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2018 Nobel Prizes in Chemistry
phage display technology

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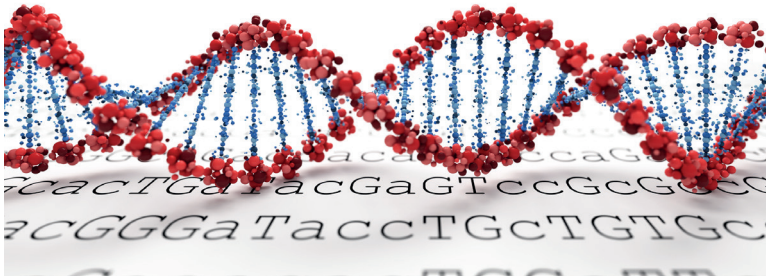

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EDITORIAL

Phage display Technology in vitro evolution. Nobel prize 2018.

Tecnología de exposición de fagos en evolución in vitro. Premio Nobel 2018.

Nelson Santiago Vispo.

DOI. 10.21931/RB/2018.03.04.1

Phage display is a laboratory methodology for the study of interactions among proteins which use bacteriophage (virus infecting bacteria) as a main tool where the phenotype can be connected with the virus genotype in the phagic particle. This technique allows a gen which encodes a protein to be cloned and merged with the gen which encodes the phage protein PIII (5 copies) or with the gen which encodes the Protein PVIII (2700 copies) and both exposed in the phage capsid. Proteins, antibodies or cloned peptides are expressed in the phage wrapping and inside we have the gen which encodes it. Libraries or collections of varieties of these molecules may be built and select the one that has the best biological activity when reacting against an immobilized target molecule, this process is called biopanning and imitates the natural selection.

Form the first seeds of live around 3,700 million years ago, almost all Earth cracks have been filled with different organisms. Life has been expanded to all ecosystems from sulfurous waters, the depths of the Marianas trench and dessert zones, because the evolution has solved a number of chemical problems. The chemical tools of life, proteins, have been optimized, changed and renewed, creating an incredible diversity.

The Chemistry Nobel Prizes of this year were inspired in the power of evolution and used the same principle, the genetic change and the selection, to develop proteins that solve chemical problems of humanity.

In 1992 I had the change and honor to work with professor Gianni Cesareni at Universidad de Torvergata in Rome, while I collaborated with the already dead and excellent researchers Ricardo Cortese and Anna Tramontano of the Institute di Ricerca di Biologia Molecolare P. Angeletti SpA (IRBM). For years' important molecules have been obtained from these libraries, used in all knowledge fields.

This year 2018, after 26 years of working with this methodology, I am proud that your researchers were chosen among those awarded with the chemistry Nobel prize. The American George Smith received the Chemistry Nobel Prize, who in 1985 invented this methodology and the British Gregory Winter who remarkably contributed to its development. The other half of the Chemistry Nobel Prize of this year was awarded to the American Frances H. Arnold (fifth woman to receive the Chemistry Nobel Prize) and who in 1993 made the first directed evolution of enzymes which are proteins catalyzing chemical reactions. Since then, the methods have been refined which are usually used to develop new catalyzers.

The Academy emphasizes that with her work the awardees have undertaken the control of evolution and have used it for better benefit of humanity. Enzymes produced by means of directed evolution are used to manufacture any type of products, from biofuel to drugs. Antibodies evolutioned by means of phage display may cure autoimmune diseases and, in some cases, cure metastatic tumors.

Many are the pharmaceutical products useful for men that have been developed with this methodology and represent a simple and economical way of improving the affinity among interactions of proteins greatly used in life sciences.

Figure 1. Nobel prize in chemistry 2018.

LETTER TO EDITOR / CARTA AL EDITOR

Bioeconomy in Ecuador.
Bioeconomia en ecuador.

Evelyn Carolina Mollocana Lara, María Belén Paredes Espinosa, Frank Alexis.

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In recent years the Ecuadorian economy has been based on exporting raw materials and agricultural products withlittle added value (USD $ 24,064 billion in 2014) and importing services and products with high added value (USD $ 24,188 billion in 2014)[1]. These conditions can make the economy vulnerable to climatic factors that affect the production of resources or foreign market dynamics[1]. Like other countries of Latin America, Ecuador is interested in moving away from a primary-export model and extractivism to reduce the number of importations and increaseexportation of products with high added value. Ecuador's environment minister expressed that bioeconomics would play a fundamental role in Ecuador's production and development when appointed in 2017[2]. Thus, two axes or main currents of bioeconomy emerge such as the direct use of biodiversity and biotechnology[3, 4]. The direct use of biodiversity is a short and medium term axis which seeks to use and commercialize natural resources in a sustainable manner. The government has pledged to take steps to mitigate and adapt to the effects of climate change, which heavily influences the bioeconomic resources of the country. In the 2017 plan for mitigation of climate change, these policies were presented for biomass conservation, environmentally friendly transportation, and new and renewable energy[5]. These policies were backed by an investment of $1.680.000.000 to enact and promote these measures. These measures were implemented through prioritizing areas to act in order to invigorate Ecuador's bioeconomy including 1) enhancing knowledge and institutional capacity, 2) sustainability in agriculture and aquaculture, 3) management and protection of biodiversity, 4) sustainable management of water, 5) adaptation of climate change, 6) clean and sustainable energy, and 7) sustainable transports.

Therefore, bioeconomy implies the sustainable use of renewable biological resources for the production of food, energy and industrial goods[6]. This is why Ecuador considers bioeconomy as a strategic activity that in a few years could come to represent 20% of GDP[7]. The recognition by its constitution that nature is subject that has its own rights and not an object with the only objective of providing profits to human beings combined with economic measures demonstrates the expectations of the role of bioeconomy in the future in Ecuador. Article 71 of the Ecuadorian Constitution establishes that nature or Pacha Mama has the right to have its existence fully respected and the maintenance and regeneration of its life cycles, structure, functions and evolutionary processes[8]. In addition, in article 414 the Ecuadorian State undertakes to adopt appropriate and transversal measures for the mitigation of climate change[8]. Nevertheless, the introduction of these articles in the Constitution does not mean limiting the actions of the State to prohibit and conserve but to promote the generation of a framework of policies that convert the vast biodiversity into a competitive advantage that boost the economy of the country. Like other Latin America countries, the model to generate links between natural resources and scientific and technological knowledge is expected to satisfy Ecuador´s needs based on sustainability and respect for nature[9]. For example, there is a project called "Bijaoplatos desechables 100% biodegradables" which consists of using bijao and banana leaves to produce disposable dishes of rapid degradation used for food expedition[10]. According to the Ministry of the Environment, Ecuador has 24 of the 27 marine and coastal ecosystems recognized worldwide, which is why it launched the project "Red de Areas Marinas y Costeras Protegidas" (Red AMCP) in order to improve conservation and the sustainable use of the country's marine and coastal resources, recognizing the potential relevance of marine biodiversity for the bioeconomy[11]. Biotechnology is part of the medium and long-term axis which seeks to produce products with a greater added value for national consumption and exportation. For example, the use of tagua for the production of nanocellulose gel with potential uses in environmental remediation and tissue reconstruction[12]. Another example is the company BiodiverseSource to commercialize cellulose from Ecuador with unique properties not available from other suppliers in the world. These are just some of the various projects currently underway in the country. The different universities, as well as different research centers in the country are aware of the opportunities that Ecuador's natural wealth gives but the success of these initiatives depends on the collaboration and mutual support of the public, private and community sectors.

In conclusion, Ecuador is moving away from a primary- export model and extractivism by invigorating and prioritizing the bioeconomy. There are several policies that the government has created and funded to make this happen. This way generating a link between natural resources and scientific and technological knowledge that can satisfy Ecuador´s needs while respecting nature.

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RESEARCHS / INVESTIGACIÓN

Gene expression profile in cervical carcinoma cells treated with HeberFERON.

Perfil de expresión génica en células de carcinoma cervical tratadas con HeberFERON.

Dania Vázquez-Blomquist[1*], Julio Raúl Fernández[1], Jamilet Miranda[2], Isabel Guillén[1], Seidy Pedroso[1], Alexander Martín[2], María Elena Ochagavia[2], José Angel Silva[3], Regla Estrada[3], Omar Gell[3], Lidia Inés Novoa[1], Daniel Palenzuela[1], Iraldo Bello[4].

DOI. 10.21931/RB/2018.03.04.3

Abstract: Interferon-alpha (IFN-α) and gamma (IFN-γ) are important cytokines with multiple functions. HeberFERON is a co-formulation of recombinant IFN-α2b and IFN-γ that shows improved pharmacodynamics properties and stronger antitumor response than individual IFNs. The aim of this study was to investigate the differentially expressed genes by HeberFERON in relation to their IFN components by a Suppressive Subtractive Hybridization (SSH) study. Two subtractive cDNA libraries were constructed from HEp-2 cells independently treated with recombinant IFN-α2b and IFN-γ for 72 hours as tester, and cells treated with HeberFERON as driver and vice versa. Near 300 cloned PCR products were sequenced and compared to the database in GenBank and BLAST. We obtained homology to 36 known proteins coding genes. Genes for ribosomal proteins and translation factors besides rRNAs 18S and 28S, cytoskeleton related proteins and proteins participating in antigen presentation and immune responses were mainly identified, using DAVID and GeneCodis tools. Validation of differential gene expression ($p< 0.05$) of genes from the main biological processes components by quantitative PCR (qPCR) showed diverse gene signature by individual IFNs or HeberFERON.

Key words: gene expression, HEp-2, interferon alpha, interferon gamma, quantitative PCR, suppressive subtractive hybridization.

Resumen: El interferón-alfa (IFN-α) y el gamma (IFN-γ) son importantes citoquinas con múltiples funciones. HeberFERON es una co-formulación de IFN-α2b recombinante e IFN-γ que muestra propiedades farmacodinámicas mejoradas y una respuesta antitumoral más fuerte que los IFN individuales. El objetivo de este estudio fue investigar los genes expresados diferencialmente por HeberFERON en relación con sus componentes de IFN mediante un estudio de Hibridación Subtractiva Supresiva (SSH). Se construyeron dos bibliotecas de ADNc sustractivas a partir de células HEp-2 tratadas independientemente con IFN-α2b recombinante e IFN-γ durante 72 horas, y las células tratadas con HeberFERON como conductor y viceversa. Cerca de 300 productos de PCR clonados fueron secuenciados y comparados con la base de datos en GenBank y BLAST. Obtuvimos homología con 36 proteínas conocidas que codifican genes. Se identificaron principalmente genes para proteínas ribosómicas y factores de traducción además de los ARNr 18S y 28S, proteínas relacionadas con citoesqueleto y proteínas que participan en la presentación de antígenos y respuestas inmunitarias, utilizando las herramientas DAVID y GeneCodis. La validación de la expresión génica diferencial ($p <0.05$) de los genes de los principales componentes de los procesos biológicos mediante PCR cuantitativo (qPCR) mostró una firma de genes diversa por IFN individuales o HeberFERON.

Palabras clave: expresión génica, HEp-2, interferón alfa, interferón gamma, PCR cuantitativa, hibridación sustractiva supresiva.

Introduction

Interferons (IFN-α, -β, -λ and -γ) are a multigene family of cytokines that possess a wide range of biological functions including antiviral, anti-proliferative, pro-apoptotic, anti-angiogenesis, anti-fibrotic, neuromodulators and other effects[1,2], through activation of related pathways[3]. These pathways involve specific IFN type I and type II receptors, which initiate activation through JAK-STAT cascades. Type I IFNs interact with the IFNα/β receptor (IFNAR) subunits composed by IFNAR1 and IFNAR2 associated with tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1); while IFN-γ binds to the IFN-γ receptor (IFNGR) receptor subunits composed by IFNGR1 and IFNGR2 associated with JAK1 and JAK2[4].

Thus, IFNs induce the expression of hundreds of IFN-regulated genes (IRGs) via the JAK-STAT pathway[5]. Some of IRGs are regulated by both types of IFNs, whereas others are selectively induced by distinct IFNs through drastic changes in genomic binding locations in a manner dependent on the combinational involvement of STAT1 and STAT2[6]. Depending of doses, treatment time and other factors, IFN-α and IFN-γ signaling may interfere or potentiate each other[7]. Contemporary studies as ChIP-chip analysis of STAT1 and STAT2 targets coupled to quantitative gene-specific PCR (ChIP-qPCR), screening of DNA microarrays or tiling arrays (ChIP-chip), or high-throughput DNA sequencing (ChIP-seq) methods have permitted a more comprehensible picture of how the complex machinery composed by transcription factors, transcriptional co-regulators, histone modifiers, and other players is working for the regulation of IFN target genes[4].

HeberFERON is a co-formulation of IFN-α2b and IFN-γ , with improved pharmacodynamics properties[8] that has demonstrated better results than the individual IFNs in the treatment of basocellular and spinocellular carcinomas[9, 10]. In an attempt to evaluate the gene expression pattern promoted by HeberFERON and potential distinctive regulation of the combination with respect to separated IFNs, we performed a SSH experiment[11] linked to qPCR in HEp-2 cell line, representing

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cervical carcinoma tissue. The understanding of the biological effects that distinguish HeberFERON from their individual components will aid to the optimal clinical application of this formulation in the future.

Materials and methods

Biological Reagents

Recombinant (r) interferons, rIFN-a2b and rIFN-g, and the pharmaceutical co-formulation of both rIFN-a2b and rIFN-g, HeberFERON, were produced at CIGB, Havana, Cuba.

Cell treatment for suppression subtractive hybridization (SSH) experiment

HEp-2 (ATCC-CCL23) cell line, (human cervix carcinoma), was grown in MEM-CANE (Gibco, USA) containing gentamycin (50 mg/mL) (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA). Cells were seeded into 75cm^2 dishes at 3-4 x10^4 cells/ mL in culture medium containing 10% FBS, incubated at 37 oC and 5% of CO_2. Twenty four hours later, the medium of the treatment groups were refreshed with rIFN-α2b (75 IU/ml), rIFN-g (5 IU/ml) or HeberFERON while the control cells received only cell culture medium with antibiotic and serum. Cells were incubated for another 72h.

Construction of SSH library

Total RNA was extracted from cells by TriReagent procedure (Sigma, USA) and DNase I treatment (Promega, USA). First-strand cDNA synthesis was carried out following SuperScript II reverse transcriptase Invitrogen kit instructions (Invitrogen, USA) from 5µg of total RNA. The second strand cDNA synthesis (dscDNA) was carried out from the first strand and a mixture with DNA polymerase I, RNase H and dNTPs (Promega, USA) at 14 ºC overnight. The dscDNA from HeberFERON -treated (population 1) and [IFN-α2b + IFN-γ]-treated (population 2) HEp-2 cells were used as tester and driver, respectively, in a first hybridization (SSH1) and they were exchanged in a second hybridization (SSH2) to obtain both genes upregulated and downregulated. Subtracted cDNA libraries were constructed using Clontech PCR-Select cDNA subtraction kit (Clontech Laboratories, USA), following the manufacturers protocol. The subtracted tester dscDNA was amplified in suppression and nested PCR to enrich only the "differential population". The nested PCR products from libraries SSH1 and SSH2 were ligated into pGEM T-Easy Vector (Promega, USA), transformed into DH10B *E. coli* cells and screened on LB plates containing ampicillin/X-gal/IPTG at standard concentrations. More than 3000 white colonies were obtained. Plasmids from selected clones were purified using a Qiagen plasmid mini kit (Qiagen, USA).

EST sequencing and bioinformatics analysis

About 300 clones were sequenced using an automated sequencer (Macrogen, Korea) and submitted to GenBank for homology analysis. Nucleic acid homology searches were performed using the BLAST program (National Institutes of Health, Bethesda, Md.). DAVID and GeneCodis were used for Gene Annotation and Functional clustering analysis[12, 13]. Web sites for IFNs, INTERFEROME V1.0 and V2.01 were consulted to find genes described as IRGs in our list (http://interferome.its.monash.edu.au/interferome/home.jspx and https://interferome-v1.erc.monash.edu.au) (October 22, 2018)[14, 15].

Quantitative PCR validation

We validated gene expression differences among treatment conditions for a subset of transcripts derived from SSH by qPCR as described[16]. The design included two biological replicates of untreated, IFN-α2b- treated, IFN-γ- treated and HeberFERON - treated cell samples; two replicates of cDNA reactions (from 1µg of total RNA) from each and three technical replicates. As a result, we had 12 data per sample per gene. Primers are listed in Table 1S (*Supplemental Materials*). Statistically significant results were considered for $p<0.05$ after reference gene normalization.

Results

Identification of differentially expressed genes after HeberFERON treatment in HEp-2 cells

From 288 clones sequenced, 215 clones were found to be highly homologous (92%-100%, E value near 0) with 36 known genes. High numbers of hits were obtained for 18S and 28S ribosomal RNAs (rRNA); four genes had more than 10 hits (RHOA, RPS21, C19orf42, and RAB7L1). Other 30 genes were also identified (Table 1).

Using DAVID and GeneCodis we annotated genes in relation to biological process, molecular function and cell compartment and obtained functional clusters (Table 2). The identified genes code for: structural proteins constituent of ribosome (RPL4, 7, 10A, 24, RPS3A, 16, 19, 21 and 27A); proteins participating in protein synthesis (EIF4A3, EEF1A1), in regulation of actin cytoskeleton (ACTG1, ACTB, RHOA) and in antigen processing and presentation and immune response (B2M, HLA-C, HLA-B, HSPD1, HSPA5, HSP90AB1).

Cross-referencing the gene list (Table 1) with the INTERFEROME V1.0 and V2.01 databases[14, 15] highlighted C2orf50 gene as the only one that had not been reported as IFN response gene in humans.

We selected transcripts from coding genes participating in the main biological processes for validation by qPCR in the HEp-2 cells untreated or treated with individual IFNs or HeberFERON. As described before[16], GAPDH and HMBS genes were the least variable and were used for qPCR normalization. Table 3 shows the factor of change for each gene in each experimental condition (treatment with IFN-α2b, IFN-γ or HeberFERON) respect to the untreated control after normalization with the two reference genes.

Using RT-qPCR fourteen genes were validated as HeberFERON response genes (Table 3). B2M and ACTB genes were up-regulated by IFN-α2b, IFN-γ and HeberFERON, confirming they are IRGs as it has been reported before[5, 17-19].

In the cases of a group of 18SrRNA, 28SrRNA, EIF4A3, EEF1A1, RPL10A, RPS3A, and RPS19 genes, non-static significant differences were detect for HeberFERON gene expression regulation (see Table 3). Conversely, both IFNs up-regulated 18SrRNA and EIF4A3 gene expressions; while RPL10A, 28SrRNA, RPS3A and RPS19 genes were upregulated solely by IFN-α2b or IFN-γ. EEF1A1 gene was the only gene down-regulated by the treatment with IFN-γ. This gene regulation behavior is an evidence of a differential gene expression pattern of HeberFERON with respect to separated IFNs. Another differential gene signature was detected for RPL4 and RPS21 or RHOA genes that were solely down-regulated or up-regulated, respectively, by HeberFERON. A more intriguing gene regulation pattern was observed for RPL7 and RPS27A genes. In both cases, the separated IFNs (IFN-α2b, no effect; IFN-γ up-regula-

Gene	GenBank No		Oligonucleotide sequence (5'....3)	Intron Spanning
GAPDH	NM_002046	R	CAAAGTTGTCATGGATGACC	Yes
		F	CCATGGAGAAGGCTGGGG	
HMBS	NM_000190	R	CCTGACTGGAGGAGTCTGGAGT	Yes
		F	GGAATGTTACGAGCAGTGATGC	
ACTB	NM_001101	R	AATGTGGCCGAGGACTTTGATT	No
		F	GGACTGGGCCATTCTCCTTAGA	
B2M	NM_004048	R	CCTGGAGGCTATCCAGCGTACT	Yes
		F	TCAATGTCGGATGGATGAAACC	
18SrRNA	NR_003286	R	GAACGCCACTTGTCCCTCTA	No
		F	CTCAACACGGGAAACCTCAC	
28srRNA	NR_003287	F	GCAAAAGCTCGCTTGATCTTGA	No
		R	CACAAGCCAGTTATCCCTGTGG	
EEF1A1	NM_002954.3	F	GTCCACCACTACTGGCCATCTG	Yes
		R	TCCAAGACCCAGGCATACTTGA	
EIF4A3	NM_004048.2	F	TCAAGCAATTTTTCGTGGCAGT	Yes
		R	TCATTTTCTCCGTCAGCCAGTC	
RPL10A	NM_080725.1	F	GCCATGAGCAGCAAAGTCTCTC	Yes
		R	CTGATCTGCAACTCCACCGTCT	
RPL4	NM_182500.1	F	CCAAGGAAGCTGTTTTGCTCCT	Yes
		R	CGGTTTCTCATTTTGCCTTTGC	
RPL7	NR_003286.1	F	CGAGGATGGCAAGAAAAGCTG	Yes
		R	TGAAGATTTGACGAAGGCGAAG	
RPS19	NR_003287.1	F	GAACCAGCAGGAGTTCGTCAGA	Yes
		R	CCAGTTCTCATCGTAGGGAGCA	
RPS21	NM_001022.3	F	CGAGTTCGTGGACCTGTACGTG	Yes
		R	GCCATTAAACCTGCCTGTGACC	
RPS27A	NM_001024.3	F	TCGTGGTGGTGCTAAGAAAAGG	Yes
		R	CGACGAAGGCGACTAATTTTGC	
RPS3A	NM_001664.2	F	GCATGGATCTTACCCGTGACAA	Yes
		R	CCAACACAGAACAGACGAAGCA	
RHOA	NM_014740.2	F	AGGCCCCTCTCCTACCCAGATA	Yes
		R	CGTTGGGACAGAAATGCTTGAC	

Table 1S. Genes evaluated and Primers information. A summary of the Gene symbols and GenBank Number (No), Sequences for both oligonucleotides (5´...3´; Forward:F and Reverse: R) and intron spanning characteristic are provided.

tion) and HeberFERON (down-regulation) promoted different regulation pattern.

The carefully observation of these transcriptional signatures identified potential antagonistic or synergistic effect of HeberFERON.

Antagonism between IFN-α2b and IFN-γ is expected for those genes where each IFN regulates the gene differently. These are the cases of: 28SrRNA, EEF1A1, RPL7, RPL10A, RPS3A, RPS19 and RPS27A genes. Another kind of antagonist was observed where both IFNs up-regulated the gene expression but HeberFERON, unexpectedly, had no effect on the mRNA expression of these genes. We have the cases of 18SrRNA and EIF4A3F as examples. Additionally, a clear antagonist effect between IFNα-2b and IFN-γ is observed for the regulation of RPL4 and RPS21 genes, where separately IFNs had no effect on regulation, while HeberFERON down-regulated the expression of both genes.

Additive or synergistic effect of the combination of both IFNs could be the cause of the significant increase in gene expression of RHOA gene by HeberFERON.

Discussion

A subtractive hybridization assays experiment was carried out in HEp-2 cell line with sensitivity to growth arrest by IFN-γ[20], IFN-α[21] and their co-formulation as HeberFERON[10]. In this study, we used this cell line as a model to firstly understand what distinguish HeberFERON from individual IFNs actions, at the transcript level.

As it has been reported in previous microarrays studies[5, 17-19] and compiled in IFN Databases INTERFEROME V1.0[14, 22] and V2.01[15], we identified HeberFERON differentially expressed genes encoding proteins that participate in Antigen processing and presentation and Immune Response (B2M, HLA-C, HLA-B, HSPD1, HSPA5, HSP90AB1), Cytoskeleton regulation (ACTB, ACTG1, RHOA) and a high proportion in protein translation (RPL10A, RPL24, RPL4, RPL7, RPS16, RPS19, RPS21, RPS27A, RPS3A, EEF1A1 and EIF4A3). High percentage of hits was for rRNAs 18S and 28S.

IFN Database INTERFEROME V1.0[14, 22] shows 69% of the IFN-g–regulated genes are also induced by type I IFNs. In com-

	Accession	Description	GENE ID	Name	No Hits
1	NR_003286.1	Homo sapiens 18S ribosomal RNA (LOC100008588)	100008588	LOC100008588	38
2	NM_001664.2	Homo sapiens ras homolog gene family, member A (RHOA), mRNA	387	RHOA	35
3	NM_001024.3	Homo sapiens ribosomal protein S21 (RPS21), mRNA	6227	RPS21	34
4	NR_003287.1	Homo sapiens 28S ribosomal RNA (LOC100008589)	100008589	LOC100008589	23
	NC_001807.4	Homo sapiens mitochondrion, complete genome			23
5	NM_024104.3	Homo sapiens chromosome 19 open reading frame 42 (C19orf42), mRNA	79086	C19orf42	18
6	NM_003929.1	Homo sapiens RAB7, member RAS oncogene family-like 1 (RAB7L1), mRNA	8934	RAB7L1	11
7	NM_182500.1	Homo sapiens chromosome 2 open reading frame 50 (C2orf50), mRNA	130813	C2orf50	2
8	NM_001020.4	Homo sapiens ribosomal protein S16 (RPS16), mRNA	6217	RPS16	2
9	NM_080725.1	Homo sapiens sulfiredoxin 1 homolog (S. cerevisiae) (SRXN1), mRNA	140809	SRXN1	2
10	NM_001101.2	Homo sapiens actin, beta (ACTB), mRNA	60	ACTB	1
11	NM_001614.2	Homo sapiens actin, gamma 1 (ACTG1), mRNA	71	ACTG1	1
12	NM_005139.2	Homo sapiens annexin A3 (ANXA3), mRNA	306	ANXA3	1
13	NM_004048.2	Homo sapiens beta-2-microglobulin (B2M), mRNA	567	B2M	1
14	NM_001344.1	Homo sapiens defender against cell death 1 (DAD1), mRNA	1603	DAD1	1
15	NM_001402.5	Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), mRNA	1915	EEF1A1	1
16	NM_014740.2	Homo sapiens eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3), mRNA	9775	EIF4A3	1
17	NM_000146.3	Homo sapiens ferritin, light polypeptide (FTL), mRNA.	2512	FTL	1
18	NM_001136557.1/ NM_001136558.1	Homo sapiens G protein-coupled receptor 107 (GPR107), mRNA	57720	GPR107	1
19	NM_003922.3	Homo sapiens hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1 (HERC1), mRNA	8925	HERC1	1
20	NM_005514.6	Homo sapiens major histocompatibility complex, class I, B (HLA-B), mRNA	3106	HLA-B	1
21	NM_002117.4	Homo sapiens major histocompatibility complex, class I, C (HLA-C), mRNA	3107	HLA-C	1
22	NM_001077442.1 /NM_001077443.1	Homo sapiens heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRNPC), transcript variant 3 and 4, mRNA	3183	HNRNPC	1
23	NM_007355.2	Homo sapiens heat shock protein 90kDa alpha (cytosolic), class B member 1 (HSP90AB1), mRNA	3326	HSP90AB1	1
24	NM_005347.3	Homo sapiens heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (HSPA5), mRNA	3309	HSPA5	1
25	NM_002156.4/ NM_199440.1	Homo sapiens heat shock 60kDa protein 1 (chaperonin)	3329	HSPD1	1
26	NM_001144831.1/ NM_007273.3	Homo sapiens prohibitin 2 (PHB2), mRNA	11331	PHB2	1
27	NM_006775.1	Homo sapiens quaking homolog, KH domain RNA binding (mouse) (QKI), transcript variant 1, mRNA	9444	QKI	1
28	NM_007104.4	Homo sapiens ribosomal protein L10a (RPL10A), mRNA	4736	RPL10A	1
29	NM_000986.3	Homo sapiens ribosomal protein L24 (RPL24), mRNA	6152	RPL24	1
30	NM_000968.2	Homo sapiens ribosomal protein L4 (RPL4), mRNA	6124	RPL4	1
31	NM_000971.3	Homo sapiens ribosomal protein L7 (RPL7), mRNA	6129	RPL7	1
32	NM_002954.3	Homo sapiens ribosomal protein S27a (RPS27A), mRNA	6233	RPS27A	1
33	NM_001006.3	Homo sapiens ribosomal protein S3A (RPS3A), mRNA	6189	RPS3A	1
34	NM_014220.2	Homo sapiens transmembrane 4 L six family member 1 (TM4SF1), mRNA	4071	TM4SF1	1
35	NM_001130145.1/ NM_006106.3	Homo sapiens Yes-associated protein 1, 65kDa (YAP1), mRNA	10413	YAP1	1
36	NM_001022.3	Homo sapiens ribosomal protein S19 (RPS19), mRNA	6223	RPS19	1

Table 1. Homology analysis results of positive clones with Gen-Bank database. GenBank Accession Number, Gene Description, Gene ID and Name are provided. Number (No) of hits for each gene is also included.

parison to type I IFNs used alone, the addition of type II IFN caused enhanced expression not only of many of the genes correlated with the direct antiviral state but also of genes involved in Antigen Presentation to cytotoxic T lymphocytes (CTLs) and Apoptosis[22].

SSH confirmed 36 differentially expressed genes from 215 sequenced clones, representing a 17%, in consistence with previous publications where the number of genes obtained by SSH represents less than 25% of the number of sequenced clones[23, 24].

The differences in gene regulation after 72 hour of incubation time with IFNs could explain, at least in part, the differential gene expression patterns detected. A recent study examining gene expression in human cervical cancer cell line found that although IFN-α and IFN-β induced comparable levels of transcription at early time points, IFN-α induced transcription declined after 8 hours[25]. This decrease was associated with the expression of the IFN stimulated gene USP18 (UBP43), which interacts with the IFNAR2 and inhibits signaling through JAK1 26. Moreover, treatment with IFN-γ for 72 hours markedly inhibits IFN-α-activated STAT1, STAT2 and STAT3; whereas a 24 hours' treatment with IFN-γ slightly enhanced IFN-α-activated STAT1[4].

