Volume 5, Issue 8 – January – June -2019

ECORFAN

Journal-Republic of Guatemala

ISSN-On line: 2410-8849

10th ANNIVERSARY ECORFAN Special Edition

ECORFAN Journal-Republic of Guatemala, Volume 5, Issue 8, January – June 2019, is a journal edited semestral by ECORFAN. Kilometer 16, American Highway, House Terra Alta, House D7 Mixco Zona 1, Republic of Guatemala. WEB: www.ecorfan.org/republicofguatemala/, journal@ecorfan.org. Editor in Chief: MARTÍNEZ-HERRERA, Erick Obed. MsC. ISSN On line: 2414-8849. Responsible for the latest update of this number ECORFAN Computer Unit. ESCAMILLA-BOUCHÁN, Imelda, LUNA-SOTO, Vladimir, Kilometer 16, American Highway, House Terra Alta, House D7 Mixco Zona 1, Republic of Guatemala, last updated June 30, 2019.

ECORFAN Journal Republic of Guatemala

Definition of Journal

Scientific Objectives

Support the international scientific community in its written production Science, Technology and Innovation in the Field of Medicine and Health Sciences, Biological and Health Sciences, Medical Mycology, Dermatology, Immunology, Human Ecology, Parasitology, Pediatric Infectious Diseases.

ECORFAN-Mexico SC is a Scientific and Technological Company in contribution to the Human Resource training focused on the continuity in the critical analysis of International Research and is attached to CONACYT-RENIECYT number 1702902, its commitment is to disseminate research and contributions of the International Scientific Community, academic institutions, agencies and entities of the public and private sectors and contribute to the linking of researchers who carry out scientific activities, technological developments and training of specialized human resources with governments, companies and social organizations.

Encourage the interlocution of the International Scientific Community with other Study Centers in Mexico and abroad and promote a wide incorporation of academics, specialists and researchers to the publication in Science Structures of Autonomous Universities - State Public Universities - Federal IES - Polytechnic Universities - Technological Universities - Federal Technological Institutes - Normal Schools - Decentralized Technological Institutes - Intercultural Universities - S & T Councils - CONACYT Research Centers.

Scope, Coverage and Audience

ECORFAN Journal Republic of Guatemala is a Journal edited by ECORFAN-Mexico S.C in its Holding with repository in Republic of Guatemala, is a scientific publication arbitrated and indexed with semester periods. It supports a wide range of contents that are evaluated by academic peers by the Double-Blind method, around subjects related to the theory and practice of Biological and Health Sciences, Medical Mycology, Dermatology, Immunology, Human Ecology, Parasitology, Pediatric Infectious Diseases with diverse approaches and perspectives , That contribute to the diffusion of the development of Science Technology and Innovation that allow the arguments related to the decision making and influence in the formulation of international policies in the Field of Medicine and Health Sciences. The editorial horizon of ECORFAN-Mexico® extends beyond the academy and integrates other segments of research and analysis outside the scope, as long as they meet the requirements of rigorous argumentative and scientific, as well as addressing issues of general and current interest of the International Scientific Society.

Editorial Board

Arbitration Committee

Assignment of Rights

The sending of an Article to ECORFAN Journal Republic of Guatemala emanates the commitment of the author not to submit it simultaneously to the consideration of other series publications for it must complement the Originality Format for its Article.

The authors sign the Authorization Format for their Article to be disseminated by means that ECORFAN-Mexico, S.C. In its Holding Republic of Guatemala considers pertinent for disclosure and diffusion of its Article its Rights of Work.

Declaration of Authorship

Indicate the Name of Author and Coauthors at most in the participation of the Article and indicate in extensive the Institutional Affiliation indicating the Department.

Identify the Name of Author and Coauthors at most with the CVU Scholarship Number-PNPC or SNI-CONACYT- Indicating the Researcher Level and their Google Scholar Profile to verify their Citation Level and H index.

Identify the Name of Author and Coauthors at most in the Science and Technology Profiles widely accepted by the International Scientific Community ORC ID - Researcher ID Thomson - arXiv Author ID - PubMed Author ID - Open ID respectively.

Indicate the contact for correspondence to the Author (Mail and Telephone) and indicate the Researcher who contributes as the first Author of the Article.

Plagiarism Detection

All Articles will be tested by plagiarism software PLAGSCAN if a plagiarism level is detected Positive will not be sent to arbitration and will be rescinded of the reception of the Article notifying the Authors responsible, claiming that academic plagiarism is criminalized in the Penal Code.

Arbitration Process

All Articles will be evaluated by academic peers by the Double Blind method, the Arbitration Approval is a requirement for the Editorial Board to make a final decision that will be final in all cases. MARVID® is a derivative brand of ECORFAN® specialized in providing the expert evaluators all of them with Doctorate degree and distinction of International Researchers in the respective Councils of Science and Technology the counterpart of CONACYT for the chapters of America-Europe-Asia- Africa and Oceania. The identification of the authorship should only appear on a first removable page, in order to ensure that the Arbitration process is anonymous and covers the following stages: Identification of the Journal with its author occupation rate - Identification of Authors and Coauthors - Detection of plagiarism PLAGSCAN - Review of Formats of Authorization and Originality-Allocation to the Editorial Board-Allocation of the pair of Expert Arbitrators-Notification of Arbitration -Declaration of observations to the Author-Verification of Article Modified for Editing-Publication.

Instructions for Scientific, Technological and Innovation Publication

Knowledge Area

The works must be unpublished and refer to topics of Biological and Health Sciences, Medical Mycology, Dermatology, Immunology, Human Ecology, Parasitology, Pediatric Infectious Diseasesand other topics related to Medicine and Health Sciences.

Presentation of Content

In the first article we present, *Antiparasitic activity of Ophiocomina nigra in Entamoeba invadens*, by SÁNCHEZ-RAMOS, Sanjuana, VALDES-SANTIAGO, Laura, CASTRUITA-DOMÍNGUEZ, José Pedro and VILLAGÓMEZ-CASTRO, Julio César with ascription in the, Instituto Tecnológico Superior de Irapuato, Universidad de Guadalajara and Universidad de Guanajuato, as a next article we present, *Phylogenetic analysis of Na^+/H^+ (NuoL/MrpA) antiporters*, by SÁNCHEZ-CALDERÓN, Lenin, CHÁVEZ-AVILÉS, Mauricio Nahuam, DÍAZ-PÉREZ, Alma Laura, GÓMEZ-LUNA, Blanca Estela, RAMÍREZ-GRANADOS, Juan Carlos, VELOZ-GARCÍA, Rafael Alejandro and DÍAZ-PÉREZ, César, with ascription in the Universidad Autónoma de Zacatecas, Instituto Tecnológico Superior de Ciudad Hidalgo, Universidad Michoacana de San Nicolás de Hidalgo and Universidad de Guanajuato, as a next article we present, *Determination of the microbiological load in organic, industrial and transfer type eggs in the central-west region of the State of Veracruz*, by JIMENEZ-HERNANDEZ, Magdalena, NAVA-VALENTE, Noemi, DEL ANGEL-CORONEL, Oscar Andrés and FRIAS-FRIAS, Rocío, with ascription in the Instituto Tecnológico Superior de Huatusco, as a next article we present, *Effects of stereotactic surgery on the anterior hypothalamus (HA) on the estrous cycle: Role of the dopaminergic system in spontaneous ovulation in the rat*, by MORÁN-PERALES, José Luis, SÁNCHEZ-GARCÍA, Octavio, GARCÍA-SUÁSTEGUI, Wendy Argelia and HANDAL-SILVA, Anabella, with ascription in the Benemérita Universidad Autónoma de Puebla.

Content

Article **ECORFAN Journal Republic of Guatemala**
June 2019 Vol.5 No.8 1-7

Antiparasitic activity of *Ophiocomina nigra* in *Entamoeba invadens*

Actividad antiparasitaria de *Ophiocomina nigra* en *Entamoeba invadens*

SÁNCHEZ-RAMOS, Sanjuana[1]†*, VALDES-SANTIAGO, Laura[2], CASTRUITA-DOMÍNGUEZ, José Pedro[3] and VILLAGÓMEZ-CASTRO, Julio César[2]

[1]*Instituto Tecnológico Superior de Irapuato*
[2]*Universidad de Guadalajara*
[3]*Universidad de Guanajuato*

ID 1st Author: *Sanjuana, Sánchez-Ramos* / **ORC ID**: 0000-0001-6835-0494

ID 1st Coauthor: *Laura, Valdes-Santiago* / **ORC ID**: 0000-0002-2943-7754

ID 2nd Coauthor: *José Pedro, Castruita-Domínguez* / **ORC ID**: 0000-0002-3834-1631

ID 3rd Coauthor: *Julio César, Villagómez-Castro* / **ORC ID**: 0000-0002-7350-2314

DOI: 10.35429/EJRG.2019.8.5.1.7 Received March 12, 2019; Accepted June 30, 2019

Abstract

Objetive: Analyze the antiparasitic activity of marine invertebrate *Ophiocomina nigra* in *Entamoeba invadens*. Methodology. In *O. nigra*, an analysis of the quantitative proximal chemical composition (moisture, ash, protein, lipids and nitrogen-free extract) was performed. In addition, the determination of the total protein pattern (SDS-PAGE 10%). On the other hand, the antiparasitic activity of *E. invadens* trophozoites was determined, which were grown in TYI medium at 28 ° C and exposed to the aqueous extract of *O. nigra* for 24 hours. Subsequently, metabolic activity (XTT assay) was determined and morphology was analyzed. Cytotoxicity tests were performed on human liver cells (Hep G2) exposed for 24 hours to *O. nigra* (XTT test) and the biomass was determined (violet crystal staining). Contribution. The antiparasitic activity of *O. nigra* in *E. invadens* and the cytotoxic effect in human liver cells was determined. There are few scientific studies of this marine invertebrate on its use in traditional medicine, so it is important to analyze its effects and therapeutic value.

***Ophiocomina nigra, Entamoeba invadens,* Trophozoites**

Resumen

Objetivo: Analizar la actividad antiparasitaria del invertebrado marino *Ophiocomina nigra* en *Entamoeba invadens*. Metodología: En *O. nigra*, se realizó un análisis de la composición química proximal cuantitativa (humedad, cenizas, proteína, lípidos y extracto libre de nitrógeno). Además, la determinación del patrón total de proteínas (SDS-PAGE 10%). Por otra parte, se determinó la actividad antiparasitaria de trofozoítos de *E. invadens* los cuales fueron cultivados en medio TYI a 28°C y expuestos al extracto acuoso de *O. nigra* por 24h. Posteriormente, se determinó la actividad metabólica (ensayo de XTT) y se analizó la morfología. Ensayos de citotoxicidad se realizaron en células hepáticas de humano (Hep G2) expuestas por 24h a *O. nigra* (ensayo de XTT) y se determino la biomasa (tinción con cristal violeta). Contribución. Se determinó la actividad antiparasitaria de *O. nigra* en *E. invadens* y el efecto citotóxico en células hepáticas humanas. Existen pocos estudios científicos de este invertebrado marino sobre su uso en la medicina tradicional, por lo que es importante analizar sus efectos y el valor terapéutico.

***Ophiocomina nigra, Entamoeba invadens,* Trofozoítos**

Citation: SÁNCHEZ-RAMOS, Sanjuana, VALDES-SANTIAGO, Laura, CASTRUITA-DOMÍNGUEZ, José Pedro and VILLAGÓMEZ-CASTRO, Julio César. Antiparasitic activity of *Ophiocomina nigra* in *Entamoeba invadens*. 2019, 5-8: 1-7

* Correspondence to Author (email: sansanchez@itesi.edu.mx)
† Researcher contributing first author.

Introduction

The fragile stars (Ophiuroidea) are marine invertebrates belonging to the Equinodermata edge, within this class of the Echinoderms is Ophiocomina nigra, which is a dark colored star, with a round, flat central disk up to 2.5 cm in diameter. The five thin and flexible arms are approximately five times the diameter of the disc in length (Baharara & Amini, 2015).

Marine invertebrates have evolved in complex ways, to overcome these challenges they have a defense system that is based on an innate immune system that includes humoral and cellular responses (Nam, et al., 2015), but like all other invertebrates they lack a vertebrate adaptive immune system (Li, Blencke, Haug, & Stensvåg, 2014). Echinoderm lectins have been attributed the function of agglutinating erythrocytes and inhibiting the adhesion of bacteria. In addition, the presence of antimicrobial peptides (AMP's) which have cytotoxic, antibacterial, antifungal and antiparasitic activity.

The innate immune system of echinoderms also has several antimicrobial components, such as lysozyme (Li, Blencke, Haug, & Stensvåg, 2014). For all the above, marine invertebrates have been studied during the last years and bioactive molecules with potential therapeutic use have been isolated.

This research aims to analyze the composition of the marine invertebrate *O. nigra* and evaluate its antiparasitic activity in Entamoeba invadens. E. parasdens reptile invadens, has been used as a study model as it presents similarity with Entamoeba histolytica (causative agent of human amebiasis) with respect to its morphology, life cycle, physiology and pathogenesis (Geiman and Ratcliffe., 1936; Mc Connachie, 1995; Diamond et al., 1978).

Material and methods

Aqueous extract of *O. nigra*

The dried starfish was obtained from an herbal shop, subsequently its size was measured and pulverized. A stock of aqueous extract was prepared with the powdered sample and to remove undissolved material it was centrifuged at 15294 rcf for 10 min.

Electrophoresis in denaturing conditions 10%

To obtain the total *O. nigra* proteins, a total homogenate in 2% SDS was performed in phosphate buffer at pH 7.0 plus a mixture of protease inhibitors (Complete Mini-Roche), then the separation of the proteins in 10% polyacrylamide gels under denaturing conditions (SDS-PAGE), using the technique described by Laemmli (1970). The gel was stained with silver for protein visualization, image acquisition was performed on a ChemiDoc MP System-BIORAD using Image LabTM software (BIORAD).

Quantitative proximal chemical composition

The chemical composition was performed according to Kirk R.S. et al. (nineteen ninety six).

Determination of humidity. The sample (1 g) was placed in an oven (Novatech E145-AIA) at 100 - 105 ° C for 8h, then it was weighed to determine the% humidity using the following equation:

% Humidity = initial sample weight - dry sample weight / initial sample weight x 100

Total dry ash method. The dehydrated sample (1 g) was calcined in a flask (Terlab MA12D) at 550 ° C for 2h, then the weight was recorded and determined:

% Ash on dry basis = ash weight / sample weight x 100

Direct extraction method with organic solvent for lipid determination. The dehydrated sample (1 g) placed in a cellulose thimble was placed in a leach. 110 mL of petroleum ether were placed in a flat-bottomed ball flask and assembled with a soxhlet and placed in an extraction equipment (Novatech VH-6) at a temperature of 100 ° C adapted to a recirculator (ECO 30) at 7 ° C for 8 h. The condensation rate was 3-6 drops / sec. After extraction, the thimble was removed, the solvent was evaporated from the flasks and placed in an oven (NovatechE145-AIA) at 100 ° C for 30 min, cooled in a desiccator and the weight recorded.

ISSN-On line: 2414-8849

The determination was made using the equation:

% Crude fat = weight of the flask with fat - weight of the flask / sample weight x 100

Quantification of raw fiber. The sample (1 g) previously dehydrated and degreased was digested with two drops of octyl alcohol and 200 mL hot 0.255 N sulfuric acid and boiled for 30 min (Craft FC-600). Subsequently, it was filtered on linen cloth and washed with boiling distilled water, at the end 200 mL of hot 0.313 N sodium hydroxide was added and boiled for 30 min. The samples were filtered by suction through a gooch crucible with thin layers of asbestos in the bottom. Finally, they were washed with 15 mL 95% ethyl alcohol and dried at 105 ° C for 12 h and weighed. Then, they were incinerated at 600 ° C for 1h in a flask (Terlab MA12D) and the sample was weighed.

% Crude fiber = incinerated sample weight / sample weight x 100

Protein Determination The Kjeldahl micrometer (Novatech RJR0549F2) was used, which was based on wet combustion of the sample by heating with concentrated sulfuric acid in the presence of metal catalysts, reducing organic nitrogen to ammonia. The ammonia was titrated with 0.05 N hydrochloric acid and the protein percentage was calculated by the following equation, using the conversion factor (6.25) of nitrogen to protein.

% Protein = (mL HCl) (N) (0.01401) (Factor) (100) / g sample

Determination of the nitrogen free extract (ELN). It was determined with the equation:

% ELN = 100% - [(% ash) + (% protein) + (% lipids) + (% fiber)]

Cultivation of Entamoeba invadens and liver cells and exposure to *O. nigra*

The maintenance of E. invadens trophozoites was performed in TYI-S-33 medium at 28 ° C. HepG2 human liver cells (ATCC® HB-8065 ™) were cultured in DMEM medium supplemented with 10% fetal bovine serum and maintained at 37 ° C with an atmosphere of 5% CO2. The cell line was manipulated with the type 1 biosafety level requirements to which it corresponds.

The amoebas or liver cells were exposed to the aqueous extract of O nigra for 24 hours at different concentrations: 31.25, 62.5, 125, 250 and 500 mg / mL, at the end of the incubation time the mitochondrial metabolic activity and biomass were determined.

Mitochondrial metabolic activity

XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -2-h-tetrazolium-5-carboxanilide) was added to the amoebas or liver cells exposed to *O. nigra* at a concentration of 0.25 mg / mL in Menadiona (Sigma) at 0.1 mM and will be incubated for 90 min at 37 ° C in the dark. The reduction of salt to water-soluble formazan crystals, a biochemical process performed by mitochondrial dehydrogenases of viable cells, will be measured at 490 nm in a microplate spectrophotometer (Epoch ™ BioTek).

Biomass Determination

Samples were fixed with 99% methanol for 15 min at room temperature. The methanol was washed with PBS (Phosphate buffer solution pH 7.2) and the 0.001% violet crystal was added for 5 min. Staining with the violet crystal allowed the interaction of the cationic dye with the negatively charged cell components. Subsequently, 33% acetic acid was added for 10 min for dye dissolution. The absorbance at 570 nm was measured at a recovered supernatant in a microplate spectrometer (Epoch ™ Biotek).

Results

Ophiocomina nigra is a marine star with thin and flexible arms, its coloration is homogeneous from dark brown to black; It is a species of marine invertebrate and dry specimens of an average size of 20 cm used in this study were purchased from a herbalist's shop (Fig. 1).

Figure 1 *Ophiocomina nigra*

ISSN-On line: 2414-8849

O. nigra has been used in traditional medicine by oral consumption, previously pulverized and added to food. Therefore, a bromatological analysis was performed observing that the proximal chemical composition that makes up this organism corresponds to 7.8% proteins, 4.2% lipids, 2.57% crude fiber, 1.2% nitrogen free extract (ELN), 82.25% ashes and moisture 1.59 % (Fig. 2).

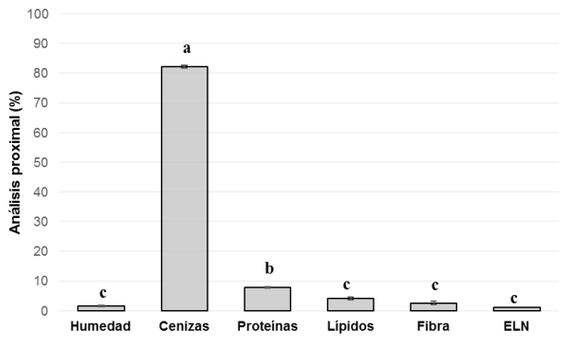


Figure 2 Proximal chemical composition of *O. nigra*

At the different parameters a comparison of means (n = 3) was performed by the Tukey method (* $p \leq 0.05$), the different letters indicate a significant difference. ELN, nitrogen free extract.

The analysis of the total protein profile of *O. nigra* indicates a wide range of proteins with a Mr ≥ 200-16 kDa (Fig. 3), where it can be observed that there are some proteins with greater intensity.

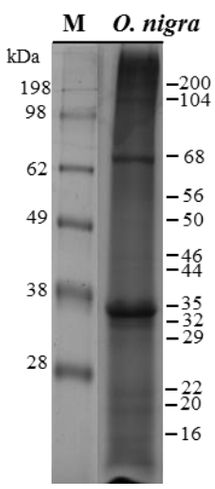


Figure 3 Total *O. nigra* proteins

The antiparasitic activity attributed to *O. nigra* was tested using E. invadens as an in vitro model. Therefore, E. invadens trophozoites were exposed with the aqueous extract of *O. nigra*, observing that the antiparasitic activity was 50.29% at the concentration of 31.25 mg / mL. In contrast, it was observed that at a higher concentration of *O. nigra* the antiparasitic activity was less than 27% (Fig. 4), however, it should be mentioned that from the concentration of 62.5 mg / mL a cellular damage was observed therefore a change in the morphology of the trophozoite, loss of adhesion and cellular debris (Fig. 5A). It is suggested that the damage caused to trophozoites caused the release of reducing agents of E. invadens and as a consequence the increase in the determination of metabolic activity was observed, a method based on the reduction of XTT to formazan (Fig. 5B).

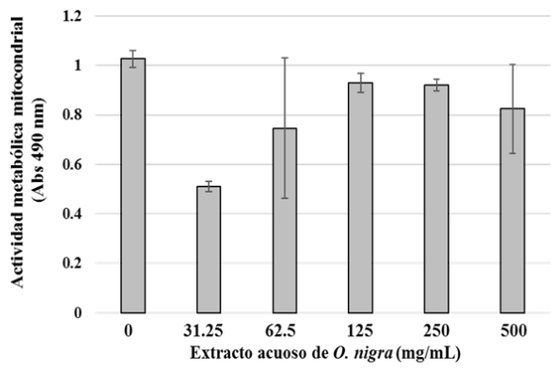


Figure 4 Determination of the antiparasitic activity of *O. nigra* in trophozoites of E. invadens

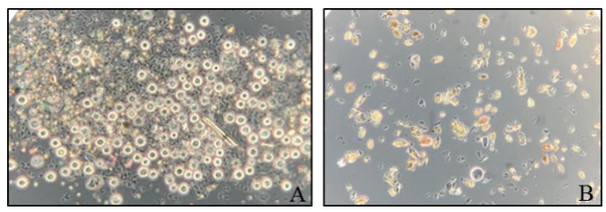


Figure 5 Morphology of E. invadens after 24h of exposure with the aqueous extract of *O. nigra*

A. The loss of the cell monolayer, the formation of trophozoite clusters, cell debris and loss of morphology (A) are observed, later when washing with phosphate buffer solution (pH 7.2), the O fragments are observed nigra and some trophozoites at the bottom of the culture plate (B). 20X

ISSN-On line: 2414-8849

On the other hand, the cytotoxic activity of the aqueous extract of *O. nigra* was determined in a culture of human liver cells, observing that regardless of the concentration of *O. nigra*, the mitochondrial metabolic activity was higher than the liver cells that were not exposed (Fig. 6), due to cell damage and the release of reducing compounds. This cellular damage was also observed when analyzing the decrease in biomass dependent on the concentration of *O. nigra*; at the highest concentration (500 mg / mL) a 59.26% loss of biomass was obtained, indicating the loss of adhesion and cell junctions, therefore, the lack of maintenance of the cell monolayer (Fig. 7) . These results indicate a cytotoxic effect caused by *O. nigra* in liver cells.

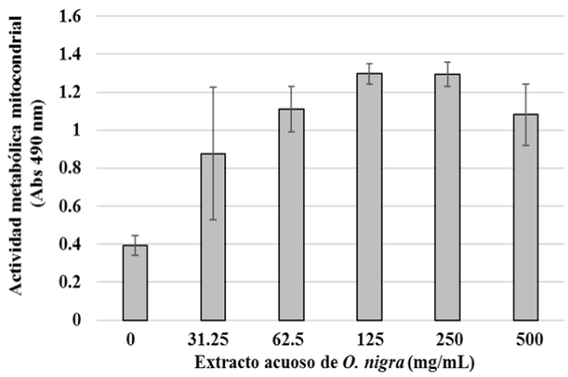


Figure 6 Determination of the cytotoxic activity of *O. nigra* in human liver cells

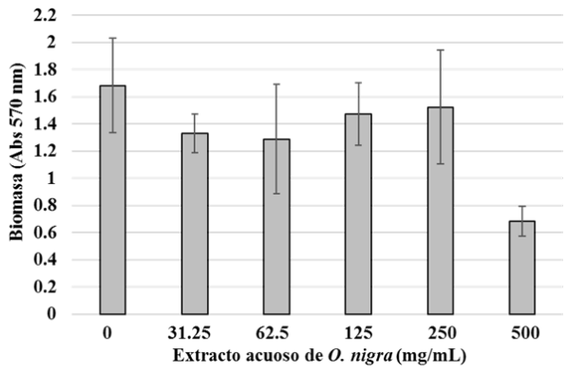


Figure 7 Biomass of liver cells exposed to *O. nigra*

Discussion

In traditional medicine, echinoderms such as Oreaster reticulate, Echinaster echinophorus, Luidia senegalensis, Mellita quinquiesperforata, Echinometra lucunter and Echinaster brasiliensis are used in Brazil against asthma, alcoholism, bronchitis, diabetes and heart disease.

This therapeutic potential is due to the ethnopharmacological information of natural products (Marmouzi, et al., 2017). The route of administration reported is oral, by preparing an infusion or direct addition to powdered roasted echinoderm foods. In this sense, it was relevant to perform an analysis of the proximal chemical composition which indicated a higher percentage of proteins (7.8%) compared to lipids, crude fiber and nitrogen free extract.

In traditional medicine, echinoderms such as On the other hand, there are the presence of some bioactive substances in fragile stars and it has been shown that they can play an important role in cancer therapy (Baharara & Amini, 2015), another invertebrate with activity Antitumor is the sea cucumber Holothuria nobilis attributing a more potent anticancer activity compared to drugs used in the treatment of cancer such as Taxol, Etoposide and Ara-C (Layson, Rodil, Mojica, & Deocaris, 2014).

In *O. nigra*, the anticoagulant activity of acid mucopolysaccharides in the mucous secretions has been demonstrated, in addition to the glycoproteins present in the secretion and the cell surface it has been reported to inhibit the adhesion of human neutrophils to endothelial cells, proposing a mechanism of Competitive inhibition of this interaction Oreaster reticulate, Echinaster echinophorus, Luidia senegalensis, Mellita quinquiesperforata, Echinometra lucunter and Echinaster brasiliensis are used in Brazil against asthma, alcoholism, bronchitis, diabetes and heart disease.

