zn mass fraction, as shown in Table 4, agree well with the medians of mean values reported by other researches for the human thyroid, including samples received from persons who died from different non-thyroid diseases [25-42]. The obtained means for Ag and Co are an order of magnitude lower while the mean for Sr is an order of magnitude higher than the median of previously reported data. However, they are inside the ranges of previously reported data. A number of values for chemical element mass fractions were not expressed on a dry mass basis by the authors of the cited references. Hence we calculated these values using published data for water 75% [56] and ash 4.16% on dry mass basis [57] contents in thyroid of adults.

Effect of age on chemical element contents

A statistically significant age-related increase in Br, Ca, Co, Fe, Rb, Sb, and Zn mass fraction was observed in thyroid of females when two age groups were compared (Table 5). In second group of females with mean age 66.3±2.7 years the mean of Br, Ca, Co, Fe, Rb, Sb, and Zn mass fraction in thyroids were 2.19, 1.93, 1.96, 1.58, 1.43, 1.55, and 1.68 times, respectively, higher than in thyroids of the first age group (mean age 30.9±3.1 years). A statistically significant increase in Ca, Co, Rb, Sb, and Zn was confirmed by the positive Pearson's coefficient of correlation between age and mass fractions of these elements (Table 6, Figure 1). In addition to these a significant increase in Se and decrease in Cl mass fraction with increasing of age was shown by the Pearson's coefficient of correlation between age and mass fractions of the elements (Table 6, Figure 1). As per author's current information, no published data referring to age-related changes of Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fractions in human thyroid is available.

Role of chemical elements in malignant transformation of the thyroid

The Br is one of the most abundant and ubiquitous of the recognized trace elements in the biosphere. Inorganic bromide is the ionic form of bromine which exerts therapeutic as well as toxic effects. An enhanced intake of bromide could interfere with the metabolism of iodine at the whole-body level. In the thyroid gland the biological behavior of bromide is more similar to the biological behavior of iodide [58]. Therefore, a goitrogenic effect of excessive bromide level in the thyroid of old females may be assumed.

In addition to the elevated Br level, an age-related increase and excess in Ca mass fractions in thyroid tissue may contribute to harmful effects on the gland. There are good reasons for such speculations since many reviews and numerous papers raise the concern about role of Ca in the prostate, breast, lung and other organ malignant transformation [59-84]. Calcium ions Ca^{2+} are central to both cell proliferation and cell death [62]. Changes in cytosolic Ca^{2+} trigger events critical for tumorigenesis, such as cellular motility, proliferation and apoptosis [64]. An increased growth rate of cells is correlated with an increase in the intracellular calcium pool content [59, 60]. Moreover, increases in cytosolic free Ca2+ represent a ubiquitous signalling mechanism that controls a variety of cellular processes, including not only proliferation, but also cell metabolism and gene

transcription [63]. Indeed, an increased level of Ca content in the thyroid tissue of old females reflects an increase in the intracellular calcium pool. Thus, an increase of Ca content in tissue and organs with age is a key feature in etiology of many benign and malignant tumors, including thyroid goiter and cancer.

An age-related increase and excess in Co, Fe, Rb, Sb, and Zn mass fractions in thyroid tissue may contribute to harmful effects on the gland. There are good reasons for such speculations since many reviews and numerous papers raise the concern about toxicity and tumorigenesis of the metals [85-104]. Each of the metals is distinct in its primary mode of action. Moreover, there are several forms of synergistic action of the metals as a part of intracellular metabolism, during which several reactive intermediates and byproducts are created [85, 86, 91]. These reactive species are capable of potent and surprisingly selective activation of stress-signaling pathways, inhibition of DNA metabolism, repair, and formation of DNA crosslinks, which are known to contribute to the development of human cancers [86, 105, 106]. In addition to genetic damage via both oxidative and nonoxidative (DNA adducts) mechanisms, metals can also cause significant changes in DNA methylation and histone modifications, leading to alterations in gene expression [89, 90, 105]. In vitro and animal tumorigenic studies provided strong support for the idea that metals can also act as co-carcinogens in combination with nonmetal carcinogens [105].

