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Effects of iron deficiency on the ovarian cycle. Experimental model

Efectos de la carencia de hierro en el ciclo ovárico. Modelo experimental

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Abstract

Iron is a vital trace element involved in more than 400 chemical reactions and is a structural component of several proteins and enzymes. It is even an indispensable cofactor for hormone synthesis, forming the heme group of cytochromes necessary for the structure of steroid hormones. It has been experimentally demonstrated that iron deficiency anemia alters the ovarian cycle; however, it is not known whether iron deficiency can alter the ovarian cycle without reaching the anemia level. Aim: to determine the effects of iron deficiency on the ovarian cycle. Methods: a rat model of iron deficiency from gestation to adulthood (70 days postnatal) was used. Ten adult female rats with iron deficiency were used to obtain samples for vaginal cytology. Samples were analyzed microscopically to determine the phases of the ovarian cycle based on the most abundant cell type. Contribution: Iron deficiency leads to a shortening of the metestrus/diestrus phase and a lengthening of the proestrus; this could lead to fertility changes associated with variations in the duration of the phases of the ovarian cycle.

Iron, Ovarian cycle, Female

Resumen

El hierro es un elemento traza necesario para la vida, actúa en mas de 400 reacciones químicas y forma parte estructural de diversa proteínas y enzimas. Es incluso un cofactor indispensable para la síntesis de hormonas y conforma el grupo hemo de citocromos requeridos para la formación de hormonas esteroideas. Se ha demostrado experimentalmente que la anemia por deficiencia de hierro altera el ciclo ovárico, sin embargo, se desconoce si padecer deficiencia de hierro sin llegar a niveles de anemia, puede alterar el ciclo. Objetivo: determinar el impacto de la deficiencia de hierro sobre el ciclo ovárico. Metodología: se empleó un modelo de deficiencia de hierro en rata, desde la gestación hasta la edad adulta (70 días postnatales). Diez ratas hembras adultas deficientes de hierro se emplearon para obtener muestras para citología vaginal. Las muestras fueron analizadas al microscopio para establecer las fases del ciclo ovárico con base en el tipo celular más abundante presente. Contribución: la deficiencia de hierro induce el acortamiento de la fase de metaestro/diestro e incrementa proestro; esto podría causar alteraciones de fertilidad vinculadas a las variaciones en la duración de las etapas del ciclo ovárico.

Hierro, Ciclo ovárico, Hembras

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Introduction

Iron is abundant throughout the universe as a trace element in nuclear stable forms; on Earth it is about as abundant as oxygen. Iron-containing ferrous compounds were likely available for the development of organisms during the first billion years on the planet (Williams, 2012). Early metabolic processes contained iron ions (II) that oxidized oxygen ions released from organic compounds. Over time, cells began to use oxidized iron ions to deliver oxygen. The evolution of iron transfer systems in eukaryotes and multicellular organisms may have coincided with the increase in oxygen concentration on Earth 2.5 billion years ago (Williams, 2012). As the systems evolved, iron was involved not only in oxygen transport, but also as a cofactor in redox reactions, cell replication, and respiration, as well as in the electron transport system (Toxqui et al., 2010).

It is known that about 400 chemical reactions are occurring in the body that depends on iron as a catalyst for the formation of its products. Most of these reactions would be affected by decreasing iron levels in the body. (Ganz, 2003). Free iron is toxic to biological processes and contributes to oxidative stress, making biological iron transfer systems very challenging. (Frazer & Anderson, 2014). The total body iron content in a person of 1.70 meters tall and weighing 70 kilograms is estimated at 3.5 to 4 grams for women and 4 to 5 grams for men. (Beard, Dawson, & Pinero, 1996). Most of the body's iron is recycled from red blood cells, the rest is supplied through food. (Ganz & Nemeth, 2006). There are two main types of dietary iron: a) heme iron or heme group is formed by an iron atom in the ferrous state, Fe2, divalent, linked to a protoporphyrin molecule consisting of four pyrroles linked by methyl bonds forming a tetrapyrrole ring. This is the prosthetic group of various proteins and enzymes such as hemoglobin and cytochromes. Heme iron is present in red meat and animal foods. (B. Silva & Faustino, 2015; Stuart et al., 2003). (b) Non-heme iron, organic or ferric iron of trivalent character, Fe3, occurs in nature as part of inorganic salts. It is found in large quantities in vegetables, especially in green leaves such as spinach. (Stuart et al., 2003). Humans absorb between 15 and 25% of heme iron (Monsen et al., 1978) and 5 to 10% of non-heme iron (Monsen, 1988).

