Research Article   **Open Access**

The Potential Effect of L-arginine on Mice Placenta

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Summary

L-arginine-nitric oxide pathway has emerged as novel regulators of several vital roles in the reproductive function which comprise pregnancy events, such as placental development. This study was done to pharmacologically enhance the performance of female reproductive system by using L-arginine powder as forerunner of nitric oxide. The study protocol consists of total number of 96 pregnant mice divided equally into two main groups (48 animals per group) and handled as follows: 1st Control group given normal saline orally daily and 2nd L-arginine dosed group 200 mg/kg BW 20% orally daily, both groups were randomly divided into four subgroup according to dosed period of pregnancy term, the dosed periods were 1-15 days, 7-15 days, 7-21 days and 15-21 days.

Several parameters were evaluated and displayed the following results: L-arginine concentration in uterine tissue was elevated in association with increased body, uterine, placenta and fetus weights. That presumably was controlled by an increase food and water intakes. Hormonal levels (estrogen and progesterone) mainly at 7-21 days and 15-21 days of gestation dosed periods. Those results showed histological and stereological profile which illustrated the activity and enlargement of placental layers acquaintance with increasing blood vessels (angiogenesis and vasodilation) and vascular density (%) especially in 7-21 and 15-21 of dosed gestation periods led to an increase placental volume and geometric parameters (cm), weight (gm) and proportional thickness (cm), vascular density, and blood vessels. Fetal traits parameters, displayed significant statistical values of fetuses and weights in all gestation periods expressed at 15-21 days as the best results. Also, increases other parameters: blood volume, steriometry values, histological assessments and alkaline phosphatase and lactogens values. The endpoints of this study presented the L-arginine donated NO which was capable of increasing remodeling blood supply and improvement of some reproductive phenotypic properties of animal models and significant number of fetuses viability.

Introduction

L-arginine was an essential amino acid found in proteins of the animal body and different sources of foods. L-arginine derived nitric oxide converted by catalyzed enzymes nitric oxide synthase. L-arginine had emerged as an important intracellular and intercellular messenger (Nitric oxide-cGMP) controlling many physiological processes [1].

L-arginine and derived nitric oxide plays important roles in numerous biochemical reactions in the body including ammonia detoxification by formation of urea, hormonal stimulation such as pituitary stimulus lead to release of growth hormone and pancreatic release of glucagon and insulin [2] and immune modulation in which it improved the immune status [3] in those suffering from sepsis, burn and trauma [4]. L-arginine donated nitric oxide was indisputable that such a polyvalent molecule could play a decisive role in the male and female reproductive system. Nitric oxide was first recognized in the reproductive system by [5]. Presently the L-arginine play an important role in male fertility which were provoke penile erectile [6-8], enhancement sperm motility [7,9,10] and also play positive position in sexual hormone regulation and ovarian function such as ovulation [11,12] . The role of NO in the uterus was suppression of myometrial contractility during pregnancy [13-17] in other explored extension held and fixed the facts participation of sexual and neuronal behaviors in reproductive fitness in L-arginine dosed mice [18].

Few ideas in the literature revealed the effect of L-arginine on placental efficiency and angiogenic process and therefore would lead to the placental growth and fetal development [6,19]. Therefore, if L-arginine was a potent vasodilator that gives a speculation lead to increase the weight of the fetus by placental competence and fitness [6,20]. So L-arginine-NO was upset the perinatal mortality and superior numbers of live fetuses [6,13,21].

According to philosophies and speculation of literatures and thesis that donation the positive appearance on functional placenta functions and the fetus's fitness, which rendering this study and aimed to evaluation of functional morphometric and stereology profile alteration in mouse placenta subjected to and explored the best time of maximal beneficial effect.

Material and Methods

Animals

This experiment was carried out at the department of physiology, College of Veterinary Medicine, University of Baghdad. Healthy adult female mice were obtained from the animal house of the pharmacology censorship center, ministry of health, Baghdad-Iraq.

Virgin female mice (8-10 weeks) with weight range of 30-35 gram were used in this study. Induction of pregnancy after 2 weeks of acclimatization mated for 48 hours female to male ratio (2:1) then separated and examined to detect pregnancy by observing vaginal mucus seal and/or vaginal smear to ensure of positive mating. These

animals were kept under 20-25ºC in an air-conditioned room and light/dark cycle of 12 hours daily.