The high level of Se content found just in the thyroid gland of old males cannot be regarded as pure chance. The seleno-protein characterized as Se-dependent glutathione peroxidase (Se-GSH-Px) is involved in protecting cells from peroxidative damage. This enzyme may reduce tissue concentration of free radicals and hydroperoxides. It is particular important for the thyroid gland, because thyroidal functions involve oxidation of iodide, which is incorporated into thyreoglobulin, the precursor of the thyroid hormones. For oxidation of iodide thyroidal cells produce a specific thyroid peroxidase using of physiologically generated hydrogen-peroxide (H_2O_2) as a cofactor [107]. It follows that the thyroid parenchyma must be continuously exposed to a physiological generation of H_2O_2 and in normal conditions must be a balance between levels of Se (as Se-GSH-Px) and H_2O_2. Thus, it might be assumed that the elevated level of Se in thyroid of old females reflects an increase in concentration of free radicals and hydroperoxides in female gland at age about 60 years and above.

All the samples were obtained from deceased citizens of Obninsk. Obninsk is the small nonindustrial city not far from Moscow in unpolluted area. None of the subjects include in this study had suffered from any systematic or chronic disorders before their sudden death. The normal state of thyroid gland was confirmed by morphological examination. Thus, our data on Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn mass fractions in intact thyroid may indicate normal values for females of urban population of the Russian Central European region.

CONCLUSION

The combination of energy dispersive X-ray fluorescent analysis and instrumental neutron activation analysis with high resolution spectrometry of short- and long-lived radionuclides is a useful analytical tool for the non-destructive determination of chemical element content in the thyroid tissue samples. This combination allows determine the mean of content for 20 chemical elements: Ag, Br, Ca, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Na, Rb, Sb, Sc, Se, Sr, and Zn.

Our data elucidate that there is a statistically significant increase in Br, Ca, Co, Fe, Rb, Sb, Se, and Zn mass fraction, as well as a decrease in Cl mass fraction in the normal thyroid of female during a lifespan. Therefore, a goitrogenic and carcinogenic effect of inadequate Br, Ca, Co, Fe, Rb, Sb, Se, and Zn level in the thyroid of old females may be assumed.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between two authors. VZ collected thyroid samples, designed the EDXRF and INAA of samples, and carried out the statistical analysis of results. SZ managed the literature searches, wrote the first draft of the manuscript, and translated the manuscript into English. Both authors read and approved the final manuscript.

