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ORIGINAL RESEARCH ARTICLE

Morphological markers of hypoxia in the fetal kidney with placental insufficiency treated with neuro-EPO: study in rats.

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ABSTRACT

Foundation: intrauterine growth restriction constitutes a complication of pregnancy. Newborns with this condition are exposed to an increased risk of perinatal and postnatal morbidity and mortality.

Objective: to evaluate morphological markers of hypoxia in fetal and kidney development, using a model of placental insufficiency treated with human erythropoietin with low sialic acid content (neuro-Epo) in rats.

Methods: three groups of gestated rats from the Wistar line were used. A control group (group I) and two experimental groups (groups II and III) with six rats each. Rats of groups II and III had uterine artery ligation on day 16 of pregnancy (E 16). Group III from E16 to E19 was administered a dose of 0.5 mg / kg / day of neuro-Epo subcutaneously and group II was administered placebo. On the 20th day of gestation the fetuses and their placentas were weighed. The fetuses' size and cephalic diameters were measured. Morphometric and histological features in the fetal kidney were studied with hematoxylin-eosin staining and PAS. A qualitative histopathological analysis of their cell types was performed.

Results: fetuses with intrauterine growth restriction did not improve growth markers. Hypoxia lesions were found in the fetal kidney of the untreated IUGR group that improved by administering neuro-Epo.

Conclusions: the administration of neuro-Epo only showed reparative and protective effects on histological alterations caused by hypoxia in the fetal kidney.

Keywords: hypoxia; fetal hypoxia; kidney; placental insufficiency; erythropoietin; rats; Wistar

1. Introduction

Intrauterine growth restriction (IUGR) is a complication of pregnancy. Newborns with IUGR are at increased risk of perinatal and postnatal morbidity and mortality.(1) It is also associated with the development of chronic adult diseases with metabolic disorders.(2)

In IUGR there is hypoxia with a marked increase in oxidative stress markers in the placenta and fetus. As a consequence, alterations occur in the structure and function of various vital molecules

such as proteins, lipids and DNA. This occurs in situations such as maternal anemia, hypertension, preeclampsia, gestational diabetes, adaptation of the pregnant woman to life at high altitudes with consequences for the mother and the newborn.[3] The most frequent cause of IUGR is placental insufficiency where the fetus is exposed to chronic hypoxia.[4]

Current studies indicate that adverse intrauterine and immediate postnatal influences could lead to vasoconstriction in peripheral organs, including the renal territory. This can generate structural and functional alterations in the kidney due to fetal hypoxia.[5] These alterations have been proposed as possible causes to explain the appearance of chronic diseases associated with this organ, such as arterial hypertension[6] and renal failure.[7] For this reason it is important to study the kidney in patients with a history of IUGR.

Research conducted in IUGR reports renal changes such as a reduction in the number of nephrons; neuroendocrine changes causing dysregulation of the hypothalamic-pituitary-adrenal axis; and vascular changes with endothelial dysfunction and reduced density of arterioles and capillaries.[6,7] In addition, a decrease in the number of glomeruli with an increase in apoptosis has been observed in rats; the possible cause of the decrease in the number of glomeruli could be an increase in the expression of apoptosis.[7]

To prevent and diminish the effects of oxidative stress and hypoxia in IUGR, several treatments have been used with contradictory results.[1] The authors suggest that it is necessary to evaluate possible efficient therapeutic options to overcome or prevent hypoxia caused by placental insufficiency, since a large number of children are born with intrauterine growth retardation and their diagnosis is made at late stages of gestation.[3]

Recently it has been shown that EPO (Erythropoietin) and its specific receptor (EPOR) have effects in several non-hematopoietic tissues such as in some tumors, in cells of the central nervous system,[8] in endothelial cells,[9] in epithelial and tubular cells of the kidney,[10] in the trophoblast and in decidual cells.[11] This hormone promotes angiogenesis, inhibition of apoptosis, and has anti-inflammatory effects, thereby protecting these cell types from the effects of hypoxia.[8]