HeberFERON antagonizes the effect of IFN-α2b and IFN-γ on the expression of 18SrRNA, EIF4A3 (helicase that promotes tumorigenesis[27]), RPL4 (inhibitor of normal physiological

Category	Term	Count	p Value	Genes
Annotation Cluster 1	**Enrichment Score: 8.309666159633796**			
GOTERM_BP_FAT	GO:0006414~translational elongation	10	2.10E-13	EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
KEGG_PATHWAY	hsa03010:Ribosome	9	5.65E-10	RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
SP_PIR_KEYWORDS	protein biosynthesis	9	6.17E-10	EIF4A3, EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPS21, RPS27A
SP_PIR_KEYWORDS	ribosomal protein	9	6.17E-10	RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
GOTERM_BP_FAT	GO:0006412~translation	10	8.97E-09	EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
Annotation Cluster 2	**Enrichment Score: 3.741836482838242**			
SP_PIR_KEYWORDS	protein biosynthesis	9	6.17E-10	EIF4A3, EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPS21, RPS27A
GOTERM_BP_FAT	GO:0042254~ribosome biogenesis	5	9.41E-05	EIF4A3, RPS19, RPS16, RPL7, RPL24
Annotation Cluster 3	**Enrichment Score: 1.258818852316191**			
GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	5	0.007511	ACTG1, ACTB, RHOA, RPL24, RPS27A
KEGG_PATHWAY	hsa04810:Regulation of actin cytoskeleton	3	0.206259	ACTG1, ACTB, RHOA
SP_PIR_KEYWORDS	cytoskeleton	3	0.285929	ACTG1, ACTB, RHOA
GOTERM_CC_FAT	GO:0005856~cytoskeleton	3	0.850258	ACTG1, ACTB, RHOA
Annotation Cluster 4	**Enrichment Score: 0.3007635785334165**			
GOTERM_BP_FAT	GO:0006955~immune response	3	0.403777	HLA-C, HLA-B, HSPD1, B2M
Annotation Cluster 5	**Enrichment Score: 0.22659942488551563**			
KEGG_PATHWAY	hsa04612:Antigen processing and presentation	4	0.003912	HSP90AB1, HLA-C, HLA-B, HSPA5, B2M

Table 2A. Gene Annotation Clustering by DAVID. Each annotation cluster has an Enrichment Score associated, a Category and Term from the Databases consulted, a list of Genes included in categories and the p value associated.

Biological Process	Number	Hyp_c	Genes
Gene expression	11	8.93E-13	RPS16,HNRNPC,RPL7,RPS19,EEF1A1,RPS21,RPL24,RPS27A,RPS3A,RPL4, RPL10A
Antigen processing and presentation	5	1.01E-08	B2M,HLA-C,HLA-B,HSP90AB1,HSPA5
Immune response	3	1.18E-08	B2M,HLA-C,HLA-B
Regulation of actin cytoskeleton	3	1.18E-08	RHOA,ACTG1,ACTB

Table 2B. Gene Annotation Clustering by GeneCodis. Each Biological Process included a Number and list of Genes. P values were obtained through Hypergeometric analysis corrected by FDR method, showing the significance of each process (Hyp_c).

levels of p53[28]), RPL7 (associated with an increased risk factor at early stages of colon recto carcinoma development[29]), RPS27A (promotor of proliferation, cell cycle progression and inhibitor of apoptosis in solid tumors, advanced-phase chronic myeloid leukemia (CML) and acute leukemia (AL) patients[30, 31]), and RPS21 (its reduction is coupled to antitumor effect of ruthenium compound[32]). Anti-cancer effect of HeberFERON at blocking translation would be more effective when multiple intervening factors can be inhibited in combination.

Antagonism between IFN-α and IFN-γ has been reported by several authors. The antagonism could involve regulation of IFN receptor expression, as observed by Rayamajhi et al, when IFN type I reduced the expression of IFNGR1 in macrophages infected with *L. Monocytogenes*, with the corresponding suppression of host responsiveness to IFN-γ[33]. In macrophages, interferon consensus sequence binding protein (ICSBP) mRNA and protein are strongly induced by IFN-γ, but only marginally by IFN type I. When both IFNs are present, IFN type I antagonizes IFN-γ-induced ICSBP mRNA and protein synthesis[34].

Regulation of phosphorylation of transcriptional factors involved in IFN type I and type II signaling could also explain the antagonism observed for HeberFERON with respect to separated IFNs. For example, overexpression of protein tyrosine phosphatase Shp1 in endothelial cells abrogated IFN type I signaling through a GAS site, suggesting a role of level of Shp1 on the interference between IFN types I and II signaling pathways[35].

The increased gene expression of RHOA stimulated by HeberFERON could indicate an additive or synergic effect. IFN-α[36] and IFN-γ[37] have been involved in the reorganization of the cell cytoskeleton through RHOA, with impact in the cell growth. The upregulation of RHOA by HeberFERON could be beneficiated from the described crosstalk between both type of IFNs[38] and the further regulated expression of STAT1 via c-Jun-mediated production of basal levels of IFN-β[39]. In this context, we could remark the facts that STAT1 and the stimulation of c-Jun expression could be involved in the regulation of RHOA gene expression[39, 40].

The diverse mode of gene regulation revealed in this work by the combination of IFN-α2b and IFN-γ (HeberFERON), is congruent with the recent study of the ENCODE project performed on genomic binding sites suggesting that transcription factors often show different co-association patterns in binding sites, and the binding of one transcriptional factor affects the preferred binding partners of others[41]. Furthermore, efficient transcriptional activation of STAT1 target genes requires posttranslational modification of STAT1 and the recruitment of coactivators and histone and chromatin modifying complexes[4].

As part of this work we obtained the gene C2orf50 regulated by individual IFNs or their combination that was not previously described as IRGs in humans[14, 15]. This gene is poorly characterized [https://www.uniprot.org/uniprot/Q96LR7] and the understanding of their participation in the mechanism of action of HeberFERON could be an interesting point in the future.

In spite of our study examined only a small number of

		IFN Alpha		IFN gamma		HeberFERON		
Function/Process	**Gene**	**FC**	**p**	**FC**	**p**	**FC**	**p**	
Ribosome/ translation								
	18SrRNA	**1,89**	0.000	**1,67**	0.000	**-1,03**	0.760	**
	28SrRNA	1,33	0.075	**1,39**	0.020	1,03	0.810	
	EIF4A3	**1,26**	0.002	**1,35**	0.005	**-1,02**	0.764	**
	EEF1A1	-1,27	0.092	**-1,47**	0.011	-1,15	0.303	
	RPL4	-1,04	0.523	1,04	0.574	**-1,25**	0.006	**
	RPL7	-1,08	0.223	**1,25**	0.000	**-1,22**	0.000	**
	RPL10A	**1,15**	0.021	1,18	0.114	-1,02	0.689	
	RPS3A	-1,11	0.447	**1,47**	0.007	-1,09	0.488	
	RPS19	1,09	0.482	**1,38**	0.022	-1,11	0.358	
	RPS21	1,04	0.591	1,19	0.062	**-1,35**	0.000	**
	RPS27A	-1,02	0.834	**1,31**	0.005	**-1,19**	0.004	**
Immune Response								
	B2M	**2,60**	0.000	**4,60**	0.000	**5,62**	0.000	
Cytoskeleton								
	ACTB	**1,42**	0.001	**1,94**	0.000	**1,28**	0.002	
	RHOA	-1,06	0.436	-1,05	0.520	**1,56**	0.000	**

Table 3. Gene expression (mRNA) following treatment with IFNα (Alpha), IFNγ (gamma) and the combination HeberFERON. Factor of Change (FC) respect to untreated cells, calculated by REST 2009, and the p values associated to each comparison are shown. In Bold and Shadows formats we show genes which have statistically significant differences ($p < 0.05$). Genes are grouped by Function and Process. Double asterisks pointed to the genes showing new regulation pattern of HeberFERON respect to IFNα (Alpha) and IFNγ (gamma).

IRGs, it suggests that compared with transcriptional patterns of separated IFNs, HeberFERON induces a unique transcriptional signature after 72 hour of cell treatment. The meaning of this new signature should be taken into account for clinical translation.

Competing interests and Funding

The authors declare that they have no competing interests. All Authors are (were) employees of the Center for Genetic Engineering and Biotechnology (CIGB), Havana where IFNa2b, IFNg and HeberFERON are produced. The study was financed by CIGB.

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RESEARCHS / INVESTIGACIÓN

Decolorization of a textile effluent and methylene blue by three white rot fungi (WRF), at pilot and laboratory scale.

Decoloración de un efluente textil y azul de metileno usando tres hongos de la pudrición blanca (HPB), a escala piloto y laboratorio.

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Abstract: Textile industries produce a great quantity of water contamination by dyes. Such contamination is a serious environmental problem because dyes diminish oxygen and light penetration to the water body, producing severe damages to aquatic ecosystems and their biota, besides many dyes are toxic and carcinogenic. White rot fungi (WRF) are beneficial organisms in bioremediation due to their powerful ligninolytic extracellular non-specific enzymes, which degrade not only wood but also a great variety of complex toxic substances, including hydrocarbons, industrial dyes, etc. In this research, we evaluated the decolorization effect by three decomposing wood mushrooms, *Pleurotus pulmonarius, Trametes versicolor* and *Lentinus crinitusin* a textile effluent and methylene blue dye. For the industrial effluent at pilot scale, *P. pulmonariusgave* best results with 40% decolorization after seven days. The other two fungi did not give good results in this same experiment, but *T versicolor* reached 97% decolorizationin methylene blue, at 400 mg/L in laboratory scale, equally *P. pulmonarius* and *L. crinitusproduced* similar but slightly less decolorization. Those results letus conclude that the fungal strains used here have good potential as a tool in industrial colored water bioremediation, especially *P pulmonarius* and *T. versicolor*.

KeyWords: Water decolorization, textile dyes, white rot fungi, *Pleurotus, Trametes* and *Lentinus*.

Resumen: Las industrias textiles producen gran cantidad de aguas contaminadas con colorantes. Esto es un grave problema medioambiental, porque los colorantes disminuyen la penetración de oxígeno y luz a los cuerpos de agua, lo que afecta negativamente el ecosistema y su biota, además muchos colorantes son bastante tóxicos, cancerígenos y teratogénicos. Los hongos de la pudrición blanca (HPB) son organismos capaces de degradar muchas sustancias tóxicas, gracias a su poderosos sistema de enzimas lignolíticas, que por su baja especificidad, degradan no solo la madera, sino también una gran variedad de sustancias complejas, como hidrocarburos, colorantes industriales, etc. En esta investigación, se evaluó tres hongos descomponedores de madera: *Pleurotus pulmonarius, Trametes versicolor* y *Lentinus crinitus*, para decolorar un efluente textil y el colorante azul de metileno. Para el efluente industrial a escala piloto, los mejores resultados arrojaron decoloración del 40% en 7 días de tratamiento con *P. pulmonarius*, y menores valores producidos con *T. versicolor* y *L. crinitus*. El colorante azul de metileno a 400 mg/L, escala de laboratorio, fue decolorado en un 97% por *T. versicolor* en 7 días, para valores similares aunque ligeramente inferiores con los hongos *P. pulmonarius* y *L crinitusen* este mismo experimento. Los anteriores resultados permiten concluir que las cepas fúngicas usadas son potencialmente útiles en procesos de biorremediación de aguas contaminadas con colorantes industriales, especialmente *P. pulmonarius* y *T. versicolor*.

Palabras Claves: Decoloración de aguas, colorantes textiles, hongos de la pudrición blanca, *Pleurotus pulmonarius*.

Introduction

The textile industry is one of the most important productive sectors in Colombia, with more than 450 textile producers, representing more than five percent of total national exportations[1]. Although this sector is so productive, it threats the environment seriously because it generates a lot of water contamination, especially by using synthetic dyes, which most of them are toxic and carcinogenic[2].

There is polluted more than 7x10^{8}kg of 10.000 different industrial dyes in a year, most of them used in fabrics dying. Some estimations how that 10-15% of those dyes used in dying processes are discharged to water streams[3].

When a natural water stream was set with dyes, there are produced various negative consequences in it, like diminishing of oxygen solubility, less light penetration, among others. All those consequences stop aquatic photosynthesis, so there is a direct inhibition of aquatic biota growth. Usually, the most common values of dye concentration in industrial effluents are around 300 mg/L, but the color in water is very notable even at a concentration of 1 mg/L.[4, 5].

For diminishing color in water, there are various physical and chemical methods, such as adsorption[3], oxidation[6], chemical degradation, photo-catalysis, ozonation, and others[7]. Although those methods offer high color removal percentages, they have various disadvantages like their high costs, their tendency to produces significant quantities of sludge, and some of them generate other compounds even more toxic than dyes[8, 9].

One of the most attractive alternative to reduce color in industrial effluents is the use of white rot fungi (WRF),which are organisms capable to feed from very hard vegetal tissues like wood, because of their powerful ligninolytic enzymes (laccases, lignin peroxidases, manganese peroxidases, and others), which break up the main components of wood: lignin and cellulose,[2, 10]. As those enzymes are substrate non-specific, many investigators have researched WRF not only to degrade

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synthetic dyes but a great variety of complex toxic substances like hydrocarbons[11]. Besides the use of such organisms for water decolorization is said to be environmentally friendly, and less expensive than physical and chemical methods[8, 12]. In this sense, many recent papers demonstrate the high efficiency of water decolorization effected by various species of WRF on a great variety of industrial dyes. For example, using *Pleurotus spp*[10, 14]; *Trametes versicolor*[9, 10, 13-17] and *Lentinus crinitus*[2, 14, 18, 19].

Methylene blue is a standard dye, mostly used by industries like textile, paper, rubber, plastics, leather, cosmetics, pharmaceutical and food industries[3]. This dye is a toxic substance to water microbiota and humans[20], equally it is known as a teratogenic substance[21]. The decolorization of methylene blue at laboratory scales have been researched by using various physical and chemical methods[20], as well as by using few WRF[22, 7],nevertheless there are very few papers showing decolorization of real industrial effluents in non-sterile conditions[24], equally there are no published data about methylene blue decolorization in non-sterile conditions by using the specific fungi used here: *Pleurotus pulmonarius, Trametes versicolor* and *Lentinus crinitus*.

The purpose of this paper is to present the water decolorization obtained in a real textile colored effluent and methylene blue, at pilot and laboratory scales respectively, by using the WRF *Pleurotus pulmonarius, Trametes versicolor* and *Lentinus crinitus*.

Materials and methods

Microorganisms and propagation

Three fungi *Pleurotus pulmonarius, Trametes versicolor* and *Lentinus crinitusused* in the decolorization experiments were obtained from the strain collection at the Universidad Catolica de Oriente mycology laboratory. The preserved strains in tube cultures under mineral oil[11].The cultures were transferred to Petri dishes with solid medium [G33] PDA (potato dextrose agar, OXOID) in sterile conditions and incubated at 24°C until full colonized. The cultures obtained were maintained at 4°C and used to produce mycelium using as substrate 2 kg hay (*Digitaria decumbens*) blocks, with 65% humidity, in polypropylene bags, under sterile conditions, with 20 days' incubation at 24°C. The full-colonized mycelium blocks, were used to evaluate decolorization in the industrial colored effluent or two concentrations of methylene blue, according to the following experiments.

Decolorization experiment at pilot scale

This experiment was set at the water treatment plant "La Cimarrona", Marinilla, Colombia, which receives and purifies various industrial colored effluents, from various dyed fabrics manufacturers. This colored industrial effluent (table 1) was used to evaluate the decolorization by the three fungi grown in the way into a pilot reactor, and two different liquid-mycelium contact ways: 1) submerged entirely blocks or [G38] 2) percolation of the liquid through a mycelial block (see figure 1).

Into the reactor (150-liter plastic drums) were put 50 liters of the colored effluent and it is respective 2 kg mycelium block, which was either submerged entirely or suspended into a stainless still basket, 20 cm above the liquid level. For both methods, a recirculation of liquid was set constantly using an aquarium pump, with discharge at 80 cm height, caudal of three L/min, passing over the mycelium basket (contact method one), or falling directly to the mother liquid (contact method two). This set was let at ambient temperature (17 °C average in Marinilla, Colombia), kept from rain and sun. A control test was made using the same reactor and circulation system, with the same effluent but without any mycelium. Experiments and control were made by triplicate. Daily samples were taken during seven days to measure decolorization. After the seventh day, the mycelium blocks were put in a humid shade place (60% relative humidity, 17°C), to register fruiting occurrence during the next ten days.

Decolorization at in vitro scale

Similar mycelium grown in was used to inoculate Erlenmeyer's containing either the industrial colored effluent or

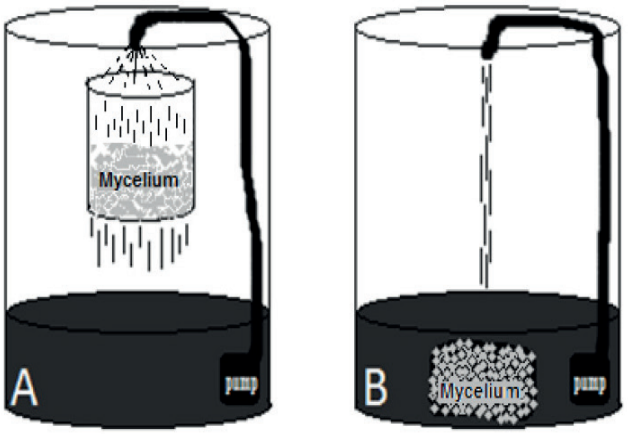


Figure 1. Pilot scale reactors for decolorization of industrial colored effluent assays were 150 liters' plastic drums containing 50 liters of effluent, in two contact ways. A: effluent recirculated through the mycelium block suspended in a basket. B: Totally submerged mycelium block under the water, both had the same recirculation system.

methylene blue (Analytical, Vasna, India) at 100 or 400 mg/L dissolved in non-sterile tap water. Every 250 ml Erlenmeyer contained 100 ml of the colored liquid and 2 g of the mycelium. The controls were the effluent and both methylene blue concentrations without mycelium. All flasks were incubated on a rotary shaker at 120 rpm, 24°C for seven days, with experiments mounted by triplicate.

Analytical part and Statistics

The decolorization data from the experiments were obtained according to the method published by (26), which quantifies the reduction of the absorbance obtained from the first liquid (without fungi treatment) to some period after fungi treatment. A spectrophotometer (spectroquant pharo 400, Merck) was used to record the absorbance spectra for both the effluent and methylene blue. A wavelength was chosen for everyone, to use it in monitoring the decolorization (figure 2). Daily absorbance measures were taken, and decolorization percentage was calculated using the following equation:

$$Color\ removal\ \% = \frac{A_i - A_f}{A_i} * 100$$

Here A_i is absorbance of the colored solution without treatment and A_f is absorbance after a period of contact with the fungi.

All data of treatments were analyzed by variance analysis ANOVA, along with comparisons by Tukey multiple range test, using Statgraphics Centurion XVI 16.1.18. All graphics were

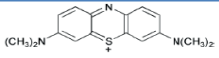
	Methylene blue	Colored industrial effluent
Chemical composition.		A complex dyes and salts mixture, in water from fabrics dying process.
pH of initial solutions used for decolorization with mycelium.	7,5	7
Conductivity	-----	5000 µS/cm
Wavelength used to register decolorization	λ=659 nm	λ=554 nm

Table 1. Some characteristics of methylene blue and the industrial colored effluent used in decolorization experiments.

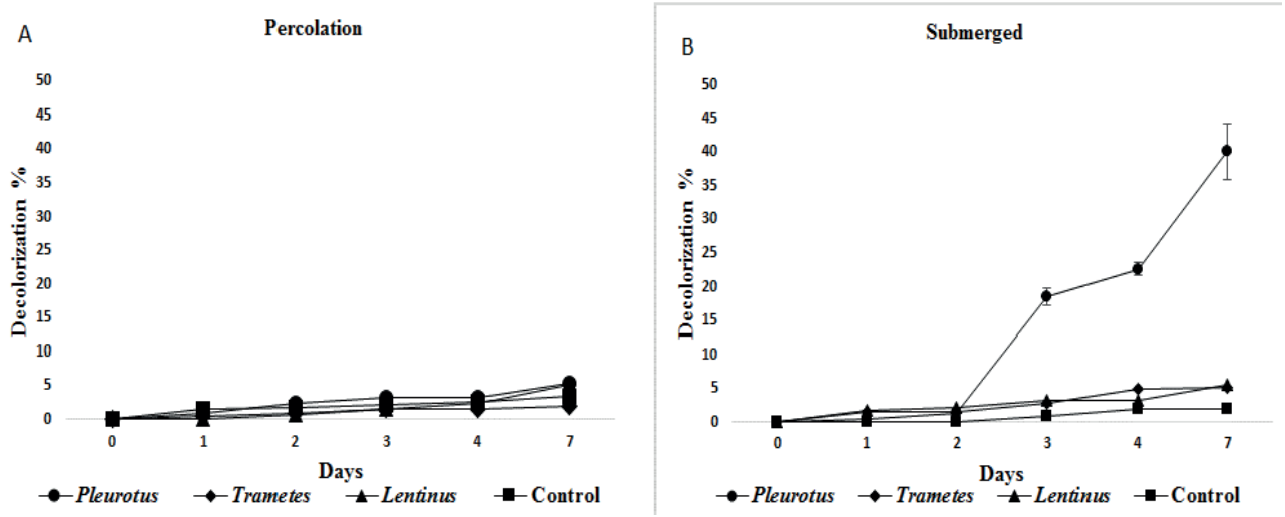


Figure 2. Absorbance spectra of the colored industrial effluent (A), and methylene blue (B), images are photos from the machine´s display. In Y is absorbance, X represents wavelength.

made using excel 2013.

Results

Decolorization of colored industrial effluent at pilot scale

After seven days, the effluent was decolorized efficiently only when mycelium blocks were set entirely submerged, with best values of 40,1% by *Pleurotus*, that is statistically different from *Trametes* (5,1%), *Lentinus* (5,5%) and the control (2%), (figure 3).

On the contrary, all three mushrooms set in percolation method produced very low decolorization with all three mushrooms, with average media values of 5, 5; 1,9and 5,2 % with *Pleurotus, Trametes* and *Lentinus* respectively (fig 3). These results are not statistically different from the control (3,4%), evaluated with Tukey multiple range tests ($P \leq 0,05$).

Ten days after finished the decolorization experiments, 100% of *Pleurotus* blocks produced fruiting, while *Trametes* and *Lentinus* did not produce fruiting.

Decolorization at in vitro scale

Better decolorization results were obtained for industrial effluent at *in vitro* tests, than in pilot scale tests by all three fungi, and once again *Pleurotus* showed the best values with 46%, statistically different than *Trametes* (19,7%), *Lentinus* (16,5%) and the control (8,5%) (Figure 4).

In both methylene blue concentrations, there were outstanding decolorization efficiencies, in almost all cases superior to 80% with all three fungi species at seventh day, with statistically differences to control tests. Trametesshowed the best results than the other two fungi, in both concentrations tested, with 95% in 100 mg/L, and 97% in 400 mg/L. (figure 5).

Discussion

These experiments corroborate the potential of WRF in water bioremediation; in this case, our experiments consisted in decolorization of a real industrial colored effluent in non-sterile conditions, with promising results. An evaluation which has been poorly explored[18, 23, 25].

This colored industrial effluent was decolored up to 40.1% only by *Pleurotus*, when its biomass set submerged entirely, while in percolation the decolorization failed. *Trametes* and *Lentinus* did not exert significant decolorization in both contact methods. At *in vitro* scale the results were similar, though slightly superior (*Pleurotus*: 46%; *Trametes*: 19,7%, and *Lentinus*:16,5%), The decolorization value with *Pleurotus* obtained here is slightly superior to the obtained by (24), of 40% decolorization in a real non-sterile effluent after 8 days with *Bjerkandera*, and 65% in the same affluent, but sterilized.

The similar decolorization values of industrial effluent at both scales by *Pleurotus* coincide with (27), who obtained scarce different production levels of Manganese peroxidase in 5 and 30 Liter reactors, using *Nematoloma frowardii*. This led us to speculate that decolorization of industrial effluents with *Pleurotus* are easily scalable. Equally (27) explains that optimal scalability of ligninolytic enzymes production is attained when is a sheer effect [G74] is kept as low as possible in bigger reactors. Both scales used here had no shear effect

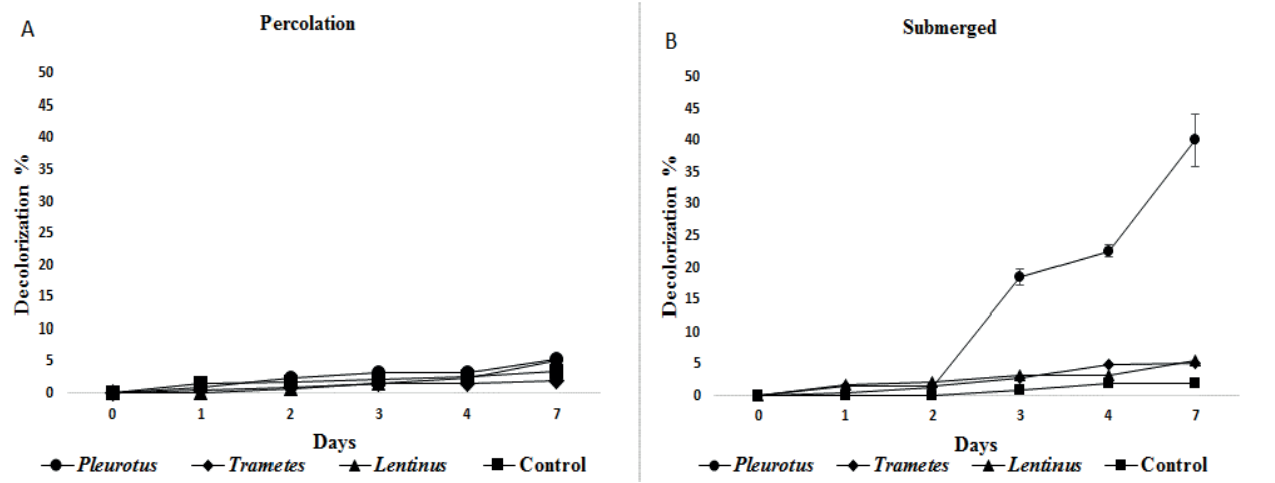


Figure 3. Mean decolorization values obtained with the funguses for the industrial effluent with A)percolation and B) submerged mycelia during seven days.

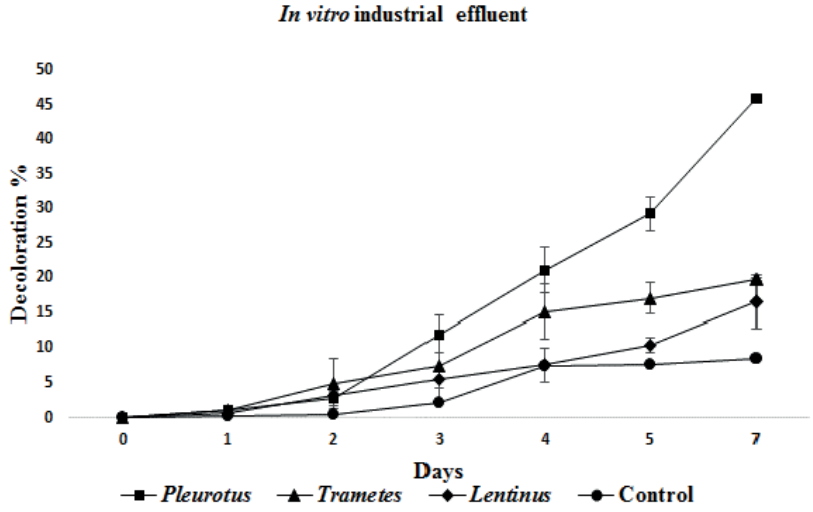


Figure 4. Mean decolorization obtained for the industrial effluent at *in vitro* experiments.

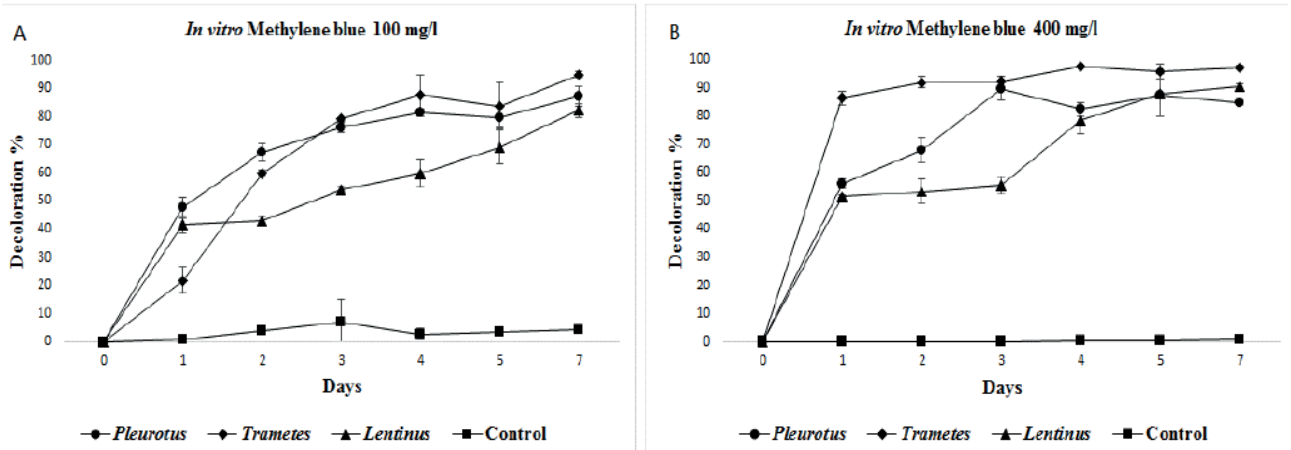


Figure 5. Absorbance spectra of the colored industrial effluent (A), and methylene blue (B), images are photos from the machine´s display. In Y is absorbance, X represents wavelength.

because in pilot scale agitation was by recirculating the liquid, and at in vitro only was used an orbital shaker.