REFERENCES

1. Gesing A. The thyroid gland and the process of aging. Thyroid Res. 2015; 8(Suppl 1): A8.
2. Mitrou P, Raptis SA, Dimitriadis G. Thyroid disease in older people. Maturitas. 2011; 70: 5-9.
3. Kwong N, Medici M, Angell TE, Liu X, Marqusee E, Cibas ES, et al. The influence of patient age on thyroid nodule formation, multinodularity, and thyroid cancer risk. J Clin Endocrinol Metab. 2015; 100(12): 434-440.
4. Mazzaferri E. Management of a solitary thyroid nodule. NEJM. 1993; 328: 553-559.
5. Smailyte G, Miseikyte-Kaubriene E, Kurtinaitis J. Increasing thyroid cancer incidence in Lithuania in 1978-2003. BMC Cancer. 2006; 11(6): 284.
6. Olinski R, Siomek A, Rozalski R, Gackowski D, Foksinski M, Guz J, et al. Oxidative damage to DNA and antioxidant status in aging and age-related diseases. Acta Biochim Pol. 2007; 54: 11-26.
7. Minelli A, Bellezza I, Conte C, Culig Z. Oxidative stress-related aging: A role for prostate cancer? Biochim Biophys Acta. 2009; 1795: 83-91.
8. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol. 2010; 38: 96-109.
9. Järup L. Hazards of heavy metal contamination. Br Med Bull. 2003; 68: 167-182.
10. Zaichick V, Zaichick S. Role of zinc in prostate cancerogenesis. In: Anke M, et al, eds. Mengen und Spurenelemente, 19 Arbeitstagung. Jena, Friedrich-Schiller-Universität, 1999: 104-115.
11. Zaichick V. INAA and EDXRF applications in the age dynamics assessment of Zn content and distribution in the normal human prostate. J Radioanal Nucl Chem. 2004; 262: 229-234.
12. Zaichick V. Medical elementology as a new scientific discipline. J Radioanal Nucl Chem. 2006; 269: 303-309.
13. Toyokuni S. Molecular mechanisms of oxidative stress-induced carcinogenesis: from epidemiology to oxygenomics. IUBMB Life. 2008; 60: 441-447.
14. Gupte A, Mumper RJ. Elevated copper and oxidative stress in cancer cells as a target for cancer treatment. Cancer Treat Rev. 2009; 35: 32-46.
15. Lee JD, Wu SM, Lu LY, Yang YT, Jeng SY. Cadmium concentration and metallothionein expression in prostate cancer and benign prostatic hyperplasia of humans. Taiwan Yizhi. 2009; 108: 554-559.
16. Zaichick V, Tsyb A, Vtyurin BM. Trace elements and thyroid cancer. Analyst. 1995; 120: 817-821.
17. Zaichick V, Choporov Yu. Determination of the natural level of human intra-thyroid iodine by instrumental neutron activation analysis. J Radioanal Nucl Chem. 1996; 207(1): 153-161.
18. Zaichick V, Zaichick S. Normal human intrathyroidal iodine. Sci Total Environ. 1997; 206(1): 39-56.
19. Zaichick V. Iodine excess and thyroid cancer. J Trace Elem Exp Med. 1998; 11(4): 508-509.
20. Zaichick V. In vivo and in vitro application of energy-dispersive XRF in clinical investigations: experience and the future. J Trace Elem Exp Med. 1998; 11: 509-510.
21. Zaichick V, Iljina T. Dietary iodine supplementation effect on the rat thyroid 131I blastomogenic action. In: Anke M, et al, eds. Die Bedentung der Mengen- und Spurenelemente. 18. Arbeitstangung. Jena, Friedrich-Schiller-Universität, 1998: 294-306.
22. Zaichick V, Zaichick S. Energy-dispersive X-ray fluorescence of iodine in thyroid puncture biopsy specimens. J Trace Microprobe Tech. 1999; 17: 219-232.
23. Zaichick V. Human intrathyroidal iodine in health and non-thyroidal disease. In: Abdulla M, et al, eds. New aspects of trace element research. London and Tokyo, Smith-Gordon and Nishimura, 1999: 114-119.
24. Zaichick V. Relevance of and potentiality for in vivo intrathyroidal iodine determination. In: Yasumura S, et al, eds.

In vivo body composition studies. Ann NY Acad Sci. 2000; 904: 630-632.