This therapeutic potential is due to the ethnopharmacological information of natural products (Marmouzi, et al., 2017). The route of administration reported is oral, by preparing an infusion or direct addition to powdered roasted echinoderm foods. In this sense, it was relevant to perform an analysis of the proximal chemical composition which indicated a higher percentage of proteins (7.8%) compared to lipids, crude fiber and nitrogen free extract. In this study, exposure of liver cells to *O. nigra* indicated a cytotoxic effect, although it should be noted that a total aqueous extract of *O. nigra* was used, where other bioactive molecules of this echinoderm were probably present. In the case of inhibition of the adhesion of bacteria to the substrate, the formation of large aggregates has been observed suggesting adhesion to the *O. nigra* glycoprotein molecule (Bavington C. et al., 2004).

ISSN-On line: 2414-8849

Similar results were obtained in the E. invadens exposure test to *O. nigra*, where trophozoite aggregates and loss of substrate adhesion were observed. Various bioactive molecules have been isolated from marine invertebrates, such as glycoside steroids consisting of saponins, cyclic steroidal glycosides, monoglycosides and steroidal diglycosides (Sumithaa, Banu, & Parvathi, 2017), terpenes, sulfated sterols, carotnoid sulfate, phenylpropaides , naphthoquinones, (Baharara & Amini, 2015), ketones and aldehydes (Thao, et al., 2014) and AMP's (Li, Blencke, Haug, & Stensvåg, 2014). In the case of saponins, it is considered one of the main constituents of some herbal drugs, research related to its existence in the marine environment has been attractive to scientists (Amini, Nabiuni, Baharara, Parivar, & Asili, 2014). Thanks to this interest, a steroidal saponin called ophiurosaponin has been identified in Ophiopholis mirabilis extracts (Wnag, Xue, Zhen, & Guo, 2014). Therefore, the use of bioactive molecules of echinoderms with a therapeutic value is attractive.

Conclusion

The antiparasitic activity of *O. nigra* was determined in E. invadens and a cytotoxic effect in human liver cells**.**

References

Amini, E., Nabiuni, M., Baharara, J., Parivar, K., & Asili, J. (2014). Hemolytic and cytotoxic effects of saponin like compounds isolated from Persian Gulf brittle star (Ophiocoma erinaceus). Journal of Coastal Life Medicine, 2 (8), 614-620.

Avant, P. (2008). Ophiocomina nigra Black brittlestar. Recuperado el 2018, de The Marine Biological Association of the United Kingdom: https://www.marlin.ac.uk/species/detail/1706

Baharara, J., & Amini, E. (2015). The Potential of Brittle Star Extracted Polysaccharide in Promoting Apoptosis via Intrinsic Signaling Pathway. Avicenna Journal of Medical Biotechnology, 7 (4), 151-158.

Balakrishnan, D., Bibiana, A. S., Vijayakumar, A., Santhosh, R. S., Dhevendaran, K., & Nithyanand, P. (2015). Antioxidant Activity of Bacteria Associated with the Marine Sponge
Bavington CD, Lever R, Mulloy B, Grundy MM, Page CP, Richardson NV, McKenzie JD. 2004 Anti-adhesive glycoproteins in echinoderm mucus secretions. Comp Biochem Physiol B Biochem Mol Biol. 2004, 139(4): 607-617

Bavington CD, Lever R, Mulloy B, Grundy MM, Page CP, Richardson NV, McKenzie JD. 2004 Anti-adhesive glycoproteins in echinoderm mucus secretions. Comp Biochem Physiol B Biochem Mol Biol. 2004, 139(4): 607-617

Kim, C.-H., Go, H.-J., Oh, H. Y., Park, J. B., Lee, T. K., Seo, J.-K., y otros. (2018). Identification of a novel antimicrobial peptide from the sea star Patiria pectinifera. Developmental and Comparative Immunology, 86, 203-213.

Kiran, N., Siddiqui, G., Khan, A. N., Ibrar, K., & Tushar, P. (2014). Extraction and Screening of Bioactive Compounds with Antimicrobial Properties from Selected Species of Mollusk and Crustacean. Journal of Clinical & Cellular Immunology, 5 (1).

KIRK R.S., Sawyer, R y Egan H. "Composición y Análisis de Alimentos de Pearson". Segunda edición. Editorial CECSA. México 1996.

KIRK R.S., Sawyer, R y Egan H. "Composición y Análisis de Alimentos de Pearson". Segunda edición. Editorial CECSA. México 1996.

Layson, R. J., Rodil, M. C., Mojica, E.-R. E., & Deocaris, C. C. (2014). Potential Anti-cancer and Anti-bacterial Activities of Philippine Echinoderm Extracts. The Journal of Tropical Life Science, 4 (3), 175-181.

Li, C., Blencke, H.-M., Haug, T., & Stensvåg, K. (2014). Antimicrobial peptides in echinoderm host defense. Developmental and Comparative Immunology, 49 (1), 190-197.

Marmouzi, I., Tamsouri, N., Hamdani, M. E., Attar, A., Kharbach, M., Alami, R., y otros. (2017). Pharmacological and chemical properties of some marine echinoderms. Brazilian Jorunal of Pharmacognosy .

Nam, B.-H., Seo, J.-K., Lee, M. J., Kim, Y.-O., Kim, D.-G., An, C. M., y otros. (2015). Functional analysis of Pacific oyster (Crassostrea gigas) b-thymosin: Focus on antimicrobial activity. Fish & Sellfish Immunology, 45, 167-174.

Sumithaa, R., Banu, N., & Parvathi, D. V. (2017). Novel Natural Products from Marine Sea

Stars. Current Trends in Biomedical Engineering & Biosciences, 2 (24).

Vergara, W., & Rodríguez, A. (2016). Nutritional Composition of Sea Cucumber Isostichopus sp. Natural Resources, 7, 130-137.

Wnag, R., Xue, X., Zhen, J., & Guo, C. (2014). Antioxidant and Antimicrobial Activity of Ophiurasaponin Extracted from Ophiopholis mirabilis. Journal of Chemistry.

ISSN-On line: 2414-8849

Article **ECORFAN Journal Republic of Guatemala**
June 2019 Vol.5 No.8 8-15

Phylogenetic analysis of Na^+/H^+ (NuoL/MrpA) antiporters

Análisis filogenético de los antiportadores Na^+/H^+ (NuoL/MrpA)

SÁNCHEZ-CALDERÓN, Lenin[1]†, CHÁVEZ-AVILÉS, Mauricio Nahuam[2], DÍAZ-PÉREZ, Alma Laura[3], GÓMEZ-LUNA, Blanca Estela[4], RAMÍREZ-GRANADOS, Juan Carlos[4], VELOZ-GARCÍA, Rafael Alejandro[4] and DÍAZ-PÉREZ, César[4]*

[1]*Doctorado en Ciencias Básicas. Universidad Autónoma de Zacatecas.*
[2]*Instituto Tecnológico Superior de Ciudad Hidalgo.*
[3]*Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo.*
[4]*Universidad de Guanajuato, Campus Celaya-Salvatierra, Departamento de Ingeniería Agroindustrial.*

ID 1er Author: *Lenin, Sánchez-Calderón* / **ORC ID**: 0000-0002-4141-0386, **CVU CONACYT ID**: 39883

ID 1er Coauthor: *Mauricio Nahuam Chávez-Avilés* / **ORC ID**: 0000-0002-6588-6653, **CVU CONACYT ID**: 211462

ID 2do Coauthor: *Alma Laura, Díaz-Pérez*

ID 3er Coauthor: *Blanca Estela, Gómez-Luna* / **ORC ID**: 0000-0001-6345-0461, **CVU CONACYT ID**: 101592

ID 4to Coauthor: *Juan Carlos, Ramírez-Granados* / **ORC ID**: 0000-0001-6460-6472, **Researcher ID Thomson**: S-5874- 2018, **CVU CONACYT ID**: 167866

ID 5to Coauthor: *Rafael Alejandro, Veloz-García* / **ORC ID**: 0000-0002-6493-5708, **Researcher ID Thomson**: S-5809-2018, **CVU CONACYT ID**: 163099

ID 6to Coauthor: *César, Díaz-Pérez* / **ORC ID**: 0000-0001-7847-1062, **Researcher ID Thomson**: X-5157-2019, **CVU CONACYT ID**: 101579/

DOI: 10.35429/EJRG.2019.8.5.8.15 Received March 10, 2019; Accepted June 30, 2019

Abstract

Objectives: Sodium/proton (Na+/H+) antiporters NuoL/MrpA-like proteins are important in monovalent cations homeostasis, ATP synthesis, are involved in growth using low concentrations of acetate, and in management of protons during methane production. To learn more about the evolutive origin and biological relevance of this protein, in this work a phylogenetic analysis of the NuoL/MrpA superfamily of proteins was done. Methodology: Phylogeny reconstruction was done with 596 NuoL/MrpA proteins and 39 MrpD-NuoM/N proteins. The algorithms used were minimum evolution and maximum likelihood, using MEGA program. Additionally, a conserved domain analysis was done. Contribution: NuoL/MrpA superfamily and their homologous proteins, MrpD-NuoM/N, form two paralogous groups. The NuoL/MrpA superfamily consists of two families. Family NuoL consist of arqueal, bacterial and eukaryotic proteins of around 600 aa in size. Family MrpA are formed by proteins from bacteria and archaea, with a 600 to 850 aa in size. Using the phylogenetic analysis and conserved domain analysis, a superfamily NuoL/MrpA evolution model was proposed.

Phylogeny, antiporters Na^+/H^+, NuoL/MrpA

Resumen

Objetivos: Las proteínas antiportadores de Na^+/H^+ similares a NuoL/MrpA son importantes para la homeostasis de cationes monovalentes, la correcta síntesis de ATP, el crecimiento a bajas concentraciones de acetato, y se piensa que es importante el manejo de los protones durante la producción de metano. Para conocer más sobre origen evolutivo y relevancia biológica de estas proteínas, en este trabajo se llevó a cabo un análisis filogenético de la superfamilia de proteínas NuoL/MrpA. Metodología: La filogenia se efectuó usando 596 proteínas NuoL/MrpA y 39 MrpD-NuoM/N. Los algoritmos de reconstrucción usados fueron el de mínima evolución y máxima verosimilitud, en el programa MEGA. Además, se llevó a cabo un análisis de dominios conservados (CDD). Contribución: Las proteínas NuoL/MrpA y sus homólogos, MrpD-NuoM/N, forman dos grupos parálogos. La superfamilia NuoL/MrpA consta de dos familias. La familia NuoL que está formada por proteínas de arqueas, bacterias y eucarióticas de alrededor de 600. La familia MrpD, formada por proteínas de 600 a 850 aa de bacterias y arqueas. Mediante el análisis filogenético y de dominios conservados, se propone el modelo evolutivo que dio origen a la superfamilia antiportadores NuoL/MrpA.

Filogenia, antiportadores Na^+/H^+, NuoL/MrpA

Citation: SÁNCHEZ-CALDERÓN, Lenin, CHÁVEZ-AVILÉS, Mauricio Nahuam, DÍAZ-PÉREZ, Alma Laura, GÓMEZ-LUNA, Blanca Estela, RAMÍREZ-GRANADOS, Juan Carlos, VELOZ-GARCÍA, Rafael Alejandro and DÍAZ-PÉREZ, César. Phylogenetic analysis of Na^+/H^+ (NuoL/MrpA) antiporters. ECORFAN Journal-Republic of Guatemala. 2019, 5-8: 8-15

* Correspondence to Author (email: cesar.diaz@ugto.mx)
† Researcher contributing first author.

Introduction

The Na + / H + antiporters similar to NuoL / MrpA are a group of secondary transporters that use the motive force of the proton gradient to displace positive cations such as sodium, which are widely distributed in organisms in the three phylogenetic domains of life, Bacteria, Archaea and Eukarya (Masahiro Ito, Morino, & Krulwich, 2017; Jasso-Chávez, Diaz-Perez, Rodríguez-Zavala, & Ferry, 2017).

The main physiological role of these transporters is the maintenance of intracellular pH homeostasis and the flow of cations such as Na +, K + and Li +, in addition these proteins play an important physiological role in bacteria regulating cation homeostasis by means of Na + / H + (Morino, Suzuki, Ito, & Krulwich, 2014), of K + / H + anti-support (Putnoky et al., 1998), pH homeostasis (Blanco-Rivero, Leganes, Fernandez-Valiente, Calle, & Fernandez-Pinas, 2005), resistance to bile salts (J. Dzioba-Winogrodzki et al., 2008), arsenite oxidation (Kashyap, Botero, Lehr, Hassett, & McDermott, 2006), pathogenesis (J. Dzioba-Winogrodzki et al., 2008) and energy conservation (Blanco-Rivero, Leganes, Fernandez-Valiente, & Fernandez-Pinas, 2009). The majority of cation / proton anti-carriers are monomers, however, NuoL / MrpA anti-carriers are complexes of several subunits and have been classified as an independent family in the carrier classification system called cation: proton antiporter-3, or CPA3 (Masahiro Ito et al., 2017).

NuoL is part of the respiratory complex I, which is the main proton entrance into the respiratory chains of mitochondria of most eukaryotes and some bacteria (Steimle et al., 2011). The resolved crystal structure of complex I shows that NuoL helps in the translocation of protons (Baradaran, Berrisford, Minhas, & Sazanov, 2013; Efremov & Sazanov, 2011), interacting with the NuoM, NuoN and NuoJ subunits through one arm formed by its C-terminal (Figure 1A). In the genomes of Bacillus and methanogenic archaea Methanosarcina acetivorans, Mrp complexes have been found, formed by the MrpABCDEFG subunits (Jasso-Chávez et al., 2017; TH Swartz, S. Ikewada, O. Ishikawa, M. Ito, & TA Krulwich , 2005), while in Vibrio cholerae the Mrp system is formed by the MrpABCDEFG subunits, since the MrpA and MrpB subunits are merged (Judith Dzioba-Winogrodzki, Winogrodzki, Krulwich, Boin, & Dibrov, 2009). The MrpA protein is the A subunit of the Mrp complex, which has the function of translocating Na + / H +, is involved in the synthesis of ATP and cell growth by converting acetate into methane and carbon dioxide (Jasso-Chavez, Apolinario, Sowers, & Ferry, 2013), it has also been seen that this protein is functional independently of the MrpABCDEFG complex in M. acetivorans (Jasso-Chavez et al., 2017).

MrpA is a membrane protein formed by 20 transmembrane segments (STM), which has a conserved structure with the anti-carrier subunit, NuoL. The N-terminal end of MrpA contains the anti-support domain, in addition to a protruding alpha helix that forms an arm that interacts with the MrpA subunit. The structure model of MrpA predicts that the mechanism of proton translocation is through STM 5, 7, 8 and 12, in addition to the fact that transport-related amino acids are conserved (Jasso-Chavez et al., 2017). The C-terminal end of MrpA contains another domain that is similar to the NuoJ subunit (Figure 1B), and possibly is part of the proton transport channel (Baradaran et al., 2013; Kao et al., 2005).

It is widely known that the subunits of the I NuoL, NuoM and NuoN complex are homologous to each other, as are the MrpA and MrpD subunits, suggesting a common origin for these proteins, in addition, there is a greater similarity between MrpA and NuoL, and between MrpD and NuoM / N, which between the subunits of its same complex, suggesting that a functional difference between them, which has been tested experimentally (Sperling, Górecki, Drakenberg, & Hägerhäll, 2016). It has also been proposed that the subunits of the Mrp complex arose from an ancestor similar to Complex I (Mathiesen & Hagerhall, 2003), however, an exhaustive analysis has not been carried out in this superfamily of transporters to know the evolutionary origin of these proteins. This is why in this work the reconstruction of the phylogeny of the Na + / H + anti-carrier superfamily similar to NuoL / MrpA was carried out and an analysis of its members to elucidate its evolutionary history.

ISSN-On line: 2414-8849

Methodology to be developed

Obtaining the working group

First, a sequence search was carried out using the NuoL proteins of Escherichia coli (accession number: P33607) and MrpA of M. acetivorans (accession number: AAM07911) on the GenBank platform using the Blastp program (Altschul et al., 1997) against the nr and swiss-prot protein bases, the BLOSUM 62 substitution matrix was used, and an expectation cut-off value (e) of 5x10-10 was taken. To narrow down the search, only nr database proteins that had the complete NADH-Ubiquinone / plastoquinone (pfam00361) domains were taken into account. Redundant proteins and fragments were removed using the guide trees generated by Clustalw (Thompson, Gibson, & Higgins, 2002). After five debug iterations, a working group of 635 sequences was obtained (39 MrpD and 596 MrpA).

Reconstruction of phylogeny

The 635 work group sequences were aligned using the Clustalw program. The alignment obtained was corrected by hand using the alignments generated with Blastp of each sequence as a reference. The correction was carried out using the Seaview program (Gouy, Guindon, & Gascuel, 2009). The MEGA version 6 program (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was used for the reconstruction of phylogeny. Phylogenetic trees were reconstructed with the Minimum Evolution (ME) and Maximum Likelihood (ML) algorithms using the JTT matrix and the LG model plus 2.0 range as methods for estimating evolutionary distance. As a statistical test, a Bootstrap analysis of 1000 repetitions was performed for each tree. The taxonomy of the organism from which each sequence came from to verify the phyllogenetic data was verified. To place the root of the tree was used as an external group to MrpD proteins.

Domain Analysis

The analysis of the domains that make up the 635 sequences was carried out using the CDD conserved domain search platform (Marchler-Bauer et al., 2014), using the standard values.

To facilitate the analysis, scripts were generated in Perl 5.0 to generate images of the location of the domains from the results table of the CDD platform.

Results

Reconstruction of the phylogeny of the MrpA family

To know the evolutionary relationships and the origin of MrpA proteins, a phylogenetic analysis was carried out using the NuoL protein of Escherichia coli and MrpA of M. acetivorans as model proteins.

After the search and purification process, a working group of 635 sequences similar to NuoL / MrpA was obtained, within this group it was observed that there was a small subgroup of 39 sequences that were 300 amino acids smaller than the model protein, in In this subgroup, MrpD-NuoM / N proteins were identified, as already mentioned, MrpD-NuoM / N proteins are homologous to NuoL / MrpA proteins, which do not have a domain at the C-terminal end, so these proteins were not excluded from the analysis, since they can be used as an external group to root the phylogenetic tree. Excluding MrpD-NuoM / N proteins, a group of 596 proteins belonging to the Na + / H + (NuoL / MrpA) anti-carrier superfamily were found, of which 47 come from archaea, 376 from bacteria and 173 from eukaryotes, confirming that this is an ancestral family.

The phylogenetic tree shows three main groups well supported by high Bootstrap values (> 96). One of the groups consists exclusively of the proteins identified as MrpD-NuoM / N, none of these is mixed in any group of NuoL / MrpA proteins, which leads us to conclude that, although they are homologous proteins, they form a paralogue group to the NuoL / MrpA superfamily, as previously suggested (Mathiesen & Hagerhall, 2002; Moparthi et al., 2014). The other two groups, which form the family of cation / proton anti-carriers (Figure 2), were named group I (NuoL Family) and group II (MrpD Family).

The NuoL family consists of 196 members, mainly eukaryotic; It is divided into two subgroups, called IA and IB.

ISSN-On line: 2414-8849

In subgroup IA are eukaryotic proteins encoded in mitochondria of NuoL metazoans (ND5, NADH dehydrogenase 5), including human NuoL (P03915). It has been found that certain mutations in the NuoL protein have been related to various diseases, since they affect oxidative metabolism (Lodi et al., 2000; Pulkes et al., 1999; Talia H Swartz, Sayuri Ikewada, Osamu Ishikawa, Masahiro Ito, & Terry Ann Krulwich, 2005). Subgroup IB contains mainly eukaryotic proteins from chloroplasts, and from fungal mitochondria, although there is also a small group of 16 bacterial proteins and one archaea protein.

Within the bacterial proteins, the Escherichia coli NuoL protein (P33607) is located, whose three-dimensional structure has been resolved (PDB: 3RKO, subunit L), from which the proton expulsion mechanism has been better understood in the respiratory complex I (Efremov & Sazanov, 2011). The archaeological protein in this group is about the FpoL subunit of the Methanosarcina mazei Gö1 oxido-reductase F420 (Baumer et al., 2000), this result shows that the F420H2 oxido-reductases proteins are orthologs of the NADH dehydrogenases and paralogs of the MrpD proteins.

The MrpA family, consisting of 400 members, is divided into four subgroups, named IIA-D (Figure 2). Subgroups IIA, IIB and IIC are formed exclusively by bacterial proteins, while the IID group only contains archaea proteins (Figure 2). The Vibrio cholera MrpA protein (WP_001911623) is located in subgroup IIB, the Bacillus subtilis MrpA protein (Q9K2S2) is in the IIC group, and the M. acetivorans archaeus MrpA protein is in the IID group, these three proteins they have been associated with the resistance of these organisms to salts (J. Dzioba-Winogrodzki et al., 2009; M. Ito, Guffanti, Oudega, & Krulwich, 1999; Jasso-Chavez et al., 2013).

The fact that these proteins are in different groups suggests that members of this family have these same physiological functionalities. The M. acetivorans MrpA protein has also been associated with methanol metabolism and ATP synthesis (Jasso-Chavez et al., 2013; Rohlin & Gunsalus, 2010), so it is feasible that more members of the archaea MrpD family also have this functionality.

Analysis of the domains of MrpA proteins

It was observed that the distribution of the MrpA family tree shows a metabolic distribution, suggesting duplication events and subsequent divergence of the family. The MrpD-NuoM / N and NuoL / MrpA proteins vary in size due to an extension of the carboxyl end in the NuoL / MrpA family. To obtain more information about the evolutionary events in this superfamily, an analysis of the domains of the 635 sequences obtained in the search for homologues was carried out.

When analyzing the domains that make up this protein superfamily, it was observed that all members, both MrpD-NuoM / N and NuoL / MrpA, share the NADH-Ubiquinone / plastoquinone domain (pfam00361), and that the extension in the carboxyl terminal of NuoL / MrpA proteins range from 50 to 350 amino acids (Figure 3). It was observed that NuoL / MrpA proteins could be divided into two groups according to their size, which coincide with the families found in the phylogenetic tree. Group I (NuoL Family) contains proteins from 550 to 750 amino acids, Group II (MrpD Family), encompasses members that vary from 700 to 850 amino acids.

Group I contains the NuoL-1 subgroups, which is made up of proteins that have the pfam06455 domain (called C-terminal of the NADH dehydrogenase subunit 5), the NuoL-2 subgroup, which contains a F subunit domain of the NADH dehydrogenase (pfam01010), and finally subgroup I-3, whose carboxyl terminus contains another uncharacterized domain of about 100 to 200 amino acids (Figure 3). Group II contains only one type of protein whose terminal carboxyl end is similar to the proteins of the NuoL-3 subgroup, but also contains an mnhB domain (COG2111) (Figure 2).

Evolutionary model of the Na + / H + family of anti-carriers

The phylogenetic tree topology suggests that the MrpD-NuoM / N and NuoL / MrpA proteins arose from a duplication event from a single, small ancestral protein, with a NADH-Ubiquinone / plastoquinone domain (pfam00361).

ISSN-On line: 2414-8849

A primitive NuoL / MrpA protein lineage was generated that gave rise, after a fusion event with the mnhB domain (COG2111) to the larger archaea and bacteria MrpA-1 proteins. In the same way, the original lineage in a first diversification gave rise to the NuoL-3 group proteins, which are found in archaea and bacteria. Subsequently, this lineage, through a specialization event, gave rise to the NuoJ proteins present in eukaryotic organelles, suggesting that they arose before the symbiosis phenomena that gave rise to the mitochondria and chloroplasts of eukaryotic cells (Figure 4).

Acknowledgments

Indicate if they were funded by any Institution, University or Company.

Conclusions

The phylogenetic analysis of the Na + / H + (NuoL / MrpA) anti-carrier superfamily showed us that this is an ancestral superfamily, composed of at least 596 members, forming a group of paralogs with the MrpD-NuoM / N family. The phylogenetic tree shows a clear division of the NuoJ and MrpA families, which probably arose from a diversification event. NuoL proteins have formed specialized groups, highlighting the NuoL proteins of mitochondria and chloroplasts, which have a bacterial origin. MrpA proteins were also fused with an mnhB protein, to give rise to current MrpA.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research, 25*(17), 3389-3402.

Baradaran, R., Berrisford, J. M., Minhas, G. S., & Sazanov, L. A. (2013). Crystal structure of the entire respiratory complex I. *Nature, 494*(7438), 443-448. doi:10.1038/nature11871

Baumer, S., Ide, T., Jacobi, C., Johann, A., Gottschalk, G., & Deppenmeier, U. (2000). The F420H2 dehydrogenase from Methanosarcina mazei is a Redox-driven proton pump closely related to NADH dehydrogenases. *J Biol Chem, 275*(24), 17968-17973. doi:10.1074/jbc.M000650200

Blanco-Rivero, A., Leganes, F., Fernandez-Valiente, E., Calle, P., & Fernandez-Pinas, F. (2005). *mrpA*, a gene with roles in resistance to Na^+ and adaptation to alkaline pH in the cyanobacterium *Anabaena* sp. PCC7120. *Microbiology, 151*(Pt 5), 1671-1682.

Blanco-Rivero, A., Leganes, F., Fernandez-Valiente, E., & Fernandez-Pinas, F. (2009). *mrpA* (all1838), a gene involved in alkali and Na^+ sensitivity, may also have a role in energy metabolism in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Plant Physiol., 166*, 1488-1496.

Dzioba-Winogrodzki, J., Winogrodzki, O., Krulwich, T. A., Boin, M. A., & Dibrov, P. (2009). The Vibrio cholerae Mrp system: cation/proton antiport properties and enhancement of bile salt resistance in a heterologous host. *Journal of molecular microbiology and biotechnology, 16*(3-4), 176-186.

Dzioba-Winogrodzki, J., Winogrodzki, O., Krulwich, T. A., Boin, M. A., Hase, C. C., & Dibrov, P. (2008). The *Vibrio cholerae* Mrp system: cation/proton antiport properties and enhancement of bile salt resistance in a heterologous host. *J. Mol. Microbiol. Biotechnol., 16*, 176-186.