This, combined with other foods that modify iron absorption, makes it´s difficult to calculate the dietary intake of this element (Monsen et al., 1978; Hallberg, 1981); in addition to the variations in their requirements during the different stages of life and physiological status, as shown in Table 1.

Age	mg/day	Adults	mg/day
6-12 months	0.96	Adults men	0.86
13-24 months	0.61	Pregnant 1 trimester	0.8
2-5 years	0.7	Pregnant 2 y 3 trimester	6.3
6-11	1.5	Breastfeeding women	1.3
12-16 girls	2.0*	Women of reproductive age	2.3
12-16 boys	1.8	Post menopausal women	0.96

Table 1 Daily iron requirements by age group
*Source: Adapted from Abbaspour and Hurrell 2014 (8). * The requirements by sex between 12-16 years may vary according to the age of menarche, in which case 2.3 mg per day will be contemplated in females*

It is estimated that 1.6 billion people worldwide suffer from anemia, of which approximately half are caused by iron deficiency (ID), so this nutritional deficiency should not be extrapolated to the prevalence of anemia (Viteri, 1998). The most affected age groups are represented by children and pregnant women (McLean, Cogswell, Egli, Wojdyla, & de Benoist, 2009); with menstrual losses being the main cause of ID in women of childbearing age and gastrointestinal bleeding in postmenopausal women and adult males (McIntyre & Long, 1993). ID is associated with: headache, pallor, fatigue, dyspnea, alopecia, and cognitive dysfunction (Lopez, Cacoub, Macdougall, & Peyrin-Biroulet, 2016). This nutritional deficiency also affects reproduction, pregnancy, and childbirth (Burke, Leon, & Suchdev, 2014). Returning to the heme group, it is known that some of the cytochromes differ from each other along with their chemical structures, which ensures that the cytochromes have variable reducing potentials and are located at different points in the respiratory chain to optimize electron transfer. Cytochrome P450 is a completely different group of enzymes; instead of oxidizing their substrates, they incorporate a molecular atom; therefore, they are monooxygenases, not oxidases like the other cytochromes.

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Currently, of the fifty-seven human P450s, fourteen are known to metabolize cholesterol. One of them, CYP51, is involved in its biosynthesis and thirteen in its degradation. Of these thirteen, seven P450s are involved in bile acid biosynthesis (7A1, 27A1, 46A, 7B1, 39A1, 8B1, and 3A4) and six in steroid hormone biosynthesis (11A1, 17A1, 21, 19A1, 11B1 y 11B2) (Pikuleva, 2006). The biosynthesis of steroid hormones, including mineralocorticoids, glucocorticoids, estrogens, and androgens, accounts for the daily elimination of about 50 mg of colesterol (Turley & Dietschy, 1982). Of the six P450s involved in steroidogenesis, only one, CYP11A1, is expressed in steroidogenic tissues (adrenal glands, ovaries, testes, placenta, and brain) (Mellon & Griffin, 2002); Of the six P450s involved in steroidogenesis, only one, CYP11A1, is expressed in steroidogenic tissues (adrenal glands, ovaries, testes, placenta, and brain); catalyzes the conversion of cholesterol to pregnenolone; in turn, CYP11A1, CYP 17A1, CYP 21, CYP 19A1, CYP 11B1 and CYP 11B2 act on pregnenolone to form steroid hormones. CYP11A1 enzymatic activity has also been detected in the pancreas and skin (Morales, Cuellar, Ramirez, Vilchis, & Diaz-Sanchez, 1999). Hence the importance of iron in the synthesis of steroid hormones, in addition to being a cofactor for the synthesis of neurotransmitters, such as dopamine and serotonin, which stimulate at the hypothalamic level the secretion of gonadotropin-releasing hormone (GnRH) and thus the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Unger et al., 2007).

Many reports on the effects of ID at the gestational level and in infants have been carried out (Burke et al., 2014; Gambling & McArdle, 2004; Idjradinata & Pollitt, 1993; Lozoff, Jimenez, & Smith, 2006; Lozoff, Kaciroti, & Walter, 2006), but little information is available on the effect of ID on follicular development and ovulation during the ovarian cycle. The ovarian cycle is regulated by two pituitary-derived gonadotropins: FSH and the luteinizing hormone, LH. FSH induces follicular development to the preovulatory stage as a prelude to producing and releasing a mature oocyte. FSH increases CYP19A1 expression in granulosa cells to produce estradiol-17β (E2) from androgens produced in intrathecal cells (Robker & Richards, 1998; J. M. Silva & Price, 2000).