25. Zhu H, Wang N, Zhang Y, Wu Q, Chen R, Gao J, et al. Element contents in organs and tissues of Chinese adult men. Health Phys. 2010; 98(1):61-73.
26. Vlasova ZA. Dynamics of trace element contents in thyroid gland in connection with age and atherosclerosis [in Russian]. Proceedings of the Leningrad Institute of Doctor Advanced Training 1969; 80: 135-144.
27. Salimi J, Moosavi K, Vatankhah S, Yaghoobi A. Investigation of heavy trace elements in neoplastic and non-neoplastic human thyroid tissue: A study by proton - induced X-ray emissions. Iran J Radiat Res. 2004; 1(4): 211-216.
28. Boulyga SF, Zhuk IV, Lomonosova EM, Kanash NV, Bazhanova NN. Determination of microelements in thyroids of the inhabitants of Belarus by neutron activation analysis using the k0-method. J Radioanal Nucl Chem. 1997; 222: 11-14.
29. Reddy SB, Charles MJ, Kumar MR, Reddy BS, Anjaneyulu Ch, Raju GJN, et al. Trace elemental analysis of adenoma and carcinoma thyroid by PIXE method. Nucl Instrum Methods Phys Res B. 2002; 196: 333-339.
30. Woodard HQ, White DR. The composition of body tissues. Brit J Radiol. 1986; 708: 1209-1218.
31. Katoh Y, Sato T, Yamamoto Y. Determination of multielement concentrations in normal human organs from the Japanese. Biol Trace Elem Res. 2002; 90(1-3): 57-70.
32. Tipton IH, Cook MJ. Trace elements in human tissue. Part II. Adult subjects from the United States. Health Phys. 1963; 9: 103-145.
33. Ataulchanov IA. Age-related changes of manganese, cobalt, coper, zinc, and iron contents in the endocrine glands of females [in Russian]. Problemy Endocrinologii. 1969; 15(2): 98-102.
34. Neimark II, Timoschnikov VM. Development of carcinoma of the thyroid gland in person residing in the focus of goiter endemic [in Russian]. Problemy Endocrinilogii. 1978; 24(3): 28-32.
35. Zabala J, Carrion N, Murillo M, Quintana M, Chirinos J, Seijas N, et al. Determination of normal human intrathyroidal iodine in Caracas population. J Trace Elem Med Bio. 2009; 23: 9-14.
36. Forssen A. Inorganic elements in the human body. Ann Med Exp Biol Fenn. 1972; 50: 99-162.
37. Kortev AI, Dontsov GI, Lyascheva AP. Bio-elements in human pathology [in Russian]. Sverdlovsk, Middle-Ural Publishing-House, 1972.
38. Soman SD, Joseph KT, Raut SJ, Mulay CD, Parameshwaran M, Panday VK, et al. Studies of major and trace element content in human tissues. Health Phys. 1970; 19: 641-656.
39. Teraoka H. Distribution of 24 elements in the internal organs of normal males and the metallic workers in Japan. Arch Environ Health. 1981; 36: 155-165.
40. Boulyga SF, Becker JS, Malenchenko AF, Dietze H-J. Application of ICP-MS for multielement analysis in small sample amounts of pathological thyroid tissue. MCA. 2000; 134: 215-222.
41. Fuzailov Yu M. Reaction of human and animal thyroids in the conditions of antimony sub-region of the Fergana valley [in Russian]. In: IX All-Union Conference on Trace Elements in Biology. Kishinev, 1981: 58-62.
42. Kvicala J, Havelka J, Zeman J, Nemec J. Determination of some trace elements in the thyroid gland by INAA. J Radioanal Nucl Chem. 1991; 149: 267-274.
43. Zaichick V. Sampling, sample storage and preparation of biomaterials for INAA in clinical medicine, occupational and environmental health. In: Harmonization of health-related environmental measurements using nuclear and isotopic techniques. Vienna, IAEA, 1997: 123-133.
44. Zaichick V. Losses of chemical elements in biological samples under the dry ashing process [in Russian]. Trace Elem Med. 2004; 5: 17-22.
45. Zaichick V, Zaichick S. INAA applied to halogen (Br and I) stability in long-term storage of lyophilized biological materials. J Radioanal Nucl Chem. 2000; 244: 279-281.
46. Zaichick V, Zaichick S. Instrumental effect on the contamination of biomedical samples in the course of sampling. J Anal Chem. 1996; 51(12): 1200-1205.
47. Zaichick V, Tsislyak Yu V. A simple device for bio-sample lyophilic drying [in Russian]. Laboratornoe Delo. 1978; 2: 109-110.