The different decolorization values of *Pleurotus* from *Lentinus* and *Trametes* may by partly because of *Pleurotus pulmonarius* has the best tolerance to the specific chemical composition of the industrial effluent used thanTrametes and Lentinus. In that sense, (28) report high tolerance to the toxic phenol for (22) *Pleurotus* species, grown in agar and in non-sterile wheat straw soaked in that substance. Likewise (13) found the ability of *Pleurotus ostreatusto* survive and do decolorization in very high concentrations of three reactive industrial dyes, with the laccase as the predominant enzyme, which increased its activity as increasing dye concentration to 2000 mg/L. On the other side, possibly some salts present in the effluent inhibits stronger the enzymes in *Trametes* and *Lentinusthan* in *Pleurotus*. This effluent comes from various

industrial fabrics dying processes. Thus it is composed of a very complex mixture of various unknown dyes and salts[24]. In this respect, (15) found high decolorization rates with *Pleurotus*, *Trametes* and *Phanerochaete* for single dyes in salinity conditions, but they found terrible results when salt concentration reached 20 g/L because under this condition there is strong enzyme inhibition exerted by the high osmotic pressure. Also (29) found *in crinitus* better activity of laccase and manganese peroxidase, when the culture medium was supplemented with Mn2+, ethanol or Cu2+, then those salts may act as cofactors for some enzymes related to the process. Contradictorily, (24) attribute their good decolorization results in a real effluent by *Bjerkandera* to the salinity contained in such effluent, but they do not report salt concentrations. The papers mentioned above shows the different tolerance of the fungi to salinity, which may explain the different decolorization values shown here by the three fungi.

For methylene blue, which was assayed here only at *in vitro* in shake flasks, there were excellent decolorization values, ranging from 80 to 95%, with all three fungi, in both concentrations assayed (100 and 400 mg/L). It seems more natural for the fungi to effect decolorization when setting with single dyes, as frequently reported by others, like (17) who obtained 92% in 9 days with *Coriolus* (=*Trametes*) in Everzol Turquoise Blue G;[8] achieved 99% in blue 19 after 15 days;[30] reached 92% in Reactive Black 5 (non-sterile), 8 days with Trametes;[2] 90% decolorization of reactive blue 220 in10 days using Lentinus crinitus; and[13] 97% with *Trametes* pubescens and *Pleurotus ostreatusin* reactive blue 19. However, the novelty in our results is the demonstration of good decolorization for methylene blue in high concentrations, and non-sterile conditions by the WRF tested, and the same goal is possible for real colored industrial effluents at bigger scales, especially with *Pleurotus*.

Methylene blue was decolorized (95%) with significantly best results than the industrial colored effluent 46% by *Pleurotus*. Maybe these differences are due to the indigenous bacteria in the effluent, interfering with a more optimal enzyme expression by the fungi. This same trend was observed by (24), who obtained 70% decolorization four diluted dyes in eight days, while they obtained only 40% in a real effluent at non-sterile conditions using *Bjerkandera*. The authors found six million contaminating bacteria per milliliter in the effluent; then decolorization was reduced by competition between fungi and bacteria. Similarly, (25) reports bacterial contamination as the main responsible factor to the high reduction in decolorization efficiency under non-sterile conditions, after various cycles with immobilized *Trametes versicolor*.

Ten days after finishing the pilot-scale experiment with the industrial colored effluent, we observed fruiting in all *Pleurotus mycelium* blocks, with the same result for submerged and percolation blocks (100% fruited blocks in both experiments). This result is following (11) who reported *Pleurotus ostreatus* grown in sawdust and used for bioremediation of polluted soil with 2% hydrocarbons, showing regular fruiting after four weeks, and high contaminants reduction. This fungus like other edible cultivated mushrooms is known by its response to a sudden change in humidity, resulting in a flush of mushrooms even in 20 days old inoculated substrate. In this study, the ability to produce fruiting after seven days soaked in dyes contaminated water by *Pleurotus*, confirms its high resistance to chemical substances, and it is potential as a tool for water bioremediation. *Trametes* and *Lentinus* did do not fructify at all, but maybe the time for fruiting in those fungi is longer than the studied here. For example, *Trametes versicolor* inoculated in apple plants, produce fruiting after six months[31].

On the other side, hey used here as support for the mycelium influenced positively the excellent results obtained here for Pleurotus in the effluent and all tree fungi in methylene blue. That substrate like many similar gramineous plants have a high proportion of lignin and cellulose, so they are usedtraditionally to produce many edible mushrooms commercially[11]. That was the main argument to use hay for mycelium propagation in the present decolorization experiments. Some authors report repeatedly superior results in decolorization processes when using immobilized WRF compared with suspended mycelium, especially when using organic supports[12, 30, 32] because those supports maintain a constant growing active mycelium, capable of producing the ligninolytic enzymes for a prolonged period. For example,(33) found that *P. chrysosporium* enhanced its decolorization by using sunflower seed shells as support, which influenced both the type and proportion of the produced enzymes responsible of dye decolorization. Equally, (34) report higher lignin peroxidase and Manganese peroxidase production in *Lentinus crinitus* grown in banana shoots-leaves compared to banana fruits-leaves, mainly because to the difference in lignin-cellulose content in those materials. Therefore, this is a valid argument to use lignin-cellulosic composed substrates, as support to produce decomposing wood enzymes from WRF, as an economically viable alternative, for different biotechnological goals, including dyed water bioremediation.

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RESEARCHS / INVESTIGACIÓN

Determinación del coeficiente de absortividad específico del factor de crecimiento epidérmico humano recombinante.

Determination of the specific absorptivity coefficient of the recombinant human epidermal growth factor.

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Resumen: Se determina el coeficiente de absortividad específico del factor de crecimiento epidérmico humano recombinante (EGFhr) mediante una metodología que utiliza la técnica de análisis de aminoácidos como método de cuantificación de las proteínas. Se empleó la albúmina de suero bovino (BSA) como proteína modelo para la evaluación de la ejecución de la metodología. Se demostró mediante análisis estadístico de los resultados que el coeficiente de absortividad específico de la proteína modelo coincide con alta precisión con el valor informado para esta proteína en la literatura. La metodología implementada se aplicó al EGFhr que contiene dos especies proteicas de 51 y 52 aminoácidos. El valor del coeficiente de absortividad específico de las dos especies no presenta diferencias y puede considerarse un único valor, lo cual resulta de mucha utilidad en el contexto productivo y de desarrollo de un producto biofarmacéutico.

Palabras Claves: cuantificación, albúmina bovina de suero, factor de crecimiento epidérmico y coeficiente de absortividad específico.

Abstract: The specific absorptivity coefficient of the recombinant human epidermal growth factor (EGFhr) was determined by a methodology that uses the technique of amino acid analysis as a method of protein quantification. Bovine serum albumin (BSA) was used as a model protein for the evaluation of the execution of the methodology. It was demonstrated by statistical analysis of the results that the coefficient of specific absorptivity of the model protein coincides with high precision with the value reported for this protein in the literature. The methodology implemented was applied to EGFhr that contains two protein species of 51 and 52 amino acids. The value of the coefficient of specific absorptivity of the two species does not present differences and can be considered as a single value, which is very useful in the productive and development context of a biopharmaceutical product.

KeyWords: quantification, serum bovine albumin, epidermal growth factor and specific absorptivity coefficient.

Introducción

El conocimiento del coeficiente de absortividad molar de los productos biofarmacéuticos de origen proteico es fundamental. Esta constante físico química se emplea en la medición de la concentración de proteína en las distintas etapas del proceso productivo, ya sea para estimar el rendimiento del producto durante su producción o para evaluar su potencia y por lo tanto llevar a cabo una formulación correcta[1].

En la determinación experimental del coeficiente de absortividad de una proteína el paso crítico es la determinación de la concentración de la solución de proteína. Los métodos empleados con mayor frecuencia para la determinación de la concentración de soluciones de proteína son: análisis de aminoácidos[2, 3], determinación de nitrógeno total por el método de Kjeldahl[4], el método de peso seco[5, 6, 7], el método de Edelhoch[8] y los métodos colorimétricos como las técnicas de Bradford y del ácido bicinconínico (BCA)[9, 10]. De estos, la técnica de análisis de aminoácidos es el método más preciso y directo, con excelente linealidad y reproducibilidad, no depende de la abundancia en la proteína de residuos aromáticos, no emplea proteínas de referencia y es un método preciso a bajas concentraciones de proteína. Adicionalmente el método también se utiliza como criterio de identificación molecular de proteínas en bases de datos[11].

Los otros métodos para determinar la concentración de una solución de proteína son útiles cuando se trata de muestras con altas concentraciones de proteína. Sin embargo, la utilidad está limitada ya que algunos dependen del contenido de aminoácidos aromáticos, el error asociado a ellos es considerable y dependen de la comparación con un estándar conocido de proteína. Por otra parte, las técnicas de ligandos coloreados tienen un rango dinámico limitado lo que hace que se tengan que preparar y analizar diluciones a partir de la muestra problema hasta que la concentración se ubique dentro de la curva de calibración realizada.

A pesar de las evidentes ventajas de la técnica de análisis de aminoácidos la mayoría de los coeficientes de absortividad informados en la literatura están determinados por métodos predictivos o son el resultado de determinaciones experimentales que emplean métodos de cuantificación de proteínas diferentes a la técnica de análisis de aminoácidos. Una explicación de esta contradicción se basa en el hecho de que la técnica de análisis de aminoácidos requiere de equipamiento costoso, de la ejecución de la técnica por un analista experimentado, del control de las posibles fuentes de contaminación y es un procedimiento trabajoso.

La metodología para la determinación del coeficiente de absortividad específico de proteínas implementada comprende la preparación de soluciones seriadas de la proteína pura, la determinación de la concentración de las soluciones y la medición de la absorbancia a 280 nm. La pendiente de la

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recta de la regresión lineal de la absorbancia en función de la concentración es el coeficiente de absortividad específico (a_s). Con el propósito de minimizar el sesgo en los resultados estos ensayos son realizados con réplicas y en días diferentes[12].

En este trabajo se informa el coeficiente de absortividad específico del EGFhr determinado empleando la técnica de análisis de aminoácidos para la determinación de la concentración de proteína. La metodología se implementó empleando una proteína modelo, la BSA, de concentración, composición aminoacídica y coeficiente de absortividad conocidos. Esto permitió evaluar la exactitud y la precisión de la metodología implementada.

Materiales y métodos

BSA: para la evaluación de la exactitud y precisión de la metodología empleamos BSA (Pierce, EEUU). A partir de la preparación comercial se prepararon diluciones seriadas a 0.80, 0.60, 0.40 y 0.20 mg/mL en agua. A cada una de estas soluciones se le midió la absorbancia a 280 nm y se determinó la concentración de proteínas mediante la técnica de análisis de aminoácidos. Los experimentos se realizaron tres veces en días independientes y por triplicado para cada solución de trabajo. Para el cálculo de la concentración de proteína se empleó el valor de peso molecular de 66 430 Da.

EGFhr: partimos de una solución de proteína a una concentración estimada de 0.71 mg/mL con pureza estimada por cromatografía líquida de alta eficacia en fase reversa de 97.73%. Esta preparación contiene dos especies proteicas, las cuales se denominan en este trabajo EGFhr-51 y EGFhr-52 y tienen una masa molecular teórica de 5952 Da y 6065 Da, respectivamente.

Cromatografía líquida de alta eficacia en fase reversa: la purificación de las especies del EGFhr se llevó a cabo en una columna de fase reversa C18 (Vydac, EEUU) de dimensiones 10 x 250 mm. Se empleó un controlador de gradiente LKB 2152 (Pharmacia, Suecia) el cual regula el funcionamiento de dos bombas LKB 2150 (Pharmacia, Suecia). La temperatura de la columna se mantuvo constante en 37oC por medio de un horno (Knauer, Alemania). Para la elusión de las muestras se utilizó un gradiente entre la solución A: ácido trifluoroacético (Pierce, EEUU) al 0.1% en agua MilliQ y la solución B: ácido trifluoroacético (Pierce, EEUU) al 0.05% en acetonitrilo (Merck, Alemania). El gradiente empleado fue: de 0 a 10 min 20% de B, a los 12 min 25% de B, a los 60 min 32% de B, a los 66 min 20% de B y a los 96 min 2% de B. El flujo de trabajo fue de 0.8 ml/min. Ambos solventes se desgasificaron con un desgasificador ERC 3310 (Erma, Japón). La detección se realizó a 226 nm utilizando un monitor de longitud de onda variable LKB 2151 (Pharmacia, Suecia). Estimación de la concentración de proteína: la determinación preliminar de la concentración de proteína de las soluciones de las especies EGFhr-51 y EGFhr-52 se realizó mediante la técnica colorimétrica de BCA. Se empleó el protocolo en placas de 96 huecos del sistema comercial BCA Protein Assay Kit (Pierce, EEUU). La curva patrón se construyó con soluciones de diferente concentración en el rango de 0.15 a 1.66 mg/mL de la preparación comercial de BSA. Las determinaciones se realizaron por triplicado. La lectura se realizó a 580 nm.

Solución de norleucina (nLeu): en un tubo Eppendorf de 1.5 mL se pesaron 1.40 mg de nLeu y se disolvieron en 1.30 mL de solución tampón de carga (pH 2.23 y citrato de litio 0.20 mol/L) al analizador automático de aminoácidos (Biochrom Ltd Cambridge Amersham-Pharmacia, Inglaterra). La concentración final de esta solución es 8.83 nmol/µL.

Soluciones de trabajo de EGFhr-51 y EGFhr-52: las proteínas puras se disolvieron en agua libre de aminoácidos y se estimó la concentración de proteína por el método colorimétrico de BCA. A partir de estas soluciones se prepararon las diluciones seriadas de trabajo a concentraciones de 0.80, 0.60, 0.40 y 0.20 mg/mL. A cada una de estas soluciones se le midió la absorbancia a 280 nm y se realizó la determinación de la concentración de proteína mediante la técnica de análisis de aminoácidos. Este procedimiento fue realizado dos veces en días independientes y por duplicado para cada solución de trabajo. Las soluciones de trabajo fueron preparadas en el momento de realizar los experimentos.

Muestras para análisis de aminoácidos: en el caso de la BSA se mezclaron volúmenes de las soluciones de trabajo y de la solución de nLeu en una relación molar 1:36 y se evaporaron en evaporador rotatorio a sequedad en ampolletas de vidrio previamente pirolizadas. En el caso de EGFhr-51 y EGFhr-52 la relación molar con el estándar interno nLeu fue de 1:2 e igualmente estas mezclas se evaporaron mediante el procedimiento antes descrito.

Hidrólisis ácida total: a las ampolletas de vidrio que contenían las muestras secas se añadieron 100 µL de HCl 6N, conteniendo 0.1% de 2-mercaptoetanol y 0.1% de fenol. Se sellaron a vacío mientras se mantenían sumergidas en nitrógeno líquido y se hidrolizaron a 110 oC por 24 horas. La mezcla de hidrólisis se secó a vacío en evaporador rotatorio y los aminoácidos libres se redisolvieron en 120 µL de la solución tampón de aplicación al analizador.

Estándar de aminoácidos libres: el estándar de aminoácidos libres empleado para la calibración del analizador automático y la cuantificación de los hidrolizados de las proteínas es una solución comercial (Pierce, EEUU) que contiene todos los α-aminoácidos a una concentración de 500 nmol/mL, excepto cistina que está a una concentración de 250 nmol/mL. Se inyectaron en cada cápsula 20 µL o sea 10 nmol de cada α-aminoácido y 5 nmol de cistina.

Cromatografía de intercambio iónico: se empleó un analizador automático Biochrom 20 (Biochrom Ltd Cambridge, Amersham-Pharmacia, Inglaterra). Los aminoácidos libres de los hidrolizados de las proteínas se separaron mediante cromatografía de intercambio iónico en una columna de alta eficiencia (4.6 x 150 mm y 9+/- 0.5 µm de tamaño de partícula) rellena con un intercambiador catiónico, utilizando iones litio como contraión. La elusión se realizó con una serie de soluciones tampón de diferente pH y fuerza iónica según las instrucciones del fabricante. La obtención post-columna de los derivados coloreados se realizó mediante la reacción con ninhidrina y la detección de los derivados se realizó a 570 nm y 440 nm. Los residuos aminoacídicos se identificaron por su tiempo de retención mediante la comparación con el estándar de aminoácidos libres analizado en idénticas condiciones. La adquisición y procesamiento de los datos se realizó con el programa PEAK MASTER versión 4.10 (HARLEY SYSTEMS, Inglaterra).

Cálculo de la cantidad de proteína analizada (CPA): el cálculo de la cantidad de proteína presente en el análisis se realizó dividiendo la cantidad detectada (nmol) en el análisis entre el valor teórico de residuos para determinado aminoácido en la secuencia de la proteína. Los valores teóricos por residuo aminoacídico se tomaron de la secuencia de aminoácidos de las especies del EGFhr y la BSA, respectivamente.

Control de la cuantificación por análisis de aminoácidos: como control de la técnica se realizó la determinación de la concentración de proteína a la BSA sin diluir y se empleó nLeu

como estándar interno. Para la hidrólisis se prepararon ampolletas de vidrio pirolizadas que contenían 3 nmol (199.26 µg) de BSA y 110.37 nmol (12.45 µg) de nLeu. Igualmente, esta mezcla se evaporó en evaporador rotatorio. El experimento se realizó por triplicado.

Espectrofotometría: la absorbancia de las soluciones de trabajo de BSA y de EGFhr-51 y EGFhr-52 se determinó en un espectrofotómetro Spectronic Génesis 2 (Spectronic Instruments, Alemania) empleando 1mL de cada solución de trabajo, en una cubeta de cuarzo de 1 cm de paso óptico. La lectura de la densidad óptica de las soluciones de trabajo se realizó a 280 nm contra el solvente empleado para su disolución (agua).

Evaluación estadística de los resultados

El método desarrollado para combinar los resultados de los distintos experimentos está basado en la combinación de dos procedimientos empleados para diferentes fines: el primero, es la estimación de una pendiente común a partir de la combinación de los pares de valores de abscisas y ordenadas de las regresiones independientes[13]. El segundo, es sopesar la combinación de las pendientes de acuerdo a la precisión de la estimación de las pendientes a promediar y si existen diferencias estadísticas significativas entre ellas o no[14].

El método consiste en determinar las pendientes de las diferentes réplicas experimentales empleando pesos iguales a la unidad. Después de obtener las pendientes para cada una de las réplicas se obtiene la regresión combinada de las réplicas que forman parte de un mismo experimento (día de ensayo). Para ello, el primer paso consiste en determinar si existen diferencias significativas entre las pendientes que se promedian mediante una prueba donde se compara el incremento del cuadrado medio residual de la regresión conjunta sopesada, causado por el uso de una sola pendiente, con el cuadrado medio residual combinado de las regresiones independientes[15]. Cuando no existen diferencias significativas entre las pendientes los pesos de los puntos de cada una de las réplicas se calculan como el inverso del cuadrado medio residual de la regresión de esa réplica, normalizado a la media de los pesos. En caso de detectarse diferencias significativas entre las regresiones a promediar los pesos se calculan como el inverso de la suma del cuadrado medio residual de esa réplica y el cuadrado medio residual entre las réplicas, normalizando también a la media. En el caso de diferencias significativas entre las réplicas, la diferencia entre los coeficientes de peso para los diferentes puntos es atenuada por la existencia de un término común en el denominador: el cuadrado medio de la diferencia entre pendientes. Los pesos fueron normalizados en todos los casos para permitir la comparación posterior de las pendientes obtenidas para cada uno de los ensayos. Los resultados de los ensayos fueron promediados de forme idéntica a la ya descrita para promediar las réplicas.

En el caso del cálculo del coeficiente de absortividad específico del EGFhr, se obtuvieron de forma independiente los resultados para el EGFhr-51 y el EGFhr-52 y luego fueron promediados entre ellos. En este caso, la comparación de las pendientes obtenidas para cada una de las especies se empleó como criterio de igualdad entre sus coeficientes de extinción. La media fue calculada siguiendo el mismo procedimiento. Con el fin de evaluar el método experimental y la calidad de los resultados, se calcularon los coeficientes de variación intra-réplica, entre réplicas y entre ensayos, así como los límites de confianza de la pendiente estimada para cada caso.

Resultados y discusión

Control de la cuantificación de proteínas por análisis de aminoácidos

Para evaluar la exactitud de la determinación de la concentración de proteína se seleccionó una preparación comercial, de concentración conocida: la BSA. Como control interno del proceso de cuantificación se empleó un aminoácido no común en la estructura de las proteínas: la norleucina. Este aminoácido es estable durante la hidrólisis ácida y puede ser cromatográficamente separado del resto de los aminoácidos proteicos. El empleo de este estándar interno permite valorar la recuperación del material proteico al finalizar el procedimiento completo de hidrólisis y minimizar la variabilidad entre las determinaciones. En el cálculo de la concentración de proteína se consideraron la recuperación del estándar interno, las diluciones realizadas y los volúmenes de partida.

En el experimento empleamos un único método de hidrólisis. Todos los residuos aminoacídicos fueron detectados con este método excepto Met, Cys y Trp. Para el cálculo de la CPA se consideró el rendimiento de Asx, Glx, Ala, Val, Ile, Leu, Tyr y Phe y se calculó la CPA promedio. Los resultados de la determinación de la concentración de la preparación comercial de BSA sin diluir (Tabla 1). La exactitud de la determinación aparece expresada mediante la desviación estándar, el coeficiente de variación porcentual y el error porcentual, este último calculado con relación al valor teórico de la concentración de la preparación comercial de BSA. Usualmente el coeficiente de variación porcentual es considerado aceptable hasta el 10%[16]. El valor de concentración experimental promedio 2.02 mg/mL coincide de manera satisfactoria con el valor teórico 2.00 mg/mL; este resultado demuestra que la cuantificación de proteínas mediante la técnica de análisis de aminoácidos resulta precisa y fiable para la determinación delcoeficiente de absortividad específico de proteínas.

Teóricamente el rendimiento de un solo aminoácido presente en una muestra de proteína recombinante, cuyas composiciones aminoacídicas y masa molecular son bien conocidas, puede ser suficiente para la determinación de la concentración. Sin embargo, el uso del rendimiento promedio de varios residuos, especialmente de aquellos con un comportamiento más estable durante la hidrólisis ácida, garantiza la cuantificación con mayor exactitud y permite ponderar el comportamiento particular de los diferentes aminoácidos en el análisis cuantitativo.

El método de hidrólisis empleado es el más común para hidrolizar proteínas, previo al análisis de sus aminoácidos constituyentes. Sin embargo, la técnica de hidrólisis ácida puede contribuir significativamente a la variación de los resultados del análisis debido a las diferencias en la estabilidad de los γ -aminoácidos que se encuentran en las proteínas. Algunos residuos como Cys y Trp son completamente degradados bajo las condiciones ácidas de hidrólisis y la Met su oxida. Por otra parte, Ser y Thr son significativamente degradados (>30%) durante la hidrólisis. En los resultados del análisis de aminoácidos influyen además los fondos que aportan los aminoácidos libres provenientes de las más diversas fuentes, en particular Gly, Ser y Ala, observándose para los residuos afectados un aumento en el valor experimental y en el coeficiente de variación. Estos residuos aminoacídicos no se recomiendan para el análisis cuantitativo. La técnica de análisis de aminoácidos debe ser rigurosamente controlada en cada laboratorio de manera que estas particularidades no tengan un mayor impacto

en la cuantificación de las proteínas por sobreestimación o subvaloración de las cantidades reales. El grupo de aminoácidos seleccionados para el calculo de la CPA resultó adecuado y permite calcular la concentración de una solución de proteína con un 0.83% de error (Tabla 1).

Para determinar el coeficiente de absortividad de proteínas con escaso grado de caracterización empleando la técnica de análisis de aminoácidos para la cuantificación, es necesario emplear una batería de métodos especializados junto a la hidrólisis con ácido clorhídrico 6N para la determinación cuantitativa de todos los aminoácidos presentes en la secuencia de la proteína[17, 18]. Esta estrategia aumenta considerablemente el número de muestras a analizar, el tiempo de trabajo y los costos del experimento.

Determinación del coeficiente de absortividad específico de proteínas

Albúmina bovina de suero: proteína modelo

La metodología empleada para la determinación del coeficiente de absortividad específico de las proteínas comprende la determinación precisa de la concentración de soluciones de proteína de diferente concentración mediante la técnica de análisis de aminoácidos y la medición de sus absorbancias a 280 nm. Los datos de la concentración para cada solución de proteína se correlacionan con su absorbancia, se determina la pendiente de la relación lineal en el rango de concentraciones estudiadas y esa pendiente es la constante de absortividad específica[12].

El coeficiente de absortividad específico de la BSA se determinó a partir de soluciones de trabajo obtenidas por dilución seriada de la preparación comercial. Para el cálculo de la concentración de proteína de cada solución de trabajo se empleó como base de cálculo de la CPA el mismo grupo de aminoácidos empleados en el control de la cuantificación de la BSA sin diluir. Se realizaron tres experimentos en días diferentes (día 1, día 2 y día 3) y 3 réplicas para cada solución de trabajo por experimento. Este diseño permitió calcular la variación de los resultados dentro del curso del mismo análisis y la variación interdía, ambas constituyen criterios de precisión de la metodología. En la tabla 2 se presentan los resultados del cálculo de la pendiente para cada réplica, para cada ensayo y la media de los 3 experimentos realizados. Se indica además si se observaron diferencias significativas o no entre los resultados a promediar. Las diferencias significativas observadas entre las réplicas del tercer ensayo y entre los resultados de los ensayos demuestran la utilidad de realizar varios ensayos con réplicas en cada uno, a fin de evitar el sesgo en los resultados. La precisión de los resultados se evaluó mediante el coeficiente de variación (Tabla 3). En todos los casos el CV es menor que 2%, menor que el de las técnicas analíticas empleadas de forma rutinaria en la cuantificación de proteínas[16].

El valor de coeficiente de absortividad específico para la BSA determinado experimentalmente en este trabajo, 0.6758 mL mg^{-1} cm^{-1} , se comparó con otros valores informados en la literatura. Wetlaufer[19] predijo un as de 0.6607 mL mg^{-1} cm^{-1}, Gill y von Hippel[20] predijeron un as de 0.6517 mL mg^{-1} cm^{-1} y Pace y colaboradores[21] un as de 0.6462 mL mg^{-1} cm^{-1}, aproximadamente 2.2%, 3.6% y 4.4% menores que nuestro resultado experimental, respectivamente. Nozaki determinó el as de BSA empleando concentraciones calculadas por el método de peso seco y el valor obtenido fue 0.6257 mL mg^{-1} cm^{-1}, que se diferencia aún más de nuestro resultado experimental: -7.4%[22].

Estos datos indican que los métodos predictivos y el método de peso seco sobreestiman la concentración de BSA y consecuentemente aportar valores de as inferiores. La determinación de concentración mediante el método de peso seco puede sobreestimar el valor de concentración debido a la presencia de sales no volátiles o de moléculas de agua enlazadas a moléculas de proteína. Los modelos predictivos de Gill y von Hippel[20] y Pace y colaboradores[21] emplean coeficientes de absortividad experimentalmente derivados de muchas proteínas y también pudieran ser el resultado de la contaminación con sales o agua. El modelo predictivo encuentra aplicación por su sencillez. Sin embargo, este modelo presenta limitaciones: requiere la presencia de residuos de Trp, Tyr y Cys, así como el conocimiento del número de estos residuos en la secuencia de la proteína y supone implícitamente que las características de absorbancia de Trp, Tyr y Cys determinadas para el modelo son equivalentes en otras condiciones experimentales.

Por el contrario, el análisis de aminoácidos provee una medida absoluta de los aminoácidos libre de interferencias. Anders y colaboradores[3] demostraron que la determinación de as mediante la cuantificación de los aminoácidos con comportamiento estable durante la hidrólisis ácida es precisa. Ellos obtuvieron para la BSA un valor de as que difiere del valor obtenido en nuestro trabajo en un 2%. Esta diferencia está dentro del rango de error probable entre diferentes laboratorios para esta técnica[16].

Los resultados obtenidos demuestran que la metodología implementada en nuestro trabajo con la BSA como proteína modelo y que emplea la técnica de análisis de aminoácidos para la determinación de la concentración de proteínas permite determinar el coeficiente de absortividad específico con precisión y nos permite el empleo de la metodología para la determinación de esa constante físico-química para otras proteínas de interés biofarmacéutico.