Dzioba-Winogrodzki, J., Winogrodzki, O., Krulwich, T. A., Boin, M. A., Hase, C. C., & Dibrov, P. (2009). The Vibrio cholerae Mrp system: cation/proton antiport properties and enhancement of bile salt resistance in a heterologous host. *J Mol Microbiol Biotechnol, 16*(3-4), 176-186. doi:10.1159/000119547

Efremov, R. G., & Sazanov, L. A. (2011). Structure of the membrane domain of respiratory complex I. *Nature, 476*(7361), 414-420. doi:10.1038/nature10330

Gouy, M., Guindon, S., & Gascuel, O. (2009). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular biology and evolution, 27*(2), 221-224.

Ito, M., Guffanti, A. A., Oudega, B., & Krulwich, T. A. (1999). mrp, a multigene, multifunctional locus in Bacillus subtilis with roles in resistance to cholate and to Na+ and in pH homeostasis. *J Bacteriol, 181*(8), 2394-2402.

Ito, M., Morino, M., & Krulwich, T. A. (2017). Mrp antiporters have important roles in diverse bacteria and archaea. *Frontiers in microbiology, 8*, 2325.

Jasso-Chavez, R., Apolinario, E. E., Sowers, K. R., & Ferry, J. G. (2013). MrpA functions in energy conversion during acetate-dependent growth of *Methanosarcina acetivorans*. *J. Bacteriol., 195*(17), 3987-3994. doi:JB.00581-13 [pii]
10.1128/JB.00581-13

Jasso-Chávez, R., Diaz-Perez, C., Rodríguez-Zavala, J. S., & Ferry, J. G. (2017). Functional role of MrpA in the MrpABCDEFG Na+/H+ antiporter complex from the archaeon Methanosarcina acetivorans. *Journal of bacteriology, 199*(2), e00662-00616.

Kao, M.-C., Di Bernardo, S., Nakamaru-Ogiso, E., Miyoshi, H., Matsuno-Yagi, A., & Yagi, T. (2005). Characterization of the membrane domain subunit NuoJ (ND6) of the NADH-quinone oxidoreductase from Escherichia coli by chromosomal DNA manipulation. *Biochemistry, 44*(9), 3562-3571.

Kashyap, D. R., Botero, L. M., Lehr, C., Hassett, D. J., & McDermott, T. R. (2006). A Na^+:H^+ antiporter and a molybdate transporter are essential for arsenite oxidation in *Agrobacterium tumefaciens*. *J. Bacteriol., 188*(4), 1577-1584. doi:10.1128/jb.188.4.1577-1584.2006

Lodi, R., Montagna, P., Cortelli, P., Iotti, S., Cevoli, S., Carelli, V., & Barbiroli, B. (2000). Secondary'4216/ND1 and 13708/ND5 Leber's hereditary optic neuropathy mitochondrial DNA mutations do not further impair in vivo mitochondrial oxidative metabolism when associated with the 11778/ND4 mitochondrial DNA mutation. *Brain, 123*(9), 1896-1902.
Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., . . . Hurwitz, D. I. (2014). CDD: NCBI's conserved domain database. *Nucleic acids research, 43*(D1), D222-D226.

Mathiesen, C., & Hagerhall, C. (2002). Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and bona fide antiporters. *Biochim Biophys Acta, 1556*(2-3), 121-132.

Mathiesen, C., & Hagerhall, C. (2003). The 'antiporter module' of respiratory chain complex I includes the MrpC/NuoK subunit -- a revision of the modular evolution scheme. *FEBS Lett., 549*(1-3), 7-13.

Moparthi, V. K., Kumar, B., Al-Eryani, Y., Sperling, E., Gorecki, K., Drakenberg, T., & Hagerhall, C. (2014). Functional role of the MrpA- and MrpD-homologous protein subunits in enzyme complexes evolutionary related to respiratory chain complex I. *Biochim Biophys Acta, 1837*(1), 178-185. doi:10.1016/j.bbabio.2013.09.012

Morino, M., Suzuki, T., Ito, M., & Krulwich, T. A. (2014). Purification and functional reconstitution of a seven-subunit Mrp-type Na^+/H^+ antiporter. *J. Bacteriol., 196*, 28-35.

Pulkes, T., Eunson, L., Patterson, V., Siddiqui, A., Wood, N. W., Nelson, I. P., . . . Hanna, M. G. (1999). The mitochondrial DNA G13513A transition in ND5 is associated with a LHON/MELAS overlap syndrome and may be a frequent cause of MELAS. *Annals of neurology, 46*(6), 916-919.

Putnoky, P., Kereszt, A., Nakamura, T., Endre, G., Grosskopf, E., Kiss, P., & Kondorosi, A. (1998). The pha gene cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K^+ efflux system. *Mol. Microbiol., 28*(6), 1091-1101.

Rohlin, L., & Gunsalus, R. P. (2010). Carbon-dependent control of electron transfer and central carbon pathway genes for methane biosynthesis in the Archaean, Methanosarcina acetivorans strain C2A. *BMC Microbiol, 10*, 62. doi:10.1186/1471-2180-10-62
Sperling, E., Górecki, K., Drakenberg, T., & Hägerhäll, C. (2016). Functional differentiation of antiporter-like polypeptides in complex I; a site-directed mutagenesis study of residues conserved in MrpA and NuoL but not in MrpD, NuoM, and NuoN. *PloS one, 11*(7), e0158972.

Steimle, S., Bajzath, C., Dörner, K., Schulte, M., Bothe, V., & Friedrich, T. (2011). Role of subunit NuoL for proton translocation by respiratory complex I. *Biochemistry, 50*(16), 3386-3393.

ISSN-On line: 2414-8849

Swartz, T. H., Ikewada, S., Ishikawa, O., Ito, M., & Krulwich, T. A. (2005). The Mrp system: a giant among monovalent cation/proton antiporters? *Extremophiles*, *9*(5), 345-354.

Swartz, T. H., Ikewada, S., Ishikawa, O., Ito, M., & Krulwich, T. A. (2005). The Mrp system: a giant among monovalent cation/proton antiporters? *Extremophiles*, *9*(5), 345-354.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

Thompson, J. D., Gibson, T., & Higgins, D. G. (2002). Multiple sequence alignment using ClustalW and ClustalX. *Current protocols in bioinformatics*, 2.3. 1-2.3. 22.

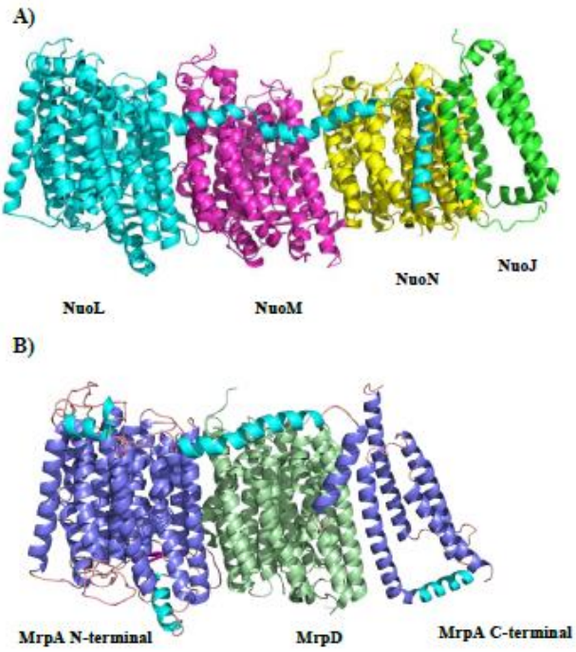


Figure 1 Three-dimensional structure of MrpAD and NuoLMNJ proteins. A) NuoLMNJ subunits of E. coli respiratory complex I (PDB: 3RKO). The anti-carrier proteins are linked by the arm that is part of the NuoL subunit. You can see the similarity between the LMN subunits. B) Molecular model of Mrp proteins of M. acetilovorans. The anti-carrier proteins, MrpA and MrpD, are similar to NuoL and NuoMN proteins respectively. MrpA contains an extra domain, which is similar to the NuoJ subunit

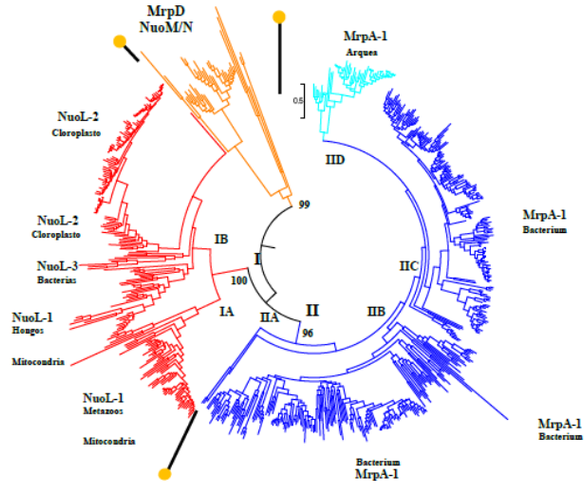


Figure 2 Phylogeny of the anti-carrier superfamily Na + / H + NuoL / MrpD. The NuoL / MrpD superfamily consists of two main groups. The NuoL family is marked as group I and the MrpD family is indicated as group II. Bootstrap values are shown in the main groups. The scale represents 0.5 amino acid substitutions per site. The tree was rebuilt using the ML method with a 1000-repetition Bootstrap

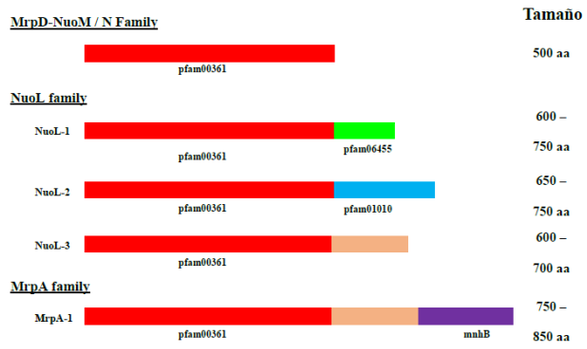


Figure 3 Arrangement of domains in the superfamily of anti-carriers Na + / H + NuoL / MrpD. The distribution of superfamily domains reflects the phylogenetic distribution. The domain of NADH-Ubiquinone / plastoquinone (pfam00361, red) is observed, conserved in the superfamily, as well as the different domains that form the extension of the C-terminal, and that generate a difference in the size of the family proteins

ISSN-On line: 2414-8849

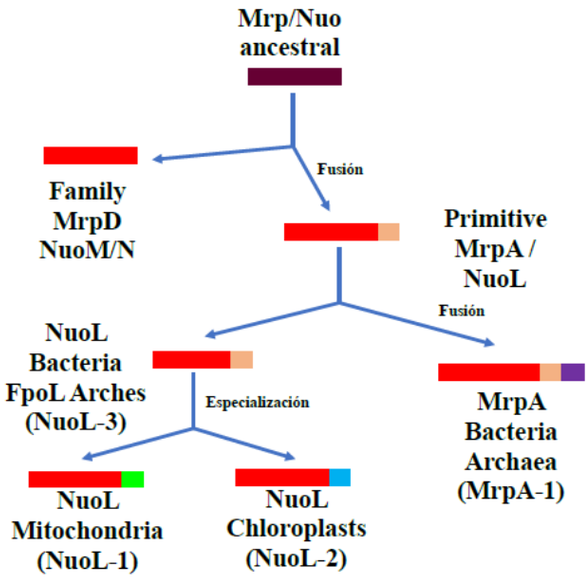


Figure 4 Evolutionary model of the origin of the superfamily of the anti-carriers Na + / H + NuoL / MrpD. The events of diversification, fusion and specialization that possibly gave this superfamily and that are proposed from the phylogenetic tree topology and the distribution of conserved domains are shown

ISSN-On line: 2414-8849

Article **ECORFAN Journal Republic of Guatemala**
June 2019 Vol.5 No.8 16-26

Determination of the microbiological load in organic, industrial and transfer type eggs in the central-west region of the State of Veracruz

Determinación de la carga microbiológica en huevo tipo orgánico, industrial y de traspatio en la región centro-occidente del Estado de Veracruz

JIMENEZ-HERNANDEZ, Magdalena†*[1], NAVA-VALENTE, Noemi[1], DEL ANGEL-CORONEL, Oscar Andrés[1] and FRIAS-FRIAS, Rocío[2]

[1]Tecnológico Nacional de México-Instituto Tecnológico Superior de Huatusco. Programa de Maestría en Ingeniería[1]. Av. 25 Poniente no. 100 Col. Reserva Territorial, Huatusco, Veracruz. México. C.P. 94100
[2]Tesista de la División de Ingeniería en Industrias Alimentarias. Instituto Tecnológico Superior de Huatusco. Tecnológico Nacional de México. Av. 25 Poniente no. 100 Col. Reserva Territorial Huatusco, Veracruz. México C.P. 94100

ID 1er Autor: *Magdalena, Jimenez-Hernandez* / **ORC ID**: 0000-0003-1988-5753, **CVU CONACYT ID**: 904566

ID 1er Coautor: *Noemi, Nava-Valente* / **ORC ID**: 0000-0002-1598-5821, **CVU CONACYT ID**: 332980

ID 2do Coautor: *Oscar Andrés, Del Angel-Coronel* / **ORC ID**: 0000-0002-0848-907X, **CVU CONACYT ID**: 226585

ID 3er Coautor: *Rocío, Frias-Frias*

DOI: 10.35429/EJRG.2019.8.5.16.26 Received March 10, 2019; Accepted June 30, 2019

Resumen

El objetivo del presente trabajo fue determinar la carga microbiológica en huevo de traspatio, tipo orgánico e industrial de la región centro-occidente del estado de Veracruz. Se evaluó la presencia de microorganismos patógenos en los tres sistemas de producción y se determinó la calidad microbiológica de cada uno de estos sistemas. Lo cual es de suma importancia considerando que en huevo con alta carga microbiológica, pueden estar presentes microorganismos como *Salmonella spp S. enteritidis*, etc., los cuales son considerados agentes causales de infecciones entéricas en la población. Aunado a lo anterior, los sistemas de explotación antes mencionados, representan una fuente de ingreso económico relevante para las familias que habitan esta región. Para el análisis microbiológico, se tomaron diferentes muestras de cascaron y de la parte interna (clara y yema). Se evaluó la presencia de *Salmonella spp* aplicando el procedimiento establecido en la Norma Oficial Mexicana NOM-114-SSA1-1994. Además se realizaron pruebas físicas para evaluar si los defectos físicos en huevo pueden correlacionarse con la presencia de *Salmonella spp* u otras bacterias. **Contribución:** Actualmente es poca la información que existe sobre la calidad microbiológica, en huevo proveniente de los diferentes sistemas de explotación que se consumen en la región centro-occidente del estado de Veracruz, particularmente para el sistema orgánico y de traspatio.

Salmonella spp. **Huevo de Traspatio, Huevo Tipo Orgánico**

Abstract

The objective of this work was to determine the microbiological load in backyard egg, organic and industrial type of the central-western region of the state of Veracruz. The presence of pathogenic microorganisms in the three production systems was evaluated and the microbiological quality of each of these systems was determined. Which is of the up most importance considering that in eggs with high microbiological load, microorganisms such as *Salmonella spp*, *S. enteritidis*, etc., may be present, which are considered causal agents of enteric infections in the population. In addition to the above, the aforementioned exploitation systems represent a source of economic income relevant to the families that inhabit this region. For microbiological analysis, different samples of shell and the internal part (white and yolk) were taken. The presence of *Salmonella spp* was evaluated by applying the procedure established in the Official Mexican Standard NOM-114-SSA1-1994. In addition, physical tests were performed to assess whether physical defects in eggs can be correlated with the presence of *Salmonella spp* or other bacteria. **Contribution:** Currently, there is little information on microbiological quality, in eggs from the different exploitation systems consumed in the central-western region of the state of Veracruz, particularly for the organic and backyard system.

***Salmonella spp*, Backyard egg, Organic type egg**

Citation: JIMENEZ-HERNANDEZ, Magdalena, NAVA-VALENTE, Noemi, DEL ANGEL-CORONEL, Oscar Andrés and FRIAS-FRIAS, Rocío. Determination of the microbiological load in organic, industrial and transfer type eggs in the central-west region of the State of Veracruz. ECORFAN Journal-Republic of Guatemala. 2019, 5-8: 16-26

* Correspondence to Author (email: magdalena_jimenez@itshuatusco.edu.mx)
† Researcher contributing first author.

Introduction

In 2017, Mexico ranked the first place of consumption per capita of the Mexican is 22.7 Kg, almost one egg a day. (National Poultry Farmers Union, 2018). Mexico ranks as the fourth largest egg producer worldwide, after China (National Poultry Farmers Union, 2018). Eggs that are produced industrially or traditionally can be contaminated internally or externally (Minorl et al 1984). Enterobacteria of the genus Salmonella spp. They have been reported as pathogens for humans and in wild and domestic animals, poultry such as chickens. There are more than 2,500 enteric Salmonella serovars of which approximately 250 have been isolated from poultry, and around 40 serovars are the most commonly found (World Health Organization, 2017).

In eggs, Salmonella species can be found in the shell, but they can penetrate inside if adequate conservation conditions are not maintained, (Carbajal, 2006). To reach this food these bacteria use different routes, one of them is when they are present in the chicken where the egg comes from, here the contamination occurs internally, (Suárez M.et al., 2000). The other possibility is that the microorganism is present in the shell and its origins can be: animals, people who handle, distribute or are in contact with eggs, (Méndez, et al., 2011).

In this work the microbiological load of different egg production systems was determined, such as, backyard, organic and industrial type, in order to know the importance of each of them, as well as the health risks that may arise. This will improve their production, applying Good Poultry Practices (BPA), raising the quality of the egg produced in each of the aforementioned systems. The different samples used for the analysis were taken from the internal and external part of the different egg batches. (hull, white and yolk).

The presence of Salmonella spp was evaluated by applying the procedure established in Standard NOM-114-SSA1-1994. On the other hand, physical tests were carried out to assess whether the presence of physical defects in organic, industrial and backyard eggs can be correlated with the presence of Salmonella spp. or other bacteria.

The risk in backyard egg production, without BPA and without proper management in bird feeding, can present a high microbial load including pathogenic bacteria, as is the case in production systems that develop in the target region. study. In addition to the above, this system represents an economic income for the families that are engaged in this activity, so it is important to evaluate its microbiological quality.

Materials and methods

The experimental phase began with the collection of organic, industrial and backyard eggs that are produced or marketed in the central-western region of the state of Veracruz. Egg collection was carried out in different parts of the area, for example: backyard were collected in Atlanca, Tenexcalco, Tehuipango, Acatepec Chalchiltepec, all these locations belong to the Municipality of Huatusco, Coauixtlauac, belonging to the municipality of Zongolica, Potrerillo market , from the municipality of Orizaba. While the organic type egg was found in the Costco super market, the brands that were purchased were Vegetatarian from the state of Jalisco, certified organic egg (Finca Guayacán) certified by bio-agricet, the closest market to this region is located in Xalapa, other organic type eggs were obtained at the Chilcuahutla Farm, the industrial egg was purchased at the Aurrera supermarket of the commercial chain belonging to the municipality of Tomatlán and at the municipal capital of Huatusco.

On each collection point, a batch of 12 eggs was analyzed, from this, 3 samples were obtained per batch, each sample contained 4 eggs. The analysis that was elaborated to the egg included two stages that began with the measurement of external variables (shell) later internal variables were measured (white and yolk). To determine the presence of salmonella spp, the procedure established by Standard NOM-114-SSA1-1994 was used. Method for the determination of Salmonella spp in food.

The analysis that was elaborated to the egg included two stages that began with the measurement of external variables (shell) later internal variables were measured (white and yolk). To determine the presence of salmonella spp, the procedure established by Standard NOM-114-SSA1-1994 was used. Method for the determination of Salmonella spp in food

ISSN-On line: 2414-8849

Physical analysis

As part of the physicochemical analyzes, each egg was weighed with an analytical balance (Electronic Analytical Balance Mod. 20002). Subsequently, the diameters of each egg, larger diameter (polar diameter) and smaller diameter (equatorial diameter) were measured for this purpose a vernier (Truper) was used, these data serve to know the size of the egg. Egg diameter data were used to determine the volume and surface area of each egg according to the methodology and equation proposed by Narushin (2005)

The response variables were: egg weight, larger diameter (DMa), smaller diameter (DMe). DMa and DMe measurements were used to determine the theoretical volume coefficient (Kv) and surface coefficient (Ks), according to the Narushin methodology (2005) with expressions:

Kv = 0,6057 - 0,0018 * (DMe)

Ovoscopy: The technique used to observe the state of eggs is ovoscopy, which consists of keeping the egg backlit for study in a dark room or box. With the present technique, the internal state of each egg was observed, obtaining corresponding data:

Air chamber: With the air chamber we can know the freshness of the egg if the air chamber is larger, its freshness is lower and vice versa. This variable was measured by placing a ruler on the upper left, with which the diameter of the air chamber was obtained and its size determined.

Position of the yolk: it was obtained with the observation of its position, by means of the ovoscope that allows visualizing the internal part of the egg without reaching its destruction.
Fractures: Fractures with greater size and thickness were observed with the naked eye, through the ovoscope the smallest ones were visualized by placing the egg against the light and turning at the same time to observe possible fractures.

Determination of the microbiological load for shell, white and egg yolk.

The microbiological analysis of the egg samples was performed according to NOM 114-SSA1-1994, in which 25.0 g sample is considered as an analytical unit, in a 1: 9 proportion of sample / broth. This amount can be varied as long as the same 1: 9 ratio is maintained in the pre-enrichment medium. The work was carried out under a laminar flow hood (Figursa Industry Mod.CFH-90).

Lactose broth was prepared as a pre-enrichment medium for culturing bacteria, to achieve a stable physiological condition in Salmonella spp. present in the samples.

For the pre-enrichment of the shell in lactose broth, sterile bags were used, placing in each bag 4 eggs without breaking them and pouring the amount of 36ml of lactose broth, maintaining the same 1: 9 ratio (4:36) of sample / broth.

The eggs remained 5 minutes in the bag promoting so that the impurities of the shell remain in the lactose broth. It was left to incubate (Memmert Incubator Mod. 30-1060) for 24 hours at a temperature of 37 ° C
After 24 hours of incubation the samples were observed to detect the possible growth of bacteria

Análisis de la clara y yema

For this step a batch of 12 eggs was used from which 4 eggs were taken from each sample in triplicate. They were poured into test tubes, to which numbers 1-4, 5-8 and 9-12 were assigned, according to the number of eggs contained in each sample.

4 eggs were poured into a beaker and perfectly homogenized.

15 ml were taken, to make a proportional analysis, for which 135 ml of lactose broth were taken, this proportion remained 15: 135 sample / broth.

150 ml flasks previously sterilized were used for this phase.

The sample was seeded in the lactose broth and incubated for 24 hours at a temperature of 37 ° C / 24 hours. (Memmert Incubator Mod. 30-1060).

ISSN-On line: 2414-8849

Differential Staining (Gram Staining)

Samples from each of the bags previously incubated by the Gram stain technique were analyzed in triplicate and determined whether they corresponded to Salmonella spp.

Selective enrichment for clear and yolk shell

After 24 hours of incubation of the analysis of the shell, white and yolk in medium lactose pre-enrichment broth, a selective enrichment was prepared, the selective sterile cystine selenite medium was prepared which is specific for the growth of Salmonella spp, It is used to increase populations of Salmonella spp and inhibit other microorganisms.

Subsequently, 9 ml of selective medium (selenite cystine) were taken per sample and added to each test tube.

Subsequently, from each bag, a 1 ml sample was taken with a micropipette.

It was poured into test tubes containing 9 ml of the cystine selenite medium.

The thread of the tubes was perfectly closed, sealing them with tape to prevent the substance from spilling and each tube was labeled. Subsequently, it was stirred to properly homogenize the sample to the selective medium. Finally, the tubes were incubated at 37 ° C for 24 hours.

Figure 1 Comparison of tubes in different phases, Witness, Pre enrichment and Selective enrichment
Source: Self Made

Finishing the microbiological study in the shell, it is destroyed, its thickness is measured and the same procedure is repeated with the samples of white and yolk.

Multiple tubes for shell, white and yolk

To perform this method, a phosphate buffer was prepared, according to NMX-F-286-1992, which was sterilized at 120 ° C. Three test tubes were prepared with 9 ml of phosphate buffer, a 1000 µl aliquot of the sample previously incubated with cystine selenite was added. Subsequently, the same procedure was repeated with the tube already homogenized, taking an aliquot of 1000 µl of the tube and poured into a new test tube with 9 ml phosphate buffer, serial dilutions 0.1, 0.01 and 0.001 were prepared. Subsequently, they were incubated at 37 ° C for 24 hours. The optical density (OD) of each suspension at a wavelength of 620 nm was determined. Calibrating the device with a phosphate buffer blank. The measurements were taken every hour, being a total of 4 readings.

Pouring on plate

The solid medium (bright green agar) was prepared and sterilized at 121 ° C. The plates were subsequently prepared under laminar flow hood (Figurine Industry Mod. CFH-90). Samples with selenite were diluted 0.1 ml in phosphate buffer tubes.

This dilution allowed to obtain a better concentration of the sample and to observe quantifiable colonies. Once the bright green bile agar solidified, a 10 µl aliquot of the previously diluted sample was taken and poured into the Petri dishes. The cultures were incubated at 37 ° C for 24 hours.

If after 24 hours of incubation, no growth is observed, another 24 hours are expected, according to NOM-210-SSA1-2014. After the growth of the plaque bacteria, the colonies that were grown were counted, then the colonies that developed on the plates were counted; The following equation was used to determine the UFC Colony Formation Units / ml:

$$\frac{ufc}{ml} = \frac{number\ of\ plaque\ colonies}{dilution\ factor\ x\ plate\ volume}$$

ISSN-On line: 2414-8849

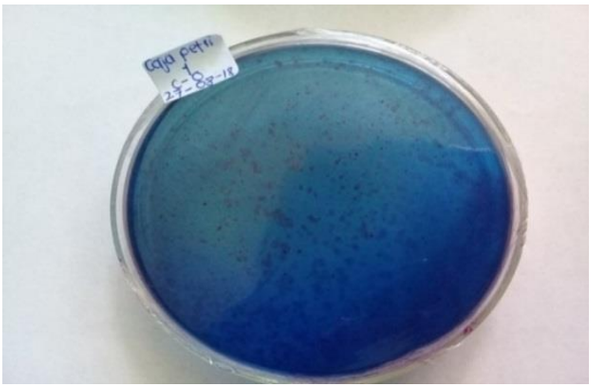


Figure 2 Plate incubated for 24 hours, in which colonies are observed
Source: Self Made

Results and Discussion

Physical analysis of organic, industrial and backyard type eggs

The results of the variance analysis did not show significant statistical differences (P≤0.05) in the volume, surface and weight parameters. The volume of the three types of eggs is statistically similar, but in the backyard egg there is a notable variability in the standard deviation, the same happens in the surface parameter. Regarding the recorded weight of the different types of eggs, it is observed that they are not significantly different, according to the Tukey mean comparison test (P≤0.05).

