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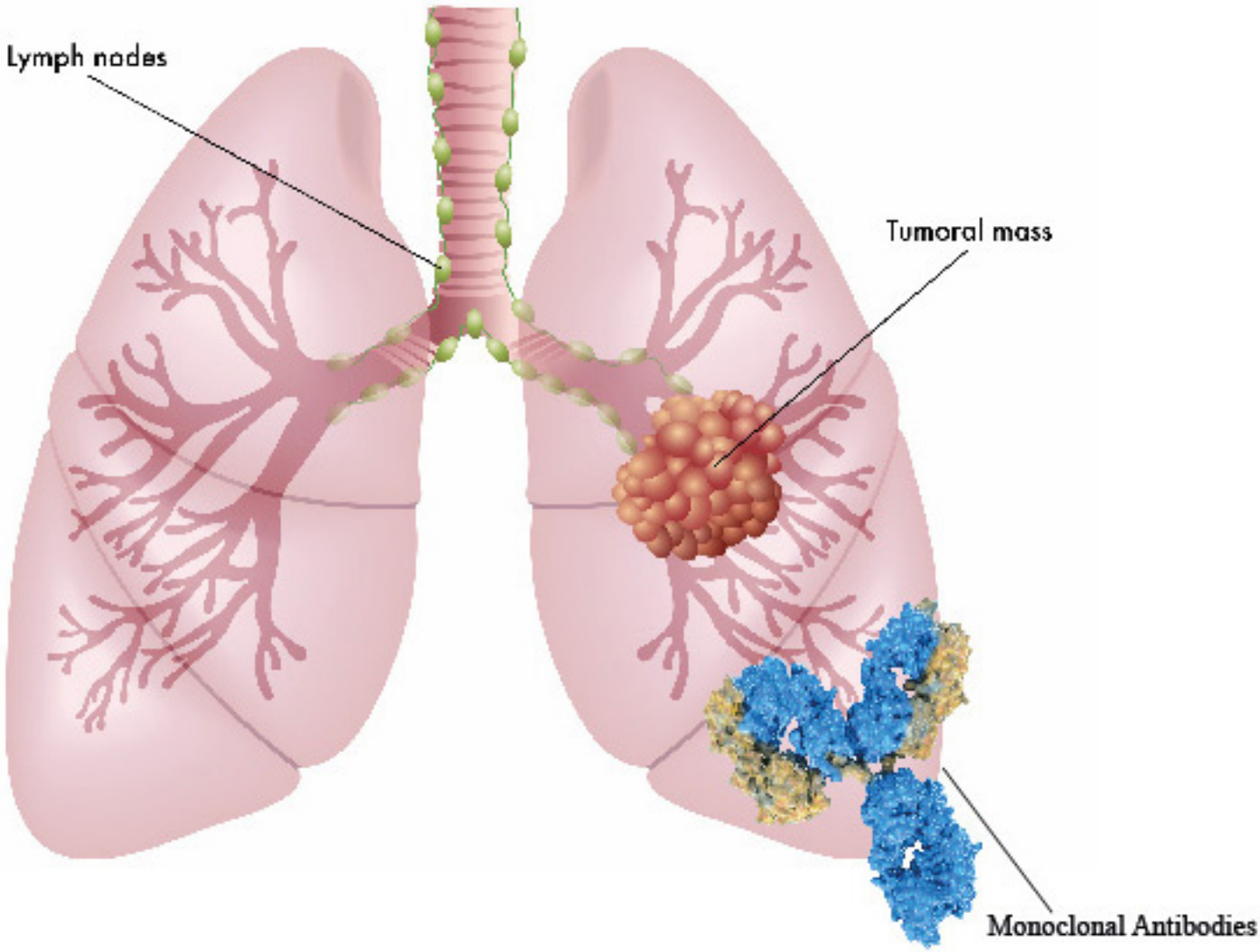
Regulations on human genome editing and stem cell research in Latin America

Non-small cell lung cancer (NSCLC)



Bioinspired systems: A new upcoming research master program at Yachay Tech University in Ecuador

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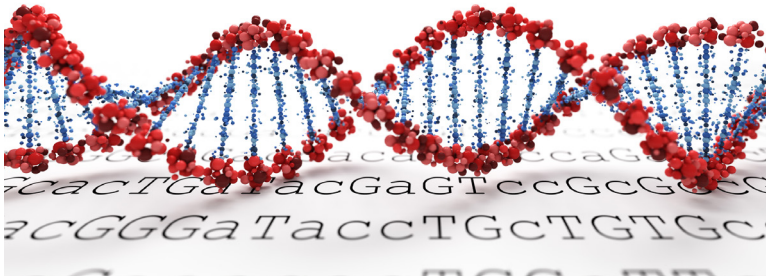

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EDITORIAL

Bioinspired systems: A new master program at Yachay Tech University in Ecuador.

Ming Ni.

DOI. 10.21931/RB/2019.04.03.1

Throughout history, people use different types of biomaterials to repair or replace damaged human tissues. Ancient Egyptians used golden wires to replace the missing teeth; Ancient Mayans used nacre or mother of pearl to achieve the same goal[1, 2]. Fast forward to the 20th century; Sir Ridley used Perspex as the material for intraocular lenses[1]. Many surgeons pioneered using synthetic polymeric membranes as the material for kidney dialyzers[1]. The modern time of biomaterials has come. Science, instead of try-and-error, has been developed to study biomaterials systematically. Professor Larry Hench, who is the inventor of Bioglass©, came up a classification of three generations of biomaterials, starting from bioinert materials to bioactive materials, to the combining of bioactive materials and resorbable materials[3]. Most recently, other scientists thought we are upgrading to the fourth generation of biomaterials, which interact with human bodies at molecular, cellular, and tissue levels. As everyone can see, our understanding of biomaterials has been evolved quickly over the past decades. Professor Buddy Ratner at the University of Washington pointed out the importance of surface science in the field of biomaterials[1]. The medical devices and implants with proper surface modification could outperform the non-surface-modified devices and implants. For example, hip and joint implants with proper textures could avoid dislodging problems. Surface chemistry, stiffness, and texture play important roles to control stem cell fate, and eventually, the performance of the medical devices and/or implants[1]. With the advance of nanotechnology, we could control and manipulate cell-biomaterial interaction at the molecular level or nanoscales. More and more evidence has shown that nanotopographic features affect cell performance. Professor Jan Boer developed a topo-chip that enables us to study topographic impacts on cells systematically and with high-throughput[5].

Bioinspired systems represent another approach to find out the optimal biomaterials as medical devices and/or implants. Bioinspired systems are nothing new to us. For example, radar has been developed as we learned from bats; lotus effect inspired us to develop self-cleaning building materials. As biomaterials, bioinspired systems have already been well studied and successfully applied in many fields. Geico's feet inspired us to develop new types of bioglues. Chameleon gave us an excellent example of a photonic crystal. Taken advantages of these bioinspired systems, we could develop superior biomaterials for medical devices and/or implants. Ecuador is a small country in South America but famous for its biodiversity: it has 10% of plants in the whole world. Plastics, as a byproduct for the oil industry, was high-tech in the 1960s. Many medical implants and devices are plastic-based, such as hip, knee, contact lenses, and many more. However, over the last few decades, plastics are everywhere, from the Arctic Ocean to the Antarctic. These plastics last for more than 50 years to completely degrade and present a bigger threat to marine life. As such, more and more researchers start to investigate natural

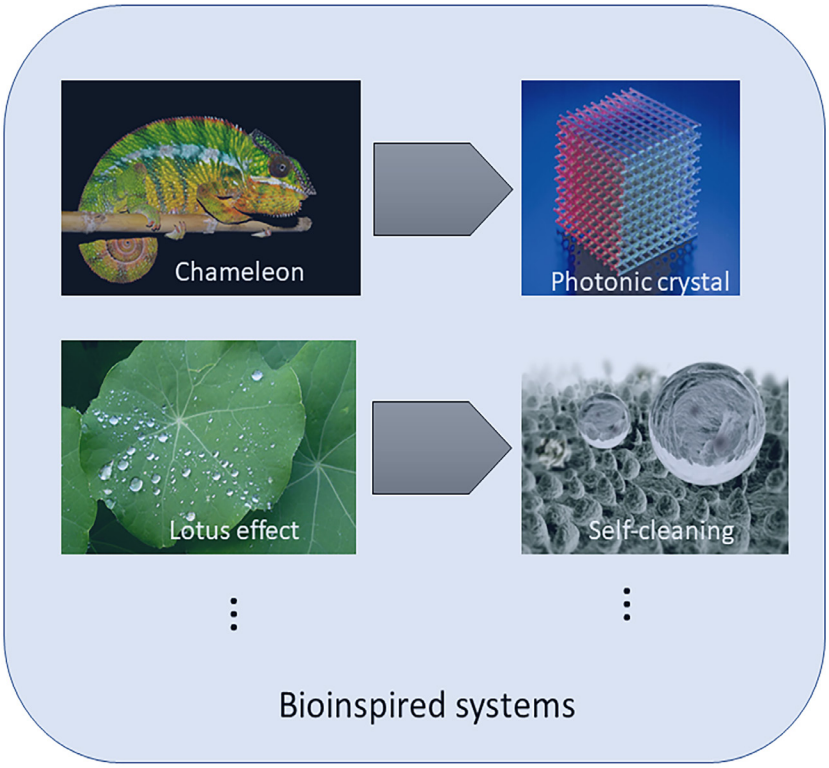

Figure 1. Mother nature gives us countless examples of bioinspired systems. For example, the skin of chameleon contains nanocrystals. By rearranging these nanocrystals, chameleon is capable to change color in response to environmental change. As another example, lotus effect inspires the development of self-cleaning materials.

plant materials to replace plastics. Now in Singapore, people use banana leaves instead of plastics for food packaging. Biodiversity in Ecuador provides us limitless arsenal for bioinspired systems and eventually a new generation of biomaterials for medical devices and/or implants. At Yachay Tech University in Ecuador, we are offering a new master program emphasis on bioinspired systems. Our aim is to educate young talents in Ecuador to use the unlimited resource of natural plant materials in Ecuador and to explore their applications in medical devices and/or implants.

Bibliographic references

1. B. D. Ratner, A. S. Hoffman, F. J. Schoen, and J. E. Lemons, Biomaterials Science: An Introduction to Materials in Medicine (Elsevier Academic Press, 2004).
2. M. Ni and B. D. Ratner, "Nacre surface transformation to hydroxyapatite in a phosphate buffer solution," Biomaterials 24(23), 4323–4331 (2003).
3. L. L. Hench, "Bioactive Glass Bone Grafts: History and Clinical Applications," in book: Handbook of Bioceramics and Biocomposites, DOI: 10.1007/978-3-319-12460-5_5 (2016).
4. C. Ning, L. Zhou and G. Tan, "Fourth-generation biomedical materials," Materials Today, 19(1), 2-3 (2017).
5. H. V. Unadkat, M. Hulsman, K. Cornelissen, B. J. Papenburg, R. K. Truckenmüller, A. E. Carpenter, M. Wessling, G. F. Post, M. Uetz, M. J. Reinders, D. Stamatialis, C. A. van Blitterswijk and J. de Boer, "An algorithm-based topographical biomaterials library to instruct cell fate," Proc Natl Acad Sci USA ,108(40):16565-16570 (2011).

LETTER TO EDITOR / CARTA AL EDITOR

Global divisions of health; bioethical principles, practices and regulations on human genome editing and stem cell research in Latin America.

Abril Saldaña Tejeda.

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Recent genetic technologies have uncovered the urgent need for global governance of health that can guarantee an ethical consensus on human genome editing and stem cell research. Although the majority of gene-transfer trials have been located in the Americas and Europe, the regulation of human somatic cell genome editing is generally limited in Latin America and largely informed by ethical concerns about genetically modified plants and animals, biopiracy, biosecurity, and use of stem cells for clinical care. Few jurisdictions in the region (i.e., Chile, Panama, Ecuador, and Colombia) have explicitly addressed somatic genome editing. Jurisdictions often address concerns regarding the use of new biotechnologies (i.e., CRISPR-Cas9) for human "enhancement" purposes rather than the prevention or cure of serious medical conditions[1].

Cases such as the 'CRISPR babies' allow us to foresee some of the most pressing ethical concerns. On November 25th, 2018, a Chinese scientist He Jiankui announced the birth of the world's first genetically engineered children, prompting a general condemnation of his actions for contravening an international scientific moratorium on all modifications of the germline nuclear genome for clinical application in human reproduction. The case of the 'CRISPR babies' has uncovered some of the potential implications of global governance of health that shapes but is also deeply dependent on national contexts. His action highlighted the need for a serious discussion about the uneven effects of the making of knowledge and technology on developed and developing countries. For instance, several scientists from top universities in the US were aware of He Jianjui's work and have been widely criticized for their silence[2]. As Sleeboom-Faulkner[3] suggests, when looking at stem cell interventions, 'idealized notions of ethics are not feasible for many stem cell scientists in low-and middle-income countries.'

To some extent, the absence of a robust regulation or clear ethical guidelines in Latin America has been the result of a general lack of consensus among scientific and non-scientific communities regarding human nature or the moral status of embryos. However, little or no regulation, in practice, means permissiveness[4, 5]. The fact that some technologies (e.g., genome transfer technologies) are being applied in countries such as Mexico to evade US rules frames the urgency of discussing global divisions relating to bioethical principles, practices and regulations[6]. The region must start consolidating multidisciplinary networks to consolidate consensus on the ethics of human genome editing and stem cell research, we must assess the implications of a geographical and discursive distance between those places where bioethical frameworks are produced (global north) and those where the actual practice of human genome editing (research and trials) could be potentially happening. We must learn from the few countries with jurisdictions in Latin America that have explicitly addressed somatic genome editing (i.e., Mexico, Panamá, Ecuador, and Chile) and assess the basic conditions for regulatory frameworks to flourish in the region and for a consensus that would care for the wellbeing of its population.

Current racialized aspects of health might complicate even further bioethical discussions in the region. For instance, the technoscientific entity of the 'Mexican genome' is being re-branded with pan-ethnic labels such as 'Latin American,' 'Latino,' and in some cases 'Hispanic.' These flexible categorizations seem to follow a commercial logic in which the intended size of the market influences whether the findings or benefits of clinical applications are presented as targeted at national or regional populations, or in the case of Latinos/Hispanics, as ambiguous populations that are hard to delimit[7]. In this context, current racialized notions of risk predisposition

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linked to genetic ancestry[8], might have implications that go beyond national borders, and that must be discussed alongside bioethical frameworks on human genome editing and stem cell research. Admixture populations are often presented as sites were racialized and medically interesting gene variants can be found, making them relevant sites for research, especially when ethical controls are minimal and this might reinforce the racialization of disease[9]. Countries of the South must not be seen as places where research and trials can take place that would be ethically impossible or difficult in the North. Similarly, southern populations must not be seen as less important in a human sense or less deserving of ethical care.

Bibliographic references

1. National Academies of Sciences, Engineering, and Medicine (NASEM). 2017. Human Genome Editing: Science, Ethics, and Governance. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/24623.
2. Kofler, Natalie. 26 February 2019. Why were scientists silent over gene-edited babies? Nature https://www.nature.com/articles/d41586-019-00662-4
3. Sleeboom-Faulkner, M. E. (2016:76). The large grey area between 'bona fide'and 'rogue'stem cell interventions—Ethical acceptability and the need to include local variability. Technological Forecasting and Social Change, 109, 76-86.
4. Isasi, Rosario, Erika Kleiderman, and Bartha Maria Knoppers. "Editing policy to fit the genome?." Science 351.6271 (2016): 337-339.
5. Pérez Carbajal y Campuzano, Hilda. "ANÁLISIS DE LA REGULACIÓN EN MATERIA DE DONACIÓN DE ÓRGANOS EN LA REPÚBLICA MEXICANA." Revista de la Facultad de Derecho de México67.269: 199-228.
6. Darnovsky, Marcy 5/29/19 The Wrong Way to Make Policy About Heritable Genome Modification. The Hill Available at https://thehill.com/opinion/healthcare/445937-the-wrong-way-to-make-policy-about-heritable-genome-modification?
7. Vasquez, E. E., & Deister, V. G. (2019). Mexican Samples, Latino DNA: The Trajectory of a National Genome in Transnational Science. Engaging Science, Technology, and Society, 5, 107-134.
8. Saldaña-Tejeda, A., & Wade, P. (2018). Obesity, race, and the indigenous origins of health risks among Mexican mestizos. Ethnic and Racial Studies, 41(15), 2731-2749.
9. Saldaña-Tejeda, A., & Wade, P. (2019). Eugenics, epigenetics, and obesity predisposition among Mexican Mestizos. Medical Anthropology, 1-16.

RESEARCHS / INVESTIGACIÓN

Composite of chitosan and Bentonite as coagulant agents in removing turbidity from Ismailia canal as water treatment plant.

A. Marey.

DOI. 10.21931/RB/2019.04.03.3

Abstract: The use of chemical coagulants is not suitable due to health and economic considerations. In the last time, the use of natural additives that are biocompatible, are biodegradable, have low toxicity and are from renewable resources attracted the attention of many types of research due to their high ability to retain different pollutants from wastewaters. Ismaili Canal is one of the most important tributaries of the River Nile in Egypt. This study successfully proved the effectiveness of the combination of chitosan and bentonite as a coagulating agent in the (Ismailia canal) for the wastewater treatment process. Chitosan and Bentonite were known as coagulant agents, thus were used in this study. Laboratory tests were conducted to test the water quality based on turbidity values and basic drinking water parameters. Chitosan and Bentonite can be applied on (Ismailia canal) resulting in a turbidity removal 97.7% at 30 minutes. The optimal dosage of bentonite(clay) combined with chitosan(polymer) was 5 and 1mg/L at PH=7.4.

Key words: Chitosan; Bentonite; wastewater; Ismailia canal.

Introduction

There are many research studies highlight the bio sorbent ability of chitosan and their composites for the pollutants from wastewaters such as heavy metal ions, organochloride pesticides, suspended solids, turbidity, organic oxidized substances, fatty and oil impurities or textile wastewater dyes. The natural water becomes wastewater after different usage, and finally, complete the hydrological cycle. The water becomes wastewater due to population growth, urbanization, industrialization; sewage from household, institutions, hospitals, industries, etc. wastewater can be destructive for the public because it contains a variety of organic and inorganic substances, biological substances, toxic inorganic compounds and the presence of toxic materials. The natural polymers are most efficient that provide several benefits such as prolific, except for physical and chemical changes from the treated water. Chitosan is the second largest biopolymer after cellulose. Chitosan (CS) is the N-DE acetylated derivative of chitin. It is characterized by high hydrophobicity and many hydroxyls and amino groups. It is environmentally friendly biodegradable and biocompatible material[1]. CS has minimum toxicity, and it is highly available in nature. Because of its high hydrophilicity and many amino and hydroxyl groups, CS presents good compatibility with clay minerals. As known adsorbent, CS is widely used to remove heavy, transition metals and dyes from wastewaters because the amine group (-NH_2) and hydroxyl group(-OH) on the polymer chain of CS can adsorb both cationic and anionic molecules[2].

Bentonite has a strong adsorption capacity because of its large surface area and surface energy. In this study, we focused on the Bentonite pre-treatment. Bentonite used in this study were both intercalated and without treatment. By intercalating process, the interlayer of Bentonite might be wider, and the chitosan could enter into the layers of Bentonite structure. The use of bentonites can improve the size and the density of the formed flocs which can increase its flocculation rate and at the same time can promote the adsorption of organics[3] for a given level of "contaminants removal." The use of bentonites can help to reduce the required dosage of chitosan and thus, the treatment costs. The objective of this study first evaluated the efficiency of different chitosan treatments for the removal of dissolved and colloidal materials .the possible efficiency improvement on using chitosan derivatives or by combined addition of the native chitosan with anionic bentonite microparticles. Ismailia Canal was constructed in 1862 by two agreements between the Egyptian government and the Suez Canal Company for creating waterway between the Nile and the Suez Canal. Ismailia Canal is one of the most branches of the Nile River in Egypt. It is the main source of drinking and irrigation water in many cities. The canal has its inlet from the Nile at Cairo and runs directly to the east to the town of Ismailia passing the governorates of Cairo- Kalioubeya-Sharkeya and Ismailia. Ismailia Canal is one of the most important tributaries of the River Nile in Egypt .along the canal there are several sources of pollution industries in the region of Cairo as well as agricultural run-offs in the Eastern part. Measuring of a specific physic-chemical parameter in the contaminated aquatic ecosystem is important in determining its effects on the living organisms inhabiting that environment[4]. The purpose of the present work was to remove the turbidity in Ismailia Canal wastewater by the composite of Chitosan-Bentonite Coagulant.

Materials and methods

Materials

The wastewater used in this study was obtained from the Ismailia Canal (Mostourd branch): Turbidity (TSS) is (100.7) NTU, PH= (8.32), Temperature= (24.04oC). Samples of canal water were taken in a one-gallon container and transported to the laboratory.1 M NaOH, 1% acetic acid. Tools used in this study include beaker glass, stir bar, PH.

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Coagulants

Chitosan (Deacetylated chitin: poly-[1-4]-B-glucosamine). (C6H11NO4) N with a minimum 85% deactyl prepared from crab shells was obtained from ACROS ORGANICS Company. It was in the form of a pale brown powder soluble in dilute acetic acid, hydrochloric acids. With molecular weight 100.000 -300.000. The bentonite powder is prepared with chemical reaction by dissolving the 50gm bentonite to 100 ml oh HCl at 10M. The magnetic stirrer was employed to mix the solution at 300 rpm and temperature 70oC.

Preparation of Chitosan-Bentonite Composite

The most common way of preparing the chitosan/bentonite composite materials is by dissolving chitosan (CS) in an acidic (acetic, hydrochloric, formic) solution.The bentonite is first swelled in distilled water and then added to the chitosan solution followed by stirring — the PH of the resulting solution adjusted with sodium hydroxide (or) hydrochloric acid. The chitosan-bentonite beads are filtered and washed with water to remove excess sodium hydroxide (or) hydrochloric acid, then dried in the oven until the weight becomes constant, then grinding the particles[5].

In some cases, before or after the mixing of bentonite with the chitosan solution, they are cross-linking agents are used to enhancing the mechanical strength and stability of chitosan in acidic media[5].

The most favorable concentration weight ratio of the natural polymer to natural clay of bentonite is in between 1:5 to 1:20[6] to achieve a good removal; the optimum dosage needs to be studied. The term “primary coagulant” refers to the main coagulant which formed a metal salt (bentonite) added alone or being added together with the secondary coagulant as “coagulant aid”. The term “coagulant aid” refers to a polymer substance[6] anionic particles of bentonite are electrostatically attracted by the protonated amino groups of chitosan[7]. This reaction facilitates the neutralization of the anionic charges which can bind together and settle rapidly by the effect of gravity[8].the natural coagulant currently is very famous and being used in most water treatment plants. It is proven that low doses of the natural polymer of chitosan together with bentonite as coagulant aid can perform better in the removal of particulate matter and color from water[8].the chitosan coated bentonite composite was prepared by dissolving 1g of deacetylated chitosan into 100 ml of 5% hydrochloric acid — the solution in for 6 hours. Then added 5g of bentonite slowly and the mixture was stirred for 3 hours and then added 1ml of sodium hydroxide aqueous solution dropwise until PH=7 to precipitate the dissolved chitosan into bentonite. Then the mixture was vacuum filtered and dried in an oven overnight at 600C.

In this study we prepare (4 beakers) were filled with 500ml water from (Ismailia Canal).then the composite was added with different dosages and mixed at 100 rpm for 5minutes. Then reduce the speed to 50 rpm for 15 minutes. Then the mixer is turned off, and flocs are allowed to settle for 30 minutes[9].

XRD analysis

The XRD is a fast resolution technique used to identify the crystal phase of a crystalline material and can provide information about the morphology.

In most studies[10, 11, 12, 13], the prepared chitosan/bentonite composites showed different diffraction patterns from the two pure components (chitosan and bentonite). It was found that the diffraction peak of chitosan/bentonite slightly shifted to a lower angle compared to the one of raw bentonite. Also, a decrease in the intensity of this peak was observed [Figure 1]. This decrease can be attributed to a slight deformation of silicate layers and a decrease in the crystallinity caused by the interaction of bentonite with chitosan.

Surface functional group	Wavenumber (cm^{-1})		
	Bentonite	Chitosan	Composite
O-H stretching	3600	3456	3453
Amide	–	1625	1638
C-N stretching	–	1485	1503
Si – O	1044	–	1048
O – Si – O asymmetric stretching	825	–	817
Si – O, deformation and bending	709	–	715
O – Si – O asymmetric bending	503	–	510

Table 1. Functional groups of chitosan, bentonite and composite coagulants.

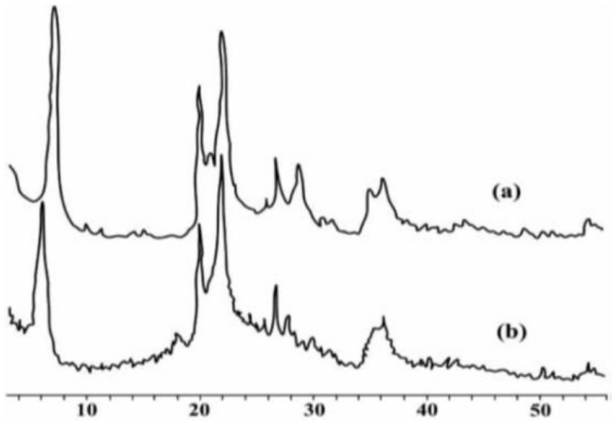

Figure 1. FXRD patterns of (a) Bentonite; (b) chitosan/bentonite beads[14].

SEM analysis

The Scanning Electron Microscope (SEM) is a common technique for characterization of the surface morphology and physical properties of the adsorbent. It is used to determine the particle shape, size distribution, and porosity. Fig (1) displays SEM pictures of raw bentonite and chitosan-bentonite composite before the adsorption process. The roughness and irregular surface morphology of the raw bentonite illustrated on the shape (a) shows a more porous material. Upon modification to form chitosan-bentonite, the sharp edges observed on the raw bentonite material are smoothened, and the distinguished gray areas have darkened. This could be an indication of a successful coating of the chitosan onto the bentonite material. And in Fig (2) shows SEM images of unmodified bentonite (a) usually show bulk agglomerated particles tightly bound together by intermolecular forces. While SEM images of chitosan usually show that its surface is tight and porous (b). but in (C) appears that bentonite is covered by chitosan which shows dense accumulation, where the uneven surface is covered by many peaks and activities, because chitosan molecules interact with bentonite on the surface.

Results and Discussion

Turbidity is an important parameter to be analyzed because it is a parameter that can be clearly seen. Increasingly clear water processing results (percent close to 100% means the better the results processing.

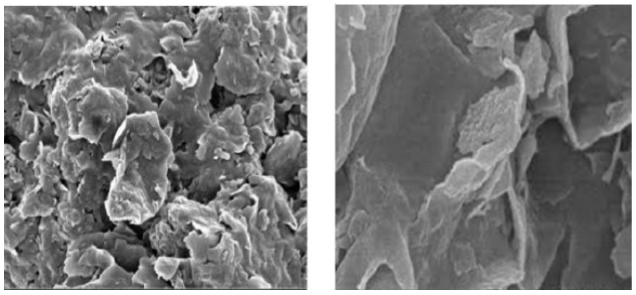

Figure 2. A Characterization of Bentonite (a) –Chitosan Composite (b). (SEM analysis)[15].

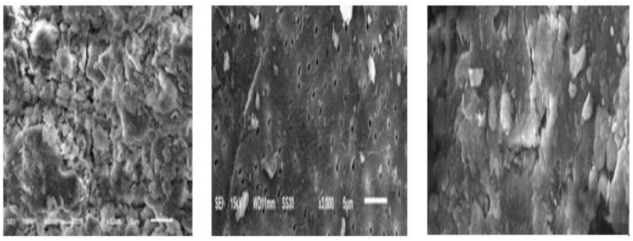

Figure 3. Example of SEM analysis of (a) bentonite; (b) chitosan and (c) complex bentonite/chitosan[16].

Effect of PH

The turbidity removals for Chitosan-Bentonite coagulation at different PH, in the experiment we adjusting the PH from 4 to 10, using the dosage of 500mg/l with mixing time 15 minutes. The difference in PH from 4 to 8 is not very much. the coagulants provide the best result of turbidity removal when the condition is acidic or slightly basic, which shows with 97.7%effeciency. The turbidity removal is decreased or distorted when the PH is over 8.this is because the positive charges on the chitosan-bentonite surface decreases as PH increases, so will contribute to the decreasing charge of both chitosan and bentonite to attract the negatively charged suspended particles.

PH	Turbidity Removal (%)
4	96
5	96.3
7	97
7.4	97.1
10	30

Table 2. Effect of PH with Turbidity Removal percentage (%).

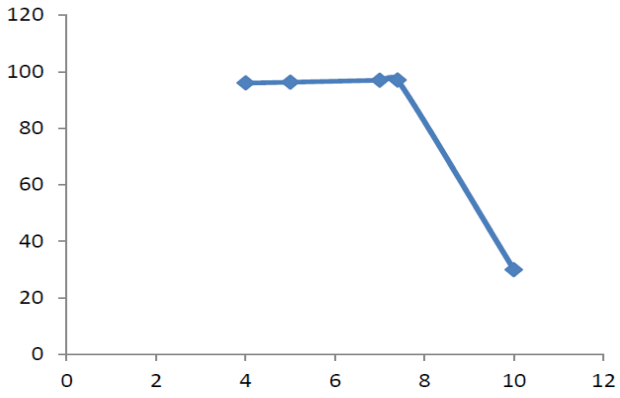

Figure 4. Shows Effect of PH with Turbidity Removal percentage (%).

Effect of contact Time (min)

The effect of mixing time with a concentration of 500mg/L with PH=7.4 with coagulant concentration (chitosan-bentonite) is 1:5. It was conducted with 120 rpm from 10 to 20 minutes and slowly from 40 to 60 and remained under settling time of 30 minutes to allow the settlement. When analyzing the data represented that 97.1% of turbidity reduction achieved in 30 minutes (optimum condition). By analyzing the reason, at lower agitation time, for example, 10 minutes, the collisions between the coagulant and suspended particles are low and will lead to lower flocculation rate[17]. Instead, if the mixing time is too long, the flocculation chains tend to stop, the small size flocs are not dense to settle down in raw water, indirectly cause the sample to be turbid again[17].

Contact time (min)	Turbidity Removal (%)
10	85
20	92,3
30	97
40	96.8
50	95
60	90

Table 3. Effect of contact time with Turbidity Removal percentage (%).

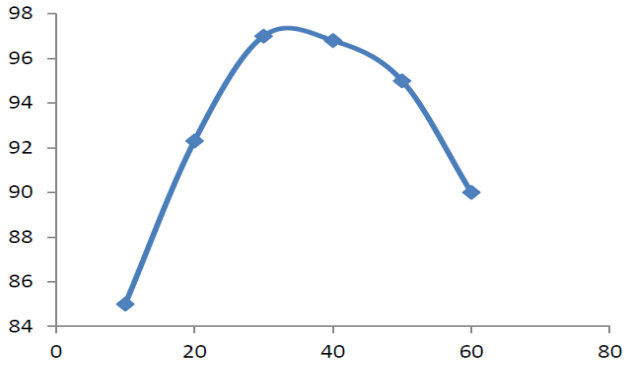

Figure 5. Shows Effect of contact time with the Turbidity Removal percentage (%).

The Effect of Bentonite: Chitosan Ratio on turbidity removal.

To study the Effect of ratio bentonite to chitosan 5:1, 5:5, and 1:5. At optimum PH=7.4 and optimum time 30 minutes. From various mixture ratios of Chitosan-Bentonite, the result shows that 5:1 is the optimum ratio with the highest turbidity removal efficiency of 97%. Effective coagulation was achieved with much lower doses of chitosan for complete neutralization of bentonites charge[18]. This means, Bentonite has less positive charge than the chitosan; one particle of chitosan is enough to attract and bind five particles of Bentonite to perform the best coagulant agent. Overdosing of chitosan causes slightly decreased in removal efficiencies, due to reversal surface charge .when the chitosan dosage exceeds the saturation of polymer bridging and the remain of chitosan destroy the polymer bridge between particles and the remaining increase in residual[19].

Composite Ratio (%) Bentonite : chitosan	Turbidity Removal (%)
5:1	97
5:5	65
1:5	30

Table 4. Effect of Bentonite:Chitosan Ratio on turbidity removal.

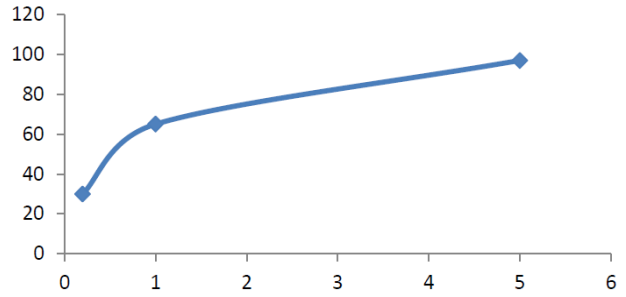

Figure 6. Effect of Bentonite: Chitosan Ratio on turbidity removal.

Conclusions

This study successfully proved the effectiveness of the combination of chitosan and bentonite as a coagulating agent in Ismailia canal as a water treatment plant. The optimal conditions were determined based on turbidity removal. In this research, the optimum conditions of the synthesis of composite chitosan-bentonite were at a weight ratio of bentonite/ chitosan 5:1 and time 30 minutes and an optimal PH of 8. Coagulation with chitosan-Bentonite successfully removed the turbidity with the efficiency of 97.7%. On these conditions, more chitosan can interact with the matrix of bentonite to form a chitosan-bentonite composite. The chitosan located on the outer surface of the bentonite and don't enter in the layer structure of bentonite.

Bibliographic references

1. Chang MY, Juang RS. Adsorption of tannic acid, humic acid, and dyes from water using the composite of chitosan and activated clay. J Colloid Interface Sci. 2004; 278: 18–25.
2. Chatterjee T., Chatterjee S., Seung Han Woo. Enhanced coagulation of bentonite particles in water by a modified chitosan biopolymer, Department of Chemical Engineering, Hanbat National University, Korea, 2009.
3. Guo J, Chen S, Liu L. Adsorption of dye from wastewater using chitosan-CTAB modified bentonites. J Colloid Interface Sci. 2012; 382: 61–66.
4. Hariani PL, Fatma F, Riyanti F. Adsorption of Phenol Pollutants from Aqueous Solution Using Ca-Bentonite/Chitosan Composite. J Mns dan Lingkung. 2015; 22: 233.
5. Hassan M. A., Tan P. L., Zainura Z. N. Coagulation and flocculation treatment of wastewater in textile industry using chitosan,Faculty of Chemical and Natural Resources Engineering, UTM, 2008.
6. Huang R, Liu Q, Zhang L, et al. Utilization of cross-linked chitosan/bentonite composite in the removal of methyl orange from aqueous solution. Water Sci Technol. 2015; 71: 174–182.
7. Huang R, Zheng D, Yang B, et al. Preparation and characterization of CTAB-HACC bentonite and its ability to adsorb phenol from aqueous solution. Water Sci Technol. 2011; 64: 286–292.
8. Mbakop S, Nthombeni N, Onyango M.S. Evaluation of Chitosan-Bentonite Composite Performance towards Remediation of Sulphate Containing Effluent. Annual Conference on Sustainable Research and Innovation. 2016; 4-6.
9. Murcott S. E, Donald R. Herleman F. Method of drinking water treatment with natural cationic polymers, Massachusetts,Institute of Technology, Cambridge, 1996.
10. Noor Z. Z., Hassan M. A, Lim S. H., Zaini U. Removal of boron from industrial wastewater by chemical precipitation using chitosan, Faculty of Chemical and Natural Resources Engineering, UTM, 2008.
11. Pan, J. R., Chih P., Huang, S. C., Chen, Ying C. Evaluation of modified chitosan biopolymer for coagulation of colloidal particals. Colloids and Surfaces A: Physicochemical and Engineering Aspects 1999; 147: 359-364.
12. Pereira FAR, Sousa KS, Cavalcanti GRS, et al. Chitosan-montmorillonite biocomposite as an adsorbent for copper (II) cations from aqueous solutions. Int J Biol Macromol [Internet]. 2013; 61: 471–478.
13. R. Nicu, R. Miranda, E. Bobu, A. Blanco, Improved efficiency of chitosans with bentonites forthe retention and drainage of pulp suspensions. Submittted to Bioresources (2013).
14. Sakaew S, Umpuch C. Removal of Azo Dyes from Aqueous Solution by using Chitosan-coated- Montmorillonite clay. 2011; 46: 172-178.
15. Shellshear, M. S. Urban stormwater treatment using chitosan. B. Eng. In civil Engineering Thesis, Faculty of Engineering and Surveying, University of Southern Queensland, 2008.
16. Syafalni, Abustan, Farhana. Raw water treatment using Bentonite-Chitosan as coagulant, Water Science & Technolgy: Water Supply, 2012; 480-487.
17. Wan Ngah WS, Ariff NFM, Hanafiah MAKM. Preparation, characterization, and environmental application of crosslinked chitosancoated bentonite for tartrazine adsorption from aqueous solutions. Water Air Soil Pollut. 2010; 206: 225–236.
18. Wan Ngah W S et al 2011 Carbohydr.Polym.83 1446-1456.
19. Wrona EJ, Cash KJ. 1996. The ecosystem approach to environmental assessment: moving from theory practice Agu.Ecosys.5, 89-97.