Factor de crecimiento epidérmico humano recombinante

Stanley Cohen en 1962 aisló por primera vez el factor de crecimiento epidérmico (EGF), una proteína con 53 residuos aminacídicos, a partir de las glándulas submaxilares de ratón macho adulto[23]. Esta proteína es un factor de crecimiento y sus principales propiedades físico-químicas han sido determinadas[27]. Su efecto biológico consiste en la modulación de la proliferación celular por medio de la activación del receptor tirosina quinasa del EGF y la proteína quinasa C. El EGF posee un gran potencial en la aplicación clínica, especialmente en la reparación de las heridas cornéales después de la cirugía, así como en el tratamiento de quemaduras y úlceras[27].

Existen informes en la literatura del coeficiente de absortividad del EGF. Se informa un valor de 3.09 mL mg^{-1} cm^{-1} para el EGF aislado de las glándulas submaxilares de ratón macho adulto[23] y de 3.025 mL mg^{-1} cm^{-1} para el EGF humano[24], ambos con 53 aminoácidos en su estructura. La secuencia de esta proteína en ambas especies no es idéntica, pero si lo es el contenido de aminoácidos con cadenas laterales aromáticas. La molécula objeto de nuestro estudio es una preparación heterogénea compuesta por dos especies proteicas de 51 y 52 aminoácidos, que se obtienen además en proporciones variables entre los lotes de producción. Aun cuando la diferencia entre estas especies consiste en un residuo de leucina[27] aminoácido que no aporta significativamente a las características espectrofotométricas de las proteínas, la determinación experimental del coeficiente de absortividad es indispensable para demostrar la existencia de un único valor de coeficiente de absortividad específico en esta molécula y utilizar este valor para la determinación de la concentración total de proteínas de los lotes. Por otra parte, la determinación experimental del coefi-

ciente de absortividad específico es un requisito exigido por las agencias reguladoras para la caracterización de los productos biofarmacéuticos de uso en humanos[1].

Como resultado del proceso de purificación mediante cromatografía líquida de alta eficacia en fase reversa de la preparación de EGFhr se obtuvieron dos picos mayoritarios. El que eluye a menor tiempo de retención se identificó como EGFhr-51 y a mayor tiempo como EGFhr-52[27]. Las fracciones obtenidas se liofilizaron. Las preparaciones puras de ambas proteínas se disolvieron en agua libre de aminoácidos. A partir de estas soluciones y considerando la concentración estimada por BCA se preparó las soluciones seriadas de trabajo. A cada solución de trabajo a diferentes concentraciones se le determinó la concentración de proteínas mediante la técnica de análisis de aminoácidos, en experimentos ejecutados en días diferentes. En los dos experimentos se registró la absorbancia de las soluciones de trabajo de las especies EGFhr-51 y EGFhr-52 en el rango entre 200 y 400 nm. La absorbancia de las muestras fue leída contra el blanco y no fue necesario hacer correcciones a 330 nm para restar el efecto de la dispersión de la luz[28]. El máximo de absorbancia se registró a 280 nm. Un factor importante al momento de preparar las diluciones seriadas fue que las lecturas de absorbancia a la longitud de onda seleccionada para la determinación del coeficiente de absortividad quedaran dentro del rango de linealidad del espectrofotómetro empleado. En la composición aminoacídica del EGFhr están presentes varios residuos que contribuyen considerablemente a su absorbancia a 280 nm. Por esa razón fue necesario hacer diluciones de las soluciones de trabajo. Los valores obtenidos se corrigieron por el factor de dilución.

El coeficiente de absortividad específico se determinó como la pendiente de la recta de regresión lineal de los valores de absorbancia a 280 nm en función de la concentración de las soluciones, obtenida experimentalmente. El valor de la pendiente, la desviación estándar, el coeficiente de correlación y los límites de confianza determinados para ambas especies en cada experimento se muestran en la tabla 4. En todos los casos, el coeficiente de determinación, numéricamente igual al cuadrado del coeficiente de correlación, fue superior a 0.97, lo que demuestra la calidad del ajuste lineal. Los valores de los coeficientes de variación de la pendiente demuestran la precisión de su determinación.

En la tabla 5 se muestran los resultados de las pendientes combinadas de las 2 réplicas de cada día y de los 2 días para las 2 especies moleculares estudiadas. Los altos valores de probabilidad obtenidos, mucho mayores que 0.05, demuestran que no existen diferencias significativas entre las pendientes estudiadas, lo que evidencia la consistencia de los resultados de los análisis. La diferencia entre este resultado y el obtenido para la BSA, donde si se observaron diferencias significativas, son debido a la ligera disminución de la precisión en la estimación de las réplicas para el EGFhr. Por otra parte, la homogeneidad de los resultados obtenida para el EGFhr redunda en un aumento de la precisión de las pendientes combinadas, de forma tal que los coeficientes de variación para el EGFhr-51 y EGFhr-52 son muy similares a los obtenidos para la pendiente combinada de la BSA.

La tabla 6 presenta el promedio entre los resultados obtenidos para ambas especies moleculares. El valor de probabilidad de que las diferencias observadas entre el valor obtenido para el EGFhr-51 y el EGFhr-52 fueran debidas al azar resultó igual a la unidad. La prueba de significación de la diferencia entre las pendientes se convierte en un instrumento para determinar si los coeficientes de absortividad de ambas especies moleculares son iguales. En este caso por tratarse de especies diferentes, se compara el incremento de la varianza residual contra la varianza residual debida a la variabilidad entre ensayos. El valor obtenido demuestra que no existen diferencias prácticas entre los valores de los coeficientes de absortividad específico de las 2 especies moleculares en estudio del EGFhr.

Conclusiones

El coeficiente de absortividad específico del EGFhr que contiene dos especies proteicas de 51 y 52 aminoácidos, determinado mediante el uso de la técnica de análisis de aminoácidos para la cuantificación de proteína, es 3.1125 mL mg^{-1} cm^{-1}, con límites del intervalo de confianza al 95 % desde 3.0340 mL mg^{-1} cm^{-1} hasta 3.1911 mL mg^{-1} cm^{-1}. El coeficiente de variación obtenido para el coeficiente de absortividad fue de 1.22%. La prueba de significación de la diferencia entre las pendientes demuestra que no existen diferencias entre los valores de los coeficientes de absortividad específico de las 2 especies moleculares en estudio del EGFhr y se justifica completamente el empleo de un coeficiente de absortividad común.

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Muestra	Réplica 1	Réplica 2	Réplica 3
C teórica (mg/mL)	2.0	2.0	**2.0**
C experimental (mg/mL)	1.95	1.88	**2.21**
C promedio (mg/mL)		**2.02**	
DE		**0.18**	
CV (%)		**8.87**	
Error (%)		**0.83**	

C - concentración. DE – desviación estándar. CV (%) – coeficiente de variación porcentual. Error (%) = ((VE-VT)/VT) *100, donde VE es el valor experimental y VT el valor teórico de la concentración de la proteína.

Tabla 1. Control de la cuantificación por análisis de aminoácidos. Determinación de la concentración de proteína de la preparación comercial de BSA mediante la técnica de análisis de aminoácidos..

Experimento	Réplica			Ensayo		Media general	
	Pendiente (a_s)	Límite Inferior (a_s)	Límite Superior (a_s)	Pendiente Límite Superior Límite Inferior (a_s)	¿Diferencias significativas?	Pendiente Límite Superior Límite Inferior (a_s)	**¿Diferencias significativas?**
Día 1	0.6785	0.6674	0.6897	0.6789	No	0.6758	**Si**
	0.6791	0.6412	0.7170	0.6726			
	0.6816	0.6514	0.7119	0.6853			
Día 2	0.6895	0.6597	0.7193	0.6844	No	0.6689	
	0.6826	0.6670	0.6982	0.6762			
	0.6861	0.6532	0.7191	0.6926			
Día 3	0.6503	0.6104	0.6902	0.6565	Si	0.6827	
	0.6471	0.6366	0.6576	0.6457			
	0.6711	**0.6549**	**0.6873**	**0.6673**			

a_s – coeficiente de absortividad específico (mL mg-1 cm-1).

Tabla 2. Determinación del coeficiente de absortividad especifico de la BSA (as). Resultados del cálculo de la pendiente para cada réplica, para cada ensayo y la media general de los 3 experimentos realizados.

Experimento	CV intra réplica (%)	CV combinado intra réplica para cada ensayo (%)	CV combinado intra réplica General (%)	CV entre réplicas (%)	CV combinado entre réplicas (%)	CV entre ensayos (%)
Día 1	0.5164	1.2212	1.1606	0.2425	0.9093	1.2682
	1.7534					
	1.3938					
Día 2	1.3571	1.1950		0.5009		
	0.7185					
	1.5093					
Día 3	1.9287	1.0656		1.9844		
	0.5090					
	0.7591					

CV – coeficiente de variación (%).

Tabla 3. CIndicadores estadísticos de la calidad de la regresión. Coeficiente de variación intra réplicas, entre réplicas y entre ensayos.

Especie EGFhr-51				
	Día 1 réplica 1	Día 1 réplica 2	Día 2 réplica 1	**Día 2 réplica 2**
Pendiente	2.9380	3.0953	3.2118	**3.2376**
DE	0.0621	0.0786	0.0533	**0.1191**
CV (%)	2.1137	2.5393	1.6595	**3.6787**
r^2	0.9932	0.9902	0.9957	**0.9827**
Límite Inferior	2.7405	2.8452	3.0423	**2.8586**
Límite Superior	3.1355	3.3455	3.3813	**3.6166**
Especie EGFhr-52				
	Día 1 réplica 1	Día 1 réplica 2	Día 2 réplica 1	**Día 2 réplica 2**
Pendiente	2.8553	3.1292	3.2459	**3.2413**
DE	0.0518	0.1022	0.1481	**0.1043**
CV (%)	1.8142	3.2660	4.5627	**3.2178**
r^2	0.9949	0.9827	0.9710	**0.9853**
Límite Inferior	2.6905	2.8040	2.7747	**2.9094**
Límite Superior	**3.0201**	**3.4543**	**3.7171**	**3.5731**

Tabla 4. Determinación del coeficiente de absortividad especifico de las especies EGFhr-51 y EGFhr-52. Valores de la pendiente de la regresión lineal, desviación estándar (DE), coeficiente de variación, (CV), coeficiente de correlación (r2) y límites de confianza obtenidos empleando el valor de concentración de las soluciones de trabajo determinado experimentalmente mediante la técnica de análisis de aminoácidos y el valor de absorbancia a 280 nm.

Tabla 5. Determinación del coeficiente de absortividad especifico de las especies EGFhr-51 y EGFhr-52. Valores de la pendiente de la regresión lineal, desviación estándar (DE), coeficiente de variación, (CV), coeficiente de correlación (r2) y límites de confianza obtenidos empleando el valor de concentración de las soluciones de trabajo determinado experimentalmente mediante la técnica de análisis de aminoácidos y el valor de absorbancia a 280 nm.

Parámetros	EGFhr-51		EGFhr-52	
	Día 1	Día 2	Día 1	Día 2
Probabilidad	0.6617	1.0000	0.4327	**1.0000**
Pendiente	3.0145	3.2256	2.9820	**3.2436**
DE	0.0549	0.0620	0.0732	**0.0836**
CV (%)	1.8212	1.9221	2.4547	**2.5774**
r^2	0.9882	0.9880	0.9779	**0.9782**
Límite Inferior	2.8845	3.0790	2.8090	**3.0460**
Límite Superior	3.1444	3.3723	3.1550	**3.4411**
Probabilidad	0.5663		**0.6015**	
Pendiente	3.1138		**3.1109**	
DE	0.0483		**0.0633**	
CV (%)	1.5512		**2.0348**	
r^2	0.9825		**0.9694**	
Límite Inferior	3.0109		**2.9759**	
Límite Superior	**3.2167**		**3.2459**	

Parámetros	Media EGFhr-51 y EGFhr-52
Probabilidad	**1.0000**
Pendiente	**3.1125**
DE	**0.0385**
r^2	**0.9772**
Límite Inferior	**3.0340**
Límite Superior	**3.1911**

Tabla 6. Resultados de la combinación de pendientes del EGFhr-51 y el EGFhr-52. Junto a la pendiente se muestra la probabilidad de que las diferencias entre las dos pendientes promediadas sean obtenidas por azar, la desviación estándar de la pendiente combinada (DE), el coeficiente de determinación (r2) y los límites de confianza.

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RESEARCHS / INVESTIGACIÓN

Trimethyltin-induced cerebellar damage on adult male Wistar rats.

Trimetil estaño induce daño cerebral en ratas machos adultas Wistar.

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Abstract: This research work was done to investigate the acute toxicological effect of trimethyltin chloride on the cerebellum of Wistar rat. Ten adult male Wistar rats were used for the study. The animals were grouped into two: Group A and B, with five adult male Wistar rats in each group. Group A serves as the trimethyltin (TMT) group, while group B serves as the normal saline (NS) group. 3mg/kg of trimethyltin chloride was administered to animals in the TMT group, while 1.0mls of normal saline was administered to the animals in the NS group via intraperitoneal route for 3 hours respectively. The animals were sacrificed at the Histology Laboratory, University of Ilorin, using 25mg/kg of ketamine administered intramuscularly to anesthetize the animals; followed by perfusion fixation through the heart. The brains were harvested, and the tissues were processed and stained using H & E, and crystal violet stains. The cerebellar cortex and nissl substances of the cerebellum were analyzed and showed a mild distortion in the layers of the cerebellar cortex. Biochemical analysis was undertaken to investigate the disruption of the oxidative status in the animal tissue, using Super Oxide Dismutase (SOD). Oxidative stress was found to increase significantly ($p < 0.05$) in the TMT groups compared with the NS group, because the SOD activity decreased more in the brain homogenates of the TMT group. The result demonstrated that trimethyltin exerts its toxic effect by promoting oxidative stress in the brain and this may affect normal brain functioning and growth.

KeyWords: trimethyltin , Wistar rat, cerebellar cortex, nissl substances.

Resumen: Este trabajo de investigación se realizó para investigar el efecto toxicológico agudo del cloruro de trimetil estaño en el cerebelo de la rata Wistar. Se utilizaron diez ratas Wistar macho adultas para el estudio. Los animales se agruparon en dos: Grupo A y B, con cinco ratas Wistar macho adultas en cada grupo. El grupo A sirve como grupo trimetil estaño (TMT), mientras que el grupo B sirve como grupo salino normal (NS). Se administraron 3 mg / kg de cloruro de trimetil estaño a animales en el grupo TMT, mientras que se administraron 1,0 ml de solución salina normal a los animales en el grupo NS por vía intraperitoneal durante 3 horas respectivamente. Los animales se sacrificaron en el Laboratorio de Histología, Universidad de Ilorin, usando 25 mg / kg de ketamina administrada por vía intramuscular para anestesiar a los animales; seguido por la fijación de la perfusión a través del corazón. Los cerebros se recolectaron y los tejidos se procesaron y se tiñeron con H&E y tintes de violeta cristal. Se analizaron la corteza cerebelosa y las sustancias nissl del cerebelo y mostraron una leve distorsión en las capas de la corteza cerebelosa. Se llevó a cabo un análisis bioquímico para investigar la alteración del estado oxidativo en el tejido animal, utilizando la superóxido dismutasa (SOD). Se encontró que el estrés oxidativo aumentaba significativamente ($p < 0,05$) en los grupos de TMT en comparación con el grupo de NS, porque la actividad de SOD disminuyó más en los homogeneizados de cerebro del grupo de TMT. El resultado demostró que la trimetilestina ejerce su efecto tóxico al promover el estrés oxidativo en el cerebro y esto puede afectar el funcionamiento y el crecimiento normal del cerebro.

Palabras Claves: trimetilestaño, rata Wistar, corteza del cerebelo, nissl sustancias.

Introduction

Trimethyltin (TMT) is a neurotoxin that affects the functions of the Central Nervous System (CNS) causing signs of intoxication such as tremors and hyper excitability in animals[1]. A neurotoxin is a substance that inhibits the function of neurons throughout the brain and the nervous system[1]. Exposure to neurotoxins can cause dizziness, nausea, loss of motor control, paralysis, difficulty with vision, seizures, and even coma or death.

Trimethyltin (TMT) belongs to a member of the organotin class of compound widely used in industry as plasticizers of polyvinyl chloride products[2] and as biocides incorporated in molluscicides, insecticides, fungicides, and bactericides[3]. Individuals working in industries where hazardous chemicals are being produced are at a higher risk of exposure.

The brain is an integral part of the body whose function helps to regulate other parts of the biological system. Any damage or form of stress experienced in the brain may have a serious impact on the entire organism[4]. Exposure of the cerebellum to hazardous chemicals could lead to neurodegenerative diseases and hypoxic damage which can result in damage to Purkinje cells in the cerebellar cortex; leading to the development of cerebellar ataxia in which there is poor coordination of voluntary movement[5].

TMT has been shown to induce neurotoxicity and oxidative stress. Oxidative stress is a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses[6]. It is necessarily a "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them"[7]. TMT can increase the production of reactive oxygen species (ROS), and nitric oxide (NO) which are often associated with the processes of cell apoptosis. The disruption of the oxidative status in the living organism can be prevented by cellular antioxidants[8].

Toxic doses of TMT chloride are capable of disturbing the natural oxidation/reduction balance in cells through mechanisms originating from their complex oxidative/radical

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reactions with endogenous oxidants. These reactions produce effects on cellular antioxidant systems, cellular membranes, and membrane-dependent redox-sensitive enzymatic systems. This may produce a variety of toxic effects, which lead to the cells death[8]. The predicted lethal dose of TMT for humans is probably 3 mg kg^{-1} (15.1 mumol kg^{-1}); however, a lesser dose of this amount may be required to produce neuronal damage[9].

The results of a study carried out by Wang[1] suggested that acute exposure to TMT, induced brain cell apoptosis in the telencephalon, optic tectum and cerebellum, suggesting that TMT exposure in the environment may affect behaviors, sensory and motorial learning based on the observation of cell apoptosis in the cerebral regions of S. marmoratus.

Bioaccumulation of TMT in the biological system may pose a serious challenge to public health. The highly reactive oxidant, superoxide anion (O_2) was investigated in this study, using Superoxide dismutase (SOD). SOD is an antioxidant that is intimately involved in the prevention of cellular damage - a common pathway for cancer, aging and a variety of diseases[10]. Antioxidants interact with free radicals and safely terminate the chain reactions before vital molecules are damaged. The study aims to identify the possible toxicological effect of trimethyltin (TMT) on the histo-architecture and biochemical composition of the cerebellum which may pose a serious challenge to public health.

Materials and methods

Ten adult male Wistar rats were used in the study. Their weights ranged from 150 - 200g. The animals were grouped into two: Group A and B, with five adult male Wistar Rats in each group. Group A serves as the trimethyltin(TMT) group, while group B serve as the normal saline (NS) group. The animals were caged to acclimatize for one day. After that, 3mg/kg of trimethyltin chloride was administered to animals in the TMT group, while 1.0mls of normal saline was administered to the animals in the NS group via intra-peritoneal route for 3 hours respectively. The animals were sacrificed using 25mg/kg of ketamine administered intramuscularly to anesthetize the animals; followed by perfusion fixation through the heart to prevent postmortem effect during the cause of harvesting the brain.

The tissue of the cerebellum for biochemical analysis was homogenized in 5% sucrose, and stored in antifreeze. The tissues for histological analysis were excised after sacrifice and stored in paraformaldehyde. Tissue processing was done on paraffin wax embedded tissue blocks and mounted on a glass slide.

Groupings and Administration of Drugs

A single dose of 3mg/kg of Trimethyltin was administered to the animals in the TMT group, while the animals in the NS group received a single dose of 1.0ml of normal saline via intra-peritoneal route using a needle and syringe.

Table 1. Grouping and Treatments.

Groups (N=10)	Administration of drugs	Route
A (N = 5)	Trimethyltin 3mg/kg (single dose)	Intra-peritoneal
B (N = 5)	Normal saline 1.0ml (NS Group)	Intra-peritoneal

Morphometry and histological techniques

The rats were sacrifice using 25mg/kg of ketamine and all the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education were observed. The brains were dissected out. The length and width of the brain were measured using a ruler whereas the weight was measured using weighing balance. The tissues were fixed in paraformaldehyde and processed using routine H and E histological techniques, and crystal violet stains.

Superoxide Dismutase Assay

Enzyme activity of superoxide dismutase was assayed according to the method of Mistra and Fridovich[11], using reagent kit produced by Randox Laboratories Ltd.

Statistical Analysis

The statistical analysis was done using Microsoft Office Excel 2007. The student t-test (Paired Two Sample) was used to analyze the differences in the body weight before and after the experiment; while the t-Test for unpaired two samples was used to determine the morphometric differences across the groups and $P < 0.05$ was considered as the level of significance.

Results

Physical Observation and Body Weight Analysis

Physical examination of the animals shows normal activities in both the TMT group (Group A) and in the NS group (Group B). Table 4.0 shows the mean body weights in both groups before and after the experiment. The statistical analyses of the animals' body weight (Table 4.0) show no statistically significant differences ($p > 0.05$) before and after the experiment in group B (NS group), but a statistically significant difference ($p < 0.05$) was observed in the group A (TMT group).

Morphometric Analysis

The gross morphological examination of the brain shows no clear differences in the weight and width of the brain in both groups. Table 4.1 shows statistical analysis of the variables (weight, length, width, and brain/body ratio). The result however, shows no clear difference in the brain/body ratio, brain weight and width in both groups ($p > 0.05$), but a statistically significant difference ($P < 0.05$) in the brain length within the groups.

Histological Examination

Hematoxylin and Eosin staining technique were used to demonstrate the general histo-architecture of the cells, while the crystal violet staining technique was used to demonstrate granulations (endoplasmic reticulum and ribosomes) in the cells. The following results were observed in Fig. 1A and Fig. 1B:

Crystal violet staining is shown in Fig. 2, 3 A and B. The

Groups	Before Experiment Mean ±SEM (body weight)	After Experiment Mean ±SEM (body weight)	P value
A (n = 5)	185.6 ± 18.389	181.96 ± 18.230	0.0311
B (n = 5)	148.4 ± 12.089	135.08 ± 11.977	0.2411

Table 2. Statistical analysis (t-Test: Paired Two Sample) for body weight (g) of the animal.

Variables (Brain)	Group A (n = 5) Mean ±SEM	Group B (n = 5) Mean ±SEM	P value
Weight (g)	0.96 ± 0.196	1.08 ± 0.215	0.6915
Length (cm)	2.42 ± 0.086	2.16 ± 0.068	0.0467
Width (cm)	1.42 ± 0.073	1.22 ± 0.124	0.2112
Brain/Body Ratio (g)	0.005178 ± 0.001	0.005949 ± 0.001	0.5749

Table 3. Statistical analysis of the brain weight, length, width and brain/body ratio.

S/N	Group A Mean ±SEM SOD Activity (Unit/ml)	Group B Mean ±SEM SOD Activity (Unit/ml)	P value
1	705 ± 3.302	778.8 ± 7.067	0.0001

Table 4. Statistical analysis of SOD activity in brain homogenates of the two groups.

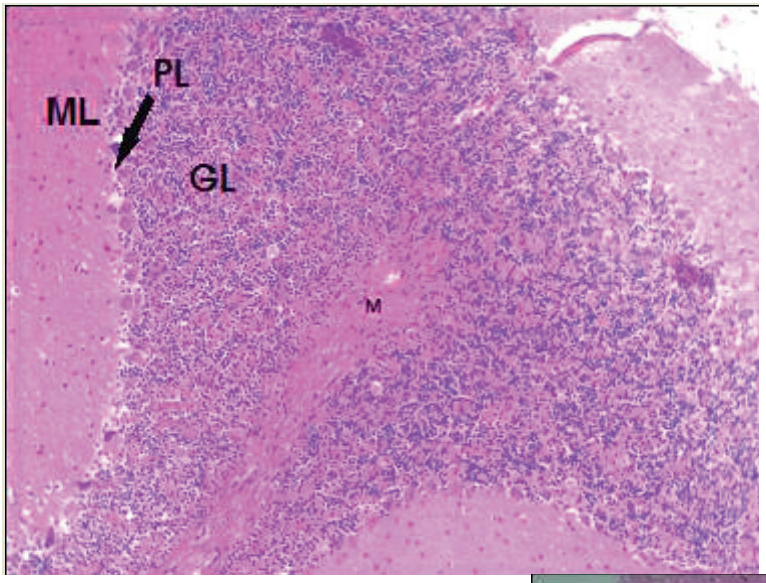

Figure 1A. ANS group (1ml of normal saline), H & E x 100, ML- Molecular layer, PL – Purkinje Layer, GL – Granular Layer, M – Central medulla of white matter.

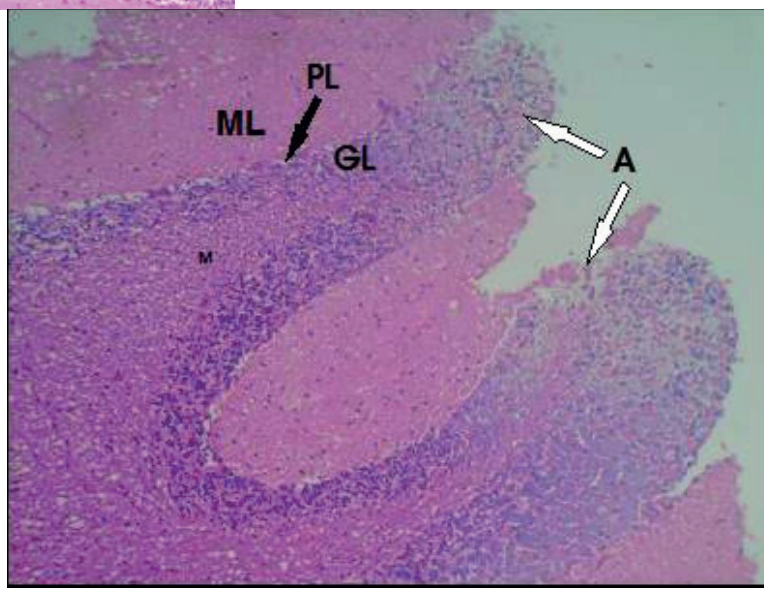

Figure 1B. TMT group (3mg/kg of TMT), H & E x 100, A – A partially stained area of the the cerebellar cortex.

The photomicrograph (Fig. 1A) of the cerebellar cells in group B (1 ml of normal saline) shows the section of the cerebellar cortex consisting of the molecular (ML) and granular layers (GL). In between, them is the Purkinje layer (PL). The central white matter (M) is also visible. The layers of the cerebellar cortex appeared clear as seen in the normal cerebellum. The photomicrograph (Fig. 1B) of group A (3mg/kg ml of TMT) shows an abnormal cellular tissue morphology of the three layers of the cerebellum. A partially stained area in the cerebellar cortex can be seen.

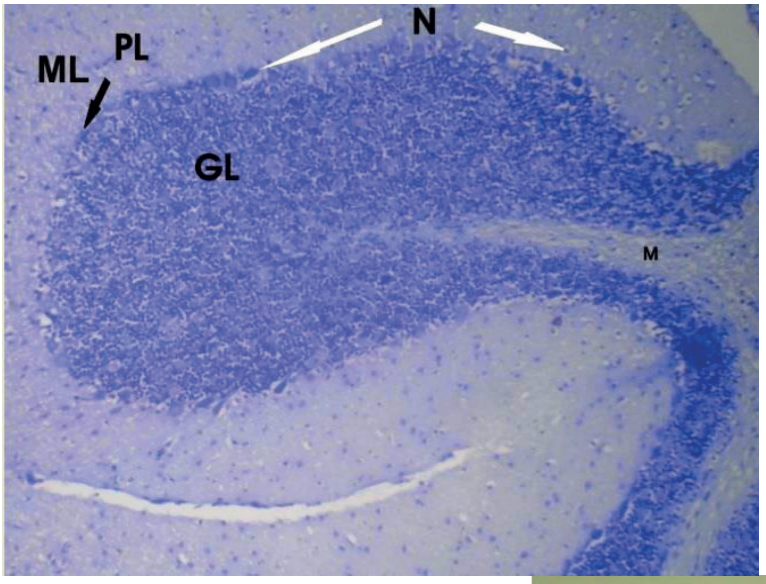

Figure 2A. NS group (1ml of normal saline), CFV x 100, ML- Molecular layer, PL – Purkinje Layer, GL – Granular Layer, M - Central medulla of white matter, N – Nissl substances.

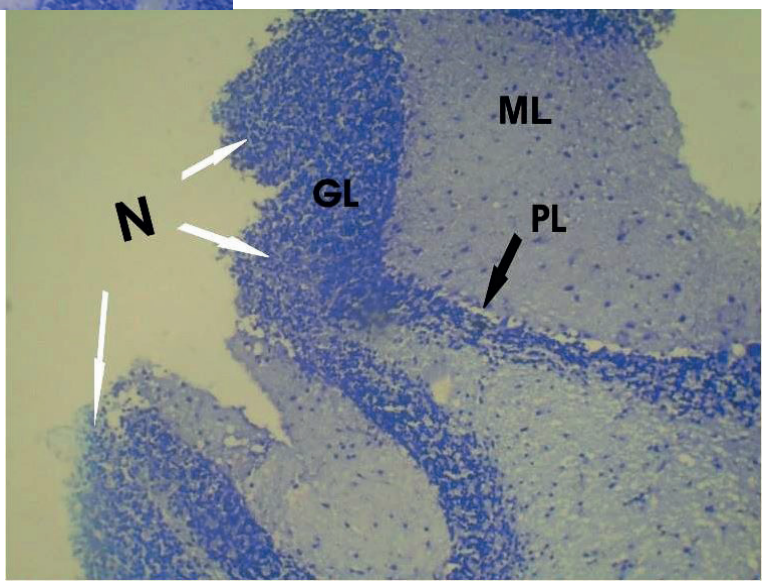

Figure 2B. TMT group (3mg/kg of TMT), CFV x 100, N – Nissl substances in abundance and clustered in areas of distortion.

photomicrograph (Fig. 2 A & 3 B) of group B (1ml of Normal saline) shows nissl substance present as usual in the cerebellar cortex (N). The photomicrograph (Fig. 2 B & 3 A) of group A (3mg/kg of TMT) also shows the presence of nissl substances strongly stained, and clustered mainly in the granular layer of the cerebellar cortex.