Study protocol

Ninety six female mice were randomly divided into two main equal groups held as follows; Control and L-arginine treated group. Each group was divided into 4 subgroups according to L-arginine administration: 7-2 days; 7-15; 7-21 and 15-21 days of pregnancy.

The pregnant mice of the control subgroups were given normal saline orally 0.1 ml/10 g by modified stomach tube and sacrificed at the end of the dosed period. The L-arginine dosed subgroups were orally administrated at dose of 200 mg/kg BW/per day, the amount of dosed L-arginine was adjusted individually according to the body weight. Treated mice were sacrificed at the end of the experiment. Pregnant mice in each group were anesthetized using diethyl ether before being sacrificed the placentas were dissected and excised; fetuses were carried out and taken to record their parameters and blood samples were collected for analysis by direct cardiac puncture.

Experimental parameters: The reflection of loading dose of L-arginine on placental function and their shading of fet uses development, the parameter derived to assess placental changes are described.

Fetal and placental weights and geometric values: After sacrificed the pregnant mice the excised uterus was transferred to watch glass and dissected carefully to extract the fetuses and placentas by incising the uterine horn and then weighing the fetuses and placentas using electrical scale, the morphometric analysis of changes in placentas as well as geometrical distances were recorded using a vernier scale.

Umbilical cord length: The umbilical cord was dissected from the maternal tissue and placental and fetal parts were measured using a vernier scale.

Placental thickness, area and volume: The thickness of placenta was measured of the pregnant mice and the placenta was extracted.

After measuring the major and minor diameters to obtain the mean diameter, the surface area of the placenta was calculated (Placental surface area=PM 2/4), and the volume of the placenta (Volume=p/6M³), P: perimeter, M: mean diameter, finally, the ratio between both was calculated (Ratio=3/2M).

Blood collection for hormonal assays: Blood samples were obtained by cardiac puncture from each anesthetized animal by using disposable insulin syringes. Samples were centrifuged at 2500 rpm for 15 minutes and serum samples were stored in a freezer at -18 C° until use.

Placentas tissue histology

Sample collection for stereological assessment: The entire placentas were gently removed and cleaned from adherent attachment after severing the umbilical cords and expulsion the fetuses from it. They were placed in normal saline solution then fixed in Bouin's fluid. Tissues, after 20 hr. fixation were embedded in paraffin wax, serial sections (5 μm thick) mounted on glass slides and were stained with Heamatoxylin and Eosin [22]. Every five sections were examined to obtain an overall picture of the changes, if any.

Placental tissue stereometrical assessment: The nuclei of the giant cells from the placentas were assessed stereometrically by oculometer scale. The sections were examined with a light microscope attached with fitted camera. Fifty nuclei per animal were measured [23].

o **Relative volume of the placental giant cells:** To assess the placental giant cells on a percent basis we used the technique of Chalkley [24] formula: Vv=(Pn+Pct)/Pt, Where Pn are the points counted in the nucleus of the structure, Pct are the points counted in the cytoplasm and Pt is the total points.

o **Absolute volume of the placental giant cells:** Absolute volume was calculated by the following formula: V=Vv. W/ Wv, Where Vv is the relative volume, W is the weight of the placenta, and Wv is the specific total mean weight per volume of the placental tissue.

o **Relative nuclear volume:** Relative nuclear volume was calculated by the following formula: Vvn=Pn/(Pn + Pct) . [2M/(2M+3t)], Where Pn and Pct are number of points falling on the cell nucleus and cytoplasm, respectively, M is the mean nuclear diameter, and t is the thickness of the histological section.

o **Relative cytoplasm volume:** Relative cytoplasm volume was calculated by the following formula: Vvcyt=1–Vvn, Where Vvn is the relative nuclear volume

o **Nucleus/cytoplasm ratio:** The nucleus/cytoplasm ratio was calculated by the following formula: N/C=Vvn/Vvcyt

o **Nucleus, Cytoplasm and mean giant cell volume:** The above parameters were calculated by the following formula: Vct=Vvn/N/C and Vcell=Vn + Vct, Where Vn is the nuclear volume and Vct is the cytoplasm volume.

o **Numerical density of giant cells:** The numerical density of giant cells was calculated by the following formula: Nv=(Vv/Vcell).

o **Number of giant cells per placenta:** The number of giant cells per placenta was calculated by following formula: Np=(Nv. W/v)

Body weight and uterine weight: The pregnant mice weight were daily recorded using sensitive scale starting first day till the last day of the treatment The uterus of each animal was isolated at the end of the experiment and weight was recorded.