E2 increases the expression of follicular development marker genes including Ccnd2, a cell cycle regulator gene to induce follicular development. When dominant follicles reach a preovulatory state, E2 induces transient LH secretion from the pituitary gland (Wesson, Miller, & Ginther, 1980). LH induces the expansion of cumulus oocytes complex (COC), the resumption of meiosis in oocytes from phase I to metaphase II (oocyte maturation), and ovulation (Su, Wigglesworth, Pendola, O'Brien, & Eppig, 2002). Kim et al, 2018 showed that iron-containing mineral compounds affect menstrual cycle and hormone concentration in humans (Kim et al., 2018). Other research reveals that 50% of the patients studied who suffer from anemia present amenorrhea (Pafumi et al., 2011). There is only one report that analyzed the ovarian cycle, follicular development, and ovulation in mice suffering from anemia (Tonai et al., 2020). However it is not known what effects iron deficiency, without reaching the degree of anemia, has on the ovarian cycle.

Methodology

Animals and diet

Wistar rats were maintained under standard vivarium conditions: 12:12 light/dark cycle (lights on at 05:00 a.m.), temperature controlled at 22 ± 2°C and free access to food and water. To obtain the study subjects, twenty dam rats of 3 months of age or 250 grams of weight were used. Fourteen days prior to mating conditions, ten rats were subjected to an iron-deficient diet, consisting of 10 ppm FeSO4 (Laboratory diet AIN-76W/10). The other ten rats received a control diet with 100 ppm FeSO4 (AIN-76W/100 laboratory diet). Twenty-one days post-mating, the offspring were obtained. At weaning, 21 postnatal days after birth, ten female offspring from iron-deficient dams and ten female offspring from the control group were randomly separated and fed the same diet as their mothers until reaching adulthood at postnatal day 70.

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Exfoliative cytology

There are several techniques to perform exfoliative cytology. The following procedure is based on a modification of the technique used in the article "Determination of the stages of the ovarian cycle" (Martínez, 2005). Cytological samples were taken from all control and iron-deficient females at the same time (7:00 am), using the following materials: Pasteur pipettes, 0.10% PBS, slides, coverslips, and Papanicolaou stain.

Procedure

- The rat was positioned on a surface holding it by the base of the tail, elevating it, thus leaving the front paws on the surface and the visualization of the vaginal introitus.
- The Pasteur pipette was loaded with 2 µL of PBS at 0.10%.
- The tip of the loaded pipette was introduced into the vaginal introitus, discharging the content and suctioning; without removing the pipette, the content was discharged and suctioned again.
- The pipette was withdrawn and the sample was applied on a slide and allowed to dry.
- The sample was stained using the Papanicolaou technique.
- The sample was observed with the aid of a microscope identifying the phase of the ovarian cycle.

The data were collected, and an analysis of the possible changes observed during the entire ovarian cycle in each rat was performed, followed by a comparison of the cycles of each study group.

Ethical considerations

The present investigation was subjected to the guidelines of NOM-062-ZOO-1999; technical specifications for the production, care, and use of laboratory animals.

Experimental subjects were kept in captivity, with appropriate handling according to the standard in its category B: experiments that cause minimal discomfort or stress, e.g., momentary restraint of the animal for clinical observation purposes; blood sampling; and injection of substances by intravenous, subcutaneous, intramuscular, intraperitoneal, or oral routes. Acute studies are those without animal survival when the animal is completely anesthetized. use of euthanasia methods with rapid unconsciousness of the subject; for example, an overdose of anesthetics. Short periods of abstinence from water or food are equivalent to what could occur naturally.

Results

Classification of ovarian cycle stages according to pattern and cellular predominance
Depending on the cellular predominance, each stage of the ovarian cycle can be identified, being in the metestrus/diestrus stages where there is the greatest predominance of leukocytes, in the proestrus of epithelial cells, and in the estrus of squamous cells. After obtaining and processing the sample as detailed in the methodology, the smears were observed under the microscope and it was found that in control and iron-deficient rats, the cellular patterns are similar. Figure 1 shows the cellular pattern obtained from control subjects, where the cellular predominance in each stage can also be seen.

