Explor Anim Med Res,
Vol.13, Issue - 1, 2023, p. 49-53
DOI : 10.52635/eamr/13.1.49-53

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

LEPTIN INCREASES NITRIC OXIDE LEVEL VIA INCREASE IN iNOS EXPRESSION IN EARLY PREGNANT MOUSE UTERUS

Kishor Kumar DG, Pashupathi M[1], Anjali[2], Manjit Panigrahi[3], Madhu CL, Kesavan M, Thakur Uttam Singh, Dinesh Kumar, Subhashree Parida*

Received 18 November 2022, revised 30 May 2023

ABSTRACT: Leptin is an adipokine hormone secreted primarily from the white adipose tissue and leptin levels are usually elevated in obese individuals. Leptin is known to mediate the relaxation of the uterus in the pregnant mouse by increasing Nitric Oxide (NO) synthesis in late pregnancy. The present study aimed to assess the effect of a pathological concentration of leptin (3 nM) on NO synthesis in the uterus of early pregnant mice and also to evaluate its effect on the transcription of genes responsible for NO synthesis. mRNA expression studies using real-time PCR revealed that leptin (3 nM) increased the relative expression of the inducible Nitric Oxide synthase (iNOS) gene which also resulted in increased NO levels. The results suggest that higher leptin levels during early pregnancy may increase NO levels in the uterus which may result in a net decrease in uterine contractions further leading to complications in pregnancy.

Key words: Early pregnancy, Leptin, Nitric Oxide, Nitric Oxide synthase.

INTRODUCTION

Leptin is an adipokine released from the white adipose tissue. Initially, it was proposed as an anti-obesity hormone for its role in the regulation of body weight by reducing appetite and feed intake (Schwartz *et al*. 1999, Ahima and Flier 2000, Kwon *et al*. 2016). In addition, its role is now clear in diverse biological functions like thermogenesis, angiogenesis, hematopoiesis, osteogenesis, and chondrogenesis. Leptin also affects the functions of the reproductive, cardiovascular, and immune systems (Wauters *et al*. 2000, Caprio 2001, Hynes and Jones 2001). Besides regulating uterine contractions, leptin is implicated to regulate the functions of the ovary, maturation of the oocyte, development of the embryo, implantation, and placentation (Cervero *et al*. 2005, Dos *et al*. 2012). Leptin level increases remarkably in obese pregnant females. Obesity during pregnancy increases the risk of various pregnancy-related complications, including miscarriage, endometriosis, ectopic pregnancy, and stillbirth (Malasevskaia *et al.* 2021).

Leptin induces uterine relaxation in humans, rats, and mice, particularly in late pregnancy (Moynihan *et al*. 2006, Mumtaz *et al*. 2015, Srinivasan *et al*. 2021). The relaxation of the uterus is mediated by inducing the release of nitric oxide (NO) in a receptor-dependent manner through the JAK-STAT pathway (Srinivasan *et al*. 2021). Proper contractions of the uterus during early pregnancy are necessary for the precise positioning of the embryo in the uterine cavity and thus for embryo implantation (Fanchin and Ayoubi 2009). Hyperleptinemia in overweight and obese individuals may cause improper relaxation of the uterus. Abnormal relaxation during early pregnancy may lead to impaired positioning of the embryo impeding the implantation process. This study was undertaken to study the effect of 3 nM leptin (concentration reported in obesity, Barrichon *et al*. 2015) on NO synthesis in the uterus and also to investigate its effect on the transcription of genes responsible for NO synthesis in the early pregnant mouse.

Division of Pharmacology and Toxicology; [1]Division of Animal Biochemistry, [2]Division of Physiology and Climatology, [3]Division of Animal Genetics and Breeding, IVRI, Izatnagar, Bareilly, U.P. India.
**Corresponding author. e-mail: subhaparida1210@gmail.com*

MATERIALS AND METHODS

Animals

The experiments were carried out in apparently healthy virgin female Swiss albino mice (8-10 weeks old weighing approx. 25 - 30 g) procured from the laboratory animal resource section of the institute and were housed under standard housing conditions. An acclimatization period of 7 days was maintained before commencing the experiments. Animals were provided with *ad libitum* standard rodent feed and potable drinking water during the study period. Handling animals and other maneuvers were carried out as per the guidelines laid by CPCSEA (Committee for Control and Supervision on Experiments on Animals) (CPCSEA 2003). Permission of the Institutional Animal Ethical Committee has been taken for the study.