Prolactin assay

Placental tissue preparation: The procedure for dissecting the placenta and its separation into junctional and labyrinth zones was similar to that previously described for the mouse with the aid of a dissecting microscope; 10-20 X magnification. The tissues were collected and washed with Hank's balanced salt solution without Ca²⁺ and Mg²⁺.

The tissues were immediately frozen on solid CO_2 and stored frozen at 25°C until further processing for placental lactogen and alkaline phosphatase assays. The tissues were homogenized in a Brinkman polytron tissue homogenizer for 60 sec at a setting of 6-5 in a Tris-saline buffer (l0 mM-Tris, 150 mM-NaCl, 1 mM-phenylmethylsulphonyl fluoride, pH 8.2). Aliquants of the homogenates were precipitated with perchloric acid, centrifuged at 4000 g. Supernatants from the centrifugation were used for assessment of placental lactogen and alkaline phosphatase activities.

Placental Lactogen assays and measurement: Placental lactogen was measured with a modification of the procedure described by

Shiuetai. Briefly, the prolactin receptor source was mammary gland membranes isolated from the lactating rabbit. Ovine prolactin (NIAMDD-OPRL-15) was used for radio-iodination and as a reference standard for the radio receptor assay. Radio iodination was accomplished with the solid-phase reagent 'Iodo-Gen' as described by Markwell and Fox. The radio iodinated hormone was purified by gel filtration on Sephadex G-100. The specific activity of the radio-iodinated ovine prolactin ranged from 55 to 95 pCi^g. The buffer for the radioreceptor assay was 25 mM-Tris-HCl, pH 7.6, containing 10 mM-CaCl$_2$ and 0.5% bovine serum albumin. The remainder of the procedure was similar to the method developed by Shiuetai. The sensitivity of the assay ranged from 0.1 to 0.2 ng/tube and within- and between assay coefficients of variation were 7% and 11%, respectively.

Alkaline phosphatase assay

Alkaline phosphatase activity was determined as previously described by Lowry. The procedure measures the cleavage of *P*-nitrophenyl phosphate to *P*-nitrophenol in a 1 M-2-amino-2-methyl-l propanol buffers at pH 10.3. Aliquants of the placental homogenates were appropriately diluted with phosphate-buffered saline. 50-µ1 sample was added to tubes placed in an ice bath, followed by the addition of 200 µ 8 mM-disodium p-nitrophenyl phosphate, 1 M-2-amino-2-methyl-l-propanol, pH 10.3. The reaction vessels were then incubated for 30 min at 37°C. The reaction was stopped by placing the tubes in an ice bath and adding 750 µ 0.25 N-NaOH. Samples were then read by spectrophotometry at 410 nm. A standard curve of *P*-nitrophenol from 1 to 50 nmol was generated. Results were expressed in nanomoles of *P*-nitrophenol released per mg protein per min or per placenta per min. The within- and between-assay coefficients of variations were 5% and 10%, respectively.

Results and Discussion

The effect of L-arginine on placental weight

The L-arginine treatment caused significant (P<0.05) increase of placental weight of treated groups in periods 1-15, 7-15, 15-21 and 7-21 days of pregnancy as compared with the control groups (Table 1). Also, the 15-21 days of treatment was significantly higher (P<0.05) than other periods of the treatment groups. Furthermore, the high value of placental weight in L-arginine treated group might be due to increase of the umbilical blood flow velocity and decreased the umbilical cord length by the role of L-arginine-NO system induced to the vasodilation this might coincides with our results that showed in Table 8 due to nitric oxide of maternal circulation and that might be cross the nitric oxide to the placenta and dilate the placental villous vasculature with increased number of villous vessels [25], dilating lumens and thinner muscular appearance of vessel walls [26]. Also this result was agreement with Myatt et al. [27] they attributed the dilatation of placental villous