48. Zaichick V, Tsislyak Yu V. A modified adsorptive and cryogenic lyophilizer for biosample concentrations [in Russian]. Laboratornoe Delo. 1981; 2: 100-101.
49. Zaichick V, Zaichick S. A search for losses of chemical elements during freeze-drying of biological materials. J Radioanal Nucl Chem. 1997; 218: 249-253.
50. Zaichick S, Zaichick V. Method and portable facility for energy-dispersive X-ray fluorescent analysis of zinc content in needle-biopsy specimens of prostate. X-Ray Spectrom. 2010; 39: 83-89.
51. Zaichick S, Zaichick V. The Br, Fe, Rb, Sr, and Zn content and interrelation in intact and morphologic normal prostate tissue of adult men investigated by energy dispersive X-ray fluorescent analysis. X-Ray Spectrom. 2011; 40: 464-469.
52. Zaichick V. Applications of synthetic reference materials in the medical Radiological Research Centre. Fresen J Anal Chem. 1995; 352: 219-223.
53. Zaichick S, Zaichick V. The effect of age and gender on 37 chemical element contents in scalp hair of healthy humans. Biol Trace Elem Res. 2010; 134: 41-54.
54. Zaichick S, Zaichick V. The effect of age on Ag, Co, Cr, Fe, Hg, Sb, Sc, Se, and Zn contents in intact human prostate investigated by neutron activation analysis. J Appl Radiat Isot. 2011; 69: 827-833.
55. Korelo AM, Zaichick V. Software to optimize the multielement INAA of medical and environmental samples [in Russian]. In: Activation analysis in environment protection. Dubna, Joint Institute for Nuclear Research, 1993: 326-332.
56. Katoh Y, Sato T, Yamamoto Y. Determination of multielement concentrations in normal human organs from the Japanese. Biol Trace Elem Res. 2002; 90: 57-70.
57. Schroeder HA, Tipton IH, Nason AP. Trace metals in man: strontium and barium. J Chron Dis. 1972; 25: 491-517.
58. Pavelka S. Radiometric determination of thyrotoxic effects of some xenobiotics. Rad Applic. 2016; 1: 155-158.
59. Legrand G, Humez S, Slomianny C, Dewailly E, Vanden Abeele F, Mariot P, et al. Ca^{2+} pools and cell growth. Evidence for sarcoendoplasmic Ca^{2+}-ATPases 2B involvement in human prostate cancer cell growth control. J Biol Chem. 2001; 276(50): 47608-47614.
60. Munarov L. Calcium signalling and control of cell proliferation by tyrosine kinase receptors. Int J Mol Med. 2002; 10: 671-676.
61. Prevarskaya N, Skryma R, Shuba Y. Ca^{2+} homeostasis in apoptotic resistance of prostate cancer cells. Biochem Biophys Res Commun. 2004; 322: 1326-1335.
62. Capiod T, Shuba Y, Skryma R, Prevarskaya N. Calcium signalling and cancer cell growth. Calcium signalling and disease. Subcell Biochem. 2007; 45: 405-427.
63. Roderick HL, Cook SJ. Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival. Nat Rev Cancer. 2008; 8: 361-375.
64. Flourakis M, Prevarskaya N. Insights into Ca^{2+} homeostasis of advanced prostate cancer cells. Biochim Biophys Acta. 2009; 1793: 1105-1109.
65. Feng M, Grice DM, Faddy HM, Nguyen N, Leitch S, Wang Y, et al. Store-independent activation of Orai1 by SPCA2 in mammary tumors. Cell. 2010; 143: 84-98.
66. Yang H, Zhang Q, He J, Lu W. Regulation of calcium signaling in lung cancer. J Thorac Dis. 2010; 2(1): 52-56.
67. McAndrew D, Grice DM, Peters AA, Davis FM, Stewart T, Rice M, et al. ORAI1-mediated calcium influx in lactation and in breast cancer. Mol Cancer Ther. 2011; 10(3): 448-460.
68. Zaichick S, Zaichick V. INAA application in the age dynamics assessment of Br, Ca, Cl, K, Mg, Mn, and Na content in the normal human prostate. J Radioanal Nucl Chem. 2011; 288: 197-202.
69. Zaichick V, Nosenko S, Moskvina I. The effect of age on 12 chemical element contents in intact prostate of adult men investigated by inductively coupled plasma atomic emission spectrometry. Biol Trace Elem Res. 2012; 147: 49-58.
70. Feng MY, Rao R. New insights into store-independent Ca($^{2+}$) entry: secretory pathway calcium ATPase 2 in normal physiology and cancer. Int J Oral Sci. 2013; 5: 71-74.
71. Zaichick V, Zaichick S. INAA application in the assessment of chemical element mass fractions in adult and geriatric prostate glands. J Appl Radiat Isot. 2014; 90: 62-73.