Induction of pregnancy

After the acclimatization period of 7 days, female mice were mated with the same age group males in the ratio of 1:1. Mating was confirmed by the presence of a copulatory plug in the vagina of female mice. The day of the appearance of the copulatory plug was considered pregnancy day 1. Followed by mating, pregnant females were separated from males and maintained till the 5th day of gestation.

In vitro studies with uterine tissue explants

On the fifth day of pregnancy, female animals (n = 5) were sacrificed by cervical dislocation under anesthesia. After carefully removing the adhering fat, the foeti, and placental tissue, uterine tissues in the form of longitudinal uterine strips were collected aseptically from the horns of uteri in sterile PBS for explant culture. Tissues were collected from the same segment of uterine horn in each animal. Uterine explants of 1-2 mm^3 were prepared aseptically inside laminar airflow and maintained in a medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (ABAM). After an incubation period of 30 min, uterine explants were treated with leptin (Rat leptin, R&D Systems, USA) at the concentrations of 0.3 nM (physiological leptin concentration reported in pregnant females) and 3 nM (pathological leptin concentration reported in obese pregnant females) (Barrichon *et al*. 2015) and incubated for 6h under 5% CO2.

mRNA expression studies

Tissue pieces were collected before (0h) and after (6h) 3 nM leptin treatment in RNAlater solution and stored at -80°C for assessing leptin-induced changes on the expression nitric oxide synthase (NOS) isoforms, eNOS, and iNOS, using real-time PCR. The specific primers for the genes were designed by online IDT software (Table 1) (Srinivasan *et al*. 2021).

RNA Isolation and cDNA synthesis

Total RNA in each sample was isolated using RNeasy Plus Mini Kit (Cat. no. 74104, Qiagen, Germany) following the manufacturer's protocol. RNA concentration and purity were assessed with the help of a Nanodrop UV spectrophotometer (Coleman Technologies Inc, USA). Samples showing the values of the A260/A280 ratio between 1.9 and 2.2 and the A260/A230 ratio of >1.5 were further utilized for cDNA synthesis. cDNA (≈ 2 µg) was synthesized on the same day using a High Capacity RNA-to-cDNA Kit (Cat. No. 4387406, Applied Biosystems, USA) following the manufacturer's instructions.

Real-time PCR conditions

Initial incubation at 95°C for 10 min; 40 cycles of amplification with denaturation at 95°C for 35s; annealing at 60°C for 30s and extension at 72°C for 30s using SYBR Green Master mix (Cat. no. 4309155, Applied Biosystems, USA). The total reaction mixture (10 µl); 2x SYBR Green Master mix - 5 µl, Forward Primer 0.5 µl, Reverse Primer 0.5 µl, cDNA 1 µl and nuclease-free water 3 µl. Mouse GAPDH was used as an internal control for the analysis of changes in gene expression (Step One Plus Rea-time PCR Machine, Applied Biosystems, USA). Based on the CT values, the relative expression of each gene was calculated.

Measurement of nitric oxide

Explants were also collected for estimation of NO levels before (0h) and after (6h) leptin (0.3 and 3 nM) treatment in sterile tubes and stored at -80°C. NO levels were measured in the form of nitrite by a method described by Zhang *et al.* (1999), using Griess Reagent (Sigma Aldrich, USA). Tissues were homogenized in PBS as 10% homogenate and supernatants were collected after centrifugation with 10000 RPM at 4°C. 100 µl of supernatant was added to a clean tube containing 100 µl of Griess Reagent. The tubes were incubated for 20 min at room temperature after thorough mixing. After 20 min of incubation, absorbance was measured at 545 nm in a spectrophotometer (Eppendorf, USA). Unknown concentrations of nitrite in the samples were estimated from a standard curve plotted from known standard concentrations of sodium nitrite using the linear regression formula and expressed as µ moles/g of tissue.

Data analysis

The data generated was expressed as mean±S.E.M. mRNA expression data were expressed as 2^{-dct} values with GAPDH as the housekeeping gene. Comparison between groups was made by unpaired t-test with Welch's correction (Ruxton 2006). Comparison among the groups for NO levels was made by two-way ANOVA followed by Tukey's post hoc test. All the statistical analyses were done by GraphPad Prism software version 9.0 (Pavithra *et al.* 2022). $p<0.05$ was considered significant for all the results.