Gestation periods[3] (days)	L-arginine treated groups	Control groups[2]
1-15	0.109 ± 0.015 Aa	0.103 ± 0.040 Aa
7-15	0.117 ± 0.020 Aa	0.104 ± 0.022 Ab
7-21	0.133 ± 0.011 Ba	0.116 ± 0.028 Ab
15-21	0.187 ± 0.029 Ba	0.141 ± 0.031 Bb

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant (p<0.05) differences among gestations periods; Small letters denoted significant (p<0.05) differences among L-arginine and control groups

Table 1: Effect of L-arginine[1] loading dose on means placental weights g at in different gestation periods of pregnant mice[4].

Gestation periods[3] (days)	L-arginine treated groups	Control groups[2]
1-15	1.25 ± 0.601 Aa	0.93 ± 0.111 Ab
7-15	1.01 ± 0.144 Ba	0.86 ± 0.048 Ab
7-21	0.94 ± 0.085 Ba	0.20 ± 0.012 Bb
15-21	0.53 ± 0.079 Ca	0.11 ± 0.010 Bb

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant (p<0.05) differences among gestations periods; Small letters denoted significant (p<0.05) differences among L-arginine and control groups

Table 2: Effect of L-arginine[1] loading dose on means umbilical cord length cm at different gestation periods of pregnant mice[4].

Gestation periods[3] days	L-arginine treated groups	Control groups[2]
1-15	0.23 ± 0.034 Aa	0.14 ± 0.028 Ab
7-15	0.20 ± 0.064 Aa	0.12 ± 0.041 Ab
7-21	0.11 ± 0.005 Ba	0.08 ± 0.073 Bb
15-21	0.09 ± 0.011 Ba	0.06 ± 0.055 Bb

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant (p<0.05) differences among gestations periods; Small letters denoted significant (p<0.05) differences among L-arginine and control groups

Table 3: Effect of L-arginine[1] loading dose on means thickness of placenta cm at different gestation periods of pregnant mice[4].

Gestation period[3] (days)	L-arginine treated groups	Control groups[2]
1-15	0.31 ± 0.007 Aa	0.24 ± 0.049 Ab
7-15	0.40 ± 0.001 Ba	0.31 ± 0.019 Bb
7-21	1.02 ± 0.266 Ca	0.41 ± 0.053 Cb
15-21	1.31 ± 0.113 Da	0.64 ± 0.012 Db

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital etiters denoted significant (p<0.05) differences among gestations periods; Small etiters denoted significant (p<0.05) differences among L-arginine and control groups

Table 4: Effect of L-arginine[1] loading dose on means fetal weight gm at different gestation periods of pregnant mice[4].

vasculature due to the nitric oxide found in the endothelium of the umbilical, chorionic plate and stem villous vessels appears to contribute the maintenance of basal vascular tone and to attenuate the action of vasoconstrictors such as endothelin (ET-1) and thromboxane [28].

The placental weight increase may be attributed to the high level of L-arginine during gestation period 7-21 as compared with other treated groups and also with control groups suggested these changes might be due to enhanced placental angiogenesis through their donation of L-arginine and growth during this period, thereby promoting an optimal intrauterine environment throughout pregnancy [29]. The dramatically increase placental blood flow and blood volume (Tables 2, 5 and 6) increased the nutrients that transfer from maternal to the placental blood and then enter to the fetus. Subsequently increased the placental and fetal weight [30]. That explains the superior results of L-arginine at period 15-21 days as compared with control group in period.

Others attributed superior placental weight at gestation period 7-21 and 15-21 day due to enlarged placental volume at same period gestation time (Table 6) that through maximized placental transport capacity in late periods of gestation. That occurs presumably under provocation effect of L-arginine nitric oxide by encouragement to rise

Gestation periods[3] (days)	Control groups[2]	L-arginine treated groups
1-15	0.102 ± 0.028 Aa	0.184 ± 0.008 Ab
7-15	0.164 ± 0.011 Ba	0.193 ± 0.010 Bb
7-21	0.169 ± 0.025 BCa	0.222 ± 0.022 Cb
15-21	0.138 ± 0.016 Ca	0.179 ± 0.010 Db

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant ($p<0.05$) differences among gestations periods; Small letters denoted significant ($p<0.05$) differences among L-arginine and control groups

Table 5: Effect of L-arginine[1] loading dose on blood volume gm at different gestation periods of pregnant mice[4].