Biochemical Examination

Superoxide dismutase (SOD) was employed to assess oxidative stress following exposure to trimethyltin. The biochemical examination has shown that (SOD) activity in brain homogenate of the TMT group decreased significantly compared with the NS group. Oxidative stress was found to increase ($p < 0.05$) significantly. The following results were observed:

The statistical analysis of the biochemical examination shows a statistically significant difference ($P < 0.05$) in SOD activity within the groups.

Discussion

The mechanism of action of trimethyltin chloride has shown to be toxic at 3mg/kg dose following short term administration. A significant decrease in the body weight ($p < 0.05$) is observed in the TMT group.

The histo-architecture of the cerebellar tissues in Group A (TMT group) shows a partially stained area of the cerebellar cortex in the H & E stain and clumping of the nissl substances in the Crystal violet stain, indicating abnormal layers of the cerebellar cortex. It had been reported that TMT administration induces significant behavioral alterations[12] and brain apoptosis in the cerebellum[1]. According to Wang *et al.*[1] TMT exposure in the environment may affect behaviors, sensory and motorial learning.

Cerebellar apoptosis results from exposure of the cerebellum to hazardous chemicals which could lead to hypoxic damage, and may result in damage to Purkinje cells in the cerebellar cortex. This can lead to the development of cerebellar ataxia depicted by poor coordination of voluntary movement[5]. Also, depression, deficits in the ability to experience emotions, and behavioral difficulties are commonly seen in patients with cerebellar lesions[13]. In the present study, the effect produced by TMT chloride on the histology of the cerebellum occurred mildly at the three layers of the cerebellar cortex.

Increased production of reactive oxygen species (ROS) has been linked with increased oxidative stress levels, which is also associated with the process of cell apoptosis[1]. Organisms

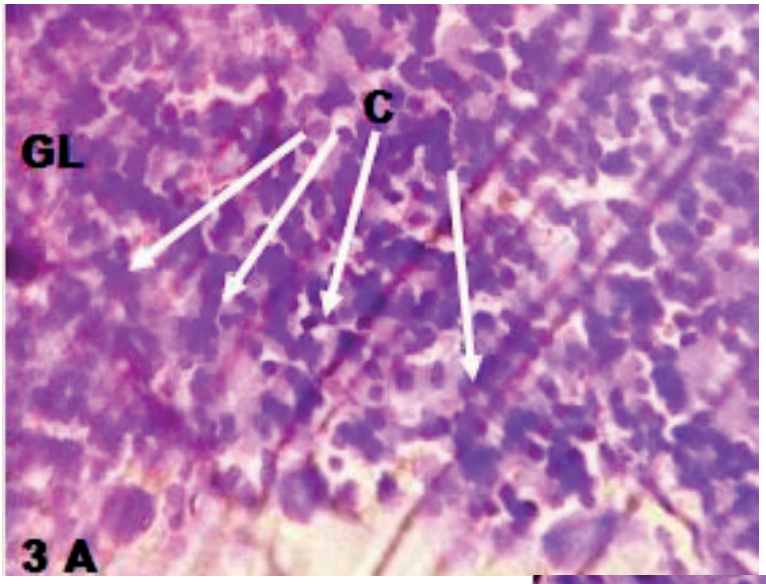

Figure 3A. TMT group (3mg/kg of TMT), CFV x 400, CA - Nissl substances in intensely stained and clustered, GL - Granular layer.

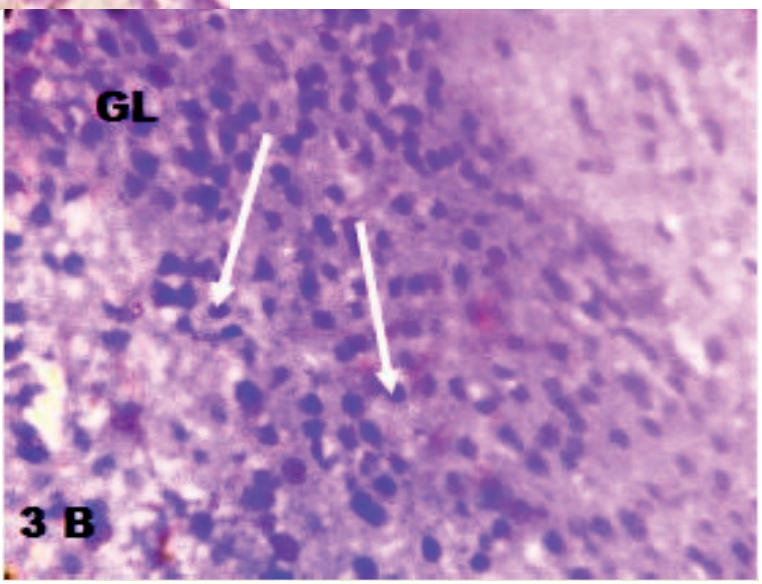

Figure 3B. NS group (1ml of normal saline), CFV x 400, Arrow – Presence of Nissl substances.

have evolved the mechanism to counteract the effect of radicals generated in the biological membrane. This mechanism involves the antioxidant system such as glutathione reductase, glutathione peroxidase, superoxide dismutase (SOD) amongst others. The antioxidant system's functions to modify the highly reactive free radicals to form the less reactive intermediate which no longer pose a threat to the cell[4].

For good biological integrity to be maintained there must be a balance between oxidation and antioxidant's level in the system. Oxidants such as superoxide anions ($O_{2.}$) may attack the membranes of the brain cells thereby causing oxidative stress. The biochemical examination has shown that (SOD) activity in brain homogenate of the TMT group decreased significantly compared with NS group. This decrease may be as a result of the imbalance between oxidants and antioxidants level in favor of the oxidants[4]. Similarly, in a study to investigate the role of oxidative stress in Purkinje cell neurotoxicity of ethanol-treated rat, results showed a decrease in the activities of SOD[14]. SOD is an active enzyme that can cause dismutation of superoxide anions produced during oxidative stress in cells[4].

Metabolism in the brain has been associated with the production of Reactive Oxygen Species (ROS)[4]. Thesignificant increase in ROS in the TMT group compared with the NS group may be associated with trimethyltin poisoning. An important source of ROS is oxidative metabolism of xenobiotics[4]. ROS is controlled by the antioxidant defense systems, which appears to maintain low concentrations rather than complete elimination. Oxidative stress occurs in a cell or tissue when the concentration of ROS generated exceeds the antioxidants capability of that cell[15]. Hence it is suggested that animals in the TMT group experienced oxidative stress.

Conclusions

Short term single dose administration of Trimethyltin (TMT) has no adverse effect on the brain weight of the cerebellum of adult male Wistar rat. However, some changes were observed in the body weight and brain length of the animal, as well as mild cellular distortion in the layers of the cerebellar cortex.

The biochemical analysis suggested an increase in oxidative stress as a result of the production of more reactive oxygen species (ROS) in the TMT group, given that SOD activity in brain homogenate of the TMT group decreased significantly compared with NS group, proving that trimethyltin increases oxidative stress.

Recommendations

It is recommended that individuals in both develop and developing nations who work in industries where TMT is used as plasticizers of polyvinyl chloride products and biocides incorporated in insecticides; should be more cautious with trimethyltin use in the environment, as well as its use with regards to health safety standards especially in humans. Indiscriminate short term exposure to TMT may not have immediate obvious health effect; however, bioaccumulation of TMT in the long-term may be very damaging.

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RESEARCHS / INVESTIGACIÓN

Effect some soil properties (organic matter, soil texture, lime) on the geochemical phosphorus fractions.

Efecto de algunas propiedades del suelo (materia orgánica, textura del suelo, cal) sobre las fracciones geoquímicas de fósforo.

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Abstract: Phosphorus (P) is essential to all known life forms because it is a key element in many physiological and biochemical processes. Soil properties due to soil formation processes and land use have an impact on the availability of phosphorus in soil and on the change in geochemical fractions. The phosphorus fractions are important for assessing their soil status and understanding the soil chemistry that affects soil fertility. Taken 43 soil samples, were selected from agricultural fields of Isparta - Southeast of Turkey, to determine the availability of phosphorus and change in fractions. Relations of phosphorus fractions with soil properties were revealed by analysis of variance, Pearson correlation analysis Descriptive physicochemical analyzes and sequential phosphorus fractionation were performed in the soil. These fractions are; sodium bicarbonate ($NaHCO_3$-P) , sodium hydroxide (NaOH-P), citrate bicarbonate dithionite (CBD-P), hydrochloric acid (Ca-P), and residual (Res-P) phosphorus. The results showed that the fractionations (Ca-P, 597.05 mg kg^{-1}) (Res-P, 259.54 mg kg^{-1}) were high for respectively. It was determined that the fractal distribution of these fractions changed according to the land use pattern.

KeyWords: Phosphorus (P), soil properties, hydrochloric acid, Pearson correlation analysis.

Resumen: El fósforo (P) es esencial para todas las formas de vida conocidas porque es un elemento clave en muchos procesos fisiológicos y bioquímicos. Las propiedades del suelo debido a los procesos de formación del suelo y el uso de la tierra tienen un impacto en la disponibilidad de fósforo en el suelo y en el cambio en las fracciones geoquímicas. Las fracciones de fósforo son importantes para evaluar el estado del suelo y comprender la química que afecta la fertilidad. Tomadas 43 muestras de suelo, fueron seleccionadas de campos agrícolas de Isparta - Sureste de Turquía, para determinar la disponibilidad de fósforo y el cambio en las fracciones. Las relaciones de las fracciones de fósforo con las propiedades del suelo se revelaron mediante análisis de varianza, análisis de correlación de Pearson. Se realizaron análisis fisicoquímicos descriptivos y fraccionamiento de fósforo secuencial en el suelo. Estas fracciones son; bicarbonato de sodio ($NaHCO_3$-P), hidróxido de sodio (NaOH-P), ditionito de bicarbonato de citrato (CBD-P), ácido clorhídrico (Ca-P) y fósforo residual (Res-P). Los resultados mostraron que los fraccionamientos (Ca-P, 597.05 mg kg^{-1}) (Res-P, 259.54 mg kg^{-1}) fueron altos para respectivamente. Se determinó que la distribución fractal de estas fracciones cambiaba de acuerdo con el patrón de uso de la tierra.

Palabras Claves: Fósforo (P), propiedades del suelo, ácido clorhídrico, análisis de correlación de Pearson.

Introduction

Phosphorus is an essential element for plant growth, crop production and quality. Due to low P availability in soil and low fertilizer use efficiency, farmers often apply P fertilizers in excess of plant requirements. Plants take up only 10-20% of the P applied with fertilizers in the year of application because the majority of applied P is rapidly fixed or precipitated into poorly available forms[24]. Many soil constituents react with P to convert it into unavailable forms and the dominance of individual fraction is largely controlled by soil properties. The amount of P fertilizer needed depends not only on the crop P requirement, but also on the amount of extractable soil P and the P fixing capacity of the soil. Discrimination between phosphorus fractions, as well as between soils, is generally found to be statistically significant at the reliable level. The Ca-P fraction contains a significant amount of CB-P (phosphorus impregnated by carbonates), CBD-P (phosphorus occluded is hydroxyl oxides and iron oxides) and Al-P + Fe-P (phosphorus occluded is aluminum and iron) and Residual-P fractions[10]. The Ca-P fraction, determined as the predominant inorganic phosphorus fraction in the rice paddy, has been found to decrease continuously due to the decrease in $CaCO_3$ content of soils[10]. Phosphate ions also become unusable by forming precipitates by combining with elements such as Ca, Mg, Al and Fe in the environment[9, 10]. The plant varieties and genotypes grown in the soil showing phosphorus deficiency often have a root system of eaves extending to the side and to the side of the soil[11]. It has been found that the increase in Al-P and Fe-P in acidic soil is mainly due to the reductive solubility and the predominance of Occluded-P forms, less than the reduction in Ca-P[20]. Marked variation in the different forms of inorganic P is a function of genetic differences among soils[4]. Organic matter, calcium carbonate and sesquioxides appear to be guiding factors in determining the distribution of forms of P[3]. Saltali *et al.*[18] have shown that the organic and inorganic P fractions decrease significantly. Dieter *et al.*[5], the proportional amount of inorganic and organic phosphorus extracted with 0.5 M $NaHCO_3$ and extracted with anion exchange membrane rated Labil / mobile P varied between 4.7% and 11.4%. Achat *et al.*[1] the results show the dominant role of aluminum and iron oxides and organic carbon in the control of the dynamics of phosphate ions in acidic and non-acidic soils. Alovisi *et al.*[2] showed that the effect of inorganic fertilizer on the moderate

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and labile P fractions was high. The $NaHCO_3$-Po fraction in the organic P fractions was observed to be the only fraction that contributed to the plant nutrition.

In the light of the available literature, this study tried to show the effects of soil properties and applied agricultural practices, descriptive statistics and Pearson correlation on phosphorus fractions in Isparta soil.

Materials and methods

Sampling and Site Description

Taken 43 soil samples, were selected from agricultural fields of Isparta - Southeast of Turkey, to determination of phosphorus availability and change in fractions. Surface soil samples were air-dried and sieved through 2 mm mash openings, Then the soil properties are determined by the methods used: organic matter (OM) by modified Wakley-Black wet oxidation with potassium dichromate ($K_2Cr_2O_7$), soil reaction and electrical conductivity by means of pH-meter and Wheatstone bridge in the saturated paste, cation exchange capacity (CEC) by using the method of sodium acetate (1 M pH 8.2) saturation and ammonium acetate (1 M pH 7.0) replacement, Lime equivalents of soils were determined by volumetrically measuring the carbon dioxide minerals from the treatment with 10% HCl (W / V) by means of Scheibler calcimetry[16]. Soil texture was determined by Bouyoucos hydrometer[6]. The summary of soil properties was given in Table 1.

Determination of Phosphorus Fractions

The soils were subjected to sequential extraction procedures of modified version of Soils, Hedley *et al.*[7], Kuo[12] ,procedures. The reagents and operationally defined chemical fractions were as follows:

1. Labile or plant available P ($NaHCO_3$-P): this was partitioned into organic and inorganic $NaHCO_3$-P fractions
2. Moderately labile P (NaOH-P) the residues from the previous sequence were treated with 50 mL of 0.1 M NaOH at 1:50 for 17 h. This was also partitioned into organic and inorganic NaOH-P.
3. Reducible P (Fe-P): extracted with citrate-bicarbonate-buffer system (CBD-P).
4. Ca-bound P (Ca-P): extracted with 1 M HCl for 1 h on a shaker.
5. Residual P (Res-P) the residues from the forgoing fractionations were acid digested with concentrated HNO3- HCl mixture (3:1, V/V).

After the sequential procedures, the phosphorus concentrations of the supernatant obtained were calorimetrically determined using the method of Murphy and Riley (15) at 880 nm wavelength.

Statistical Analyses

Descriptive statistical analyzes were applied to the Physico-chemical properties and phosphorus fractions of soil (concentration and proportional values) in the SPSS 22 package program. Then, the correlation between the soil properties and phosphorus fractions was examined by conventional correlation analysis.

Results and Discussion

Descriptive Statistics

The basic descriptive statistics for different phosphorus fractions shown in Table 2. In Table 2, the majority of the fractions showed typical normal distribution and their skewness and / or kurtosis value ≤ 2 x standard errors. The available P to plant ($NaHCO_3$-Po, $NaHCO_3$-Pi, NaOH-Pi and NaOH-Po) shows high positive skewness. This stimulation indicates that there is an excessive increase in this fraction, especially in some soils, due to the effects of the practices and / or soil formation processes generally done in the soil. This is an expected situation when the size of the study area and the similarities in the soil formation processes are taken into consideration, in particular the similarity of the climate and the mainstream in general.

Positive skewness data means that there is a tendency to enrich for P fractions in some soils with significantly higher analytical values than the majority of soils. This can show that fertilization P is converted to a high positive fraction. Depleted-P from the primary mineral structure and/or fertilizer-P was likely to accumulate in Ca-P and/or CBD-P depending on the abundance of the Fe/Al oxides or carbonate minerals and governing thermodynamic conditions. This situation is expected when Ca-P compounds are thought to be more stable in alkaline calcareous conditions and have lower Fe solubility[13, 21].

Given low skewness, it may indicate extinction due to conversion or loss of the relevant fraction. Res-P fraction is the low skewness. Much of the organic matter-induced activity has been stripped away at earlier stages (in particular, alkali extraction steps such as NaHCO3 and NaOH). There is still a small fraction of organic matter that is not alkaline hastened

Table 1. Descriptive statistics of the soils properties. (N: 43).

Soil Properties	Min	Max	Mean	Std. deviation	Variance	skewness	Kurtosis
OM %	0.51	4.95	2.11	1.01	1.02	1.08	1.45
pH	6.78	8.03	7.65	0.32	0.10	-1.29	0.80
EC ($\mu S\ cm^{-1}$)	105.20	762.00	288.26	129.87	16867.37	1.20	2.89
Lime %	0.75	35.57	11.11	10.06	101.15	1.04	0.30
Sand ($g\ kg^{-1}$)	6.00	695.00	390.95	166.36	27674.80	-0.31	-0.30
Silt ($g\ kg^{-1}$)	118.00	667.00	249.88	106.68	11381.26	1.69	4.66
Clay ($g\ kg^{-1}$)	170.00	699.00	359.22	121.44	14747.83	0.71	0.68
CEC ($cmol\ kg^{-1}$)	17.30	62.90	33.59	10.27	105.41	0.88	0.69

P-Fractions mg kg^{-1}	Min	Max	Mean	Std. Deviation	Variance	Skewness		Kurtosis	
						Statistic	Std. Error	Statistic	Std. Error
$NaHCO_3$-Pt	5.17	117.21	24.16	26.79	717.44	2.46	0.37	5.14	0.72
$NaHCO_3$-Pi	3.91	96.68	20.69	23.79	566.19	2.40	0.37	4.76	0.72
$NaHCO_3$-Po	0.27	20.52	3.47	4.48	20.04	2.59	0.37	7.33	0.72
NaOH-Pt	32.80	257.83	77.84	46.11	2126.37	2.42	0.37	6.54	0.72
NaOH-Pi	30.71	226.63	72.02	43.59	1899.90	2.31	0.37	5.48	0.72
NaOH-Po	0.55	31.20	5.82	8.31	69.02	2.13	0.37	3.64	0.72
CBD-P	65.25	312.60	132.34	50.52	2552.26	2.03	0.37	5.85	0.72
Ca-P	200.23	1385.27	597.05	277.51	77009.64	0.98	0.37	0.60	0.72
Res-P	99.52	490.49	259.54	111.31	12390.58	0.50	0.37	-0.84	0.72
Total-P	551.95	2621.16	1192.93	449.95	202450.53	1.62	0.37	3.28	0.72

Table 2. Descriptive statistics in the absence of transformation to phosphorus fractions (N: 43).

but which can be oxidized, a relatively low skewness factor in the data set.

The averages of determined P fractions (Table 2, Fig 1) were: $NaHCO_3$-Pt 24.16 mg kg^{-1} ; $NaHCO_3$-Pi 20.69 mg kg^{-1} ; $NaHCO_3$-Po 3.47 mg kg^{-1} ; NaOH-Pt 77.84 mg kg^{-1} ; NaOH-Pi 72,02 mg kg^{-1} ; NaOH-Po 5.82 mg kg^{-1} ; CBD-P 132.34 mg kg^{-1} ; Ca-P 597.05 mg kg^{-1} ; Res-P 259.54 mg kg^{-1} . Ca-P Fraction, low skewness coefficient 0.98 and kurtosis coefficient 0.60. This means that with continuous fertilization, this fraction generally increases (can be observed from the concentration values) but it may lead to a reduction in the proportion of this fraction in areas where composting is relatively uncommon or underdeveloped.

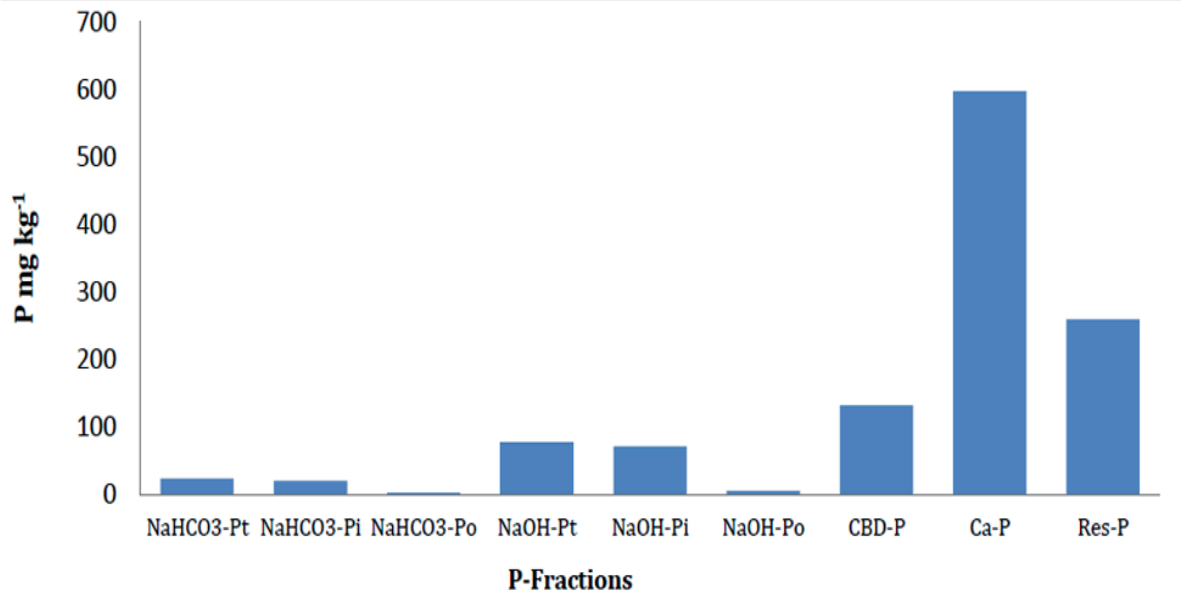
Relationships between Phosphorus Fractions and Soil Properties

Some soil Physico-chemical properties, Pearson correlation coefficients of P fractions given in Tables 3 and 4. The positive correlation between available phosphorus fractions of the soils NaHCO3-Pi fraction and OM (r = 0.581**), EC (r = 0.476**), sand (r = 0.329*), NaHCO3-Pt (r = 0.993**), NaOH-Pt (r = 0.358*), NaOH-Pi (r = 0.397*). The fertilization program can explain positive correlation. The decrease in the content of clay increases the amount of available phosphorus, which can be considered to be related to adsorption mechanisms. . Maranguit *et al.*[14] found High positive correlation was observed between the available P fractions (H_2O-Pi, $NaHCO_3$-Pi and Po) and the total organic P amount and carbon content.

$NaHCO_3$-Po was found to be positively correlated with OM (r =0. 590**). Positive correlation indicates that the increase in organic matter in the soil may be the result of increased fertilization of the soil, which is usually related to the presence of perennial plants, especially fruit trees in the field. Actually, Alovisi *et al.*[2] have shown that inorganic P fertilizers significantly increase moderate and labile P fractions. On the other hand, available phosphorus has reported that the most contribution is from the organic P fractions to the $NaHCO_3$-Po fraction.

NaOH-Pi Fraction positively correlated with OM (r = 0.609**), NaHCO3-Pt (r = 0.361*), NaHCO3-Pi (r = 0.397*), NaOH–Pt (r = 0.968**), CBD-P (r = 0.421*) and Total-P (r = 0.563**). Positive relationship is the function of the adsorption mechanisms, as previously mentioned, with the surface bound to this fraction (usually bound to Fe and Al, OH surfaces) and the decrease in clay quantity. Uygur *et al.*[22] found significant relationships between Fe and Al and NaOH-Pi that could be extrapolated by CBD. In this respect, surfaces of oxide minerals are an important component in the adsorption of mineral P[8, 23, 25].

Figure 1. The averages of phosphorus fractions (N: 43).



P Fractions	OM	Lime	pH	EC	Sand	silt	Clay	CEC
NaHCO₃-Pt	0.615**	0.155	-0.075	0.456**	0.309*	-0.157	-0.285	-0.064
NaHCO₃-Pi	0.581**	0.129	-0.065	0.476**	0.329*	-0.177	-0.297	-0.067
NaHCO₃-Po	0.590**	0.243	-0.107	0.200	0.095	-0.001	-0.129	-0.028
NaOH-Pt	0.615**	0.109	-0.224	0.221	0.286	-0.173	-0.241	-0.066
NaOH-Pi	0.609**	0.129	-0.189	0.252	0.277	-0.143	-0.254	-0.046
NaOH-Po	0.217	-0.074	-0.253	-0.100	0.139	-0.209	-0.007	-0.124
CBD-P	0.574**	0.108	-0.120	0.138	0.126	-0.044	-0.134	-0.030
Ca-P	0.497**	0.335*	0.263	0.320*	0.041	0.086	-0.133	0.116
Res-P	0.220	-0.112	-0.086	-0.006	-0.134	0.085	0.108	0.392*
Total-P	0.625**	0.232	0.073	0.311*	0.102	0.015	-0.154	0.144

** Correlation is important at 0.01 level (2-way)
* Correlation is important at 0.05 level (2-way)

Table 3. Pearson correlation between phosphorus fractions and some soil properties matrix (N: 43).

P Fractions	NaHCO₃-P (mg kg⁻¹)			NaOH-P (mg kg⁻¹)			CBD-P	Ca-P	Res-P	Total-P
	Pt	Pi	Po	Pt	Pi	Po	(mg kg⁻¹)			
NaHCO₃-Pt		0.993**	0.12	0.33	0.361*	0.01	0.22	0.09	0.02	0.31
NaHCO₃-Pi	0.993**		0.00	0.358*	0.397*	-0.02	0.24	0.09	0.05	0.32
NaHCO₃-Po	0.12	0.00		-0.19	-0.28	0.23	-0.21	-0.04	-0.29	-0.14
NaOH-Pt	0.33	0.358*	-0.19		0.968**	0.446*	0.405*	0.31	-0.18	0.560**
NaOH-Pi	0.361*	0.397*	-0.28	0.968**		0.21	0.421*	0.29	-0.06	0.563**
NaOH-Po	0.01	-0.02	0.23	0.446*	0.21		0.08	0.16	-0.490**	0.18
CBD-P	0.22	0.24	-0.21	0.405*	0.421*	0.08		-0.04	0.01	0.26
Ca-P	0.09	0.09	-0.04	0.31	0.29	0.16	-0.04		0.26	0.920**
Res-P	0.02	0.05	-0.29	-0.18	-0.06	-0.490**	0.01	0.26		0.32
Total-P	0.31	0.32	-0.14	0.560**	0.563**	0.18	0.26	0.920**	0.32	

** Correlation is important at 0.01 level (2-way)
* Correlation is important at 0.05 level (2-way)

Table 4. Pearson correlation matrix between phosphorus fractions (N: 43).

NaOH-Po Fraction positively correlated with NaOH-Pt (r = 0.446*). Positive relationship can be explained mainly for different reasons: i) Because NaOH is alkaline, it can extract organic matter in considerable quantities, ii) Fractions of organic matter or soil organic matter, such as humic acid and fulvic acid, are in competition with phosphate ions for the adsorption surfaces of the soils. Yi-Chao *et al.*[26] showed that $NaHCO_3$ and organic P extractable with extractable inorganic P and NaOH are significantly associated with miner P fertilization.

CBD-P Fraction positively correlated with OM(r = 0.574**), NaOH–Pi (r = 0.421*). Positive relationship can be explained by fertilization practices in general. The more fertilizer applied to any soil, the higher the availability of fractions (CBD-P, Ca-P and Total-P) in the fractions with high availability in the short run (fractions extractable with $NaHCO_3$ and NaOH) and in the long run at low fractions for buffering the high quantity fractions.

Ca-P fraction in soils was correlated positively with OM (r = 0.497**), Lime (r = 0.335*), EC (r = 0.320*) andTotal-P (r = 0.920**). The relationship between Ca-P and the extractable phosphorus fractions OM can be explained by the plant cover-induced fertilization practices, positive correlations with other properties can be considered because of phosphorous fertilization applied in the region. Actually, negative correlations were found between Ca-P fraction and clay properties in this study. It is thought that the soil components may cause clay adsorption and / or precipitation reactions instead of the Ca-P fraction of P added to the soil when the soil components are high. Patiram *et al.*[17] found that the different forms of P in acidic soils did not change significantly in the fractions of Al-P and Ca-P. Tandon[20], found Ca-P in the Indian soil represents about 40-50% of total P in calcareous and neutral soils.

Res-P fraction in soils was correlated positively with CEC (r = 0.392*). The relationship with the CEC points to an increase in the ability to retain P in soil formation processes due to high clay minerals. Shukla *et al.*[19] and Uygur et al. 21 found

that the residual fraction in the calcareous soils was in relatively high quantities.