72. Zaichick V, Zaichick S. Determination of trace elements in adults and geriatric prostate combining neutron activation with inductively coupled plasma atomic emission spectrometry. Open J Biochem. 2014; 1: 16-33.
73. Pavithra V, Sathisha TG, Kasturi K, Mallika DS, Amos SJ, Ragunatha S, et al. Serum levels of metal ions in female patients with breast cancer. J Clin Diagn Res. 2015; 9: BC25-BC27.
74. Zaichick V, Zaichick S, Davydov G. Differences between chemical element contents in hyperplastic and nonhyperplastic prostate glands investigated by neutron activation analysis. Biol Trace Elem Res. 2015; 164: 25-35.
75. Zaichick S, Zaichick V. Prostatic tissue level of some androgen dependent and independent trace elements in patients with benign prostatic hyperplasia. Androl Gynecol Curr Res. 2015; 3: 3.
76. Zaichick V, Zaichick S. The bromine, calcium, potassium, magnesium, manganese, and sodium contents in adenocarcinoma of human prostate gland. JHOR. 2016; 2: 1-12.
77. Zaichick V, Zaichick S. Variations in concentration and distribution of several androgen-dependent and -independent trace elements in nonhyperplastic prostate gland tissue throughout adulthood. J Androl Gynaecol. 2016; 4(1): 1-10.
78. Zaichick V, Zaichick S. Prostatic tissue levels of 43 trace elements in patients with prostate adenocarcinoma. CCO. 2016; 5(1): 79-94.
79. Zaichick V, Zaichick S. Levels of 43 trace elements in hyperplastic prostate tissues. BJMMR. 2016; 15(2): 1-12.
80. Zaichick V, Zaichick S. Prostatic tissue level of some major and trace elements in patients with BPH. J Nephrol Urol. 2016; 3(1): 025.
81. Zaichick V, Zaichick S. Age-related changes in concentration and histological distribution of Br, Ca, Cl, K, Mg, Mn, and Na in nonhyperplastic prostate of adults. EJBMSR. 2016; 4(2): 31-48.
82. Zaichick V, Zaichick S. Age-related changes in concentration and histological distribution of 18 chemical elements in nonhyperplastic prostate of adults. WJPMR. 2016; 2(4): 5-18.
83. Zaichick V, Zaichick S. The comparison between the contents and interrelationships of 17 chemical elements in normal and cancerous prostate gland. J Prostate Cancer. 2016; 1(1): 105.
84. Zaichick V, Zaichick S, Rossmann M. Intracellular calcium excess as one of the main factors in the etiology of prostate cancer. AIMS Mol Sci. 2016; 3(4): 635-647.
85. Sunderman FW. Mechanism of metal carcinogenesis. Biol Trace Elem Res. 1979; 1: 63-86.
86. Snow ET. Metal carcinogenesis: mechanistic implications. Pharmacol Ther. 1992; 53: 31-65.
87. Toyokuni S. Role of iron in carcinogenesis: cancer as a ferrotoxic disease. Cancer Sci. 2009; 100: 9-16.
88. Martinez-Zamudio R, Ha HC. Environmental epigenetics in metal exposure. Epigenetics. 2011; 6: 820-827.
89. Tokar EJ, Benbrahim-Tallaa L, Waalkes MP. Metal ions in human cancer development. Met Ions Life Sci. 2011; 8: 375-401.
90. Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. Heavy metal toxicity and the environment. EXS. 2012; 101: 133-164.
91. Koedrith P, Kim H, Weon JI, Seo YR. Toxicogenomic approaches for understanding molecular mechanisms of heavy metal mutagenicity and carcinogenicity. Int J Hyg Environ Health. 2013; 216: 587-598.
92. Tabrez S, Priyadarshini M, Priyamvada S, Khan MS, Na A, Zaidi SK, et al. Gene-environment interactions in heavy metal and pesticide carcinogenesis. Mutat Res. 2014; 760: 1-9.
93. Zaichick V, Zaichick S. Role of zinc in prostate cancerogenesis. In: Anke M, et al, eds. Mengen und Spurenelemente. 19. Arbeitstagung. Jena, Friedrich-Schiller-Universität, 1999: 104-115.
94. Zaichick V. INAA and EDXRF applications in the age dynamics assessment of Zn content and distribution in the normal human prostate. J Radioanal Nucl Chem. 2004; 262: 229-234.
95. Zaichick S, Zaichick V. The effect of age on Ag, Co, Cr, Fe, Hg, Sb, Sc, Se, and Zn contents in intact human prostate investigated by neutron activation analysis. J Appl Radiat Isot. 2011; 69: 827-833.
96. Zaichick S, Zaichick V, Nosenko S, Moskvina I. Mass fractions of 52 trace elements and zinc trace element content ratios in intact human prostates investigated by inductively coupled plasma mass spectrometry. Biol Trace Elem Res. 2012; 149: 171-183.
97. Zaichick V, Zaichick S. Use of INAA and ICP-MS for the assessment of trace element mass fractions in adult and geriatric prostate. J Radioanal Nucl Chem. 2014; 301: 383-397.