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RESEARCHS / INVESTIGACIÓN

Diet Composition and Feeding Strategy of Nemipterus japonicus Bloch, 1791 from Tha-baw-seik, Myanmar.

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Abstract: Food composition of *Nemipterus japonicus* was studied by analyzing about 600 specimens from Tha-baw-seik (Lat. 14° 05' and long. 98° 05' E), Myanmar during October 2016 to September 2017. Based on the stomach content analysis, the relative importance index (IRI value) was calculated, and the predator feeding strategies were conducted during the study period. This study showed that *N. japonicus* is a generally demersal carnivore, and its trophic spectrum was composed of 23 food items. Among them, algae had not been described as a food item for *N. Japonicus* in the other previous studies. Crustaceans were the most dominant diet in the guts in term of the IRI value. Also, fish items were commonly the most dominant food followed by prawn, crab, and *Acetes spp* in all length groups but sex variation had no significant food changes with their IRI value.

Key words: Relative importance index, feeding strategies, Tha-baw-seik and *Nemipterus japonicus*.

Introduction

Nemipterid fishes are a demersal fish resource, found on mud or sand bottoms, usually in schools, feeds mainly on small fishes, crustaceans, mollusks (mainly cephalopods), polychaetes and echinoderms. Nemipterid fishes have a small mouth opening, villiform teeth only on the jaws, soft gill racker with bristles, small stomach and long intestine adapted for small preys. The adaptation is also significant in preventing the escape of the prey[1].

The nemipterid fishes are one of the most important economic groups of marine fishes in Myanmar. From the commercial point of view, all species are treated as one group under the name 'Threadfin', locally known as 'ShweNgar or Ngar Ni.' It yields 3040.45 tonnes, and by percentage, it had 5.89% of the Myanmar marine fish landings in Yangon, the business city of Myanmar[2].

The study of the feeding habits of fish and other animals based upon the analysis of stomach content has become a standard practice[11]. Stomach content analysis provides important insight into fish feeding patterns, and quantitative assessment of food habits is an important aspect of fisheries management. Moreover, food and feeding habit of fish are important biological factors for selecting a group of fish for culture in ponds to avoid competition for food among themselves and live in association and to utilize all the available food[12].

It is virtually impossible to gather sufficient information about food and feeding habit of fish in their natural habitat without studying its gut contents. All knowledge on the food and feeding habit of fishes provide keys for the selection of species in culture and the importance of such information is necessary for successful fish farming. The food habit of different fish varies from month to month. This variation is due to changes in the composition of food organisms occurring at different seasons of the year.

The threadfin breams constitute an important group of commercial fishes in Tha-baw-seik, Myanmar. Tha-baw-seik is one of the large fishing landing areas in the southern part of Myanmar and it is located in Lat. 14° 05' and long. 98° 05' E. Some observations on the feeding habits of Nemipterids were made from different parts of the world; nevertheless the available information is rather meager and scantly.

In Myanmar, there was only one literature available about food and feeding habits of *N. japonicus*. Sabai Soe 2014[8] observed on gut contents of threadfin breams (*genus- nemipterus*) in myeik waters, Myanmar. In this study, seven species of *Nemipterus* were observed during the study period.

The objectives of the present study were to provide information on the diet composition and feeding strategy of *N. japonicus* in Tha-baw-seik landings mainly based on their general stomach content analysis.

Materials and methods

Sample collecting and laboratory procedures

Around 600 specimens of *N. japonicus* were collected during October 2016 to September 2017 from the fish landings at Tha-baw-seik (Lat. 14° 05' and long. 98° 05' E) (figure 1). The standard length of the samples was recorded and ranged 100-140 mm in length and 141-190 mm in length and 191-220 mm in length. The stomach of the fishes was dissected with the help of simple scissors. The stomach contents were taken into a Petri dish, and the food items were identified under the binocular microscope, and they were counted and weighted in an electronic balance.

The relative importance index (IRI)

Several methods employed for analyzing the food habits of fishes reviewed by Bowen[3] to quantify the feeding preference of fishes were applied in the present study.

For the nemipterid fishes, the relative importance index was found most suitable and hence is adopted in the present study.

The methods used in the present study are:

$$\text{Frequency of Occurrence, } O_i = \frac{J_i}{P}$$

Where J_i is the number of fish containing prey i and P is the number of fish with food in their stomach.

$$\text{Percent by number, } N_i = \frac{N_i}{\sum_{il} N_i}$$

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Where N_i is the number of food category i

$$\text{Percent by weight, } W_i = \frac{W_i}{\sum_{il}^{Q} Wi}$$

Where W_i is the weight of food category i

From these three indices, the relative importance of prey items was calculated using the Index of Relative Importance (IRI)[4]. The IRI was calculated for each prey as:

Index of relative importance, IRI = (% Ni +% Wi) %Oi; Where, Ni, Wi, and Oi represent percentages of number, weight or volume and frequency of occurrence prey respectively.

Cluster analysis

ACluster analysis was done to find out the similarities between groups. The most commonly used clustering technique is the hierarchical agglomerative method. The results of this are represented by a tree diagram or dendrogram with the X-axis representing all predators and Y-axis defining the similarity level at which the predators are fused. Bray-Curtis coefficient[14] was used to produce the dendrogram. The following formula calculated the coefficient:

$$Sjk = 100\left\{1 - \frac{\sum_{i=1}^{i''} |Y_{ij} - Y_{ik}|}{\sum_{i=1}^{i''} (Y_{ij} + Y_{ik})}\right\}$$

Where Y_{ij} represents the entry with i^{th} row and j^{th} column of the data matrix, i.e. the %IRI for the i^{th} prey in the j^{th} predator. Y_{ik} is the %IRI for the ith prey in the kth predator; 'min' stands for, the minimum of two values and ∑ represents the overall rows in the matrix.

Besides, the significance of the difference between the IRI value of male and female was tested by One Way ANOVA[13].

Predator feeding strategies

Prey- specific abundance is represented by the following equation:

$$P_i = (\sum S_i / \sum S_{ti})\ x\ 100$$

Where P_i is prey- a specific abundance of prey type I, S_i total stomach contents (number) comprised of prey I, and S_{ti} is the total stomach content of only the fish with prey item i in their stomach[5]. According to Amundsen et al5, the interpretation of the feeding strategy diagram can be obtained by examining the distribution of points along the diagonals and axes of the graph. The diagonal from the lower left to the upper right corner provides a measure of prey importance, with dominant prey at the top, and rare or unimportant prey at the lower end. The vertical axis represents the feeding strategy of the predator in terms of specialization or generalization. Predators have specialized on prey positioned in the upper part of the graph, whereas preys positioned in the lower part have been eaten only occasionally (generalization). Prey points located at the upper left of the diagram would be indicative of specialization by individual predators, and those in the upper right would represent specialization of the predator population. Prey with high specific abundance and low occurrence (upper left) were consumed by a few individuals displaying specialization, whereas prey with a low abundance and a high occurrence (lower right) were eaten occasionally by most individuals.

Results

Prey of N. japonicus in terms of frequency of occurrence (%FO), gravimetric (% W), numerical (%N), and index of relative importance (IRI).

The total 23 food items which are classified into eight general categories were included in the trophic spectrum of *N. Japonicus*. Among them, crustacean with % IRI 70.98 dominated over all other food categories and was comprised of *Acetes spp* with % IRI 25.06, crab with % IRI 13.69, prawn with % IRI 29.46, lobster with % IRI 0.038 and *squilla* with % IRI 2.74. The subsequently abundant food category was fish constituted chiefly by immature ones of *Nemipterus spp* with % IRI 0.148, *Bregmaceros spp* with % IRI 7.77, *Secutor spp* with % IRI 0.14, *Sauridas spp* with % IRI 0.115, *Platycephalus spp* with % IRI

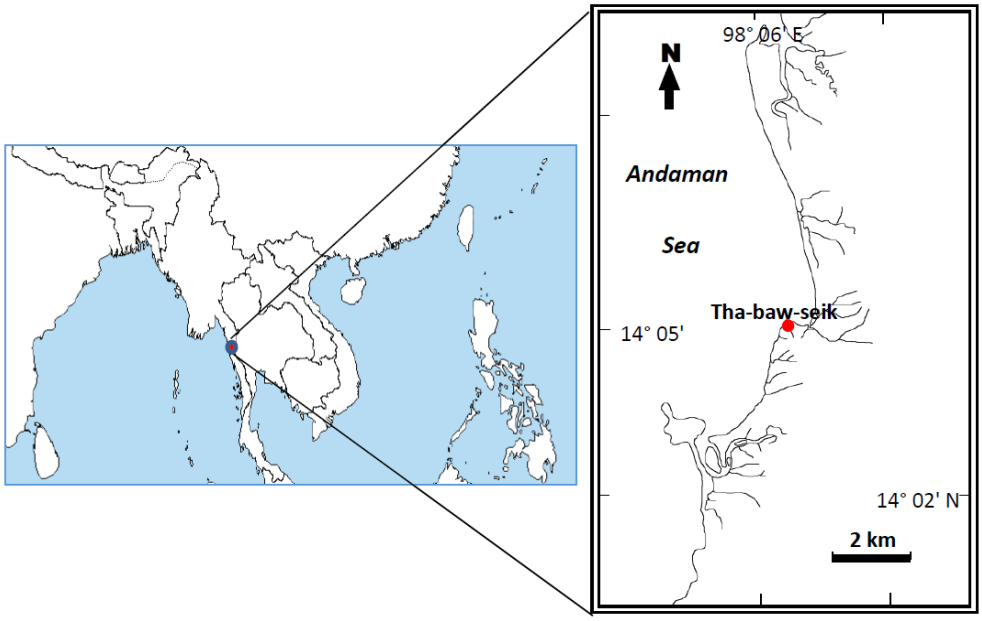

Figure 1. The map of Tha-baw-seik, myanmar.

0.332, *Cynoglossus spp* with % IRI 0.08, *Stolephorus spp* with % IRI 2.04, fish larvae with % IRI 0.38 and unidentified fishes with % IRI 3.53. The additional food items were cephalopod comprised of *Loligo* with % IRI 1.722 and *Sepia* with % IRI 0.855 and unidentified squids with % IRI 0.013, polychaetes with % IRI 0.54, nematodes with % IRI 0.892, the brittle star with % IRI 0.521, algae with % IRI 0.348 and miscellaneous items with % IRI 9.409.

In terms of frequency of occurrence, the prawn was observed 15.8% of the total stomach followed by *Acetes spp* with 14% and crab with 13%. Moreover, they were the most frequently. As for the weight, prawn with 17.17% and *Acetes spp* with 15.28% were mostly consumed by *Nemipterus japonicus* followed by *Bregmaceros spp* with 8.43% which is one item of the fish category. When considering the abundance, *Acetes spp* with 33.11% produced the largest part of the diet, followed by crab with 13.22% and prawn with 11.35% (table. 1 and fig.2

In different length groups of *N. Japonicus*, the contribution of crabs ranged from 19.08% in the smallest group and 18.73% in the medium group to 9.81% in the largest group. Therefore, there was a sharp decrease in IRI value in the largest group. Prawn was one of the major diets for every length sizes of fishes which ranged from 16.14% in the smallest groups and 15.85% in the median length groups to 19.81% in the largest groups (table 2).

Of the three different length groups, *Acetes spp* was consumed by the smallest length group of fishes the most with 14.04% of IRI value and subsequently, IRI value of this item was 15.19% in the medium length group and 10.407% in the largest length group. Fish was the most dominant food, with 30.33% in the largest length groups of *N. Japonicus* followed by 18.29% in the median length groups and 17.39% in the small length groups. The lobster was absent in the smallest length group and largest length group, but they were found only in the median length group with the lowest IRI value, 3.703%. Presence of *squilla* was the highest 11.11% in the largest length groups and lowest 4.37% in the smallest length groups. Contribution of squids ranged 10.14% in the smallest length groups, 3.65% in the median length groups and 3.703% in the largest length groups. Polychaetes ranged from 2.89% in the smallest length group to 5.097% in the median length groups, but they were absent in the largest length groups. Nematodes were only present in the smallest length groups with 4.34%. The fragments of brittle star constituted 1.449% in the smallest length groups, 3.65% in the median length groups and 3.703% in the largest length groups. Algae were only present in the median length groups with 2.43%. Miscellaneous item was found in all the length groups, which ranged from 8.69%. in the smallest groups and 7.407% in the largest groups to 9.75%.in the medium groups (table 2).

Comparison of IRI value in different sexes indicated that prawn was the most favorite food item in both sexes with 18.33% in male and 18.64% in female. Fishes were the second abundant food in both sexes with 16.66% in male and 14.705% in female. The next abundant food item was a miscellaneous item with 17.5% in male and 17.64% in female. The lobster was present in males with 0.833%. The lowest IRI values of males were lobster, and brittle star with 0.833% and the lowest IRI values of males was algae, with 0.989% (table 3).

Similarity analysis between different length groups can be found in the figure. 4. The similarity between the smallest group and the largest group had 67.93 %. These groups shared diets such as *Acetes spp.*, benthic crabs, and prawns. Moreover, the similarity between medium and largest groups was observed at 72.31%.

The significance of the difference between the IRI values of males and females was tested by analysis of variance. Since the null hypothesis H0: μA=μB and H0 is not rejected (p = ≥ 0.05), there is no significant difference in the relationship between males and females (table 4).

Predator feeding strategies

The feeding strategy of *N. japonicus* was found by plotting prey- specific abundance against the frequency of occurrence in figure 3. There were 23 different prey types represented by points. Based on the interpretation of Amundsen *et al.* 1996[5] were important food of *N. japonicus*, whereas some species of fishes, lobster and squilla were unimportant. Also, *N. japonicus* were specialized on fish larvae, polychaetes, unidentified fishes and algae, etc. but some species of fish, crab, prawn, *Acetes spp.*, *squilla*, and lobster were eaten only occasionally. Although fish larvae, polychaetes, unidentified fishes and algae were consumed by a few individual predators, crab, prawn, *Acetes sp.*, *Bremaceros spp*, etc. were eaten by most individual predators. Therefore, though the abundance of certain prey items like fish larvae and polychaetes was very high in the ecosystem, their occurrence was meager in the diet. The analysis showed that *N.* has a specialized feeding strategy focusing on crustaceans, especially Crab, *Acetes spp*, and Prawn, which they consumed in very large quantities. *Bregmaceros spp* and Misc. item was the next most often found prey items on which *N. japonicus* specialized. Therefore, they were consumed occasionally by most individuals. Though the abundance of certain prey items like fish larvae and polychaetes was very high in the ecosystem, their occurrence was meager in the diet.

Discussion

The present study revealed that *N. japonicus* is a benthic carnivore that relies mainly on benthic crustaceans and fishes. Rao and Rao[6] described food items as *squilla*, crabs, prawns, teleosts, cephalopods, amphipods, polychaetes and other miscellaneous items in that order of importance from the gut of *N. japonicus*. Krishnamurthy[7] described *N. japonicus* is actively predacious and possibly a sight feeder, feeding on crustaceans, mollusks, annelids and echinoderms. Moreover, about the results by SabaiSoe[8], the food composition of *N. japonicus* consist of *Solenocera spp.*, *Stolephorus spp.*, *Squilla*, Crabs and Miscellaneous. According to the results conducted in the present study, the following food items were observed in the guts of fishes: fish comprised with *Nemipterus spp.*, *Bregmaceros spp.*, *Secutor spp.*, *Sauridas spp.*, *Platycephalus spp.*, *Cynoglossus spp.*, *Leiognathus spp.*, *Stolephorus spp.*, fish larvae and unidentified fishes, crustacean comprised with *Acetes spp.*, crab, prawn, lobster and *Squilla*, cephalopod comprised with *Loligo*, *Sepia* and unidentified squids, polychaetes, nematodes, brittle stars, algae and miscellaneous items. Therefore, the food items of the present study were almost the same as the previous studies of *N. japonicus.* Russel 1990[1] observed that cephalopods mainly squid and cuttlefish formed dominant food followed by finishes and other benthic crustaceans. In contrast, the present study was observed that benthic crustaceans were the most dominant food.

Of the crustacean items, *Acetes pp*, prawn and crab were the favorite food of *N. japonicus* with the highest IRI value, but *Squilla* and lobster were also found occasionally with the lowest IRI value. Fish ranked in the second dominant food category of *N. japonicus*. Among them, *Bregmaceros spp* was observed with the important quantities, and other species

Food items	N%	O%	W%	IRI	IRI%
Fish					
Nemipterus spp.	0.68	1.05	0.75	1.474	0.148
Bregmaceros spp.	6.105	8.41	8.43	77.07	7.77
Secutor spp.	0.63	0.708	1.064	1.38	0.14
*Sauridas*spp.	1.028	0.517	0.227	1.145	0.115
*Platycephalus*spp.	0.479	0.94	3.007	3.306	0.332
Cynoglossus spp.	0.483	0.799	1.17	1.418	0.14
Leiognathus spp.	0.34	1.01	0.45	0.801	0.08
Stolephorus spp.	2.51	3.553	4.989	20.23	2.04
Fish larvae	2.01	1.989	0.89	3.7992	0.383
Unidentified fishes	4.676	5.85	5.18	35.06	3.534
Crustacean					
*Acetes*spp.	33.11	14.09	15.28	248.60	25.06
Crab	13.22	13.95	8.78	135.82	13.69
Prawn	11.35	15.8	17.71	292.306	29.46
Lobster	0.221	0.41	0.38	0.379	0.038
Squilla	1.642	3.115	8.2	27.21	2.74
Cephalopod					
Loligo	1.735	2.73	5.62	17.08	1.722
Sepia	1.55	2.08	3.317	8.48	0.855
Unidentified squids	0.104	0.175	0.16	0.336	0.013
Polychaetes	1.433	2.21	1.77	5.359	0.54
Nematodes	5.633	3.59	0.89	8.854	0.892
Brittle stars	1.18	2.17	1.83	5.17	0.521
Algae	1.08	5.81	0.407	3.455	0.348
Misc. items	8.73	8.96	9.43	93.34	9.409

Table 1. Prey of *N. japonicus* collected from the Tha-baw-seik coastal areas in terms of frequency of occurrence (%FO), gravimetric (% W), numerical (%N), and index of relative importance (IRI).

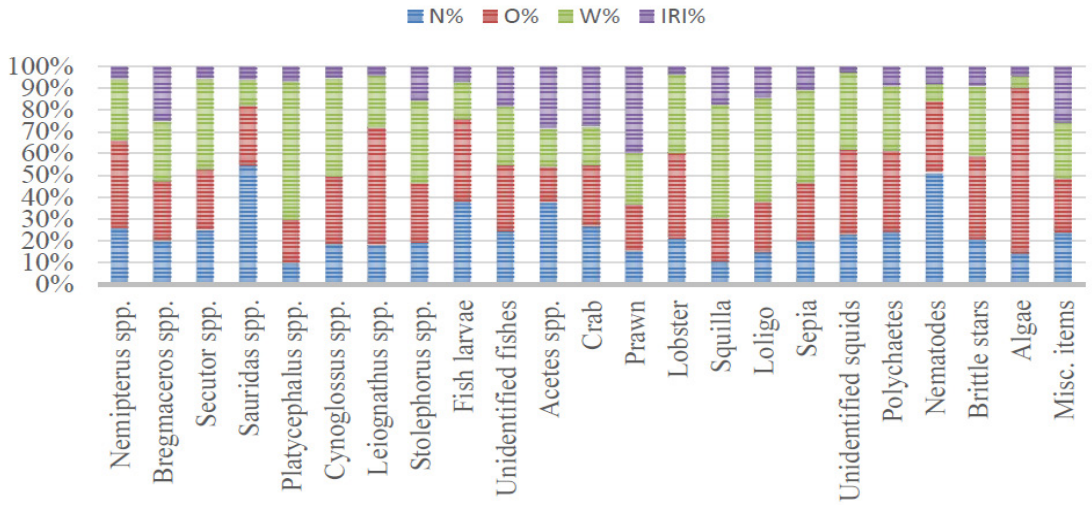

Figure 2. Prey of *N. japonicus* collected from the Tha-baw-seik coastal areas in terms of relative importance index (IRI).

had the negligible quantities. The studies of Manojkumar[9] and Abdu Rahiman[10] made the fish prey items identify up to the species level but the present study could not be done because of their digestion.

Loligo and *Sepia* were considerably found but *Loligo* was more abundant than *Sepia*. The miscellaneous items comprised of debris, rubbish, unidentified bones, unidentified shells, unidentified hairs, and silts were found very often. However, polychaetes, nematodes, brittle stars, and algae were negligible food or minor food in some months.

As for predator feeding strategies, Abdu Rahiman[10] showed that *N. japonicus* most often specialized on crustacean mainly *S. choprai*, benthic crabs, unidentified fishes, *A. indicus* and *L. durance* in his study whereas the present study found that crab, *Acetes spp*, prawn, *Bregmaceros spp* and Misc. items represented the specialization of the predator population.

In all length groups, fishes were commonly the most dominant food followed by prawn, crab and *Acetes spp*. According to the IRI values, *N. Japonicus* were generally increased with the increasing length of them in fishes, prawn, and *squilla* while the values were decreased with the decreasing length in *Acetes spp*, crab, squid and miscellaneous item. A large percentage of fishes, prawn, and squilla in the largest length groups indicate they alter in feeding towards fish, prawn and *squilla* items as length increases. On the pieces of evidence from the report of Rao and Rao 1991, squilla dominated for all length groups followed by crabs and prawns. A large proportion of prawns mainly *S. choprai* and *A. indicus*, *L. duvauceli* and fish, which are active mobile benthic organisms recorded in large length groups indicated predatory behavior of *N. japo-*

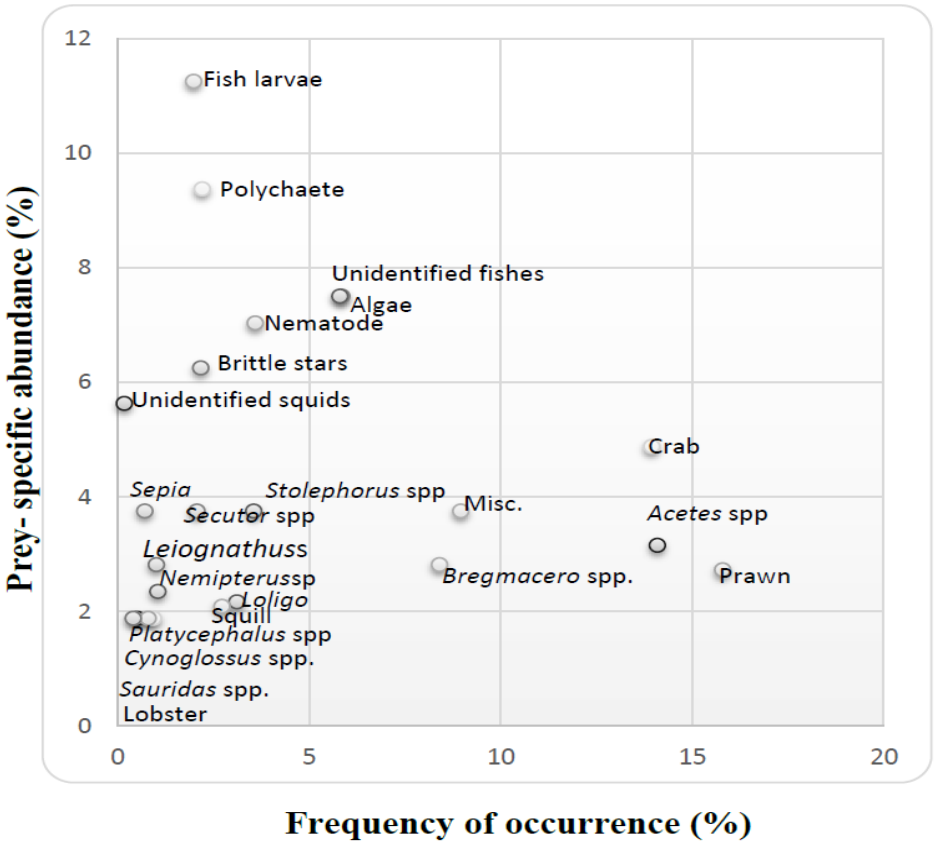

Figure 3. Feeding strategy diagram for *N. japonicus*. Prey- specific abundance (%) plotted against frequency of occurrence of food items in its diet.

	N. japonicus		
Food items	**smallest group** (100-140 mm)	**median group** (141-190 mm)	**largest group** (191-220 mm)
Fish	**17.39**	**18.29**	**30.33**
Nemipterus spp.	0.96	3.26	6.72
Bregmaceros spp.	8.77	5.36	16.38
Secutor spp.	0	0.42	0
Sauridas spp.	*5.38*	1.13	0
Platycephalus spp.	0	1.53	0
Cynoglossus spp.	0	0.26	0
Leiognathus spp.	0	0	0.93
Stolephorus spp.	0	2.15	1.36
Fish larvae	0	0.55	0.69
Unidentified fishes	2.28	3.62	4.25
***Acetes* spp.**	14.04	15.19	10.407
Crab	19.08	18.73	9.81
Prawn	16.14	15.85	19.81
Lobster	0	3.703	0
Squilla	4.37	7.37	11.11
Squid	10.14	3.65	3.703
Polychaetes	2.89	5.097	0
Nematodes	4.34	0	0
Brittle stars	1.449	3.65	3.703
Algae	0	2.43	0
Misc. items	8.69	9.75	7.407

Table 2. Ontogenetic variation in %IRI of different prey of *N. japonicus*.

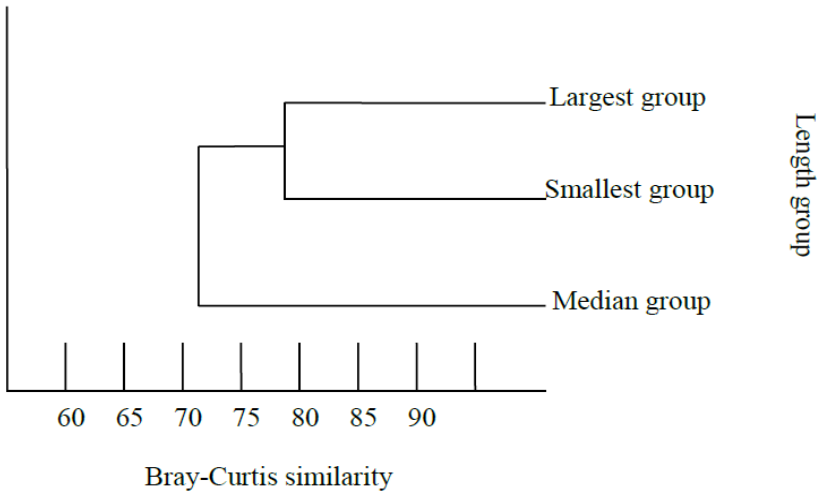

Figure 4. Dendrogram based on %IR: values of different length groups of *N. japonicus* using Bray-Curtis similarity.

Food items	Male	Female
Fish	**16.66**	**14.7**
Nemipterus spp.	2.56	0
Bregmaceros spp.	4.98	9.38
Secutor spp.	0.39	0
Sauridas spp.	1.22	1.26
Platycephalus spp.	1.34	1.55
Cynoglossus spp.	0.26	0
Leiognathus spp.	0.13	0
Stolephorus spp.	1.56	0
Fish larvae	0.76	0
Unidentified fishes	3.46	2.51
***Acetes* spp.**	10	8.823
Crab	17.5	20.588
Prawn	18.33	17.64
Lobster	0.833	0
Squilla	5	4.893
Squid	3.33	2.941
Polychaetes	3.33	5.882
Nematodes	1.66	2.941
Brittle stars	0.833	2.941
Algae	2.5	0.989
Misc. items	17.5	17.64

Table 3. Sex variation in %IRI of different prey of *N. japonicus*.

ANOVA						
Sources	SS	df	MS	F_{cal}	P value	
Between Groups	0.149	1	0.149	0.0	0.95	
Within Groups	1534.9	40	38.37			
Total	1535.09	41				

Not significant at 5% level

Table 4. Comparison of IRI value between male and female of N. japonicus from Tha-baw-seik, Myanmar.

nicus on benthic animals. Compared with the present study, it is proved that almost all food items observed in the guts were only the benthic organisms. Moreover, it was also found that the sex variation in IRI value of N. Japonicus had no significance with their different food items.

Conclusions

Acetes spp., Crab, Prawn and *Squilla* were the favorite food of *N. japonicus.* Regarding preyed fishes, it seems to eat *Bregmaceros spp* as its major food. It cannot be denied that *N. japonicus* is the kind of benthic fish because almost all food items were only the benthic organisms. Moreover, it can be concluded that their food items can be their associated animals. Therefore, the species found in their guts may be distributed along the Tha-baw-seik and its adjacent coastal waters.

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Bibliographic references

1. Russell, B.C. Nemipterid fishes of the world. (Threadfn breams, whiptail breams, monocle breams, dwarf monocle breams, and coral breams) Family Nemipteridae. An annotated and illustrated catalogue of nemipterid species known to date. FAO Fish.Synops, 1990. 12(125): 1-149.
2. FAO Fisheries and Aquaculture Department. Fishery and Aquaculture Country profiles. The Republic of the Union of Myanmar. Food and Agriculture Organization of the United Nations. 2012. 20p.
3. Bowen, S. Quantitative description of the diet, In: B. R. Murphy and D. W, Willis, (Eds). Fisheries Techniques, American Fisheries Society, Bethesda, MD, 1996.513-532.
4. Pinkas, L.M., S. Oliphant and I.L.K. Iverson. Food habits of albacore, bluefin tuna and bonito in Californian waters. Calif. Fish Game, 52: 1971. 1-105.
5. Amundsen, P. A., H. M. Gabler& F. J. Staldvik. A new approach to graphical analysis of feeding strategy from stomach contents data - modification of the Costello (1990) method. Journal of Fish Biology, 1996. 48: 607-614
6. Rao, D.M and K.S. Rao. Food and feeding behaviour of Nemipterusjaponicus (Bloch) populations off Visakapatanam, South India. J. Mar. Biol. Ass. India., 1991. 33 (1&2): 335-345.
7. Krishnamurthy, B. Biology of the threndfin bream, Nemipterusjaponicus (Bloch). Indian J. Fish., 1971. 18(1&2): 1-21.
8. SabaiSoe. Obeservation on gut contents of threadfin breams (genus- nemipterus) in myeik waters. Unpublished Master of Science Thesis. Department of Marine Science. Myeik University. 2014.
9. Manojkumar, P.P., Pavithran, P.P and Ramachandran, N.P. Food and feeding habits of Nemipterusjaponicus (bloch) from Malabar coast, Kerala. Indian J. Fish. 2015. 62 (1): 64-69.
10. Abdu Rahiman, KP. Studies on food and feeding of marine demersal finfishes with special preference of trophic interactions. Unpublished Ph.D Dissertation. Under the faculty of Marine Science. Central marine fisheries research institute, Cochin. 2006.
11. Hyslop, E.J. Stomach contents analysis: a review of methods and their application. J. Fish. Biol., 17:411-429. 1980.
12. Dewan, S. and Shaha, S. N. Food and feeding habits of Tilapia nilotica (L.) (Perciformes: Cichlidae). II. Diel and seasonal patterns of feeding. Bangladesh J. Zool. 7(2): 75-80. 1979.
13. Snedecor, G.W. and Cochran, W.G. Statistical Methods. Oxford and IBH Publishing Company, New Delhi India. 593pp. 1967.
14. Bray, J.R. and J.T. Curtis. An ordination of the upland forest communities of Southern Wisconsin. Ecological Monographs, 27, 325-349. 1957.

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RESEARCHS / INVESTIGACIÓN

Chemical diversity and antibacterial activity of volatile compounds from two Centrolobium paraense Tul. varieties.

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Abstract: This work presents a comparative study of the chemical composition of the essential oils obtained from two varieties of Centrolobium paraense (orinocense EO o and paraense EO p) from Venezuela. GC analyzed the oils, and the constituents were identified by GC-MS and retention indices. Eighteen compounds were identified in EO o, which made up 88.9 % of the oil, but only sixteen compounds were identified in EO p, which made up 95.5 % of the total oil. β-caryophyllene (35.1 %) and -humulene (35.3 %) are the main constituents of EO p. The main constituents of EO o are α-humulene (24.8 %), β-caryophyllene (14.3 %), caryophyllene oxide (18.33 %), and humulene epoxide II (16.86 %). The biological activity of both oils was assayed. They were found to be equally active against Staphylococcus aureus and Enterococcus faecalis, with MIC of 100 and 600 µL/mL respectively. This is the first report describing the chemical composition of the essential oil of these species and their antibacterial activity.

KeyWords: antibacterial activity, caryophyllene oxide, Centrolobium paraense, essential oil, humulene epoxide II, α-humulene, β-caryophyllene.

Introduction

The neotropical genus *Centrolobium* Mart. ex Benth (Leguminosae, Papilionoideae) comprises seven trees species: C. *robustum, C. microchaete, C. tomentosum, C. ochroxylum, C. sclerophyllum, C. paraense*, and *C. yavizanum* which grow in Brasil, Bolivia, Ecuador, Peru, Colombia, The Guianas, Panama, Trinidad and Venezuela between 50 and 350 meters above sea level. Some *Centrolobium* species are highly valued because of their enduring and beautiful wood, which has an orange-yellow color with dark red or black stripes[1]. In Venezuela *Centrolobium paraense* is the only *Centrolobium* species that have been described, which is popularly known as "cartán" or "Colorado".

Because its wood is highly appreciated *C. paraense* has been overexploited to the point that it is becoming scarce and it is considered to be in danger of extinction according to the ideas presented on the book "Libro Rojo de la Flora Venezolana"[2]. Since research on this valuable tree is very scarce it was considered convenient to start a research program in order to devise methods to increase its population.

Two varieties have been identified within this species: *C. paraense var. paraense* and *C. paraense var. orinocense.* Taxonomic differences between both varieties are difficult to establish, mainly regarding the shape of the leaves at their base and the amount of indument at their surface[1, 3].

Previous studies on the secondary metabolites present on the wood of *Centrolobium species*, (*C. paraense* included), reported diarylheptanoids and isoflavonoides such as centrolobol, centrolobine and methylcentrolobine[4, 5, 6, 7]. Our laboratory is making an overall study of *C. paraense*, which includes physico-chemical and anatomical aspects of its wood[8, 9]. Since no reports have been found on the chemical composition of the essential oil of the leaves, we have obtained, by hydrodistillation, the essential oils from the leaves of both varieties and compare their compositions. At the same time, their possible biological activity against human pathogens has been tested.

Materials and methods

Plant material

Fresh aerial parts of *C. paraense var. orinocense* and paraense (laminar portion) were collected in May 2014, from plants growing wild in similar environmental conditions near to Upata locality, state Bolívar, Venezuela at 345 m above sea level. Dr. Elio Sanoja made botanical identification. Voucher specimens were deposited at the MERF herbarium, Faculty of Pharmacy, University of Los Andes Merida, Venezuela (Luis Beltrán Rojas 063 and 064 *paraense* and *orinocense* respectively). The extraction conditions were identical for both species.

Isolation of essential oils

Aerial parts of both varieties were collected separately from random points on the trees under investigation. Leaves laminar portion (300 g) from each variety were separated and subjected to hydrodistillation in a Clevenger-type apparatus for 4h. The quantity of essential oil was measured directly in the extraction burette of the apparatus and content (%) was calculated as volume (mL) of essential oil per 100 g of plant material (v/w %). The oil sample was dried over sodium sulfate (Na_2SO_4), and stored in a dark vial at 4ºC until they were analyzed chromatographically.

Gas Chromatography GC

Analyses were performed using a Perkin-Elmer Autosystem gas chromatography equipped with an FID detector and

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data-handling system. A 5 % phenylmethyl polysiloxane fused-silica capillary column was used (30 m x 0.25 mm i.d., film thickness 0.25 m; HP-5, Hewlett-Packard, CA, USA). The oven temperature was programmed from 60 °C to 260 °C, rising at 4 °C/min. The injector and detector temperatures were 200 °C and 280 °C, respectively. The carrier gas was helium at 0.8 mL/min. The sample (1.0 µL) was injected using a split ratio of 10:1. Retention indices were calculated concerning C_8-C_{24} *n*-alkanes. The percentage composition of the oil was calculated by the normalization method from the GC peak areas. The percentage composition of the oil was calculated by the normalization method from the GC peak areas.

Gas chromatography-mass spectrometry

GC-MS analyses were carried out on a Model 5973 Hewlett-Packard GC-MS system fitted with an HP- 5MS fused silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm, Hewlett-Packard). The oven temperature program was the same as that used for the HP-5 column for GC analysis; the transfer line temperature was programmed from 60 °C to 260 °C; rising at 4 °C/min, source temperature, 230 °C; quadrupole temperature 150 °C; carrier gas, helium adjusted to a linear velocity of 34 cm/s; ionization energy, 70 eV; scan range, 40 to 500 amu; 3.9 scans/s. Sample (1.0 µL) was injected using a Hewlett-Packard ALS injector with a split ratio of 50:1. The identity of the oil components was established from their GC retention indices, by comparison of their MS with those of standard compounds available in the laboratory, and by a library search (Nist 05 and Wiley MS Data Library, 6th edn)[10, 11].