Conclusions

Descriptive analyzes have shown that although some similarities exist in the soil, some soils show distinct differences. The averages of determined P fractions were: $NaHCO_3$-Pt 24.16 mg kg^{-1}; $NaHCO_3$-Pi 20.69 mg kg^{-1} ; $NaHCO_3$-Po 3.47 mg kg^{-1} ; NaOH-Pt 77.84 mg kg^{-1} ; NaOH-Pi 72,02 mg kg^{-1} ; NaOH-Po 5.82 mg kg^{-1} ; CBD-P 132.34 mg kg^{-1} ; Ca-P 597.05 mg kg^{-1} ; Res-P 259.54 mg kg^{-1} .

It can be concluded that the division of phosphate fertilizers between the geochemical fractions of phosphorus is largely related to the organic matter, the calcium carbonate equivalent and the soil texture content in the soil.

In order to increase the use of phosphorus in the soil structure, which show different behaviors according to the changing soil properties, plant fertilization and reduce P contamination, it is necessary to determine the form and amount of fertilization system according to soil properties carefully.

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RESEARCHS / INVESTIGACIÓN

Evaluación de los reguladores de crecimiento (Kinetina y Ácido giberélico) para acelerar la germinación de Gynoxys verrucosa.

Evaluation of growth regulators (Kinetin and gibberellic acid) to accelerate the germination of Gynoxys verrucosa.

J S Cueva & H P Lucero M.

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Resumen: Las especies de bosque nativas de los altos Andes son esenciales para el funcionamiento del ecosistema, un ejemplo es la maleza *Gynoxys verrucosa*, que también es reconocida por sus propiedades para el tratamiento del cáncer. La presente investigación apunta a contribuir al vacío que existe con respecto a su germinación y para ello se emplea técnicas de cultivos *in vitro*. La metodología involucrada en una selección previa de semillas que se sometieron al método de desinfección estándar, aplicando posteriormente reguladores de crecimiento (100 ppm) a diferentes tiempos de exposición. (12-06-24 h), se sembraron posteriormente en viales con cultivo Murashige y Skoog (1962) en cuanto a los resultados se evaluó el efecto de la adición de reguladores del crecimiento de las plantas (kinetina y ácido giberélico) sobre la respuesta de germinación a 15 días se observó una tasa de germinación del 60% con Kinetin y en 17 la tasa de germinación fue del 40% con giberelinas. Los datos se evaluaron utilizando un análisis de varianza (Anova) en bloques aleatorios.

Palabras clave: *Gynoxys verrucosa*, micropropagación, cinetina, ácido giberélico, germinación.

Abstract: Native forest species of the high Andes, are essential to the functioning of the ecosystem, an example is *Gynoxys verrucosa* Weed, which is also recognized by its properties for the treatment of cancer the present research aims to contribute to the vacuum that exists with regard to its germination and for this is employed technical of *in vitro* crops. The methodology involved in a previous selection of seeds, these were subjected to the standard method of disinfection (70% alcohol for 1 min, 20% hypochlorite for 5 min and 2% of Benomil) subsequently applied growth regulators (100ppm) at different exposure times (12-06- 24 h), they were subsequently planted in vials with culture Murashige and Skoog (1962) in terms of the results was evaluated the effect of the addition of regulators of plant growth (kinetin and gibberellic acid) on the germination response to 15 days was observed 60% germination rate with Kinetin and at 17 was 40% germination rate with Gibberellins. The data were evaluated using an analysis of variance (Anova) in random blocks..

Key words: *Gynoxys verrucosa*, micropropagation, kinetin, acid gibberellic, germination.

Introducción

Las especies arbustivas nativas de la zona alto andina, son de mucha importancia dentro de las comunidades rurales[1], debido a que varias especies vegetales presentan ciertas propiedades que han ayudado al tratamiento de innumerables enfermedades[2]. La alta diversidad de plantas que tiene nuestro país, ha contribuido a que las distintas comunidades que han habitado y habitan en esta región, hayan adquirido una gran profundidad de conocimientos sobre las plantas que crecen en su entorno, fundamentalmente en términos de uso y su ecología[3].En las provincias de Loja y Zamora Chinchipe las familias más utilizadas para la preparación de remedios caseros son: *Asteraceae, Laminaceae, Solanaceae, Fabaceae, Onagraceae* y *Apiaceae*[4].

Gynoxys verrucosa Wedd, quien pertenece a la familia de las Asteraceaes, comúnmente conocida como guangalo[5, 6], es utilizada tradicionalmente en las comunidades indígenas del sur para el tratamiento de infecciones de la piel y la cicatrización de heridas por aplicación directa de las hojas en la piel[4]. Resientes investigaciones fitoquímicas del extracto de las hojas mostraron resultados prometedores por su elevada capacidad de luchar contra células tumorales leucémicas; convirtiéndose con esto *G. verrucosa* Wedd en una fuente de metabolitos secundarios con potencial antineoplásico[2, 5, 6].

Sin embargo, normalmente en los estudios con especies medicinales se observa que no se aplican principios de biología reproductiva y mucho menos en cuanto su germinación[8]. Esto se debe a que los métodos convencionales representan un problema al momento de propagar especies forestales[7, 8]. La biotecnología aporta con métodos para optimizar el tiempo y el espacio; dentro de ellas, los cultivos *in vitro* son utilizados para propagar plantas en grandes cantidades. Es por ello que la micropropagación es una técnica de cultivos *in vitro* que hace posible alcanzar este objetivo[7].

En la actualidad es muy escasa la información de *G. verrucosa* Wedd, pero ciertas características como el florecimiento y la maduración de los frutos que es de aproximadamente de dos meses; además presenta una capacidad de germinación baja[9]. Nos llevó a considerar algunas investigaciones que resaltan la necesidad de aplicar métodos químicos para incrementar la respuesta germinativa de las semillas; ya que la germinación de semillas y el crecimiento de plántulas pueden ser controladas por aplicación exógena de reguladores de crecimiento vegetal, ciertas concentraciones fisiológicas podrían actuar como promotoras o inhibidoras de ambos procesos[9].

[1] Proyecto "Distribución geográfica, asociaciones micorrícicas, propagación y diversidad genética y química de especies vegetales de interés medicinal en la región Sur del Ecuador", Universidad Técnica Particular de Loja, Área Biologíca, Departamentos de Ciencias Naturales y de Química, San Cayetano Alto, Calle Paris, Loja, Ecuador

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Dentro de los reguladores del crecimiento más estudiados en lo que se refiere a germinación, se encuentran el ácido giberélico (AG3); pero en la actualidad se han evaluado otras hormonas como las kinetinas, las cuales se han empleado para estimular la germinación de semillas de diferentes especies forestales, observando resultados prometedores[1]. En consecuencia, la presente investigación se planteó como objetivo general "Evaluar reguladores de crecimiento vegetal en la disminución del tiempo hasta la germinación de *G. verrucosa* Wedd" y como objetivos específicos: Evaluar el efecto de la kinetina y el AG3 en la aceleración de la germinación de *G. verrucosa* Wedd y obtener individuos sanos a partir de la germinación *in vitro*.

Materiales y métodos

Área de estudio

El desarrollo de la presente investigación se llevó a cabo en el laboratorio de fisiología vegetal del Departamento de Ciencias Naturales de la Universidad Técnica Particular de Loja.

Material vegetal

El material vegetal (semillas) se obtuvo de diferentes poblaciones en la provincia de Loja (Yangana, Villonaco, Celica). El material vegetal que se colectó, consistía en frutos maduros próximos a la dehiscencia.

Desinfección de las semillas

Con las semillas colectadas se realiza un proceso previo a la desinfección, el cual consiste en la separación de las semillas vanas y las semillas buenas (semillas secas de semillas "llenas" respectivamente); estas fueron colocadas en cajas Petri y se mantuvieron a temperatura ambiente, para luego ser desinfectadas; para lo cual se fusionó un protocolo estándar modificándolo en cuanto a sus concentraciones y el proceso consiste en: agitación por 1 min con alcohol al 70% (Las semillas son colocadas en un tubo falcón de 50mL), seguido de enjuague con agua destilada estéril; lavado en hipoclorito de sodio comercial diluido al 20% con una gota de jabón líquido por 5 minutos y un lavado con agua destilada estéril; para finalizar la desinfección y como escarificante se agrega agua oxigenada al 10% de igual manera se agita por 5min y se procede a enjuagar con agua estéril 10-11.

Tratamiento de las semillas

En cuanto al tratamiento de las semillas se consideraron diferentes concentraciones de reguladores de crecimiento, como: kinetina (Kin) (citoquininas) (100-200-300 ppm) y ácido giberélico (AG3) (giberelinas) (100-200-300 ppm). A los cuales una vez cumplido el proceso de desinfección, se le agrega tratamiento por tiempos establecidos de 6, 12 y 24 horas por cada tratamiento, al efectuar el tiempo establecido retiramos el tratamiento y se prepara 2g de Benomil en 100ml de agua destilada, el cual se agrega en cada tubo falcón que cumplió su tiempo de exposición[10, 11].

Siembra

El medio de cultivo *in vitro* utilizado es el Murashigue & Skoog, 1962 (MS), el cual se prepara sin suplementos. La siembra se realiza en condiciones asépticas, en una cámara de flujo laminar, usamos una micropipeta de 5ml, debido a que las semillas son pequeñas para la siembra; se colocarán 10 semillas en cada frasco con medio de cultivo MS, así también haremos uso de pinzas esterilizadas para fijar las semillas en el medio. Finalmente, los frascos serán correctamente etiquetados y colocamos en el cuarto de crecimiento hasta su germinación.

Diseño experimental

El diseño experimental fue de bloques completos al azar. Como factores se consideró Reguladores de Crecimiento Vegetal (RCV), tiempo de exposición al tratamiento, proveniencia de la semilla. Los RCV fueron kinetina (KIN) y giberélina (AG3) Como se observa en la Tabla 1. Los tiempos de exposición fueron 6, 12 y 24 horas. Cada tratamiento consistió de 5 frascos. En cada frasco se sembraron 10 semillas.

Tratamiento	Tiempos de exposición (Horas)		
	6	12	24
KIN	T6-K	T12-K	T24-K
GIB	T6-G	T12-G	T24-G
CONTROL	C6	C12	C24

Tabla 1. Diseño experimental planteado para la evaluación de los tratamientos y sus periodos.

Análisis estadístico

A cada frasco que germinó se contará el numeró de semillas germinadas, para luego evaluar si existe un efecto positivo de los reguladores de crecimiento (KIN y AG3) en la germinación; para los individuos sanos (individuos que sobrevivieron después de la germinación) se le coloca a cada frasco el valor de 1. Para el análisis de cada uno de los datos se evaluó con una prueba estadística de ANOVA, la prueba de Brown-forsythe y la prueba de Barteltt en el programa estadístico (Prism versión 6.2) que nos permitió estimar el mejor regulador de crecimiento y en qué tiempo de exposición.

Resultados

Para responder nuestro primer objetivo, el manejo de las semillas sometidas a tratamientos con reguladores de crecimiento la tabla 2, nos da un resumen de los resultados obtenidos de un total de 392 frascos sembrados *in vitro*, de los cuales 112 frascos han dado respuesta de germinación. El regulador de crecimiento que mayor respuesta ha tenido es Kinetina presentando un total de 185 plántulas germinadas, en 15 días; Así también el Ácido Giberélico del mismo modo presenta resultados prometedores, a partir de 17 días después de la siembra se observa germinación en un total de 100 plántulas.

Normalmente se ha observado que los frascos control o blanco mostraron baja germinación; los pocos frascos que germinaron fue en 25 días. La figura 1, muestra el comportamiento que se dio con la aplicación de reguladores de crecimiento; la pruebas de ANOVA, la prueba de Brown-forsythe y la prueba de Barteltt, basadas en las medias y la varianza de nuestros datos nos arrojan un $P < 0.05$, con lo cual podemos definir qué existe diferencias significativas, ya sea en los tratamientos frente a sus controles; y en cuanto al mejor tratamiento se observa que es KIN de 24H con un total de 84 plántulas germinadas, el más bajo 12h KIN.

Al evaluar una mayor concentración de reguladores, en la figura 2, se observa la disminución del patrón de germinación, a pesar que existe diferencias significativas, se determinó que el porcentaje de germinación es bajo con el aumento de la concentración de RCV, las KIN que anteriormente en concen-

TRATMIENTO	TIEMPOS EXPOSICIÓN	FRASCOS GERMINADOS	N° SEMILLAS GERMINADAS POR FRASCO	N° FRASCOS CONTAMINADOS
KIN	24H	29	84	15
KIN	12H	11	32	0
KIN	6H	29	69	0
AG_3	24H	18	41	5
AG_3	12H	13	33	0
AG_3	6H	11	26	0

Tabla 2. Síntesis de resultados de germinación.

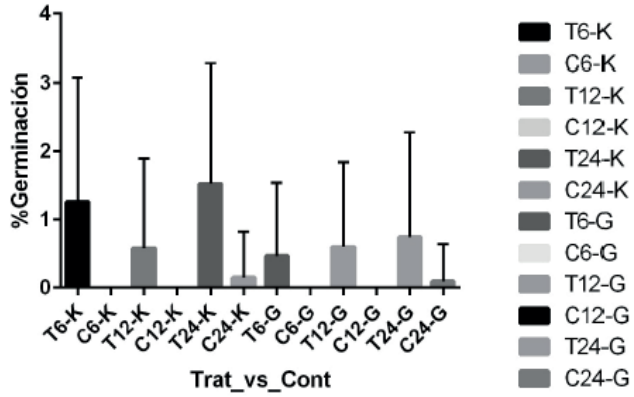

Figura 1. Comportamiento de los tratamientos frente a sus controles.

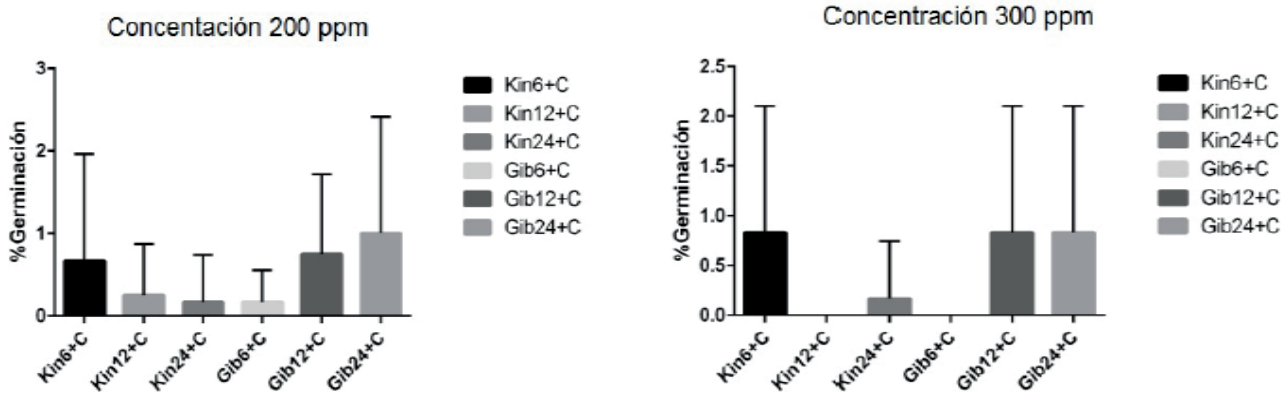

Figura 2. Respuesta de germinación con aumento de concentración de 200 y 300 ppm.

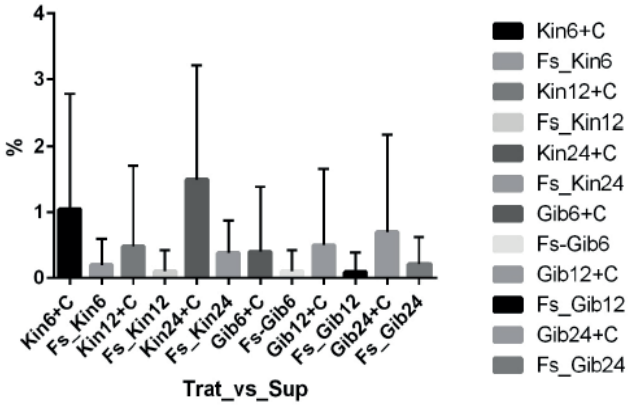

Figura 3. Evaluación de la supervivencia (Tratamientos frente a la supervivencia).

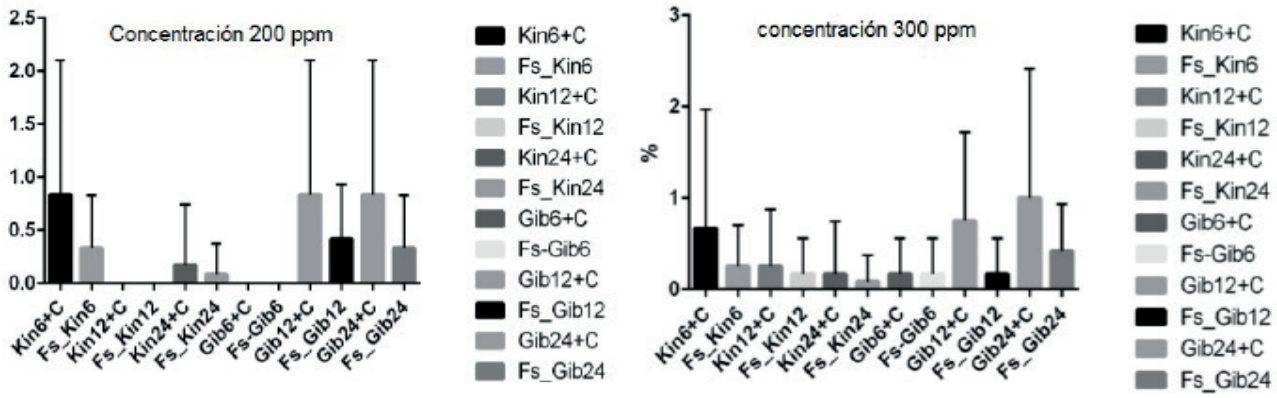

Figura 4. Tratamientos frente a la supervivencia en concentración de 200 y 300 ppm.

Figura 5. Formación de callos, el primero con KIN 24h y el segundo de AG_3 24h.

traciones de 100 ppm fueron positivas para dar respuesta a la germinación, en este caso al aumentar la concentración hay tratamientos que no estimulan la germinación, sin embargo esto no sucede con las AG3, que se observa respuesta germinativa con 200 y 300 ppm.

Habitualmente se observó que las plántulas crecen en el primer mes después de la germinación sin ningún problema, sin embargo, después de 30 días, algunas plántulas sufren necrosamiento en sus tejidos mientras que otras se contaminan. Es por esta razón en el presente estudio para dar respuesta al segundo objetivo, se evalúalo a cada individuo que sobrevivió.

Por lo tanto lo que se evaluó, fue la comparación de los datos de germinación que se obtuvo por tiempo y tratamiento frente a los que lograron sobrevivir; en la figura 4, se muestra la reducción de la supervivencia (Fs_Kin/Gib), lo que indica que la supervivencia no está relacionada con los tratamientos; como se indicó anteriormente el mejor tratamiento fue KIN, sin embargo no fue tan exitosa en cuanto a la supervivencia, todo lo contario a lo que sucede con AG3, gran parte de los individuos que germinaron si logran sobrevivir; se apreció el mismo patrón en las concentraciones de 200 y 300 ppm, figura 4 en donde se corrobora que AG_3 responde mejor a la supervivencia.

Como una respuesta positiva de los individuos que sobrevivieron, tomando en cuenta que no fue parte del cumplimiento de los objetivos; en la figura 5, se observa la obtención de callos a partir de las semillas que lograron sobrevivir, en cada uno de los tratamientos en sus diferentes tiempos de exposición, se consiguió formar callos, sin embargo cabe mencionar que son más exitosos los que estuvieron expuestos con los tratamientos de AG_3.

Discusión

Los resultados obtenidos nos han permitido determinar la efectividad de los reguladores de crecimiento, en cuanto a la aceleración de la en la germinación, como se observa en la Tabla 2, el porcentaje de germinación por frasco en cada tratamiento es bajo, relativamente hablando ya que en la naturaleza la estrategia de producir muchas semillas, estrategias "K" y "R" representan porcentajes de germinación menores a 10%[12]. Tomando en cuenta que el material vegetal con el que se trabajó fue obtenido directamente del campo, es decir que son variedades naturales no seleccionadas; la mayoría de estudios "*in vitro*" emplean semillas comerciales, es decir aquellas que han sufrido un tipo de selección.

Normalmente la familia *Asteraceae* presenta la característica de producir una gran cantidad de semillas con fines de lograr mayor supervivencia, pero no todas poseen reservas energéticas. Lo que provoca el deterioro de las semillas, por lo que la calidad de la misma se ve afectada por daños mecánicos, humedad de la semilla, patógenos y muchos otros factores que afectan su recuperación[13].

Por lo tanto, la metodología con la que se trabajó en el presente estudio fue un aspecto muy importante, ya que está directamente relacionada con la latencia de la semilla, de igual manera los tratamientos que se llevaron a cabo con el

fin de acelerar el proceso de germinación o promover el establecimiento de plántulas. Se considera que varias hormonas y compuestos nitrogenados pueden ayudar a romper la dormancia bajo ciertas condiciones, y pueden tener al mismo tiempo un impacto directo sobre la germinación[11].

La pérdida de la capacidad de germinación podría estar relacionada con los cambios en el balance endógeno de los reguladores del crecimiento vegetal en la semilla, por lo que se recomienda de un suministro de éstas para estimular la germinación; se han reportado algunos estudios con reguladores de crecimiento, entre las que se destacan se encuentra las Giberelinas (AG_3), pero en la actualidad se han visto resultados prometedores con Citoquininas (Kimetina)[10].

Las primeras investigaciones *In vitro* con *G. verrucosa* fueron a partir de estacas para la obtención de brotes, en el cual se observó una respuesta de 68% en un periodo de 60 días, con alto riesgo de contaminación[14]. Pero para evitar esto se trabajó con semillas, las cuales se obtuvo respuesta en 15 y 17 días con la utilización de Reguladores de crecimiento vegetal (RCV).

Las diferencias mostradas en la Figura 2, destacan que KIN respondió favorablemente en el tiempo de germinación, según algunos estudios en el que aplicaron ensayos con kinetinas (KIN) observaron que dichas hormonas presentan mejor respuesta al estrés biótico[15]; esto se debe a que éstas promueven respuesta de defensas al momento de germinar, lo que favores a la activación de las semilla. Comprobando con esto que las KIN ejercen su acción principalmente en las semillas que están bajo condiciones de estrés[16].

No obstante, el efecto de AG_3 también respondió de una manera positiva, del cual se obtuvo resultados en 17 días, y de igual manera son efectivas en semillas que sufren condiciones de estrés o latencia. El problema está en la concentración que se trabaje, debido a que aún no hay estudios que definan una concentración adecuada, para obtener resultados más prometedores. Se considera que el efecto de las Giberelinas y las Kinetinas no es muy claro y en algunos casos es contradictorio, esto se debe a las concentraciones con las que se trabaje; por lo que es necesario investigar función de RCV para *Asteraceaes*[17].

En cuanto a la respuesta germinativa se podría decir que ha sido efectiva, en un porcentaje de 45% de todos los ensayos realizados; normalmente por frasco germinan de 2 a 3 semillas. Lo máximo que se ha observado ha sido de 6 semillas, pero no es tan común, en consecuencia se consideró realizar ensayos con una mayor concentración entre 200 y 300 ppm, con el fin de aumentar el número de semillas germinadas. Sin embargo, se observa que con la adición de RCV no incrementa de manera significativa la germinación de semillas (Figura 3); se piensa que con el aumento de las concentraciones de RCV se inhiben procesos internos de las semillas, con lo cual se afecta a la germinación[18].

Consecuentemente al evaluar la supervivencia se observa que se da un proceso antagónico con las KIN, lo que no sucede con AG3. Se considera que esto es por el medio (MS) que se utilizó; pues, la permanencia de los diferentes minerales que componen la fórmula del MS tiene una duración diferente entre sí. Es así que las concentraciones de amonio: nitrato pueden volatilizarse en un tiempo relativamente corto; mientras que los compuestos a base de Potasio y fósforo tendrían un mayor tiempo de permanencia. Por lo tanto, se especula que esa sea la razón para las respuestas percibidas en los cultivos[19].

Se señala para obtener una mayor viabilidad en la supervivencia de las semillas, hay que considerar evaluar factores como la temperatura, luz humedad. Debido a que son estos los activan o desactivan a los RCV. Ciertos estudios mencionan que se observa mayor supervivencia en condiciones alternando luz y oscuridad, dichas condiciones permiten la activación del metabolismo de las AG3, lo que favorece a la supervivencia a las plantas que fueron inducidas con este RCV[20].

Por otro lado, podemos definir que tanto las condiciones manejadas in vitro como la humedad de los frascos en los que se sembraron, el periodo de luz al que estuvieron sometidas las semillas, y los RCV aplicados de manera externa. Representaron ser las adecuadas como se observa en la figura 6, el estímulo brindado por dichas condiciones, son significativas para lograr un proceso de desdiferenciación para la obtención de callos.

A pesar que en la mayoría de estudios el suministro de hormonas se realiza directamente al medio de cultivo. En el presente estudio se lo desarrollo con un enfoque diferente (acelera la germinación) obteniendo resultados hasta este punto. Por consiguiente, queda la oferta abierta, para el desarrollo de las etapas faltantes, debido al éxito de esta investigación.

Conclusiones

Aparentemente se podía considerar que fue un porcentaje bajo de germinación; teniendo presente que las semillas fueron seleccionas directamente de sus sitios naturales. Donde *G. verrucosa* Weed posee la característica de producir un gran número de semillas; pero esto no garantiza un éxito de germinación. A lo que, si podemos atribuir la respuesta de germinación, es al manejo adecuado del protocolo de desinfección ya que nos permitió una escarificación correcta de la semilla. Así también la aplicación de los RCV, a pesar que aún no existe una concentración adecuada para trabajar con especies arbustivas. Tomando en cuenta los resultados que se obtuvieron en cuanto a la aplicación se recomienda trabajar con una concentración menor a 100 ppm; ya que como se observó al momento de aumentar la concentración en algunos casos se inhibía la germinación.

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REVIEW / ARTÍCULO DE REVISIÓN

Benefits in Latin America and the Caribbean about production of Cavendish AAA banana resistant to black Sigatoka.

Beneficios para América Latina y el Caribe de la producción de banano Cavendish AAA resistente a la Sigatoka negra.

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Abstract: The production of banana *Cavendish AAA* in Latin America and the Caribbean (LAC) constitutes a major source of income in GDP (gross domestic product) of each country. *The Muse spp. AAA* Group *Cavendish* is exposed to multiple pests and foliar diseases; whose control increases the economic value for crop maintenance. The black Sigatoka caused by the fungus *Mycosphaerella fijiensis* is one of the most representative conditions. In LAC the conventional method of treatment against black Sigatoka is the use of fungicides, which affect human health and the environment. For this reason, an efficient alternative to increase the resistance of the plants to the black Sigatoka, is the use of techniques of molecular biology that allows the creation of Genetically Modified Organisms (GMO). This paper reviews the identification of genes in the Musa Grain Nain and Williams to increase resistance to the fungus *Mycosphaerella fijiensis*. It also details the alternative techniques for modifying banana bulbs as the use of CRISPR/Cas9 for gene modification would be a powerful tool to achieve this goal because it shows successful results in the treatment of Phytoene desaturate (PDS) that causes albinism and dwarfism in plants. On the other hand, it is also analyzed the possible introduction of GMO bulbs in the main banana exporting country of the world, Ecuador, describing the possible competitive advantages that the country would obtain against the international market.

Key words: Genetically Modified Organism (GMO), *Musa spp. Cavendish AAA*, *Mycosphaerella fijiensis*, pests and diseases.

Resumen: La producción de bananos *Cavendish AAA* en América Latina y el Caribe (ALC) es una fuente importante de ingresos en el PIB (producto interno bruto) de cada país. La *musa spp.* Grupo *AAA Cavendish* está expuesto a múltiples plagas y enfermedades foliares, cuyo control aumenta el valor económico para el mantenimiento de los cultivos. La Sigatoka negra causada por el hongo *Mycosphaerella fijiensis* es una de las condiciones más representativas. En ALC, el método convencional de tratamiento contra la Sigatoka negra es el uso de fungicidas, que afectan la salud humana y el medio ambiente. Por esta razón, una alternativa eficiente para aumentar la resistencia de las plantas a la Sigatoka negra es el uso de técnicas de biología molecular que permiten la creación de organismos genéticamente modificados (OGM). Este artículo revisa la identificación de genes en Musa Grain Nain y Williams para aumentar la resistencia al hongo *Mycosphaerella fijiensis*. También detalla técnicas alternativas para modificar los bulbos de banano, ya que el uso de CRISPR / Cas9 para la modificación genética sería una herramienta poderosa para lograr este objetivo, debido a que muestra resultados exitosos en el tratamiento del desatato de fitoeno (PDS) que causa albinismo y enanismo en las plantas Por otro lado, también se analiza la posible introducción de bulbos de OGM en el principal país exportador de banano del mundo, Ecuador, que describe las posibles ventajas competitivas que el país obtendría contra el mercado internacional.

Palabras clave: Organismos Genéticamente Modificados (OGM), *Musa spp. Cavendish AAA, Mycosphaerella fijiensis*, pests and diseases.

Introduction

Bananas are one of the most important crops in the world, only in 2015 world banana exports reached 18.6 million tons[1]. The majority of exports from Central and South America are directed at the North American markets, Western Europe, Japan and Russia[2]. There are a lot of varieties of banana, however the species Banana *Canvendish AAA*, of the Zingiberales Order, family *Musaceae* and genus *Musa*[3], It is the most commercialized and consumed tropical fruit around the world[4]. The annual production of Banana Cavendish globally is 50 billion tons and the largest producers are India and the Philippines, which produce around 60 tons per hectare[5]. There are some varieties of this banana species, but the most relevant clones are: *Dwarf Cavendish, Grande Naine*, *Lacantan* and *Williams*[6].