RESULTS AND DISCUSSION

Leptin at a concentration of 3 nM significantly increased ($p< 0.05$) the relative expression of the iNOS gene; whereas it did not affect the relative expression of the eNOS gene after 6h incubation compared to vehicle-treated explants (Fig. 1). Uterine explants treated with 0.3 nM leptin showed no significant difference in nitric oxide levels compared to vehicle-treated explants whereas nitric oxide levels were significantly increased ($p<0.05$) in 3 nM leptin-treated uterine explants after 6h of stimulation (Table 2 and Fig. 2). Muscular contractions in the uterus during estrus are essential to enhance the passage of spermatozoa through the uterine cavity following successful mating to facilitate its advancement toward the place of fertilization (Suarez and Pacey 2006). After ovulation, the uterine contractions start to reduce and reach a peak relaxant state in the mid-luteal phase which helps in proper embryo positioning in the uterine cavity and thus facilitates embryo implantation (Fanchin and Ayoubi 2009). At term, the uterine contractions suddenly increase to expel the fetus from the uterine cavity (McEvoy and Sabir 2022). Abnormal uterine contractions during any of these stages may lead to reproductive problems in animals and humans like abortions, implantation failure, and dystocia (Norman *et al*. 1991, Bellver and Simon 2018, Kissler *et al*. 2020). The Nitric oxide system in the uterus helps to maintain uterine quiescence throughout pregnancy to aid in the development of the fetus and maintain pregnancy. Nitric oxide synthase enzymes (NOS) are also reported to be expressed in uterine tissue (Buhimschi *et al*. 1995). The NOS enzyme has two main isoforms, constitutive (cNOS) and inducible (iNOS) (Lowenstein *et al*. 1994). NO, which is produced by NOS enzymes, has a marked relaxant action on the pregnant uterus (Yallampalli *et al*. 1993). Leptin at a pathological concentration (3 nM) significantly increased the relative expression of the iNOS gene and increased NO levels which may result in a net decrease in uterine contractions in early pregnancy. Leptin has been reported to increase the serum levels of NO in a dose-dependent manner in Wister rats (Frühbeck 1999) and in the basal hypothalamus and the anterior pituitary gland in male rats by activating NOS (Yu *et al*. 1997). Leptin is also reported to induce vasodilation by activating eNOS in endothelial cells (Vecchione *et al*. 2002) and iNOS in vascular smooth muscle cells thereby resulting in enhanced NO bioavailability in peripheral blood vessels (Rodriguez *et al*. 2007). Similarly, Becerril *et al.* (2018) reported that the activation of iNOS by leptin is vital for the production and release of tenascin C in adipose cells which mediates adipose tissue inflammation and fibrosis. Otero and co-workers (2003) stated that leptin enhanced the expression

Table 2. Effect of leptin (0.3 and 3 nM) on nitric oxide synthesis in the early pregnant uterus after 6h stimulation.

Leptin Concentration(nM)	Level of nitric oxide after 6h (μ moles/g tissue)
Vehicle	0.14 ± 8.92
0.3	0.15 ± 7.29
3	0.21 ± 12.47*

*$p<0.05$ as compared to respective vehicle control.

Table 1. Sequence of primers used in real-time PCR analysis.

Name	Primer Sequence	Amplicon Size	Annealing temperature (°C)	Gene Accession No.
GAPDH	F:5' CTGCACCACCAACTGCTTAG -3' R:5' - GGGCCATCCACAGTCTTCT - 3'	120 bp	60	NM_001289726.1
eNOS	F:5' - GCATCACCAGGAAGAAGA-3' R:5' - CAGTCTCAGAGCCATACAG-3'	120 bp	60	NM_021838.2
iNOS	F:5' - CACAGTCCTCTTTGCTACT-3'R: 5' -CCAAGGTGTTTGCCTTATAC-3'	125 bp	60	NM_012611.3

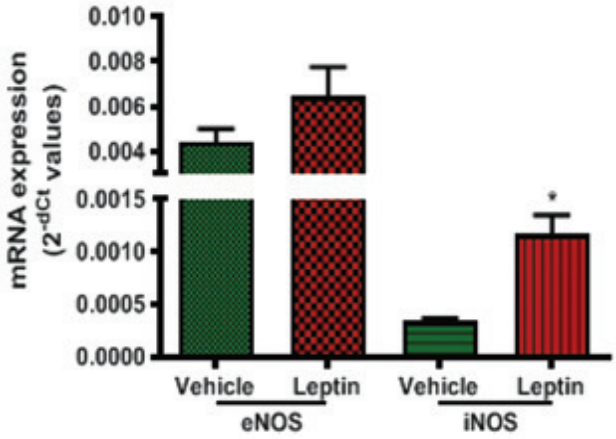

Fig. 1. Effect of 3 nM leptin on mRNA expressions of eNOS and iNOS genes in the early pregnant uterus after 6h stimulation. [p<0.05 as compared to respective vehicle control].