In more recent years, the renoprotective effect of this molecule has been studied.[12] In the adult kidney, EPO receptors have been found at vascular and other sites, especially in tubular cells.[12] Experimental studies both in vitro and in vivo modeling diseases such as diabetic nephropathy and nephrotic syndrome have shown that when EPO is administered, inflammatory podocyte damage and albuminuria improve.[9]

In Cuba [2007] a patent was obtained for a recombinant human erythropoietin with low sialic acid content (neuro-EPO), which has therapeutic utilities superior to the acidic one because it has greater affinity with its receptor; it eliminates the risk of producing erythropoiesis and favors angiogenesis, cell proliferation and inhibition of apoptosis in areas of cerebral ischemia[13].

Research has shown that EPO has cytoprotective effects in several hypoxic tissues.[10-13] It is necessary to know its effect during gestation with hypoxia since there is no research at present regarding its use in this type of patients, so we propose a possible treatment with human recombinant Erythropoietin with low sialic acid content in a model of placental insufficiency to evaluate fetal development and the reparative and protective effects in the fetal kidney with hypoxia.

The objective of this work is to evaluate morphological markers of hypoxia in fetal development and in the kidney, using a model of placental insufficiency treated with human Erythropoietin with low sialic acid content (neuro-Epo) in rats.

2. Methods

Research conducted at the Victoria de Girón

Institute of Basic Preclinical Sciences.

Eighteen adult Wistar rats weighing between 200 and 230 grams (g) from the National Center for the Production of Laboratory Animals (CENPALAB) were used. These were kept with free access to water and food, with a temperature of 22 to 24 degrees Celsius, continuous ventilation and light/dark cycles of 12 hours each.

When the female rat was in the estrous phase of the sexual cycle, mating was performed. The following day, copulation was verified by vaginal lavage, taking the presence of spermatozoa as day zero of gestation.

On day 16 of gestation the rats were weighed and randomly distributed into three groups, a control group (group I) with 6 rats and the two experimental groups (groups II and III) with 6 rats each. The rats in groups II and III underwent surgery on day 16 of gestation. Both uterine arteries were ligated causing placental insufficiency and IUGR in the offspring [14].

Group I dams were not surgically intervened. Group III rats, after uterine artery ligation, were treated with neuro-Epo at a dose of 0.5mg/kg/day from day 16 to day 19 of gestation. Euthanasia was performed on day 20 of gestation under anesthesia. A laparotomy was performed on the mother to obtain the uterine horns. Once extracted, the fetuses and placentas were taken.

All placentas and their fetuses were weighed on a digital balance (gr). The placental weight/fetal weight index was calculated by dividing the placental weight by the fetal weight. Subsequently, the size of the fetus was measured from the crown of the head to the end of the tail using a millimeter paper. Anteroposterior diameter and biparietal diameter were also measured using a caliper.

The fetus underwent a laparotomy and the kidneys were removed and fixed in 10% buffered formalin. The renal tissue was embedded in kerosene, cut and stained with hematoxylin eosin and PAS. The samples were photographed with a Motic camera coupled to a Motic microscope. The images digitized with Motic plus 2 software were transferred to a computer, the analysis of the samples was performed with the help of Image J software.

Renal morphometry was performed at 10x magnification and the following variables were measured: kidney length (mm) from the upper pole of the kidney to the lower pole, transverse kidney length (mm) from the renal pelvis to the contralateral side, renal medulla length(mm)from the renal pelvis to the inner edge of the cortex, and renal cortex length was calculated by subtracting the renal medulla length from the transverse kidney length.(16)

The number and area of glomeruli were determined in 10 fields per slide. The morphology of the renal corpuscle was analyzed using hematoxylin and eosin (HE) and periodic acid Shiff (PAS) stains at 40x and 100x magnification, taking into account the morphology of the epithelial cells or podocytes and the organization of the mesangial cells with their extracellular matrix.