Gestation periods[3] (days)	L-arginine treated groups	Control groups[2]
1-15	0.71 ± 0.059 Aa	0.32 ± 0.061 Ba
7-15	1.23 ± 0.206 Ab	0.37 ± 0.010 Ba
7-21	1.5 2 ± 0.175 Ac	0.90 ± 0.043 Bb
15-21	1.58 ± 0.222 Ac	0.96 ± 0.197 Bb

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant ($p<0.05$) differences among gestations periods; Small letters denoted significant ($p<0.05$) differences among L-arginine and control groups

Table 6: Effect of L-arginine[1] loading dose on placental volume cm at different gestation periods of pregnant mice[4].

Gestation Periods[3] days	L-arginine treated groups	Control groups[2]
1-15	0.59 ± 0.044 Aa	0.20 ± 0.005 Ab
7-15	1.32 ± 0.201 Ba	0.33 ± 0.039 Bb
7-21	1.90 ± 0.115 Ca	0.40 ± 0.079 CBb
15-21	2.17 ± 0.265 Da	0.67 ± 0.022 Db

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital etiters denoted significant ($p<0.05$) differences among gestations periods; Small letters denoted significant ($p<0.05$) differences among L-arginine and control groups

Table 7: Effect of L-arginine[1] loading dose on placental surface area cm at different gestation periods of pregnant mice[4].

of extraction rate for both oxygen and substances per unit of uterine blood or umbilical blood from the arterio-venous concentration difference [30].

De Boo et al. [31], Reynolds et al. [32] Faber and Thornburg [33] and Meschia [34] reported results that L-arginine derived NO into animal during 7-21 days and 15-21 days periods of gestation increased protein accretion, this presumably led to increase the placental weight and also fetal weight.

Furthermore, L-arginine-NO pathway transport against the concentration gradient (active transport), in which they activate carrier proteins in microvilli of the placental membrane led to partially factor promoted increasing placental weight, That speculated by Logic et al. [35] for amino acids turnovers and exogenous transport.

So, the placental weight showed at the gestation periods 1-15 and 7-15 appeared lesser than other L-arginine treated groups due to placental thickness at this periods was greater and lesser its permeability to cross high amounts of nutrients and oxygen and also level of L-arginine less than other periods which coincides with our results that showed in Table 3. But in gestation periods 7-21 and 15-21 the placental weight increased more than other L-arginine treated groups and control groups attributed to the natural diminution of

placental thickness due to high level and important role of L-arginine-NO pathway in inducing vasodilatation led to augmentation of permeability with greater placental diffusion thus increased the nutrients and oxygen that cross into the placenta which were coincides with our results that showed in periods 7-21 days and 15-21 days of gestation, therefore led to increased placental weight [36-39].

The effect of L-arginine on fetal weight

The L-arginine treatment caused significant increase ($P<0.05$) of fetal weight of treated groups from period 1-15, 7-15, 7-21 and 15-21 days of pregnancy as compared with the control groups Table 4. Also the period of treatment 15-21 days is significant ($P<0.05$) increased as compared with other treatment periods.

The fetal weight increase presumably due to contribution of nitric oxide in placental vasculature [26], its important regulator of placental perfusion, Therefore enhanced fetal growth, this led to improve the fetal-maternal circulation by vasodilation, subsequently increased the volume and deceased viscosity of the blood in fetal-maternal circulation [40].

Furthermore, Lampariello et al. [41] attributed the fetal growth and increased birth weight of newborns might be due to the nitric oxide improve the utero-placental blood flow and thereby increase oxygen delivery to the fetus, also its effect on the pregnancy and neonatal by indirect influencing the utero-placental circulation. Thureen et al. [42] recorded the L-arginine had influenced mechanism of hyper synthesis and release of insulin from B cells aggregated pancreatic island that directed our attitude to direct attribution rise of fetal weight might be play the L-arginine essential role in the acceleration of fetal growth by stimulation of insulin secretion utilized circulating glucose.