The thickness of the shell in the three types of egg production systems showed significant statistical differences (P≤0.05). The backyard eggs showed a greater thickness of the shell, while the organic eggs showed a lower thickness, those that presented an intermediate value were the eggs from the industrial production system.

In a study conducted by Hernández-Bautista et al., (2013) the properties of physical parameters of the eggs of 4 lines of hybrid hens were evaluated, of which 40 were housed in individual cages where food was provided and 80 were raised When fully grazing, a similar behavior in the parameters of egg weight and egg diameter was observed in these two systems, which may be due to the age of the birds. This explains the data that were obtained in the analysis of variance and the variability that is observed in the backyard eggs when not knowing exactly the age of the hen.

The aforementioned study also points out that the eggs of grazing chickens showed a better shell thickness, possibly due to the lower laying rate of grazing hens favors the increase in shell thickness.

In the analysis of variance with respect to the thickness of the shell of the organic type egg reflects a smaller thickness of the shell according to Muriuri and Harrison (1991). The high ambient temperature increases the chicken's body temperature by reducing the quality of the shell (shell thickness). According to Roland (1977) in the United States, the total egg produced by shell problems (thin shell and fractured) is not collected mainly in the southern states. The organic type eggs that were manipulated in the experimental phase are from organic farms in Mexico located in the north of the country with a warm climate that reduces the quality of the shell, which produces a smaller thickness.

According to Muriuri and Harrison (1991), the high ambient temperature reduces the quality of the shell. The organic type eggs manipulated for the experimental phase are from ecological farms in northern Mexico with a warm climate that reduces the quality of the shell that produces a smaller thickness of it.

Egg ovoscopy of three production systems

The technique used to observe the state of eggs is ovoscopy.

The results obtained in the analysis of variance in table 1, showed no statistically significant differences (P≤0.05). The air chamber in the three types of production systems is statistically equal, only a notable variability in the standard deviation in the backyard egg is observed.

Type of egg	Air chamber (cm)
Industrial	2.492±0.373 [a]
Organic	2.584±0.719 [a]
Backyard	2.474±2.479 [a]

* Means with the same letter in each row are not significantly different according to the Tukey mean comparison test (P≤0.05)

Table 1 Determination of the air chamber in industrial egg, organic type and backyard

ISSN-On line: 2414-8849

According to the previous data, no statistically significant differences were shown (P≤0.05) but with a wide variability. The variability observed in the backyard egg can be due to or attributed to the fact that they have a lower laying rate and therefore, the way in which the eggs are collected is different compared to the industrial eggs Juárez and Ochoa (1995).

Regarding the fractures in the ovoscopy of the industrial and backyard eggs, no fractures were observed while in the organic type eggs there was a percentage of 9.37% of fractures, this is consistent with the previous results in the physical tests. of the shell thickness where a smaller thickness is observed with respect to the other egg production systems, consequently a greater fragility.

Microbiological analysis in eggshell

To determine the microbiological load in eggshell, first a pre-enrichment was previously described in the methodology. The results of the analysis of variance showed significant statistical differences (P≤0.05) between the microbiological load observed in the different types of eggs evaluated. Table 2.

For the 0.1ml dilution it was observed that the industrial eggs were the ones with the lowest microbiology load (0.117 absorbance units), while in the backyard eggs and organic eggs the highest values of this variable were recorded (0.421 and 0.368 units , respectively).

With respect to the 0.01 dilution, significant differences were also observed (P≤0.05) between the three types of eggs, the industrial type again being the one with the lowest microbiological load value, while the backyard eggs maintained the highest concentration for this treatment; in the case of organic type eggs, these recorded an intermediate microbiological load in relation to the previous types.

Finally, for the dilution to 0.001 the industrial type eggs showed the highest values of microbiological load with respect to the organic and backyard types that were statistically equal according to the Tukey test (P≤0.05).

Dilution (ml)	Type of Egg * (Absorbance Units)		
	Backyard	Organic	Industrial
0.1	0.421±0.148 a	0.368±0.078 a	0.117±0.0155 b
0.01	0.053±0.014 a	0.048±0.010 ab	0.028±0.0051 b
0.001	0.011±0.004 b	0.012±0.006 b	0.038±0.0275 a

* Means with the same letter in each row are not significantly different according to the Tukey mean comparison test (P≤0.05).

Table 2 Determination of the microbiological load by DO620 spectrophotometry for organic, industrial and backyard eggs

According to the previous results, the lower concentration of microorganisms in industrial-type eggs can be attributed to the hygiene conditions with which this type of production system is managed, since it is characterized by a total control of food, health and cage care, as well as the handling of bird times and movements to avoid stress during the laying season (SAGARPA., 2009).

On the other hand, the management and control in the production of organic type egg and backyard is less strict, since in both cases the flocks of hens are freely grazed and there is little control over the laying sites. According to Cuca-García et al., (2011).

Microbiological analysis in egg white and yolk

The determination of the microbiological load of the white and yolk was made starting with a pre-enrichment of the sample, following with an enrichment in a selective medium such as selenite cystine incubated for 24 hours and consequently the realization of multiple tubes to determine the optical density at a wavelength of 620nm.

Table 3 shows the results of the analysis of variance showed significant statistical differences (P≤0.05) between the microbiological load observed in the white and yolk of the different types of eggs evaluated. For dilution 0.1 the analysis of variance showed no statistically significant differences (P≤0.05) between the three types of egg, the microbiological load of the backyard egg and organic type showed great variability.

In the 0.01 dilution, statistically equal results were observed according to the Tukey test (P≤0.05).

ISSN-On line: 2414-8849

Finally, in the 0.001 dilution, statistically significant results were shown (P≤0.05), the highest microbiological load in this dilution was the industrial one (0.0490 ± 0.034 a), the one with the lowest microbiological load was the organic one (0.0122 ± 0.005 b) while the backyard was at an intermediate point (0.0168 ± 0.031 ab)

Type of Egg * Absorbance Units			
Dilution	**Backyard**	**Organic**	**Industrial**
0.1	0.3229±0.283 a	0.2274±0.156 a	0.0626±0.023 a
0.01	0.0416±0.033 a	0.0639±0.090 a	0.0306±0.006 a
0.001	0.0168±0.031 ab	0.0122± 0.005 b	0.0490±0.034 a

* Means with the same letter are not significantly different according to the Tukey mean comparison test (P≤0.05)

Table 3 Determination of the microbiological load in egg white and yolk by DO620 spectrophotometry for backyard, industrial and organic type eggs

According to the results discussed above; no statistically significant differences were shown (P≤0.05) but it was observed in the backyard egg and organic type there is great variability in the standard deviation. According to Mancera (2005) in a study conducted in Mexico City to identify Salmonella enteritidis in eggs for consumption. 131 isolates considered in 12 different bacterial genera were obtained:

Acinetobacter spp., Alcaligene spp., Bacillus spp., Branhamella spp., Edwardsiella spp., Hafnia spp., Klebsiella spp., Serratia spp., Shigella spp., Staphylococcus spp., Yersinia spp., obtaining a strain of Salmonella enteritidis, which was classified as not typifiable. This would partially explain the results obtained in Table 3 where similar microbiological loads were observed in the 0.1ml and 0.01ml dilutions of the three egg production systems, which does not mean that the inside of the egg is contaminated with Salmonella spp, it is possible There are also other types of bacteria that are not pathogenic. In this regard, the results of the analysis of variance of the microbiological load of the exterior and interior of organic type eggs did not show significant statistical differences (P≤0.05), in the results of the microbiological load of the shell. In the results of the white and yolk the results of the analysis of variance showed significant statistical differences (P≤0.05), the lowest microbiology load was found in dilution 0.1 (0.2274 ± 0.15 b).

	Dilution		
	0.1	**0.01**	**0.001**
Shell	0.3679±0.07 a	0.0476±0.01 a	0.0.117±0.006 a
Clara and Yema	0.2274±0.15 b	0.0639±0.09 a	0.0122±0.005 a

* Means with the same letter are significantly different according to the Tukey mean comparison test (P≤0.05)

Table 4 Microbiological load outside and inside of organic egg by spectrophotometry DO_{620}

This can be attributed to the fact that hens that are raised in this type of production have access to the outside. Access to pasture, despite not being specifically required for organic birds, allows birds to forage in search of plants, insects, and generally results in better health (Baier, 2015). Preventive practices and healthy living conditions, such as keeping feeding systems and drinking fountains clean, are critical for reducing diseases and the presence of pathogenic organisms such as Salmonella spp and E. coli (Baier, 2015).

	Dilution		
	0.1	**0.01**	**0.001**
Shell	0.4207±0.14 a	0.0532±0.01 a	0.0115±0.004 a
Clara and Yema	0.3229±0.28 a	0.0416±0.03 a	0.0168±0.031 a

* Means with the same letter are not significantly different according to the Tukey mean comparison test (P≤0.05)

Table 5 Microbiological load outside and inside backyard egg by spectrophotometry DO_{620}

The comparison of the internal and external bacterial growth of the backyard eggs was made from the dilutions shown in Table 5. The optical density was determined by spectrophotometry at a wavelength of 620 nm. The results of the analysis of variance did not show significant statistical differences (P≤0.05) between the microbiological load inside and outside the backyard egg and the corresponding dilutions. The above data can be attributed to the fact that under backyard conditions there is little or no sanitary management, the backyard bird feeding consists of what the birds can collect as leaves, tender herbs, fodder, insects, food leftovers, fruits and tortilla which should be the day to avoid digestive diseases (Cuca-García et al., 2011). According to a study conducted in Ecuador in the city of Loja, the presence of salmonella spp. In backyard chicken eggs marketed in different parts of that city, the presence of Salmonella spp.

ISSN-On line: 2414-8849

In the microbiological analysis of suspicious samples, the confirmation disc was applied, its result being negative, despite this the egg if it contains a microbiological load in this study and genotyping was no longer continued. The results shown in table 5 present a microbiological load, this does not mean that it is salmonella spp. if there are other microorganisms present in the sample, which can cause interference, for example, between gram negative and positive species, multiple forms of the O chain or O antigen can be found, it is very variable in its composition between different families, species and even within the same species of gram-negative bacteria. However, despite the specificity of the antibodies (responsible for recognizing the component of interest) for a particular antigen (component of interest), nonspecific binding with other bacteria can occur.

There are studies that indicate that this phenomenon in gram-negative bacteria may be due to the antigenic similarity of lipid A among gram-negative bacterial species (Mutharia, 1984). The microbiological load of the exterior and interior of the industrial egg was determined by spectrophotometry, whose optical density was measured at a wavelength of 620 nm.

The results of the analysis of variance showed significant statistical differences (P≤0.05) in the microbiological load of the shell in the 0.1ml dilution (0.1170 ± 0.015 a) the highest microbiological load was shown while in the others they were of lesser load and similar . In the interior of the industrial egg in the white and yolk, no significant differences were found according to the Tukey mean comparison test (P≤0.05).

	Dilution		
	0.1	**0.01**	**0.001**
Shell	0.1170±0.015 [a]	0.0285±0.005 [b]	0.0382±0.027 [b]
Clara and Yema	0.0626±0.023 [a]	0.0306±0.006 [a]	0.0489±0.034 [a]

* Means with the same letter are not significantly different according to the Tukey mean comparison test (P≤0.05).

Table 6 Microbiological load outside and inside of industrial egg by spectrophotometry DO_{620}.

Table 6 of the microbiological load of the industrial egg showed statistically significant differences in the shell according to the Tukey mean comparison test (P≤0.05).

In a study carried out in Mexico City on consumption eggs (Industrial) Of the 400 samples taken from different companies, 131 bacterial isolates were obtained, 88 being yolk (67%), 26 of white (20%) and 17 of shell with membranes (13%).

Comparison of the number of colonies present in plaque by the method of pouring in plaque inside and outside the egg

The present comparison was obtained from the realization of multiple tubes using only the 0.1 ml dilution. This dilution was made to have a better concentration of the culture, obtaining isolated colonies that can be quantified and proportional to the start culture, consequently a medium of solid culture (bright green agar) to manipulate it in the plate pouring process according to NOM-114-SSA1-1994, this bright green agar allows quantifying the colonies present in the culture. The entire procedure was handled in the laminar flow hood to avoid cross contamination. The results presented in table 7 did not show statistically significant differences (P≤0.05). The three types of eggs have a statistically equal number of colonies, but a standard deviation with a wide variability in the two internal and external variables (shell, white and yolk) in backyard eggs.

	Type of egg		
	Organic type	**Backyard**	**Industrial**
Shell	96.28±85.79 [a]	112.09±85.09 [a]	18.33±9.46 [a]
Yolk and white	105.96±64.06 [a]	114.00±105.82 [a]	22.50±23.85 [a]

* Means with the same letter per row are not significantly different according to the Tukey mean comparison test (P≤0.05).

Table 7 Determination of the number of colonies present in each production system

The results showed no statistically significant differences (P≤0.05). All three types of eggs have a statistically equal number of colonies. About industrial eggs, work was found on the type of microorganisms present in the interior and exterior (shell, white and yolk) while in the organic types no work was found on microorganisms present in them, only their physical and sensory properties. In a study by Neira Solís (2016) About the microbiota in eggs and derivatives: identification and development, in this work we studied two production systems the backyard and the industrial.

ISSN-On line: 2414-8849

Commercial eggs showed great variability in the shell microbiota, being higher in backyard eggs than in industrial eggs, in no case was a genus of salmonella spp found, with respect to transport through the shell it was observed that microorganisms tend to adhere to the surface of the egg and can easily cross the natural protective barriers and thus multiply inside the egg, in the yolk the microorganisms find a liquid medium where they multiply easily, for this reason in the white and yolk there is a greater number of colonies than in the shell

Comparison of the number of colonies per plate discharge at different collection points

The present comparison of numbers of colonies in organic eggs from different collection points was obtained from the 0.1 ml dilution. This dilution was made to have a lower and better concentration of the culture, then a solid culture medium was made to analyze the samples on account. viable (plate pouring technique) according to NOM-114-SSA1-1994, the bright green agar (solid medium) allows quantifying the colonies present in the culture. All the analysis was performed under laminar flow hood to avoid cross contamination. This comparison was made to identify the collection point with the highest risk of contamination.

The results of the analysis of variance showed statistically significant differences in the eggshell, the highest value of the number of colonies occurred in the Finca de Chilcuahutla4 (202.50 ± 3.54 a) and the lowest in the Finca de Guayacán (10b). Regarding Clara and yolk, the analysis of variance did not show statistically significant differences, but with a notable variability in the Chilcuahutla Estate (125 ± 141.42 a).

Collection Points				
Variables	**Chilcuautla Farm**	**Jalisco**	**Huatusco**	**Guayacán Estate**
SHELL	202.50±3.54 [a]	186±91.5 [a]	48.38±27.9 [b]	10 [b]
WITHE AND YEMA	125±141.42 [a]	124.4±50.43 [a]	105.0±67.64 [a]	78.75±49.8 [a]

* Means with the same letter per row are not significantly different according to the Tukey mean comparison test (P≤0.05).

Table 8 Comparison of the number of organic type colonies in the differentcollection points

In the organic type system of the shell variable there is variability between the collection points as it has a high value and a very low value, the Guayacán Estate has an organic certification provided by bio-agricert Annex1.as well as the Jalisco for the certification of cage free hen. The Finca Chilcuahutla is certified by Rainforest and Amsa Starbooks despite this it has a high number of colonies.

In the backyard eggs there was great variability in the different collection points. In the case of the shell, it did not show statistically significant differences (P≤0.05). The data are statistically equal, the collection point of Atlanca presented a greater number of colonies (238.50 ± 54.45 a) but at the same time the various points presented very high standard deviations.

In the case of the white and yolk the results of the analysis of variance showed significant statistical differences (P≤0.05). The one with the highest number of colonies was Tehuipango (242.50 ± 10.61 a), the ones that indicated the lowest number of colonies were Mercado de Potrerillo (10 bc) and Chalchiltepec (20.00 ± 7.07 c). The results obtained in table 9 show collection points with a number of colonies that are too high and others that are too low or zero, this variability was presented in the clear and yolk of the different collection points, this can be attributed to the care that each place gives birds including feeding and laying sites.

Collection points (Number of colonies)							
Variables	**Atlanca**	**Tenexcalco**	**Tehuipango**	**Potrerillo Market**	**Coauixtlahua**	**Chalchiltepec**	**Acatepec**
Shell	238.50 ± 54.45 [a]	187.50± 17.68 [a]	112.17± 99.41 [a]	99.00 [a]	83.33± 80.36 [a]	56.25±58.34 [a]	47.83± 48.18 [a]
Clara and yolk			242.50±10.61 [a]	10.00 [bc]	212.50± 88.39 [ab]	20.00± 7.07 [c]	60.00±39.69 [bc]

* Means with the same letter per row are not significantly different according to the Tukey mean comparison test (P≤0.05).

Table 9 Comparison of the number of backyard egg colonies

Conclusions

The three egg production systems that were compared showed statistically equal physical parameters, such as volume, surface and weight. Only the shell thickness parameter was different, the one with the smallest shell thickness was the organic type egg. In the microbiological analysis of the shell of these egg production systems, a lower microbiological load was observed in the industrial eggs, while in the backyard and organic type eggs the microbiological loads were similar. Backyard eggs showed great variability in the standard deviation.

ISSN-On line: 2414-8849

In the case of the microbiological analysis of the yolk of the three production systems, no statistically significant differences were shown. The three production systems indicated similarities in the microbial load. Organic and backyard type eggs recorded a wide variability in the standard deviation, this happened because they had too many values dispersed with respect to the average, they included quite low and very high values.

In the comparison of the number of colonies present in plaque by the method of pouring in plaque of the interior and exterior (shell, white and yolk) of the egg the results that were observed in the production systems were statistically equal, with a notable variability in egg Backyard and Organic this is attributed to the fact that in some collection points no colonies were found, in others low values were located while some had very high values. The white and yolk in the different production systems presented a higher value of the microbial load than the shell, the yolk is a nutrient rich medium where microorganisms multiply easily.

Organic type eggs have a low microbial load, the birds are cared for so that the eggs have a lower load or there is no microbial load, there are rules to take care of this type of system but not all ecological farms meet these requirements, for For this reason, farms were found where there were eggs with a high number of colonies and with a low or zero value, another factor that affects this type of egg was the fractures that were observed as it could be attributed to the presence of microorganisms.

Something similar happened with the Traspatio eggs, although they are higher than the organic ones, they are statistically equal, since they show variability between the collection points. This helps us to know the state of each production system, with this we can conclude that the Traspatio system can be improved, to reduce the microbiological load since at several collection points there was no microbial load or it was low everything depends on the care and Chicken feed. With the aforementioned we can improve the Backyard system to help the economy of families that depend on this practice. Until now, the amount of microorganisms present in the types of eggs consumed in the state of Veracruz had not been determined, particularly for organic and backyard production systems.

References

Baier, A. (2015). *Hoja de Datos: Producción de Aves Orgánicas para Carne y Huevos.* Estados Unidos: Especialista en Agricultura NCAT.

Carbajal, A. A. (2006). Calidad nutricional de los huevos y relación con la salud. *revista de nutricion practica*, 73-76.

Cuca-García J.M.1, G.-A. D.-P. (2011). Producción y Manejo de Aves Domesticas. *Universidad Autónoma Chapingo*, 237.

Juárez, A. y. (1995). Rasgos de producción de huevo y calidad de cáscara en gallinas criollas de cuello desnudo, en climatropical. . *Archivos de Zootecnia.*, 79-84.

Mancera Martínez Arturo, J. V. (2005). Identificación de Salmonella Enteritidis en huevo para consumo en la ciudad de México. *Técnica Pecuaria en México*, 229-237.

Méndez, I., Badillo, C., Ortíz, G., & Faccini, A. (2011). Caracterización microbiológica de Salmonella en alimentos de venta callejera en un sector universitario de Bogotá. *Médicas UIS*, 24:26-33.

Minorl., L. A. (1984). *Serology of salmonella.* london: bergman.

Muriuri, H. K. (1991). Effect of peripheral foot cooling on metabolic rate and thermoregulation on fed and fasted chicken hens ina hot environmet. *Poultry Sci.*, 74-79.

Mutharia, L. C. (1984). Monoclonal Antibodies Specific for Escherichia coli J5 Lipopolysaccharide: Cross-Reaction with OtherGram-Negative Bacterial Species. . *American Society for Microbiology, Infection and Immunity* , 631-636.

Narushin, V. (2005). Production, Modeling, and Education Egg Geometry Calculation using the Measurements ofLength and Breadth. *Poultry Science*, 482-489.

Neira Solis, C. (2016). *Microbiota en huevos y derivados: identificacion y desarrollo.* oviedo, españa.

Organizacion Mundial de la Salud, O. (septiembre de 2017). Obtenido de www.who.int/mediacentre/factsheets/fs139/es/

ISSN-On line: 2414-8849

Roland, D. (1977). The extent of unconllected eggs due to indadecuate shell. *Poultry Sci.*, 1517-1521.

SAGARPA, S. (1ª Edición, 2009). Manual de Buenas Prácticas Pecuarias Producción de Huevo para Plato. *Subdirección de Promoción y Regulación de Inocuidad Pecuaria*.

Suárez, M. y. (2000). Presencia de Salmonella serovariedad Enteritidis en productos de origen avícola y su repercusión en salud pública . *Iatreia*, 13,238-245.

Union Nacional de Avicultores, U. (2018). *Situacion de la avicultura Mexicana.* http://www.una.org.mx/index.php/panorama/situacion-de-la-avicultura-mexicana.

ISSN-On line: 2414-8849

Article **ECORFAN Journal Republic of Guatemala**
June 2019 Vol.5 No.8 27-50

Effects of stereotactic surgery on the anterior hypothalamus (HA) on the estrous cycle: Role of the dopaminergic system in spontaneous ovulation in the rat

Efectos de la cirugía estereotáxica de abordaje al hipotálamo anterior (HA) sobre el ciclo estral: Papel del sistema dopaminérgico en la ovulación espontánea en la rata

MORÁN-PERALES, José Luis†*, SÁNCHEZ-GARCÍA, Octavio, GARCÍA-SUÁSTEGUI, Wendy Argelia and HANDAL-SILVA, Anabella

Benemérita Universidad Autónoma de Puebla-Departamento de Biología y Toxicología de la Reproducción, Instituto de Ciencias

ID 1st Author: *José Luis, Morán-Perales* / **ORC ID**: 0000-0002-2823-2829, **Researcher ID Thomson**: S-5803-2018**, arXiv Author ID**: doctor_moran, **PubMed Autor ID**: moranperales, **CVU CONACYT ID**: 207096

ID 1st Coauthor: *Octavio, Sánchez-García* / **ORC ID**: 0000-0002-2710-8084, **Researcher ID Thomson**: S-6739-2018, **PubMed Autor ID**: sanchezgarcia, **CVU CONACYT ID**: 367319

ID 2st Coauthor: *Wendy Argelia, García-Suástegui* / **ORC ID**: 0000-0001-5223-3189, **Researcher ID Thomson**: S-6831-2018, **PubMed Autor ID**: garcia-suastegui, **CVU CONACYT ID**: 48932

ID 3st Coauthor: *Anabella, Handal-Silva* / **ORC ID**: 0000-0002-6915-5655, **Researcher ID Thomson**: S-6799-2018, **PubMed Autor ID**: anabellahandal, **CVU CONACYT ID**: 210819

DOI: 10.35429/EJRG.2019.8.5.27.50 Received March 10, 2019; Accepted June 08, 2019

Abstract

We evaluated the function of dopaminergic receptors (DAR) of the anterior hypothalamus (AH) on the estral cycle (EC) regulation and spontaneous ovulation by a single microinjection (MI) with the dopaminergic antagonist haloperidol (HLP) in adult rats. One hundred thirty nine rats that exhibit forth-day estral cycles (cyclic animals: CA) received a stereotaxic surgery (STXS) on the right, left or both AH sides and were distributed in three different groups with a MI of 1 µL of: HLP (15 µg) or dimethyl-sulfoxide (vehicle) or other false MI group. All the animals with STXS were sacrificed in next vaginal estrus (VE) exhibited and the ova shed (OS) counted. In sixteen AC, the OS were counted at VE and forming a control group. The STXS affected the animals EC: just 59/139 exhibited a short EC (SEC) with 4.6±0.1 days compared with 80/139 that exhibited a long EC (LEC) of 13.6±0.2 days. False or HLP MI diminished OS just in animals exhibiting a SEC. STXS affects neuroendocrine processes controlling EC length when cutting dorsal connections to AH. The DAR of the AH participate on ovarian mechanisms of follicular selection.

Anterior hypothalamus, Stereotaxic Surgery, Hypothalamic dopaminergic system

Resumen

Se evaluó el antagonismo de los *receptores a dopamina* (RDA) en el *hipotálamo anterior* (HA) en la regulación del ciclo estral (CE) y la ovulación espontánea mediante la *microinyección* (MI) de *haloperidol* (HLP) en ratas adultas. Ciento treintainueve ratas con CE regular de cuatro días (*animales cíclicos*: AC), recibieron *cirugía estereotáxica* (CETX) en el lado derecho, izquierdo o en ambos lados del HA y se distribuyeron en tres grupos con: 1µL MI de HLP (15µg) o dimetilsulfóxido puro (vehículo) y otro grupo con MI falsa. Todos los animales con CETX se sacrificaron en el siguiente *estro vaginal* (EV) y se contó el *número de ovocitos liberados* (NOL). Como grupo control, en 16 AC se les contó el NOL al EV. La CETX afectó el CE de los animales: solo en 59/139 se presentó un *CE Corto* (CEC) de 4.6±0.1 días, pero en 80/139 la duración del *CE Largo* (CEL) fue de 13.6±0.2 días. Las MI falsas y con HLP disminuyeron el NOL únicamente en los animales que presentaron CEC. La CETX afecta procesos neuroendocrinos que controlan la duración del CE cuando se seccionan vías de conexión dorsales al HA. Los RDA del HA inciden en mecanismos ováricos de selección folicular.