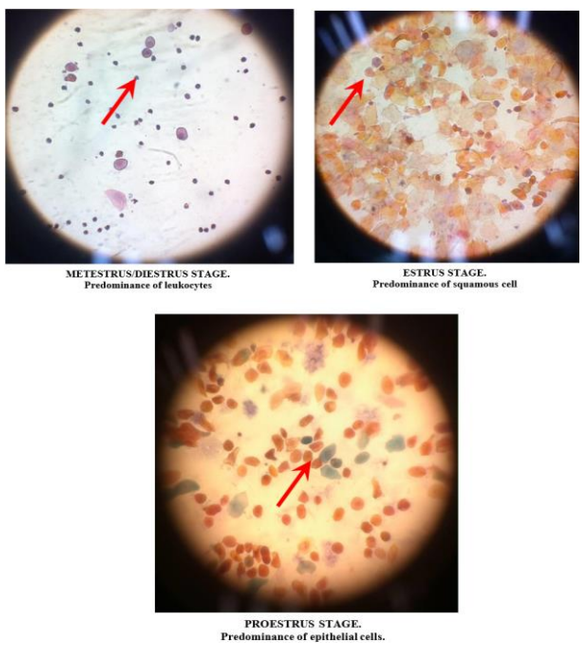

Figure 1 Typical cell patterns that are seen during the ovarian cycle

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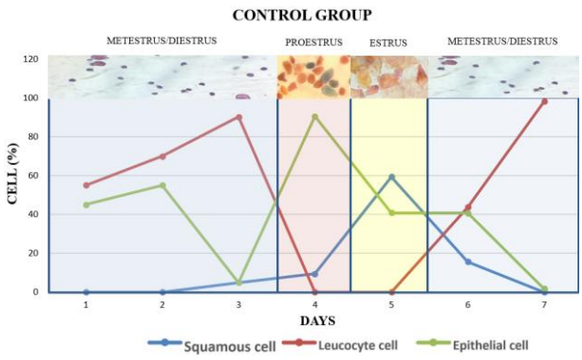
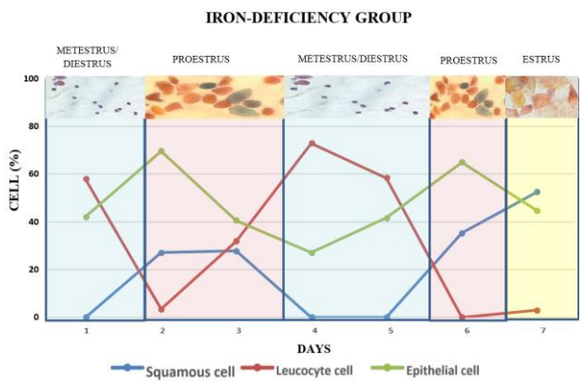
As previously mentioned, the ovarian cycle begins in the estrus stage, which is the cellular desquamation. In women, it is translated as menstruation. This is followed by the metestrus/diestrus stage in which ovulation occurs and few epithelial cells and abundant leukocytes are seen. Finally, in the proestrus stage, there is an increase in epithelial cells. In this regard, it was found in control rats (Graphic 1) and in iron-deficient rats (Graphic 2) that the sequence of stages of the cycle is not altered.

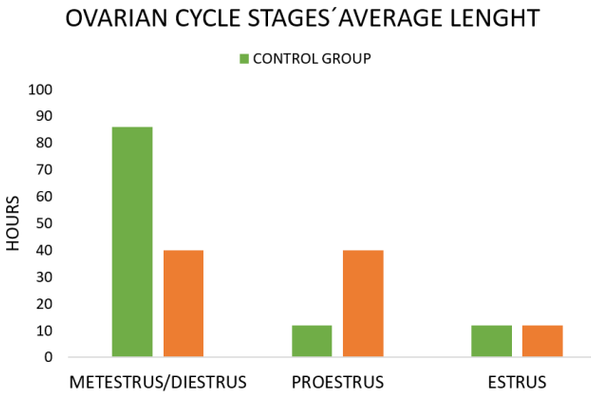


Graphic 1 Cell percentage obtaine by exfoliative cytology in rats from the control group



Graphic 2 Cell percentage was obtained by exfoliative cytology in rats from the iron deficient-group

In experimental models, the average duration of the metestrus/diestrus stage is 86 hours, proestrus 12 hours, and estrus 12 hours. The proestrus stage is seen to lengthen in iron-deficient females; in these individuals, the period might last up to 42 hours (Graphic 3). However, the metestrus/diestrus phase is shortened to 40–42 hrs. Despite these changes, the total duration of each cycle is between 108 and 110 hours.