For the statistical analysis of the results, a database was created in Microsoft Excel and processed with the GraphPadPrim5 program. The t-test was used for variables with normal distribution and the Kruskal-Wallis nonparametric test (for multiple independent variables) was used for variables without normal distribution. Differences were considered significant when the p values were equal to or less than 0.05.

3. Results

The analysis of fetal growth variables showed that the control group (group I) presented a greater number of live fetuses, greater weight, greater length and greater biparietal diameter (BP diameter) with significant differences with respect to the experimental groups. There were no significant differences between the experimental groups with respect to these variables. The antero-posterior

diameter (AP diameter) of the head behaved the same in the three groups. The placenta weight/fetal weight index was higher in the treated IUGR group with significant differences with respect to IUGR and control. This indicates that placentas are less efficient (Table 1).

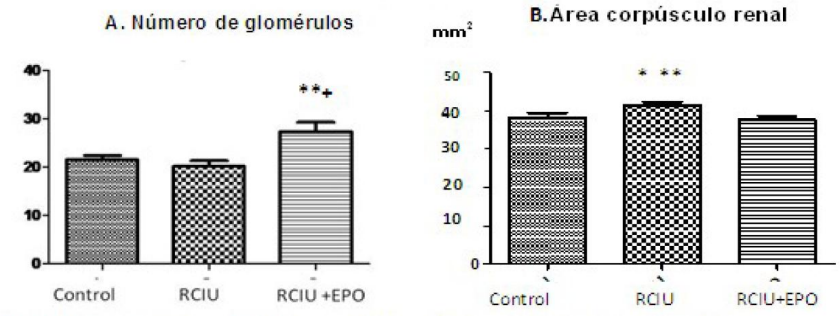
Table 1. Fetal growth variables at day 20 of gestation.

Reproductive variables	Control M + DS	IUGR M + DS	RCILKEPO M+DS
Number of live fetuses			
Weight	3,9±0,8*+	2,9±0,9	2,9±0,4
Size	40±4,3*+	30±4,5	35±4,7
AP Diameter	0,9±0,1	0,9±0,1	0,9±0,1
Diameter BP	0,8±0,1*-	0,6±0,2	0,6±0,1
Fetal weight/weight index placenta	0,17±0,03	0,19±0,04	0,2±0,04*

*Significant difference p<0.05 between group I and group II (Kruskal-Wallis test).

+Significant difference p<0.05 between group I group III (Kruskal-Wallis test).

**Significant difference p<0.05 between group I and group III (Kruskal-Wallis test)



+Significant difference p<0.05 between IUGR+EPO group with respect to control group (t-test).

**Significant difference p<0.05 between IUGR+EPO group with respect to IUGR (t-test).

*Significant difference p<0.05 between control group and IUGR group (t-test).

Figure 1. Number and areas of glomeruli by group.

Morphometric variables in histological sections of the kidney such as: measurements of the renal medulla, renal cortex, kidney length and transverse length of the kidney showed no significant difference between the groups.

Regarding the number of glomeruli by groups, the group with IUGR treated with neuro-Epo presented a greater number of glomeruli with significant differences with respect to the control group and the group with IUGR. There were no significant differences between the latter group and the control group.

In the areas of the renal corpuscles, it was observed that the IUGR group presented a greater area with significant differences with respect to the control and the IUGR group treated with neuro-Epo. There were no significant differences between the control group and the IUGR treated group (Figure 1).

When comparing the control group in photomicrograph A and the IUGR group in photomicrograph B, a detachment of podocytes towards the intercapsular space was observed in the IUGR group. In the center of the glomerulus there is increased disorganization of the mesangial cells with their extracellular matrix. In the treated IUGR group represented in microphotography C, the renal structures can be observed very similar to the control (Figure 1).

In the same figure when examining the proximal convoluted tubules in the control group and the IUGR group, in the IUGR group there was a flattening of the tubular epithelial cells and denudation or detachment of the cells with an increase in the tubular lumen. In the treated IUGR group, smaller proximal convoluted tubule lumina

and less loss of tubular epithelial cells were observed. These characteristics are more similar to the control.