Hipotálamo anterior, Cirugía Estereotáxica, Sistema dopaminérgico del hipotálamo

Citation: MORÁN-PERALES, José Luis, SÁNCHEZ-GARCÍA, Octavio, GARCÍA-SUÁSTEGUI, Wendy Argelia and HANDAL-SILVA, Anabella. Effects of stereotactic surgery on the anterior hypothalamus (HA) on the estrous cycle: role of the dopaminergic system in spontaneous ovulation in the rat. ECORFAN Journal-Republic of Guatemala. 2019, 5-8: 27-50

* Correspondence to Author (email: moranperales@yahoo.com.mx)
† Researcher contributing first author.

Introduction

The reproductive functions are the result of the integration of multiple phenomena in which the organs and systems of the body participate, where the nervous system is the main leader of the organism. Its role is essential because the application or suppression of various stimuli: surgical, pharmacological, mechanical, electrical or even biological, in organisms, invariably affect the nervous information and the ability of the organs to respond to them. Thus, it has been widely shown that the nervous system plays a crucial role in the control of reproductive function. In the female, the release of viable gametes is a cyclic event that is subject to nervous control and in turn, immersed in a sequence of immune, neuroendocrine and endocrine phenomena that lead to ovulation (Erickson, 1995).

Gonadotropin secretion is regulated by gonadotropin-releasing hypothalamic hormone: GnRH, whose secretion is controlled by different neurotransmitter systems: amino acids (glutamate, aspartate, glycine, amino-butyrate), amines biogenic (adrena-lina, norepinephrine, serotonin, dopamine and histamine), acetylcholine and various neuro-peptides (opioids, encephalin, substance P, neuropeptide Y, vasoactive intestinal peptide, angiotensin II, among many others) (Kordon et al, 1994) . It has been widely documented that these neuronal communication systems are modulated by the action of sex steroids produced in the gonads (Fink, 1988).

In rodents, the neurons that secrete GnRH are preferentially located in the septal-preoptic-suprachiasmatic region and in the medial-basal hypothalamus (McCann et al, 1978). According to the information available, the participation of the various neurotransmission systems in the regulation of GnRH secretion varies during the estrous cycle because during this cycle the production of sexual steroids is also variable (Miyake, 1988).

We have shown that dopamine is a key signal for the regulation of the rat estrous cycle, since the antagonism of its receptors, at the systemic level (Domínguez et al, 1987), locally within the anterior hypothalamus (Morán & Domínguez, 1995) or within the ovary (Venegas et al, 2015), it is able to offset the estrous cycle and induce a delay in the spontaneous ovulation of the rat.

Justification

Although, our group has worked in recent years analyzing the role of the ovarian dopaminergic signal (Letras et al, 2016; González et al, 2016; Guzmán Herrera, 2018; Venegas Meneses et al, 2017) it remains to be clarified what it is the functional role of dopamine that projects towards the rostral areas of the rat's hypothalamus and that probably participates in the control of gonadotropin secretion and ovarian function at different times of the estrous or ovarian cycle. Therefore, the present work shows the effects of the surgical approach to the anterior hypothalamus in order to analyze the role of dopamine through the pharmacological antagonism of its receptors in this brain region, where the phasic discharge of GnRH is controlled. The works of our group mentioned above, show the important participation of this chemical signal in the control of reproductive function and indicate that attention should be paid to the hormonal status of the female, since it is clear that the function of dopamine is variable throughout the reproductive cycle. Experimental evidence leads us to the reflection of taking into account that when the role of central and peripheral dopaminergic systems in reproductive function is analyzed, attention is required at the moment in which the function of dopamine will be studied. Thus, in the present study we sought to carry out a research work focused on the analysis of dopaminergic antagonism in the anterior hypothalamus on each day of the rat estrous cycle.

Problem

The surgical technique used in our experimental models to place implants (Morán & Domínguez, 1995; Morán & Domínguez, 1997) or to perform micro-injections of dopamine antagonists in the anterior hypothalamus (Méndez & Morán, 2001b), poses the methodological difficulty for to ensure that the animals managed to maintain an intracerebral guide cannula that had to remain in the skull throughout the experiment and in addition to taking into account a period of several days for the adaptation of the animals to said cannula, in this work it was decided to reevaluate the Role of dopamine more simply, supported by a high-precision microinjection technique to administer the dopamine antagonist haloperidol on the right and / or left side of the anterior hypothalamus of adult rats that exhibited regular four-day estrous cycles (cyclic animals) .

ISSN-On line: 2414-8849

The purpose of the present work was to induce the blocking of dopaminergic receptors one or both sides of the anterior hypothalamus and analyze how spontaneous ovulation is affected and the duration of the estrous cycle, as an indirect indicator of gonadotropin secretion and thus, confirm whether the dopaminergic system of the anterior hypothalamus has a lateralized involvement on the day of estrus.

Objectives

General objective

To analyze the effects of local microinjection on the right, left, or both sides of the anterior hypothalamus of the dopamine receptor antagonist haloperidol on gonadotropin secretion, estimated by the duration of the estrous cycle, and spontaneous ovulation of the adult rat.

Specific objectives

To analyze the effects of local haloperidol microinjection on the right, left or both sides of the anterior hypothalamus at 1:00 p.m. on estrus on the duration of the estrous cycle in adult rats with regular cycles of four days duration.

To analyze the effects of local haloperidol microinjection on the right, left or both sides of the anterior hypothalamus on spontaneous ovulation in the same animals.

Theoretical framework

In the rat, the hypothalamus occupies the ventral half of the diencephalon; It constitutes about 10% of the volume of the brain and contains a large number of circuits that contribute to the maintenance of body homeostasis (Kupfermann, 1985). It extends laterally from the walls of the third ventricle to the lateral hypothalamic grooves. It is delimited face-caudally by the optic chiasma (anterior limit) and by the mammillary bodies (posterior limit) (Simerly, 1995).

Taking the third ventricle as a reference, three regions have been described: periventricular, lateral and medial. The periventricular region is that which surrounds the third ventricle.

The lateral one extends from the descending columns of the fornix to the prosencephalic medial fascicle, the medial is located in the middle of the other two regions and contains most of the neuronal clusters called nuclei (Simerly, 1995).

Based on the distribution of its nuclei, the hypothalamus is divided into three areas: anterior, middle and posterior. The anterior hypothalamus contains the preoptic (medial and lateral), suprachiasmatic, supraoptic and anterior nuclei. The middle hypothalamus is formed by the medial, ventromedial, arcuate, tuberal and periventricular nuclei. The posterior hypothalamus contains the mammary nuclei (premamillary, lateral, medial, supramamilar, and intercalated and posterior) (Simerly, 1995).

GnRH is a decapeptide whose primary structure is: PiroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2; It is stored in the nerve terminals that converge in the middle eminence. Under the action of certain stimuli, the neurohormone is released into the blood capillaries of the primary plexus of the hypothalamic-pituitary portal system (Barry et al, 1985; Palkovits, 1980). Its release is given in response to action potentials that culminate with the entry of extracellular calcium through voltage-regulated and ligand-regulated Ca ++ channels (Fink, 1979 and 1988).

Once GnRH is released into the hypothalamic-pituitary portal system, the decapeptide binds to specific receptors in the gonadotropic membrane (Silverman, 1988). This hormone-receptor complex triggers the synthesis of second messengers that promote the synthesis and secretion of gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Conn) , 1994; Feder, 1981; Fink et al, 1983).

In the rat and other small mammals, GnRH is synthesized by specialized hypothalamic neurons (GnRH-ergic neurons) that are predominantly located in the preoptic, anterior hypothalamus and middle hypothalamus regions (McCann et al, 1978). The axons of these GnRH-ergic neurons project towards the middle eminence where the peptide is stored and released under the indirect action of sex steroids that influence different neurotransmitter systems (Kordon et al, 1994).

ISSN-On line: 2414-8849

The hormone is released into the primary plexus vessels of the hypothalamic-pituitary portal system and transported by blood to the adenohypophysis (Pierce, 1988). In the female rat, the pattern of GnRH secretion varies during the estrous cycle as does the population of its receptors (Miyake, 1988).

The synthesis and release of GnRH is modulated by the action of steroid hormones (estrogens, progestogens and androgens) and regulated by different neurotransmitters (amino acids, biogenic amines, acetylcholine and neuropeptides) (Fink, 1979; Kordon et al. 1994).

Numerous experimental evidence has shown the importance of the preoptic and hypothalamic anterior areas (generically referred to as the anterior hypothalamus hereafter) in the regulation of mammalian reproductive function (Barraclough & Wise, 1982; Barraclough, 1983; Clemens et al, 1976; Kalra, 1974; McCann et al, 1978).

In the rat, the electrolytic destruction of the anterior hypothalamus, covering the suprachiasmatic region, results in a lack of ovulation, the development of a polycystic ovary without luteal bodies and persistent vaginal estrus. This allowed us to suggest that the neural centers that regulate follicular development and ovulation are found in the anterior hypothalamus (Everett, 1939; Hillarp, 1949).

In another experiment, rats were used which were induced to block ovulation with an injection of sodium pentobarbital on the day of the proestrus. The electrical stimulation in the anterior hypothalamus of these animals resulted in the pre-ovulation gonadotropin release and induction of ovulation, an effect that was not observed in animals with pre-optic-tube section (Everett & Radford, 1961; Tajasen & Everett , 1967). These results suggest that the neural centers that regulate the release of LH, essential for ovulation to occur, are possibly located in the anterior hypothalamus.

In the hemiatrated animal, bilateral lesions of the anterior hypothalamic area or frontal deafferentation between the anterior pituitary and hypothalamic areas blocked compensatory ovarian hypertrophy (D'Angelo & Kravats, 1960; Hàlasz & Gorski, 1967).

These evidences suggest that the neural structures related to the increase in the plasma concentration of FSH, which would occur after hemicraction, are located outside the pituitary area and are probably located in the anterior hypothalamic area.

In the castrated female rat, deafferentation between the anterior hypothalamus and the arcuate nucleus results in the decrease in the plasma concentrations of FSH and LH, while in the intact animal said deaferentation does not block ovulation on the morning of estrus. vaginal, even when there are prolonged periods of right-handed. These results were accompanied by the decrease in GnRH content in the middle eminence (Kalra, 1974). Based on these studies, it has been postulated that the anterior hypothalamus is closely related to the tonic and cyclic release of gonadotropins and that the integrity of the nerve pathways that arrive at the hypothalamus from the anterior part is essential for normal estrales cycle regulation.

These results were corroborated in animals with bilateral lesions of the preoptic area (Clemens et al, 1976). In that study, the ovaries showed fresh luteal bodies as a sign of recent ovulation. When the lesion covered the anterior hypothalamic area, a state of persistent vaginal estrus was observed, with ovulation block and polycystic ovarian formation. Based on these results, it was suggested that nerve connections located in the anterior hypothalamus are necessary for the animal to have normal vaginal cycles and for ovulation to occur.

On the other hand, several studies have shown that the participation of the cholinergic and catecholaminergic systems in the regulation of endocrine and neuroendocrine mechanisms that culminate with ovulation varies during the rat's estrous cycle, which depend on the time and day of the estrous cycle in which these neurotransmission systems are disrupted (Dominguez et al, 1982, 1985 and 1987).

The doses of reserpine (inhibitor of the catecholamine recapture system) necessary to block ovulation are minimal during the days of estrus and right-handed 1; these doses must be doubled to obtain the same effect during the days of right-handed and pro-atrial (Dominguez et al, 1985).

ISSN-On line: 2414-8849

Likewise, the blockage of cholinergic systems with atropine (Dominguez et al, 1982), of the noradrenergic receptors with propranolol and dopaminergic with haloperidol (Dominguez et al, 1987) also produce differential effects on the processes that culminate with ovulation that depend on the phases of the estrous cycle in which neurotransmission is interrupted; In general, the effect of the drugs was more severe when they were administered during the first half of the estrous cycle.

Dopamine is an endogenous catecholamine whose role as peripheral and central neurotransmitter has been widely described (Tohyama & Takatsuji, 1998). Dopamine, at the peripheral level has great importance in the carotid body. Small, highly fluorescent cells are found along the paravertebral spine and regulate some of the cardiovascular functions of dopaminergic drugs. For example, the activation of dopamine receptors induces a positive ionotropic effect. Dopaminergic neurons also innervate the renal and gastrointestinal arteries where dopamine induces vasodilation. This action is coupled with the positive ionotropic action in the heart (Carvey, 1998; Litter, 1988).

Dopaminergic neurons that project towards the hypothalamus have their somas concentrated in four regions: A11, A12, A13, A14 (Bjöurklound & Nobin, 1973):

1) A_{11}: somas located in the posterior hypothalamus and the ventral thalamus; these somas are the main source of spinal dopaminergic innervation.
2) A_{12}: somas located in the arcuate nucleus (TIDA) and in the periventricular nucleus (THDA); project their axons to the outer line of the middle eminence and to the intermediate lobe of the pituitary gland.
3) A_{13}: somas located in the medial uncertain area; project their axons to the anterior hypothalamus, the dorsomedial nucleus of the hypothalamus and the posterior nucleus.
4) A_{14}: somas located in the rostral portion of the periventricular nucleus; project their axons to the preoptic, periventricular, suprachiasmatic and lateral septal nuclei.

The dopaminergic soma A11, A13 and A14 are generically referred to as incertohyotothalamic system (Bjöurkloud & Nobin, 1973; Bjöurkloud et al, 1975). Dopaminergic soma A13 and A14 reach areas of the hypothalamus that contain GnRH-ergic somas, so their functions could be involved with the control of the secretion of FSH and LH (Sanhera et al, 1991b).

There is a great diversity of dopamine agonist and antagonist drugs that can act more or less selectively on a type or subtype of receptor. Haloperidol is a drug belonging to the group of butyrophenones widely used as a neuroleptic agent in the treatment of some mental illnesses (Baldessarini, 1989). They are synthetic compounds whose fundamental structure consists of a chain of three carbon atoms attached to a ketone group which in turn is linked to a benzene group; This fundamental structure is called butyrophenone and in this there is a fluorine atom in the para position of the phenylketonic group (Figure 1).

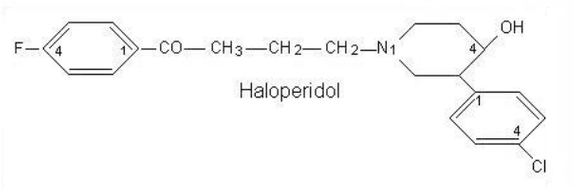


Figure 1. Structure of haloperidol, an antagonist of dopamine receptors. It binds to D1 and D2 receptors, although with greater affinity to the latter.

The action of butyrophenones in experimental animals causes a decrease in motor activity and at high doses produces a state of immobility and catalepsy. In the autonomic nervous system, butyrophenones have an α-adrenergic or sympatholytic blocking action and cause the relaxation of the nictitating membranes in the cat, decrease in blood pressure in rats and antagonize the pressor effect of adrenaline and norepinephrine. They also have anticholinergic action (Litter, 1988).

Its main physical characteristics are: white crystalline powder, odorless; slightly bitter; molecular weight of 375.88 g / mol; practically insoluble in water (0.02 mg / ml); soluble in acidic solutions (eg 3 mg / ml in 0.1N HCl) and in non-polar solvents (chloroform, ketone and benzene); completely soluble in ethanol, methanol and dimethylsulfoxide.

ISSN-On line: 2414-8849

Given its non-polar characteristics, it easily crosses the biological membrane so it can be easily absorbed through the skin. Haloperidol works by blocking dopamine receptors in the hypothalamus and especially in the pituitary gland at the level of the limbic system and the striatum (Litter, 1988).

Based on different pharmacological studies, haloperidol has been shown to interact antagonistically with both the receptors of the DA1 subtypes (with a relative potency in the order of μM) and those of the DA2 subtype (with a relative potency in the order of the nM).

Dominguez et al. (1987) evaluated the effects of a dopamine blocker at different stages of the estrous cycle on ovulation and the distribution of the follicular population of the ovaries of the adult rat. In this experiment, rats with regular four-day estrous cycles were used, which were injected with the haloperidol dopamine receptor antagonist (2.5 mg / kg weight; i.m.). Groups of animals received a single dose of the drug at 1:00 p.m. of estrus, right-handed, right-handed or proestro and all of them were sacrificed on the morning of the expected estrus.

Haloperidol blocked ovulation significantly when it was administered in estrus and right-handed but was less effective when applied in right-handed and 2-proestro; the follicular population of the ovaries showed a delay in their development and growth that depended on the day of the estrous cycle in which the pharmacological blockade of the dopamine receptors was performed, being more serious these alterations in the animals that received treatment in the first half of the estrous cycle.

These results allowed us to suggest that the dopaminergic information in the first half of the estrous cycle is essential for the sequence of neuroendocrine and endocrine events that culminate in ovulation to be carried out in a normal way. On the other hand, apparently in the second half of the estrous cycle the participation of this system is unclear, since only in half of the animals treated with the drug was ovulation blocked. These results were confirmed in a recent reevaluation, using a novel model of local ovarian antagonism (Venegas et al, 2015).

Morán & Domínguez (1995) showed that the unilateral blockade of dopaminergic receptors in the anterior hypothalamus on different days of the estrous cycle produces effects that depend on the phase of the cycle in which the pharmacological blockade of dopamine receptors is performed with haloperidol crystals In this experimental model, also the implant with haloperidol on the right or left side of the anterior hypothalamus performed at 1:00 p.m. of the estrus and right-handed was absolutely effective in blocking ovulation; The implant of the drug at 13:00 h of the right-handed 2 produced unclear results, since only 40% of the animals managed to ovulate, but unlike the study by Dominguez et al. (1987) described in previous lines, the implant Unilateral with haloperidol in the anterior hypothalamus performed at 1:00 p.m. of the proestro was ineffective in preventing ovulation.

These results allowed us to suggest that the dopaminergic information generated in this region of the hypothalamus during the first half of the estrous cycle is necessary for neuroendocrine and endocrine events that culminate with ovulation to be carried out properly and that there is a possibility that different dopaminergic systems are associated in the regulation of the estrous cycle and ovulation, since the effects of the central block in the second half of the rat estrous cycle are different from those of the systemic block.

Based on other experimental evidence, there is controversy about the involvement of the dopaminergic system in the neuronal activity of the hypothalamus nuclei directly related to the regulation of gonadotropin secretion. According to some authors, hypothalamic dopaminergic systems would exert an inhibitory effect on gonadotropic secretion (Choudhury et al, 1974; Ramírez et al, 1984; Tasaka et al, 1985), while for others it would be stimulant (Clemens et al, 1976; MacKenzie et al, 1988 and 1989; Sanhera et al, 1991; Schneider & McCann, 1970; Vijayan & McCann, 1978; Weiner & Ganong, 1978), or who do not participate significantly in this function (Sawyer & Clifton , 1980). From the results obtained in our laboratory it can be suggested that the differences observed in these studies are based on peculiarities of the experimental models used, since it has been commented that the functions of dopaminergic systems in ovulation control vary during the cycle estral (Domínguez et al, 1987; Morán and Domínguez, 1995).

ISSN-On line: 2414-8849

All these antecedents allow us to suggest that dopamine is a fundamental chemical signal for the control of various brain functions, however there is some controversy about its role in neuroendocrine mechanisms that are coupled to the functioning of the Hypothalamus-Pituitary-Ovary axis, particularly as regards its effect on the control of the secretion of GnRH, gonadotropins: FSH and LH, and ultimately on the ovarian mechanisms that lead to ovulation. We have mentioned in previous lines that much of the dopaminergic information related to the anterior hypothalamus comes from the medial uncertain area of the thalamus. Their somas are located in areas A11, A13 and A14 and express ovarian steroid receptors that regulate the synthesis of dopamine (Sanhera et al, 1991a).

Based on these studies, it is suggested that dopaminergic symptoms of the medial uncertain area could be involved in the mechanisms of retrocontrol of sexual steroids on the central nervous system that participates in the control of gonadotropin release and sexual behavior of the female rat (MacKenzie et al, 1988; MacKenzie et al, 1989; Wilson et al, 1991). The electrolytic destruction of the somas of areas A11, A13 and A14 inhibits the pre-release of LH and prolactin, without affecting the discharge of FSH (Sanhera et al, 1991b).

We have also mentioned that the dopaminergic system participates differentially in the neuroendocrine and endocrine mechanisms that culminate with ovulation, since its variable participation throughout the estrous cycle of the adult rat depends on the time of day when the blockage is performed. Pharmacological of dopamine receptors. Systemic blockade of dopaminergic receptors during estrus and right-handed 1 inhibited ovulation of animals by suppressing the pre-release of LH in 100% of animals; in right-handed 2, a partial ovulation block was observed, which depended on the time the antagonist was injected; while its administration at 13:00 h of the proestro reduced the ovulatory response of the animals to 50% without modifying it when it was administered in the morning or at night (Domínguez et al, 1987). The unilateral implant with haloperidol crystals at the level of the suprachiasmatic region of the anterior hypothalamus produced similar results, with the difference that the implant at 13:00 h on the day of the proestrus did not prevent the ovulation of the animals (Morán & Domínguez; 1995).

These results allow us to suggest that the participation of dopaminergic systems in the control of spontaneous ovulation is different and that the generalized systemic blockade of these systems affects the discharge of gonadotropins by different mechanisms than that regulated by the previous hypothalamus. In another study, it was shown that there is also some degree of lateralization of the dopaminergic system of the anterior hypothalamus on the day of estrus. Implantation of a 1: 1 mixture of haloperidol and cholesterol crystals at 1:00 p.m. from the estrus on the right side of the anterior hypothalamus blocked ovulation on the morning of the expected estrus, but did not do so in animals with implants in the left side (Morán & Domínguez; 1997). The results of our working group using the experimental model with permanent cannulas inserted in the skull of the animal throughout the experiment, through which implants or micro-injections of the dopamine antagonist were deposited, have opened new paradigms in the study of neuroendocrine regulation of ovulation, since surgical methods and techniques for addressing the anterior hypothalamus invariably lead us to mechanically section the dorsal pathways to this CNS structure.

In these experimental models, the implantation of the cholesterol and haloperidol crystals was performed using an internal cannula of greater length (7.8 mm long) than the permanent cannula (4 mm long) used as a guide for the final implant (Morán & Domínguez ; 1995 and 1997). Since unilateral implants with pure cholesterol crystals in both studies significantly affected ovulation in the morning of the expected estrus, the possibility was raised that the dorsal thalamic pathways to the anterior hypothalamus that were sectioned by the passage of the internal cannula could be involved in the regulation of the estrous cycle and participate in the mechanisms that control ovulation (Morán & Domínguez; 1995 and 1997).

In order to analyze the role of the dorsal thalamic pathways to the anterior hypothalamus, we have recently replaced the intracerebral implant technique with crystals with intracerebral microinjection techniques using permanent cannulas of greater length and it was observed that ovulation was blocked in 100% in animals that received a unilateral microinjection of haloperidol (15 µg; 0.3 µg / min; ic)

ISSN-On line: 2414-8849

On the right or left side of the anterior hypothalamus through a permanent 4 mm long cannula compared to 60% ovulation in animals who received said microinjection through a permanent 6 mm cannula (Méndez Bermúdez & Morán Perales, 2001; Méndez & Morán, 2001b; Morán, 2003; Morán et al, 2004). These results allowed us to confirm that the integrity of the dorsal pathways to the anterior hypothalamus is necessary for the proper integration of the neuroendocrine signals that regulate the estrous cycle and ovulation.

In summary and according to our background, we can affirm that at the beginning of the estrous cycle the participation of dopamine systems in the control of gonadotropin secretion and the estrous cycle plays a crucial role and presents a certain degree of laterality.

The purpose of this work was to test whether the pharmacological blockade of dopamine receptors on one or both sides of the anterior hypothalamus on the afternoon of estrus day will cause different effects on the duration of the estrous cycle and on the ovarian function of the adult rat , which will be reflected in differences in the ovulatory capacity of each ovary.

Research Methodology

Biological Material

Adult female rats of the CII-ZV strain, with a body weight of 200-250 grams, were maintained under controlled lighting conditions (14 h light / 10 h dark; lights from 05:00 to 19:00 h) and with free access to water and balanced food. All the animals used in this work, as well as the experimental methodologies in relation to animal welfare, conformed to the Official Mexican Standard NOM-062-ZOO-1999.

General Procedures

In order to record the estrous cycle in each animal, all of them were taken vaginal smears daily between 09:00 and 10:00 h. Vaginal smears were placed on a glass slide and stained using the hematoxylin-eosin technique to determine the stage of the estrous cycle in each animal and its vaginal cyclic pattern (Luna, 1975) (Figures 2 and 3).

In all experiments, only those animals that had at least three consecutive four-day cycles (cyclic animals) were used: estrus, right-handed, right-handed and proestrous. These animals of regular estrous cycle were assigned to the different experiments.

Stereotactic Surgery

Between 11:30 and 12:30 h animals with regular estrous cycles and vaginal estrus were sedated with ketamine (25 mg / kg weight, im; Anesket, Agropecuaria SA de CV) and three minutes later anesthetized with sodium pentobarbital (40 mg / kg, ip; Anesthesia; Smith Kline Norden of Mexico). Once immobile, the animals were mounted on a SAS 4100 stereotactic device (ASI Instruments Inc.) (Figure 4).