During the process of obtaining the banana the plant can be affected by pests, the most relevant condition is Black Sigatoka, a foliar disease of *Musaceae*, caused by the fungus *Mycosphaerella fijiensis*[7]. The disease reduces the photosynthetic efficiency of the plant, because the pathogen destroys the leaf area by the action of the phytotoxin, whose substance prevents the passage of electrons in the chloroplast membrane, causing foliar tissue necrosis[8]. The direct consequence is the reduction of the size and weight of the bunch, which represents a reduction in crop production. When the fungus is in favorable conditions and there is no chemical control of the pest, the disease can reduce the obtaining of bananas 35% to 50% in crops[9]. Currently the control of this pest is carried out only with the use of fungicides, which represent high costs during the production process. Therefore, the best way to reduce

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production costs would be the use of molecular biology techniques to generate resistance against this disease.

Identification of resistant genes to the black Sigatoka in banana Musa Cavendish AAA

The genes that generate resistance to black Sigatoka in the main banana varieties cultivated for export, known as 'Grande Naine' and 'Williams', belong to the monospecific triploid bananas (AAA) of the Cavendish sub-group have been identified through multiple investigations which are detailed below.

Cavendish (Musa spp. AAA group) cv. Grand Nain

Inedible cultivars, resistance to diseases is necessary to maintain the quality of the final product[10]. In banana Cavendish Grand Nain[11] the resistance to Black Sigatoka develops through the expression of the ThEn-42 endocytinase gene, obtained from Trichoderma harzianum (fungus that is also used as a fungicide) together with the grape stilbene synthase (StSy). This gene manipulated in transgenic banana plants requires the control of the 35S promoter and the PR-10 inducible promoter, respectively. In addition, it is necessary to add the gene of superoxide dismutase Cu (antioxidant defense), Zn-SOD of tomato, under the control of the ubiquitin promoter to improve the elimination of free radicals generated during the fungal attack[11, 12].

Cavendish (Musa spp. AAA group) cv. Williams

In banana Cavendish William it has been identified that the gene of chitinase is responsible for resistance to *Mycosphaerella fijiensises*[13]. To determine the research, it was carried out in a subtractive cDNA library after the inoculation of conidia in greenhouse conditions[14]. The vectors containing the promoters for the expression of the gene in banana are inserted in front of the reporter gene β-glucuronidase (uidAINT, Gus) of the plasmid Pcambia1391Z (pESKUL1 and pESKUL7)[15]. So for its expression, pESKUL1 contains the promoter 12-1, while pESKUL7 contains the promoter 85-1[16].

Methods used for genetic transformation

Transformation of Musa cv. Grain Nain mediated by Agrobacterium tumefaciens

The embryogenic cultures can be transformed through the Agrobacterium tumefaciens whose result is the production of non-chimeric stable plants. Recent research indicates that a greater combination of antifungal genes gives greater protection to black Sigatoka[17]. Particularly, the combination of the endocytinase gene (ThEn-42), the stilbene synthase (StSy) gene together with the Cu-Zn chloroplast and the superoxide dismutase (Cu, Zu-SOD) gene achieve this purpose.

The initiation of embryogenic callus was performed with immature male flowers[18] obtaining embryogenic tissue[11] where embryo maintenance and maturation was performed[19]. The regeneration of them was carried out in a basal MS medium supplemented. The regenerated mature plants were rooted in the same medium[11], then hardened and transferred to the greenhouse. Plasmid YC39 containing the ThEn-42 endocytinasase gene from Trichoderma Harzianum[20] was cloned between a 35S CaMV constitutive promoter, the AMV enhancer and the NOS terminator. The StSy gene was cloned under the control of the PR-10 promoter[11, 21]. While under the control of the ubiquitin promoter, the Cu gene, Zn-SOD from tomato was cloned[14]. The terminators were inserted into the binary vector pGA 429[11].

Genomic DNA was isolated from the leaves of banana plants grown *in vitro*[11]. A Southern blot analysis was performed to confirm the stable integration of the transgenes. The DNA fragments containing the nptII gene were amplified from the plasmid with the same sets of primers used for the PCR analysis, and used as a hybridization probe in the Southern blot membrane. Endocytinase activity was measured in leaf samples of transformed and wild-type plantain plants using the fluorescence bioassay of umbelliferil[20].

Use of CRISPR / Cas9 for genetic modification in Musa cv. William

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / Cas9 (CRISPR-associated protein9) genome editing technology has shown great promise for quickly addressing emerging challenges in agriculture[22]. Currently, CRISPR / Cas9 is used only to improve the characteristics of bananas, with the aim of increasing product quality in the market. However, CRISPR / Cas9 could be applied to enhance the mechanisms of resistance to foliar diseases of this important tropical crop[23]. For example, recent research has used the CRISPR / Cas9 gene editing system to deliver a self-resolving polycistronic guide RNA (GRNA) designed to target the gene for the enzyme phytoene desaturase (PDS), key in the carotenogenic pathway. PDS causes albinism and dwarfism by altering the biosynthesis of chlorophyll, carotenoids and gibberellins, in the *Cavendish* cultivar "Williams". In this way it is verified that the CRISPR / Cas9 is a tool that will allow the development of resistance to diseases[24].

Analysis of the production of the conventional and transgenic bulbs

Latin America and the Caribbean (LAC) is the largest banana export region, accounting for 66% of *Cavendish's* world exports[25]. There are seven banana species that are produced in LAC, of which 3 are *Cavendish*[26]. Therefore, the volume of production in LAC of the *Cavendish* type is 20 million tons[27]. The average price of the Cavendish banana has a progressive increase[28] due to the demand in the international markets. In the Caribbean, prices have increased considerably above the regional average, reaching more than US $ 1,000 per ton in Jamaica. However, countries like Peru show a value of US $100 per ton[28]. An influence for the price difference are the conditions of the banana as quality, size, etc. which underlie the development of the plants and therefore their exposure to pests.

Table 1. Dates of exportation, production and importation of *Musa spp.* around the world.

Dates of banana around the world in 2017					
Exportation[25,42]		Production[27,43]		Importation[44]	
21 million tons of exportable offer		113 million tons produced		22 million tons excluding the Dominican	
Country	%	Country	%	Country	%
Ecuador	31	India	25	United States	22
Costa Rica	12	China	12	Russia	7
Guatemala	12	Indonesia	6	Germany	7
Colombia	9	Brazil	6	Belgic	7
Belgium	6	Ecuador	6	United Kingdom	5
Netherlands	3	Philippines	3	China	5
Honduras	3	Angola	3	Japan	5
United States	3	Guatemala	3	Netherlands	4
Mexico	3	Tanzania	3	India	4
Ivory Coast	2	Rwanda	3	Francie	3
Others	16	Others	27	Others	31

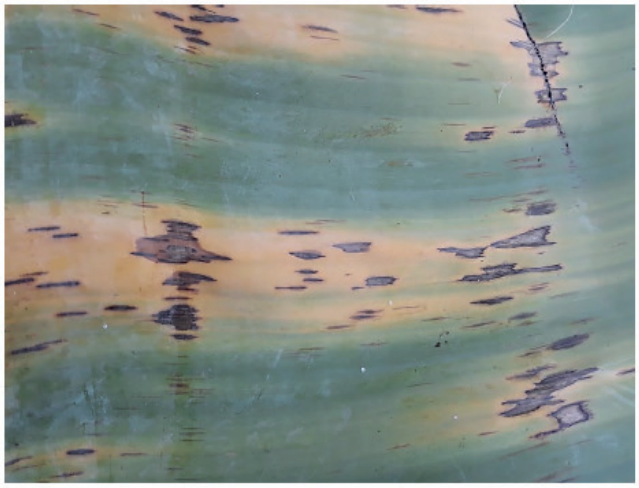

Figure 1. Close-up of a wild type leaf of the m*usa spp. Cavendish AAA*. Note the necrotic regions surrounded by the yellowing of the leaves, the signs of Black Sigatoka damage. Exemplary located in the San Marcos farm at 1km from the Mocache, Province of Los Ríos, Ecuador.

For this reason, important research centers such as the Honduran Foundation for Agricultural Research (FHIA) in Costa Rica and Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) in Brazil develop improved hybrids (FHIA-21, BLS-resistant plantain-like hybrid) with success[29, 30] in resistance to black sigakota. In order to reduce the use of fertilizers which generate high maintenance costs, and affect the properties of the soil. However, the large-scale adoption of these hybrids is not always guaranteed due to differences in taste and processing qualities (e.g. see[31]).

The use of resistant GMOs is recognized as one of the solutions to reinforce the potential of genomic studies of bananas and pathogens[32]. Although in LAC, it has not worked in particular, certain clones from the Philippines, Java, Malaysia, Bali, Papua, New Guinea and Solomon Islands with a genetic improvement program[34]. For example, genetic engineering in Uganda develops cultivars resistant to local pests and diseases, with improved agronomic attributes that are acceptable to consumers[33, 36]. This is a possible solution to the problem of paying more than US $ 200 per hectare per year in the maintenance of the crop. Uganda could generate potential annual benefits ranging from approximately US $ 179 million to US $ 365 million[35]. For this reason, the banana improvement program pursues a holistic approach, which combines cultivars for diseases and integrated pest management (IPM)[34]. This includes clean propagules, biological control and better crop management techniques for successful production[35].

Possible advantages of producing banana GMO Cavendish in Ecuador

In Ecuador there are approximately 166,972 hectares of bananas, located in the Littoral region, particularly in the provinces of Los Ríos, Guayas and El Oro[37]. The loss of banana production is 50% due to pests and foliar diseases, the most representative of which is Black Sigatoka[38]. In Ecuador, Black Sigatoka is controlled by aerial spraying[39]; however, climate variability has caused the disease to behave differently at the national level[37]. As a consequence, the most affected province is Los Ríos, with losses of up to 74% of production[37]. In this province, between 25 and 29 cycles of fumigation per hectare of plantation are applied annually; whose approximate cost is five hundred dollars per cycle[40]. In the control of the black Sigatoka, systemic fungicides such as strobilurins and triazoles are used that keep the pathogen sensitive[39]. However, the consequences of its use harm both human health, because they generate respiratory, endocrine, skin problems, etc., as well as the environment[40].

An alternative mechanism to control Black Sigatoka is the use of GMO[37]. However, article 401 of the Political Constitution of the Republic of Ecuador declares the country free of bulbs and transgenic crops, which would mean that GMOs are not circulating in Ecuador at present, and any exception to this article must be justified by the Presidency of the Republic of Ecuador[41]. Therefore, the application of techniques that genetically modify the banana plant are restricted.

Improving the production of the *Cavendish AAA* banana by conventional methods is difficult, due to the high sterility of the female gametes, the polyploidy that exists in most varieties, and the long periods of the growth cycle[40]. The production in Ecuador of a genetically modified Cavendish banana with resistance to Black Sigatoka, is the best alternative to avoid production losses[37] and the use of fungicides[40] that harm human health and the environment. Therefore, it is expected in the future, greater openness on the part of the Ecuadorian legislative bodies to establish regulatory frameworks, allowing the research and use of genetic engineering technology as a practical alternative for the improvement of crops in Ecuador.

Conclusions

The *Cavendish* banana is a product of relative importance in the economy of the LAC countries. Current methods of protection against black Sigatoka are functional but not efficient. For this reason, the use of molecular biology tools for the edition of genes resistant to pathogens would radically decrease the use of fungicides. In this way, the high costs of maintaining banana crops would be reduced, as well as contributing to the ecology. The application of GMOs in African countries has shown comparative competitive advantages with the hybrid bulbs used in LAC.

Cultures of *Musa spp. AAA Cavendish* cv. *Grand Nain* and *Williams* contain manipulable genes that through efficient methods increases the resistance to the fungus *Mycosphaerella fijiensis*. One of the most efficient techniques to generate resistance to black Sigatoka is the use of agrobacterium tumefaciens as a conventional method of genetic transformation. In particular, the use of recent innovative techniques such as CRISPR / Cas9 for gene modification would be a powerful tool to generate high resistance to diseases caused by fungi, maintaining banana quality and decreasing the high maintenance costs of LAC crops. In Ecuador monocultures predominate, especially banana; the area of greatest production is the littoral region, which is affected by foliar diseases, especially Black Sigatoka. To control this pest requires the indiscriminate use of agrotoxins,

which decrease the fertility of the soil and harm human health. If the Ecuadorian territory is promoted the research and development of technologies to genetically modify the *Musa spp. AAA Cavendish*, the high maintenance costs of these crops would be reduced, potentially increasing the competitive advantage of Ecuador in the international market.

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CASE REPORTS / REPORTE DE CASO

Cáncer hereditario de colon no polipósico asociado a adenocarcinoma de endometrio, piel actínica y consanguinidad. A propósito de un caso.

Hereditary non-polyposis colon cancer associated with endometrial adenocarcinoma, actinic skin and consanguinity. A case report.

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Resumen: El síndrome de Lynch es el cáncer colorrectal hereditario no asociado a poliposis y que se relaciona con los tumores malignos no colorrectales. Se han descrito como sitios extrarrectales los tumores malignos de endometrio, mama, ovario, intestino delgado, estómago y vías urinarias. El objetivo fue dar a conocer un interesante caso de asociación de cáncer de colon no polipósico y de endometrio en una familia con antecedentes de cáncer en tres generaciones. Se presenta una paciente de 55 años de edad, blanca, con piel actínica y antecedentes de carcinoma de colon derecho operada a los 44 años de edad de $T_2N_0M_0$ (hemicolectomía derecha y quimioterapia con 5-Fluoruracilo) y que ahora, a los 55 años de edad, comienza a presentar sangramiento vaginal resultando un adenocarcinoma de endometrio $T_{1c}N_0M_0$ y se interviene quirúrgicamente (histerectomía con doble anexectomía y radioterapia con Cobalto 60 a una dosis de 50 Gray). La evolución es favorable con respuesta completa y un Intervalo libre de enfermedad de 18 años y 3 meses. Se confecciona el árbol genealógico dados los antecedentes de cáncer. Además, presenta comorbilidades como obesidad exógena. La importancia del trabajo estriba en que se presenta un caso clínico inusual de síndrome de Lynch II donde se asocia un carcinoma de colon derecho con adenocarcinoma de endometrio, obesidad exógena y piel actínica en una paciente con historia familiar de cáncer y consanguinidad.

Palabras clave: neoplasias/cáncer de colon, adenocarcinoma de endometrio; factores de riesgo/ obesidad; cutáneos/ piel actínica; consanguinidad/ endogamia.

Abstract: The Lynch syndrome is a hereditary non-polyposic colorectal cancer and it is related with to non-colorectal cancer. Non-colorectal cancer sites as malignant endometrial, breast, ovary, small intestine, stomach and urinary tract have been described. The aim of this study was to present an interesting case of association no polyposic colon cancer and endometrial cancer in a family with a history of cancer in three generations. It is report a patient diagnosed 55 years old, white, actinic skin and a history of colon carcinoma operated right at 44 years old $T_2N_0M_0$ (right hemi colectomy and chemotherapy 5Fluoruracilo) and now, at 55 years old, start to have vaginal bleeding resulting $T_{1c}N_0M_0$ endometrial adenocarcinoma and intervenes surgically (hysterectomy with double oophorectomy and radiotherapy with 60 Cobalt 50 Grays) was reported. Clinical complete response and disease-free interval of 18 years and 3 months was reported. The genetic tree given the history of cancer is performed. Exogenous obesity as comorbidities was also presented. It is important to report a case of Lynch II syndrome where right colon carcinoma associated with endometrial adenocarcinoma, exogenous obesity and actinic skin in a patient with familial cancer and consanguinity.

Key words: neoplasms/ colon cancer, endometrial adenocarcinoma; risk factors/ obesity; cutaneos/ actinic skin; consanguinity/ inbreeding.

Introducción

Originalmente el síndrome de Lynch se caracteriza por la asociación del cáncer colorrectal hereditario no polipósico (CCHNP) con el adenocarcinoma de endometrio[1]; se han descrito como otros sitios no colorrectales asociados con el CCHNP los tumores malignos de endometrio, mama, ovario, intestino delgado, estómago y vías urinarias[2]. En este síndrome se han identificado mutaciones en uno de cuatro genes reparadores de los desajustes en hMLH1, hMSH2, hMSH6 o el hPMS2, con carácter autosómico dominante.

El riesgo estimado para desarrollar cáncer de colon entre las mujeres es del 40 al 60 % contra el 80 % entre los hombres. El riesgo de cáncer de endometrio en mujeres con el síndrome de Lynch sobrepasa el riesgo de cáncer colorrectal[4]. Se reportan en la literatura médica casos portadores de este síndrome no asociados a consanguinidad, obesidad y piel con daño actínico como este caso.

Se reporta una paciente operada de CCHNP, que recibió tratamiento adyuvante con quimioterapia; a los diez años se diagnosticó un adenocarcinoma de endometrio. La paciente es obesa y tiene un fototipo 1 de piel con daño actínico. Se identifica su árbol genealógico y se comprueba consanguinidad entre dos familias con cáncer de colon en tres generaciones (abuelo, tío y paciente). También padecieron de tumores malignos de estómago, sarcoma de partes blandas y tumor maligno cerebral.

Se obtuvo el consentimiento de la paciente para reportar su cuadro clínico cuidando su identidad. El objetivo es mostrar la asociación del cáncer de colon hereditario no polipósico con el adenocarcinoma de endometrio en una familia con consanguinidad, también hubo asociación con obesidad exógena y piel

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fototipo 1 con daño actínico.

Presentación del caso

Paciente de 55 años de edad, femenina, raza blanca, obesa, con piel actínica, operada a los 44 años por adenocarcinoma de colon derecho. Recibió posteriormente seis ciclos de quimioterapia con 5-fluoruracilo a 450 mg/m^2 por cinco días, cada tres semanas. Recibe seguimiento sistemático en consulta externa hasta que en febrero de 2009 presenta sangrado vaginal de moderada cuantía. Se realiza biopsia por legrado diagnóstico en dos ocasiones siendo el resultado negativo de células neoplásicas. En julio de 2009, se detectan varios miomas uterinos por ultrasonografía ginecológica. El 20 de julio de 2009 se realizó una histerectomía abdominal con doble anexectomía. En la pieza quirúrgica se diagnostica un adenocarcinoma de endometrio y se clasifica como un T1cN0M0.

Cronograma

En mayo de 1999 se realiza hemicolectomía derecha por adenocarcinoma de colon que infiltra hasta la muscular en el Hospital Universitario Provincial Camilo Cienfuegos de Sancti Spiritus. Se clasifica como un $T_2N_0M_0$. Posteriormente es remitida al Servicio de Oncología del propio hospital en junio de 1999 y recibe seis ciclos de quimioterapia con 5-fluoruracilo a 450 mg/m^2 por cinco días infusión continua, cada tres semanas. Continúa en seguimiento de consulta hasta enero de 2009 que debuta con sangrado vaginal de moderada cuantía y anemia por lo que se remite al Hospital Materno-Infantil en febrero de 2009 donde se realiza legrado diagnóstico. El resultado de la biopsia 2009-B-35 resulta endometrio secretor.

La paciente continúa sangrando y se ingresa en la sala de Oncología en marzo 2009 para estudio. Al examen físico mediante tacto vaginal se describen útero y anexos sin alteraciones. Cuello sano. También el ultrasonido ginecológico muestra un útero que mide 65 por 40,2 por 55 mm, con grosor endometrial de 9 mm. Riñones y vejiga normales.

Se realizaron estudios analíticos: hemoglobina 134 g/L, eritrosedimentación de 30 mm, grupo O Rh positivo, creatinina 86 mmol/L, ácido úrico 404 mmol/L, glicemia 5,8 mmol/L, tiempo de coagulación 8 minutos, conteo de plaquetas 203 por 10^9 / L.

En el propio mes se repite el ultrasonido ginecológico donde describen varios miomas uterinos. Se repite el legrado diagnostico. Biopsia 09-B-35: endometrio secretor. El 18 de Julio de 2009 se ingresa la paciente en el Servicio de Cirugía General para tratamiento quirúrgico realizándose histerectomía total abdominal con doble anexectomía. El 22 de Julio de 2009 causa alta hospitalaria.

A la semana reingresa debido a un absceso de la herida quirúrgica. El cultivo bacteriológico de la herida no señala crecimiento bacteriano. Recibe tratamiento con una tríada de antimicrobianos cefotaxima-gentamicina-metronidazol por siete días. Anemia de 100 g/L.

Se recibe el informe de biopsia de la pieza quirúrgica de histerectomía.

2009-B-5701: adenocarcinoma de endometrio que invade el tercio interno y parte del externo del miometrio, cercano a la serosa (grado II de Scarff). Cuello con signos de metaplasia escamosa del exocervix, ovarios con cambios atróficos.

Luego de realizar estudios de extensión se clasifica como un $T_{1c}N_0M_0$ y como un estadio Ic de la FIGO. Ingresa para tratamiento con radiaciones. Recibe gammaterapia con Cobalto 60 externo 25 aplicaciones y dosis total de 50 Grays. A las quince aplicaciones hace episodio de radiodermitis húmeda. Por esta causa se suspende el tratamiento por una semana y luego concluye sin otra alteración.

Discusión

El principal hallazgo de la investigación es la descripción de una paciente con el síndrome de Lynch en una familia con cáncer de colon no polipósico en tres generaciones por vía del abuelo materno. En el árbol genealógico padecieron de cáncer de colon el abuelo materno, un tío varón y la paciente reportada. Por otra parte, dos tíos maternos, hembra y varón, padecieron de cáncer de estómago, un tío con sarcoma de partes blandas. La madre de la paciente falleció a causa de un tumor maligno cerebral. Una fortaleza del estudio es que pudo obtenerse la información acerca de la familia y detallar la localización de los tumores primarios en tres generaciones mediante la anamnesis. Además, se pudo confeccionar el árbol genealógico de esta familia. También ante el síntoma sangrado vaginal, a pesar de las biopsias negativas obtenidas por legrado en dos ocasiones, se insistió en la búsqueda del tumor de endometrio. La posibilidad de este síndrome, se confirmó por la evidencia obtenida mediante el método clínico y los antecedentes patológicos familiares de cáncer. Como debilidad del estudio tenemos la ausencia de marcadores genéticos (hMLH1, hMSH2, hMSH6 o el hPMS2) no disponibles en la provincia para corroborar el diagnóstico. De todas maneras, cuando se describe el síndrome de Lynch originalmente el método fue bastante similar[5].

El significado del estudio y los posibles mecanismos e implicaciones para la clínica consisten en que este síndrome se hereda con carácter autosómico dominante. Los individuos con este síndrome heredan un alelo no funcional por lo cual la reparación del ADN es defectuosa en el tejido diana[6]. Las mutaciones en las líneas hMLH1 y hMSH2 ocurren en el 90% de los casos con síndrome de Lynch. El criterio inicial de este síndrome (Ámsterdam I) se basó en la clínica enfocada en el cáncer de colon. Con posterioridad fue revisado e incluyó la asociación de otros tipos de cáncer (Ámsterdam II). El criterio incluye tres o más cánceres relacionados con el síndrome, dos que afecten generaciones sucesivas o un tumor diagnosticado antes de los 50 años de edad[7]. En el presente caso cumplió con los criterios para el diagnóstico.

Lindor M N *et al* recomiendan para las mujeres de familias con riesgo del síndrome de Lynch la realización de colonoscopia cada dos años a partir de los 20-25 años de edad. En los casos con cáncer familiar recomienda hacerlo diez años antes de la edad del caso más joven diagnosticado de cáncer en la familia. En las familias que expresan el MSH6, hacerla a partir de los 30 años de edad. A estas mujeres también les recomiendan la muestra del endometrio, el ultrasonido transvaginal y citología urinaria. Además de estos procederes invasivos, proponen esos autores indagar en la historia familiar y el examen físico a partir de los 21 años en las mujeres. Las resecciones colorrectales y la histerectomía profiláctica no se recomiendan, sólo en mujeres con maternidad satisfecha[8]. Se difiere en la presente investigación de los métodos invasivos por varias razones. Primero: estos procederes diagnósticos y terapéuticos no están exentos de eventos adversos y causan molestias innecesarias en algunos casos. En el caso presentado la anemia progresiva y la falsa imagen sonográfica de miomas uterinos provocó la histerectomía. Segundo: son procederes caros y su disponibilidad requiere a veces viajar a instituciones lejanas.

La recomendación para estudios posteriores pudiera ser la

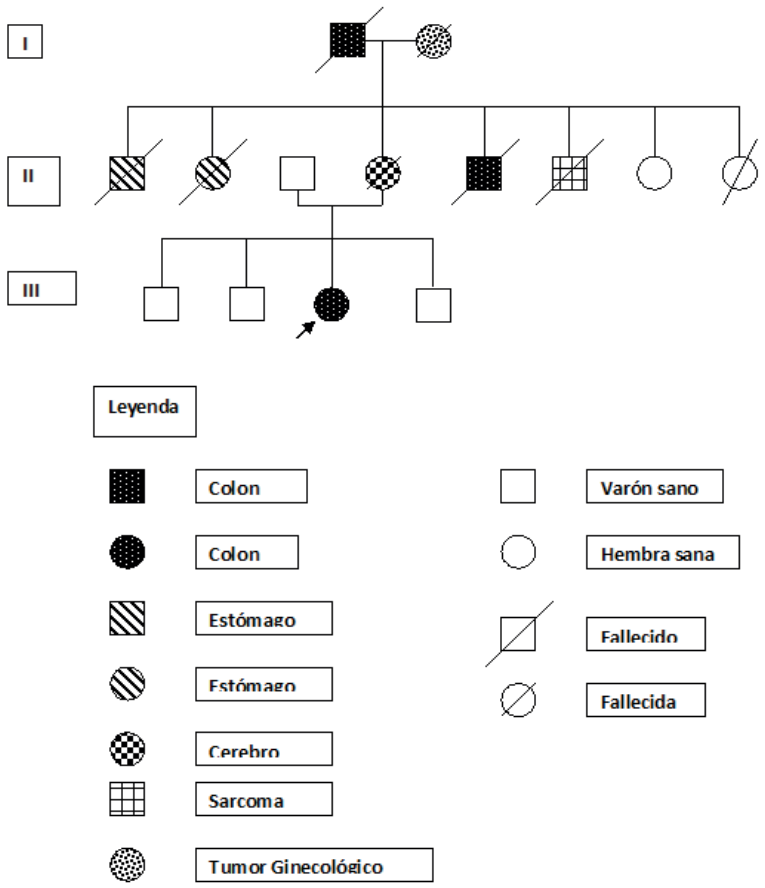

Figura 1. Árbol genealógico del cáncer de colon hereditario no polipósico en tres generaciones.

acuciosidad del diagnóstico del síndrome de Lynch mediante el método clínico. Se haría a punto de partida del debut del cáncer de colon en mujeres de familias con tumores malignos de colon no polipósico. Este grupo de pacientes con algunos factores de riesgo para adenocarcinomas de endometrio como la tríada obesidad, hipertensión y diabetes mellitus o algunos de estos inducen a la búsqueda del síndrome de Lynch[9].

Otra variante sería la búsqueda inversa del cáncer de colon a punto de partida del adenocarcinoma de endometrio en mujeres de familia con cáncer de colon hereditario. De todas maneras, la detección de cualquiera de las dos variantes pudiera influir en la sobrevida de las mujeres con síndrome de Lynch con una terapéutica eficaz y pertinente[10].

Conclusiones

Se presenta un caso clínico en el cual se asocia, por primera vez, el carcinoma de colon derecho no polipósico hereditario con el adenocarcinoma de endometrio, consanguinidad, obesidad exógena y piel actínica.

Conflicto de intereses

No existen conflictos de intereses

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NEWS AND VIEWS / NOTICIAS Y OPINIONES

Blockchain: A technological tool for sustainable development or a massive energy consumption network?

Blockchain: ¿Una herramienta tecnológica para el desarrollo sostenible o una red de consumo eléctrico/energético masivo?

Nicolas Serrano Palacio.

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Abstract: The blockchain term appeared online in 2008 and has created impact on public institutions, private companies, startups, etc. It is a way to record information and its main application since inception has been as a new means of exchange between two parties. Blockchain is a highly secured and decentralized accounting system that allows direct exchange between two unknown parties without the necessity for a trusted third party, and it implies a new operation model for businesses and institutions. This new model could be a useful tool to solve global issues as income inequality and contribute to the accomplishment of the sustainable development agenda proposed for the United Nations for all countries in the world.

Key words: Blockchain, Bitcoin, Energy Consumption, Sustainable development, Income inequality.

Resumen: El término Blockchain apareció en línea en el 2008 y ha creado un impacto en instituciones públicas, compañías privadas, emprendimientos, etc. Blockchain es una manera de almacenar información y su aplicación principal desde el inicio ha sido crear medios de intercambio entre dos entidades. Blockchain es un sistema de contabilidad altamente seguro y decentralizado que permite un intercambio directo entre dos entidades desconocidas sin la necesidad de una tercera entidad en la cual confiar. Eso implica un nuevo modelo de operaciones para negociones e instituciones. El modelo puede ser una herramienta útil para resolver problemas globales como la desigualdad de ingresos además de cumplir con la agenda de desarrollo sostenible propuesta por las Naciones Unidas para todas las naciones miembro.

Palabras clave: Blockchain, Bitcoin, Consumo eléctrico/energético, Desarrollo sostenible, Desigualdad de ingresos.