In otherwise, administration of L-arginine increased the number of born alive or live litter birth weight in pregnant animal during the periods 7-15 days and 7-21 days of gestation, as reported previously [42], an increased in plasma concentrations of L-arginine likely resulted in enhancement of placental transport from mother to fetus. This would provide adequate amounts of L-arginine from the pregnant animal to their fetuses, thereby supporting their optimal metabolism and growth during the periods 7-15 days and 7-21 days of gestation of most rapid fetal growth as compared with other period in same group and also with control group. Indeed, results of a recent study showed that L-arginine infusion into animal during 7-21 days and 15-21 days periods of gestation increased protein accretion in fetus [31], therefore, increased the fetal weight.

Roberto et al. [43] and Salvemini et al. [44] attributed the decreased in the results of fetal weight in the early period (1-15) of gestation due to reduce corpus luteum (CL) number due to the nitric oxide caused upset of CL through exaggeration of the biochemical event derived prostaglandin F2α (PGF2α) synthesis by up regulating expression of cyclooxygenase enzyme that key enzyme for (PGF2α) synthesis which considered a luteolytic agent, therefore reduce CL number and thereby resulting impairs the progesterone production, also this lack of progesterone production reduce the availability of amino acids, subsequently reduce protein synthesis which important for fetal growth, also CL was the main source for progesterone required for establishment and maintenance of pregnancy and increased the fetal weight [45].

Furthermore, the administration of L-arginine and in control group but the L-arginine more pronounce accelerated fetal weight reception gain through certain 7-15 days and15-21 days periods of

gestation, which coincides with the period of rapid fetal growth [46], thereby increased the weight in this periods. Previous reports had shown that uterine capacity starts to become limiting for embryonic survival at as early 1-15 days period of gestation, thereby affecting fetal growth [47] and leading to losses of viable fetuses. This was in agreement with the finding of this study that almost all born dead were fully formed. Administration of L-arginine reduced the number of dead born, probably due to an improved uterine environment capacity for fetal growth and development [48].

The effect of L-arginine on blood volume

The blood volume displayed significant increase in blood volume at all periods of gestation in L-arginine dosed groups as compared with control groups whereas the superior values occur in 15-21day of gestation in L-arginine dosed group showed in Table 5.

Dong and Yallamplalli [49] attributed increased blood volume might be induced vasodilation that regulates by intrinsic ability of L-arginine-nitric oxide system to regulate cytosolic $[Ca^{++}]$, that L-arginine-NO system activity indicated an upset in cytosolic $[Ca^{++}]$ enable reduction of contraction cellular events associated with restricted of their tonicity partially, the placental vasculature expressed NOS [50] that had been presence in stem villious of placenta modulated NO production and enhanced activity NO synthase by L-arginine substrate or under systematic loading L-arginine which led to play a local role in controlling of placental blood volume [51].

That demonstrated the sequel of NO vasodilator effect provoked blood outflow then blood flow by relaxation of the blood vessels wall [52]. In addition to previous studies which were in agreement with our results indicated that progesterone [6] might be regulates the c.GMP effectors system for relaxation of blood vessels, and then led to increase blood demand through enlarging the blood vessel capacity then blood volume [53].

Furthermore, attributed increase blood volume might be due to increase estrogen during early pregnancy that initiated a receptor-mediated event that activated NOS and probably (nNOS) [6,54] to produce NO this NO increased and persist smooth muscle cyclic guanosine monophosphate (c.GMP) which activated a c.GMP-dependent kinase that encouraged velocity of Ca^{++} activated K^+ channel activity and decreased Ca^{++} inflow by voltage gated Ca^{++} channel, resulting in vasodilation and quiescence during pregnancy [50]. Thus, an increase of NO availability might be included as one of the mechanisms through which estrogen reduces arterial wall thickness and increases vessel distention ability.