Graphic 3 Duration of the metestrus/diestrus (shortening) and proestrus (lengthening) stages in the study groups

Discussion

According to the cellular predominance, the cellular patterns in each stage of the ovarian cycle were analyzed. When evaluating the results, it can be seen that the cellular pattern in both: control and iron-deficient females is similar, which indicates that the deficiency of this trace element does not affect this level. The same occurs when observing the sequence of the stages of the cycle, since no alterations in the order of the phases were observed between the study groups. It is interesting to mention that variations were found in the duration of the metestrus/diestrus and proestrus phases in iron deficient females, which may justify its indirect participation in hormonal regulation (M. Hidiroglou 1979). It has been described that iron intervenes in the synthesis of some neurotransmitters, such as dopamine and noradrenaline, which use it as a cofactor for tyrosine hydroxylase and tryptophan hydroxylase in the case of serotonin. These neurotransmitters stimulate the secretion of gonadotropin-releasing hormone (GnRH) at the hypothalamic level (Unger et al., 2007). Thus, alterations in GnRH pulses could be indirectly modified by iron deficiency and lead to problems in the release of the two main gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anterior pituitary; however, further studies are lacking. Both LH and FSH promote sex steroid synthesis in association with follicular growth and ovulation. It is worth mentioning that FSH stimulates follicle maturation to favor fertilization and LH intervenes in follicle release. Therefore, iron deficiency could intervene in alterations that impact fertility processes.

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Changes in the length of the phases of the cycle were found in the metestrus/diestrus and proestrus stages, which show a lengthening and shortening, respectively. These changes are probably caused by estrogenic overstimulation, which is influenced by defects at the hypothalamic level in the stimulation of GnRH, which controls FSH and LH pulses, but more research is needed to confirm this. Our group has reported that iron-deficient subjects, both females and males, uptake lower levels of this trace element in gonads, indicating the presence of possible alterations in its function since iron is an essential element for cellular homeostasis (Vieyra-Reyes, Oros-Pantoja, Torres-Garcia, Gutierrez-Ruiz, & Perez-Honorato, 2017).

This could potentially be involved in the advancement of changes in the stages of the ovarian cycle, leading to the development of reproductive issues like those seen in iron-deficient patients.

On the other hand, both iron overload and iron depletion (data not yet published by our research group) favor oxidative stress at central and peripheral levels, which could lead to the development of reproductive alterations. In addition, about the brain, it has been demonstrated that iron deficiency alters synaptic plasticity; therefore, it would be advisable to study whether these alterations in the phases of the cycle are caused by plastic variations at the hypothalamic level. In addition, it has been published that iron excess favors the disruption of follicular development and steroidogenesis, however, there are no data regarding iron deficiency itself.

Due to the findings demonstrated in the present study, it is observed that iron deficiency can be a constant that alters fertility processes. For this reason, it is important to perform a timely and accurate diagnosis of iron levels, mainly in women of childbearing age.

In Mexico, even in our days, the culture orients and values women based on procreation and family formation, where maternity is a sociocultural aspect of categorical influence in daily life. In both the public and private sectors, the cost of medical care generated by the presence of alterations in the menstrual cycle and infertility is high and, unfortunately, not linked to iron levels in the body.

Therefore, the present study has social scopes that could be taken into account in clinical practice guidelines; innovation and research development scopes, through intentional search protocols for the aforementioned alterations; and clinical scopes that could be put into practice through diagnostic tests from menarche or before the onset of active sexual life, thus contributing to the reduction of costs in the health sector.

Conclusions

Due to changes in the length of the ovarian cycle's stages, iron deficiency shortens the metestrus/diestrus phase's duration and lengthens the proestrus, which may affect fertility.

Perspectives

- Determine hormone levels to identify the point at which the hypothalamic-pituitary-ovarian axis is altered.
- Identify dopamine, norepinephrine, and serotonin levels and correlate them to variations in the ovarian cycle.
- Establish the conception rate in females with iron deficiency.
- Measure the histological expression of gonadotropin receptors in reproductive organs.

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