Antimicrobial Assay

The antibacterial activity and minimum inhibitory concentration (MIC) were evaluated by the agar disk diffusion method described by Velasco *et al.* (2007)[12]. The bacterial strains used in experiments were as follows: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25992), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 23357). Every bacterial inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 µL of essential oil and then incubated at 37 ºC for 24 h. Positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Oxacillin®, Vancomycin®, Tobramycin®, Cefepime® and Aztreonam®. The minimal inhibitory concentration (MIC) was determined only with microorganisms that displayed inhibitory zones. MIC was determined by dilution of the essential oil in dimethyl sulphoxide (DMSO) by pipetting 10 µL of each dilution onto a filter paper disc. Dilutions of the oil within a concentration range of 10-350 µg/mL were also carried out.

Statistical analysis

Data obtained from antibacterial activity were expressed as mean values. The statistical analyses were carried out employing one way ANOVA, results with P <0.05 were considered to be statistically significant. A statistical package (SPSS version 19.0) was used for the data analysis.

Results and Discussion

The results reported here on the essential oil chemical compositions of these varieties grow in Venezuela are novel, and show an interesting chemical diversification. Gas chromatographic analyses of both essential oils tested showed different composition. The yields of essential oil from *orinocense* (EO_o) and *paraense* (EO_p) were in the range of 0.25 - 0.22 %, respectively. EO_p showed a yellowish oil, and EO_o was a colorless oil. The identified components, percentages composition, and their Retention indices (RI) values listed in order of elution time on the HP 5 capillary column are reported in Table 1. A total of eighteen compounds were identified in the oil of the *orinocense* variety, which represents 88.9% of the oil. In the case of the *paraense* variety only sixteen constituents were identified, which in this case, represented 95.5 % of the total oil.

All the identified compounds from the essential oil from both varieties were sesquiterpenes. The main constituents of the *paraense* variety were β-caryophyllene (35.1 %) and α-humulene (35.1 %), which, with the contribution of other six minor constituents add up to the total sesquiterpene hydrocarbon fraction (77.4 %). Eight minor constituents, which account for 18.0% of the *paraense* variety oil, are oxygenated sesquiterpenes, being nerolidol (4.6 %) the most abundant one. In the oil of the orinocense variety, α-humulene (24.8 %), β-**ββ** (14.3 %) were the most abundant sesquiterpene hydrocarbons, the contribution of other seven minor compounds added up to a total sesquiterpene hydrocarbon fraction of 44.3 %. Oxigenated sesquiterpenoids made, in this case 44.6 % of the oil. Caryophyllene oxide (17.6 %) and humulene epoxide II (16.3 %) were the most abundant oxygenated sesquiterpenes in the oil of the *orinocense* variety, which along with seven other minor oxygenated constituents made a total oxygenated sesquiterpene fraction of 44.7 %.

In spite of this fact, both oils are different because the *paraense* variety contains 77.4 % of hydrocarbon sesquiterpenes and only 18 % of oxygenated sesquiterpenes. On the other hand, the *orinocense* variety oil contains the same proportion of hydrocarbon sesquiterpenes (44.3%) and oxygenated sesquiterpenes (44.6 %). β-caryophyllene and α-humulene, the compounds that dominate the hydrocarbon fraction of both oils, are twice as abundant in the *paraense* variety oil. On the other hand caryophyllene oxide and humulene epoxide II are six times more abundant in the oxygenated fraction of the *orinocence* variety oil than in the paraense variety oil. Finally ɑ-copaene, *cis* calamenene, and Epi-ɑ-Cadinol, which are minor constituents of the *orinocense* variety, are not present in the *paraense* variety oil. On the other hand δ cadinene, a minor constituent of the *paraense* variety oil is absent from the *orinocense* oil.

Table 2 shows the results obtained from the antibacterial evaluation of both essential oils (EO_o and EO_p). The results revealed that the oil possessed antibacterial activity with varying magnitudes towards different strains. The essential oils were active only against *S. aureus* and *E. faecalis* and completely ineffective against *E. coli*, *K. pneumoniae* and *P. aeruginosa*. This behavior is possible because Gram-negative bacteria are less susceptible to essential oils than the Gram-positive strains because the former possess an outer membrane surrounding the cell membrane which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering[13].

Biological activity of both oils was statistically superior (P<0.05) on the bacterial growth of *S. aureus* compared with *E. faecalis* with inhibition halos of 13 mm and 8 mm, respectively (Table 2). The MIC value of the oils was 100 µL/mL on *S. aureus* and 600 µL/mL against *E. faecalis*. These results suggest that the oils were more active against *S. aureus*. Although both oils (EO_o and EO_p) possess different chemical composition, the antimicrobial activity of the essential oils did not show statistically significant differences (p=0.841) against *S. aureus* (Ta-

N°	Component	$IR_{Lit.}$	IR_{Exp}	*Centrolobium* varieties	
				EO_o	EO_p
1	Cyclosativene	1368	1372	0.7	0.7
2	α-copaene	1376	1382	0.5	0.6
3	β-caryophyllene	1418	1428	14.3	35.1
4	β-copaene	1430	1437	0.3	-
5	α-humulene	1454	1466	24.8	35.3
6	γ- muurolene	1478	1486	1.2	1.2
7	α-selinene	1494	1496	0.4	0.5
8	α-muurolene	1500	1509	1.7	2.6
9	δ cadinene	1524	1522	-	1.4
10	Cis calamenene	1528	1530	0.5	-
11	Nerolidol	1564	1566	4.4	4.6
12	Caryophyllenyl alcohol	1570	1573	0.34	1.28
13	Caryophylene oxide	1581	1586	17.6	2.9
14	1,5,8,8-tetramethyl-3,7-cycloundecadien-1-ol	1607	1596	0.9	2.3
15	Humulene epoxide II	1608	1610	16.3	2.6
16	Epi-α-Cadinol	1640	1642	0.8	-
17	α-muurolol	1646	1649	1.6	1.3
18	β-eudesmol	1650	1654	1.4	1.2
19	α-eudesmol	1654	1657	1.2	1.8
	Class composition				
	Sesquiterpene hydrocarbons (%)			44.3	77.4
	Sesquiterpene /oxigenate (%)			44.6	18.0
	Total Identified (%)			88.9	95.4

Notes: Retention indices in literature (IR_{Lit}), Retention indices experimental (IR_{Exp}), Essential oil *Centrolobium paraense* var. *orinocense* (EO_o),: Essential oil of *Centrolobium paraense* var. *paraense* (EO_p

Table 1. Chemical composition of the essential oil (%) of two varieties of *Centrolobium paraense* from Venezuela.

Microorganisms	Inhibition zone (mm)*							EO_o MIC µL/mL	EO_p MIC µL/mL
	EO_o	EO_p	Reference compounds						
			OX	VA	TOB	AZT	CEP		
Staphylococcus aureus (ATCC 29923)	13*	13*	23*					100	**100**
Enterococcus faecalis (ATCC 29212)	8*	8*		20*				600	**600**
Escherichia coli (ATCC 25922)	NA	NA			26*			NT	**NT**
Klebsiella pneumoniae (ATCC 23357)	NA	NA				30*		NT	**NT**
Pseudomonas aeruginosa (ATCC 27853)	NA	NA					32*	NT	**NT**

Notes: EO_o: Essential Oil of *Centrolobium paraense* var. *orinocense*, EO_p: Essential Oil of *Centrolobium paraense* var. *paraense*, Oxacillin® (30 µg), VA: Vancomycin® (30 µg), TOB: Tobramycin® (10 µg), AZT: Aztreonam® (30 µg), CEF: Cefepime® (30 µg), NA: Not Active, NT: Not Tested, *inhibition zone, diameter measured in mm, disc diameter 6 mm, average of two consecutive trials. MIC: Minimal Inhibitory Concentration of the essential oil, concentration range: 10-700 µL/mL.

Table 2. Antibacterial activity of essential oils of two varieties of *Centrolobium paraense* from Venezuela.

Bacterium	Analysis of variances (ANOVA)				Turkey's multiple comparisons			
	Sum of squares	Mean squares	*F*	*Sig.*	Compound		Mean difference	*Sig.*
S.aureus								
Inter-groups					EO$_a$	EO$_b$	0.250	0.841
Intra-groups	287.167	143.583	369.214	0.000		Ox	-10.250*	0.000
					EO$_b$	EO$_a$	-0.250	0.841
Total	3.500	0.389				Ox	-10.500*	0.000
					Ox	EO$_a$	10.250*	0.000
	290.667					EO$_b$	10.500*	0.000
E.faecalis								
Inter-groups					EO$_a$	EO$_b$	-0.2500	0.674
Intra-groups	360.500	180.250	1081.50	0.000		Va	-11.75*	0.000
					EO$_b$	EO$_a$	0.2500	0.674
Total	1.500	0.167				Va	-11.50*	0.000
					Va	EO$_a$	11.750*	0.000
	362.000					EO$_b$	11.500*	0.000

Notes: EO_o: Essential Oil of *Centrolobiumparaense* var. *orinocense*, EO_p: Essential Oil of *Centrolobiumparaense* var. *paraense*. Ox (Oxacillin®), Va (Vancomycin®). (*)The difference in means is significant at the level of *Sig.* p= 0.05.

Table 3. ANOVA and Turkey's multiple comparisons of biological activity of essential oils of *Centrolobium* (EO_o, EO_p), against *S. aerus* and *E. faecalis*.

ble 3). Similar behavior was observed on *E. faecalis* (*P*=0.674). The statistical analysis indicated that the inhibitory effect of the essential oils against the two bacteria tested was in most cases not as strong as that of the reference compounds. (Oxacillin® and Vancomycin® respectively).

The abundance, interaction mechanism, and presence of several reactive chemical components in the essential oils of both varieties, could have applications in the pharmaceutical and chemical industries. Previous investigations have shown that individual compounds detected in the essential oil of C. paraense show important biological activity. For example, β-caryophyllene has anti-inflamatory, anticarcinogenic, antibiotic, antioxidant, and local anesthetic activities[14]. The α-humulene showed anticancer activity[15], anti-inflammatory effects[16] and mixed with con β-caryophyllene increases its cytotoxicity against human tumour cell lines in vitro[14]. Nerolidol has an impact on the protein prenylation, and it can reduce adenomas in rats[17]. Caryophyllene oxide in combination with other sesquiterpenes showed higher anticancer activity against several cancer cell lines as human lung carcinoma, human colon adenocarcinoma, human leukemia cancer, human cervical adenocarcinoma, human gastric cancer, and human stomach cancer[18, 19, 20]. The specific antibacterial activity of both oils on the tested microorganisms could be attributed to its high sesquiterpene content and the synergistic and antagonistic effects of these compounds. However, further studies are needed to obtain a better understanding of their biological activity.

Conclusions

This article is the first report on the differences in the chemical composition of the essential oil of these two varieties of *Centrolobium paraense* and their antibacterial activity, information that could be used with chemotaxonomic purposes in this genus. Results suggest that essential oils from both varieties of *C. paraense* could contribute to the control of infections caused by *S. aureus* and *E. faecalis*.

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Bibliographic references

1. Pirie m., klitgaard b., pennington r. (2009). revision and biogeography of centrolobium (leguminosae - papilionoideae). systematic botany 34 (2): 345–359. doi.org/10.1600/036364409788606262
2. llamozas s., de stefano r., meier w.; riina r., stauffer f., aymard g., huber o., ortiz r. (2003). libro rojo de la flora venezolana. edit., fundación polar, provita, fundación instituto botánico de venezuela. caracas, venezuela http://www.lrfv.org/libro-rojo-de-la-flora-venezolana
3. Rudd v. (1999). centrolobium (fabaceae). in flora of the venezuelan guayana. edit., p. berry, k. yatskievych, b. holst, volume 5, eriocaulaceae–lentibulariaceae. missouri botanical garden press u.s.a. p 269-271.
4. Craveiro a., prado a., gottlieb o., de albuquerque a. (1970). diarylheptanoid of centrolobium species. phytochemistry 9 (8): 1869-1875. doi.org/10.1016/s0031-9422(00)85606-x
5. Jurd l., wong r. (1984). diarylheptanoid and other phenolic constituents of centrolobium species. austrian journal of chemistry 37(5): 1127-1133. doi.org/10.1071/ch9841127
6. Alegrio l., braz r., gottlieb o. (1989). diarylheptanoids and isoflavonoids from centrolobium species. phytochemistry 28(9):2359-2362. doi.org/10.1016/s0031-9422(00)97984-6
7. Araujo c., alegrio l., leon l. (1998). antileishmanial activity of compounds extracted and characterized from centrolobium sclerophyllun. phytochemistry 49(3): 751-754. doi.org/10.1016/s0031-9422(97)00976-x
8. Velásquez j., toro m., rojas l., encinas o. (2006). actividad antifúngica de los extractivos naturales de especies latifoliadas de la guayana venezolana. madera y bosques 12 (1):51-61. doi.org/10.21829/myb.2006.1211250
9. Morgado r., gutiérrez l., garcía p., arrioja t., toro m., gómez l., velásquez j. (2010). variación longitudinal del peso especifico en la madera de centrolobium paraense tul. (fabaceae). revista forestal venezolana 54 (1), 227-234. https://www.thefreelibrary.com/variacion+longitudinal+del+peso+especifico+en+la+madera+de...-a0303895959
10. Adams r. (2007). identification of essential oils component by gas chromatography/quadrupole mass spectroscopy. 4th ed. allured publ. corp., carol stream, il, 1-499.
11. Davies n. (1990). gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20 m. phases. journal of chromatography a 503(6):1-24. doi.org/10.1016/s0021-9673(01)81487-4
12. Velasco j., rojas j., salazar p., rodríguez m., díaz t., morales a., rondón m. (2007). antibacterial activity of the essential oil of lippia oreganoides against multiresistant bacterial strains of nosocomial origin. natural product communications 2(1):85-88.
13. verma r., padalia r., goswami p., verma s., chauhan a. darokar m. (2016). chemical composition and antibacterial activity of the essential oil of kauri pine [agathis robusta (c. moore ex f. muell.) f.m. bailey] from india. journal of wood chemistry and technology 36(): 270-277. doi.org/10.1080/02773813.2015.1137946
14. Legault j., pichette a. (2007). potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel. journal of pharmacy and pharmacology 59 (12): 1643-1647. doi.org/10.1211/jpp.59.12.0005
15. Legault j., dahl w., debiton e., pichette a., madelmont j. (2003). antitumor activity of balsam fir oil: production of reactive oxygen species induced by alpha-humulene as possible mechanism of action. planta medica, 69(5): 402-407. doi.org/10.1055/s-2003-39695
16. Passos g., fernández e., cunha da f., ferreira j., pianowski l., campos m., calixto j. (2007). anti-inflammatory and anti-allergic properties of the essential oil and active compounds from cordia verbenacea. journal of ethnopharmacology 110(2): 323-333. doi.org/10.1016/j.jep.2006.09.032
17. Wattenberg i. (1991). inhibition of azoxymethane-induced neoplasia of the large bowel by 3-hydroxy-3,7,11-trimethyl-1,6,10-dodecatriene (nerolidol). carcinogenesis 12(1):151-152. doi.org/10.1093/carcin/12.1.151
18. Sylvestre m., legault j., dufour d., pichette a. (2005). chemical composition and anticancer activity of leaf essential oil of myrica galae l. phytomedicine 12(4): 299-304. doi.org/10.1016/j.phymed.2003.12.004
19. Buchbauer g. (2010). biological activities of essential oils. in handbook of essential oils: science, technology, and applications. edit. by hüsnü can baser and gerhard buchbauer. taylor & francis group, 988p.
20. Jun n., mosaddik a., moon j., jang k., lee d., ahn k., cho s. (2011). cytotoxic activity of β-caryophyllene oxide isolated from jeju guava (psidium cattleianum sabine) leaf. records natural products, 5(3): 242-246. https://pdfs.semanticscholar.org/ea7e/2d2bf99281288ba235215de070179ca429eb.pdf

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RESEARCHS / INVESTIGACIÓN

Synthesis and characterization of a Nitrogenase-cofactor biomimetic based on molybdenum complexes with a polydentate-N_5 ligand.

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Abstract: Nitrogen fixation is an outstanding process in which atmospheric molecular nitrogen is reduced to ammonia, which is easier to assimilate by the plants. Due to the challenge to understand the nitrogen fixation process *in vivo* conditions, biomimetic compounds have been synthesized to perform the reduction of nitrogen in softest environments than in the Haber process. Thus, the purpose of this work is the synthesis and characterization of new molybdenum complexes with polydentate nitrogenated ligands and the evaluation of those complexes as possible dinitrogen reductants.

Key words: Nitrogenase, nitrogen fixation, biomimetic, molybdenum complex.

Introduction

Nitrogen fixation is a fundamental process to obtain ammonia and sustain life. There are at least three forms of nitrogen fixation in the earth: atmospheric, bacterial, and industrial production. The low concentration of ammonia is located in the troposphere, and most of it occurs in the agricultural process. By Keywords, nitrogen is fixed in the troposphere by photochemical reactions[1] of dinitrogen in the presence of lightning; however, nitrogen oxides NO_x produced in such reactions are less assimilable than ammonia or ammonium and do not contribute significantly to the nitrogen assimilation by the plants.

Haber Fritz proposed a production method of ammonia more than 100 years ago, then taken by Carl Bosch and converted into an industrial process[2]. In such a process, hydrogen required for the reduction of N_2 is synthesized by a redox reaction between CO and H_2O, nitrogen is taken from air through distillation towers, then, both gases are directed to a chemical reactor and treated with an iron catalyst at high pressure (~150 bar) and high temperatures (500-600 ºC) in order to obtain NH_3 (equation 1). Haber-Bosch process is expensive and pollutant, however, about 40% of the earth population depends on the production of fertilizers coming from this process[3].

$$3H_{2(g)} + N_{2(g)} \rightarrow 2NH_{3(g)} \qquad \text{eq. 1}$$

In biological nitrogen fixation, Nitrogenase corresponds to a complex enzyme responsible of the fixation of atmospheric dinitrogen to a reduced form of nitrogen (i.e., amino, amido, imido, azido, nitrite or ammonia). In biology, only a specific group of microorganisms contains such an enzyme to make this a successful process. The first structural description of a nitrogenase enzyme corresponds to that of *Azobacter vinelandii*[4].

The structure founded for its active site corresponds to a molybdenum (III) complex with an octahedral geometry on Mo with three sulfurs of the Fe-S cluster, two oxygen from the bidentate homocitrate and the imidazole group of a histidine (Figure 1)[5].

To contribute to a full understanding of the nitrogen fixation mechanism *in vivo*, synthesis of new functional biomimetic compounds suitable to reduce dinitrogen in softer conditions than the Haber-Bosch method have been reported. The synthesis of such nitrogenase cofactor biomimetics began with the synthesis of molybdenum-dinitrogen complexes, which were further functionalized to produce amides, hydrazide, and imido compounds because these compounds could be transformed to ammonia easily. In the first synthetic biomimetic complex, the oxidation state of molybdenum was zero, but, further investigations have improved the synthesis of new ligands, which tune the oxidation state of molybdenum (Mo^{III}, Mo^{V}, and Mo^{VI}) to increase their reactivity[6]. Besides, the nitrogenase mechanism according to the model of Thorneley and Lowe suggests that monoatomic nitrogen in the active center of the enzyme is reduced to form a nitride (N^{3-}) before the release of ammonia[7]. However, later ESEEM studies complemented the crystallographic results showed the presence of an interstitial carbon instead of a nitrogen atom[8]. Later is a clear example of the complexity of the mechanism of reduction of N_2.

As an effort to contribute to the unraveling reaction mechanism of nitrogen fixation, we will propose the synthesis and characterization of molybdenum (III) complexes with ligands oxygen and nitrogen electron donors.

Materials and methods

All the reactants were of an analytical grade of purity and used without further purification. However, 2-pirydinecarboxylaldehyde and diethylenetriamine which were distilled before the reactions. UV-Vis spectra were obtained in a Perkin Elmer Lambda 1050 spectrophotometer; cyclic voltammograms were performed in a Metrhom Autolab PGSTAT302N potentiostat and NMR was obtained in a Varian 400 MHz NMR Unity-Inova spectrometer.

Synthesis of tris (acetylacetonato) molybdenum (III)

The synthesis of tris (acetylacetonato) molybdenum (III) (Figure 3a) was performed according to the procedure reported previously[9] but with slight changes and implementing two types of synthesis by changing the presence of inert (He) atmosphere by air atmosphere. In a general procedure, 100 mg of hexacarbonyl molybdenum (0) were dissolved in 5ml of acetylacetone. This mixture was stirred and refluxed for 2 hours, after that, the reaction was kept in a warm bath at 100 °C for 1 hour more, and then let it cooled at RT. Later, the solvent was removed and finally the solid was sublimated at around

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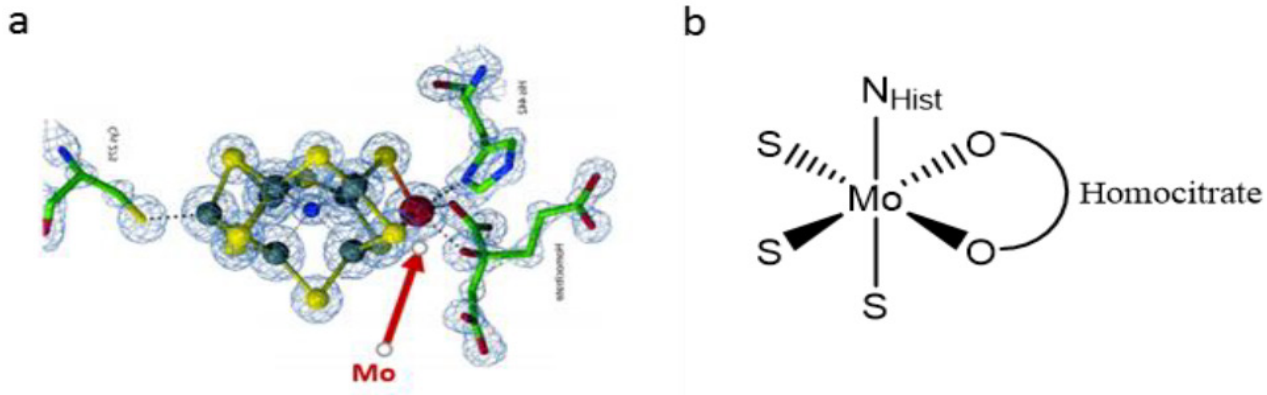

Figure 1. a) FeMo cofactor representation of A. vinelandii. Taken from Ref. 7. b) Octahedral geometry of molybdenum in the cofactor.

150°C until the remnants of hexacarbonyl molybdenum do not sublimate anymore. The later procedure was performed under the exclusion of air by helium atmosphere and air without inert atmosphere.

Synthesis of the ligand

The synthesis of penta-amine ligand 1,9-bis(2'-pyridyl)-2,5,8-triazonane was carried out according to the procedure previously reported[10, 11]. In a round flask 25 mL of anhydrous ethanol, 2 mL (0.021 mol) of 2-pyridinecarboxaldehyde and 1.13 mL (0.0105 mol) of diethylenetriamine were placed together and the reaction mixture was heated to 55 °C with constant stirring. The reaction was followed by thin layer chromatography tests and using methanol: chloroform: hexane 1: 5: 3 mixture as an eluent to verify the disappearance of the aldehyde. Then, a catalytic (Pd/C) hydrogenation of the imine obtained in such synthesis was performed (Figure 2). The reduced polyamine pentadentate ligand was used without the formation of the hydrochloride as is described in (10, 11).

Synthesis of the complex

In a round flask, 25 mL of anhydrous ethanol, 2,5 mL of a 0,42 M of the ligand and 414 mg (1,05 mmol) were placed and stirred at 60 ºC during 10 hours. After this time, a yellow-brown solid was obtained.

Results and Discussion

Two procedures were performed for the synthesis of tris (acetylacetonato)molybdenum(III), yields for both procedures (65% He, 25% air) and purity of the final product showed that helium atmosphere provides better results probably because inert sphere prevents the formation of oxo-molybdenum species.

Obtained product from the reaction under inert atmosphere was a dark purple solid (Figure 3b) with the physical characteristics of the previously reported for such compound, a melting point of 225-226 ºC compared with those obtained in the literature[9] indicates the success in the synthesis of the desired molybdenum complex.

From the reaction of 2-pirydinecarboxylaldehyde and diethylenetriamine we obtained the condensation product (imine) and the corresponding reduced amine via catalytic hydrogenation with 81% of yield. The obtained compound was characterized using NMR-H[1] and assigned unambiguously to the pentadentate ligand (Figure 4).

The yellow-brown product obtained from the reaction between tris (acetylacetonate)molybdenum(III) and the pentadentate ligand 1,9-bis(2'-pyridyl)-2,5,8-triazonane presents a UV-Vis electronic spectra with three maximum in the UV region, two of them at high energy (206, 260 nm) with large molar extinction coefficients (7,760 and 5,560 $M^{-1}cm^{-1}$) that corresponds to charge transfer electronic transitions. The other transition is located at 309 nm and has a lower extinction coefficient (890 $M^{-1}cm^{-1}$) that could be assigned to a d-d transition. In the case of our product, we expect a hexacoordinated octahedral complex, in which, five of the coordination positions are occupied by the pentadentate ligand and a labile solvent molecule in the sixth position. Thus, for an octahedral d^3 High Spin species, we expect three electronic transitions following the Tanabe-Sugano diagram[12], however, as in the case of the d^3 first row transition metals, the third transition $^4T_{1g}(P) \leftarrow {}^4A_{2g}$ for Mo (III) is expected to be at high energy and then obscured by the charge-transfer transitions. By the other hand, the literature is reported that the second and the third electronic transitions for Mo (III) HS are located in the UV region. For example, in the case of the $[Mo(H_2O)_6]^{3+}$ such transitions are located at 310 nm for the second one $^4T_{1g}(F) \leftarrow {}^4A_{2g}$ and 380 nm for the first one $^4T_{2g} \leftarrow {}^4A_{2g}$[13]. In our case, we can assign the signal at 309 nm as the first electronic transition for the d^3 species; however, the second transition is most probably obscured by the charge transfer located at 260 nm (Figure 5a).

From cyclic voltammetry (Figure 5b), we observed a reversible process and determined a midpoint E_m = 0,07 V (Fc/Fc^+) for our complex, such redox potential is in the same order that other for octahedral Mo (III) complexes, for example $MoCl_3(pyridine)_3$ shows an E^o = 0,49 VSCE [Mo(III)/Mo(IV)][14].

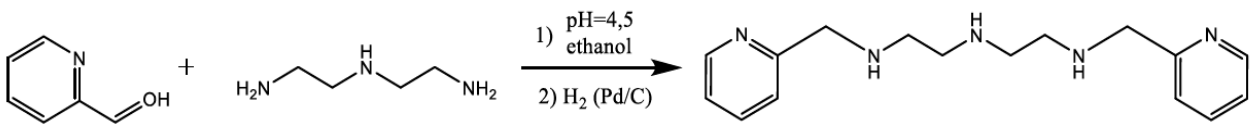

Figure 2. The reaction of Schiff base formation.

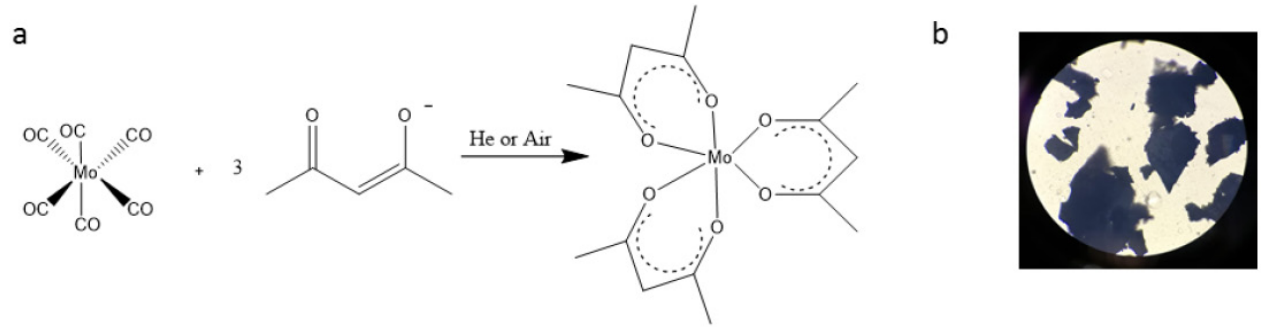

Figure 3. a) Synthesis reaction of tris (acetylacetonato) molybdenum (III) and b) Dark purple crystals obtained.

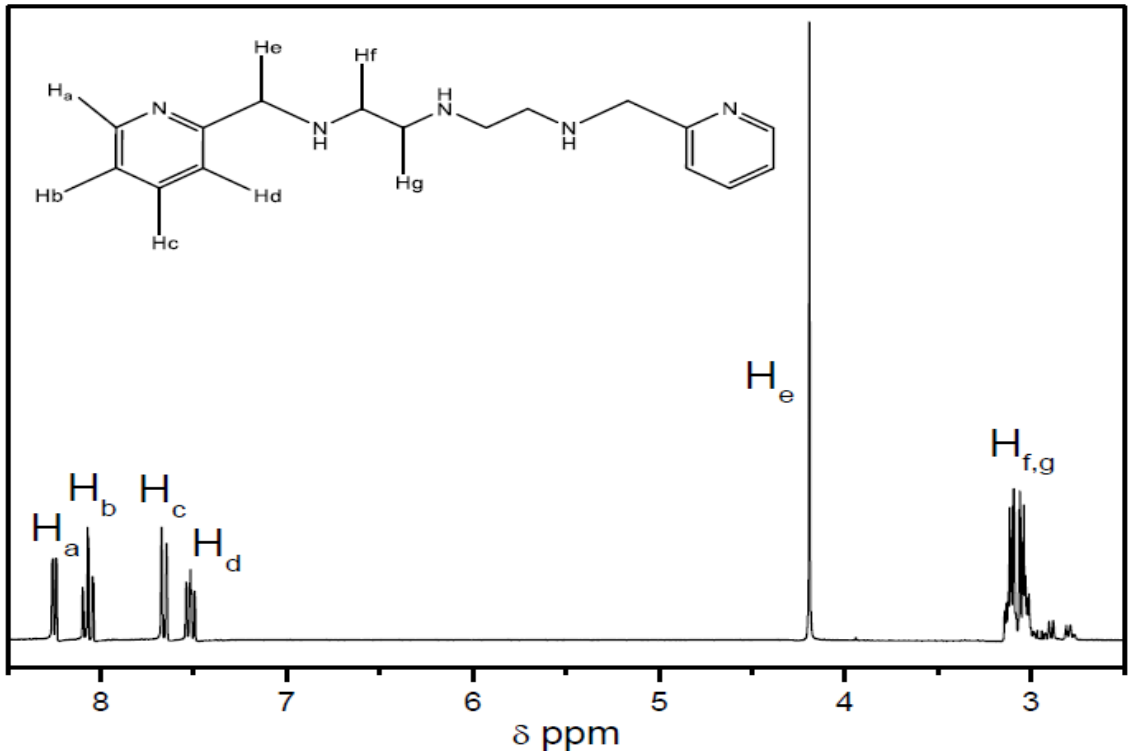

Figure 4. 400 MHz ^{1}H NMR spectra of the pentadentate ligand in D_2O.

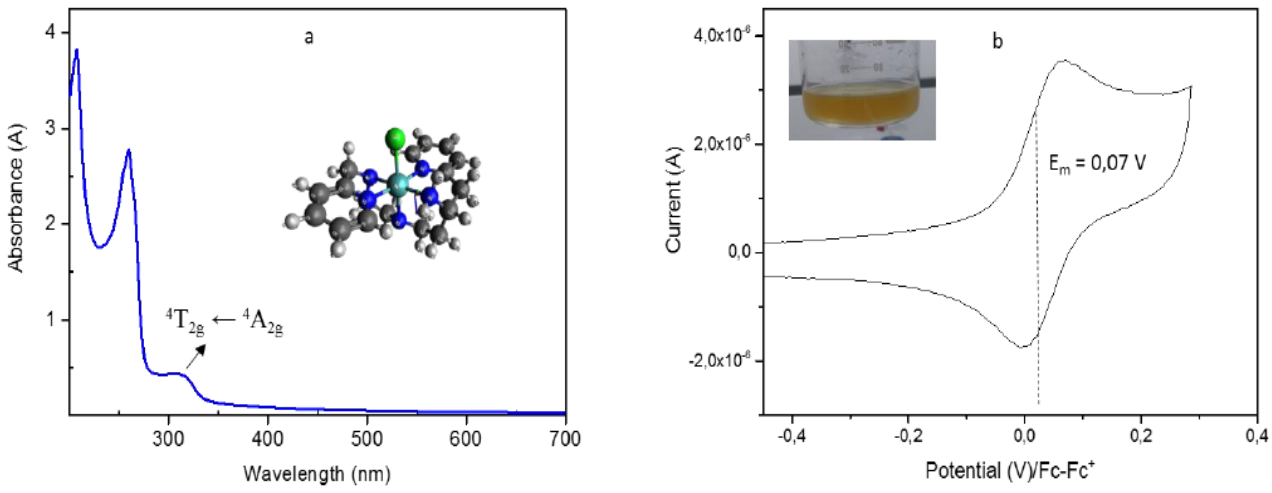

Figure 5. a) UV-vis spectrum and b) voltammogram of the molybdenum-polydentate complex in EtOH, 50 mV/s.

Conclusions

We performed the synthesis and partial characterization of a molybdenum complex coordinated with a pentadentate ligand. UV-Vis and electrochemical studies helped us to determine the oxidation state of Mo as 3+. The strategy of the synthesis was to have a complex with molybdenum in an octahedral environment with a pentadentate ligand and one labile position occupied by a solvent molecule. Such a labile position is expected to be a good site for the coordination of dinitrogen to induce the redox chemistry (nitrogen fix) between molybdenum and N_2. Further characterization of the molybdenum complex is required to perform the next step of the biomimetic system. The reactivity of the synthesized compound with N_2 is in the process of evaluation.

Acknowledgements

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Bibliographic references

1. Warneck P. Nitrogen Compounds in the Troposphere. In: Chemistry of the Natural Atmosphere. San Diego, CA: Academic; 1988. p. 422–83.
2. Leigh G.J. Haber-Bosch and Other Industrial Processes. In: Catalysts for Nitrogen Fixation Nitrogen Fixation: Origins, Applications, and Research Progress. Smith B.E., Richards R.L., Newton W.E., editors. Dordrecht: Springer; 2011.
3. Erisman J.W., Sutton M.A., Galloway J, Klimont Z, Winiwarter W. How a century of ammonia synthesis changed the world. Nature Geoscience. 2008;1(10):636–9
4. Kim J, Rees D. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science. 1992. Sep18; 257(5077):1677–82.
5. Bjornsson R, Delgado-Jaime MU, Lima FA, Sippel D, Schlesier J, Weyhermüller T, et al. Molybdenum L-Edge XAS Spectra of MoFe Nitrogenase. Zeitschrift für anorganische und allgemeine Chemie. 2014; 641(1):65–71
6. Eizawa A, Nishibayashi Y. Catalytic Nitrogen Fixation Using Molybdenum–Dinitrogen Complexes as Catalysts. In: Nitrogen Fixation Topics in Organometallic Chemistry. Nishibayashi Y., editors. Springer, Cham; p. 153–9
7. Einsle O. Nitrogenase MoFe-Protein at 1.16 A Resolution: A Central Ligand in the FeMo-Cofactor. Science. 2002Sep6; 297(5587):1696–700.
8. Spatzal T, Aksoyoglu M, Zhang L, Andrade S, Schleicher E, Weber S, Rees D, Einsle O. Evidence for Interstitial Carbon in Nitrogenase FeMo Cofactor. Science. 2011:334:940
9. Larson ML, Moore FW. Synthesis and Properties of Molybdenum(III) Acetylacetonate. Inorg Chem. 1962;1(4):856–9.
10. Raleigh C. J.; Martell A. E. Inorg. Chem. 1985; 24:142-148.
11. Saucedo-Vázquez J.P., Kroneck P.M.H., Sosa-Torres M.E. The role of molecular oxygen in the iron(III)-promoted oxidative dehydrogenation of amines. Dalton Transactions. 2015;44(12):5510–9
12. Huheey J.E., Keiter E.A., Keiter R.L. Appendix G: Tanabe-Sugano Diagrams. In: Inorganic chemistry: principles of structure and reactivity. 4th ed. New York, USA: Harper Collins College Publishers; 1993. p. A38–A39.
13. Ardon M, Pernick A. J. Molybdenum aquo ions in solution. Less Common Metals. 1977:54(1):233-241
14. Millar M. J. Am. Chem. Soc. Stable Monomeric Complexes of Molybdenum(III) and Tungsten(III). 1982:104:288-9

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RESEARCHS / INVESTIGACIÓN

Synthesis of new Carnitine Palmitoyltransferase I inhibitors derivatives of C75.