Figure 2 Subjection of the adult rats used in the experiments to obtain samples of vaginal epithelial cells and perform the estrous cycle recording (2.a and 2.b). The sample of cells is obtained with a stainless steel handle that is gently rubbed into the skin of the vagina (2.b and 2.c). The samples are placed on glass slides (smears) and allowed to dry at room temperature before they are stained by the hematoxylin-eosin technique (2.d)

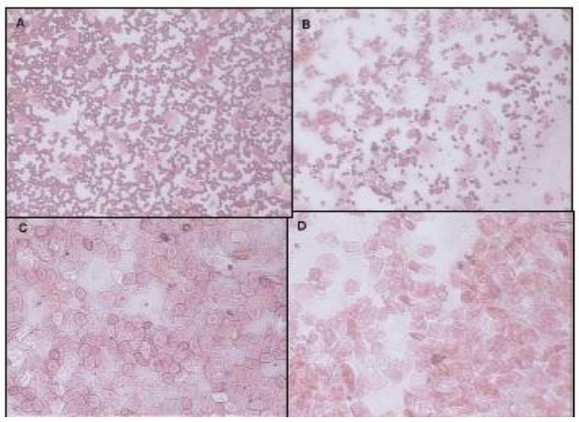


Figure 3 Characterization of the estrous cycle in an adult rat estimated by the appearance of vaginal Cytology A and B) Right, it is distinguished by the relatively high presence of leukocytes, few cubic epithelial cells with well-defined nuclei and some cells of defoliation of the vaginal epithelium; C) Proestro, is distinguished by the predominance of cubic epithelial cells with well-defined nuclei, some defoliation cells but virtual absence of leukocytes; D) Estrol, is distinguished by the abundant presence of nucleus-free cells - cornified - product of the defoliation of the vaginal epithelium, few epithelial cells with well-defined nuclei and occasionally some leukocytes.

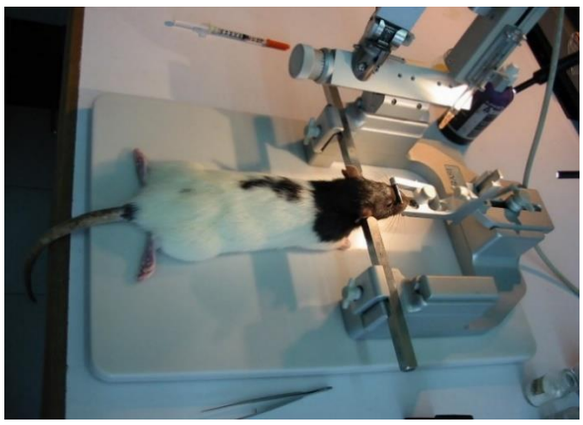


Figure 4 Mounting the animal on a standard stereotactic device. The animal remains attached to the ear bars by the auditory meatus and the muzzle by the bar of the incisors

At 13:00 on the day of estrus, the experimental groups of cyclic animals were treated as follows:

Each animal was shaved the skin of the head and a 1 cm long incision was made on the scalp with a scalpel.

The muscle layers of the skull were removed by scraping with fine-pointed tweezers. The wound was rubbed with gauze impregnated with 2% chlorhexidine antiseptic solution, followed by rubbing with 1% hydrogen peroxide solution in order to cauterize the blood vessels, to locate the anterior (bregma) and posterior (lambda) bone commissures) of the skull and mark the stereotactic coordinates.

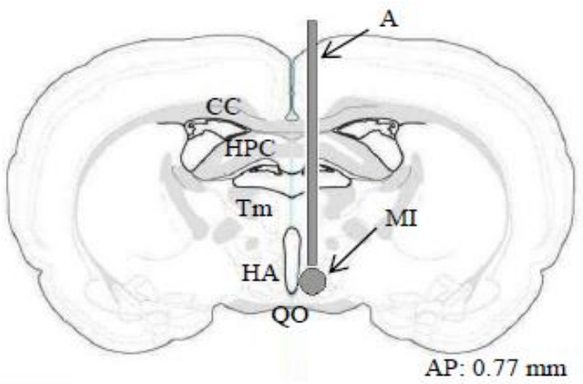


Figure 5 Coronal section of the brain of the adult rat showing the site of the unilateral or bilateral microinjection (MI) of haloperidol. The needle (A) of the microinjector passed through the thalamus and was placed just above the anterior hypothalamus. CC: cerebral cortex; HPC: hippocampus; Tm: thalamus; QO: optical chiasma; HA: anterior hypothalamus (Modified from the Stereotactic Atlas of Paxinos & Watson, 1998)

The calculation of the coordinates for the realization of the microinjections was made based on the atlas of Paxinos and Watson (1998): Antero-Posterior = + 0.77 mm; Lateral = ± 0.05 mm, with respect to the lambda commissure (Figure 5).

Once the Antero-Posterior and Lateral coordinates were located, the right and / or left side of the skull was perforated with Foredom 73B (Foredom Electric Co.) surgical drill and a stainless steel trepan (Figure 6).

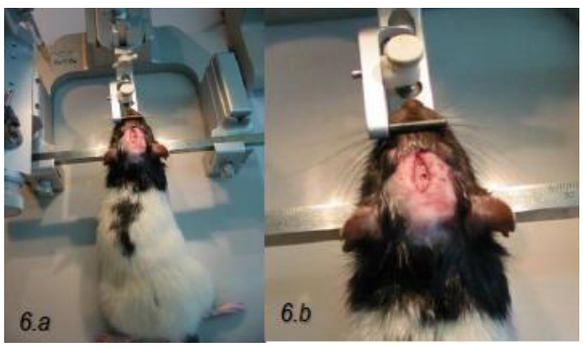


Figure 6. 6.a) Image showing the incision on the scalp of the animals and the location of the trepan. 6.b) When the meninges were exposed, the dura was cut in a cross so that the needle of the microinjector freely enters the brain of the animal

Microinjection technique

Once the trepane was performed, the meninges were visualized; The dura was cut crosswise and the microinjector needle (hypodermic needle No. 25; 0.05 mm internal diameter) was inserted connected to a nanomolar perfusion pump (Model 310; Steolting Co.) to the Vertical coordinate = - 0.72, microinjection site (Figures 5 and 7).

ISSN-On line: 2414-8849

Each animal received an intracerebral microinjection of 1μL of haloperidol solution (15 μg / μL; 5 μg / min) in dimethylsulfoxide (DMSO) on the right, left or both sides of the anterior hypothalamus. As a control group, cyclic animals were used to which a unilateral microinjection of 1μL of pure DMSO (0.33 μL / min) was applied. The total time of each microinjection covered a period of six minutes: three minutes of infusion followed by three minutes of rest, before removing the needle from the microinjector. All animals received a dose of 8000 units of penicillin i.m. during the three days following stereotactic surgery (Figure 8).

Figure 7. 7.a) Image showing the way in which the microinjection was performed by means of the nanomolar infusion pump mounted on the stereotactic apparatus. 7.b) The needle of the microinjector freely crosses the cerebral cortex and the thalamus of the animal until it reaches the anterior hypothalamus.

Figure 8. Photograph showing the equipment used to perform intracerebral microinjection of the haloperidol solution dissolved in dimethylsulfoxide in the anterior hypothalamus

Euthanasia and General Autopsy

The day after the unilateral bilateral injection, the vaginal smears were resumed and all animals were sacrificed between 09: 00-10: 00 h of the next vaginal estrus, preceded by proestrus (euthanasia was performed after a complete estrous cycle).

As a control group, cyclic animals were used that were not subjected to any surgical manipulation or experimental treatment (intact animals) and were sacrificed in the morning of estrus after four consecutive four-day estrous cycles.

Data analysis

With the data from the estrous cycle record, the relative duration of the estrous cycle was estimated and the rate of animals that presented a Short Estral Cycle (TCEC: from 4 to 6 days) or Long (TCEL: greater than 8 days) was estimated.:

$$TCEC = \frac{\textit{Number of Animals that presented Short CE}}{\textit{Number of Animals Treated in the Group}}$$

$$TCEL = \frac{\textit{Number of Animals that presented CE Largo}}{\textit{Number of Animals Treated in the Group}}$$

All animals were sacrificed between 09:00 and 10:00 in the morning of the next vaginal estrus in a carbon dioxide chamber.

At autopsy, the uterine tubes were dissected where the presence of oocytes was sought, which in their case were counted on a Stemi 2000-C stereomicroscope (Zeiss Co.). In those cases in which no fresh oocytes were observed, the ovaries were fixed in böuin solution for 24 hours and then progressively dehydrated in 70% ethanol, 96% ethanol and 100% ethanol (periods of 3 to 24, 3 and 3 hours respectively), to then place them in 2 chloroform changes (periods of 24 and 3 hours, respectively) and finally included in paraffin blocks; they were cut in series at 10 μm thick and colored according to the hematoxylin-eosin technique (Luna, 1975) and the thorough count of the fresh luteal bodies or healthy pre-follicle follicles was carried out, depending on the case. The ovaries and uterus were also dissected, and weighed on a 0.1 mg precision balance (Scientech SP-250; Scientech Co.).

ISSN-On line: 2414-8849

The weight of the organs was expressed in milligrams per 100 grams of body weight (mg / 100g weight). The presence or absence of extravasation fluid in the uterine lumen was recorded from the uterus to estimate the rate of distended uterus (TUD) as an index of estrogen or progesterone secretion (Sánchez & Domínguez, 1995):

$$TUD = \frac{\text{Number of Animals with Uterus Distant}}{\text{Number of Animals Treated in the Group}}$$

The brains of all animals with cerebral microinjection were fixed in 10% formalin solution for 48 h and then cut in the frontal plane at 40 µm thickness. These cuts were mounted in 2% jelly and then stained with 0.1% cresyl violet in order to locate the correct microinjection site (Luna, 1975) (Figure 9).

Statistic analysis

The TCEC, TCEL and TUD data were analyzed with Fisher's Exact Probability test. Data on the number of oocytes released and the days of the estrous cycle were analyzed by the Kruskall-Wallis test, followed by the Dunn multiple comparison test or with Mann-Whitney U. The relative weights of the ovaries and uterus were analyzed with simple ANDEVA followed by the Tukey-Kramer multiple comparisons test or with Student's t test, as appropriate. In all tests, those differences in which the probability is equal to or less than 5% were accepted as significant.

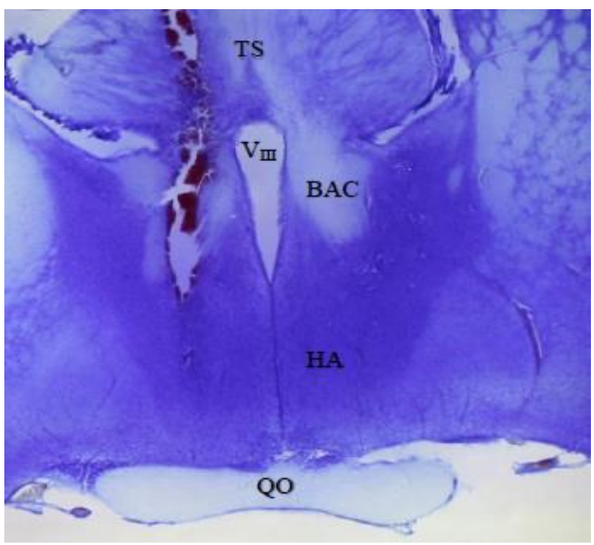


Figure 9 Coronal section of the suprachiasmatic area of the brain of the adult rat showing the area of microinjection with the haloperidol solution on the right side of the anterior hypothalamus. TS: triangular septal nucleus; BAC: bed nucleus of the previous commissure; VIII: third ventricle; HA: anterior hypothalamus; QO: optical chiasma.

Results

Effects of Stereotactic Surgery on the Estral Cycle

Surgical manipulation to perform bi-or unilateral microinjection in the anterior hypothalamus affected the animal's estrous cycle, since of the total of the animals that underwent stereotactic surgery 58% (80/139) of them significantly lengthened the cycle (Figure 10).

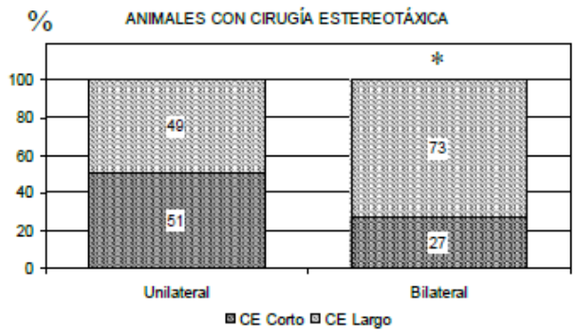


Figure 10 Percentage of animals undergoing stereotactic surgery that presented a short (CE) estrous cycle (Average Duration: 4.6 ± 0.1 days) or long (Average Duration: 13.6 ± 1.2)

Compared to the group of intact animals (absolute control), stereotactic surgery performed on one side of the animals' brains, which before the intervention fulfilled at least three consecutive four-day estrous cycles, can cause elongation. of the estrous cycle significantly (43/88). But if brain surgery is performed on both sides of the brain, the probability that the estrous cycle will be prolonged is greater (37/51).

That is, when the effect of bilateral brain surgery was analyzed compared to unilateral, it resulted in that 73% of animals with surgical manipulation in both cerebral hemispheres had an estrous cycle greater than 8 days compared to 49% of the animals with unilateral stereotactic surgery (Figure 11).

ISSN-On line: 2414-8849

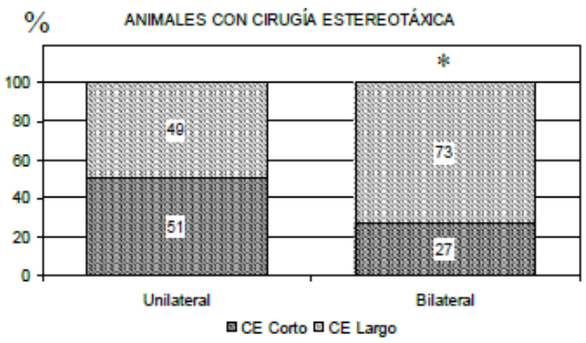


Figure 11 Comparison of the percentages of animals undergoing unilateral or bilateral stereotactic surgery in the brain of animals that presented a short (CE) estrous cycle (Average Duration: 4.6 ± 0.1 days) or long (Average Duration: 13.6 ± 1.2) (* p <0.01 compared to unilateral stereotactic surgery; Fisher's Exact Probability test)

These results indicate that surgical manipulation in both hemispheres induces more likely the increase in the duration of the estrous cycle than manipulation on only one side of the brain (Table 1).

Group	TCEC (%)	DCE (days)	TCEL (%)	DCE (days)
Absolute Control	26/26 (100%)	4.0±0.0	0/26 (0%)	- - - - -
MI-False				
HA-Right	7/17 (41%)	4.4±0.2	10/17 (59%)	13.3±0.3
HA-Left	8/16 (50%)	5.0±0.3	8/16 (50%)	13.3±0.5
HA-Bilateral	11/23 (47%)	4.0±0.1	12/23 (53%)	14.0±0.6
MI-Dmso				
HA-Right	5/13 (38%)	4.5±0.3	8/13 (62%)	14.2±1.3
HA-Left	8/11 (72%)	5.0±0.6	3/11 (28%)	12.0±2.3
HA-Bilateral	1/11 (10%) *, β	5.0±0.6	10/11 (90%) *, β	13.2±0.4
MI-HLP				
HA-Right	10/17 (58%)	4.4±0.2	7/17 (42%)	13.4±0.7
HA-Left	7/14 (50%)	4.4±0.2	7/14 (50%)	13.4±0.6
HA-Bilateral	2/17 (11%) *, α	6.0±0.0	15/17 (89%) *, α	14.2±0.5
* p <0.05 compared to all groups with false microinjection; α p <0.05 compared to unilateral microinjections in HA of the same group; β p <0.01 compared to microinjection in HA-Left of the same group (Fisher's Exact Probability Tests).				

Table 1 Short and long-term (TCEL) and half-day (TCEL) estrous cycle rates ± eem of the estrous cycle duration (DCE) in animals undergoing intracerebral microinjection (MI) of a solution of haloperidol (HLP; 10 µg / µL; 0.2 µg / min) in dimethylsulfoxide (Dmso) on the right, left side or on both sides of the anterior hypothalamus (HA) at 1:00 p.m. on the day of estrus. False MI consisted of needle insertion without touching the HA

Effects of Intra-Cerebral Microinjection on the Duration of the Estral Cycle

The global analysis of the groups indicated that the average duration of a Short Estral Cycle was 4.6 ± 0.1 days (N = 59), while the average duration of a Long Estral Cycle was 13.6 ± 0.2 (N = 80). In Table 1 it can be seen that there were no differences in the cycle duration between the groups of animals with Short Estral Cycle or with Long Estral Cycle.

In the control groups with False Microinjection no differences were observed in the rates of animals that presented a Short Estral Cycle or a Long Estral Cycle. Neither were differences observed in the groups with Unilateral Microinjection with DMSO or with haloperidol with respect to the groups with False Microinjection (Table 1).

On the other hand, in Table 1 it can be observed that in the groups with Bilateral Microinjection with DMSO or with haloperidol it caused that the rate of animals that presented Long Estral Cycle was significantly higher compared with the groups with False or Unilateral Microinjections (Bilateral Microinjection: 25 / 28 vs. False Microinjection: 30/56 or Unilateral Microinjection: 25/54, p <0.001; Fisher's Exact Probability Test).

Since the surgical manipulation affected the duration of the estrous cycle, in the analysis of the data described below it was decided to group the data in animals with Short Estral Cycle (Observed Range: from 4 to 6 days of duration) and in animals with Long Estral Cycle (Observed Range: 8 to 17 days long).

Effects of Haloperidol Brain Microinjection on the Ovulation of Animals with Short Estral Cycle

The global analysis of the effects of surgical manipulations in animals that presented a Short Estral Cycle, allow to visualize the tendency to reduce ovulation with respect to the group of intact animals (Absolute Control (N = 26): 11.7 ± 0.3 vs. False Microinjections and with DMSO (N = 40): 10.8 ± 0.5 or Microinjections with Haloperidol (N = 19): 9.7 ± 0.9; p <0.05; Kruskall-Wallis followed by Dunn's multiple comparisons test) (Figure 12).

ISSN-On line: 2414-8849

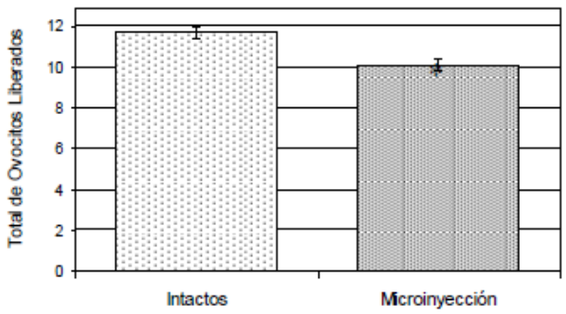


Figure 12 Mean ± E.E. of the number of oocytes released by the ovaries of intact animals and to which they were given an intracerebral microinjection of a solution of haloperidol in dimethylsulfoxide (10 µg / µL; 0.2 µg / min) or a false microinjection on the right, left or right side both sides of the anterior hypothalamus at 1:00 p.m. on estrus day (* p <0.01 with intact animal groups; Mann-Whitney U test)

Compared to the group of intact animals, Unilateral False Microinjection induced the drop in the number of oocytes released by the left ovary (Absolute Control (N = 26): 6.2 ± 0.3 vs. Unilateral False Microinjection (N = 15): 2.8 ± 0.5 ; p <0.005; Mann-Whitney U) without modifying those of the right ovary (Table 2).

	Animals That Presented Short Estral Cycle		
Group	NOL Left Side	NOL Side Right	NOL Totals
(N)	6.2±0.3	5.5±0.2	11.7±0.3
Absolute Control (26)			
MI-False	3.9±0.9 *	5.1±0.7	9.0±1.3
HA-Law (7)	1.9±0.4 *, α	5.9±0.8	7.8±0.8 *
HA-Left (8)	6.0±0.6	6.1±0.7	12.1±0.7
HA-Bilateral (11)			
MI-Dmso	5.2±0.8	5.6±0.5	10.8±0.9
HA-Right (5)	4.5±0.6 α	6.5±0.6	11.0±0.6 δ
HA-Left (8)	4	5	9
HA-Bilateral (1)			
MI-HLP	3.7±0.4 *, α	6.0±0.4	9.7±0.5 β
HA-Right (10)	5.9±0.7 α	3.9±0.5	9.8±0.6 β
HA-Left (7)	(2,4)	(7,6)	(9,10)
* p <0.05 compared to the Absolute Control and Bilateral False Microinjection (Kruskall-Wallis test followed by Dunn's multiple comparisons test); α p <0.005 compared to the contralateral ovary; β p <0.05 compared to the Absolute Control; δ p <0.05 compared to its group with False Microinjection (Tests with Mann-Whitney U).			

Table 2 Mean ± e.e.m. of the number of oocytes released (NOL) by the ovaries of the animals that underwent an intracerebral microinjection (MI) of a haloperidol solution (HLP) in dimethylsulfoxide (Dmso) (10 µg / µL; 0.2 µg / min) in the right, left or both sides of the anterior hypothalamus (HA) at 1:00 p.m. on the day of estrus. All animals were sacrificed on the morning of the next vaginal estrus after microinjection and after presenting a short-term estrous cycle (4 to 6 days). False MI consisted of needle insertion without touching the HA

This drop is such that it is reflected in the total number of oocytes released in the groups with False Microinjection on the right or left side of the anterior hypothalamus (Absolute Control (N = 26): 11.7 ± 0.3 vs. Unilateral False Microinjection (N = 15): 8.3 ± 0.7; p <0.01; Mann-Whitney U). This did not occur in the groups with False Microinjection on both sides of the anterior hypothalamus (Figure 13). There was a similar trend in the groups with Unilateral Microinjection with DMSO, in which the left ovary also decreased its ovulatory quota (Absolute Control (N = 26): 6.2 ± 0.3 vs. DMSO Unilateral Microinjection: 4.8 ± 0.5 (N = 13) ; p <0.05; Mann-Whitney U) but not reflected in the total number of oocytes (Table 2) (Figure 13).

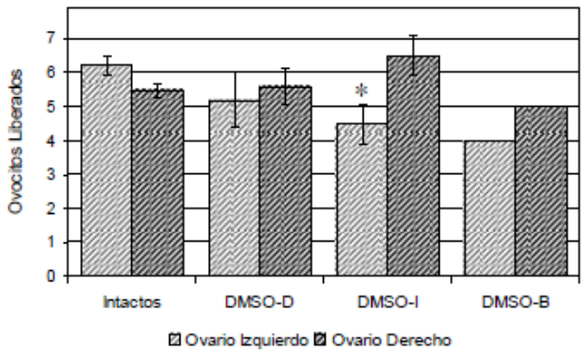


Figure 13 Mean ± E.E. of the number of oocytes released by the ovaries of animals that underwent intracerebral microinjection with dimethylsulfoxide (DMSO) (1 µL; 0.2 µL / min) on the right (-D), left (-I) side or both sides (-B) of the anterior hypothalamus at 1:00 p.m. on the day of estrus (* p <0.05 compared to the contralateral ovary and with the same ovary from the group of intact animals; Kruskall-Wallis test followed by the comparison test Dunn multiple)

Unilateral Microinjection with Haloperidol induced changes in ovulation of the ovaries that depended on the side on which dopamine receptors were blocked. Microinjection of the dopamine antagonist on the right side of the anterior hypothalamus inhibited ovulation of the left ovary, while microinjection of the drug on the left side inhibited that of the right. Both drops tend to decrease the total oocyte quota (Absolute Control (N = 26): 11.7 ± 0.3 vs. Unilateral Microinjections with Haloperidol (N = 17): 9.8 ± 0.4; p <0.05; U of Mann-Whitney) (Table 2) (Figure 14). There were no significant changes in the weight of the ovaries in the groups of animals that presented Short Estral Cycle. However, False or DMSO Unilateral Microinjection on the left side of the anterior hypothalamus tend to increase the weight of the uterus (Absolute Control (N = 26):

ISSN-On line: 2414-8849

182 ± 8 mg / 100 g weight or Right Side Microinjections (N = 12) : 188 ± 10 mg / 100 g weight vs. Microinjection Left Side (N = 16): 223 ± 11 mg / 100 g weight, p <0.05; Tests with Student's t), which correlated with the Uterus Distenced Rate In these groups. False or DMSO Bilateral Microinjections, like Haloperidol Microinjections, did not modify the weight of the uterus (Table 3).

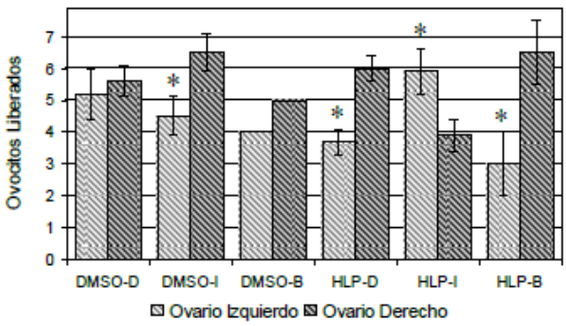


Figure 14. Mean ± E.E. of the number of oocytes released by the ovaries of animals that underwent intracerebral microinjection with haloperidol (HLP) (15 µg / µL; 3 µg / min) or with DMSO (vehicle; 1 µL; 0.2 µg / min) in the right (-D), left (-I) or both sides (-B) of the anterior hypothalamus at 1:00 p.m. on the day of estrus (* p <0.05 with the contralateral ovary Kruskall-Wallis test followed by Dunn's multiple comparison test)

Animals That Presented Short Estral Cycle					
Group (N)	OI	OD	MO	Uterus	TUD
Absolute Control (26)	12.9±0.5	12.8±0.6	25.7±0.9	182±8	5/26
MI-False					
HA-D (7)	12.8±0.8	11.9±0.6	24.7±1.4	177±12	1/7
HA-I (8)	12.9±1.3	14.6±1.6	27.5±2.7	223±16	6/8
HA-B (11)	11.6±0.7	11.5±0.5	23.1±1.0	177±9	3/11
MI-Dmso					
HA-D (5)	11.3±0.9	11.4±0.7	22.7±1.5	203±15	1/5
HA-I (8)	11.3±0.3	11.4±0.5	22.7±0.7	222±15	5/8*
HA-B (1)	(9.1)	(10.4)	(19.5)	(187)	0/1
MI-HLP					
HA-D (10)	13.7±0.6	12.9±0.7	26.6±1.1	179±8	2/10
HA-I (7)	12.9±0.-8	12.4±1.0	25.3±1.7	188±13	1/7
HA-B (2)	(12.1,11.3)	(14.1,12.8)	(26.2,24.1)	(244,182)	1/2
* p <0.05 compared to the Absolute Control and the group with MI-HLP in HA-I; ** p <0.05 compared to the other False Operations (Fisher's Exact Probability Test).					

