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Estrous Cycle, Fertility and Fetal Development in Rats with Hyperbilirubinemia

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Abstract

Objective: Decreasing in reproductive capacity has been observed in jaundiced female rats. The animals with hyperbilirubinemia present early sexual maturation, late ovulation, reduced number of corpora lutea and precocious vaginal opening. The interaction with toxic agents leads to abnormal development of the embryos. The objective is to investigate the influence of jaundice on estrous cycle, fertility, ovaries and fetus development.

Methods: 66 female rats were divided into two groups (n=33): group 1 - ligature of the biliopancreatic duct, group 2 - sham operation. These animals were paired with males. Vaginal smears were collected daily to verify pregnancy and the gestational period. The morphologic aspect of the ovaries and the corpus luteum were studied. The morphology of the fetuses were assessed. Serum bilirubins were recorded. 32 rats of the control group and 11 jaundiced rats became pregnant.

Results: The 17 rats with hyperbilirubinemia that did not become pregnant presented involutive corpora lutea and modifications in their estrous cycles, remaining in pro-estrus or estrus. The pregnant rats with hyperbilirubinemia had fetuses with abnormal development.

Conclusions: Fertilization occurs in jaundiced rats, but reproductive capacity is reduced, with irregular estrous cycles, involutive corpora lutea and abnormal fetal development.

Keywords: Hyperbilirubinemia; Fertilization; Fetus development; Estrous cycle; Corpus luteum

Introduction

Jaundice may be caused by bilirubin dysfunction, hemolysis, cirrhosis, hepatitis, biliary obstruction and other cholestases [1]. Intrahepatic cholestasis of pregnancy occurs during the third trimester and disappears after the end of the pregnancy. Its pathophysiology is still unknown, however evidences suggest that genetic causes may determine estrogenic gestational disturbances. Hepatocyte enzyme system involved in the metabolism and excretion of bilirubin seems to play a pivotal role in this condition, being associated with prematurity and stillbirth [1-5].

Viral hepatitis may be present or starts during the pregnancy. Jaundice is provoked by cholestasis and may persist for at least four weeks. Hepatitis is associated with increased rates of fetal morbidity and mortality [6]. When pregnancy occurs in cirrhotic patients spontaneous abortions and premature infants have been described [7].

Decreasing in reproductive capacity has been observed in jaundiced female rats. The animals with hyperbilirubinemia present early sexual maturation, late ovulation, reduced number of corpora lutea and precocious vaginal opening [8,9]. The harmful toxicity of hyperbilirubinemia can be demonstrated by the reducing number of embryos implantation sites and higher number of embryo resorptions [8,9]. The interaction with toxic agents leads to abnormal development of the embryos [10].

Considering that gestational complications are more frequent among women with hyperbilirubinemia. This study was conducted with the purpose to verify alterations in the fertility, ovaries and fetuses development in rats with hyperbilirubinemia.

Methods

All this study, involving animals, was approved by the Committee of Ethics Applied to Animals of the Federal University of Minas Gerais, under the number number 149/2011. Institutional guidelines for the care and treatment of laboratory animals were adhered to [11].

Sixty six sexually mature Lewis rats aged four months and whose initial weights ranged from 191 to 220 grams were used. These animals were kept in temperature and lighting environments, receiving daily water and standard normocaloric diet for rats [Nuvilab®] containing by weight: 19.0% protein, 56.0% carbohydrate, 3.5% fat, 4.5% cellulose, 5.0% vitamins and minerals, totaling 17.03 kJ/g. The animals were randomly divided into two groups:

Group 1 (n=33): ligature of the biliopancreatic duct;

Group 2 (n=33): sham operation

Vaginal smears were collected daily during seven days to determine the estrous cycle. The vaginal smear was fixed with a 1:1 alcohol-ether solution and stained by Shorr's method (Figure 1) [12].