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Abstract: Carnitine Palmitoyltransferase (CPT1) is an enzyme that catalyzes the transport of fatty acids from the cytosol into the mitochondria. CPT1 inhibition in the hypothalamus increases fatty acid levels, which produces an increased expression of anorexigenic neuropeptides, a sign of satiety. C75 acts as an antiobesity predrug. *In vivo* C75, is converted into C75-CoA adduct, which is a potent inhibitor of CPT1 and produces a loss of appetite and body weight. In this work, we present three new derivatives of C75, where the carboxylic group is replaced by a carnitine unit, malonic group, and a hydroxyl group with changes from *trans* to *cis* relative stereochemistry. Our results suggest that introducing a bigger group than carboxylic in β position or *cis* relative configuration of the lactone leads to a decrease of CPT1 inhibitory activity.

Key words: C75, CPT1, Carnitine Palmitoyltransferase 1 inhibitor, α-Methylene-γ-butyrolactones.

Introduction

Obesity is a global problem that is increasing at epidemic rates. According to the World Health Organization, in 2016, more than 1.9 billion adults were overweight, and more than 650 million are classified as truly obese[1]. Obesity is a risk factor for health as it is related to serious diseases such as type 2 diabetes mellitus, cardiovascular and Alzheimer's disease, and even some types of cancer[2]. Carnitine palmitoyltransferase 1 (CPT1), is an enzyme belonging to the family of carnitine acyltransferases whose function is to transport the long chain fatty acids coupled with coenzyme A (LCFA-CoA), from the cytosol to the mitochondria where LCFA are β-oxidized to satisfy the need for the energy required in the body[3]. Energy homeostasis is specifically regulated by the hypothalamic neurons, which can detect increased LCFA levels and regulate food intake by modulating appetite[4]. The inhibition of hypothalamic CPT1 increases cytosolic LCFA-CoA levels of hypothalamic neurons, which triggers the mRNA expression of anorexigenic neuropeptides and decreases the mRNA expression of orexigenic neuropeptides. This is a signal of satiety and nutrient abundance which produces a loss of appetite[5]. The activity of CPT1 can be modulated by its physiological inhibitor malonyl-CoA, which controls the balance between synthesis and oxidation of LCFAs[6].

C75 is an α-methylene-γ-butyrolactone, which acts as an appetite suppressor when it is coupled with coenzyme A (C75-CoA) by inhibiting CPT1 enzyme. Studies *in vivo* showed that injection of free C75 is transformed into C75-CoA and causes suppression of appetite and loss of body weight in animals[5]. The majority of the studies were carried out with a racemic mixture of C75, however (+)-C75-CoA enantiomer is responsible for the strong inhibition of CPT1 and a decrease in food intake in animals[7]. Herein, we show the synthesis of some racemic C75 derivatives and the evaluation of their CPT1 inhibitory activity with the aim of better understanding the structural requirements for stronger CPT1 inhibition.

Materials and methods

The organic synthesis was carried out by standard procedures. A thin layer, column chromatography, melting point, NMR, IR, and high-resolution mass spectrometry were used to characterize and identify the obtained products (*data not shown*).

The anorexigenic activity of the C75 derivatives was tested *in vivo* in Sprague-Dawley rats, with intracerebroventricular (i.c.v.) administrations[5]. To perform *in vitro* CPT1 inhibitory studies, all derivatives of C75 were previously converted into coenzyme A adducts. The determination of the CPT1 inhibitory activity was carried out using a radiometric method[7].

Results

We started the design of new derivatives of C75 with changes in the β position of lactone. Since malonyl-CoA is a physiological inhibitor of CPT1 and carnitine is a substrate of that enzyme, we synthesized two derivatives which substitute carboxylic group by malonic and carnitine moiety. On the other hand, the importance of relative lactone stereochemistry is unknown, and *cis*-C75 is unstable due to the migration of exocyclic double bond into endocyclic. Thus, we synthesized an alcoholic derivative of *cis*-C75, which can be compared with the already reported trans compound (UB006)[8].

The synthesis of the C75 derivatives is initiated by the reduction using BH_3: SMe_2 of the advanced acid mixture reported previously in the synthesis of C75[9] with a protected double bond as selenoether.

The mixture of alcoholic diastereomers could be separated with fewer difficulties than acids and were the straining point in the synthesis of designed derivatives. The final alcoholic product (±)-*cis*-UB006 where obtained straightforwardly by recovering the exocyclic double bond in oxidative conditions.

The malonic derivative (±)-UB001 was synthesized in

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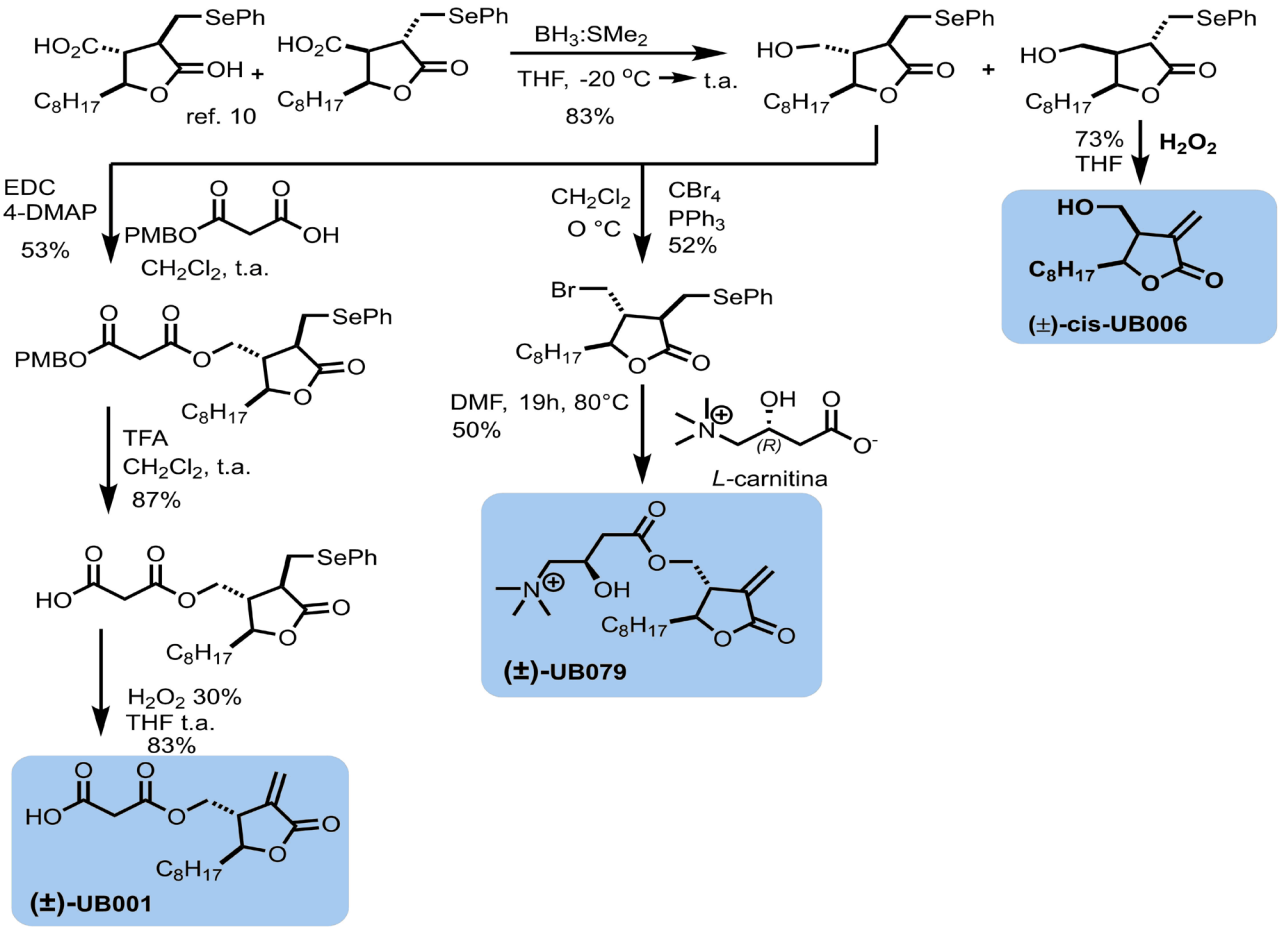

Figure 1. Synthesis of three new derivatives of C75.

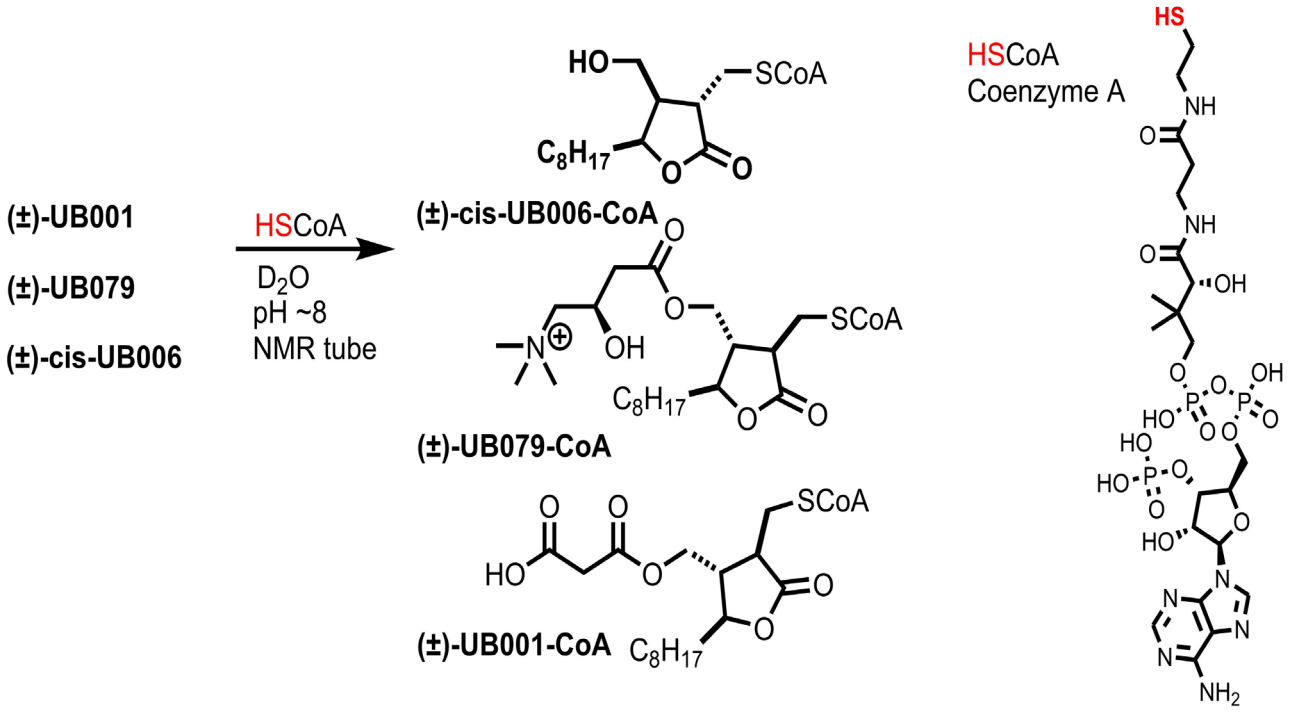

Figure 2. Preparation of the adducts of derivatives with coenzyme A.

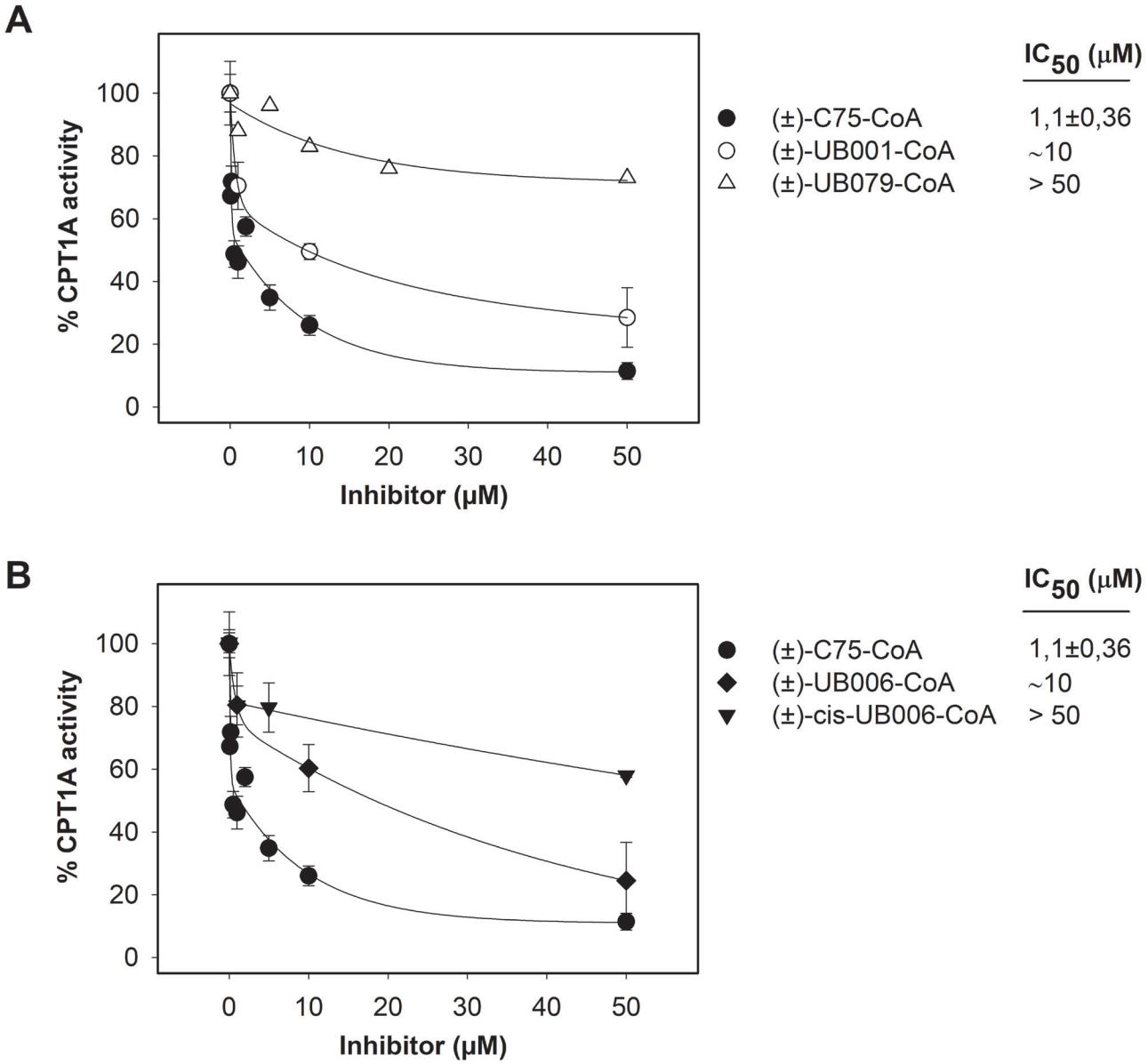

Figure 3. Effect of C75 derivatives on CPT1 activity was assayed in the presence of increasing concentrations of UB001-CoA, UB079-CoA (A), UB006-CoA, *cis*-UB006-CoA (B). C75-CoA was used as a positive control. The results are represented as the mean ± S.E.M.

three steps, condensation of starting *trans* alcohol with a monoprotected malonic acid using carbodiimide and 4-DMAP as a catalyst. Next, the benzyl group was removed with TFA. Finally, under oxidizing conditions, the double bond of the lactone was recovered obtaining the derivative UB001.

The last derivatives with carnitine moiety were obtained in two steps. The first hydroxyl group was replaced by bromine using Appel reaction conditions, and then *L*-carnitine was coupled. The reaction required relatively high temperature and in the same step double bond was formed indicating that selenoether is unstable during the heating. The product called UB079 is a mixture of diastereomers due using enantiopure carnitine, and this mixture was used for preliminary CPT1 activity studies.

To test the inhibitory power *in vitro* of the synthesized derivatives, these must be previously converted into their coenzyme A adducts. The synthesis of the coenzyme A adduct is carried out by mixing the derivative and the HSCoA in D_2O at a pH ~ 8 in an NMR tube and reaction was finished before 4h in all cases.

Once coenzyme A adduct of the derivatives was prepared, the inhibitory activity of these compounds was tested *in vitro*. The malonic derivative, UB001-CoA showed a decrease in the inhibitory activity concerning C75. Whereas, the carnitine derivative, UB079-CoA, was inactive in the inhibition of CPT1 at concentrations IC_{50} > 50 mM. On the other hand, it is observed that the alcoholic analogous, compound (±)-cis-UB006-CoA, present much worst inhibitory capacity than C75-CoA and *trans* UB006-CoA.

Of the three derivatives presented, the compound UB001-CoA was selected for *in vivo* study. For the analysis, the free C75 (positive control) and UB001 were administered by i.c.v. Injection as well as a vehicle without any drug as a control. Then, body weight and food intake were measured. The UB001, which present about 10-fold decrease of inhibitory *in vitro* activity, compared to C75, present complete loss of appetite suppressing activity (Figure 4B) when administered at the same concentration as C75 i.c.v. and did not cause a loss of body weight (Figure 4A).

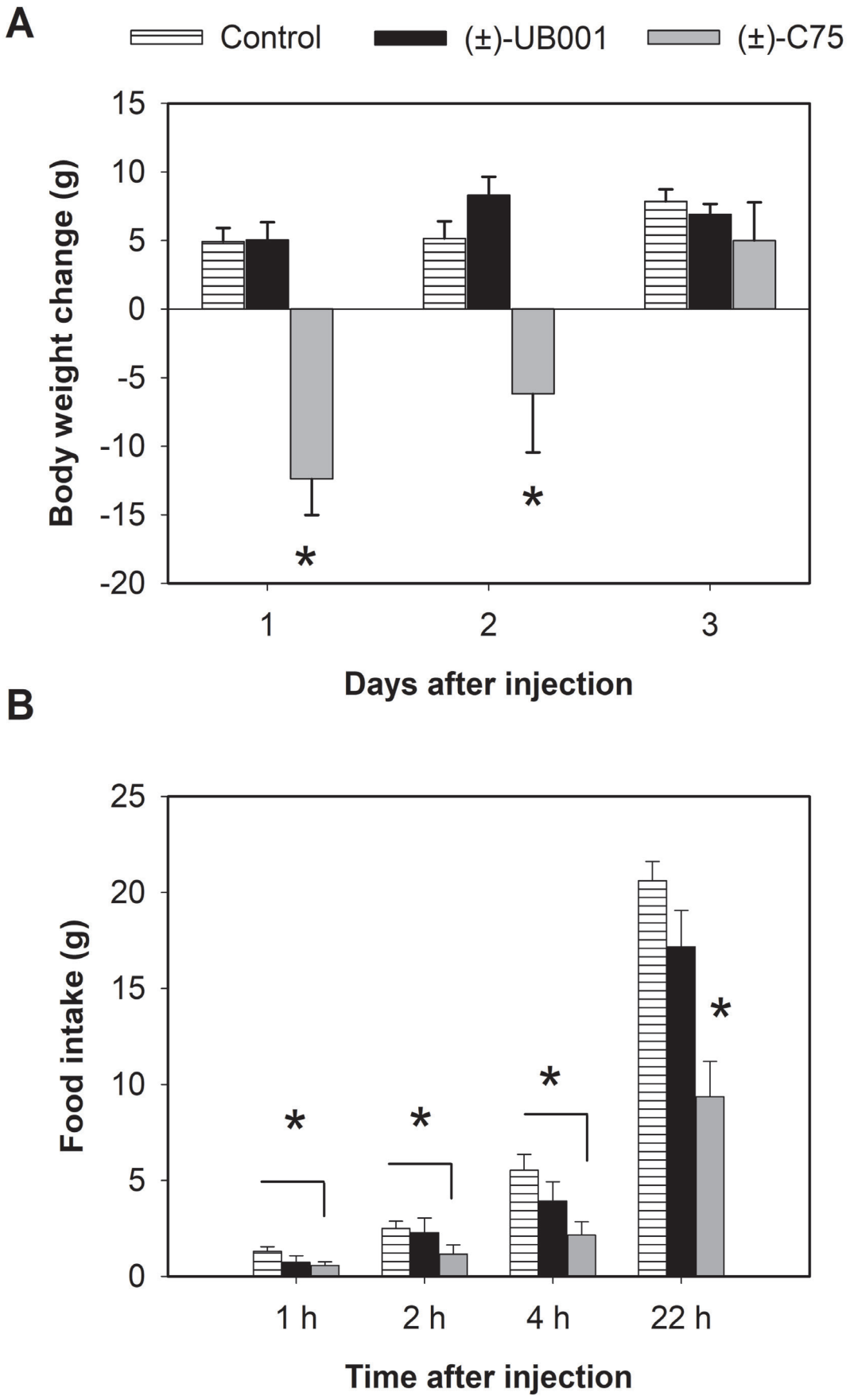

Figure 4. Effect of UB001 on body weight and food intake. (A). Accumulated food intake was measured after 1, 2, and 22 h after the injection (B). The results are expressed as the average ± S.E.M.

Conclusions

WWe conclude that the groups tested in the β position of the derivatives, the carnitine unit in UB079 and the malonic group in UB001 cause the loss of CPT1 inhibitory activity and seems that active center of that enzyme does not support bulkier group than carboxylic on the β position of the lactone. On the other hand, the change of *trans* to *cis* relative stereochemistry produces a loss of CPT1 inhibitory activity.

These new findings suggest that the length of the spacer between coenzyme A and the carboxylic group should be less than seven bonds, and it will be considered in future studies of CPT1 inhibitors derivatives of C75.

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Bibliographic references

1. Obesity and overweight. World Heal. Organ. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed 24 Mar2019).
2. Tseng Y-H, Cypess AM, Kahn CR. Cellular bioenergetics as a target for obesity therapy. Nat Rev Drug Discov 2010; 9: 465–482. doi:10.1038/nrd3138.
3. Thupari JN, Landree LE, Ronnett G V, Kuhajda FP. C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. 2002; 99: 9498–9502.
4. Wolfgang MJ, Lane MD. The Role of Hypothalamic Malonyl-CoA in Energy. 2006; 281: 37265–37269. doi:10.1074/jbc.R600016200.
5. Mera P, Bentebibel A, López-Viñas E, Cordente AG, Gurunathan C, Sebastián D et al. C75 is converted to C75-CoA in the hypothalamus, where it inhibits carnitine palmitoyltransferase 1 and decreases food intake and body weight. Biochem Pharmacol 2009; 77: 1084–1095. doi:10.1016/j.bcp.2008.11.020.
6. López M, Lelliott CJ, Vidal-Puig A. Hypothalamic fatty acid metabolism: a housekeeping pathway that regulates food intake. Bioessays 2007; 29: 248–61. doi:10.1002/bies.20539.
7. Makowski K, Mera P, Paredes D, Herrero L, Ariza X, Asins G et al. Differential pharmacologic properties of the two C75 enantiomers: (+)-C75 is a strong anorectic drug; (-)-C75 has antitumor activity. Chirality 2013; 25: 281–7. doi:10.1002/chir.22139.
8. Makowski K, Mir JF, Mera P, Ariza X, Asins G, Hegardt FG et al. (–)-UB006: A new fatty acid synthase inhibitor and cytotoxic agent without anorexic side effects. Eur J Med Chem 2017; 131: 207–221. doi:10.1016/j.ejmech.2017.03.012.
9. Sanchez C, Makowski K, Mera P, Farras J, Nicolas E, Herrero L et al. Convenient synthesis of C75, an inhibitor of FAS and CPT1. RSC Adv 2013; 3: 6564–6571. doi:10.1039/C3RA40913A.

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RESEARCHS / INVESTIGACIÓN

Comparative analysis of the effect of some organic manure on soil microorganisms.

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Abstract: This study showed that the abundance of different microbial groups was general in soil with amendments in comparison to soils without amendments. It was discovered that soils with organic manures were rich in bacteria and fungi diversity when compared with soil without organic manure, which recorded low microbial counts. *Escherichia coli* and *Staphylococcus aureus* were widely distributed in this study. The soil treatment which had Cow dung showed highest microbial count and heights for growth of maize seeds, and the compost manure soil treatment followed this, and the poultry manure soil treatment was next. This suggests that the higher the fertility in amended soils is revealed in the heights of the maize plant grown and colony counts. Plant height recorded under various amendments showed significant differences ($p<0.05$).

KeyWords: Organic manure, microorganisms, growth heights.

Introduction

One of the oldest ways to enhance soil quality for agricultural sustainability is to add to the organic amendment through increasing of manure[1]. Applying organic fertilizers is one of the critical technical ways of improving soil fertility. Organic manure provides basic nutrients for crops and improves soil physico-chemical properties; it is also able to enhance soil microbial activity of the soil, such as improving the activity of soil enzymes and increasing soil microbial biomass[2, 3]. Various environmental stresses and agricultural practices affect the quantity and nature of microorganism's species, as well as the number of individuals in the soil[5]. Environmental conditions for soil organisms favoring certain functional groups are created by different cultivation practices[6]. The absorption of fertilizing substances has a high impact on soil microbial communities which are important to agro-ecosystems, involved in key roles, such as soil aggregate formation, soil humus formation, nutrient cycling, decomposition of various compounds and other transformations[7, 8, 9]. Application of organic matter is important to cultivated soil because it enhances the rate of soil degradation and the decomposition of soil organic matter [10, 11, 12].

An example of a controlling input to the soil system and the processes within it are nutrients, for example, carbon content, cycling of nitrogen and phosphorus affect soil dynamics and agricultural production[13]. Application of organic nitrogen sources increases soil microbial population[14] compared to the inorganic form. Microorganisms and its function in soil show the soil quality and plant productivity[15]. The increasing cost of chemical fertilizers, reduction of soil micronutrients, environmental and health hazards and exorbitant prices for organically produced crops, the use of organic manure in farming has attracted a lot of attention recently[16]. Manures from livestock and poultry are necessary ways of taking back nutrients into the soil. It is better to use organic manure than mineral fertilizer due to the high cost of the latter. Organic manures can be got for free, but inorganic fertilizers can never be obtained free[17]. Crew and Peoples (2004)[18] stated that although chemical fertilizers give out their nutrients faster into the soil for productivity, their effects have resulted in negative effects in the sustainability of production. According to Savci (2012)[19], the bad effects of chemical fertilizers on the soil are not immediately seen because soils have strong buffering power due to their components, but the toxic substances are taken up by crops and cause harm to humans and animals who feed on them.

Materials and methods

Site description

The study was conducted at Michael Okpara University of Agriculture Umuahia, Abia State, Nigeria. The farm area is an agricultural soil with the typical loamy soil which is easy to cultivate on.

Experimental design

Four treatments using different manure applications were designed as follows: Poultry manure + soil (A1), Cattle manure + soil (A2), Compost manure + soil (A3), and soil alone, i.e. no manure (CT). These treatments were put into different perforated buckets respectively, and 3-4 seeds of maize were planted. The soil was gotten from agricultural farmland in the Michael Okpara University of Agriculture Umudike. The growth of the maize seedlings was monitored for 30 days by observing the heights from each treatment soil samples were taken from the topsoil (0-20cm soil).

Physico-chemical analyses of the Soil

Soil samples were also cooled, air-dried analyzed for exchangeable potassium, moisture content, pH, temperature, organic carbon, total nitrogen, and available phosphorus. These tests were also done for different treatments.

Soil microbial biomass

The total heterotrophic plate count and total fungal counts were taken on nutrient and Sabouraud dextrose agar plates respectively after incubation for 24 - 48 hours and 3-5 days. The isolates were sub-cultured and stored from which biochemical tests for characterization and identification was done for microorganisms.

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Results and Discussion

Table 1 shows that treatment A2 had the highest growth at day 30 as compared to other treatments, this could either be as a result of the type of meal these animals are fed with, which was reflected in their feces. Lin et al. (2010)[20], who documented that other types of manure promoted higher peanut yield than chicken manure. Fertilization is the most common management of agricultural soils. Organic and inorganic fertilizers are primarily used to increase crop yield[21]. Soil fertility is a necessary type of renewable natural resource[22]. A fertile soil leads to an increase in profit for farmers[23]. To maintain and increase crop productivity and sustain agriculture for the long-term, effective, and efficient approaches to slowing nutrients, removal, and returning of nutrients to the soil will be required[24]. The maintenance of soil fertility means giving back to the soil the nutrients removed from it by harvests, runoff, erosion, leaching, and other loss pathways[25].

Samples Heights (CM)				
Days	Cattle manure + soil	Poultry manure + soil	Compost manure + soil	soil alone
	A2	**A1**	**A3**	**CT**
0	0.00	0.00	0.00	0.00
5	5.21	4.23	4.83	3.41
10	14.45	9.72	10.39	8.26
15	28.01	14.41	17.75	12.34
20	43.34	27.09	34.53	29.65
25	52.74	36.18	46.51	31.94
30	68.43	53.00	60.00	41.00

Table 1. Shows four treatments using different manure applications, The growth of the maize seedlings was monitored for 30 days.

Table 2 shows that cow dung treatment (A2) had the highest total heterotrophic plate count followed by A3, then A1 and next is CT, but there was a decrease in fungal counts. This must be as a result of the diet intake of these Cattles, which is reflected in their feces. It appears that higher bacterial counts for organic manure produced better plant growth.

Bacteria isolated were *E. coli, P. aeruginosa, Klebsiella spp., Salmonella spp., Staphylococcus sp., Shigella sp., Serratia sp.*, while fungi isolated were *A. niger, A. flavus, Rhodotorula spp., Rhizopus stolonifer*, source of plant nutrients is soil microbial biomass, and it is highly correlated with soil organic carbon[26]. Soil microbial activity can be enhanced, and it is associated with high available nitrogen for plants[27].

The community of microorganisms responds to changing environmental conditions by varying individual activity[28]. Factors such as soil humidity, pH, fertilization pre-determine the number and species composition of microorganisms in the soil. Stimulation of bacteria and Actinomycetes reducing the fungal population can be achieved with the supplement of organic fertilizers. Changes in soil properties or plant and animal communities come after changes in microbial communities, and such providing an early sign of soil improvement or an early warning of soil deterioration[29]. The nutrient release, which is as a result of mineralization processes in soils gives rise to plant production using organic farming. This means that a functional soil microflora and a good quantity of available nutrients have importance in organic farming. Every farmer's motive is to fertilize the soil instead of the plant to ensure adequate nutrient mineralization present to meet his profits[29]. An important reservoir of plant nutrients such as nitrogen and phosphorus is microbial biomass, which is among the most labile pools of organic matter[30]. This biomass, when responding to environmental changes, can have major effects for the availabilities of nutrients[31].

The pH of these treatments is right for the growth of these crops. The best Soil pHs for overall availability of nutrients plant growth and microbial processes is slightly acidic to neutral (6.0-7.5). The amount of various constituents in the manure affects soil pH. There is a high concentration of NH_4-N in Liquid

Sample Code	THPC (X 10^5)	TFPC (X 10^3)
A2	5.3	2.2
A3	3.6	1.7
A1	2.8	1.4
CT	1.7	1.2

Table 2. Total count of heterotrophic plaques.

and poultry manures, and low amounts of organic matter; it is possible that NH_4- forming synthetic fertilizers, liquid, and poultry manures can reduce soil pH. Applying solid Cattle manure shifts the soil pH to neutral in acidic[32] and alkaline soils[33, 34] and this strengthens the availability of nutrients, for example, Phosphorus and micronutrients. The shift towards neutrality is best for the growth of the plant and many useful processes of microorganisms. Manure in solid form is a source

Sample	Exchangeable Potassium (K)	Moisture content (%)	pH	Temperature (°C)	Organic Carbon	Total Nitrogen	Available Potassium (P)
A1	1.32	11.11	6.6	34.0	1.10	1.26	7.70
A2	2.03	22.00	6.7	29.0	2.22	0.25	6.20
A3	2.15	20.43	6.8	32.0	3.21	0.21	6.30
CT	0.23	6.18	6.9	28.0	0.21	0.11	4.21

Table 3. Shows four treatments using different manure applications, The growth of the maize seedlings was monitored for 30 days.

of nutrients and an important soil conditioner[35].

Many soils take in potassium in a way that is sufficient enough to stop leaching, but not enough to plant roots. Soil's physico-chemical properties, soil microbial biomass, nitrogen contents, and phosphorus of soils can be improved using Organic fertilizers[2, 4]. Organic matter makes the physical characteristics of the soil better and adds the important plant nutrients to the soil[1].

Biological Oxygen Demand (BOD) for modern bathroom was higher than that of local bathroom. Chemical Oxygen Demand, Total Dissolved Solid, Total Suspended Solids, Conductivity and Dissolved Oxygen were higher for local bathrooms than modern bathrooms. Eze *et al.*, (2015)[36] recorded pH, 5.95 ± 0.41 to 6.30 ±0.42; Temperature, 26.6 ± 0.5ºC to 27.2 ± 1.6ºC; Conductivity, 34.9 ± 1.0µS/cm 106.0 ± 2.0µS/cm; total dissolved solids, 100.0 ± 3.0mg/L 600.0 ± 5.0mg/L; total suspended solids, 265.0 ± 4.0mg/L 348.0 ± 10.0mg/L, dissolved oxygen (DO), 10.35 ± 0.83mg/L - 31.6 ± 2.0mg/L, biochemical oxygen demand (BOD), 3.1 ± 0.04mg/L -14.0 ± 0.5mg/L; chemical oxygen demand (COD), 10.0 ± 0.5mg/L 20.0 ± 1.0mg/L.

In research by Noutsopoulos *et al.* (2015)[37], they recorded higher COD counts for influent sample in system A than system B. Nga'Ng' a recorded higher electrical conductivity for greywaters than drinking water and lower counts for DO and pH. Kotut et al. (2011)[38] also recorded mean counts from different greywater samples: for conductivity, 599.7-654.5 µS/cm^2, DO 3.5-5.2mgL^{-1}, pH 8.2-9.2, Temperature, 23.8-26.3, BOD5 (mgL^{-1}) 560-6250, Total Coliform counts (10^6) 2.3-6.5, Faecal Coliforms (10^5) 0.34-2.9. Wijaya and Soedjino (2018)[39] recorded higher counts for samples from Medokan Semamir and Genteng except for BOD, which was lower. Abedin and Rakib (2013)[40] also recorded that water from greywaters was higher than the standards given by Bangladesh (ECR, 1997)[41] and WHO guideline values (2004)[42].

Soil samples were also cooled, air-dried analyzed for exchangeable potassium, moisture content, pH, temperature, organic carbon, total nitrogen, and available phosphorus. These tests were also done for different treatments.

Conclusions

Organic manure achieves a high microbial load, high nutrient content for soil, and this leads to higher growth of crops. Farmers should be encouraged to use organic manure as a way of adding nutrients to the soil other than the use of fertilizers.

Bibliographic references

1. Faissal, A., Ouazzani, N., Parrado, J.R., Dary, M., Manyani, H., Morgado, B.R., Barragan, M.D. and Mandi, L, (2017). Impact of fertilization by natural manure on microbial quality of soil: Molecular approach. Saudi Journal of Biological Sciences, 24: 1437-1443.
2. Ren, Z.G., Chen, Y.S., Tang, F.Q. (1996) Effect of inorganic fertilizer combined with organic manure on the microflora and enzyme activities in paddy soil. Plant Nutrition and Fertilizer Science, 2: 279-283.
3. Bardgett, R.D., Hobbs, P.J. and Jarvis, S.C. (1999). Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 31: 1021–1030.
4. Lv, W.G., Huang, Q.W., Sheng, Q.R. (2005). The effect of organic fertilizer and organic-inorganic fertilizer application on soil enzymes activities during watermelon growing period. Journal of Nanjing Agricultural University 28: 67-71.
5. Oehl, F., Sieverding, E., Ma¨ der, P., Dubois, D., Ineichen, K., Boller, T. and Wiemken, A. (2004). Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. Oecologia, 138: 574–583.
6. Sun, R.I., Zhao, B.Q., Zhu, L.S. (2003). Effects of long-term fertilization on soil enzyme activities and its role in adjusting-controlling soil fertility. J. Plant Nutrition and Fertilizer Science, 9: 406-410.
7. Lynch, J.M. and Bragg, E. (1985). Microorganisms and soil aggregate stability. Adv Soil Sci 2:133–171.
8. Zak, J.C., Willig, M.R., Moorhead, D.L. and Wildman, H.G. (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem, 26:1101–1108.
9. Wu F, Dong M, LiuY,MaX,An L,Young JPW, FengH (2011) Effects of slong-term fertilization on AM fungal community structure and Glomalin-related soil protein in the Loess Plateau of China. Plant Soil, 342:233–247.
10. Chen, H.Q., Marhan, S., Billen, N. and Stahr, K. (2009). Soil organic-carbon and total nitrogen stocks as affected by different land uses in Baden-Württemberg (southwest Germany). J. Plant Nutr. Soil Sci., 172: 32–42.
11. Domínguez, J., Aira, M.aand Gomez-Brandon, M. (2010). Vermicomposting: earthworms enhance the work of microbes. In Microbes at Work: From Wastes to Resources, Eds. Insam H, Franke-Whittle I, Goberna M. 93–114. Springer-Verlag, Berlin.
12. Liang B, Yang X, He X, Murphy D, Zhou J 2012: Long-term combined application of manure and NPK fertilizers influenced nitrogen retention and stabilization of organic C in Loess soil. Plant Soil, 353:s 249–260. doi:10.1007/s11104-011-1028-z.