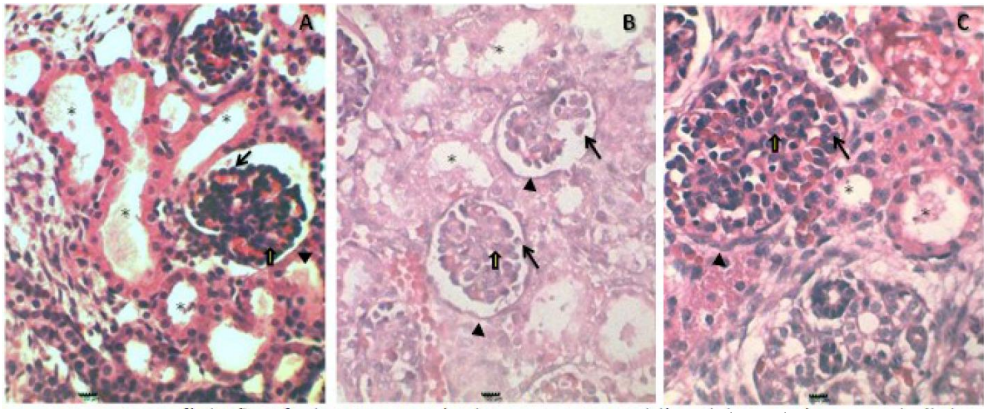


Figure 1. Photomicrograph of fetal kidneys. ABC with H/E staining and magnification at $40 x$ at day 20 of gestation. A. Control: proximal tubules (asterisk), mesangial cells (yellow arrow), podocytes attached to their membranes (black arrow). B. IUGR glomerulus (arrowhead), podocyte detachment (black arrow), dilated tubules (asterisks), disorganized mesangial cells (yellow flank).C. Podocytes attached to their membranes (black arrow), better organized tubules, recovering (asterisk arrow).

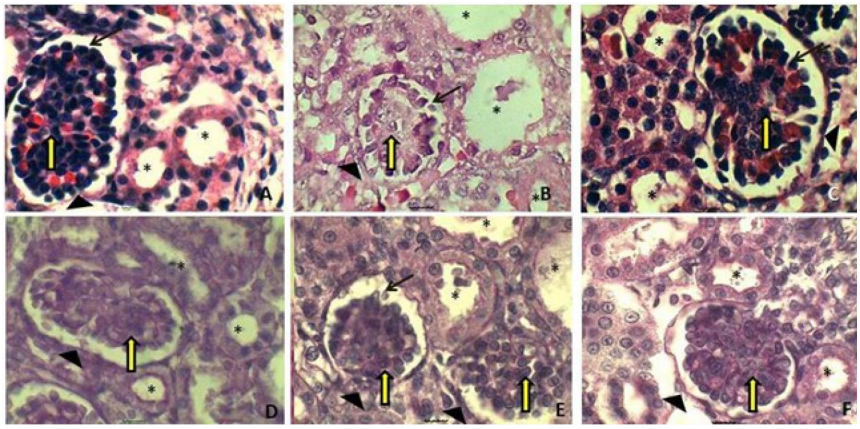


Figure 2. Photomicrograph of fetal kidneys. ABC with H/E staining magnification at (100x) on day 20 of gestation. A: shows normal structure of the renal glomerulus (arrowhead), podocytes (black arrow), mensangial cells (yellow arrow), proximal tubules (asterisk); B: shows in IUGR the glomerulus (arrowhead), podocyte detachment to the urinary space (black arrow), proximal tubules (asterisk) flattening and denudation of tubular epithelium cells, vacuolization of nuclei, disorganization mesangial cells (yellow arrow); C: shows renal glomerulus (arrowhead), podocytes (black arrow), proximal tubules with recovery of epithelial cells (asterisk). D.E.F. with PAS staining at (100x) on day 20 of gestation. D: shows glomerulus and tubules with normal architecture; E: shows proximal tubule with loss of epithelial cell nucleus (asterisk), detachment of podocytes to the urinary space (black arrow); F: shows better organization of glomerulus and tubules (signs of recovery).