Introduction

Blockchain, a distributed ledger network, has recently caught the attention of diverse sectors like industry, financial institutions, academia, online community and entrepreneurs[1] (p.11-19); it is all due to the wide range of challenges blockchain could solve. Main examples are: institutional transparency, businesses decentralization, track able information and democratizing work rewards across all members of the network by eliminating the need of a central authority that has total control[2]. Nevertheless, blockchain infrastructure consumes a massive amount of electricity[3] to properly maintain its data integrity. The two topics above may look unrelated until it is seen through a sustainable development perspective where the future of humankind depends not only on social and economic development but also on environment preservation.

The article will discuss the basic technical concepts related to the equally rewarded property of a blockchain, and how this technology is affecting another sustainable development dimension: environmental sustainability. To conclude we would make a "call to action" to the academic and research community to solve the blockchain challenges and help societies evolve towards a more sustainable era.

Equality and Sustainable Development

Sustainable development is an organizing principle for human development respecting the existence of other life forms. According to the United Nations; people, economy and environment are the three pillars for sustainable development. In turn, these three pillars relate to seventeen Sustainable Development Goals (SDG) that need to be pursued in order to reach sustainability. The goals are inter linked and working on a specific one will have an effect on others. The objective is to harmonize all efforts so that progress can be measured for all sustainability actions[4]. Blockchain, a global distributed ledger[1], could help solve economic inequality by using its decentralized properties but it also presents a problem to the environment if we look at the amount of electric power needed to maintain its networks.

Nowadays, the technology industry has been following a "winner takes it all" model[5, 6] (p. 108) where just one business is capable of practical domination over a market where all other competitors fail in the process. This model causes a few very large centralized companies who offer a product or service and get richly compensated for it as many times as demands are made by the clients; the lack of competitors helps the company to grow consistently and win more market share in the process[7] (p. 103-105). Sadly, the actual model and the concentrated wealth that it generates prevents new companies from disrupting the existing status quo in the market place with innovative solutions; mainly because the most powerful companies of the world control not only their own ecosystem but also dispose of financial and information resources that give them an unfair advantage over their small competitors[8]. This problem could lead to a world of centralized power and income inequality within the whole population. Blockchain could theoretically be a viable solution to this issue

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Figure 1. Sustainable Development Goals proposed by the United Nations on 2015.

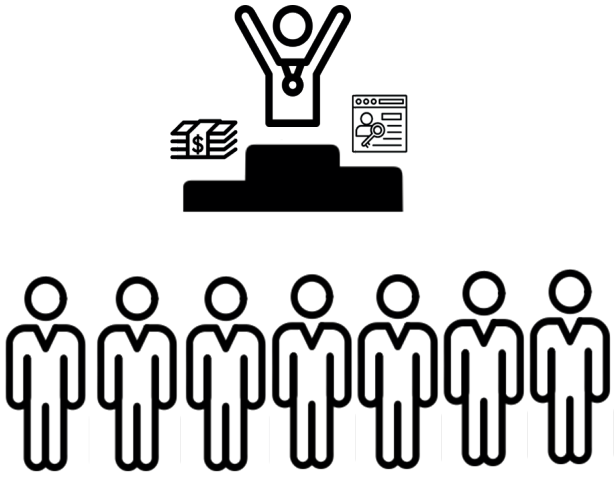

Figure 2. Winner takes it all model representation. A single company would get all the market.

because of its decentralizing and empowering nature.

The blockchain proposes a business model where every worker on the network gets equally rewarded for doing a specific task and the unhackable feature gives security consistency to the system. It also prevents control of a single central authority through transparent management of data ledgers in the network[2].

The theory behind blockchain date back to 19802 but it was not until ten years ago that the right conjunction of concepts gave it the ability to disrupt multiple sectors simultaneously and generated a new area of technological development[9].

History

The first appearance of the term "blockchain" was in 2008 on a white paper published online by Satoshi Nakamoto1. The name was a nickname; until today nobody have verified the truth identity of this person or group of people although some of the earliest Bitcoin developers had been working with him/her/them via online forums, platforms and emails[1](p. 5).

2008 as the publication year was not a random choice. The economic crisis in the US and all over the world had taken its toll over savings and businesses while bank corporations and wealthy people remained in a privileged position[10]. Bitcoin, and blockchain behind it, promoted the concept of a decentralized and distributed platform that provided a new way to exchange money using a publicly powered network rather than a central and privately controlled node[2].

From that date, blockchain research and development has been growing at a fast rate. The private sector has seen growing investment on the technology and, as a result, we can see multiple private or public blockchain platforms[11]; academia has been developing a new blockchain space backed up with all the scientific rigor that a peer review provides[12]; and the entrepreneurs and innovators have been involved as main actors in the process, developing and creating better solutions[13, 14].

In December 2017 we witnessed the explosion of the cryptocurrencies; some experts compared it to the .com mania that caused market instability in the years 2000s[15]. Two months later, people declared that the cryptocurrency era was over as, intuitively, blockchain was following the Gartner hype curve[16] (Fig. 3). But for other people, this was the end of the cryptocurrency bubble and the beginning of the blockchain era. In 2018, ten years later its inception, blockchain is well positioned as a technological important topic[9] with high hope and expectations for this relatively new concept and its potential impact on global issues such as income inequality[17].

The future of blockchain highly depends on public adoption and market penetration[18], nevertheless academia and the research community can help in the search for solutions to the challenges this technology presents. In other to understand these challenges, the researcher has to familiarize himself with the technical concepts behind it.

Blockchain Theory

To explain the concept of blockchain; it has to be compared to an account ledger where all information (in case of Bitcoin, transactions) is stored. If the ledger where held by a

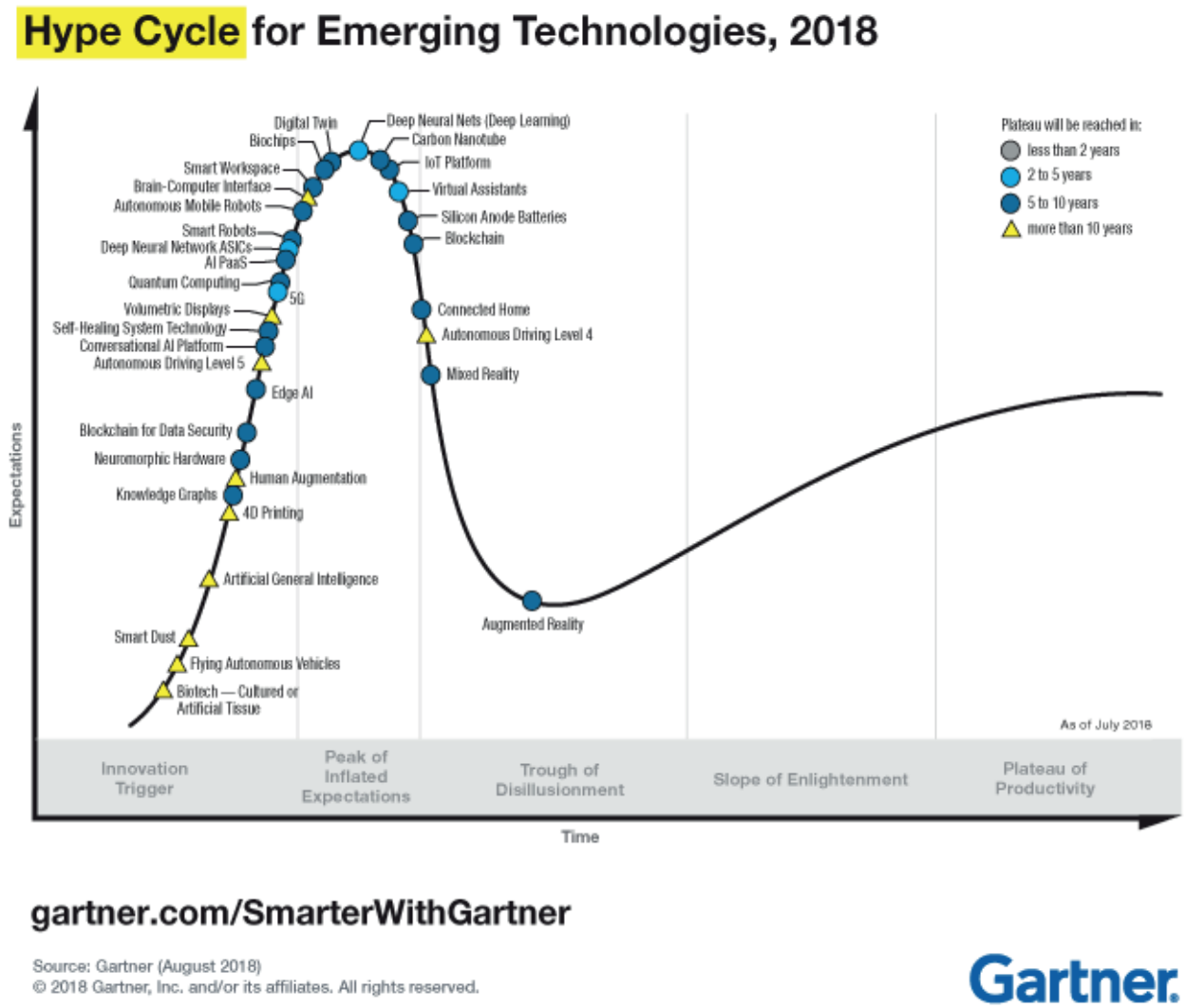

Figure 3. Hype Cycle or Gartner curve for Emergin Technologies 2018.

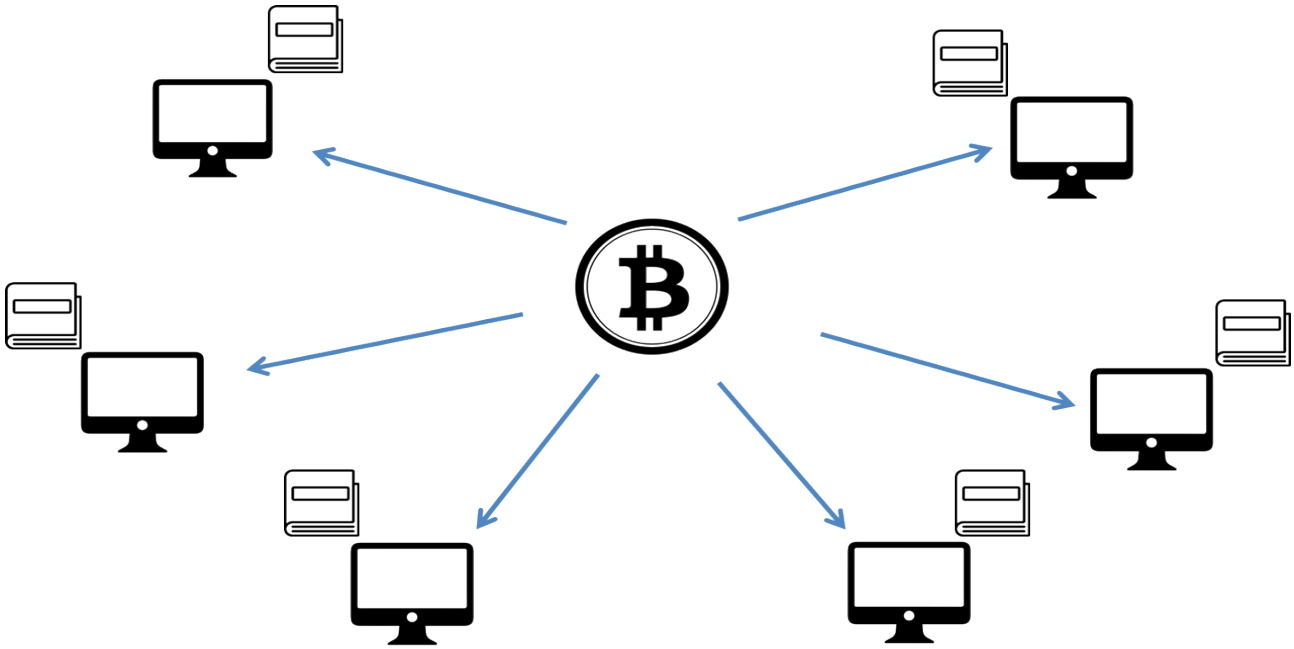

Figure 4. Simple scheme of a blockchain network where all nodes contain the same ledger.

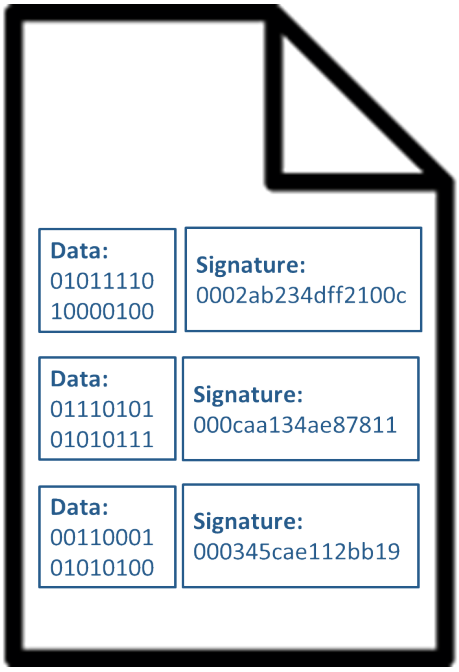

Figure 5. Ledger containing groups of data with the correspondent signature for each one.

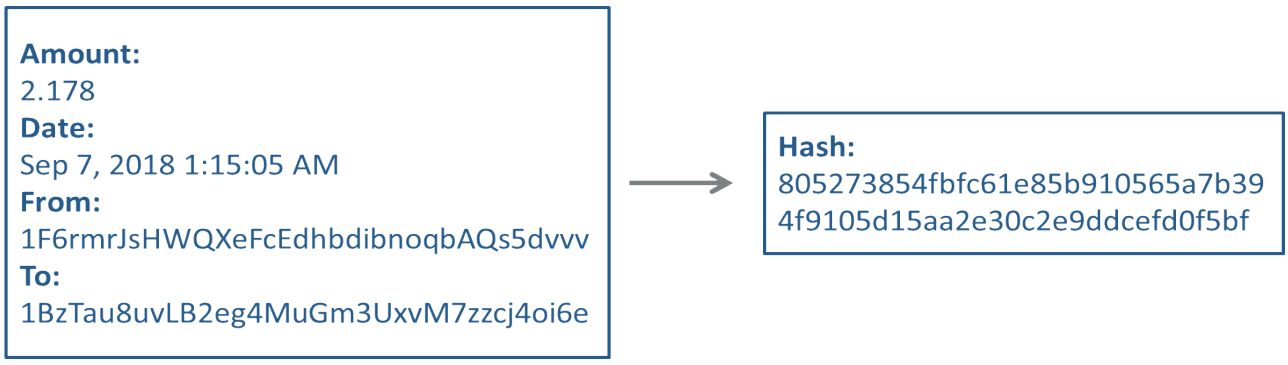

Figure 6. Hashing process to get a hash or signature by inputting transaction data into a cryptographic function.

single owner, it could be modified depending on his/her self-interests. To prevent that from happening, the ledger is distributed and maintained by several people or entities. So in the case where a participant presents a modified version of the ledger, the whole group can verify or audit the ledger and deny the corrupted ledger. Blockchain as a data structure does not only assures data veracity but also provides the historic record of the data[1, 2].

Blockchain is improbable hackable and it guarantees the veracity of the information by supplying a unique "digital signature" to every set of data stored each time period. In order to maintain the network as a decentralized structure a ledger is distributed to all the registered nodes on the network and all the transactions are tracked through each node.[2] (p.3) (Fig. 4).

Commonly the ledger created contains all the previous archived data and the digital signature of each set of data called block. The signatures guarantee the authenticity of the contained information because if, at any place of the ledger, some data changes or becomes corrupted; the signature will be completely different[1] (p. 6) (Fig. 5).

In order to create a signature or "Hash"; the system protocol takes all the data and process it through a cryptographic hash function that would convert it into a pseudo random sequence of numbers and characters[19] (p. 83-84). In the Bitcoin blockchain; multiple cryptographic functions are used such as SHA256 or RIPEMD-160 and it takes as input the following elements: sender address, receiver address, amount (represented by previous received transactions) and timestamp. The function would output the transaction signature as a hexadecimal string of 256 bits (Fig. 6).

To get the most efficient storage, the transactions signatures (called hashes) are processed with each other to build a Merkle tree and the last signature after processing the previous ones will be called "Root Hash"[2] (p. 4) (Fig. 7).

To finish, the protocol would take the root hash, timestamp and a value variable nonce to compute the "Block Hash" (Fig. 8). This process, called proof of work, endeavors to yeld a block hash that follows a specific condition. In the Bitcoin blockchain, the condition is that the hash has to have at least n zeros at the beginning of the string; with n being a difficulty variable that increases if the block hash had been found before ten minutes and decreases if the block had been found after ten minutes. Proof of work, the previously described algorithm, guarantees that each transaction is entered in a specific manner and prevents vulnerability to the double spending problem[1] (p. 8-11). The whole process is called mining.

This hash and the new data would be sent to all the nodes on the network for verification. If any data is modified at any

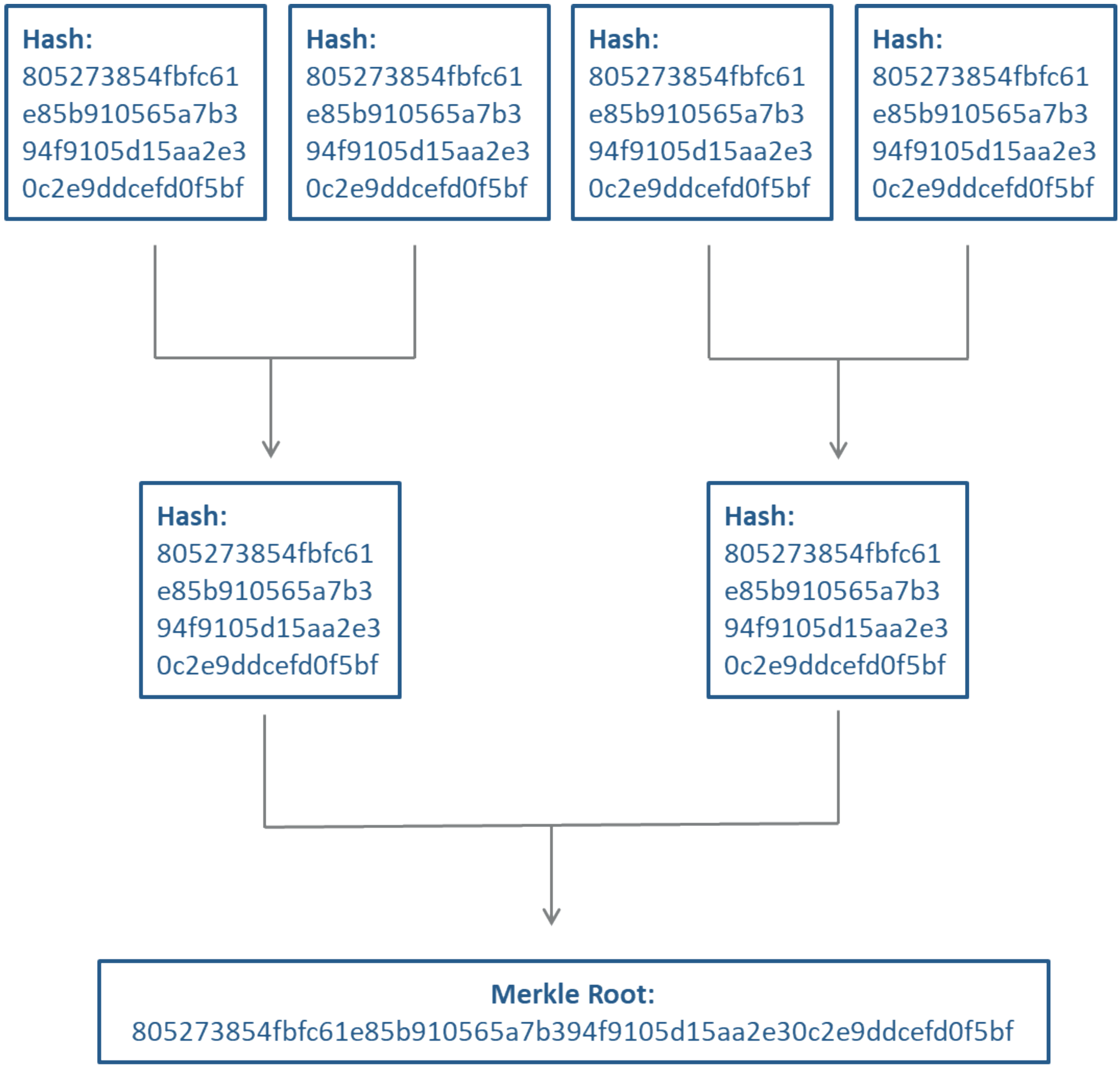

Figure 7. Merkle tree structure formed by hashes of different transactions.

time during that process, the Block Hash will be completely differently and thus the system would know that it has been compromised. The protocol would deny the wrong data and only accept the one that the majority of the network approves. With this feature, the network guarantees the veracity of the information and prevents the problem of getting the data modified by an attacker or impostor[19] (p. 5-9).

The new block hash and its information would be stored in all nodes of the blockchain. So in the future; it could be validated by any node registered on the network (Fig. 9). The participation depends on the nature of the blockchain; in a public blockchain such as Bitcoin or Etherum, people around the world can freely join the network[2] (p. 5)

The proof of work algorithm requires huge amounts of computing power and hence electric energy. As described before, the dimensions of sustainable development are interconnected; so it would be useful for the technology users to consider their energy requirement and its impact on their community.

Energy consumption problem

Alex de Vries described Bitcoin as an extremely energy-hungry process3. This is mainly because the proof of work algorithm uses exhaustive search method which consists on trying all possible permutations until getting the right answer, in the blockchain case: respecting the difficulty condition[20].

The blockchain structure allows one block creation per a determined time period. If more nodes with high computing power enter the network, the time period of block creation will decrease and hence the difficulty will increase at the future. The crypto currency revolution has attracted a lot of enthusiastic people not only to mine but to build large facilities called mining farms[21]. Inside the farms, there can found hundreds of ASIC, specialized mining computers that can try million of hashes permutations per second for proof of work procedure[22].

ASIC and GPU computing for mining Bitcoin are major trends nowadays. The whole Bitcoin network consumes the same amount of energy as the country of Ireland as a whole[3]. The compensation for joining the mining network is that each

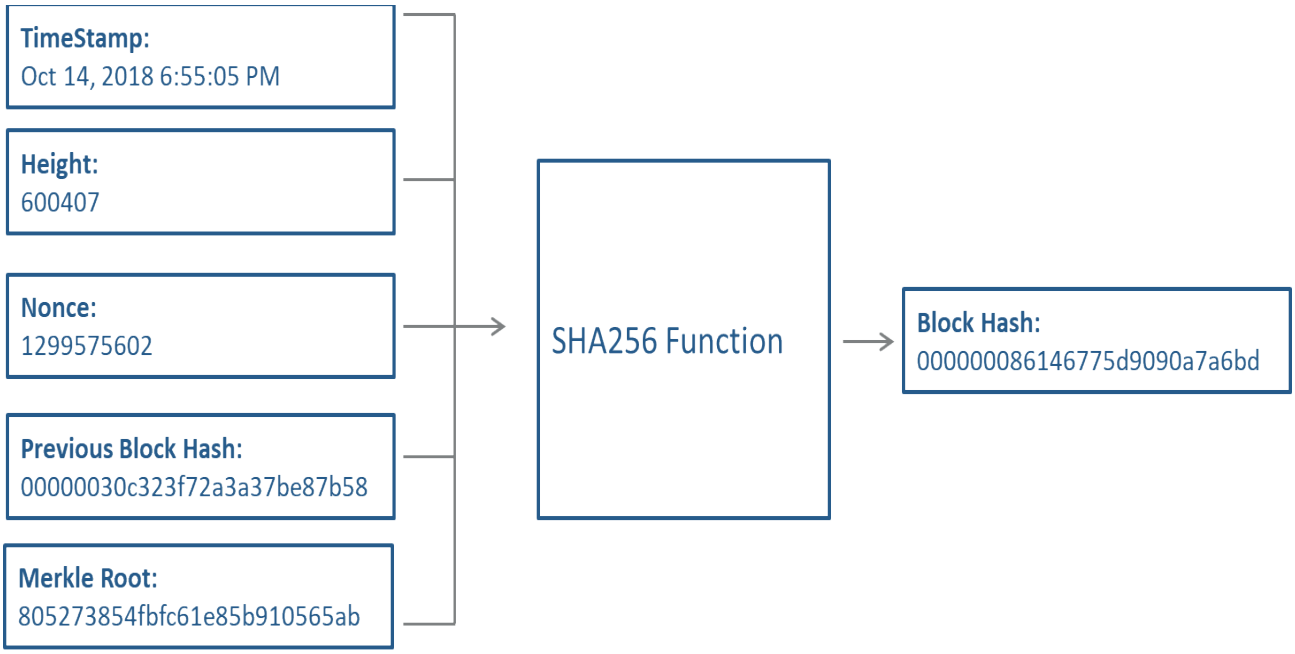

Figure 8. Process and inputs to obtain the block hash.

TimeStamp:
Oct 14, 2018 6:55:05 PM
Height:
600407
Nonce:
1299575602
Previous Block Hash:
00000030c323f72a3a37be87b58
Merkle Root:
805273854fbfc61e85b910565ab

Block Hash:
000000086146775d9090a7a6bd

Index	Amount	Date	From	To	Transaction Hash
0	0.45	Sep 7, 2018 1:15:05 AM	1F6rmrJsHWTQXeFcEdhbdi	bnoqbAQs5dvvvAA567FX110st	51B87481829E23ADF9282C4D487AE99DC16C06
1	2.00	Sep 10, 2018 12:15:00 AM	1BzTau8uvLB2eg4MuGm3Us	xvM7zzcj4oi6eUV1050LLeNM20	ADF08432826EA4103934C3D9E26C06ADFAE911
2	650.32	Sep 10, 2018 05:15:55 PM	22xTvLM7zzcj4oi6e05763JJ	xAA7eB123TYwq15209Hggstuw	8C578203D5FBEC4AB6E1D9DCE5CD2373FE9E52
3	56.11	Sep 23, 2018 9:40:00 AM	FS400824xcvHsadfwt7877	xH901221ASdtss2ptppwqQ981	6DC5484CF8A6639E80F0E09369DC5484CF8A661
4	101.11	Oct 7, 2018 11:00:12 AM	2K9uuytjsfhfTIWQasd31KKL	x82987hjAnIMaDlT0404l0V3yu	1EF688F4A92AFBCDB869C88C11CA026D432491
5	27.10	Oct 9, 2018 02:22:27 PM	487TTrt11101WADqghGHy3	BHsgjTj12783213qerFltuMpqjm	56166464CD45901D69619BF6C64915AAC10001

Figure 9. Information that would be stored in a blockchain after passing validation process.

time a new hash block is found and validated; the founding miner receives the fees for all the included transactions and 12.5 of new created Bitcoin[23]. The second amount is temporary until 21 million Bitcoin have been created; when that happen the winning miner would start receiving just the transaction fees[24]. Changes of a miner finding the right hash are random but the more computing power to process information; the more likely is for the miner to find the right hash.

Because of the large amount of computing power needed to find a right block hash, multiple middle class miners (with just one ASIC or a GPU graphic card) join a "mining pool". This organization delegates specific actions to each miner in order to have an organized procedure and increase the chances of getting the block reward. If the mining pool finds the right hash and get the fees and created Bitcoin, they will be distributed according to their group policies[25].

A common blockchain that uses the proof of work algorithm would spend huge amounts of electricity because of the network maintenance; this issue is a contributor to climate change and therefore sustainable development.

Truby[26] (2018) argues that the amount of electric energy consumed by blockchain technologies pose a serious threat to the global commitment to mitigate greenhouse gas emissions pursuant to the Paris Agreement[27]. 28% of greenhouse gasses are released by electricity production[28] and part of that is used to power up mining equipment like computers, ASICs, GPU cards and others. Greenhouse gasses form a shell that keep the heat on the earth's surface like a greenhouse that cover the plants; that is why it is called Greenhouse Effect[29] (p. 77).

Blockchain and Sustainable Development

The blockchain phenomenon shows us how two aspects of sustainable development are related and how one action in a specific SDG could cause positive and negative impact on others SDGs.

The most important social feature of Blockchain is to decentralize business and organizations. As previously described, it could generate positive impact on the income inequality problem and promote a fair economic growth for all human classes on Earth rather that keeping the "winner takes it all" model.

But to reach a sustainable future, blockchain technology would need to evolve towards an improved energy consumption profile. Yli-Huumo's article identifies seven challenges of blockchain that should be investigated and improved[18]. These challenges are: thorughput, latency, ledger size, security, wasted resources, usability, hard forks or multiple chains.

There is an interesting opportunity for research NGOs and social researchers in the blockchain field; if more expert minds come together to find solutions, the decentralizing impact of Blockchain would arrive soon to our society.

Conclusions

It is true that concepts behind blockchain have been there for some time19 but the myriad combination and specific focus of these concepts make this technology fascinating. The hype of have found an interesting technology that could solve actual issues like social equity should give us hope but also reminder us to focus on finding better methods rather than wholly glorifying new platforms without further considerations.

As a Computer Science student, I am committed to research and develop technology for the good of all society and I make a "call to action" to my fellow partners to make a contribution in this field. Academia and research are capable of taking the blockchain revolution to another level and improving some social challenges we are facing nowadays.

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