13. Barber, S.A. (1995). Soil nutrient bioavailability: A Mechanistic Approach. 2nd edn. Wiley, New York.
14. Krishnakumar, S., Saravanan, A. Natarajan, S.K. Veerabadran, V., and S. Mani, S. (2005). Microbial Population and Enzymatic Activity as Influenced by Organic Farming. Res. J. Agr and Biol. Sci. 1: 85-88.
15. Latour, A., Corberand, T.S., Laguerre, G., Allard, F., and Lemanceau, P. (1996). The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. Appl Environ Microbiol. 62: 2449–2456.
16. Ramesh, P., Singh, M. and Subbaro, A. (2005).Organic farming: Its relevance to the Indian context. Current Science, 88: 561-569.
17. Kolavalli, S. and Adam, S. (2011). Manure use in northern Ghana: Observations from a fieldtrip.http://gssp.ifpri.info/files/2011/06/manure-use-in-northern Ghana3.docx. Accessed on 4th May 2018.
18. Crews, T.E. and Peoples, M.B. (2004). Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agric. Ecosyst. Environ. 102: 279-297. doi: 10.1016/j.agee.2003.09.018.
19. Savci, S. (2004). An Agricultural Pollutant: Chemical Fertilizer. International Journal of Environmental Science and Development, 3(1): 77-79.
20. Lin, X.J., Wang, F., Cai, H.S., Lin, R.B., He, C.M., Li, Q.H. and Li, Y. (2010). Effects of different organic fertilizers on soil microbial biomass and peanut yield. 19th World Congress of soil science, Soil solutions for a changing World Brisbane, Australia, published on DVD.
21. Creechio, C., Curci, M., Mininni, R., Ricciuti, P. and Ruggiero, P. (2001). Short-term effects of municipal solid waste compost amendments on soil carbon and nitrogen content, some enzyme activities and genetic diversity. Biol Fertil Soils, 34: 311-318.
22. Sanchez, P.A., Shepherd, K.D., Soule, M.J., Place, F.M., Buresh, R.J. and Andizae, A. M.N (1997). Soil fertility replenishment in Africa: An investment in natural resource capital. In: F.E. Buresh et al. (eds). Replenishing soil fertility in Africa ASA, CSSA, SSSA, Madison, Madison, WI, USA, 1-46.
23. Fresco, L.O and Kroonenberg (1992). Time and spatial scales in ecological sustainability. Land Use Policy, 9: 155-167.
24. Gruhn, P., Goletti, F. and Yudelman, M. (2000). Integrated nutrient management, soil fertility, and sustainable agriculture: Current issues and future challenges. Food, Agriculture and the Environment Discussion paper 32. Washington, DC: IFPRI.
25. Aune, J.B. (1993). Ecological and economical requirements for sustainable land use in sub-saharan Africa. Forum for Development Studies. 2: 211-219.
26. Dikstra, F.A., Bader, N.E., Johnson, D.W. and Cheng, W. (2009). Does accelerated soil organic matter decomposition in the presence of plants increase plant N availability? Soil Biol Biochem 41:1080-1087.
27. Tu, C., Ristaino, J.B. and Hu, S. (2006). Soil microbial biomass and activity in organic tomato farming systems: Effects of organic inputs and straw mulching. Soil Biol Biochem, 38: 247-255.
28. Novak, A., Michalceivic, W. and Jakubiszyn, B. (1993). Effect of fertilization with manure, straw and biohumus on numbers of bacteria, fungi, Actinomycetes and microbial biomass in soil. Rzecz Nauki Polskiej/ AR szczecini, 57: 101-113.
29. Fliessbach, A. and Mader, P. (2000). Microbial biomass and size-density functions differ between soils of organic and conventional agricultural system. Soil Biol. Biochem, 32: 757-768.
30. Marumoto, T., Anderson, J.P.E. and Domsch, K.H. (1982). Mineralization of nutrients from soil microbial biomass. Soil Biol. Biochem. 14: 469-475.
31. Malero, S., Porras, J., Herencia, J. and Madejon, E. (2006). Chemical and biochemical properties in a silty loam soil under conventional and organic management. Soil Tillage Res. 90 (1-2): 162-170.
32. Benke, M.B., Hao, X., O'Donovan, J.T, Clayton, G.W., Lupwayi, N.Z., Caffyn, P. and Hall, M. (2009). Livestock manure improves acid soil productivity under a cold northern Alberta climate. Can. J. Soil Sci. 90: 685-697.
33. Chang, C., Sommerfeldt, T.G. and Entz, T. (1990). Rates of soil chemical changes with eleven annual applications of Cattle feed lot manure. Can. J. Soil Sci. 70: 673-681.
34. Hao, X. and Chang, C. (2002). Effect of 25 annual Cattle manure applications on soluble and exchangeable cations in soils. Soil Sci. 167: 126-134.
35. Schoenau, J., Grevers, M., Japp, M., King, T., Lipoth, S., Qian, P. and Stumborg, C. (2005). Monitoring the long-term effects of repeated manure applications on crop production, and soil and environmental quality in Saskatchewan. ADF project No. 20010276. [Online] http://www.agriculture.gov.sk.ca/apps/asf/ADFAdminReport/20010276.
36. Eze, V.C., Onwuakor, C.E. and Mgbeokwere, E. U. (2015). Comparative Analysis of the Microbiological and Physico-chemical characteristics of Greywater Sources in Off-Campus Hostels at Michael Okpara University of Agriculture, Umudike, Abia State, International Journal of Current Microbiology and Applied Sciences, 4(8): 196-205.
37. Noutsopoulos, C., Andreadakis, A., Kouris, N., Mendrinou, P. and Mantziaras, I.D. (2015). Proceedings of the 14th International Conference on Environmental Science and Technology.
38. Kotut, K., Nganga, V.G. and Kariuki, F. W. (2011). Physico-chemical and Microbial Quality of Greywater from various Households in Homa Bay Town. The Open Environmental Engineering Journal, 4: 162-169.
39. Wijaya, I.M.W. and Soedjono, E.S. (2018). Physicochemical Characteristic of Municipal Wastewater in Tropical Area: Case Study of Surabaya City, Indonesia. IOP Conf. Series: Earth and Environmental Science. 135: 1-7.
40. Abedin, S.B. and Rakib, Z.B. (2013). Generation and Quality Analysis of Greywater at Dhaka City. Environmental Research, Engineering and Management. 2(64): 29-41.
41. Environment Conservation Rule, 1997 (in Bangla) https://www.resourcedata.org/dataset/rgi-environment-conservation-rule-1997-in-bangla-
42. World Health Organization (2004). Guidelines for Drinking-water Quality. Third Edition. Volume 1. 1-540. Geneva.

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RESEARCHS / INVESTIGACIÓN

Effect of Short-term Curcumin exposure and its Modulatory Role on Acute Cadmium Hepatotoxicity.

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Abstract: Curcumin, presents chemo-preventive, antioxidant and anti-inflammatory properties, whereas cadmium is a serious contaminant due to industrial and agricultural practices. Most of the work on curcumin deals with repeated exposures for longer durations. The present study was designed to investigate the effect of a single short-term (24 hour) curcumin treatment with FDA approved dose of 100 mg/kg body weight for humans, on Male Balb/c mice liver. Further, the modulatory role of its pre-treatment on acute cadmium hepatotoxicity was also studied. Animals treated with curcumin for 24 hours were divided into two groups, and one group was sacrificed. The other group was sacrificed after additional exposure to cadmium for 18 hours with corresponding positive and negative control groups. Oxidative stress was measured using a multi-parametric biochemical approach, and histopathological changes were studied using light and electron microscopes. Administration of curcumin for 24 hours resulted in an increase in oxidative stress in liver suggesting a pro-oxidant role, which might be due to the generation of reactive oxygen species, while post-treatment with Cd resulted in a synergistic effect on oxidative stress. Concurrent marked histological alterations were observed under the light microscope in the form of basophilic depositions, Kupffer cell hyperplasia, and lobular inflammation. Electron micrographs also revealed similar features along with pronounced damage to the endothelial cell fenestrations and bile canaliculi with crystalline deposits on hepatocytic surfaces. Therefore, it was concluded that after 24-hour exposure to curcumin, it acted as a pro-oxidant in mice liver and was not found to have an ameliorative effect on acute Cd-induced hepatotoxicity.

KeyWords: Curcumin, Cadmium, acute exposure, liver, oxidative stress, pro-oxidant scanning electron microscopy, light microscopy.

Introduction

Antioxidants play an important role to nullify the deleterious effects of free radicals such as reactive oxygen species, thereby not only protecting the cells and tissues but also regulating various pathological and physiological processes. Curcumin, derived from the rhizome of the herb *Curcuma longa* (turmeric spice), is one such antioxidant which is a bioactive hydrophobic polyphenol compound. It is a safe nutritional dietary supplement that has been widely used in traditional medicine and as a spice/coloring agent since time immemorial[1].

In India and Southeast Asia, the diverse biological functions of turmeric such as antioxidant activities, anti-inflammatory, and anti-mutagenic were realized since long, and it has been used in the treatment of inflammation, skin wounds, and tumors[2]. In recent years, there is growing evidence that curcumin is a potentially important chemopreventive agent against cancer and may play a crucial role in both the prevention and treatment of various neurodegenerative diseases such as Parkinson's and Alzheimer's disease[3].

García-Niño and Pedraza-Chaverri (2014) in a review on the protective effects of curcumin against heavy metals-induced liver damage, gave an account of the various mechanisms by which curcumin confers protection. These mechanisms included suppression of oxidative stress, inflammation, and activation of stellate cells in the liver, with concomitant up-regulation of enzymes involved in detoxification and Keap1/Nrf2/ARE pathway expression[1]. Various studies on guinea pigs, rats, monkeys, and pigs have reported that on the feeding of curcumin or turmeric there were no toxic effects[4-6]. On the other hand, Deshpande *et al.*, (1998) reported that on feeding turmeric or ethanolic turmeric extract, in high doses for long-term exposure, to mice and rats, hepatoxicity was observed in the form of profuse focal necrosis along with decreased body weights[7]. Similarly, Kandarkar *et al.*, (1998), while analyzing the effects of doses of whole spice turmeric or ethanolic turmeric extract reported being cancer protective, observed coagulative necrosis with areas of parenchymal regeneration[8].

Cadmium (Cd) is a toxic heavy metal that has no physiological function in the body and causes deleterious effects on health following both acute and chronic exposures. Moreover, Cd accumulates in the body over time as in humans its biological half-life is 17-30 years[9]. Non-occupational exposure is generally *via* consumption of food and drinking water contaminated with Cd along with cigarette smoke. Occupational exposure mainly results from numerous modes of exposure, such as $CdCl_2$ utilized in paint manufacture and Cd fume inhalation in battery industry[10]. Exposure to Cd has been reported to affect a myriad of organs in humans, but most commonly it affects kidney, lung, bone and skeletal, cardiovascular, and nervous systems[11, 12].

The liver is a major organ that is involved in metabolism and detoxification reactions, through which all substances that are absorbed by the intestine pass. The liver is known to accumulate toxins, including heavy metals such as Cd following acute or chronic exposures that result in hepatotoxicity[13-15].

Tarasub *et al.* in 2008 reported that following co-treatment

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with cadmium acetate and curcumin, curcumin was unable to ameliorate oxidative damage induced due to cadmium[16]. In another study, they reported that combined treatment of curcumin and vitamin C was more effective in protection against Cd-induced hepatic injury by scave nging free radicals[17].

Hepatotoxic effects of Cadmium and beneficial effects of antioxidant compounds such as curcumin following long terms exposures have been studied in detail in previous studies. However, there is a lacuna in knowledge related to the effect of short-term treatment of curcumin on the liver and its modulatory role on subsequent treatment with cadmium chloride ($CdCl_2$) in mice. Therefore, the present study was undertaken to investigate the effect of short-term exposure to curcumin in the liver of mice. Further, the ameliorative effects of short-term pre-treatment with curcumin in acute cadmium chloride induced hepatotoxicity would be evaluated by assessment of biochemical parameters of oxidative stress and ultrastructural alterations.

Materials and methods

Chemicals

All chemicals were of analytical grade specifications and obtained from HIMEDIA Ltd, India. Cadmium chloride (CdCl2) and curcumin were obtained from Sigma Chem. Co., St. Louis, MO, USA.

Animals and Treatments

Four to six-week-old Balb/c male mice weighing 20-25 grams were procured from the Central Animal House, Panjab University, Chandigarh. Mice were kept in cages, given food and water *ad libitum* and allowed to acclimatize for seven days, maintained at 12 hours light/dark regime before experimental use.

The animals were divided into five experimental groups, each containing seven mice and the doses was administered intraperitoneally. Curcumin dose of 100 mg/kg body weight (bw) was used, as it is the FDA approved dose for humans[18]. Curcumin was dissolved in DMSO (dimethyl sulfoxide), an amphiphilic compound that increases the permeability of the membranes, for its effective uptake by the cells[19]. A single *i.p.* Injection of 100 mg curcumin/kg bw dissolved in 100 ml DMSO was administered to the group T-II and animals were sacrificed after 24 hours of exposure, and therefore, a DMSO-treated group was also studied (group T-I). Cadmium chloride was dissolved in water and dose used in this study (0.8 mg $CdCl_2$/kg bw) was two-thirds the experimentally determined LD_{50} value for intraperitoneal exposure in mice (calculated using Sun's formula, 1963)[20] to which group T-III were exposed for 18 hours.

The groups T-IV and T-V were injected 100 ml DMSO or 100 mg curcumin/kg bw dissolved in 100 ml DMSO, respectively and after 24 hours were given another treatment of 0.8 mg $CdCl_2$/kg bw and finally sacrificed after 18 hours of the second treatment following various protocols and ethical procedures.

Hepatic Biochemical Estimations

Preparation of Homogenate

10% homogenates of liver were prepared in 50mM Tris-HCl buffer (pH-7.4) using a Potter-Elvejhem homogenizer at 0-4°C. The homogenate was used for the spectrophotometric determination (using Jenway 6305 uv/vis spectrometer) of lipid peroxidation by measuring the tissue malondialdehyde (MDA) level[21], superoxide anion-SA[22], hydroperoxides-HP[23], reduced glutathione-GSH[24], and protein content[25].

Preparation of Post Mitochondrial Supernatant -PMS

Liver homogenates were centrifuged at 9,200 rpm for 10 minutes at 4°C. The supernatant was stored at -20°C and used for the estimation of activities of Superoxide dismutase-SOD[26], Catalase-CAT[27], Glutathione peroxidase-GPx[28], Glutathione reductase-GR[29], and Glutathione-S-transferase-GST[30], by continuous spectrophotometric rate determination (using Perkin Elmer Lambda 35 UV/vis spectrometer) and protein content[25].

Histopathological studies

Tissue from the liver of mice was fixed in 10% buffered formalin and processed routinely. The blocks were embedded in paraffin wax. Sections of 5-6 µm thickness were cut by rotary microtome, stained with Haematoxylin-eosin (H&E) stain and examined under a light microscope (Leica DC 100, PC I Interface Digital Camera).

Scanning electron microscopy

The liver slices were washed with phosphate buffer and fixed in 4% glutaraldehyde in phosphate buffer. They were then dehydrated in ascending acetone grades and critical point dried through transitional fluid amyl acetate. The dried samples were fixed on metal stubs with double adhesive tape for gold sputtering. The stubs so prepared were examined using JEOL JSM 6100 Scanning Electron Microscope and captured at different magnifications to study the ultrastructural features.

Statistical analysis

Student's t-test determined significance between pair of means for control and treated groups. The data were expressed as mean ± standard error of seven mice and the level of significance considered was $P < 0.05$.

Results and Discussion

Effects of short-term curcumin treatment and pre-treatment for 24 hours, followed by acute Cd exposure for 18 hours were studied in male Balb/c mice. To investigate whether the exposure to curcumin alone or followed by Cd exposure causes hepatotoxicity resulting from oxidative stress, the levels of hepatic superoxide anion, hydroperoxides, GSH, total thiols, and MDA were measured in the liver homogenates of treated mice while activities of enzymes of the antioxidant system were measured in the PMS.

DMSO treated control group T-I was compared with the treated groups T-II to T-V. The GSH and total thiol levels in the liver of group T-I were 0.80 ± 0.03 and 31.1 ± 2.1 nmoles/mg protein, respectively. Both GSH and total thiol levels decreased in all the treated groups T-II to T-V as compared to the controls (T-I). Exposure to curcumin for 24 hours (group T-II) or pre-treatment with curcumin followed by acute Cd exposure (group T-V) resulted in increased oxidative stress as seen by an increase in LPO, SA, HP and activities of antioxidant and detoxifying enzymes SOD, CAT, GPx, GR and GST, with a concomitant decrease in cellular antioxidant GSH for the groups T-II and T-V (Figs. 1-3). These

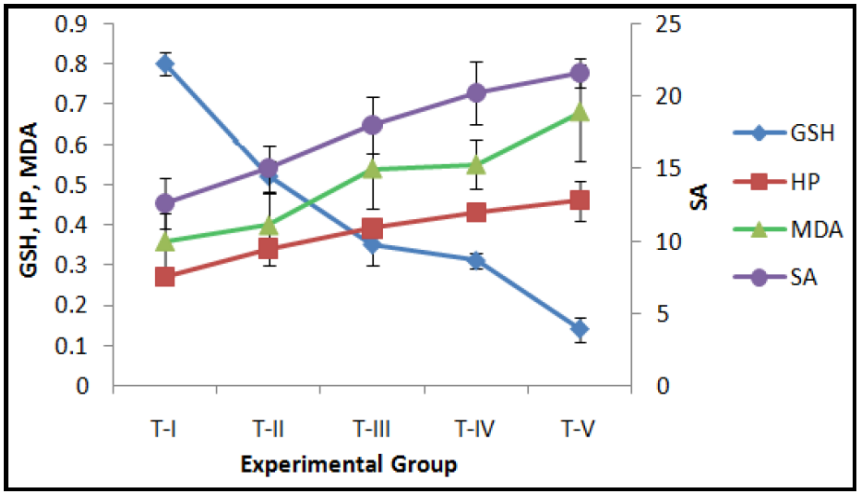

Figure 1. Effect of exposure to Curcumin or Cd or their combinations on hepatic GSH (nmoles/mg protein), Superoxide anion (SA) (nmoles/mg protein), MDA (nmoles/mg protein) and Hydroperoxides (HP) (mmoles/mg protein) levels. Data expressed Mean ± SE (n=7). Level of significance $P < 0.05$.

observations indicate pro-oxidant effects following short-term exposure to curcumin in mice. The increase in activities of antioxidant/detoxifying enzymes SOD, CAT, GPx, GR and GST following treatments with curcumin, Cd or curcumin and Cd combinations is suggestive of the activation of the cellular protective mechanisms involving antioxidant and detoxifying enzymes. However, the protective mechanisms of the cells *i.e.* antioxidant and detoxifying enzymes were insufficient to neutralize the overwhelming effect of ROS generation, thereby resulting in the observed oxidative stress for the doses and duration of exposure in the present study.

The basal MDA level, indicative of lipid peroxidation, in control liver was 0.36 ± 0.07 nmoles/mg protein. MDA was significantly increased by 1.5, 1.6 and 1.8 fold, following Cd exposure in groups T-III to T-V respectively (Fig. 1). On the other hand, total thiols showed a significant decrease of 4.7, 5.4 and 8.1 fold for Cd-exposed groups, i.e. groups T-III to T-V, respectively, irrespective of the pre-treatment with curcumin in group T-V (Fig. 2). A corresponding decrease of 2.3, 2.5, and 5.7 fold in GSH levels in groups T-III to T-V, respectively, was observed (Fig. 2). Similar increase ranging between 1.1 to 2 fold was observed in the activities of the liver antioxidant enzymes SOD, GPx, CAT and GSH pathway enzymes GPx and GR for groups T-III to T-V (Figs. 2 and 3).

Acute Cd exposure has been reported to be associated with an increase in LPO, SA and HP and disruption of the cellular antioxidant system with a subsequent decrease in GSH[31, 32]. Waisberg *et al.* (2003) had reported that Cd cannot directly generate free radicals and rather is involved in the indirect formation of ROS and RNS[33]. An imbalance between lipid peroxidation and antioxidant system results in oxidative stress. One of the main manifestations of oxidative damage is lipid peroxidation[34]. In the present study, the increase in MDA levels with a concurrent decrease in GSH and total thiols, indicates the depletion of reduced glutathione and total thiols, due to increased lipid peroxidation. The increase in the levels of hydroxyl radicals, superoxide anions, hydrogen peroxide along with the enzymes of the antioxidant system indicated that short-term exposure to curcumin and/or Cd results in oxidative stress.

Pro-oxidant effects of curcumin have also been previously reported, though mainly in terms of reactive oxygen species induced DNA damage[35-37]. Rukkumani *et al.* (2004) investigated the effect of curcumin on alcohol and poly-unsaturated fatty acid-induced oxidative stress and had also reported an increase in LPO and HP with an associated decrease in GSH following curcumin administration[38]. In a study following co-treatment with cadmium acetate and curcumin, it was reported that curcumin did not have a protective effect against Cd-induced oxidative stress[16]. Following long-term pre-exposure, to curcumin, there are reports that tissues are protected against LPO as curcumin increases intracellular glutathione concentration and acts as a powerful oxygen free radical scavenger[39].

Masuda *et al.* (1999) explained the anti-oxidative role of curcumin, suggesting a two-stage mechanism involving a radical trapping stage and radical termination stage[40]. In a subsequent study, they elaborated that radical terminations were of two types; (i) dimer formation between two curcumin radicals and (ii) a coupling product between curcumin and lipid hydroperoxide with the latter being fundamental in the antioxidant mechanism of curcumin[41]. Thus, the increase in ROS generation observed in the groups exposed to curcumin *viz.* groups T-II and T-V may be explained based on the formation of free radicals, before the radical tapping stage during the 24 hours of exposure in the present study resulting in pro-oxidant effects.

Kawanishi *et al.* (2005) reported that like many antioxidants, curcumin could be a "double-edged sword," having carcinogenic and pro-oxidant effects on one hand and anticancer and antioxidant effects on the other[42]. The antioxidant, protective and ameliorative effects of curcumin have been reported after long term, sustained exposures of curcumin by exerting effects of a potent scavenger of different ROS including superoxide anion radicals (O2-.) and hydroxyl radicals (.OH)[43].

In our previous study on dose-response effects following acute Cd exposures in mice, we reported that with an increase in $CdCl_2$ dose there were significant changes in the biochemical parameters indicative of oxidative stress, while a certain amount of Cd load was required for histopathological changes to take place in hepatic tissue[15]. The hepatic tissues of the mice administered different treatments were further investigated to identify the correlation between oxidative stress and histopathological alterations.

Liver from the control group T-I and curcumin-treated

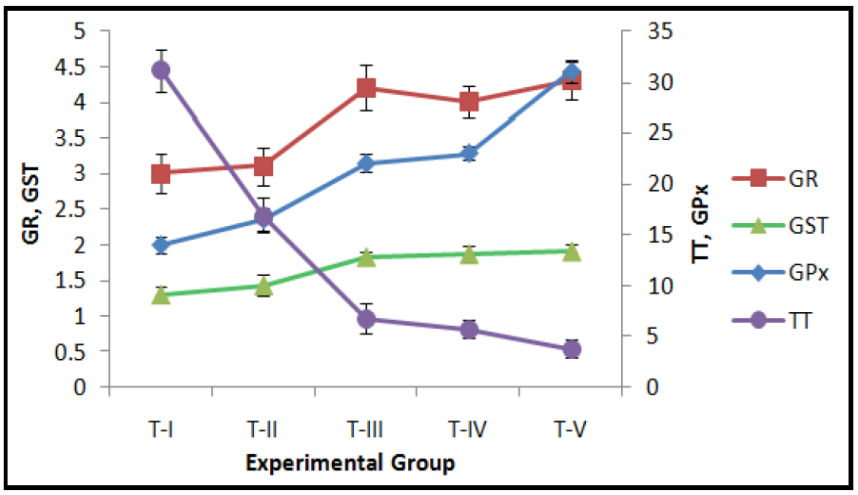

Figure 2. Effect of exposure to Curcumin or Cd or their combinations on hepatic Glutathione peroxidase (GPx) (nmoles NADPH consumed/ min./mg protein), Glutathione reductase (GR) (nmoles NADPH oxidized/min./mg protein), Glutathione-S-transferase (GST) (GSH adduct formed/min./mg protein) and Total Thiols (TT) (nmoles/mg protein). Data expressed Mean ± SE (n=7). Level of significance $P < 0.05$.

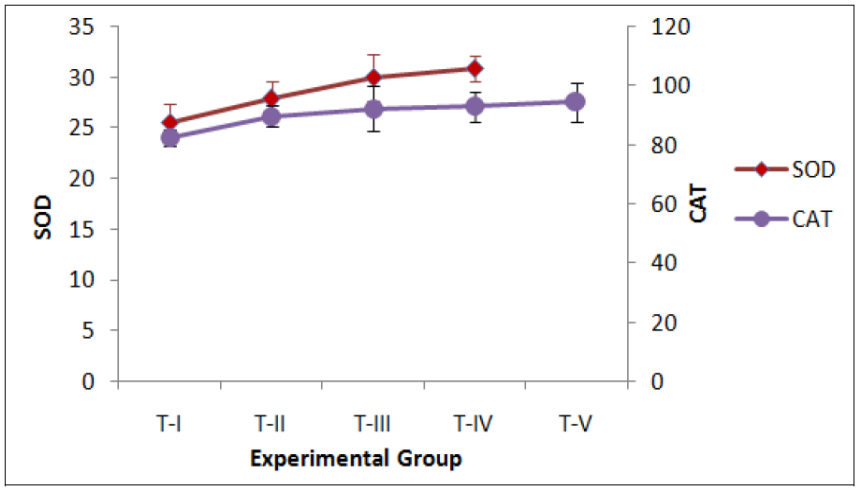

Figure 3. Effect of exposure to Curcumin or Cd or their combinations on hepatic Superoxide dismutase (SOD)- Unit#/min/mg protein (Unit# - 1 unit enzyme activity is defined as the amount of enzyme-inhibiting 50% Nitroblue tetrazolium reduction), and Catalase (CAT)- mmoles H_2O_2 decomposed/min/mg protein and Glutathione peroxidase (GPx)- nmoles NADPH consumed/min/mg protein. Enzyme activities. Data expressed Mean ± SE (n=7). Level of significance $P < 0.05$.

group T-II did not exhibit any macroscopic alterations in the morphology of hepatic tissue of mice. On the other hand, exposure to Cd (groups T-III and T-IV) or curcumin pre-treatment followed by Cd exposure (group T-V) resulted in marked alterations in the histology of the liver as observed under the light and scanning electron microscopes.

Light micrograph examination of sections of the groups T-II, T-III/T-IV, and T-V revealed signs of cell injury as compared to control mice (group T-I) that had normal morphology and occasional foci of lobular inflammation (Fig. 4A). Multiple foci of lobular inflammation by mononuclear cells and Kupffer cell hyperplasia were observed in all the other groups T-II to T-V (Figs. 4 B-D), though cell injury was more pronounced in group T-V with prominent sinusoidal dilation and giant cell transformation along with depositions of basophilic material (Fig. 4D). The morphological changes were diffused and not localized to any specific area, suggesting that Cd and curcumin acted as general hepatotoxins following short-term exposures.

Investigation of the morphological features of the livers under the scanning electron microscope revealed that the mesothelial layer of the liver was apparently normal in the control group T-I, groups T-II and T-V (Figs. 5 A, B and D), whereas hyperplasia with necrotic damage was observed in the liver of mice in the groups T-III/T-IV (Fig. 5C). Kupffer cells were seen lying over the endothelial cells lining the sinusoids in groups T-I and T-II (Figs. 6 A & B). Similar to the observations of KC hyperplasia seen in the light micrographs of groups T-II and T-V (Figs. 4 C & D), KC lying over necrotic hepatocytes were also observed on the electron micrographs (Fig. 6 C) with crystalline deposits on the hepatocytic surface in the group T-V (Fig. 6 D).

Endothelial cells lining the sinusoidal wall of the liver have characteristic fenestrations with well-defined porosity of bile canaliculi, as observed in the liver of groups T-I and T-II (Figs. 7 and 8: A and B). However, loss of endothelial cell fenestrations and blockage of bile canaliculi due to blebbing of the surface epithelia due to necrotic damage were seen in the liver of mice in the groups exposed to Cd, *i.e.* T-III/T-IV and T-V (Figs. 7 and 8: C and D).

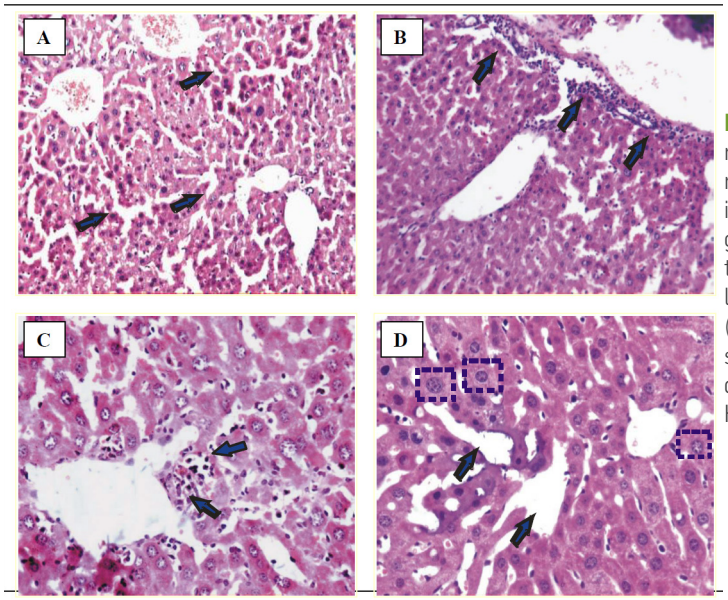

Figure 4. Micrographs of liver of treated mice. (A) Normal morphology with occasional foci of lobular inflammation in control group T-I. H & E × 550. (B) Portal tract inflammation and Mononuclear cell (MNC) infiltration in group T-III/T-IV. H & E × 550.(C) MNC infiltration around the central vein, KC (Kupffer cell) hyperplasia and locular inflammation in group T-II. H & E × 280.
(D) Hepatocytic damage with giant cell transformation, sinusoidal dilation and KC hyperplasia with basophilic deposits within heptocytes and sinusoids in group T-V. H & E × 550.

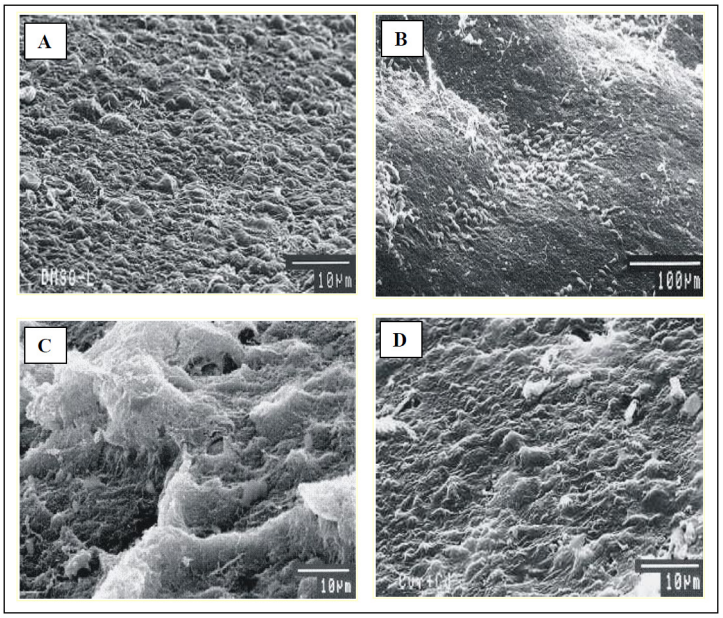

Figure 5. Scanning electron micrographs of mesothelial layer of liver of mice. (A) Group T-I (SEM 10 µm), (B) Group T-II (SEM 100 µm) and (D) Group T-V (SEM 10 µm) - Normal mesothelial layer. (C) Group T-III/IV show hyperplasia and necrotic damage of mesothelial layer (SEM 10 µm).

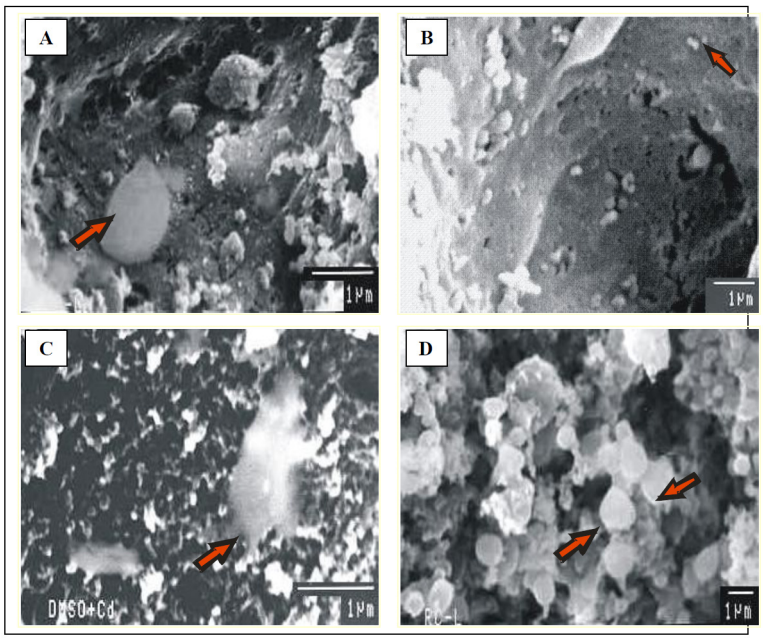

Figure 6. Micrographs of liver showing. (A) Group T-I (SEM 1µm) and (B) Group T-II (SEM 1µm) Kupffer cell lying on the surface of normal endothelial cells lining the sinusoids. (C) Group T-III/IV has Kupffer cells lying over necrotic hepatocytes (SEM 1µm). (D) Crystalline deposits observed on the hepatocyte surface group T-V (SEM 1µm).

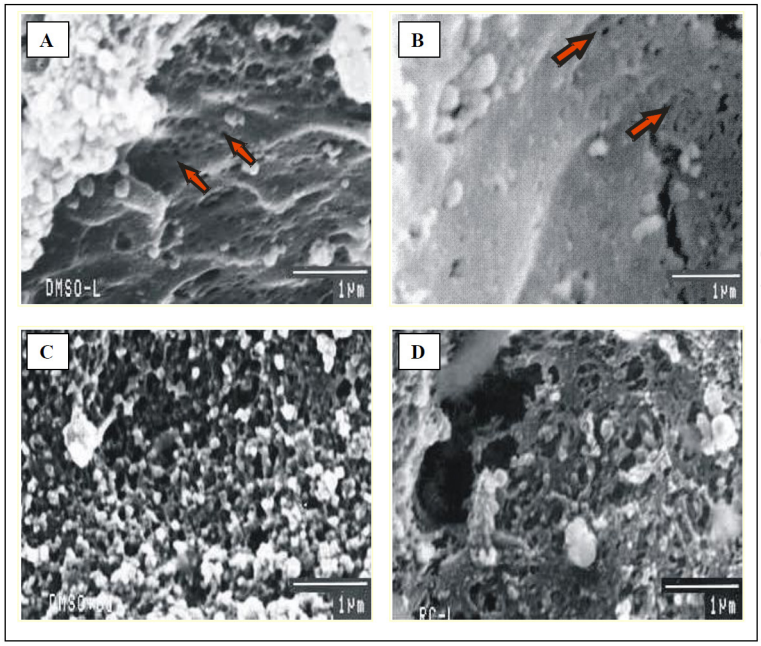

Figure 7. Scanning electron micrographs of liver of mice with (A & B) Clear sieve plate fenestrations of endothelial cells lining the sinusoids in groups T-I and T-II (C & D) Sinusoidal damage observed as necrosis and loss of fenestrations in Cd exposed groups T-III/T-IV and T-V. (SEM 1 µm).