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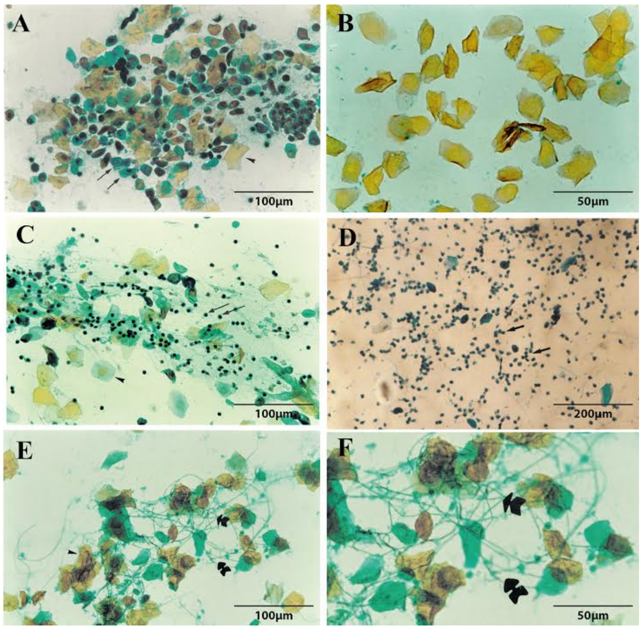

Figure 1: Vaginal smear of rat at different stages of the estrous cycle. [Shorr's] staining method, 315X]. **A:** Pro-estrus, with a predominance of nucleated epithelial cells [arrows] and few cornified cells [arrowhead]. Duration 12-14 hours. **B:** Estrus, with a predominance of squamous epithelial cells. Length from 25 to 27 hours. **C:** Metestrus, with nucleated epithelial cells [arrowhead] and many leukocytes [arrows]. Duration 6-8 hours. **D:** Diestrus, with a predominance of leukocytes [arrows]. Duration 55-57 hours. **E:** Cornified squamous epithelial cells [arrowhead] and sperm [arrows] found after copulation.

All animals of the two groups underwent median laparotomy under general anesthesia with ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg), both applied in the peritoneum. The biliopancreatic duct of the animals of group 1 was ligated twice with 5-0 silk sutures and then cut. The animals in group 2 underwent only to laparotomy and laparorrhaphy.

On the 10th postoperative day, venous blood was collected to quantify the levels of total and direct circulating bilirubin using a monomeric bilirubin kit (Merck) and an automatic chemistry analyzer (Cobas Mira Swiss). On the 23rd postoperative day, the female rats from both groups were placed with a fertile male, the ratio being 2 to 3 females for each male. Eight animals from group 2 were not placed with male.

After the pairing, vaginal smears continued to be collected daily to verify the copulation had occurred, which was determined upon the observation of spermatozoids The day on which spermatozoids were observed in the vaginal smear was considered day zero of pregnancy, after which the female rat was separated from the male rat. After the coupling, vaginal smears were collected during seven days to verify the diestrus. These animals were killed on the 12th or 20th day after copulation. The females that did not copulate were excluded from this study.

On the 20th day of gestation, the morphologic aspect and cephalo-caudal length of the fetuses (millimeters) were analyzed in both groups. Embryos vitality was determined upon observation of active movements while they were removed from the uterus.

The ovaries were removed, weighed and fixed in Bouin solution for histology. The ovaries were sectioned into 7 µm semi-serial sections which were stained with hematoxylin and eosin and Masson's trichrome stain. The number and size of the corpora lutea were determined after examining extensively each corpus luteum. The degenerated corpora lutea were not considered. The mean diameters of all the corpora lutea [micrometers] of both ovaries were determined for each animal. Liver biopsy was taken in both groups and processed for histology.

Chi-square test was used to assess the influence of hyperbilirubinemia on pregnancy Analysis of variance was employed to compare the weight of the ovaries and diamenter of the corpus luteum among the different groups (pregnancy at different stages, no pregnancy and control). Sperman correlation analysis was applied to verify the relationship among the weight of the rats, diameter of the corpora lutea and weight of the ovaries. Data was considered significant for $p<0.05$.

Results

Table 1 shows the levels of bilirubin in the animals that underwent ligature of the biliopancreatic duct. All the animals presented elevated levels of bilirubin. There was no significant statistical difference among the jaundiced rats ($p>0.05$).

Animals	Total bilirubin	Direct bilirubin	Indirect bilirubin
Control[a] (sham operation)	0.11 ± 0.04	0.06 ± 0.04	0.05 ± 0.04
Nonpregnant[b]	7.81 ± 2.68	3.78 ± 2,49	4.02 ± 1.41
Pregnant[c]	6.61 ± 2.49	3.84 ± 1.51	2.35 ± 0.98
a< b, a<c Student t test (p<0.001). b>c Student t-test (p>0.05).			

Table 1: Bilirubin levels - mg /dL (mean ± standard deviation of mean) ten days after ligature of the biliopancreatic duct associated with pregnancy and in the sham group, without ligature.