To confirm the lesions found at the level of the fetal renal cortex on day 20 of gestation, microphotographs were taken with H/E and PAS staining at 100x magnification.

Figure 2 shows the microphotographs with H/E and PAS staining at 100x magnification. Microphotograph B represents the group with IUGR, in this image we can observe the detachment of podocytes and the vacuolization of the nuclei with prominent nucleoli, all this indicates signs of hypoxia. At the level of the glomerulus the mesangial cells are disorganized; these pathologic changes of the glomerulus have been described in global focal glomerulosclerosis. In the proximal convoluted tubules the tubular epithelium is observed flattened, with loss of the cells of this epithelium in some regions of the tubule, these characteristics create a wider lumina in this structure.

When comparing the group with IUGR treated with neuro-Epo represented in microphotography C and the control group represented in microphotography A, it is observed at the glomerulus level that the podocytes present similar cellular morphology to the control group, they are attached to the basal membranes, well organized

and without apparent signs of morphological changes. Great similarity can also be observed between the mesangial cells of the two groups and the epithelial cells of the tubules.

In the treated IUGR group represented in microphotography F, signs of epithelial regeneration are observed at the level of the proximal convoluted tubules, evidenced by an increase in the number of cells and better organization of the tubule lumen. This recovery process is normal after hypoxia, and in the groups supplied with neuro-Epo these signs become evident faster in the fetal period.

4. Discussion

In the present study, uterine artery ligation is associated with a reduction in fetal measurements such as weight, length and head diameters, confirming the previously demonstrated negative effect of low blood flow on fetal growth.[15,16]

When neuro-Epo treatment was administered to pregnant rats with uterine artery ligation, fetal growth variables did not improve. This treatment was insufficient to mitigate these effects, which we could think is due to the fact that in this model there is a significant reduction of blood flow by totally occluding the arteries close to the termination of the cervix of each uterine horn. At the level of the placental tissue, severe ischemia is produced and as a result other authors have observed a great amount of resorption and variation in fetal weight.[16,17]

The placenta weight / fetal weight ratio in the period studied is higher in IUGR treated with neuro-Epo. This represents a lower contribution of grams to the fetus per gram of placenta and this decreases placental efficiency[4].

Morgado et al. studied the placental morphology of IUGR by bilateral ligation of the uterine arteries, they found differences in the areas of the placental zones of the rats in comparison with the controls and with the group treated with neuro-EPO. In the IUGR group, the size of the labyrinth area decreased, compromising the exchange between the trophoblastic tissues and the fetal vessel. In the group treated with neuro-Epo the labyrinth was similar to the control but the spongiotrophoblast cells were observed with stellate or mesenchymal form with abundant extracellular matrix.[17] These differences in the organization of the spongiotrophoblast could explain the poor functioning of the placenta and the low fetal weight in this treated group. Other authors suggest that a failure in the differentiation of other cell types of the spongiotrophoblast, such as endovascular invasive trophoblast cells, could compromise the remodeling of the uterine arteries and placental blood flow, favoring in this model the appearance of intrauterine growth retardation[18] .

In our investigation at the level of the fetal kidney there are no changes in the number of glomeruli in the IUGR group with respect to the control, these results do not coincide with what is reported in the literature. In studies performed with models of placental insufficiency that cause hypoxia with decreased renal flow, a reduction in the number of nephrons and other indicators was observed.[19] We could think that in our study the number of glomeruli does not vary because in this model the degree of IUGR is not uniform and different metabolic phenotypes appear at the renal level. The fetus is exposed to different degrees of hypoxia depending on its location in the horn,[20] and in our study the location of the fetus in the horn was not taken into account. In the same horn, fetuses are exposed to different microenvironments that produce modifications in histones and DNA methylation. This modification brings about changes in gene transcription and a predisposition to renal disease.[20] However, in the IUGR group treated with neuro-EPO, an increase in the number of glomeruli is observed, possibly as an adaptive response. This molecule could improve the organization of the glomerulus, the endothelium of the vessels, and preserve the architecture of the renal and vascular interstitium.[10]