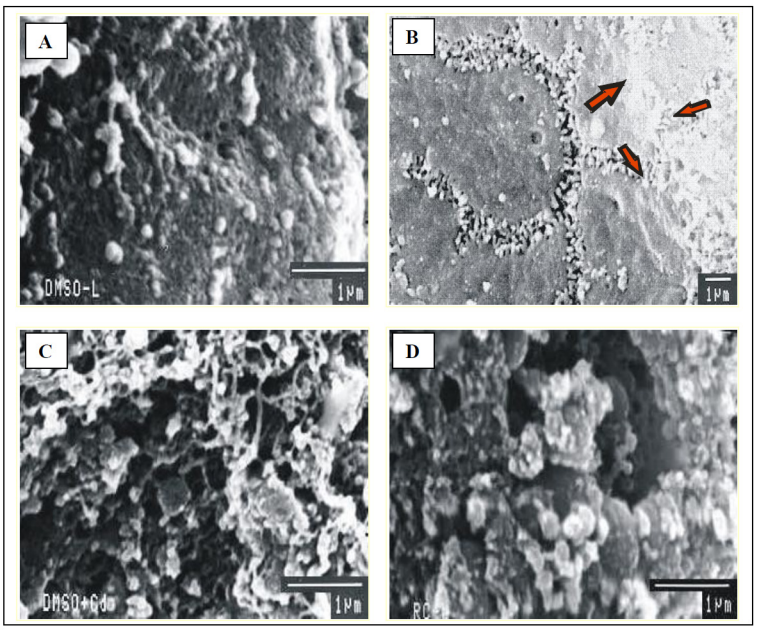

Figure 8. Scanning electron micrographs of liver of mice showing well defined porosity of hepatocytes of bile canalculi in groups T-I and T-II (A & B) and necrotic damage resulting in loss of bile bile canalculi in Cd exposed groups T-III/T-IV and T-V (C & D). (SEM 1 µm).

A plausible mechanism of curcumin and Cd-induced hepatotoxicity may be that the damage results cumulatively due to release of endogenous inflammatory mediators (ROS) and concurrent Kupffer cell and mononuclear cell activation as seen on light and electron micrographs. Blockage of the bile canaliculi and loss of endothelial cell fenestrations may further result in ischemia[44] with subsequent loss of function of hepatocytes.

Conclusions

In summary, the present results suggested that curcumin exposure for 24 hours or curcumin pre-treatment followed by Cd exposure for 18 hours, in mice resulted in increased oxidative stress that was measured in terms of increased levels of LPO, SA, HP and activities of enzymes of antioxidant and detoxifying systems with concomitant decrease in the GSH and total thiols. Corresponding histopathological alterations were observed in the liver sections in the light and electron micrographs of the treated mice, suggesting that on short-term exposure to curcumin, it acts as a pro-oxidant and has a synergistic effect on acute Cd hepatotoxicity.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Bibliographic references

1. García-Niño, W. R., & Pedraza-Chaverri, J. (2014). Protective effect of curcumin against heavy metals-induced liver damage. Food and Chemical Toxicology, 69, 182-201.

2. Iqbal, M., Sharma, S. D., Okazaki, Y., Fujisawa, M., & Okada, S. (2003). Dietary supplementation of curcumin enhances antioxidant and phase II metabolizing enzymes in ddY male mice: possible role in protection against chemical carcinogenesis and toxicity. Pharmacology & toxicology, 92(1), 33-38.
3. Ambegaokar, S. S., Wu, L., Alamshahi, K., Lau, J., Jazayeri, L., Chan, S., ... & Timiras, P. S. (2003). Curcumin inhibits dose-dependently and time-dependently neuroglial cell proliferation and growth. Neuroendocrinology Letters, 24(6), 469-469.
4. Wahlstrom, B., & Blennow, G. (1978). A study on the fate of curcumin in the rat. Acta Pharmcol. Toxicol., 43: 86-92.
5. Bhavanishankar, T.N., Shanta, N.V., Ramesh, H.P., Indira, Murthy A.S., & Sreenivasmurthy, V. (1980). Toxicity studies on turmeric (Curcuma longa): Acute toxicity studies in rats, guinea pigs and monkeys. Ind. J. Exp. Biol.,18, 73-75.
6. Bille, N., Larsen, J.C., Hansen, E.V. & Wurtzen, G. (1985). Subchronic oral toxicity of turmeric oleoresin in pigs. Food Chem. Toxicol., 23, 967-973.
7. Deshpande, S.S., Lalitha, V.S., Ingle, A.D., Raste, A.S., Garde, S.G., & Maru, G.B. (1998). Subchronic oral toxicity of turmeric and ethanolic turmeric extract in female mice and rats. Toxicol. Letters, 95,183-193.
8. Kandarkar, S.V., Sawant, S.S., Ingle, A.D., Deshpande, S.S., & Maru, G.B. (1998). Subchronic oral hepatotoxicity of turmeric in mice - Histopathological and ultrastructural studies. Ind. J. Exp. Biol., 36, 675-679.
9. Kjellström, T., & Nordberg, G. F. (1978). A kinetic model of cadmium metabolism in the human being. Environmental research, 16(1-3), 248-269.
10. Agency for Toxic Substances and Disease Registry (ATSDR). 2003 & 2012. Toxicological profile for Cadmium. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.
11. Ognjanović, B. I., Marković, S. D., Đorđević, N. Z., Trbojević, I. S., Štajn, A. Š., & Saičić, Z. S. (2010). Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: Protective role of coenzyme Q10 and Vitamin E. Reproductive Toxicology, 29(2), 191-197.
12. Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary toxicology, 7(2), 60-72.
13. Souza, V., Bucio, L., Jay, D., Chavez, E., & Gutierrez-Ruiz, M. C. (1996). Effect of cadmium on calcium transport in a human fetal hepatic cell line (WRL-68 cells). Toxicology, 112(2), 97-104.
14. Saïdi, S. A., Azaza, M. S., Windmolders, P., van Pelt, J., & El-Feki, A. (2013). Cytotoxicity evaluation and antioxidant enzyme expression related to heavy metals found in tuna by-products meal: an in vitro study in human and rat liver cell lines. Experimental and toxicologic pathology, 65(7-8), 1025-1033.
15. Sahrawat, T.R., Sobti, R.C., Sharma, S.C., Saikia, U.N., & Sharma, M.L. (2019). Hepatotoxicity on acute exposure to cadmium chloride in mice. Journal of Emerging Technologies and Innovative Research (JETIR), 6(5), 384-390.
16. Tarasub, N., Narula, K., & Ayutthaya, W.D.N (2008). Effects of curcumin on cadmium-induced hepatotoxicity in rats. Thai. J. Toxicol. 23, 100–107.
17. Tarasub, N., Junseecha, T., Tarasub, C., & Ayutthaya, W.D.N. (2012). Protective effects of curcumin, vitamin C, or their combination on cadmium-induced hepatotoxicity. Journal of basic and clinical pharmacy, 3(2), 273.
18. Chainani-Wu, N. (2003). Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). The Journal of Alternative & Complementary Medicine, 9(1), 161-168.
19. Perkins, S., Verschoyle, R. D., Hill, K., Parveen, I., Threadgill, M. D., Sharma, R. A., ... & Gescher, A. J. (2002). Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. Cancer Epidemiology and Prevention Biomarkers, 11(6), 535-540.
20. Sun, R. (1963). A forthright and practical method for comprehensive calculation of LD50. Learned J. Pharm, 10, 65.
21. Beuge, J.A., & Augst, S.D. (1978). Microsomal lipid peroxidation. Method Enzymol., 52:302-310.
22. Beutler, E., Duron, O., & Kelley, B.M. (1963). Improved method for detection of blood glutathione. J. Lab. Clin. Med., 61,882-888.
23. Elferink, J.G. (1984). Measurement of the metabolic burst in human neutrophils: a comparison between cytochrome c and NBT reduction. Research communications in chemical pathology and pharmacology, 43(2), 339-342.
24. Jiang, Z.Y., Hunt, J.Y., & Wolf. S.P. (1992). Detection of lipid hydroperoxides using the 'fox method'. Anal. Biochem., 202, 384-389.
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. Journal of biological chemistry, 193, 265-275.
26. Kono, Y. (1978). Generation of superoxide radicals during auto-oxidation of hydroxyl-amine hydrochloride an assay for SOD. Arch Biochem Biophys, 186, 189-195.
27. Luck H. (1963). Catalase. In: Methods of enzymatic analysis Ed. H.U. Bergmeyer, Academic Press, New York, pp. 885-889.
28. Paglia, D.E., & Valentine, W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. The Journal of laboratory and clinical medicine, 70(1), 158-169.
29. Horn H.D. (1971). Glutathione reductase. In: Methods of enzymes analysis. Ed. Bergmeger, H.U:Academic Press, New York, pp. 875-881.
30. Habig W.H., Pabst M.J., & Kakoby W.B. (1974) Glutathione-S-transferase. J. Biol. Chem., 249:7130-7139.
31. Sarkar, S., Yadav, P., Trivedi, R., Bansal, A. K., & Bhatnagar, D. (1995). Cadmium-induced lipid peroxidation and the status of the antioxidant system in rat tissues. Journal of Trace Elements in Medicine and Biology, 9(3), 144-149.
32. El- Maraghy, S. A., Gad, M. Z., Fahim, A. T., & Hamdy, M. A. (2001). Effect of cadmium and aluminum intake on the antioxidant status and lipid peroxidation in rat tissues. Journal of Biochemical and Molecular Toxicology, 15(4), 207-214.
33. Waisberg, M., Joseph, P., Hale, B., & Beyersmann, D. (2003). Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology, 192(2-3), 95-117.
34. Stohs, S. J., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. Free radical biology and medicine, 18(2), 321-336.
35. Ahsan, H., Parveen, N., Khan, N. U., & Hadi, S. M. (1999). Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. Chemico-biological interactions, 121(2), 161-175.
36. Kelly, M.R., Xu, J., Alexander, K.E., & Loo, G. (2001). Disparate effects of similar phenolic phytochemicals as inhibitors of oxidative damage to cellular DNA. Mutation Research/DNA Repair, 485(4), 309-318.
37. Urbina-Cano, P., Bobadilla-Morales, L., Ramírez-Herrera, M.A., Corona-Rivera, J.R., Mendoza-Magaña, M.L., Troyo-Sanromán, R., & Corona-Rivera, A. (2006). DNA damage in mouse lymphocytes exposed to curcumin and copper. Journal of applied genetics, 47(4), 377-382.
38. Rukkumani, R., Aruna, K., Varma, P.S., Rajasekaran, K.N., & Menon, V.P. (2004). Comparative effects of curcumin and an analog of curcumin on alcohol and PUFA induced oxidative stress. J Pharm Pharm Sci, 7(2), 274-283.
39. Ciftci, O., Ozdemir, I., Tanyildizi, S., Yildiz, S., & Oguzturk, H. (2011). Antioxidative effects of curcumin, - -myrcene and 1, 8-cineole against 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced oxidative stress in rats liver. Toxicology and Industrial Health, 27(5), 447-453.
40. Masuda, T., Hidaka, K., Shinohara, A., Maekawa, T., Takeda, Y., & Yamaguchi, H. (1999). Chemical studies on antioxidant mechanism of curcuminoid: analysis of radical reaction products from curcumin. Journal of agricultural and food chemistry, 47(1), 71-77.
41. Masuda, T., Toi, Y., Bando, H., Maekawa, T., Takeda, Y., & Yamaguchi, H. (2002). Structural identification of new curcumin dimers and their contribution to the antioxidant mechanism of curcumin. Journal of agricultural and food chemistry, 50(9), 2524-2530.

42. Kawanishi, S., Oikawa, S., & Murata, M. (2005). Evaluation for safety of antioxidant chemopreventive agents. Antioxidants & redox signaling, 7(11-12), 1728-1739.
43. Nanji, A. A., Jokelainen, K., Tipoe, G. L., Rahemtulla, A., Thomas, P., & Dannenberg, A. J. (2003). Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-- B-dependent genes. American Journal of Physiology-Gastrointestinal and Liver Physiology, 284(2), G321-G327.
44. Liu, J., Kershaw, W. C., Liu, Y. P., & Klaassen, C. D. (1992). Cadmium-induced hepatic endothelial cell injury in inbred strains of mice. Toxicology, 75(1), 51-62.

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RESEARCHS / INVESTIGACIÓN

Infección por Virus del Papiloma Humano y citología cérvico-vaginal en mujeres indígenas del Cañar, Ecuador.

Human Papillomavirus infection and cervico-vaginal cytology in indigenous women from Cañar, Ecuador.

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Resumen: Existen pocos estudios sobre la circulación del Virus del Papiloma Humano (VPH) en mujeres indígenas ecuatorianas. Los objetivos del trabajo fueron: determinar la circulación de VPH e identificar alteraciones citológicas en muestras cervicouterinas de mujeres indígenas ecuatorianas, así como definir el comportamiento de algunas variables sociodemográficas y clínico-epidemiológicas. Se realizó un estudio analítico de corte transversal entre julio de 2017 y septiembre de 2018, en 100 mujeres indígenas entre 15 y 55 años, residentes en el Cañar, Ecuador. Se investigó la frecuencia de infección viral, los genotipos circulantes y su asociación con determinadas variables sociodemográficas y clínico-epidemiológicas. Se obtuvo un 98% de citologías negativas para malignidad y un 2% con lesiones intraepiteliales cervicales. No se diagnosticaron casos con lesiones de alto grado. El 34% (34/100) de las muestras resultó positiva a VPH, predominando los genotipos oncogénicos. El VPH 31 fue el más frecuente en el 41,2% (14/34) de los casos, seguido por el VPH 16 en el 20,6% (7/34). Las mujeres entre 20 y 30 años de edad, tenían una probabilidad 5 veces mayor de estar infectadas con VPH (44,1%; 15/34). La frecuencia de infección fue significativamente mayor en mujeres solteras y en aquellas que refirieron haber tenido de 2 a 3 partos. La infección con VPH 16 estuvo asociada al uso de anticonceptivos hormonales, en el 57,1% (4/7) de los casos; p=0,005, RP=12,44 IC95% (2,40-64,62). La elevada prevalencia de infección por VPH oncogénico indica la necesidad de incorporar esta población indígena en los programas de detección precoz del cáncer cervicouterino.

Palabras clave: Virus del Papiloma Humano, citología cérvico-vaginal, mujeres indígenas, quechua, Ecuador.

Abstract: There are few studies on the circulation of Human Papilloma Virus (HPV) in indigenous Ecuadorian women. The aim of the study is to determine the circulation of HPV and identify cytological alterations in cervical samples of indigenous Ecuadorian women and to define the behavior of some socio-demographic and clinical-epidemiological variables. An analytical cross-sectional study was done between July 2017 and September 2018 to determine the presence of cytological alterations and HPV infection in 100 indigenous women between 15 and 55 years of age, residing in Cañar, Ecuador. The association between socio-demographic and clinical-epidemiological variables with viral infection was investigated. Was obtained a 98% of negative cytology for malignancy and 2% of cervical lesions. Cases with high-grade lesions were not diagnosed. In general, a 34% (34/100) tested was positive for HPV, predominating oncogenic genotypes. HPV 31 was the most frequent in 41.2% (14/34) of cases followed by HPV 16 in 20.6% (7/34). Women between 20 and 30 years of age were five times more likely to be infected with HPV (44.1%, 15/34). The frequency of infection was significantly higher in single women and in those who reported having 2 to 3 births. Infection with HPV 16 was associated with the use of hormonal contraceptives, in 57.1% (4/7) of the cases; p = 0.005, RP = 12.44 IC95% (2.40-64.62). The high prevalence of oncogenic HPV infection indicates the need to incorporate this indigenous population into early detection programs for cervical cancer.

Key words: Human Papillomavirus, cervico-vaginal cytology, indigenous women, Quechua, Ecuador.

Introducción

La infeccion con el Virus del Papiloma Humano (VPH) es la condicion necesaria para el surgimiento y desarrollo de cáncer cervicouterino (CaCu) y en general, es la principal causa de las neoplasias de la zona anogenital[1]. El cáncer de cuello uterino se ubica en el tercer lugar entre los cánceres de mayor incidencia a escala mundial[2]. Se han descrito cerca de 200 genotipos de VPH hasta la fecha y aproximadamente de 15 a 19 son considerados de alto riesgo (VPH-AR), de acuerdo con su potencial oncogénico[3]. El VPH tipo 16 y el 18 son los genotipos oncogénicos más frecuentemente asociados a lesiones precancerosas y al cáncer cervical[4]. De acuerdo con los pocos estudios realizados en América del Sur, además del VPH 16 y el 18, el VPH 58 es otro de los VPH-AR encontrados con mayor frecuencia en la región. Este último ha sido detectado en el centro y norte de Brasil, Argentina, Colombia y Ecuador[5].

De acuerdo con los datos del Registro Nacional de Tumores de la Sociedad de Lucha Contra el Cáncer (SOLCA, 2010), en el Ecuador, el CaCu es considerado uno de los tipos de cáncer más frecuente en la población femenina. Aproximadamente, 20 de cada 100 000 mujeres padecen esta neoplasia y se presentan 1 200 casos nuevos cada año. Según las cifras del Instituto Nacional de Estadísticas y Censos del Ecuador (INEC,

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2011), de esas mujeres, 300 fallecen debido a esta causa[6]. En Ecuador, según los datos publicados por la Organización Panamericana de la Salud, hasta el año 2013, la cobertura de la citología es muy baja, aproximadamente un 28%. Además, no están incluidas técnicas moleculares para la detección del VPH, como agente causal del CaCu y hasta la fecha, se mantiene solamente el uso de la citología convencional mediante la técnica de Papanicolaou[7].

Son muy escasas las investigaciones enfocadas a la detección de VPH y lesiones citológicas en grupos poblaciones vulnerables, desde el punto de vista socioeconómico, como las comunidades indígenas ecuatorianas. La población femenina indígena sufre las consecuencias de las deficiencias en la infraestructura sanitaria, difícil acceso a los servicios de salud, tradiciones socioculturales y religiosas propias de los pueblos indígenas originarios. No existe información suficiente sobre esta problemática en mujeres indígenas de la provincia del Cañar. Por lo antes expuesto, este trabajo tiene como objetivos determinar la circulación de VPH e identificar alteraciones citológicas en muestras cervicouterinas de mujeres indígenas ecuatorianas, así como definir el comportamiento de algunas variables sociodemográficas, clínicas y epidemiológicas.

Materiales y métodos

Tipo de investigación, diseño del estudio y contexto, universo y muestra

Se realizó un estudio descriptivo de corte transversal, entre julio y septiembre de 2017. El universo estuvo constituido por todas las mujeres indígenas entre 15 y 55 años de edad, residentes en la provincia el Cañar del Ecuador, que accedieron a participar en el estudio, en el periodo definido previamente y que cumplieron con los criterios de inclusión. La muestra quedó constituida por 100 mujeres de las cuales se tomaron muestras cervicouterinas para determinar la presencia de infección por VPH, de lesiones intraepiteliales y otras alteraciones cérvico-vaginales; así como las variables sociodemográficas, clínicas y epidemiológicas.

Criterios de inclusión y exclusión

Se incluyeron mujeres con edades comprendidas entre 15 y 55 años, sexualmente activas y que dieran su consentimiento de participación en la investigación. Como criterio de exclusión se tuvo en cuenta: embarazo en curso, haber recibido tratamientos o procedimientos ginecológicos 3 meses antes de la prueba y haber tenido relaciones sexuales 48 horas antes de la prueba o tacto genital previo. También se consideró como criterio de exclusión, el uso de ciertos fármacos que podrían afectar el resultado de la prueba citológica o de la detección viral; como la colchicina, los estrógenos, la podofilina, los progestágenos y el nitrato de plata[8].

Muestras clínicas

Las muestras clínicas para el análisis citomorfológico fueron los extendidos de células cervicouterinas, clasificados como útiles para la realización de la citología cérvico-vaginal. Las citologías satisfactorias se clasificaron, según el Sistema de Bethesda 2014[9].

Las muestras clínicas para la detección de VPH fueron colectadas inmediatamente después de realizar los extendidos de células para la realización de la citología cérvico-vaginal y consistieron en cepillados endocervicales. Una vez obtenidas las muestras, se mantuvieron en la solución comercial SurePath (Becton Dickinson, Sparks, MD, EEUU) que es destinada a la preservación, traslado y almacenamiento de muestras para citología en base líquida y para la detección viral del VPH. Las muestras se conservaron a -20 oC hasta su procesamiento.

Prueba de Papanicolaou

Los hallazgos y posteriormente la clasificación de los resultados se interpretaron utilizando la terminología del Sistema Bethesda 2014 9. Una prueba de Papanicolaou es positiva si se observan: células escamosas de significado indeterminado (ASC-US), lesión escamosa intraepitelial de bajo grado (L-SIL), células escamosas de significado indeterminado en las que no se excluye una lesión de alto grado (ASC-H), lesión escamosa intraepitelial de alto grado (H-SIL), carcinoma de células escamosas, células glandulares de significado indeterminado (ASG-US), células glandulares atípicas; posiblemente neoplásicas, adenocarcinoma endocervical *in situ* y adenocarcinoma[9].

Extracción del ADN

Se realizó la homogenización de las suspensiones celulares, mediante agitación vigorosa. La solución comercial SurePath, en la que se preservaron las muestras, es utilizada para la citología en base líquida y contiene formaldehido. Por ello, deben tenerse en cuenta tratamientos sucesivos con calor para facilitar la obtención y calidad del ADN, según se ha publicado previamente[10]. La extracción se llevó a cabo con el estuche comercial QIAamp® DNA Mini Kit (QIAGEN, Hilden, Alemania) según el protocolo para células y tejidos y las indicaciones del fabricante. El ADN obtenido se diluyó en 100 µL de tampón de elusión y se almacenó a -20º C hasta la realización de la detección de VPH.

Detección y genotipado de VPH mediante microarreglos de baja densidad

La detección y genotipado de VPH en las muestras estudiadas fue realizada utilizando el estuche comercial CLART® HPV 2 (Genómica, España), siguiendo las instrucciones del fabricante. Esta técnica detecta la presencia de los 35 genotipos de VPH con mayor importancia clínica, en muestras cervicouterinas; VPH-BR: VPH 6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, 85 y 89; VPH-AR: VPH 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82; según el criterio de la Agencia Internacional de Investigaciones del Cáncer (IARC).

Análisis de la información

Todos los datos personales, clínicos, epidemiológicos y de laboratorio de cada paciente se almacenaron en una base de datos en Excel. Para el análisis de las diferentes variables fue aplicado el paquete estadístico SPSS versión 19.0 (IBM Inc., Berkeley, CA, EEUU). La asociación entre las variables sociodemográficas, clínicas y epidemiológicas y la infección por VPH se analizó mediante tablas de contingencia y la prueba estadística de Chi cuadrado (X^2). Se consideraron significativos los valores de razón de prevalencia (RP)>1 y p<0,05; estimándose intervalos de confianza con un nivel de confiabilidad del 95%.

Aspectos éticos

Las mujeres acudieron a la convocatoria para participar en el estudio a través de la divulgación de la iglesia local del Cañar, reuniones con los dirigentes comunitarios, avisos y comunicaciones colocadas en espacios libres de la comunidad, manteniendo los aspectos éticos y legales. En todos los casos se obtuvo el consentimiento informado de las mujeres para participar en la investigación. Se comunicó que la investigación

se realizaría según los preceptos éticos de la medicina actual. Para ello se tuvieron en cuenta los principios de la Declaración de Helsinki sobre la investigación clínica en humanos[11].

Resultados y discusión

En las tablas 1 y 2 se muestran los datos sociodemográficos, epidemiológicos, la historia sexual y ginecológica de las mujeres estudiadas.

Los resultados del presente trabajo muestran que el mayor porcentaje de mujeres iniciaron sus relaciones sexuales después de los 20 años, lo que obedece a los cánones religiosos que identifican a esta población. No obstante, se ha reportado previamente, en un estudio realizado en adolescentes, que la edad de inicio de las relaciones sexuales en Ecuador tuvo una media de 15 años, tanto para hombres como mujeres[12].

En esta pesquisa, la mayor parte de las mujeres estudiadas (78%) refirió una sola pareja sexual durante toda su vida sexualmente activa. Estudios similares en comunidades indígenas de América Latina han reportado que más de un 50% de estas mujeres, con una edad promedio de 30 años, refieren una pareja sexual[13]. Este resultado pudiera estar relacionado con las características socio-culturales de esta etnia indígena, donde se obedecen rígidos preceptos en cuanto a la moral, la sexualidad y la familia[14].

Variable	Grupos o rangos	Totales N=100 (n=%)
Edad en años Mediana=40 años	<20	2
	20-30	24
	31-40	27
	41-50	27
	>50	20
Etnia indígena quechua	Si	93
Nivel educacional	Analfabeta	37
	Primaria	50
	Secundaria	10
	Universitaria	3
Situación ocupacional	Estudiante	3
	Ama de Casa	32
	Agricultora	58
	Obrera	6
	Profesional	1
Estado civil	Casada	78
	Soltera	10
	Divorciada	2
	Unión Consensual	7
	Viuda	3
Consumo habitual de cigarrillos	Si	0
Consumo de alcohol	Si	2
Consumo de drogas	Si	0
Alimentación sana	Si	82

Tabla 1. Datos sociodemográficos y epidemiológicos de mujeres indígenas residentes en la región del Cañar, Ecuador.

En relación a la prueba de Papanicolaou, la calidad de las muestras fue satisfactoria en el 100% de los casos. El 98% de las muestras fueron negativas para malignidad y en el 2% se identificaron alteraciones citológicas; un caso con ASC-US y otro con lesión de bajo grado (LSIL). No se detectaron lesiones de alto grado. Si bien las poblaciones indígenas no han sido ampliamente estudiadas tanto en Ecuador como en otros países de Latinoamérica, existen reportes donde el porcentaje de ASC-US varía entre 0,6 y 10,4%; mientras que se reporta LSIL entre 1,8 y 4,5% [15, 16].

Se debe destacar que el mayor porcentaje de las mujeres encuestadas no se habían realizado un estudio citológico anteriormente. La falta de instrucción, el poco acceso a los medios de comunicación y el habla en lengua quechua, limitan en gran medida sus conocimientos y la percepción de riesgo para su salud sexual, la cual comúnmente está sujeta a la decisión del jefe de hogar[15, 16].

De las 100 mujeres examinadas, el 34% (34/100) resultó positiva a uno o más genotipos de VPH. En general fueron identificados, 12 genotipos diferentes, con un predominio absoluto de los genotipos oncogénicos, pues en todos los casos positivos se detectó, al menos, un genotipo de alto riesgo. El VPH 31 fue el genotipo más frecuente, seguido por el VPH 16 (Figura 1).

En esta investigación la frecuencia de infección por VPH fue superior a un 30%. De acuerdo a un meta-análisis realizado en el 2010, que incluyó un millón de mujeres con citología normal, procedentes de 59 países y 5 continentes, la prevalencia de la infección cervical se mantiene en un rango de 1,6% a 25%, con una prevalencia global estimada de 11,7%, aunque fue diferente según las regiones geográficas. De hecho, la prevalencia del VPH se estimó en un 24% en África Sub-Sahariana, en el Este de Europa un 21,4% y en Latinoamérica un 16,1%. Los menores valores de prevalencia se encontraron en América del Norte (4,7%) y el Oeste Asiático (17%)[17].

Otro meta-análisis realizado por autores mexicanos incluyó el estudio de 1425 muestras de mujeres con citología normal provenientes de diferentes países, como México, Estados

Variable	Grupos o rangos	Totales N=100 (n=%)
Edad de la menarquia en años	≤14	92
	15-17	8
Edad de inicio de las relaciones sexuales en años	≤ 15	2
	16-20	81
	> 20	17
Número de embarazos	0-1	10
	2-3	45
	≥4	45
Número de partos	0-1	13
	2-3	52
	≥4	35
Número de parejas sexuales referidas en los últimos 2 años	1	78
	2-3	22
	≥4	0
Citologías cérvico-vaginales previas	Si	27
Citologías cérvico-vaginales en los últimos 5 años	Si	24
Resultado de la citología cérvico-vaginal actual	Negativa	98
	ASC-US	1
	LSIL	1
	ASC-H	0
	HSIL	0
Procesos inflamatorios en la citología cérvico-vaginal	Si	45
Grado de inflamación en la mucosa cérvico-vaginal	Leve	21
	Moderado	18
	Severo	6
Atrofia cervical	Si	25
Historia previa de ITS	Si	42
Uso habitual del condón	Si	4
Uso de anticonceptivos hormonales	Si	45
Uso de anticonceptivos hormonales en años	1	13
	2-4	23
	≥5	9
Síntomas ginecológicos*	Leucorrea	72
	Prurito	10
	Metrorragia	7
	Ninguno	22

Tabla 2. Historia sexual y datos ginecológicos de las mujeres estudiadas.

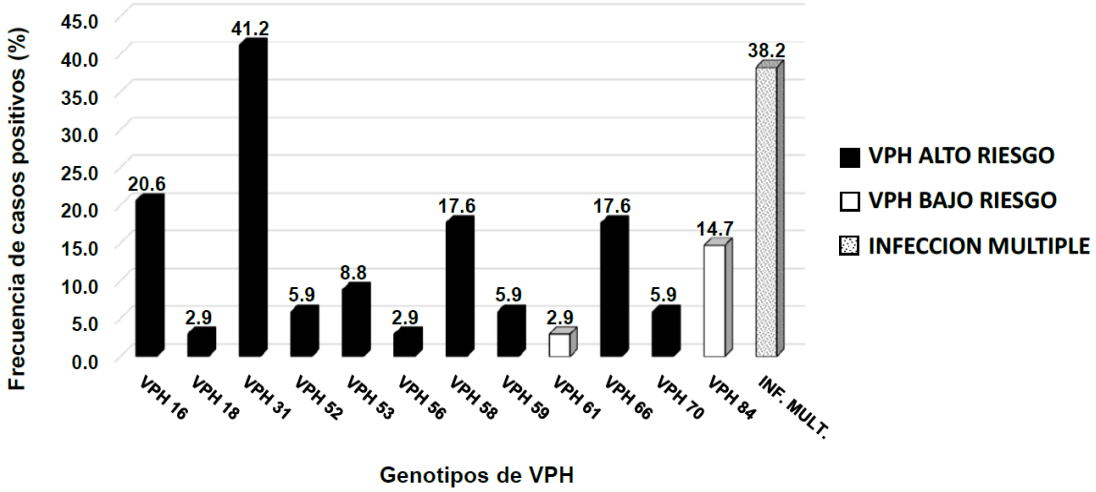

Figura 1. Frecuencia de genotipos de VPH detectados en mujeres indígenas residentes en la provincia del Cañar, Ecuador.

Variable	Grupos o rangos	Totales N=100 (n=%)	Positivo a VPH N=34 n (%)		Valor de p	RP (IC95%)
Edad en años	<20	2	2	5,9	0,113	0,33 (0,25-0,43)
	20-30	**24**	**15**	**44,1**	**0,001**	**5,00 (1,88-13,27)**
	31-40	27	9	26,5	0,565	0,96 (0,38-2,45)
	41-50	**27**	**5**	**14,7**	**0,037**	**0,35 (0,12-1,01)**
	>50	**20**	**3**	**8,8**	**0,036**	**0,28 (0,08-1,03)**
Estado civil	**Casada**	**78**	**17**	**50**	**0,002**	**0,25 (0,10-0,61)**
	Soltera	**10**	**13**	**38,2**	**0,000**	**7,55 (2,41-23,72)**
	Divorciada	2	1	3	0,567	0,97 (1,12-32,50)
	Unión Consensual	7	3	8,8	0,445	1,50 (0,32-7,12)
	Viuda	3	0	0	0,283	0,65 (0,56-0,75)
Edad de inicio de las relaciones Sexuales en años	≤ 15 16-20 **> 20**	2 81 **17**	1 31 **2**	2,9 91,2 **5,9**	0,567 0,051 **0,027**	1,97 (0,12-32,50) 3,31 (0,89-12,28) **0,21 (0,05-0,10)**
Número de embarazos	0-1 **2-3** **≥4**	10 **45** **45**	2 **23** **9**	5,9 **67,6** **26,5**	0,271 **0,001** **0,006**	0,45 (0,09-2,26) **4,18 (1,73-10,10)** **0,30 (0,12-0,74)**
Número de partos	0-1 **2-3** **≥4**	13 **52** **35**	3 **24** **7**	8,8 **70,6** **20,6**	0,289 **0,010** **0,024**	0,54 (0,14-2,12) **3,06 (1,27-7,41)** **0,35 (0,13-0,92)**
Historia previa de ITS	**Si**	**42**	**19**	**55,9**	**0,036**	**2,37 (1,01-5,52)**

Tabla 3. Variables sociodemográficas, clínicas y epidemiológicas asociadas a la infección con el Virus del Papiloma Humano en mujeres indígenas residentes en la provincia del Cañar, Ecuador.

Unidos, Canadá, Brasil, Suecia, Tanzania, Sudáfrica, Tailandia, Arabia Saudita y Australia. En dicha investigación se detectó una prevalencia de infección por VPH de 12,4 %[18]. El presente estudio muestra resultados diferentes a los obtenidos en Latinoamérica para mujeres con citología normal. Sin embargo, análisis similares en mujeres indígenas ecuatorianas reportaron porcentajes de infección por VPH hasta de un 30%[19, 20]. De la misma forma, la distribución de genotipos puede variar entre países o entre regiones de un mismo país. En esta investigación se pudo comprobar el predominio absoluto de los genotipos oncogénicos, sin embrago los resultados muestran que el genotipo más frecuente fue el VPH 31 y no el 16, que fue el segundo, seguido por los tipos 58 y 66. El hecho de que predominaran los genotipos oncogénicos y en frecuencias elevadas, es una alarma de salud para la atención sexual y reproductiva de este grupo de mujeres indígenas, las que además no asisten a la realización de la prueba citológica y no cuentan con programas gratuitos de salud.

La asociación entre las variables sociodemográficas, clínicas y epidemiológicas y la infección con VPH se observa en la tabla 3.

En esta investigación la presencia de infecciones múlti-

ples con varios genotipos de VPH fue significativamente más frecuente en mujeres entre 20 y 30 años, detectándose en el 53,8% (7/13) de los casos; p=0,013, RP=4,80 IC95% (1,43-16,15), en las mujeres solteras en el 46,2% (6/13); p=0,012, RP=5,38 IC95% (1,54-18,68) y en las que tenían antecedentes de infecciones de transmisión sexual (ITS) en un 69,2% (9/13); p=0,034, RP=3,68 IC95% (1,10-12,91).

La infección con VPH 16 estuvo asociada al uso de anticonceptivos hormonales por más de un año, detectándose el virus en el 57,1% (4/7) de los casos; p=0,005, RP=12,44 IC95% (2,40-64,62). Este genotipo fue significativamente más frecuente en las mujeres diagnosticadas con procesos inflamatorios en la citología cérvico-vaginal; 57,1% (4/7); p=0,034, RP=5,96 IC95% (1,22-29,13).

El genotipo 31, el más frecuente en las mujeres estudiadas, tuvo una prevalencia significativamente superior en las mujeres solteras; 50% (7/14), p=0,003, RP=6,82 IC95% (2,01-23,19) y en las mujeres entre los 20 y 30 años de edad; 50% (7/14), p=0,021, RP=4,10 IC95% (1,25-13,14).

No se encontró asociación entre la infección por VPH y el resultado de la citología cérvico-vaginal o prueba de Papanicolaou ya que solo se diagnosticaron dos casos con algún tipo de lesión.

Conclusiones

La cultura e idiosincrasia de las poblaciones indígenas puede influir en diferentes aspectos de la salud sexual y reproductiva, pues sus costumbres y hábitos dentro de las comunidades cerradas pueden modificar los niveles de transmisión de diversas infecciones y así el comportamiento de ciertas enfermedades de transmisión sexual que se presentan en otras poblaciones, con características diferentes. La elevada prevalencia de infección por VPH oncogénico indica la necesidad de incorporar esta población indígena del Cañar en los programas de detección precoz del cáncer cervicouterino.