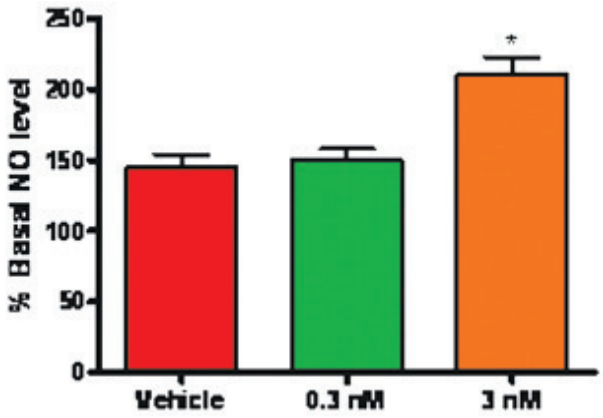

Fig. 2. Effect of 0.3 and 3 nM leptin on nitric oxide synthesis in the early pregnant uterus after 6h stimulation.
[p<0.05 as compared to vehicle control and 0.3 nM leptin].

of iNOS and NO production in human primary chondrocytes and ATDC5 cells in a dose-dependent manner along with interferon-ã. It was also suggested that leptin is involved in the promotion of ulcer healing by increasing NO levels through the up-regulation of cNOS and iNOS in the ulcer area (Konturek *et al*. 2001). Leptin is also involved in peroxynitrite-mediated oxidative stress in the steatotic liver by inducing the expression of iNOS and NADPH oxidase (Chatterjee *et al*. 2013).

CONCLUSION

Adequate uterine contractions are necessary for proper positioning and successful embryo implantation in early pregnancy (Bulletti *et al*. 2004). Higher serum levels of leptin as observed in obese women during early pregnancy may increase nitric oxide levels by inducing iNOS expression in the uterus and thus, may reduce the adequate uterine contractions contributing to ectopic pregnancy, miscarriages, and/or endometriosis.

ACKNOWLEDGMENTS

We extend our heartfelt gratitude to the Director, IVRI for providing the necessary facilities for carrying out this work.

REFERENCES

Ahima RS, Flier JS (2000) Leptin. Annu Rev Physiol 62(1): 413-437.

Barrichon M, Hadi T, Wendremaire M, Ptasinski C, Seigneuric R *et al.* (2015) Dose dependent biphasic leptin induced proliferation is caused by non specific IL 6/NF - κB pathway activation in human myometrial cells. Br J Pharmacol 172(12): 2974-2990.

Becerril S, Rodríguez A, Catalán V, Mendez-Gimenez L, Ramírez B *et al*. (2018) Targeted disruption of the iNOS gene improves adipose tissue inflammation and fibrosis in leptin-deficient ob/ob mice: role of tenascin. C Int J Obes 42(8): 1458-1470.

Bellver J, Simón C (2018) Implantation failure of endometrial origin: what is new?. Curr Opin Obstet Gynecol 30(4): 229-236.

Buhimschi I, Yallampalli C, Dong YL, Garfield RE (1995) Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. Am J Obstet Gynecol 172(5): 1577-1584.

Bulletti C, De Ziegler D, Setti PL, Cicinelli E, Polli V, Flamigni C (2004) The patterns of uterine contractility in normal menstruating women: from physiology to pathology. Ann NY Acad Sci 1034(1): 64-83.

Caprio M, Fabbrini E, Isidori AM, Aversa A, Fabbri A (2001) Leptin in reproduction. Trends Endocrinol Metab 12(2): 65-72.

Cervero A, Horcajadas JA, Dominguez F, Pellicer A, Simon C (2005) Leptin system in embryo development and implantation: a protein in search of a function. Reprod Biomed Online 10(2): 217-223.

Chatterjee S, Ganini D, Tokar EJ, Kumar A, Das S *et al.* (2013) Leptin is key to peroxynitrite-mediated oxidative stress and Kupffer cell activation in experimental non-alcoholic steatohepatitis. J Hepatol 58(4): 778-784.

Dos Santos E, Serazin V, Morvan C, Torre A, Wainer R *et al.*

(2012) Adiponectin and leptin systems in human endometrium during window of implantation. Fertil Steril 97(3): 771-778.

Fanchin R, Ayoubi JM (2009) Uterine dynamics: impact on the human reproduction process. Reprod Biomed Online 18: S57-S62.

Frühbeck G (1999) Pivotal role of nitric oxide in the control of blood pressure after leptin administration. Diabetes 48(4): 903-908.

Hynes GR, Jones PJ (2001) Leptin and its role in lipid metabolism. Curr Opin Lipidol 12(3): 321-327.