Table 3 Mean ± e.e.m. of the relative weight of the left ovary (OI), right (OD) or ovarian mass (MO) and uterus (mg / 100g body weight) and distended uterus rate (TUD) of animals that underwent Intracerebral microinjection (MI) of a solution of haloperidol (HLP) in dimethylsulfoxide (Dmso; 10µg / µL; 0.2 µg / min) on the right (-D), left (-I) or both sides (-B) of the anterior hypothalamus (HA) at 1:00 p.m. on the day of estrus. All animals were sacrificed on the morning of the next vaginal estrus after microinjection and after presenting a short-term estrous cycle (4 to 6 days). False MI consisted of needle insertion without touching the HA

Effects of Haloperidol Brain Microinjection on the Ovulation of Animals with Long Estral Cycle

False Microinjections did not modify the individual ovulation of each ovary in animals that had a Long Estral Cycle, but a relative drop in total ovulation was observed in the group with Bilateral False Microinjection (Table 4).

Bilateral Microinjection of DMSO reduced the number of oocytes released by the left ovary, but increased it in those released by the right ovary. Compared to the group with Bilateral False Microinjection, DMSO Bilateral Microinjection tends to increase the total number of oocytes released (Table 4) (Figure 15).

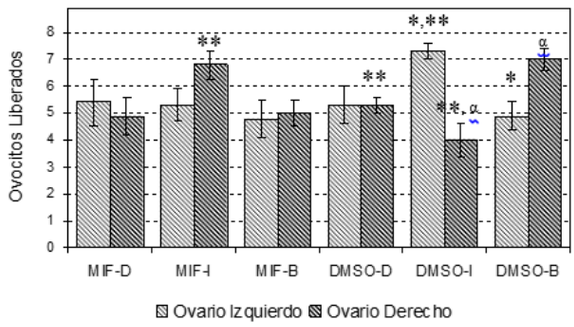


Figure 15. Mean ± E.E. of the number of oocytes released by the ovaries of the animals who underwent a false intracerebral microinjection (MIF) or with DMSO (vehicle; 1µL; 0.2 µg / min) on the right (-D), left (-I)) or on both sides (-B) of the anterior hypothalamus at 1:00 p.m. on the day of estrus (* p <0.05 compared to the contralateral ovary; ** p <0.01 compared to bilateral manipulation in the same group; □ p < 0.05 compared to your group with MIF (Mann-Whitney U test).

It was observed that the number of oocytes released by the left ovary tends to decrease and increase those of the right ovary in the groups with Unilateral Microinjection with Haloperidol (Left Ovary (N = 14): 4.6 ± 0.2 vs. Right Ovary (N = 14) : 6.3 ± 0.3, p <0.001; Mann-Whitney U test), unchanged in the group with Bilateral Microinjection of the drug (Table 4) (Figure 16).

No significant changes were observed in the weight of the ovaries or in the Uterus Dysted Rates among the groups of animals that presented the Long Estral Cycle (Table 5).

ISSN-On line: 2414-8849

Discussion of results

After stereotactic surgery, the probability that an animal with regular four-day estrous cycles loses the pattern of regularity in its subsequent cycles is very high, since 58% of cyclic animals undergoing brain surgery (80/139) significantly extend the next estrous cycle to an average of 14 days.

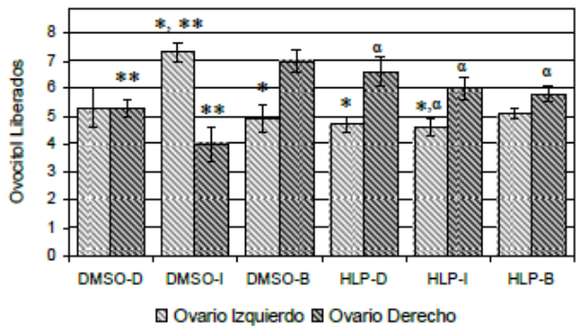


Figure 16 Mean ± E.E. of the number of oocytes released by the ovaries of animals that underwent intracerebral microinjection with haloperidol (HLP) (15 µg / µL; 3 µg / min) or with DMSO (vehicle; 1 µL; 0.2 µg / min) in the right (-D), left (-I) or both sides (-B) of the anterior hypothalamus at 1:00 p.m. on the day of estrus (* p <0.05 with the contralateral ovary; ** p <0.01 compared to bilateral manipulation in the same group; p <0.05 compared to its group with DMSO (Mann-Whitney U test)

The registration of vaginal smears in these animals showed that the entire cycle was characterized by the presence of leukocytes (vaginal right-handed) before observing the presence of the proestrus and vaginal estrus. This suggests that surgical manipulation is capable of significantly altering the neuroendocrine and endocrine mechanisms that regulate gonadotropin secretion and therefore the estrous cycle. It is feasible that the presence of the vaginal right-hand sign is a reflection of the secretion of prolactin capable of sustaining the progestogenic activity of the luteal bodies that for some reason do not return.

It is widely documented that prolactin maintains a relatively high secretion during the early stages of pseudopregnancy and pregnancy and prior to the formation of the placenta (Freeman, 1988). Prolactin indirectly inhibits the secretion of FSH and LH by prolonging the life of the corpus luteum (Smith et al, 1975 and 1976).

When estrogen concentrations have declined and remain relatively low, the increasing concentration of progesterone acts with a negative feedback effect on the anterior hypothalamus and inhibits the secretion of GnRH and thereby the secretion of gonadotropins is interrupted (Fink, 1979 and 1988 ; Fink et al, 1983). For years, it has been known that stress induces the sudden discharge of prolactin in the rat, accompanied by the suppression of the prevolvulatory peak of LH (Neill, 1970; Neill et al, 1971). This evidence is totally consistent with our observations, since more than half of the animals subject to stereotactic surgery lengthened the estrous cycle.

Animals That Presented Long Estral Cycle			
Group (N)	NOL Left Side	NOL Right Side	NOL Totals
Absolute Control (26)	6.2±0.3	5.5±0.2	11.7±0.3
MI-False			
HA-Right (10)	5.4±0.9	4.9±0.7	10.3±0.7
HA-Left (8)	5.3±0.6	6.8±0.5 $^{\delta}$	12.1±0.6
HA-Bilateral (12)	4.8±0.7	5.0±0.5	9.8±0.4
MI-Dmso			
HA-Right (8)	5.3±0.7	5.3±0.3 $^{\delta}$	10.6±0.7
HA-Left (3)	7.3±0.3 $^{\beta}$	4.0±0.6 $^{\beta,\delta}$	11.3±0.7
HA-Bilateral (10)	4.9±0.5 $^{\alpha}$	7.0±0.4 $^{\beta}$	11.9±0.5
MI-HLP			
HA-Law (7)	4.7±0.3 $^{\alpha}$	6.6±0.5	11.3±0.5
HA-Left (7)	4.6±0.3 $^{\alpha}$	6.0±0.4	10.6±0.6
HA-Bilateral (15)	5.1±0.2	5.8±0.3	10.9±0.3
** p <0.05 compared to the Absolute Control (Kruskall-Wallis test followed by Dunn's multiple comparisons test); ** p <0.01 compared to the same group with DMSO and with Haloperidol; α p <0.05 compared to the contralateral ovary; β p <0.05 compared to the other groups with microinjection in HA on the same side; p <0.05 compared to bilateral manipulation with the same treatment (Mann-Whitney U tests).*			

Table 4 Mean ± e.e.m. of the number of oocytes released (NOL) by the ovaries of the animals that underwent an intracerebral microinjection (MI) of a solution of haloperidol (HLP) in dimethylsulfoxide (Dmso; 10µg / µL; 0.2 µg / min) in the right side (-D), left (-I) or on both sides (-B) of the anterior hypothalamus (HA) at 1:00 p.m. on the day of estrus. All animals were sacrificed on the morning of the next vaginal estrus after microinjection and after presenting a prolonged estrous cycle of 12 to 17 days. False MI consisted of needle insertion without touching the HA

ISSN-On line: 2414-8849

It is also feasible that surgical stress helped increase the likelihood that the animal will lose the estrous cycle by inducing the discharge of glucocorticoids. It is a known fact that CRH produced in the hypothalamus directly stimulates the adenohypophyseal corticotrope and induces the release of ACTH and β-Endorphin, followed by the secretion of cortisol and corticosterone in the adrenal cortex (Arimura, 2000; Welsh & Johnson, 1981)

CRH is rapidly released in response to a wide variety of stressors, but it is particularly important to note that CRH stimulates its own secretion in the hypothalamus by means of a mechanism for increasing regulation of a short-loop paracrine type. It is known that CRH interrupts the release of GHRH and GnRH (Arimura 2000; McCann et al, 1983) so it is feasible that the alteration of the estrous cycle is the reflection of the interruption of the hypothalamic signal that determines secretion of gonadotropins and changes in ovulation (Kamel & Kubajak, 1987; Peppler & Jacobs, 1976; Welsh & Jonson, 1981). In addition, glucocorticoids are shown to be able to stimulate progesterone secretion in follicular cell cultures (Adshi et al, 1981).

Animals That Presented Long Estral Cycle					
Group	*OI*	*OD*	*MO*	*Uterus*	*TUD*
(N)	12.9±0.4	12.8±0.5	25.7±0.9	182±8	5/26
Absolute Control (26)					
MI-False	14.5±0.9	12.9±0.7	27.4±1.5	163±8	1/10
HA-D (10)	12.7±1.2	12.2±1.1	24.9±2.0	171±15	2/8
HA-I (8)	13.2±0.8	11.7±0.6	24.9±0.9	182±10	1/12
HA-B (12)					
MI-Dmso	11.9±0.4	12.6±0.5	24.5±0.7	187±10	4/8
HA-D (8)	11.1±1.4	13.7±1.4	24.8±1.6	191±10	2/3
HA-I (3)	11.4±0.6	11.5±0.6	22.9±1.0	187±7	4/10
HA-B (10)					
MI-HLP	12.7±0.5	13.7±0.9	26.4±1.2	189±14	3/7
HA-D (7)	13.5±0.8	13.7±0.7	27.2±1.0	177±14	2/7
HA-I (7)	11.6±0.4	12.2±0.5	23.8±0.8	167±9	4/15

Table 5 Mean ± e.e.m. of the relative weight of the right (OD), left (OI) ovaries, ovarian mass (MO) and uterus (mg / 100g body weight) by animals that underwent microinjection (MI) of a dissolution of haloperidol (HLP) in dimethylsulfoxide (Dmso; 10 µg / µL; 0.2 µg / min) on the right (-D), left (-I) or on both sides (-B) of the anterior hypothalamus (HA) at 1:00 p.m. on the day of estrus. All animals were sacrificed on the morning of the next vaginal estrus after microinjection and after presenting a prolonged estrous cycle (12 to 17 days). False MI consisted of needle insertion without touching the HA

On the other hand, when the dopamine antagonist vehicle or drug was infiltrated directly on both sides of the anterior hypothalamus, significant differences were observed depending on whether the microinjection was performed on one or both sides of the hypothalamus, since the frequency at which they occurred estrous cycle of more than twelve days duration was significantly longer in animals with bilateral microinjection than in those with unilateral microinjection. It has been shown that surgical manipulation in any of the cerebral hemispheres is able to significantly block spontaneous ovulation by inhibiting the normal pattern of GnRH secretion and consequently that of gonadotropins (Morán & Domínguez, 1995 and 1997).

Our results confirm that the dorsal pathways to the anterior hypothalamus carry information that participates in the neuroendocrine and endocrine mechanisms that lead to ovulation after an estrous cycle and allow us to suggest that the section of these pathways is capable of modifying the normal pattern of the GnRH secretion and thus the secretion of gonadotropins, necessary for the estrous cycle to develop properly.

The results in the experimental groups with false unilateral microinjection referring to ovulation lead us to affirm that the inhibition of the ovulatory capacity of the left ovary is necessarily the result of the alteration of nerve signals coming from one of the sides of the brain and that hypothetically they participate critically in the mechanisms that regulate the recruitment and follicular development of the left ovary without affecting the right ovary. There is very consistent evidence that proposes the existence of a direct nervous connection between the ovary and the CNS, particularly the hypothalamus (Advis et al, 1989; Domínguez et al, 1971; Domínguez et al, 1989; Fukuda et al, 1984; Mizunuma et al, 1983).

Recently, we have used intrahypothalamic microinjection techniques through a stainless steel cannula that remains embedded in one of the cerebral hemispheres for at least four complete estrous cycles. The results in the control groups with microinjection or false implant are opposite to our observations, since it is the right ovary that reduces its ovulatory capacity and the left one apparently compensates for it (Méndez & Morán, 2001b; Morán, 2003; Morán et al, 2004).

ISSN-On line: 2414-8849

These observations allowed us to suggest that the chronic effect caused by the presence of the permanent cannula induces a reorganization in the mechanisms that regulate ovarian function, that is, the system tends to readjust the nervous nature signals that reach the ovaries. This CNS plasticity phenomenon is evident in our group with false bilateral microinjection, since the ovulatory quota decreased significantly and it was because the left ovary also reduced its ovulant capacity.

The insertion of an empty cannula or the manipulation produced by the implantation of crystals of pure cholesterol on one side of the anterior hypothalamus is able to decrease the ovulatory rate (Morán & Domínguez, 1995 and 1997). In these experiments, the administration of GnRH or estrogen restored ovulation in 100% of the cases, which suggested that the section of the thalamus pathways would lengthen the duration of the estrous cycle by affecting gonadotropin secretion by delaying the discharge GVR preoccupation. In our animals, the section of these thalamic pathways reproduces the observations of Morán and Domínguez (1995 and 1997), which seems to confirm that the thalamic structures dorsal to the anterior hypothalamus play a significant role in the integration of neuroendocrine signals that lead to proper discharge of GnRH.

The blockade of the dopamine receptors induced by microinjection of 15 µg haloperidol on the right or left side of the anterior hypothalamus affected the development of the estrous cycle and the ovulatory response in the animals, regardless of the side on which the microinjection was performed, which suggests that dopaminergic information regarding either side of the anterior hypothalamus is important for endocrine and neuroendocrine mechanisms that culminate in rat ovulation to develop properly.

The microinjection of haloperidol on one side of the anterior hypothalamus is capable of affecting gonadotropin secretion and thereby ovarian function (Morán & Domínguez, 1995 and 1997). It is known that dopamine antagonists decisively inhibit the adenohypophyseal discharge of prolactin (Neill, 1988). Our experimental model seeks to be adequate to study the influence of the dopaminergic system on the secretion of FSH and LH without directly involving the tuberoinfundibular system that controls the provulatory discharge of prolactin.

The predominance of a typical right-handed vaginal smear in animals with micro-injection of the dopamine antagonist suggests the prolongation of the life of the corpus luteum, most likely maintained by the uncontrolled discharge of prolactin (Smith et al, 1975 and 1976). Thus, the high plasma concentrations of progesterone combined with low estrogen production, conditioned by low gonadotropin profiles, would prolong the negative feedback effect of sex steroids at the level of the hypothalamus, suppressing the pre-discharge of GnRH and therefore of gonadotropins (Keyes & Wiltbank, 1988; McNelly, 1980).

In a previous study, Morán and Domínguez (1995) showed that when a unilateral implant with 10.0 ± 0.3 µg of haloperidol crystals is placed on the right or left side of the anterior hypothalamus in four-day regular estrous cycle rats, a Effective ovulation block, which can be partially reversed at 80% efficiency when GnRH is administered in animals. The results of this study allowed us to suggest that apparently the absence of ovulation is caused by the interruption of the mechanisms that control the tonic and phasic discharge of GnRH, in which estrogens play a crucial role. Consequently, blocking the dopaminergic receptors of the anterior hypothalamus affects the discharge of gonadotropins and therefore not enough estrogen would be produced.

This added to the lack of control of the prolactin discharge would cause the follicular development to stop, in a similar way to what happens during pregnancy, fall in the production of ovarian estrogens and therefore, absence of the signs of ovulation in the morning. of the expected estrus.

In another study, Morán and Domínguez (1997) showed that particularly on the day of estrus, the dopaminergic system of the anterior hypothalamus presents laterality in the control of spontaneous ovulation, as is the case with the cholinergic system (Cruz et al, 1989). The 10.0 ± 0.4 µg implant of a 1: 1 mixture of haloperidol and cholesterol crystals on the right side of the anterior hypothalamus effectively blocked ovulation in the morning of the expected estrus but the implant placed on the left side did not.

ISSN-On line: 2414-8849

This functional laterality could possibly be related to the positive feedback effect of estrogen that requires that the right side of the anterior hypothalamus remain intact (Morán & Domínguez, 1995 and 1997) or with the asymmetric distribution of GnRH in the basal medial hypothalamus (Bakalkin et al, 1984; Gerendai et al, 1979).

In the present work, it was not considered to sacrifice the animals on the day of the expected estrus but to sacrifice them until the sign of the evident vaginal estrus (that is, the first estrus observed after the surgical interventions). In 98.6% (137/139) of the cases oocytes were found in the tubes (only two animals had to be counted the number of fresh luteal bodies). However, our observations also report changes that depend on the side of the hypothalamus in which the dopamine receptor blockade was performed. Apparently, in the animals they presented a Short Estral Cycle, a greater sensitivity to the pharmacological blockade of the right side of the anterior hypothalamus was observed that negatively affects the ovulant capacity of the left ovary accompanied by an increase in that of the right, but if the blockade of the receptors Dopamine was performed on the left side of the anterior hypothalamus, the result is the other way around. In animals with Long Estral Cycle, this cross laterality does not occur, however, the decrease in the ovulant capacity of the left ovary and increase in the right one occurs independently of the side of the hypothalamus on which the microinjection of haloperidol was performed.

Different studies have reported that for our animal strain the left ovary releases more oocytes than the right one (Domínguez et al, 1989) and that this difference is modified when the innervation of the ovaries is pharmacologically or surgically interrupted (Chávez et al, 1987; Cruz et al, 1986; Domínguez et al, 1989).

These results have postulated that the nervous information related to the ovaries participates in the modulation of the response to gonadotropins and in the control of follicular recruitment that leads to ovulation of each ovary. Our results support this hypothesis, since if changes in the ovulatory capacity of each ovary were observed as a result of anti-dopaminergic manipulations on one side of the anterior hypothalamus.

There are other studies that support our observations regarding the prolongation of the estrous cycle that follows intracerebral microinjection. In an experimental model similar to that used in the present work, we show that the first estrous cycle that follows brain surgery was significantly prolonged, but was particularly long in a group with bilateral microinjection of the EEDQ (selective antagonist of DA-1 receptors) , although subsequent estrous cycles tended to normalize with respect to the group of adult rats with four-day cycles (Meléndez and Morán, 2002). Our results agree with this background and allow us to suggest that dopamine is a necessary signal for normal estrous cycles to develop.

The results of this work support the idea that the dopaminergic system of the anterior hypothalamus participates significantly in the integration of the neural and neuroendocrine signals that regulate the estrous cycle. Clemens et al. (1976) described the effects of electrolytic destruction of the rat's anterior hypothalamus. Bilateral lesion of the medial preoptic nucleus induced the prolongation of the estrous cycle, which showed prolonged periods of vaginal right-handedness in the animals. In this same study, the dicidoma test in the uterus of other animals, proved that this lesion induces the spontaneous appearance of pseudopregnancy stages.

The ovaries of the animals showed the presence of fresh luteal bodies at the end of each period of pseudopregnancy, which covered a period of 12 to 14 days. Daily administration of the dopamine agonist lergotril mesylate in animals with bilateral lesion of the preoptic area causes periods of pseudopregnancy to be reduced to four or five days, similar to a normal estrous cycle and presence of fresh luteal bodies in the ovaries of the animals.

Kalra (1974) showed that the disconnection of the pathways that separate the anterior hypothalamus from the middle eminence also induces the appearance of long estrous cycles in the rat, without affecting ovulation in the morning of the vaginal estrus. In these animals, the loss of the estrous cycle correlates with the decrease of the GnRH content in the middle eminence, which suggested that the control of the phasic discharge of the decapeptide comes from the rostral structures of the diencephalon.

ISSN-On line: 2414-8849

These results as a whole allowed us to suggest that the structures that control the tonic and phasic discharge of gonadotropins are located in the preoptic area and that dopamine is apparently the necessary signal for the synchronization of the estrous cycle with ovarian function.

Other results of our laboratory have shown that the systemic administration of haloperidol (2.5 mg / kg body weight) during the first half of the estrous cycle in four-day regular cycle rats induces changes in the secretion of gonadotropins that cause ovulation delay. , that is, the estrous cycle extends significantly to 16.8 ± 0.6 days (Morán et al, 2001; Ramírez-Ávila, 2001; Vargas-Torres, 2002).

Conclusions

The results of this study allow us to conclude that:

Stereotactic surgery performed in rats with regular four-day estrous cycles is capable of inducing the loss of the normal pattern of vaginal cytology expression.

In a short estrous cycle, the mechanical section of the neural connections that occurs when the needle of the microinjector is introduced into only one of the cerebral hemispheres of rats with regular four-day estrous cycles induces alteration of the nerve signals that reach the ovaries. inhibit ovulation of the left ovary.

In a long estrous cycle, the mechanical section of the neural connections of both cerebral hemispheres of rats with regular four-day estrous cycles induces alteration of nerve signals that reach the ovaries and result in decreased ovulatory capacity.

In a short estrous cycle, the effect of unilateral blockade of dopamine receptors in the anterior hypothalamus inhibits ovulation of the contralateral ovary, which is reflected in the fall in the total ovulatory quota in these animals.

In a long estrous cycle, the effect of unilateral blockade of dopamine receptors in the anterior hypothalamus results in the inhibition of ovulation of the left ovary, but stimulates that of the right, which compensates for the total ovulatory quota.

References

Adshi, E. Y., Jones, P. B. C. & Shueh, A. J. W. (1981). Synergistic effects of glucocorticoids in the stimulation of progesterone production by follicle-stimulating hormone in cultured rat granulose cells. Endocrinology 109:1888-1894.

Advis, J.P., C.E. Ahmed & S.R. Ojeda (1989). Direct hypothalamic control vasoactivo intestinal peptide (VIP) levels in the developing rat ovary. Brain Research Bulletin 22:605-610.

Arimura, A. (2000). Hypothalamic Hormones. En:" Neuroendocrinology in Physiology and Medicine". Capítulo 3. Eds. P.M. Conn & M.E. Freeman. Humana Press. Pp.41-58.

Bakalkin, G.Y., V.V. Tsivezov, E.A. Sjutkin, S.P. Veselova, I.D. Novilov & O.G. Krivosheev (1984). Laterization of LH-RH in the rat hypothalamus. Brain Res 296:361-364.

Baldessarini, R.J. (1989). Las drogas en el tratamiento de los transtornos psiquiátricos. En:" Bases Farmacológicas de la Terapéutica". 7a. edición. Capítulo 19. Eds. L.S, Goodman, T.W. Rall & F. Murad. Editorial Médica Panamericana. pp 378-431.

Barraclough, C.A. (1983). The role of catecholamines in the regulation of gonadotropin secretion. Acta Morphol Hung 31:101-116.

Barraclough, C.A. & P.M. Wise (1982). The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle stimulating hormone secretion. Endocrine Rev 3:91-119.

Barry, J., G.E. Hoffman & S. Wray (1985). LHRH-containing systems. En: "Handbook of Chemical Neuroanatomy: GABA and Neuropeptides in the CNS, Part I". Vol. 4. Capítulo IV. Eds. A. Björklund & T. Hökfelt. Elsevier. Amsterdam. pp 166-215.

Björklund A. & A. Nobin (1973). Fluorescence histochemical and microespectrofluorometric mapping of dopamine and noradrenaline cells groups in the rat diencephalon. Brain Res 51:193-205

Björklund A., O. Lundvall & A. Nobin (1975). Evidence of an incertohypothalamic dopamine neuron system in the rat. Brain Res 89:2942.

ISSN-On line: 2414-8849

Carvey, M. P. (1998). Drug action in the central nervous system. Ed. M.P. Carvey. Oxford. New York. pp 214-225.

Chávez, R., M.E. Cruz & R. Domínguez (1987). Differences in the ovulation rate of the right or left ovary in unilaterally ovariectomized rats: Effect of ipsi and contralateral vagus nerve on the remaining ovary. J Endocrinol 113:397-401.

Choudhury, S, R. Sharpe, P. Brown (1974). The effect of pimozide, a dopaminergic antagonist, on pituitary gonadotrophic function in the rat. J Reprod Fert 39:275-283.

Clemens, J.A., E.B. Smalsting & B.D. Sawyer (1976). Studies on the role of the preoptic area in the control of reproductive function in the rat. Endocrinology 99:728-735.

Conn, M. (1994). The molecular mechanism of gonadotropin-releasing hormone action in the pituitary. En: "Physiology of Reproduction". 2th. Edition. Vol. I. Capítulo 32. Eds. E. Knobil & J. Neill. Raven Press. New York, pp 1815-1832.

Cruz, M. E. R. Chávez & R. Domínguez (1986). Ovulation, follicular growth and ovarian reactivity to exogenous gonadotrophins in adults' rats with unilateral or bilateral section of the vagi nerves. Revista de Investigación Clínica 38:167-171.

Cruz, M. E., L. P. Jaramillo & R. Domínguez (1989). Asymmetric ovulatory response induced by a unilateral implant of atropine in the anterior hypothalamus of the cyclic rat. J Endocrinol 123:437-439.

D'angelo, S.A & A.S. Kravats (1960). Gonadotropic hormone function in persistent estrous rats with hypothalamic lesions. Proc Soc Exp Biol Med 104:130-133.

Domínguez, R., M.E. Cruz & R. Chávez (1989). Differences in the ovulatory ability between the right and left ovary are related to ovarian innervation. En: "Growth Factors and the Ovary". Eds. A.N. Hirshfield. Plenum Press. New York. pp. 321-325.

Domínguez, R., C.M. Gaitán, S.A. Méndez & A. Ulloa- Aguírre (1987). Effects of catecholaminergic blockade by haloperidol or propranolol at different stages of the oestrous cycle on ovulation and gonadotrophin levels in the rat. J Endocrinol 113:37-44.

Domínguez, R. & L. Riboni (1971). Failure of ovulation in autografted ovary of hemispayed rat. Neuroendocrinology 7: 164-170.

Domínguez, R., L. Riboni, D. Zipitria & R. Revilla (1982). Is there a cholinergic circadian rhythm throughout the oestrous cycle related to ovulation in the rat? J Endocrinol 95: 175-180.

Domínguez, R., D. Zipitria, L. Riboni & R. Revilla (1985). Differences in the ability of reserpine and chlorpromazine to block ovulation throughout the estrous cycle of the rat. J Interdisc Cycle Res 16:63-72.