98. Zaichick V, Zaichick S. Age-related histological and zinc content changes in adult nonhyperplastic prostate glands. Age. 2014; 36: 167-181.
99. Zaichick V, Zaichick S. Differences and relationships between morphometric parameters and zinc content in nonhyperplastic and hyperplastic prostate glands. BJMMR. 2015; 8(8): 692-706.
100. Zaichick V, Zaichick S. Trace element contents in adenocarcinoma of human prostate investigated by energy dispersive X-ray fluorescent analysis. J Adenocarcinoma. 2016; 1(1): 1-7.
101. Zaichick V, Zaichick S. Trace element contents in adenocarcinoma of the human prostate gland investigated by neutron activation analysis. CROOA. 2016; 1(1): 1-10.
102. Zaichick V, Zaichick S. Variations in concentration and histological distribution of Ag, Co, Cr, Fe, Hg, Rb, Sb, Sc, Se, and Zn in nonhyperplastic prostate gland throughout adulthood. J Cell Mol Biol. 2016; 2(1): 011.
103. Zaichick V, Zaichick S. Age-related changes in concentration and histological distribution of 54 trace elements in nonhyperplastic prostate of adults. Int Arch Urol Complic. 2016; 2(2): 019.
104. Zaichick V, Zaichick S, Wynchank S. Intracellular zinc excess as one of the main factors in the etiology of prostate cancer. J Analytic Oncol. 2016; 5(3): 124-131.
105. Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. Chem Res Toxicol. 2008; 21: 28-44.
106. Chervona Y, Arita A, Costa M. Carcinogenic metals and the epigenome: understanding the effect of nickel, arsenic, and chromium. Metallomics. 2012; 4: 619-627.
107. Aaseth J, Frey H, Glattre E, Norheim G, Ringstad J, Thomassen Y. Selenium concentrations in the human thyroid gland. Biol Trace Elem Res. 1990; 24(2-3): 147-152.