Kissler K, Jones J, McFarland AK, Luchsinger J (2020) A qualitative meta-synthesis of women's experiences of labor dystocia. Women Birth 33(4): e332-e338.

Konturek PC, Brzozowski T, Sulekova Z, Brzozowska I, Duda A *et al*. (2001) Role of leptin in ulcer healing. Eur J Pharmacol 414(1): 87-97.

Kwon O, Kim KW, Kim MS (2016) Leptin signaling pathways in hypothalamic neurons. Cell Mol Life Sci 73(7): 1457-1477.

Malasevskaia I, Sultana S, Hassan A, Hafez AA, Onal F *et al*. (2021) A 21st century epidemy-obesity: and its impact on pregnancy loss. Cureus 13(1): e12417.

McEvoy A, Sabir S (2022) Physiology, Pregnancy Contractions. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.

Moynihan AT, Hehir MP, Glavey SV, Smith TJ, Morrison JJ (2006) Inhibitory effect of leptin on human uterine contractility *in vitro*. Am J Obstet Gynecol 195: 504-509.

Mumtaz S, AlSaif S, Wray S, Noble K (2015) Inhibitory effect of visfatin and leptin on human and rat myometrial contractility. Life Sci 125: 57-62.

Lowenstein CJ, Dinerman JL, Snyder SH (1994) Nitric oxide: a physiologic messenger. Ann Int Med 120(3): 27-237.

Norman JE, Thong KJ, Baird DT (1991) Uterine contractility and induction of abortion in early pregnancy by misoprostol and mifepristone. Lancet 338(8777): 1233-1236.

Otero M, Reino JJG, Gualillo O (2003) Synergistic induction of nitric oxide synthase type II: *in vitro* effect of leptin and interferon γ in human chondrocytes and ATDC5 chondrogenic cells. Arthritis Rheumatol 48(2): 404-409.

Pavithra S, Kumar DK, Ramesh G, Panigrahi M, Sahoo M *et al.* (2022) Fat augments leptin-induced uterine contractions by decreasing JAK2 and BKCa channel expressions in late pregnant rats. Cytokine 157: 155966.

Rodriguez A, Fortuno A, Gomez-Ambrosi J, Zalba G, Diez J, Fruhbeck G (2007) The inhibitory effect of leptin on angiotensin II-induced vasoconstriction in vascular smooth muscle cells is mediated via a nitric oxide-dependent mechanism. Endocrinology 148(1): 324-331.

Ruxton GD (2006) The unequal variance t-test is an underused alternative to Student's t-test and the Mann–Whitney U test. Behav Ecol 17(4) : 688-690.

Schwartz MW, Baskin DG, Kaiyala KJ, Woods SC (1999) Model for the regulation of energy balance and adiposity by the central nervous system. Am J Clin Nutr 69(4): 584-596.

Suarez SS, Pacey AA (2006) Sperm transport in the female reproductive tract. Hum Reprod Update 12(1): 23-37.

Srinivasan G, Parida S, Pavithra S, Panigrahi M, Sahoo M *et al*. (2021) Leptin receptor stimulation in late pregnant mouse uterine tissue inhibits spontaneous contractions by increasing NO and cGMP. Cytokine 137: 155341.

Vecchione C, Maffei A, Colella S, Aretini A, Poulet R *et al*. (2002) Leptin effect on endothelial nitric oxide is mediated through Akt–endothelial nitric oxide synthase phosphorylation pathway. Diabetes 51(1): 168-173.

Wauters M, Considine RV, Van Gaal LF (2000) Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 143(3): 293-312.

Yallampalli C, Garfield RE, Byam-Smith M (1993) Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. Endocrinology 133(4): 1899-1902.

Yu WH, Kimura M, Walczewska A, Karanth S, McCann SM (1997) Role of leptin in hypothalamic–pituitary function. Proc Natl Acad Sci USA 94(3): 1023-1028.

Zhang X, Recchia FA, Bernstein R, Xu X, Nasjletti A, Hintze TH (1999) Kinin-mediated coronary nitric oxide production contributes to the therapeutic action of angiotensin-converting enzyme and neutral endopeptidase inhibitors and amlodipine in the treatment in heart failure. J Pharmacol Exp Ther 288(2): 742-751.

***Cite this article as:** Kishor Kumar DG, Pashupathi M, Anjali, Panigrahi M, Madhu CL, Kesavan M, Singh TU, Dinesh Kumar, Parida S (2023) Leptin increases nitric oxide level via increase in iNOS expression in early pregnant mouse uterus.Explor Anim Med Res 13(1): 49-53. DOI: 10.52635/eamr/13.1.49-53.