Table 2 shows that percentage of pregnancy among the rats that underwent ligature of the biliopancreatic duct, which was only 39.3% (11 rats) when compared with the 92% found in the rats of the control group (23 rats) ($p=0.0002$), ($OR=17.8$) ($3.04<OR<135.55$).

	Pregnancy		
Jaundice	Yes	No	Total
Yes	11 (39.3%)	17 (60.7%)	28
No	23 (92.0%)	2 (8.0%)	25
Total	34	19	53
Chi-squared test, Or (p=0.0002)			

Table 2: Pregnancy in rats submitted or not (sham) to biliopancreatic duct ligature.

All rats that got pregnant remained in diestrus during seven days after copulation. The rats that underwent ligature of the biliopancreatic duct and did not become pregnant remained with atypical estrous cycles characterized by prolonged periods [6 days] of proestrus or estrus.

There were a total of 47 fetuses from group 1 on day 20 of gestation. Although the fetuses were the same age, they presented with different stages of development. Their cephalo-caudal length was 10 mm to 20 mm. Some of the fetuses were so small and deformed that it was not possible to measure them (Figure 2). These fetuses were inactive, suggesting that they were dead.

Figure 2: Fetuses of rats from the two studied groups fixed in Bouin's solution. **A:** Five fetuses from rats of the group 1 with ligature of the biliopancreatic duct on the 20th day of pregnancy. Observe the different sizes and the macroscopic morphology which reveals different stages of development and reduced cephalo-caudal length. **B:** Four fetuses from rats of the sham group 2 on the 20th days of pregnancy. Observe the normal development with no macroscopic difference between them.

On day 20 of gestation, the rats that did not undergo the ligature of the biliopancreatic duct conceived 40 fetuses whose external morphology indicated that they were all in the same stage of development and presented minimal differences in size (20.1 to 20.2 mm) (Figure 2). All of these fetuses showed active movements upon being removed from the uterus.

The aspect of the luteal cells is shown in Figure 3. The cells of the jaundiced pregnant rats did not differ from those of the healthy pregnant rats.

No difference was observed in the diameter of the corpora lutea among the animals with or without ligation of the biliopancreatic duct (Table 3). The animals on day 20 of gestation presented with a larger corpus luteum graviditatis when compared to those on day 12 of gestation (p=0.0001). Considering the weight of the ovaries (Table 4), the animals with 20 days of gestation presented with heavier ovaries when compared to the rats with 12 days of gestation (p=0.0001).

The weight of the animal did not affect the diameter of the corpus luteum and the weight of the ovaries (p>0.05). There is however a significant relationship between the diameter of the corpus luteum and the weight of the ovaries (p=0. 0001 and r=0.7038).

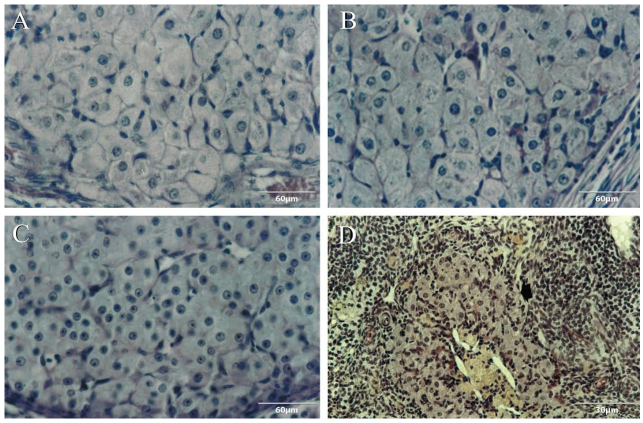

Figure 3: Corpus luteum of animals in different groups. HE staining, x 528. **A:** 12th day of pregnancy. **B:** 20th day of pregnancy, with ligation of the biliopancreatic duct. **C:** Corpus luteum of unmated rat without the biliopancreatic duct ligation and killed during the diestrus period. Observe the luteal cells with secretory activity and abundant cytoplasm containing the negative images of lipid droplets [polyhedral, rounded, central nucleus and clear nucleolus]. **D:** Corpus luteum of non-pregnant rat of group 1, with ligation of the biliopancreatic duct. Observe the large amount of fibroblasts [arrow] between the luteal cells in regression stage. Most of luteal cells have scant cytoplasm [arrowhead].