Erickson, G.F. (1995). The ovary: Basic principles and concepts. En: "Endocrinology and Matabolism". Capítulo 17. Eds. P. Felling, J.D. Boxter & L.A Frohman. 3th Edition. McGraw-Hill. New York. pp 973-1013.

Everett, J.W. & H.M. Radford (1961). Irritative deposits from stainless steel electrodes in the preoptic rat brain causing release of pituitary gonadotropin. Proc Soc Exp Biol Med 108:604-609

Everett, J.W. (1939). Spontaneous persistent estrous in a strain albino rats. Endocrinology 25:123-127.

Feder, H.H. (1981). Estrous ciclicity in mammals. En: "Neuroendocrinology of Reproduction: Physiology and Behavior". Sección III. Capítulo 10. Eds. N.T. Adler. Plenum Press. New York & London. pp 279-308.

Fink, G. (1979). Feedback actions of target hormones on hypothalamus and pituitary with special reference to gonadal steroids. Ann Rev Physiol 41:571-585.

Fink, G. (1988). Gonadotropic secretion and its control. En: "Physiology of Reproduction". 1th Edition. Vol. I. Capítulo 32. Eds. E. Knobil & J. Neill. Raven Press, New York, pp 1349-1377.

ISSN-On line: 2414-8849

Fink, G., H.F. Stanley & A. G. Watts (1983). Central nervous control of sex and gonadotropin release: Peptide and nonpeptide transmitter interactions. En: "Brain Peptides". Eds. D.T. Krieger, M.J. Brownstein & J.B. Martin. John Wiley & Sons. New York. pp. 413-435.

Freeman, M.E. (1988). The ovarian cycle of the rat. En: "Physiology of Reproduction". 1th Edition. Vol. II. Capítulo 45. Eds. E. Knobil & J. Neill. Raven Press, New York, pp. 1893-1928.

Fukuda, M., K. Yamanouchi, Y. Nakano, H. Furuya & Y. Arai (1984). Hypothalamic laterality in regulating gonadotropic function: Unilateral hypothalamic lesion and ovarian compensatory hypertrophy. Neurosci Letters 51:365-370.

Gerendai, I., W. Rotsytein, B. Marchetti & V. Scapagnini (1979). LH-RH content changes in the mediobasal hypothalamus after unilateral ovariectomy. En: "Neuroendocrinology: Biological and Clinical Aspects". Proceedings of Serono Symposia. Vol. 19. Eds. A. Polleri & R. McLeod. Academic Press. New York. pp. 97-102.

González, K., Morán, J.L., Handal A. & Reynoso A. (2016). El bloqueo farmacológico de los receptores ováricos a la dopamina altera el ciclo estral y la ovulación en la rata adulta. Revista de Sistemas Experimentales 3(7):27-45 (ISSN-2410-3950 ECORFAN) (2016).

Guzmán Herrera, N., Sánchez Gracía, O., Handal, A. & y Morán, J.L. (2018). Dopaminergic receptor type 1 antagonism in rat ovarian tissue: effects on ovulation and ovarian compensatory hypertrophy. Journal Multidisciplinary Science UTSOE. V(X):12-27 (ISSN: 2395-860X LatinIndex).

Hàlasz, B. & R.A. Gorski (1967). Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. Endocrinology 80:608-622.

Kalra, S.P. (1974). Role of estrogen in the restoration of LH release following stimulation of partially deafferentated hypothalamus in rat. Brain Res 68:297- 307.

Kamel, F. & Kubajak, C. L. (1987). Modulation of gonadotropin secretion by corticosterone interaction with gonadal steroids and mechanism of action. Endocrinology 121:561-568.

Keyes, P.L. & M.C. Wiltbank (1988). Endocrine regulation of the corpus luteum. Ann Rev Physiol 50: 465-482.

Kordon, C., S. V. Drouva, G. Martínez De La Escalera & R. I. Weiner (1994). Role of classic and peptide neuromediators in the neuroendocrine regulation of luteinizing hormone and prolactin. En: The Physiology of Reproduction. 2th. Edición. Vol. I. Capítulo 27. Eds. E. Knobil & J. Neill. Raven Press. New York, pp.1621-1681.

Kupfermann, I. (1985). Hypothalamus, limbic system and cerebral cortex: Homeostasis and arousal. En: "Principles of Neural Science". 2a. edición. Sección VIII. Capítulo 46. Eds. E.R. Kandel & J.H. Schwartz. Elsevier. New York. pp 608-625.

Letras, D., Handal, A., Diaz, A. y Morán, J. (2016). La *Sulpirida* reduce la ovulación compensadora, pero incrementa la hipertrofia compensadora del ovario derecho en la rata hemi-ovariectomizada. *ECORFAN Sistemas Experimentales* 3 (7): 46-59.

Litter, M. (1988). Farmacología del Sistema Nervioso. En:"Farmacología Experimental y Clínica". 7ª. Edición. 2ª. Parte: Farmacología Especial. Sección l. El ateneo, Buenos Aires. pp 179-429.

Luna, L.G. (1975). Manual of histology staining methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company. New York. pp 21 y 52.

MacKenzie, F.J., M.D. James & C.A Wilson (1988). Changes in dopamine activity in the zone incerta (ZI) over the rat estrous cycle and the effect of lesions of the ZI on cyclicity: Further evidence that the incertohypothalamic tract has a stimulatory role in the control of LH release. Brain Res 444:75-83.

MacKenzie, F.J., A.J. Junter, C. Daly & C.A. Wilson (1989). Evidence that the dopaminergic incertohypothalamic tract has a stimulatory effect on ovulation and gonadotrophin release. Neuroendocrinology 39:289-295.

ISSN-On line: 2414-8849

McCann, S.M., H. Mizunuma & W.K. Samson (1983). Differential hypothalamic control of FSH secretion: a review. Psychoneuroendocrinology 8:299-303.

McCann, S.M., S.M. Ojeda, E. Vijayan & R.L. Moss (1978). LH-releasing hormone (LHRH), its localization, mechanism of release and action in the anterior pituitary and CNS. En: "Clinical Psychoneuroendocrinolgy in Reproduction". Eds. L. Carenza, P. Pancheri & L. Zichella. Academic Press. New York. pp 57-69.

McNelly, A.S. (1980). Prolactin and the control of gonadotrophin secretion in the female. J Reprod Fert 58:537-549.

Meléndez, E., Morán, C. & Morán, J.L. (2002). Efectos de la microinyección de EEDQ en el hipotálamo anterior sobre el ciclo estral de la rata. Memorias del XLV Congreso Nacional de Ciencias Fisiológicas. Colima, Col. C-236.

Méndez Bermúdez A. & Morán Perales J.L (2001a). Efectos de la microinyección del implante unilateral del antagonista dopaminérgico en el hipotálamo anterior en diferentes etapas del ciclo estral sobre la ovulación espontánea de la rata adulta.

Memorias en Extenso del XI Encuentro Regional De Investigadores En Flora Y Fauna De La Región Centro-Sur De La República Mexicana. Puebla, Pue. FA-20, pp 226-230.

Méndez A. & Moran J.L (2001b). La sección de vías talámicas dorsales al hipotálamo anterior inhibe la ovulación de la rata adulta. Memorias del XLIV Congreso Nacional de Ciencias Fisiológicas, Monterrey, NL. C-7.

Mizunuma, H., L.R. De Palatis & M. McCann (1983). Effect of unilateral orchidectomy on plasma FSH concentration: Evidence for direct neural connection between testes and CNS. Neuroendocrinology.37:291-296.

Miyake, A. (1988). Control of LHRH secretion in women and female rats. En: "Neuroendocrine Control of the Hypothalamo-Pituitary System". Ed. Hiroo Imura. Karger, pp 65-76.

Moran J.L. (2003). La microinyección de Haloperidol en el lado derecho del hipotálamo anterior inhibe la ovulación del ovario ipsilateral. Memorias del XLVI Congreso Nacional de Ciencias Fisiológicas. Aguascalientes, Ags. C-19.

Moran Perales J.L, Castillo Pérez A., Rodríguez Martínez C. & Handal Silva, A. (2004). Cambios en la ovulación inducidos por la microinyección unilateral (MIU) del Haloperidol (HLP) en el Hipotálamo Anterior (HA) de la rata adulta. Memorias del XLVII Congreso Nacional de Ciencias Fisiológicas. Boca del Río, Ver. C-247. Morán, J.L. & R. Domínguez (1995). Effects of the unilateral implant of haloperidol at the preoptic-anterior hypothalamic area, ovulation. Endocrine 3:399-401.

Morán, J.L. & R. Domínguez (1997). Differences in the sensitivity of the right and left side of the preoptic anterior hypothalamic area to the effect of a unilateral implantation of haloperidol, performed on the day of oestrous, on spontaneous ovulation. Med Sci Res 25: 465-466.

Morán, J.L., Ramírez, B., Meléndez, E. & Handal, A. (2001) Efectos de la administración sistémica de haloperidol sobre la ovulación y la atresia folicular en el ovario de la rata adulta. Memorias del XLIV Congreso Nacional de Ciencias Fisiológicas. Monterrey, N.L. O-73.

Neill, J.D. (1988). Prolactin secretion and its control. En: "Physiology of Reproduction". Vol. I. Capítulo 33. Eds. E. Knobil & J. Neill. Raven Press, New York, pp. 1379-1390.

NORMA OFICIAL MEXICANA NOM-062-ZOO-1999 (1999). Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio.

Palkovits, M. (1980). Functional anatomy of the 'endocrine' brain. En: "The Endocrine Functions of the Brain". Capítulo 1. Eds. M. Motta. Raven Press. New York. pp 1-16.

Paxinos, G. & Watson, Ch. (1998). The Rat Brain: In stereotaxic coordinates. 4th. Edition, Academic Press.

Peppler, R. D. & Jacobs, J. J. (1976). The effect of adrenal lectomy on ovulation and follicular development in the rat. Biol Reprod. 15:173-178.

ISSN-On line: 2414-8849

Pierce, J.G. (1988). Gonadotropins: chemistry and biosynthesis. En: "Physiology of Reproduction". 1th Edition. Vol. 1. Capítulo 31. Eds. E. Knobil & J. Neill. Raven Press, New York, pp 1335-1348.

Ramírez, V.D., H.M. Feder & C.H. Sawyer (1984). The role of brain catecholamines in the regulation of LH secretion: A critical inquiry. En: "Frontiers in Neuroendocrinology". Volumen III. Capítulo 2. Eds. L. Martini & W.F. Ganong. Raven Press. New York. pp 27-71.

Ramírez-Ávila, B. (2001). Efectos de la administración secuencial de GnRH sobre la ovulación en ratas adultas con bloqueo farmacológico del sistema dopaminérgico. Tesis Profesional. Escuela de Biología de la BUAP.

Sánchez, M.A. & R. Domínguez (1995). Differential effects of unilateral lesions in the medial amygdala on spontaneous and induced ovulation. Brain Res Bull 38:313-317.

Sanhera, M.K, S. Grady, W. Smith, D.J. Woodward & J.C. Porter (1991a). Incertohypothalamic A13 dopamine neurons: Effect of gonadal steroids on tyrosine hydroxylase. Neuroendocrinology 53:268-275.

Sanhera, M.K., J. Anselmo-Franci & S.M. Mccann (1991b). Effect on medial zona incerta lesions on ovulatory surge of gonadotrophins and prolactin in the rat. Neuroendocrinology 53: 433-438.

Sawyer, C.H. & D.K. Clifton (1980). Aminergic innervation of the hypothalamus. Fed Proc 39:2889-2895.

Silverman, A.J. (1988). The gonadotropin-releasing hormone (GnRH) Neuronal Systems: Immunocytochemistry. En: "Physiology of Reproduction". Vol. 1. Capítulo 29. Eds. E. Knobil & J. Neill. Raven Press, New York, pp 1282-1304.

Simerly, R.B. (1995). Anatomical substrates of hypothalamic integration. En:" The Rat Nervous System". 2th. Edition. Eds. G. Paxinos. Academic Press. New York. pp 353-376.

Smith, M.S., M.F. Freeman & J. D. Neill (1975). The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescuer of the corpus luteum of pseudopregnancy. Endocrinology 96:219-226.

Smith, M.S., McLean, B., and Neill, J.D. (1976) Prolactin: the initial luteotropic stimulus of pseudopregnancy in the rat. Endocrinology, 98: pp 1370-1377.

Tajasen, T. & J.W. Everett (1967). Surgical analysis of the preoptico-tuberal pathway controlling ovulatory release of gonadotrophins in the rat. Endocrinology 81:1387-1396.

Tasaka, K., A. Miyake, T. Sakumoto & T. Aono (1985). Dopamine decreases release of luteinizing hormone releasing hormone from superfused rat mediobasal hypothalamus. J Endocrinol Invest 8:373-376.

Tohyama, M. & K. Takatsuji (1998). The catecholaminergic system. En: "Atlas of Neuroactive Substances and their Receptors in the Rat". Ed. M. Tohyama & K. Takatsuji. Oxford University Press, pp 20-33.

Vargas-Torres, L.A. (2002). Efectos del bloqueo farmacológico de la información dopaminérgica sobre el ciclo estral de la rata: Análisis de los mecanismos que inhiben la función de los ovarios. Tesis Profesional. Escuela de Biología de la BUAP.

Venegas Meneses, B., Juárez Robelo, C.E., Handal Silva, A. & Morán Perales, J.L. (2017). Efectos del bloqueo irreversible de los receptores dopaminérgicos del ovario sobre la ovulación espontánea de la rata adulta. Revista de Ciencias de la Salud 4(11): 11-23 (ISSN-2410-3551 ECORFAN).

Venegas, B., Padilla, F., Juárez, C., Morán, J., Morán, C., Rosas, N., Handal, A & Domínguez, R. (2015). Effects of ovarian dopaminergic receptors on ovulation. *Endocrine,* 50 (3):783–796.

Welsh, T. H. & Johnson, B. H. (1981). Stress-induced alterations in secretions of corticosteroids, progesterone, luteinizing hormone, and testosterone in bulls. Endocrinology. 109: 185-190.

ISSN-On line: 2414-8849

Wilson, C.A., M.D. James, J.P. Grierson & D.R. Hole (1991). Involvement of catecholaminergic systems in the zona incerta in the steroidal control of gonadotrophin release and female sexual behavior. Neuroendocrinology 53:113-123.

Acknowledgments

Our Academic Body (CA-090) thanks MVZ Francisco Ramos Collazo, director of the Claude Bernard Bioterio Autonomous University of Puebla, veterinarian assigned to the welfare of our experimental animals, all facilities and care for the development of draft.

ISSN-On line: 2414-8849

[Title in Times New Roman and Bold No. 14 in English and Spanish]

Surname (IN UPPERCASE), Name 1st Author†*, Surname (IN UPPERCASE), Name 1st Coauthor, Surname (IN UPPERCASE), Name 2nd Coauthor and Surname (IN UPPERCASE), Name 3rd Coauthor

Institutional Affiliation of Author including Dependency (No.10 Times New Roman and Italic)

International Identification of Science - Technology and Innovation

ID 1st author: (ORC ID - Researcher ID Thomson, arXiv Author ID - PubMed Author ID - Open ID) and CVU 1st author: (Scholar-PNPC or SNI-CONACYT) (No.10 Times New Roman)

ID 1st coauthor: (ORC ID - Researcher ID Thomson, arXiv Author ID - PubMed Author ID - Open ID) and CVU 1st coauthor: (Scholar or SNI) (No.10 Times New Roman)

ID 2nd coauthor: (ORC ID - Researcher ID Thomson, arXiv Author ID - PubMed Author ID - Open ID) and CVU 2nd coauthor: (Scholar or SNI) (No.10 Times New Roman)

ID 3rd coauthor: (ORC ID - Researcher ID Thomson, arXiv Author ID - PubMed Author ID - Open ID) and CVU 3rd coauthor: (Scholar or SNI) (No.10 Times New Roman)

(Report Submission Date: Month, Day, and Year); Accepted (Insert date of Acceptance: Use Only ECORFAN)

Abstract (In English, 150-200 words)

Objectives
Methodology
Contribution

Keywords (In English)

Indicate 3 keywords in Times New Roman and Bold No. 10

Abstract (In Spanish, 150-200 words)

Objectives
Methodology
Contribution

Keywords (In Spanish)

Indicate 3 keywords in Times New Roman and Bold No. 10

Citation: Surname (IN UPPERCASE), Name 1st Author†*, Surname (IN UPPERCASE), Name 1st Coauthor, Surname (IN UPPERCASE), Name 2nd Coauthor and Surname (IN UPPERCASE), Name 3rd Coauthor. Paper Title. ECORFAN Journal-Republic of Guatemala.Year 1-1: 1-11 [Times New Roman No.10]

* Correspondence to Author (example@example.org)
† Researcher contributing as first author.

Introduction

Text in Times New Roman No.12, single space.

General explanation of the subject and explain why it is important.

What is your added value with respect to other techniques?

Clearly focus each of its features

Clearly explain the problem to be solved and the central hypothesis.

Explanation of sections Article.

Development of headings and subheadings of the article with subsequent numbers

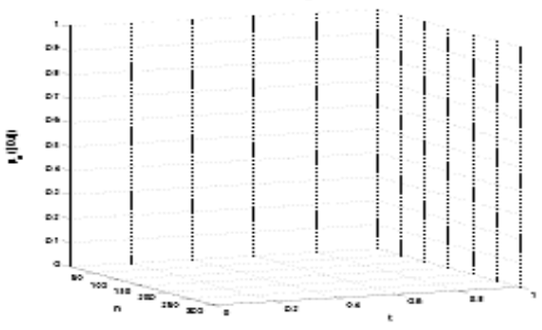
[Title No.12 in Times New Roman, single spaced and bold]

Products in development No.12 Times New Roman, single spaced.

Including graphs, figures and tables-Editable

In the article content any graphic, table and figure should be editable formats that can change size, type and number of letter, for the purposes of edition, these must be high quality, not pixelated and should be noticeable even reducing image scale.

[Indicating the title at the bottom with No.10 and Times New Roman Bold]



Graphic 1 Title and *Source (in italics)*

Should not be images-everything must be editable.

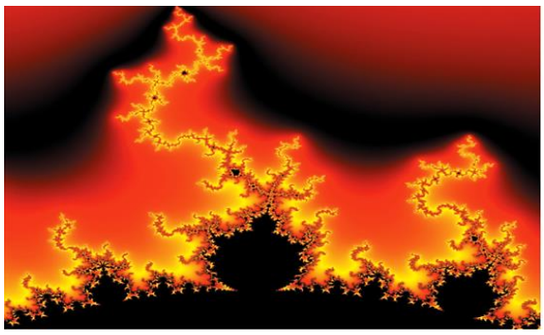


Figure 1 Title and *Source (in italics)*

Should not be images-everything must be editable.

Table 1 Title and *Source (in italics)*

Should not be images-everything must be editable.

Each article shall present separately in **3 folders**: a) Figures, b) Charts and c) Tables in .JPG format, indicating the number and sequential Bold Title.

For the use of equations, noted as follows:

$$Y_{ij} = \alpha + \sum_{h=1}^{r} \beta_h X_{hij} + u_j + e_{ij} \qquad (1)$$

Must be editable and number aligned on the right side.

Methodology

Develop give the meaning of the variables in linear writing and important is the comparison of the used criteria.

Results

The results shall be by section of the article.

Annexes

Tables and adequate sources thanks to indicate if were funded by any institution, University or company.

Conclusions

Explain clearly the results and possibilities of improvement.

ISSN: 2524-2121

References

Use APA system. Should not be numbered, nor with bullets, however if necessary numbering will be because reference or mention is made somewhere in the Article.

Use Roman Alphabet, all references you have used must be in the Roman Alphabet, even if you have quoted an Article, book in any of the official languages of the United Nations (English, French, German, Chinese, Russian, Portuguese, Italian, Spanish, Arabic), you must write the reference in Roman script and not in any of the official languages.

Technical Specifications

Each article must submit your dates into a Word document (.docx):

Journal Name
Article title
Abstract
Keywords
Article sections, for example:

1. Introduction
2. Description of the method
3. Analysis from the regression demand curve
4. Results
5. Thanks
6. Conclusions
7. References

Author Name (s)
Email Correspondence to Author
References

Intellectual Property Requirements for editing:

-Authentic Signature in Color of Originality Format Author and Coauthors

-Authentic Signature in Color of the Acceptance Format of Author and Coauthors

ISSN: 2524-2121

Reservation to Editorial Policy

ECORFAN Journal Republic of Guatemala reserves the right to make editorial changes required to adapt the Articles to the Editorial Policy of the Journal. Once the Article is accepted in its final version, the Journal will send the author the proofs for review. ECORFAN® will only accept the correction of errata and errors or omissions arising from the editing process of the Journal, reserving in full the copyrights and content dissemination. No deletions, substitutions or additions that alter the formation of the Article will be accepted.

Code of Ethics - Good Practices and Declaration of Solution to Editorial Conflicts

Declaration of Originality and unpublished character of the Article, of Authors, on the obtaining of data and interpretation of results, Acknowledgments, Conflict of interests, Assignment of rights and Distribution.

The ECORFAN-Mexico, S.C Management claims to Authors of Articles that its content must be original, unpublished and of Scientific, Technological and Innovation content to be submitted for evaluation.

The Authors signing the Article must be the same that have contributed to its conception, realization and development, as well as obtaining the data, interpreting the results, drafting and reviewing it. The Corresponding Author of the proposed Article will request the form that follows.

Article title:

- The sending of an Article to ECORFAN Journal Republic of Guatemala emanates the commitment of the author not to submit it simultaneously to the consideration of other series publications for it must complement the Format of Originality for its Article, unless it is rejected by the Arbitration Committee, it may be withdrawn.

- None of the data presented in this article has been plagiarized or invented. The original data are clearly distinguished from those already published. And it is known of the test in PLAGSCAN if a level of plagiarism is detected Positive will not proceed to arbitrate.

- References are cited on which the information contained in the Article is based, as well as theories and data from other previously published Articles.

- The authors sign the Format of Authorization for their Article to be disseminated by means that ECORFAN-Mexico, S.C. In its Republic of Guatemala considers pertinent for disclosure and diffusion of its Article its Rights of Work.

- Consent has been obtained from those who have contributed unpublished data obtained through verbal or written communication, and such communication and Authorship are adequately identified.

- The Author and Co-Authors who sign this work have participated in its planning, design and execution, as well as in the interpretation of the results. They also critically reviewed the paper, approved its final version and agreed with its publication.

- No signature responsible for the work has been omitted and the criteria of Scientific Authorization are satisfied.

- The results of this Article have been interpreted objectively. Any results contrary to the point of view of those who sign are exposed and discussed in the Article.

Copyright and Access

The publication of this Article supposes the transfer of the copyright to ECORFAN-Mexico, SC in its Holding Mexico for its ECORFAN Journal Republic of Guatemala, which reserves the right to distribute on the Web the published version of the Article and the making available of the Article in This format supposes for its Authors the fulfilment of what is established in the Law of Science and Technology of the United Mexican States, regarding the obligation to allow access to the results of Scientific Research.

Article Title:

Name and Surnames of the Contact Author and the Coauthors	Signature
1.	
2.	
3.	
4.	

Principles of Ethics and Declaration of Solution to Editorial Conflicts

Editor Responsibilities

The Publisher undertakes to guarantee the confidentiality of the evaluation process, it may not disclose to the Arbitrators the identity of the Authors, nor may it reveal the identity of the Arbitrators at any time.

The Editor assumes the responsibility to properly inform the Author of the stage of the editorial process in which the text is sent, as well as the resolutions of Double-Blind Review.

The Editor should evaluate manuscripts and their intellectual content without distinction of race, gender, sexual orientation, religious beliefs, ethnicity, nationality, or the political philosophy of the Authors.

The Editor and his editing team of ECORFAN® Holdings will not disclose any information about Articles submitted to anyone other than the corresponding Author.

The Editor should make fair and impartial decisions and ensure a fair Double-Blind Review.

Responsibilities of the Editorial Board

The description of the peer review processes is made known by the Editorial Board in order that the Authors know what the evaluation criteria are and will always be willing to justify any controversy in the evaluation process. In case of Plagiarism Detection to the Article the Committee notifies the Authors for Violation to the Right of Scientific, Technological and Innovation Authorization.

Responsibilities of the Arbitration Committee

The Arbitrators undertake to notify about any unethical conduct by the Authors and to indicate all the information that may be reason to reject the publication of the Articles. In addition, they must undertake to keep confidential information related to the Articles they evaluate.

Any manuscript received for your arbitration must be treated as confidential, should not be displayed or discussed with other experts, except with the permission of the Editor.

The Arbitrators must be conducted objectively, any personal criticism of the Author is inappropriate.

The Arbitrators must express their points of view with clarity and with valid arguments that contribute to the Scientific, Technological and Innovation of the Author.

The Arbitrators should not evaluate manuscripts in which they have conflicts of interest and have been notified to the Editor before submitting the Article for Double-Blind Review.

Responsibilities of the Authors

Authors must guarantee that their articles are the product of their original work and that the data has been obtained ethically.

Authors must ensure that they have not been previously published or that they are not considered in another serial publication.

Authors must strictly follow the rules for the publication of Defined Articles by the Editorial Board.

The authors have requested that the text in all its forms be an unethical editorial behavior and is unacceptable, consequently, any manuscript that incurs in plagiarism is eliminated and not considered for publication.

Authors should cite publications that have been influential in the nature of the Article submitted to arbitration.

Information services

Indexation - Bases and Repositories

RESEARCH GATE (Germany)
GOOGLE SCHOLAR (Citation indices-Google)
REDIB (Ibero-American Network of Innovation and Scientific Knowledge- CSIC)
MENDELEY (Bibliographic References Manager)

Publishing Services:

Citation and Index Identification H.
Management of Originality Format and Authorization.
Testing Article with PLAGSCAN.
Article Evaluation.
Certificate of Double-Blind Review.
Article Edition.
Web layout.
Indexing and Repository
ArticleTranslation.
Article Publication.
Certificate of Article.
Service Billing.

Editorial Policy and Management

16 Kilometro, American Highway, House Terra Alta, D7 Mixco Zona 1-Guatemala. Phones: +52 1 55 6159 2296, +52 1 55 1260 0355, +52 1 55 6034 9181; Email: contact@ecorfan.org www.ecorfan.org

ECORFAN Journal-Republic of Guatemala

2414 8849
ISSN 2414 - 8849

www.ecorfan.org