Referencias bibliográficas

1. zur Hausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009;384(2):260-5.
2. Human Papillomavirus and Related Diseases Report. Cuba: ICO Information Centre on HPV and Cancer, 2014.
3. de Villiers EM. Cross-roads in the classification of papillomaviruses. Virology. 2013;445(1-2):2-10.
4. Rader JS, Tsaih SW, Fullin D, Murray MW, Iden M, Zimmermann MT, et al. Genetic variations in human papillomavirus and cervical cancer outcomes. Int J Cancer. 2018.
5. Bedoya-Pilozo CH, Medina Magues LG, Espinosa-Garcia M, Sanchez M, Parrales Valdiviezo JV, Molina D, et al. Molecular epidemiology and phylogenetic analysis of human papillomavirus infection in women with cervical lesions and cancer from the coastal region of Ecuador. Rev Argent Microbiol. 2018;50(2):136-46.
6. Mancilla JC. Public expenditure in health in Ecuador Pathologica. 2013;103(1):53-60.
7. De Sanjose S. Historia de cribado en mujeres con cáncer infiltrante de cuello uterino. Gaceta Sanitaria. 2006;20:166-70.
8. Asotic A, Taric S, Asotic J. Correlation of cervical smear and pathohistological findings. Med Arch. 2014;68(2):106-9.
9. Ritu N, Wilbur DC. The Bethesda System for Reporting Cervical Cytology. Third ed. Chicago , IL. USA: Springer; 2014. 342 p.
10. Tardif KD, Pyne MT, Malmberg E, Lunt TC, Schlaberg R. Cervical Cytology Specimen Stability in Surepath Preservative and Analytical Sensitivity for HPV Testing with the cobas and Hybrid Capture 2 Tests. PLoS One. 2016;11(2):e0149611.
11. Wold-Medical-Association. Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects 2008 [Available from: http://www.wma.net/en/30publications/10policies/b3/7c.pdf].
12. Jaruseviciene L, Orozco M, Ibarra M, Ossio FC, Vega B, Auquilla N, et al. Primary healthcare providers' views on improving sexual and reproductive healthcare for adolescents in Bolivia, Ecuador, and Nicaragua. Glob Health Action. 2013;6:20444.
13. Mendoza L, Mongelos P, Paez M, Castro A, Rodriguez-Riveros I, Gimenez G, et al. Human papillomavirus and other genital infections in indigenous women from Paraguay: a cross-sectional analytical study. BMC Infect Dis. 2013;13:531.
14. Muñoz G, Mota L, Bowie WR, Quizhpe A, E. O, Spiegel JM, et al. Ecosystem approach to promoting appropriate antibiotic use for children in indigenous communities in Ecuador. Rev Panam Salud Publica. 2011;30(6):566'73.
15. Moore SP, Forman D, Pineros M, Fernandez SM, de Oliveira Santos M, Bray F. Cancer in indigenous people in Latin America and the Caribbean: a review. Cancer Med. 2014;3(1):70-80.
16. Nugus P, Desalliers J, Morales J, Graves L, Evans A, Macaulay AC. Localizing Global Medicine: Challenges and Opportunities in Cervical Screening in an Indigenous Community in Ecuador. Qual Health Res. 2018;28(5):800-12.
17. Bruni L, Diaz M, Castellsague X, Ferrer E, Bosch FX, de Sanjose S. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. J Infect Dis. 2010;202(12):1789-99.
18. Aguilar-Lemarroy A, Vallejo-Ruiz V, Cortes-Gutierrez EI, Salgado-Bernabe ME, Ramos-Gonzalez NP, Ortega-Cervantes L, et al. Human papillomavirus infections in Mexican women with normal cytology, precancerous lesions, and cervical cancer: type-specific prevalence and HPV coinfections. J Med Virol. 2015;87(5):871-84.
19. Brown CR, Leon ML, Munoz K, Fagioni A, Amador LG, Frain B, et al. Human papillomavirus infection and its association with cervical dysplasia in Ecuadorian women attending a private cancer screening clinic. Braz J Med Biol Res. 2009;42(7):629-36.
20. Cabrera JA, Cárdena OJ, Campoverde MA, Ortiz JI. Prevalencia de genotipos del papiloma virus humano en mujeres de la provincia del Azuay, Ecuador. MASKANA. 2015;6(1):79-93.

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CASE REPORTS / REPORTE DE CASO

Quistes pulmonares congénitos en recién nacido. A propósito de un caso.

Congenital lung cysts in a newborn. About a case.

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Resumen: Las malformaciones pulmonares congénitas en especial los Quistes pulmonares congénitos son un grupo heterogéneo de alteraciones del desarrollo pulmonar que pueden producirse en distintas etapas de la embriogénesis, afectando al parénquima, la irrigación arterial, al drenaje venoso o ser una combinación de ellas. Se presenta un caso clínico donde analiza las malformaciones pulmonares congénitas en un paciente recién nacido a término de peso adecuado para la edad gestacional, en mismo que al ingreso del servicio de Neonatología. Al control radiológico en el cual se visualiza una imagen de burbujas de aire atrapada en base izquierda, sospecha inicial de hernia diafragmática, el mismo que se descarta a las 24 horas, cambiando a un diagnóstico de malformaciones pulmonares congénitas Quistes pulmonares congénitos.

Palabras clave: Quistes pulmonares congénitos.

Abstract: Congenital pulmonary malformations, especially congenital pulmonary cysts, are a heterogeneous group of alterations in lung development that can occur at different stages of embryogenesis, affecting the parenchyma, the arterial supply, the venous drainage or being a combination of them. A clinical case is presented where it analyzes the congenital pulmonary malformations in a newborn patient at the term of adequate weight for the gestational age, in the same as when entering the Neonatology service. To the radiological control in which an image of bubbles of air trapped in the left base is visualized, initial suspicion of diaphragmatic hernia, the same that is discarded at 24 hours, changing to a diagnosis of congenital pulmonary malformations. Congenital lung cysts.

Key words: Congenital pulmonary cysts.

Introduction

Las malformaciones pulmonares congénitas son un grupo diverso de trastornos del desarrollo y crecimiento broncopulmonar originados en las distintas etapas evolutivas del sistema respiratorio[1, 2]. Pueden afectar la vía aérea, el parénquima pulmonar, la irrigación arterial pulmonar, el drenaje venoso pulmonar, o una combinación de ellos[3, 4].

Los Quistes pulmonares congénitos se caracteriza por un crecimiento excesivo de los bronquiales terminales, que no están conectados adecuadamente con el espacio sacular, por lo tanto se van formando unas masas muy pequeñas, tiene aparición esporádica, no hay relación con sexo, edad, raza ni con predisposición familiar; correspondería a entre 20% y 40% de todas las operaciones pulmonares por malformaciones pulmonares[5]. Por lo general es unilateral, afecta un lóbulo, en un gran porcentaje, y en la mayoría de los casos es basal. Se puede acompañar de hidrops fetal y de polihidroamnios, ya que en el periodo fetal puede comprimir estructuras vecinas, dificultar el retorno venoso al corazón, producir el hidrops o comprimir estructuras como el esófago, y puede producir polihidroamnios[6, 7].

Caso clínico

Antecedentes prenatales

Recién nacido de madre de 21 años, estudiante de instrucción superior. Producto de la primera gestación, con control prenatal regular, sin antecedentes de importancia.

Al inició el trabajo de parto, con membranas íntegras; se obtuvo por parto eutócico un recién nacido de 40 semanas de edad gestacional, de sexo femenino, con calificación de Apgar 8/9, al minuto y cinco minutos, Silverman-Andersen de cero, peso de 3540 gramos, longitud de 53 cm y exploración física normal; se pasó al alojamiento conjunto, siendo egresada a las 28 horas después en Neonatología.

Antecedentes postnatales

Se presenta un caso clínico de un recién nacido a término de peso adecuado para la edad gestacional de 9 días de vida transferido de una hospital de salud de primer nivel, que presenta dificultad respiratoria con score de downes de 3, por lo cual es ingresado a Neonatología del mismo hospital en donde es tratado y transferido con un diagnostico presuntivo de hernia diafragmática debido a un reporte radiológico en el que se observa estructuras con aire sobre el pulmón Izquierdo más destrocaría, mas colapso de pulmón derecho, con lo cual al momento del ingreso a esta unidad se repiten exámenes radiológicos una tomografía axial computadorizada (TAC) la misma que reporta:

Esta TAC fue raliazada por medio de contraste iodado por vía oral, en la misma se observa opacidad anterior a nivel del mediastino anterior en relación al timo estructuras del me-

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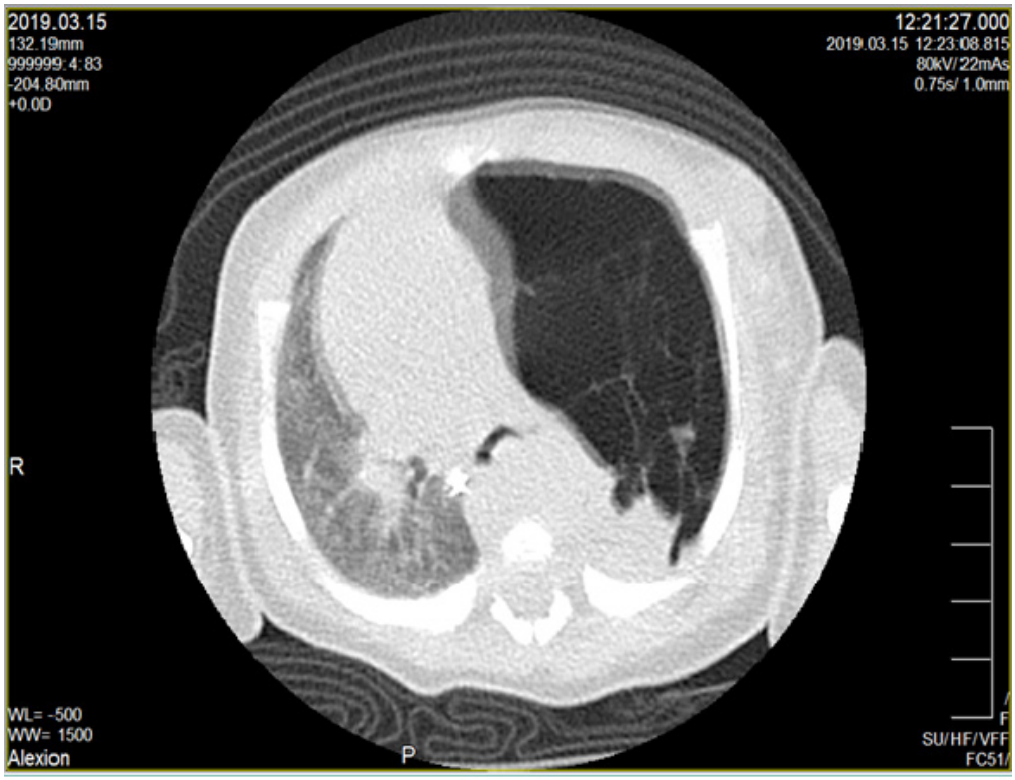

Figura 1. Corte Axial del Resonancia magnética. Se observa lección quística en pulmón izquierdo, que abarca el lóbulo medio.

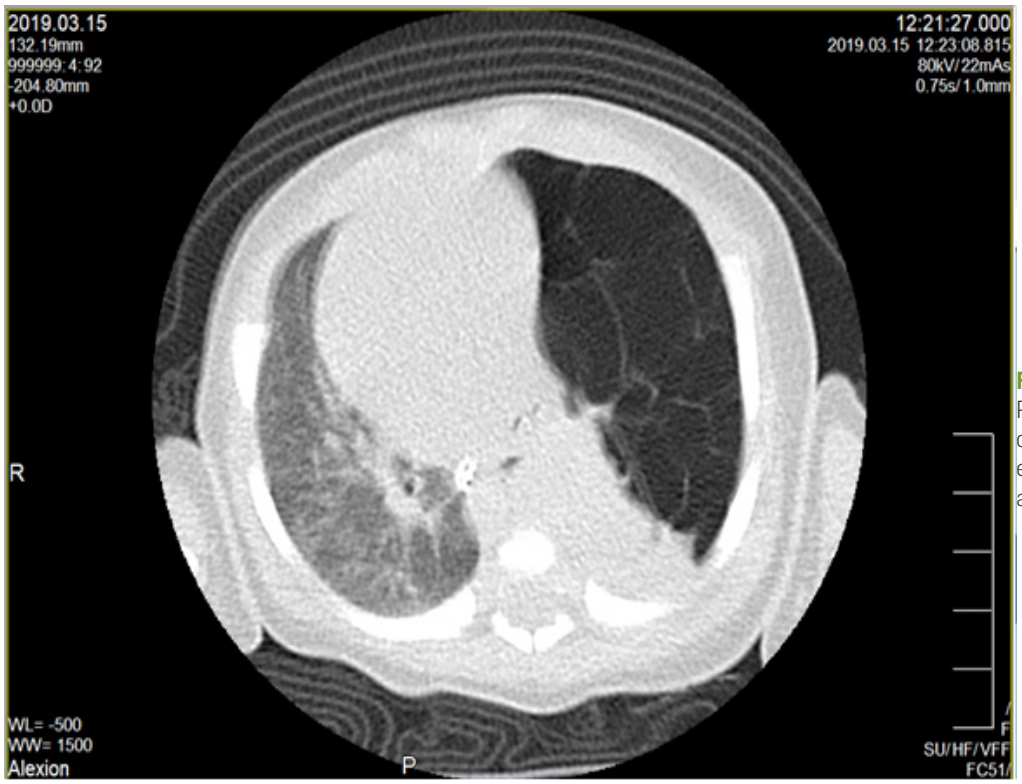

Figura 2. Corte Axial del Resonancia magnética. Se observa lección quística en pulmón izquierdo, que abarca el lóbulo superior.

diastino herniado hacia la derecha. Se evidencia parénquima pulmonar para hiliar izquierdo, imagen hiperdensa con broncograma aéreo que impresiona. El hemitorax izquierdo ocupado por imagen hipodensa densidad liquida con presencia de tabiques en posible relación a quistes. En el hemitorax derecho parénquima en vidrio deslustrado presencia de trama hiperdensa y en el hemidiafragma izquierdo se observa mala definición con no protusion de las estructuras del abdomen hacia la cavidad torácica.

Posteriormente al paciente se corrige diagnostico a quistes pulmonares congénitos y se comienza tramites de transferencia a tercer nivel para resolución quirúrgica, durante la estadía en nuestra unidad, a los 22 día de hospitalización, presenta dos paros cardiacos que se recuperan con la administración de presión positiva, se realiza gasometría misma que reporta acidosis metabólica descompensada por lo que se administra bicarbonato 20 ml iv , tras la reanimación se observa neumotórax atención en RX de control que se coloca

tubo torácico con buena resolución, a la auscultación posterior encontramos los ruidos cardiorrespiratorios normofonéticos entrada de aire regular, a las 24 horas se repite placa de RX la misma que confirma un nuevo colapso asociado a neumotórax, por lo que se decide permanecer con tubo torácico por 48 sin complicaciones posteriores por lo cual se procede al retiro del mismo.

A los 30 días de vida es transferido a una unidad de tercer nivel, donde es analizado por el departamento de cirugía pulmonar, la misma que realiza a los 34 días de vida una bullectomia apical izquierda por toracotomía, se libera la compresión el área quirúrgica, se advierte facilidad en manejo ventilatorio y mejoría hemodinámica con buena evolución, RXTX de control se observa buena expansión pulmonar.

Se recibe segmento sacular sin hilos de referencia anatómica de tejido blando que mide 6.5x4.5cm parcialmente abierto, resto de superficie membranosa con áreas hemorrágicas y congestivas, al corte superficie de aspecto esponjoso con múltiples formaciones pseudoquisticas de paredes finas amarillentas , alguna de las cuales presentan contenido liquido espumoso. sppr 3c

Se inicia terapia respiratoria. Se intenta por tres ocasiones destete de oxigeno sin éxito. A los 41 días de vida se logra destete completo de oxígeno en lo cual es dado de alta para seguimiento por consulta externa.

Exámenes microscópicos

Fragmentos de tejido en los que se observan paredes de múltiples quistes dilatadas revestidas parcialmente por un epitelio simple cubico el estroma es fibroconectivo, con vasos congestivos e infiltrado inflamatorio linfocitario predominantemente.

No se observa parénquima pulmonar conservado.

Diagnósticos finales

Recien nacido a término según pesos y longitudes biométricas CIE 10 (Q330) con síndrome de dificultad respiratoria más paro cardiaco CIE 10 (I46) y malformaciones congénitas del pulmón Izquierdo CIE 10 (Q330) con quiste pulmonar congénito del mismo pulmón CIE 10 (Q330) y neumotórax CIE 10 (J930).

Discusión

Los quistes pulmonares congénitos se originan por una alteración de la diferenciación bronquio-bronquiolo que pueden comprometer al brote del esbozo bronquiolar completo o parcialmente a los bronquios mayores (quistes centrales) o los bronquiolos (quistes periféricos) hasta los alvéolos en forma difusa (pulmón en esponja o displasia alveolar)[8, 9]. Su clasificación es muy variada, pueden ser centrales o periféricos, solitarios o múltiples, perihiliares intra o extra pulmonares, ciegos o con comunicación con el árbol bronquial incrementándose el riesgo de infecciones en el período neonatal[10].

Muchas de estas malformaciones pulmonares suelen estar asintomáticas, pero los riesgos de complicaciones como neumotórax, hemorragia y transformación maligna siempre están latentes[11].

Conclusiones

Aunque a veces está indicado el tratamiento conservador, en ocasiones es indispensable la intervención quirúrgica inmediata. la resección debe realizarse sin retraso en infantes sintomáticos y probablemente en infantes asintomáticos, a causa de la compresión del pulmón normal y el riesgo de infección[12].

Los quistes pulmonares resultan raros en la etapa neonatal y constituyen una importante condición al incrementar los riesgos de morbilidad y mortalidad presentándose de forma aislada o asociados a otras enfermedades, por ello, resulta útil el diagnóstico precoz que permitirá establecer el tratamiento de elección para cada paciente y llevar a cabo un seguimiento multidisciplinario oportuno con el objetivo de mejorar la calidad de vida.

Referencias bibliográficas

1. Sola A. Cuidados neonatales. Descubriendo la vida de un recién nacido enfermo. Argentina: Ediciones Médicas; 2011: 1002-8.
2. Abdallah B, Bouthour H, Hellal Y, Ben Malek MR, Gharbi Y, Kaabar N. Congenital pulmonary malformations: clinical, radiological and treatment features. Tunis Med [Internet]. 2011; 91(1): [aprox. 4p.].
3. Guido M, Bovenschulte H, Drebber U, Pfister R. Bronchogenic cyst mimicking ischemic heart disease. Lung India [Internet]. 2012; 29(4): [aprox. 2p.]. 4. Hadchouel-Duvergé A, Lezmi G, de Blic J, Delacourt C. Congenital lung malformations: natural history and pathophysiological mechanisms. Rev Mal Respir [Internet]. 2012; 29(4): [aprox. 10p.]. 5. Biyyam DR, Chapman T, Ferguson MR, Deutsch G, Dighe MK. Congenital lung abnormalities: embryologic features, prenatal diagnosis, and postnatal radiologic-pathologic correlation. Radiographics [Internet]. 2010; 30(6): [aprox. 7]p.].
4. Alfara J JF, López-Rodó LM, Mier Odriozola J Ml. Malformación adenomatoidea quística pulmonar de afectación bilateral en el adulto. Arch Bronconeumol [Internet]. 2008 [citado 12 Feb 2013]; 44(4):[aprox. 2p.].
5. Viejo M AN, Montes AF, Fernández DI. Neumonía redonda: una causa poco habitual de nódulos pulmonares múltiples. Arch Bronconeumol [Internet]. 2010 [citado 14 May 2013]; 46(4): [aprox. 2p.].
6. James Clayton E, Molsen Z. Lung Cysts en Mohsen Zim, MD.Pediatrics. Fourth.Edition. Edited by Little, Brown and Cambry. Boston. 2000.p. 193-194.
7. Avroy Fanaroff A, Richard Martin S. Otros trastornos pulmonares en la infancia. En: Avroy A. Enfermedades del feto y del recién nacido. La Habana: Editorial Científico –Técnica, 1985.558.
8. González Valdés JA. Malformaciones congénitas de las vías aéreas inferiores y en pulmones. En: Rojo Concepción M, González Valdés LA. Neumología. La Habana:ECIMED, 2005; t 9:1-7
9. Dosios T, Stinios J, Nicolaides P, Spyrakos S, Androulakakis E, Constantopoulos A. Pleuropulmonary blastoma in childhood. A malignant degeneration of pulmonary cysts. Pediatr Surg Int 2004; 20(12):10-2.
10. Hasiotou M, Polyviou P, Strantzia CM, Pourtsidis A, Stinios I. Pleuropulmonary blastoma in the area of a previously diagnosed congenital lung cyst: report of two cases. Acta Radiol 2004; 45:289-92.
11. Hill DA, Dehner LP. A cautionary note about congenital cystic adenomatoid malformation (CCAM) Type 4. Am J Surg Pathol 2004;28: 554- 5.
12. Katz DS, Scalzetti EM, Groskin SA, Kohman LJ, Patel LS, Landas S. Pleuropulmonary blastoma simulating an empyema in a young child. J Thorac Imaging 1995; 10:112–6.

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REVIEW / ARTÍCULO DE REVISIÓN

Pembrolizumab and Nivolumab in the treatment of Non-small cell lung cancer (NSCLC).

Camila Lissett Velastegui Gamboa and Dayanara Lissette Yánez Arcos.
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Abstract: Lung cancer is a disease difficult to treat and with low survival rates, especially non-smaller cell lung cancer (NSCLC). To treat cancer in advanced stages, new methods had arisen like immunotherapy. Pembrolizumab and nivolumab are IgG4 antibodies targeting programmed death cell receptor (PD-1) used for cancer immunotherapy, that blocks the protection that has cancer cells against the immune system. This antibody works binding and blocking the PD-1 membrane protein of T cells, which is responsible for cell recognition. If T cells cannot recognize the cells, then it would attack, so in this way, the immune system can be enhanced. Pembrolizumab and nivolumab have a variable region that is capable of recognizing the PD-1 receptor, and this plays an important role to kill cancer cells. The structure of the complex PD -1 and its ligand PD-L1 or PD-L2 reveals the structural basis of the PD-1. The interaction with a human antibody has been studied with antibody fragments revealing the molecular basis for the blockade of PD1 / PDL1-PDL2 interaction by pembrolizumab and nivolumab. Different studies involving immunotherapy have shown the remarkable results of pembrolizumab and nivolumab over current chemotherapy for cancer treatment making available a possible way for a new treatment for lung cancer. In a comparative analysis made between those immune checkpoint inhibitors had found the efficacy of pembrolizumab for treatment of NSCLC.

KeyWords: Pembrolizumab, nivolumab, lung cancer, immunotherapy.

Introduction

Around the world, there is an increase of 18.1 million new cases of cancer and 9.6 million die from this disease in 2018. Of which, the cancers with the highest incidence are lung, breast, and colorectal cancer, in turn, are found among the top five with the highest mortality. Of these, lung cancer is found to have the highest mortality, with 1.8 million deaths[1]. In the United States, 1 735 350 new cases have been reported during 2018, of which 606640 will die from this disease[2]. Currently, in the world, more than 32 million people suffer from this disease and will increase by 70% in the next 20 years[3]. The World Health `Organization has defined cancer as one of the leading causes of deaths in the world, with lung cancer having the highest number of deaths with 1.69 million in 2015[4].

In Ecuador, it constitutes a serious public health problem, with a 20% chance of acquiring cancer before 75 years of age[5]. Lung cancer is one of the most aggressive, being the one that produces the highest number of deaths per year, according to data from the National Registry of Tumors of the Fight Against Cancer Society in Ecuador (Solca), registering 384 deaths during 2014 and 753 deaths in 2012[6]. The cancer mortality rate during 2009 and 2013 for bronchial lung cancer was 11.1% and an incidence rate of 14.4%[7].

To give a solution to the problem, new and more effective treatments have been developed. One of these treatments is immunotherapy. The food and drug administration (FAO) has approved several treatments for the treatment of cancer, like Nivolumab and Pembrolizumab for untreated metastatic non-squamous non-small lung cancer[8]. The clinical trials of both had demonstrated an improvement in the overall response rate and progression-free survival[9, 10]. Nivolumab was the first PD-1 inhibitor to gain regulatory approval[9]. By the other side, Pembrolizumab was nominated for the molecule of the year in 2017 for its promising results in the treatment of cancer, as well as its versatility in the treatment of several types of cancer, such as lung cancer[11]. This review presents the data of Nivolumab and Pembrolizumab in the treatment of non-small cell lung cancer.

Types of small cell cancer

Lung cancer is one of the most common cancers and has the highest mortality rate of[12]. At diagnosis, half of the patients die within one year, and the overall surviving rate in 5 years is approximately 17.8%[13]. There are two main types of lung cancer: small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). The first account for 15% of lung cancer and 25% of deaths. This type of cancer tends to metastasize early, so operation is not useful and rarely used as treatment. Chemotherapy and radiotherapy are usually used for cancer treatment in SCLC, although the cure is not easy to achieve[14].

Non-small cell cancer is the most common type of cancer, accounting approximately 85 % of cancer diagnoses. This type of cancer is characterized by a different class of tumors[15] and can be subdivided into three main categories squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma.

Squamous-cell carcinoma accounts for 25%-30% of lung cancer and is characterized because it arrays in the epithelial cell of the bronchi. Adenocarcinoma account by 40% of all lung cancer, being the most common. This type tends to grow in peripheral of the lungs and grows slowly. Large-cell carcinoma is detected by the default of the two other possibilities because it did not show clear symptoms[13].

Types of treatment current used

The detection of stage cancer and the type of tumor are key steps at the moment to choose the treatment. Different treatment includes surgery, chemotherapy, radiotherapy, molecular targeting, and immunotherapy (15). Patients with NSCLC in stage I, II, and III commonly have surgery to remove the tumor, although to avoid the relapse, they also have

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chemotherapy and radiotherapy[16].

Approximately 40% of patients have stage IV, and there is not a cure for them due to in this stage, cancer had spread long distances. Their first line of treatment is the combination of chemotherapy based with both platinum and non-platinum compounds[17]. The treatment will depend on the performance status (PS) that is a score that estimates the ability of the patient to do some activities[18]. PS around 0 to 1 recommend platinum- doublet therapy. Patients with PS 2, recommend only one drug which is typically not platinum[19].

Radiotherapy also constitutes a type of treatment of patients with lung cancer. This treatment is important to avoid the locoregional recurrence. Because radiotherapy uses high energy to damage DNA in the cancer cell, it is used at specific sites of the body[20]. Addition of radiotherapy to chemotherapy increases the survival in 3 years approximately 5%[14].

Another therapy that is improving the survival rate is the molecular targeting of cells, in which the target against some factor that helps tumors growth is avoided. The targets are for: epidermal growth factor receptor (EGFR) which is involved in cell growth and proliferation; KRAS a mutated gene present in NSCLC; anaplastic lymphoma kinase ALK which commonly is mutated in tumors; BRAF a proto-oncogene that regulates survival and cell proliferation[13].

Finally, one of the recent therapies that are applying is immunotherapy in which the own immune system can kill tumor cells. These cells present common characteristics that made them be recognized by the immune system and can avoid being destroyed[21]. The edition of this mechanism made the cytotoxic cells able to destroy tumor cells. The problem with this therapy is that the immune system cannot identify the normal cells either, so it can also develop an autoimmune disease. In patients with advanced lung cancer is a matter of extent, their lifetime, because non-small cell lung cancer stage IV has no cure. This principle is the principle of work for pembrolizumab and nivolumab.

Immunotherapy treatment for lung cancer

Cancer cells have mutations that provide characteristic for being recognized like antigens, although, like cancer cells are cells of the body, it can also present characteristic that enables the immune system to attack. In the case of T cells, the amplitude of the response is mediated by the recognition of the T cell receptor (TCR). This recognition can be regulated by stimulatory and inhibitory signals knowing as immune checkpoints[22].

The inhibitory immune checkpoints control the function of T lymphocytes avoiding the excessive immune activation, and they are key factors in how cancer cells evade the immune system. Blockage of these immune checkpoints can enhance the activity of the T cell against cancer cells[23].

One of these immune checkpoints is the protein PD-1, that is like a gene encoding for a protein of the immunoglobulin superfamily. The expression of this gene was associated with dying thymocytes, so this protein was called programmed death receptor PD-1. After was shown that this protein is not involved in apoptosis and have an important role in immune cell tolerance; as a result, it prevents immune disease[24].

Mechanism of action

The immune system evasion is the headline procedure of cancer cell to reduce or limit the effectiveness of antitumor response of the immune system by (25) causing a localized immune suppression through activating of a key immune checkpoint[26]. The structural information of this immune checkpoint protein and its complex with ligands, antibodies, has a potentiality ability for therapeutic designing agents in the immune checkpoint targeting of cancer[27]. Effective therapies given by an adequate molecular recognition of cancer antigens benefit the rise of the new era of treatment, where the individual immune system evade the suppression and combat against them[28]. For that, this next step is crucial in advance of bettering clinical responses in a combination of immunotarget therapy, and it is the focus in researches, and it has been investigated currently[29].

The engagement of the ligand (PD-L1 or PD-L2) and its receptor PD-1 in T cells shows downregulation of TCR, made them nonresponsive for T cells[24]. The expression of PD-L1or PD-L2 is what some cancer cells do to avoid the immune system, to contrasts, this is necessary to blockage the interaction between PD-1 and these ligands[30, 31]. Nivolumab and Pembrolizumab are a humanized monoclonal antibody of IgG4 that alter the engagement between PD-1. In the case of Nivolumab blocks the interaction with the ligands PD-L1 and PD-L2 and Pembrolizumab with PD-L1[26, 32].

Structure of nivolumab

The binding interaction between the crystal structure of nivolumab (PD – 1) and its binding *section* (PD – L1) suggest a competitive binding caused by steric clash to abrogating caused by the interaction[33]. The steric clash of the binding interaction of protein is the result of atomic overlapping caused by the repulsing binding of the Van der Waals[34]. In the case of nivolumab structure, the b-sheet of the interaction of PD – 1/ PD – L1 is dominated by N – loop. The variable loop (VL) chain interact on the surface overlapping the surface with PD – L1[33]. As a result of this interaction, the complex PD – 1 / PD – L1 result of having a strong interaction due to the formation of new binding formation.

One fact of the interaction of nivolumab with its receptors (PD – L1) is the presence of glycosylation protein as an important role in the post-translational modification, important clue for any biological process, as it N- loop. Which is independent of nivolumab interaction, that implies a branch of tumor cell studies, considering that the expression of PD – 1 in tumor cells also include glycan modifications. The importance of maintaining the protein glycosylation formed in the nivolumab complex is that the alteration of these patterns could result in an incomplete synthesis and neo – synthesis processes in tumor cells. On the other hand, the N – loop dominated binding in nivolumab indicates the flexibility if N – a loop of PD – 1, open new ruts of researches on PD – 1/ PD – L1 blockade antibody design[33].

The interaction of the nivolumab with its corresponding receptor is shown in the type and number of receptors involved. In figure 1 are shown the formation of hydrogen bonds between nivolumab and PD – 1 in red and van der Waal interaction in pink, with a total of residues involved in bridge formation of 18 residues. In the same way, figure 1 show residues involved in the interaction of nivolumab and PD – 1; green for the heavy chain and orange for the light chain, having a total of 15 residues in total[29].

Structure of Pembrolizumab

The crystallized structure of the immunoglobulin IG4 pembrolizumab presents particular properties of this subclass, like as immune checkpoint inhibitory activity[26]. This ability allows it to inhibit the interaction among the programmed

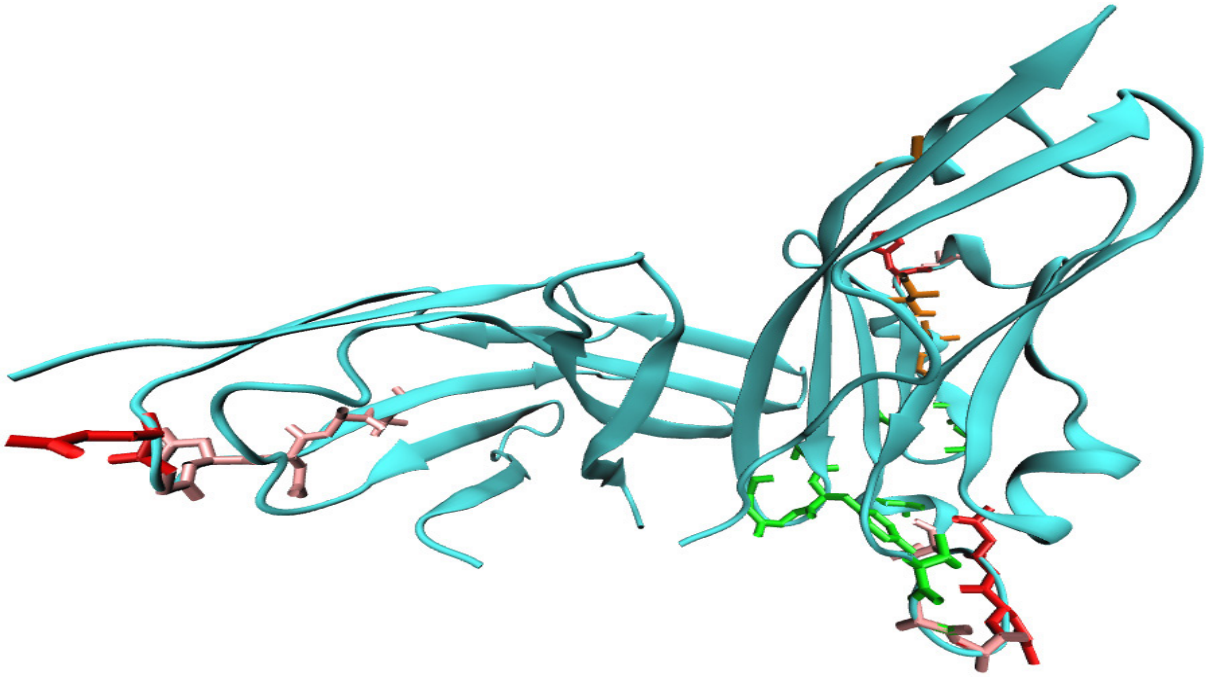

Figure 1. Initial state of the immunoglobulin nivolumab. Representation of the antibodies nivolumab (PDB:4ZQK). The initial conformation state of the immunoglobulin shows the amino acid of the heavy and light chain.

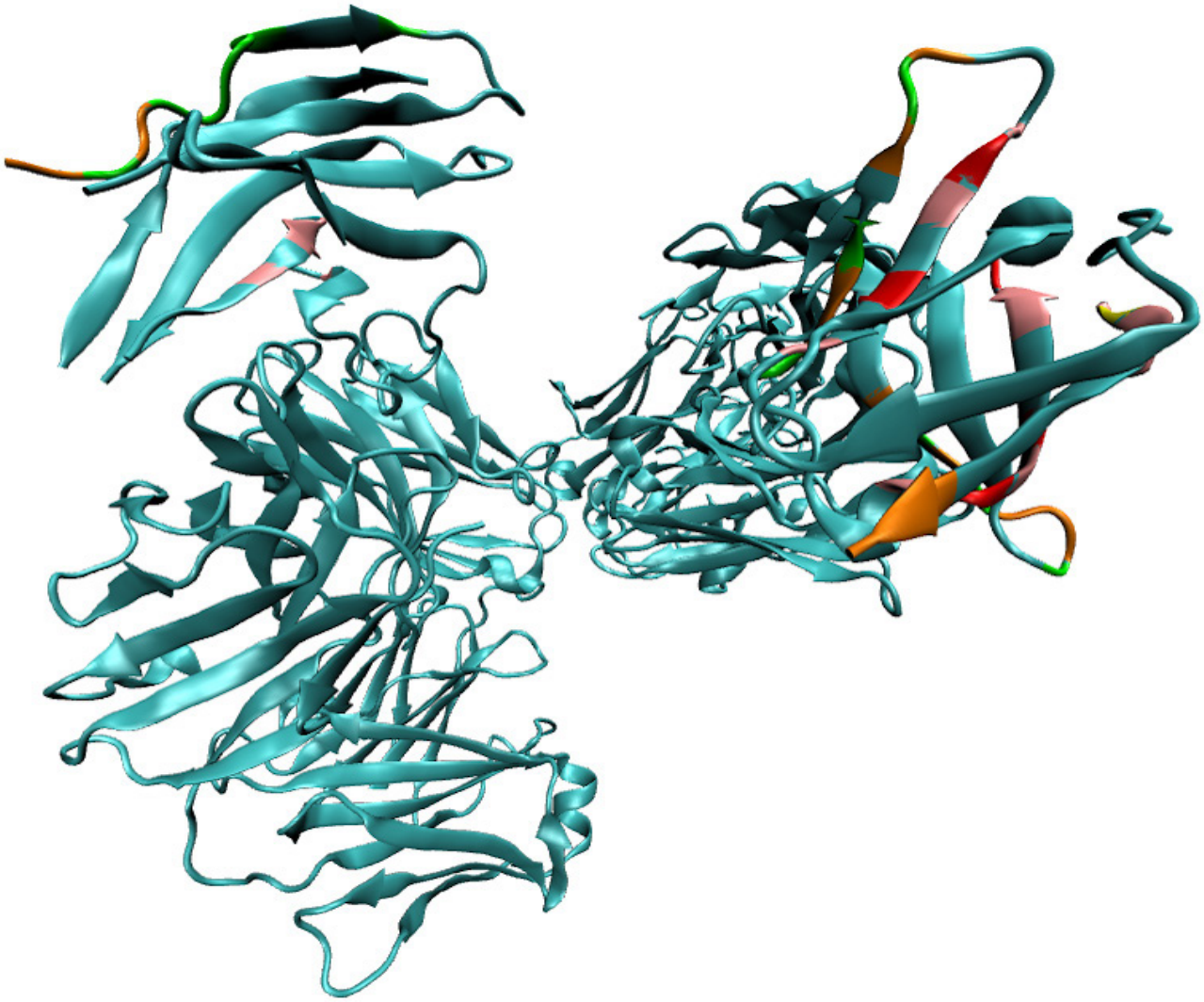

Figure 2. Initial state of the immunoglobulin pembrolizumab. Representation of the antibodies pembrolizumab (PDB:5JXE). The initial conformation state of the immunoglobulin shows the amino acid of the heavy and light chain.

death cell death protein (PD -1) and T-cell[28]. Nowadays the interaction of immune checkpoints and its complexes, like as ligands antibodies and small molecules is deeply study as targeting therapy of cancer[27].

The study of molecular identification or the crystal structure of cancer antigens is the focus of some researches due to the *new approach for effectives therapies*. Nowadays is currently study the PD1 receptor in cancer immunotherapy[28].

The complex interaction of PD – 1 pembrolizumab have a complex crystallized structure of 1.2 Å[29] with an asymmetrical Y shape and symmetrical high regions[26]. The complete structure suggests the mechanism in which the antibody block interaction of PD – 1/PD – L1 is given through outcompeting; in other words, it's more successful than the competitor[28]. The epitope region of the pembrolizumab with PD – 1, such as other type of antibodies, is conformed of discontinues segments. Furthermore, the binding of the complex PD – 1/ pembrolizumab produce a conformational change in the BC loop and the FG loop, making the binging with PD – L1 incompatible[29].