Although the number of glomeruli did not

decrease, we found an increase in the area of the renal corpuscle in the IUGR group. This confirms what was found in other experimental studies in rats with intrauterine growth restriction models. They observed glomerular hypertrophy, with sclerotic changes, with increased collagen deposition in the tubular interstitium and inflammation that can trigger glomerulosclerosis,[21] and subsequently these modifications could develop severe renal diseases.[22]

The histopathological study of the kidney showed glomerular and tubular damage. At glomerular level there was a detachment of epithelial cells (podocytes), a disorganization of mesangial cells and their extracellular matrix, these alterations have been described in ischemia caused by hypoxia accompanied by destructive lesions such as necrosis and apoptosis. At the molecular level there is a decrease in ATP and an increase in intracellular Ca, which activates proteases and phospholipases causing lesions in the cytoskeleton and cell membranes, in addition to an increase in reactive oxygen forms, activating the caspases cascade that produces necrosis and apoptosis in epithelial cells as well as in mesangial cells and tubular epithelium cells.[22] At the level of the tubules there is denudation of the cells, widening of the tubular lumen with loss of the microvilli. In the kidneys of fetuses treated with neuro-EPO, a better organization is appreciated in the tissue damaged by hypoxia, although still in a focal way glomerular and tubular lesions are appreciated by hypoxia.

The tissue protection of EPO has been demonstrated by increased angiogenesis, its anti-inflammatory and anti-apoptotic effect. Studies have shown that EPO suppresses the inflammatory effect of reperfusion on the kidney by reducing reactive oxygen species through inhibition of polymorphonuclear cells and neutrophil infiltration. In addition, it reduces the production of proinflammatory cytokines such as TNF alpha (tumor necrosis factor alpha) and interleukin 6 and stimulates the expression of anti-inflammatory cytokines such as interleukin 10.[23]

Other in vitro studies of umbilical vein endothelial cells and renal proximal tubule epithelial cells in humans, together with in vivo studies in rat kidneys, with the administration of rhEPO, found an increase in HIF-1α, VEGF (vascular endothelial growth factor) and BMP7 (bone morphogenetic protein 7), VEGF (vascular endothelial growth factor) and BMP7 (bone morphogenetic protein 7), which stimulated cell proliferation, decreasing tubular hypoxia and the quantification of the number of apoptotic cells (nuclear fragmentation labeling technique) (TUNEL).[24]

In another mouse model in which bilateral occlusion of the renal artery was performed and rhEPO was administered, a decrease in hypoxia and renal damage, repression of apoptosis and upregulation of the anti-apoptotic gene of the Bcl-2 family were observed[10]. All these studies confirm the renoprotective character of EPO.

In our investigation the EPO molecule is not exactly the same as that used in other studies. So far there are contradictions regarding the permeability of this molecule in the placental membrane, however, studies performed in ischemia and perfusion models in rat fetuses treated with rhEPO, increased the permeability of radioactive rhEPO across the placental membrane and the blood-brain barrier under conditions of hypoxia and fetal ischemia, Although it was not possible to identify this molecule in fetal tissues, its cytoprotective character against damage caused by hypoxia at the level of the fetal kidney was evidenced in our study.

Erythropoietin is gaining ground as a therapeutic option during pregnancy. However, more studies are needed to establish the timing of drug application and dose adjustment,[26] as well as its impact on postnatal life.

It can be concluded that treatment with neuro-Epo does not favor fetal growth in the

offspring of pregnant rats with IUGR, however it exerts reparative and protective effects on the histological alterations caused by hypoxia in the fetal kidney.

Conflict of interest: No conflict of interest.

Contribution:

Methodological conception: Nínive Núñez López.

Data analysis and interpretation: Nínive Núñez López, Yaima Nova Bonet, Laura López Marín, Yordanca Morgado Gamboa.

Write or critically review the document: Aida María Suarez Aguiar, Laymit Alonso Padilla, Nínive Núñez López.

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