		Descriptive measurements (micrometers)			
Days (d) of pregnancy	**N**	**Minimum**	**Maximum**	**Mean**	**Standard-deviation of mean**
Group 1 (12 d)[a]	6	243.3	388.4	315.5	48.8
Group 1 (20 d)[b]	5	4480	501	476.8	22.4
Group 2 (12 d)[c]	13	280	334.7	311.6	21
Group 2 (20 d)[d]	10	409.8	509.9	475	38.5
Control (non pregnant)[e]	8	271.3	329	290.8	22.1
b=d>a=c=e, analysis of variance (p=0.0001).					

Table 3: The diameter of the *corpora lutea* of female rats.

		Descriptive measurements (grams)			
Days (d) of pregnancy	**N**	**Minimum**	**Maximum**	**Mean**	**Standart-deviation of mean**
Group 1 (12 d)[a]	6	0.05	0.1	0.07	0.0237
Group 1 (20 d)[b]	5	0.08	0.16	0.112	0.0327

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Group 2 (12 d)[c]	13	0.05	0.09	0.0712	0.0164
Group 2 (20d)[d]	10	0.09	0.12	0.1033	0.0103
Control (non pregnant)[e]	8	0.04	0.07	0.055	0.0131
b=d>a=c=e, analysis of variance (p=0.0001).					

Table 4: Weight of the ovaries in all groups.

Discussion

The rat is a good model for studying reproduction phenomena because it has an estrous cycle that is characterized by a short luteal phase. This allows studies to be conducted during a short period of time [13-16]. The vaginal smears confirmed normal estrous cycles, which were considered for the inclusion of the female rats in this study. Corpora lutea were not observed in the ovaries of the rats with abnormal cycles. By using a knowingly fertile male, confirmed by previous mating, the female rats could be incriminated for the unsuccessful fertilization.

There are no recent publications on this subject, however, based on our previous results [17,18] and in this current study, it is worth to suppose that hyperbilirubinemia does not interfere with the development of luteal cells during pregnancy. The histology of the ovaries of the jaundiced rats which did not develop pregnancy suggests anovulation due to an absence of functioning corpora lutea [17,18]. The fact that these animals remained in one phase of the estrous cycle [pro-estrus or estrus] reinforces the hypothesis of anovulation.

The pregnant animals that underwent ligature of the biliopancreatic duct remaining in diestrus indicates that hyperbilirubinemia does not inhibit the secretory function of the corpora lutea graviditatis in these animals. This finding is reinforced by the results demonstrating no difference in the diameter of the corpora lutea and weight of the ovaries between the groups with and without hyperbilirubinemia [17,18].

In this study the vaginal ephitelium of some jaudiced rats became cornified. This modification could be caused by the hyperbilirubinemia, which increases free estrogen in plasma by displacing it from albumins binding site [19,20].

The possible mechanism related to reproductive disorders in women with liver diseases are still not elucidated. Some authors ascribe to the hypothalamic-pituitary axis a role in the sex steroid metabolism dysfunction in the liver [2,5,7]. Based on the present study, possible endocrine changes may be linked to the estrogen metabolism and transport. A permanent cornification of the vaginal epithelium was observed, and this phenomenon may be associated with hyperbilirubinemia, which increases free estrogen in plasma by releasing this hormone from albumin binding [19,20].

The results of the present study demonstrated severe disorders of the fetal development associated with hyperbilirubinemia. An eventual enhancement of the maternal bilirubin transport through placenta may be related with the etiopathogenesis of the fetal damage [21,22]. However, previous study has demonstrated in monkeys that direct bilirubin does not pass through placenta [23].

Hyperbilirubinemia may have interfered with fertilization, which could explain why some of the animals that copulated did not become pregnant. Another possibility could be an absence of the initial development of the ovum or implantation abnomalies. Imperceptible abortions may have occurred due to multiple malformations, as were detected in the present study. Considering that all the fetuses tend to be in the same stage of development before birth, it is possible that those with abnormal growth ceased their development in the initial phases of intrauterine development. Yeary did not find alterations in the pregnancy of rats with jaundice caused by ligature of the biliopancreatic duct on the 9th day of pregnancy [21]. This favorable result may be explained by cholestasis, which occurred in the second stage of embryonic development or due to the short period of hyperbilirubinemia.

Conclusion

Fertilization occurs in the presence of obstructive jaundice, although the reproductive capacity is impaired and the estrous cycles become irregular. The vaginal epithelium remains cornified and the corpora lutea degenerates. In presence of pregnancy, the corpora lutea graviditatis are not altered and their size increase progressively, however the fetal development is severely compromised.

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Declaration of Conflicting Interests

The authors declare no conflicts of interest with respect to the research, authorship and publication of this article.

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