The complex interaction of the pembrolizumab with its corresponding PD – 1 is shown in the type and number of receptors involved. This interaction has more variety of bond formation and residues involved than nivolumab, showing more stability of interaction. In figure 1 are shown the bond formation between pembrolizumab and PD – 1 with hydrogen bond in red, van der Waal interaction in pink, water mediated in yellow and salt bridges in grey; with a total of residues involved in bridge formation of 33 residues. In the same way, figure 2 show residues involve in the interaction of pembrolizumab and PD – 1; green for the heavy chain and orange for the light chain, having a total of 29 residues in total[29].

Nivolumab and Pembrolizumab in lung cancer

Nivolumab and Pembrolizumab are currently being used in the treatment of Non-small Cell Lung cancer due to the great improvement that represents the use of presents in the treatment of cancer in comparison with other types of therapies. All these data are clearly shown in the clinical trials that had made with Nivolumab and Pembrolizumab[35]. This review analyzes the clinical trials made with nivolumab or pembrolizumab vs chemotherapy. In addition to that this review also includes an analysis of a clinical trial in which nivolumab and pembrolizumab were compared directly.

Nivolumab and Pembrolizumab vs. platinum-based therapy

How it's better than other treatment

Several medicine proposals are available for researches to preprocess, named checkmate for nivolumab researches and keynote for pembrolizumab researches, with variable results. We aimed to compare different medicine for non-small cell lung cancer available for nivolumab and pembrolizumab and compare those results with available chemotherapy treatments.

The comparison results are resume in table 1. Where can be demonstrated that treatment with pembrolizumab (50% tumor cell expression) for NSCLC treatment have longer progression-free survival (PFS) and mean overlap survival (OS) compare with chemotherapy treatment (platinum-based).

In the other hand, not significant longer PSF and OS where show between nivolumab and chemotherapy treatment at 5% level expression- Table 1 is of current cancer treatment for NSCLC; (immune checkpoint blockades vs chemotherapy) by comparing the results on clinical trials.

The results of these trials clearly show that Pembrolizumab is better in the treatment of NSCLC because it increases the survival rates, adverse effects are less, and objective response is higher. Pembrolizumab has many benefits over chemotherapy and can be considered to treat advanced non-small cell lung cancer that has over 50% of the presence of PD-L1 in tumor cells.

Nivolumab and Pembrolizumab

The study present was an experiment with patients with advanced non-small cell lung cancer in stage IV, which 230 were treated with nivolumab and 41 with pembrolizumab to evaluated their secondary effects and their effectiveness. The differences in the number of patients treated with every drug are due to eligibility criteria. The drugs were administrated according to standard doses 3mg/kg every two weeks in nivolumab and 2mg/kg every three weeks in the case of pembrolizumab. The result of this experiment is summarized in Table 2[36].

The results showed better results for pembrolizumab than nivolumab, although these results are statistically limited due to the differences in the number of patients treated with each drug. In addition to that, there were some parameters to

	IMMUNOTHERAPY					CHEMOTHERAPY				
	Type of medicament	Range of patients	PFS (Months)	OS (Months)	Treatment-related adverse events	Type of medicament	Range of patients	PFS (Months)	OS (Months)	Treatment-related adverse events
Nivolumab	checkmate 017	135	3.5	9.2	58%	docetaxel	137	2.8	6 ~24%	86%
	checkmate 057	291	2.3	12.2	10%	docetaxel	290	4.2	9.4 ~39%	54%
	checkmate 026	211	4.2	14.4	71%	platinum-based	212	5.9	13.2	92%
Pembrolizumab	keynote -010	345	5.2	17.3	16%	platinum-based	346	4.1	8.2	35%
	Keynote - 024	154	10.3	*80.2%	26.6%	platinum-based	151	6	*72.40%	53.3%

Table 1. Comparison based on the rate of success of different treatment. The variation on the number of patients considered for each study is not taken as a major influence in the general comparison of the result of the clinical trials. The histology was also take in count. The number of death during the experiment was not taking in count due to the influence of diverse adverse effects implied of the medicament that may affect or influence on the development of cancer.
NDA = No Data Available; PFS = Median Progression Free Surviva; OS = Median Overall Survival.
*The median overall survival was not reached in both groups, so it was estimated the percentage of patients alive at six months.

Clinical Trials Results		
Characteristics	**Pembrolizumab**	**Nivolumab**
PFS (Performance Free-Survival)	5.7 months	13.5 months
OS (Overall Survival)	9.2 months	13.5 months
(irAE)Immune related-adverse events	10%	4.9%
Incidence of Pneumonitis	4.8%	14.6%
Treatment-related deaths	3	0
Discontinuation of PD-1 antibody due to irAE.	28 patients	4 patients
The objective response rate	44.8%	27.8%
Last follow-up survival of treated patients which had died	62.2%	46.6%

Table 2. Comparison of clinical between Pembrolizumab and Nivolumab in which the patients have NSCLC stage IV and were assigned to each group.

assign the patient to the treatment, so there exists some bias. There exist a wider range to patients that can be treated with Nivolumab than Pembrolizumab.

Another important aspect to take in consideration these treatments is the cost. In the case of Nivolumab the overall cost is $103,220[37], Pembrolizumab 160,000 and chemotherapy 73,000[38]. Although a better measure is the incremental cost-effectiveness ratio (ICER) that denotes the necessary payment for an additional year for these treatments. The ICER for nivolumab is $117,857, for chemotherapy $185,802 and for pembrolizumab is $98,421 (34). As we can see, pembrolizumab has better cost-effectiveness for NSCLC than nivolumab although the cost of pembrolizumab is higher.

Conclusions

Pembrolizumab and Nivolumab had shown better results in the treatment of non- small cell lung cancer in comparison with chemotherapy. This could be because both drugs improve the capacity of the immune system to recognize and attack tumor cells. Nivolumab had less adverse effects than chemotherapy, although the general survival did not have significant differences. By the other side, Pembrolizumab shows better results in survival and adverse effects in comparison with chemotherapy. Pembrolizumab and Nivolumab are anti-PD-1 receptors, although there are some structural differences that in the clinical trial had shown better results for Pembrolizumab in patients with PD-L1 expression over 50%. Future research should include a direct comparison between both drugs with the same conditions and number of patients for both groups.

Bibliographic references

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;
2. National Cancer Institute. Estadísticas del cáncer. 2018;20:1–5. Available from: https://www.cancer.gov/espanol/cancer/naturaleza/estadisticas
3. Perraso V. 10 Gráficos Para Entender El Grave Impacto Del Cáncer En El Mundo. Bbc. 2016;http://www.bbc.com/mundo/noticias/2016/02/160203_c.
4. World Health Organization. Cáncer [Internet]. Cáncer. 2018. Available from: http://www.who.int/es/news-room/fact-sheets/detail/cancer
5. Ministerio de Salud Publica del Ecuador. Estrategia nacional para la atención integral del cáncer en el Ecuador [Internet]. 2017. Available from: https://aplicaciones.msp.gob.ec/salud/archivosdigitales/documentosDirecciones/dnn/archivos/ac_0059_2017.pdf
6. González S. Cáncer de pulmón, uno de los más agresivos en Ecuador. Diario La Hora [Internet]. 2017;1–3. Available from: https://lahora.com.ec/noticia/1102046320/cc3a1ncer-de-pulmc3b3n-uno-de-los-mc3a1s-agresivos-en-ecuador
7. Cordero FC, Ayala PC, Maldonado JY, Montenegro WT. Trends in cancer incidence and mortality over three decades in Quito-Ecuador. Colomb Med. 2018;
8. FDA. Pembrolizumab (Keytruda) 5-10-2017 [Internet]. 2017. p. 10–1. Available from: https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm558048.htm
9. Sundar R, Cho BC, Brahmer JR, Soo RA. Nivolumab in NSCLC: Latest evidence and clinical potential. Therapeutic Advances in Medical Oncology. 2015.
10. FDA. FDA Approves Pembrolizumab in Combination With Chemotherapy for First-Line Treatment of Metastatic Nonsquamous NSCLC [Internet]. 2018. p. 1–2. Available from: https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm624659.htm
11. Couzin-Frankel J. A cancer drug's broad swipe. Science (80-). 2017;358(6370):1520–1.
12. Khuder SA, Lally CA, Flannery JT, Calle EE, Flanders WD, Heath CW, et al. Effect of cigarette smoking on major histological types of lung cancer: a meta-analysis. Lung cancer [Internet]. 2001;31(2–3):139–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11165392
13. Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. Transl Lung Cancer Res. 2016;
14. Kalemkerian GP. Small Cell Lung Cancer. Semin Respir Crit Care Med. 2016;37(5):783–96.
15. Gridelli C, Rossi A, Carbone DP, Guarize J, Karachaliou N, Mok T, et al. Non-small-cell lung cancer. Nat Rev Dis Prim. 2015;
16. Howington JA, Blum MG, Chang AC, Balekian AA, Murthy SC. Treatment of Stage I and II Non-small Cell Lung Cancer. Chest [Internet]. 2013 May 1;143(5):e278S-e313S. Available from: https://doi.org/10.1378/chest.12-2359
17. Ramalingam S, Belani C. Systemic Chemotherapy for Advanced Non-Small Cell Lung Cancer: Recent Advances and Future Directions. Oncologist [Internet]. 2008;13(Supplement 1):5–13. Available from: http://theoncologist.alphamedpress.org/cgi/doi/10.1634/theoncologist.13-S1-5
18. West H, Jin JO. Performance status in patients with cancer. JAMA Oncology. 2015.
19. Masters GA, Temin S, Azzoli CG, Giaccone G, Baker S, Brahmer JR, et al. Systemic Therapy for Stage IV Non–Small-Cell Lung Cancer: American Society of Clinical Oncology Clinical Practice Guideline Update. J Clin Oncol [Internet]. 2015;33(30):3488–515. Available from: https://doi.org/10.1200/JCO.2015.62.1342
20. Amini A, Yeh N, Gaspar LE, Kavanagh B, Karam SD. Stereotactic body radiation therapy (SBRT) for lung cancer patients previously treated with conventional radiotherapy: A review. Vol. 9, Radiation Oncology. 2014.
21. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. Science (80-) [Internet]. 2011 Mar 25;331(6024):1565 LP – 1570. Available from: http://science.sciencemag.org/content/331/6024/1565.abstract
22. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature Reviews Cancer. 2012.
23. Mahoney KM, Freeman GJ, McDermott DF. The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma.

Clin Ther [Internet]. 2015 Apr 1;37(4):764–82. Available from: https://doi.org/10.1016/j.clinthera.2015.02.018
24. Haanen JBAG, Robert C. Immune Checkpoint Inhibitors. In: Progress in Tumor Research [Internet]. 2015. p. 55–66. Available from: https://www.karger.com/DOI/10.1159/000437178
25. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the Treatment of Non–Small-Cell Lung Cancer. N Engl J Med [Internet]. 2015;372(21):2018–28. Available from: https://doi.org/10.1056/NEJMoa1501824
26. Scapin G, Yang X, Prosise WW, McCoy M, Reichert P, Johnston JM, et al. Structure of full-length human anti-PD1 therapeutic IgG4 antibody pembrolizumab. Nat Struct Mol Biol. 2015;
27. Zak KM, Grudnik P, Magiera K, Dömling A, Dubin G, Holak TA. Structural Biology of the Immune Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2. Structure [Internet]. 2017 Aug 1;25(8):1163–74. Available from: https://doi.org/10.1016/j.str.2017.06.011
28. Tavares ABMLA, Lima Neto JX, Fulco UL, Albuquerque EL. Inhibition of the checkpoint protein PD-1 by the therapeutic antibody pembrolizumab outlined by quantum chemistry. Sci Rep. 2018;
29. Lee JY, Lee HT, Shin W, Chae J, Choi J, Kim SH, et al. Structural basis of checkpoint blockade by monoclonal antibodies in cancer immunotherapy. Nat Commun. 2016;
30. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. N Engl J Med [Internet]. 2016;375(19):1823–33. Available from: https://doi.org/10.1056/NEJMoa1606774
31. Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunès JA, et al. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. Int Immunol. 2010;
32. Robert C, Long G V., Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in Previously Untreated Melanoma without BRAF Mutation. N Engl J Med. 2015;
33. Tan S, Zhang H, Chai Y, Song H, Tong Z, Wang Q, et al. An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat Commun. 2017;
34. Verma V, Sprave T, Haque W, Simone CB, Chang JY, Welsh JW, et al. A systematic review of the cost and cost-effectiveness studies of immune checkpoint inhibitors. J Immunother Cancer. 2018;
35. Pabani A, Butts CA. The current landscape of immunotherapy for the treatment of metastatic non-small-cell lung cancer. Curr Oncol. 2018 Jun;25(Suppl 1): S94–102.
36. Ksienski D, Wai ES, Croteau N, Fiorino L, Brooks E, Poonja Z, et al. Efficacy of Nivolumab and Pembrolizumab in Patients With Advanced Non–Small-Cell Lung Cancer Needing Treatment Interruption Because of Adverse Events: A Retrospective Multicenter Analysis. Clin Lung Cancer [Internet]. 2018; Available from https://doi.org/10.1016/j.cllc.2018.09.005
37. Andrews A. Treating with Checkpoint Inhibitors-Figure $1 Million per Patient. Am Heal drug benefits. 2015;
38. Georgieva M, da Silveira Nogueira Lima JP, Aguiar Jr. P, de Lima Lopes Jr. G, Haaland B. Cost-effectiveness of pembrolizumab as first-line therapy for advanced non-small cell lung cancer. Lung Cancer [Internet]. 2018 Oct 1;124:248–54. Available from: https://doi.org/10.1016/j.lungcan.2018.08.018

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NEWS AND VIEWS

Stem cell activity in the repair of cardiovascular tissues.

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Abstract: Stem cells can become different types of cells and have the potential to divide and self-renew. There are two types of stem cells, first the embryonic stem cells and second the adult stem cells, both help in regeneration or repair tissues of an organism, for this reason, the stem cells are being used to renew the world of medicine. Stem cells are obtained from three sources: the first can be our own body that where certain organs still have some cells still not completely differentiated. The second source is the embryos when they are in the blastocyst phase (between five to fourteen days from conception), and the third source can be in the cells of the skin, liver or another cell type that have been modified to behave like embryonic stem cells. With this therapy, we would find ourselves before an inexhaustible source to repair the tissues and organs that were damaged in our bodies. One of the main causes of mortality in heart failure, but with the help of cell therapy has been studied the repair of cardiac tissue with the stem cell transplant. The objective of the cellular transplantation is that the transplanted cells in the heart tissue manage to regenerate, renewed, and repair any part of the heart tissue damaged.

KeyWords: Stem cell, cardiomyocytes, hematopoietic cells, autologous, bone marrow.

Introduction

One of the fields of medicine that has caused more expectation in recent years has been cell therapy with stem cells. Stem cells in our body have the ability to self-renew, allowing our organs to repair the damaged tissue. These cells can be classified into embryonic stem cells, obtained from the embryo, adult stem cells found in the tissues of the adult organism, and induced pluripotent stem cells (iPSC) are adult cells that have been rescheduled genetically, the three types of cells have the ability for self-renewal and differentiation themselves in any of the types of specialized cell. Stem cells can be obtained in the human body in certain organs, in embryos or in the cells of the skin, liver or another cell type that have been modified to behave like embryonic stem cells.

Heart failure has been one of the diseases with the highest mortality rate[1], it is for this reason that it has sought to thousands of treatments that help to avoid or reduce deaths from heart failure. Among the most effective treatments is the heart transplant, however, is one of the treatments that carry more risk for the patient because many times they have to wait for a heart for years and with a fairly restricted lifestyle. Besides, when it is going to perform the transplant , there is a risk of death during the surgery and after it because of the rejection that the body can suffer when receiving a foreign organ. In recent years it has been shown that the heart has cells with the ability to renew, therefore it has been proposed as a type of treatment cell therapy with cells from the same organism to decrease the range of rejection of the body.

Stem cells may be donated from the same patient (autologous transplants) or may be donated by another individual that is compatible with the human leukocyte antigen (allogeneic transplants). There are several ways to transplant stem cells, and they can be: 1. intravascular: by intravenous route or 2. It is injected directly into the cardiac muscle: by trans-epicardial route or by infusion intracoronary or by injection through the coronary veins. The field of regenerative medicine is very extensive and is still in the process to be a completely feasible method, and that can save lives not only from people with heart tissue damage but to any other area such as neurological diseases, pulmonary or hepatic.

STEM CELLS: Definition

Stem cells differ from other cells because they have two essential characteristics. First, these are cells with the capacity to auto-renew through cell division, but without being specialized. Second, to be cells not specialized can become any cell specifies of tissues or organs with special functions. They have the potential to divide, also in many tissues; they can regenerate to repair or replace other cells that have died while the person or animal is alive. There are two types of stem cells, "from their origin we divide the stem cells in embryonic (derived from the embryo either the blastocyst or the gonadal crest) and adults (derived from one of the tissues Adults) "[2].

Embryonic stem cells

After 4 or 5 days of fertilization, the cells of the embryo have been divided, forming a new structure called blastocyst constituted with around 500 cells. Cells that form the blastocyst are responsible for giving rise to all cell types, systems, tissues, and organs of the individual that is beginning to form. Embryonic stem cells are found in the internal cell mass of the blastocyst, can divide continuously and then have the ability to differentiate to form any tissue of the organism. The characteristics of embryonic stem cells are the same as cancer cells, according to Aj & Science[3]. Human embryonic stem cells (hESCs) share several characteristics with cancer cells, including upregulation of oncogene expression, increased proliferation, genomic instability, and elevated telomerase activity. For this reason, embryonic stem cells are used for the field of regenerative medicine, challenging researchers because they try to control cell differentiation to obtain any tissue or regenerate any organ[4].

Adult stem cells

Adult stem cells are cells found in different tissues, but these cells are not differentiated. Adult stem cells in the body are used to repair the tissue in which they reside, fulfilling the two main functions of the embryonic stem cells can self-renew and can differentiate to produce the cell types of the

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tissue or organ. The functions are significantly lower than the embryonic cells; however, several studies have been achieved with adult stem cells because they also have a function of trans differentiation, "every time it seems more evident than adult stem cells are capable of generating mature cells from tissues derived from different embryonic layers"[2].

In each tissue or organ of our body there is a small number of stem cells that allow the tissue to regenerate, but in a limited way, therefore, when these cells are lost the tissue loses the ability to repair. The investigation of adult stem cells has achieved to find this type of cells in the brain and heart and with this finding has been able to apply cell transplantation as a therapy to regenerate damaged tissue healing many diseases that are caused by tissue damage.

Obtaining the stem cells

There are different types of stem cells, the same ones that can be obtained in different ways: Adult Stem Cells or specific can be found in our own body in certain organs although they are in small numbers. Embryonic stem cells as its name say it is found in embryos. These embryos can be donated voluntarily by different patients in fertility clinics. And induced pluripotent stem that is in the cells of the skin, liver, or another cell type that have been modified to behave like embryonic stem cells.

Embryonic stem cells are those that can be extracted from the embryos; these cells are pluripotent which means that they are not yet cells of a specific tissue or organ and it can be converted into any tissue or organ of the human body. These embryonic cells are often obtained from different fertility clinics where fertilized ovules are found from some in vitro fertilization technique from different donors. Embryonic cells should be extracted when the embryo has few days of its creation, i.e. it is in a state of the blastocyst. This blastocyst has two parts an outer layer of cells that formed the placenta and the internal cell mass (inner layer of cells) these are the non-specialized cells, and it is not differentiated yet. In this way, the internal cell mass must be extracted in a culture plate containing a nutrient-rich broth (culture medium)[5].

Adult stem cells are those undifferentiated cells that are among cells that if they are differentiated tissue or organ. The function of these cells is to maintain and repair the tissue they are in. The amount of these cells in the different organs is not very high, but through years of research has been finding more in different tissues and organs. As well as hematopoietic stem cells, these cells can be obtained from the bone marrow, blood or umbilical cord. In the case of bone marrow cells, they are obtained through punctures in both iliac crests (located in the hip bone); this procedure will be carried out under general anesthesia and lasts 2 to 3 hours[6]. In the case of the extraction of stem cells in the peripheral blood should be initiated with the administration of a growth factor (approximately four days before extraction). This medicine helps stem cells leave the bone marrow to the blood, once the cells are in the blood is necessary to perform the apheresis (the process by which extract blood from the donor of a vein of the elbow). This blood is processed on a machine that separates the stem cells of the others and proceeds to return the remainder through another vein in the other arm[7].

Induced pluripotent stem cells (IPSC) are the adult cell that has been genetically reprogrammed, possessing pluripotent characteristics. These are derived from cells that initially were not pluripotent. Therefore, these cells can be obtained as adult stem cells[7].

Tissue damage in the cardiovascular system

The heart is one of the first organs that are formed in the fetus, cells called cardiomyocytes to form the cardiac muscle and according to Nadal-Ginard[8], the cardiomyocytes are generated from a cell precursor that divides and gives rise to groups of cells of the same type. Cardiomyocytes are cells responsible for generating contraction and relaxation in the heart; in other words, they are responsible for the heartbeat[9]. The cells of the muscle heart (cardiomyocytes) are cells with the potential to multiply during the development of the fetus until the human being comes to the 3 or 4 months of postnatal life, then it is assumed that the human being already has the

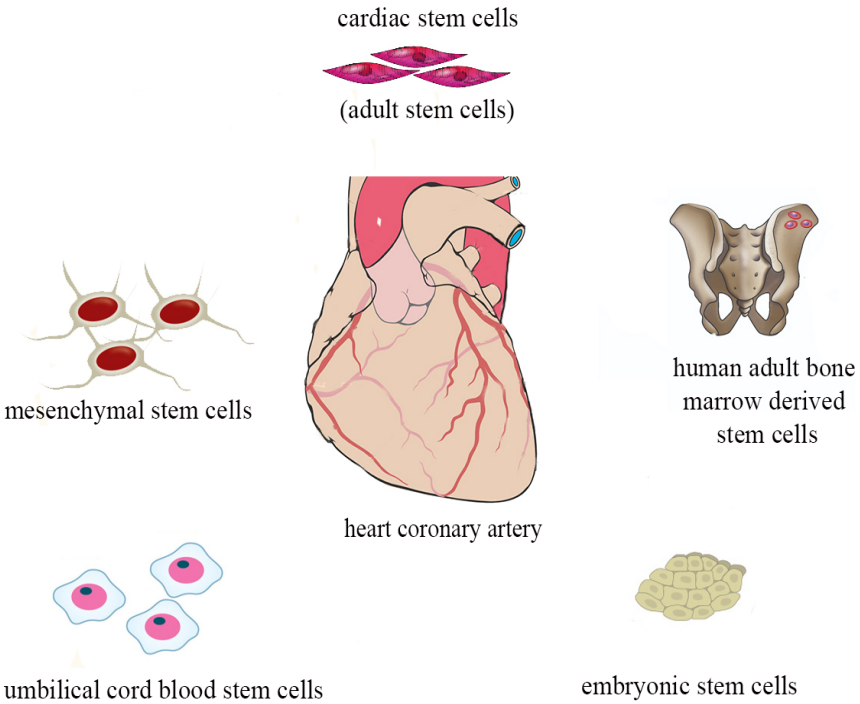

Figure 1. Types of stem cells used to repair cardiac tissue.

maximum number of cardiomyocytes and from there lost cells will not recover or regenerate[8, 10].

Cardiovascular diseases are the diseases that cause most deaths in the world, "accounting for more than 17 million deaths every year and accounting for 31% of all global deaths"[11]. The World Health Organization claims that cardiovascular disease is due to heart and blood vessel disorders; one of the most important and most dangerous diseases is heart failure. Every year more people die because of this disease than any other, for this reason, thousands of treatments have been sought to reduce deaths, however, when treatments such as drugs or therapies do not work, the heart transplant is used.

Although the heart transplant seems to be the best treatment, it is also the riskiest during and after surgery. Also, Park and Yoon[11] say "heart transplantation is currently the only definitive treatment; however, it is limited by lack of donors, potential graft rejections, and various side effects resulting from immunosuppression." For this reason, other ways of repairing the patient's heart have been investigated without the need for a complete replacement, and a form has been therapy or cell transplantation.

The heart has the potential to regenerate their tissues, but in a limited way and depending on the speed of its degeneration, therefore, one of the best ways to help with the regeneration of muscle cardiac is transplanting stem cells from the same heart as cardiomyocytes or myoblasts (cells from the muscle-skeletal).

How transplanting the stem cells

Before talking about the ways to perform stem cell transplantation, it is necessary to mention the two types of bone marrow transplantation that can be found:

Autologous transplants: In these transplants, an individual donates his or her stem cells from his or her bone marrow to the treatment[12].

Allogeneic transplants: Instead, in this transplant, the patient receives the stem cells from another person's bone marrow for treatment[12]. Of course, this type of transplant has certain complications as the patient must find a donor with the compatible bone marrow. The patient should be compatible with the Human Leukocyte Antigen (HLA) because they are some specific proteins in white blood cells and other cells that make the type of tissues different in each. For this reason, a patient with a higher percentage about HLA will have a lower probability of the occurrence of side effects[13].

There are several pathways for stem cell transplantation but first must select what type of stem cells are to be used in transplantation, can be embryonic, adult or pluripotent-induced stem cells. In this case, will explain the transplantation of hematopoietic stem cells obtained from the bone marrow. The pathways for this transplantation may be: intravascular or injected directly into the heart muscle.

1. Intravascular this can be performed by intravenous route.

The intravenous route:

This procedure is simple and consists of entering stem cells by an injection into the blood using a central venous catheter. The problem in this pathway lies in the fact that the stem cells entered can be directed toward other organs which reduce greatly the number of stem cells that should reach the myocardium and them join together to generate the regeneration[6].

2. Injected directly into the heart muscle.

This process is usually used in diseases such as cardiomyopathy, or coronary heart disease. This process can be done a trans-epicardial route or by infusion intracoronary or by injection through the coronary veins.

Trans-epicardial route:

In this process, stem cells are inserted directly in the myocardium (affected area and its edges). According Siminiak T, Fiszer D, Jerzykowska O: "In humans, this pathway has been used so far in conjunction with Revascularization surgery or bypass, or during the placement of a mechanical ventricular device"[14]. By this means the cells to enter directly to the affected area and will have more precision to make the process successful. However, this process has a small limitation because it can have risks in surgery and by the use of anesthesia. As a result, it is usually only performed to individuals who are going to have a revascularization surgery[6].

Intracoronary Infusion:

In this process the cells are inserted by an intracoronary catheter and depending on the exact area in which it was inserted, the cells will be distributed by the coronary vascular bed or placed in a specific area of the myocardium[6].

Injection through coronary veins:

This method uses a catheter that has an ultrasound system placed at the tip of the catheter (this is used to guide the catheter) and an expandable needle through which the cells are inserted into the myocardium. The cells are injected more deeply in parallel to the ventricle wall. This process is still risky and complicated[6].

Clinical trials

In several experimental studies have been using different types of stem cells, in this clinical study was sought to identify what type of stem cell is most suitable for transplantation in cardiac diseases (Table 1).

Embryonic cells seem to be the most suitable because of their ability to differentiate. However, they face many ethical problems and in different studies are prone to be carcinogenic. According to Dorticós E and Hernández P: "It is known that in the bone marrow there is a very heterogeneous cellular population, although the various types of progenitor cells, as well as the mechanisms of control of their function and differentiation, are not yet well understood. Hematopoietic progenitors (CD34), endothelial precursors (CD 133), mesenchymal cells (stromal) (CD34-), others called a lateral population, and the multipotent adult progenitor cells (MAPC) are found. Therefore, of the different types of stem cells, those coming from the bone marrow seem to be, so far, those that have shown greater ability to differentiate towards cardiac muscle fibers or endothelial cells"[6]. For this reason, we have observed that stem cells obtained from bone marrow have been taken into account in several clinical trials in recent years.

In a clinical study performed on 55 patients with dilated cardiomyopathy, it was divided into two groups, 28 patients would have CD34+ transplantation, while 27 patients would not have transplantation with stem cells and would only serve as a control group. The two groups received granulocyte-colony stimulating factor (G-CSF) therapy, but only in the first group were collect cells CD34+ by a technique called apheresis, then patients in this group received intracoronary transplantation of autologous CD34+ cells. In both groups,

Types	Transformation into cardiomyocytes	Viability	Need for immunosuppression	Development of some cancer	Bioethical problems
Embryonic cells	+	+ -	+ -	++	++
Hematopoietic stem cell	+	++	-	-	-
Allogenic myoblast	+	+ -	+	¿	+ -
Autologous myoblasts	+	+	-	-	-

Table 1. Stem cells used in cardiac regeneration.

Disease	Type of cells	Patients	Purpose	Results	Year
Acute myocardial infarction[15]	BMSC	20	Viability Security ESL	Yes Safe SI Significant reduction	2002
Acute myocardial infarction[16]	BMSC	69 Random	Viability Security LVEF	Yes Safe with no deaths SI	2004
Myocardial infarction [17]	G-CSF Injection peripheral blood	27	Viability Efficiency	Yes Yes SI	2004
Recent myocardial infarction [18]	CF 133+, Bone marrow	35	Viability Security	Yes Safe	2004
Acute myocardial infarction [19]	BMSC	67 Random	Feasibility Security LVEF	Yes No safe SI	2006

Table 2. Clinical studies with hematopoietic stem cells injected by infusion intracoronary. BMSC: bone marrow-derived stem cell; G-CSF: granulocyte-colony stimulating factor; ESL: end-systolic left; LVEF: Left ventricle ejection fraction; SI: Significant improvement.

the same evaluation parameters were taken like an increase in the left ventricular ejection fraction (LVEF), an increase in 6-minute walk distance and measured plasma levels of NT-proBNP. At the beginning of the clinical trial, all 55 patients had the same age, gender, left ventricular ejection fraction (LVEF), and NT-proBNP levels were the same. However, after one year of the study, the parameters of each group were evaluated and according to Vrtovec[20] the following results were obtained: An increase in LVEF (from 25.5± 7.5% to 30.1± 6.7%), an increase in 6-minute walk distance (from 359± 104 m to 485± 127 m), and a decrease in NT-proBNP (from 2069± 1996 pg/mL to 1037± 950 pg/mL). Also during the clinical trial, it was possible to demonstrate the decrease in mortality in patients with stem cell transplantation, two patients died by cardiac failure which represents a percentage of 7%, in contrast in the control group died 8 patients; it means 30 % of 27 patients died. The clinical trial showed the autologous hematopoietic stem cell transplantation as a technique to improve the function of the left ventricle, thus improving the lifestyle of patients suffering from dilated cardiomyopathy.

Conclusions

The cellular self-renewal not only of the heart but of any organ will allow giving a turn to the medicine allowing advancement in the disciplines like the genetics and cellular biology. In advances in regenerative medicine, clinical trials have been conducted practically for all types of tissues and organs, but some of them without results. In cell therapy, stem cells are ones indicated to be transplanted by their ability to self-renew and differentiation, helping to generate new healthy cells and in this way to replace the damaged or dead tissue cells. There are three types of stem cells, embryonic stem cells, adult and pluripotent; however, transplantation of these cells entails ethical problems. Embryonic stem cells by obtaining them from the embryo cause more conflict with society.

On the other hand, with the adult stem cells and pluripotent does not pose any more problem than the informed consent of the person from which the cells are extracted. Of the different types of stem cells that we can obtain, the best is the adult stem cells specifically the hematopoietic stem cells obtained from the bone marrow since in several trials it has been seen that these cells differentiate into cells of the tissue damaged. One of the organs most studied for cell transplantation is the heart. The heart is an organ in a continuous process of death and renewal. Therefore the cultivation of stem cells of the own cardiac tissue as the cardiomyocytes represents an innovative form of treatment for heart failure. However, current studies on cell therapy applied to cardiac tissue, show a favorable differentiation towards cardiomyocytes when hematopoietic stem cells are obtained from the bone marrow.

Bibliographic references

1. Trainini J, Cichero D, Cardiol NB-RA, 2002 undefined. Cardioimplante celular autólogo. SacOrgAr.
2. Prósper F, Gavira JJ, Herreros J, Rábago G, Luquin R, Moreno J, et al. Trasplante celular y terapia regenerativa con células madre Cell transplant and regenerative therapy with stem cells. An Sist Sanit Navar. 2006;29(2):219–34.
3. Aj B, Science B. Chapter 12: 2000;1869:127–42.
4. Castagnino JM. Células madre embrionarias. Acta bioquímica clínica Latinoam. 2005;39(3):277–8.
5. Rippon HJ, Bishop AE. Embryonic stem cells. Cell Prolif [Internet]. 2004 Feb [cited 2018 Nov 27];37(1):23–34. Available from: http://doi.wiley.com/10.1111/j.1365-2184.2004.00298.x
6. Centro Nacional de Información de Ciencias Médicas. E, Hernández Ramírez P. Revista cubana de hematología inmunología y hemoterapia. [Internet]. Vol. 22, Revista Cubana de Hematología,

Inmunología y Hemoterapia. Centro Nacional de Información de Ciencias Medicas, Ministerio de Salud Publica; 2006 [cited 2018 Nov 27]. 0-0 p. Available from: http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0864-02892006000100003
7. Herreros González J, Prósper Cardoso F, Alegría Ezquerra E. Utilización de células madre para la regeneración miocárdica en la insuficiencia cardíaca. Rev Española Cardiol [Internet]. 2003 Oct 1 [cited 2018 Nov 27];56(10):935–9. Available from: http://www.revespcardiol.org/cgi-bin/wdbcgi.exe/cardio/mrevista_cardio.fulltext?pident=13052378
8. Nadal-Ginard B. Inducción de nuevos cardiomiocitos en el corazón adulto : futuro de la regeneración miocárdica como alternativa al trasplante. Rev Española Cardiol. 2001;54(5):543–50.
9. Woodcock EA, Matkovich SJ. Cardiomyocytes structure, function and associated pathologies. Int J Biochem Cell Biol. 2005;37(9):1746–51.
10. Bigalli D, Bico Uribe JA, Gossio Landoni EE. Cardioimplante celular para reparar tejido cardiaco: un nuevo concepto terapeutico?^ies. Rev urug cardiol. 2005;20(3):158–70.
11. Park M, Yoon Y. Cardiac Regeneration with Human Pluripotent Stem Cell-Derived Cardiomyocytes. Korean Circ J. 2018;48(11):974.
12. Cuadros Celorrio M, Sarmiento González-Nieto V, Villegas Portero R. Células madre en pacientes cardíacos [Internet]. [cited 2018 Nov 27]. Available from: http://www.aetsa.org/download/publicaciones/antiguas/AETSA_2007-02-08_Celulas_Madre.pdf
13. Valdés Chavarri M, Pascual Figal D, Prósper Cardoso F, Moreno Montañés J, García Olmos D, Barcia Albacar JA. Medicina regenerativa con células madre adultas. Rev Clin Esp [Internet]. 2005;205(11):556–64. Available from: http://dx.doi.org/10.1016/S0014-2565(05)72638-2
14. Siminiak T, Fiszer D, Jerzykowska O. Percutánea trans-coronaria-venosa trasplante de autólogas esqueléticos mioblastos en el tratamiento de poste - infarto deterioro de la contractilidad miocárdica: la ... Eur Hear [Internet]. 2005 [cited 2018 Nov 27]; Available from: https://academic.oup.com/eurheartj/article-abstract/26/12/1188/524870
15. Assmus B, Schächinger V, Teupe C, Britten M, Lehmann R, Döbert N, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation [Internet]. 2002 Dec 10 [cited 2018 Nov 27];106(24):3009–17. Available from: https://www.ahajournals.org/doi/10.1161/01.CIR.0000043246.74879.CD
16. Chen S, Fang W, Ye F, Liu Y, Qian J, ... SS-TA journal of, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Elsevier [Internet]. [cited 2018 Nov 27]; Available from: https://www.sciencedirect.com/science/article/pii/S0002914904004485
17. Kang H, Kim H, Zhang S, Park K, Lancet HC-T, 2004 U. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis. Elsevier [Internet]. [cited 2018 Nov 27]; Available from: https://www.sciencedirect.com/science/article/pii/S0140673604156894
18. De Bondt P, Van Haute I, Lootens N, Heyndrickx G, Wijns Jozef Bartunek W, Vanderheyden M, et al. Promotes Cardiac Recovery After Recent Myocardial Infarction: Feasibility and Safety Intracoronary Injection of CD133-Positive Enriched Bone Marrow Progenitor Cells. 2005 [cited 2018 Nov 27]; Available from: http://circ.ahajournals.org/content/112/9_suppl/I-178
19. Janssens S, Dubois C, Bogaert J, Lancet KT-T, 2006 U. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Elsevier [Internet]. [cited 2018 Nov 27]; Available from: https://www.sciencedirect.com/science/article/pii/S0140673605678610
20. Vrtovec B, Poglajen G, Sever M, Lezaic L, Domanovic D, Cernelc P, et al. Effects of Intracoronary Stem Cell Transplantation in Patients With Dilated Cardiomyopathy. J Card Fail. 2011;17(4):272–81.

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