The loading of L-arginine capable of elevating the NO levels in CNS and reproductive organs; placenta, that NO had triggered, regulator and demined of gonadotrophin releasing hormone (GnRH) that involvement the stimulation and regulation of Luteinizing hormone (LH) released production, these findings are supported by [55] and increased postpone CL-Progesterone functionally.

Furthermore, the anatomical localization of NO neurons in close proximity to GnRH neurons in hypothalamus these adjacent between neuron may be regulated physiologically and regulator manner of GnRH secretion that increase blood volume [56].

At the endpoint may give an impression to say increase blood content (volume) which produced raise in placental weight and volume, finally it is reflected to increase physiological demand of fetal growth and fetal vital processes.

The morphometric parameter of placenta

Placental volume and surface area: The L-arginine loading dose caused significant (P<0.05) increase of placental volume and surface area in treated groups at periods 1-15 days , 7-15 days, 7-21 days and 15-21days of pregnancy as compared with the control groups Tables 6 and 7. The group treatment at 15-2 days presented higher significant (P<0.05) of placental volume and surface area than other periods of L-arginine treated groups.

The increased of the placental volume which might be through the L-arginine-NO pathway enhance the intrauterine growth trophoblast cells and labyrinth zone volume due to excessive provocation and produced of NO in the placental stem villous that encouragement the micro blood vessels network vasodilation .

Furthermore, the placental volume and placental surface area increased might be due to the labyrinth zone encouragement in their density was considered principle site in hemotrophic exchange "nutrient, hormones, ions, waste and water" between maternal and fetal parts [57,58] associated directly with increase functionally placental fluids in their layers these give a true impression geometrically exaggerated in dimensional and out line, this results was coincided with histological appearance through hyperplasia and hypertrophy in placental zones with comparable with control showed increased patches and multiple area increased of giant cells.

In otherwise, the NO induced vasodilation that promote compensatory mechanism and utilized reserved blood volume to increase blood flow in the placental layers and also the NO play a key role in angiogenesis of placenta [19,29]. NO is a proangiogenic growth factors in the systemic circulation, rise NO production led to stimulate new vessels growth and thus increasing total vessel length and subsequently, augmentation of permeability with greater placental diffusion due to increased Labyrinth zone surface area and decreased inter-hemal membrane thickness with an increased diffusion capacity necessary for exponential growth of embryonic tissues thus increased the nutrients and oxygen that cross into the placenta [36-39] that led to increase in geometric and enlarged morphometric of placental volume and placental surface area.

Giles et al. [25] demonstrated and explained the relationship between placental enlargement and vasculogenic functional remodeling and histological convention as dramatic influenced by L-arginine-NO metabolic pathway and their sequel due to increase the number of villous vessels and dilated the placental villous vasculature led to the expansion of the fetal capillary volume continues until at least embryonic day 18.5, would need for continually greater volumes of fetal blood to exchange with the maternal circulation to obtain enough nutrient and oxygen for fetal growth then increased the blood flow in this vessels and subsequently increased the placental volume and surface area [26].

The placental volume and surface area results under L-arginine loading dose was coincided with Babaei et al. [59] suggestion could use for attribution the increased of placental volume might be due to L-arginine-NO pathway which had important role in vasculogenesis (formation of blood vessels from mesoderm precursor cells) and angiogenesis (creation of new vessels from a pre-existing blood supply) in the placental villi which led to develops the labyrinth layer that was critical for maternal-fetal exchange, subsequently increased the placental volume and surface area. Finally, the placental enlargement was positive directly proportional with increased fetal weight and

increased survival of fetuses (viable fetus).

Histological assessment of placenta: In both control and L-arginine dosed groups the giant cells and placental volumes were positivly increased parallel in period 15-21 days of gestation but the L-arginine dosed group showed superior values than control group that attributed to normally cessation and attained in placental volume at 16 days in control group [60] (Table 6) whereas, placental volume in L-arginine loading doses 15-21 day of gestation increase may be due to:

First, increase maximized stereological volume of giant cells of labyrinth zone (Table 7).

Second, increase giant cell volume (%) that provoked their endocrine releasing factor or hormones [61]. These promotion effects and locally control of labyrinth and junctional zones caused differentiation of trophoblast glycogen cell which finally increased volume indirectly [61].

Third, L-arginine-NO system facilitated migration of trophoblast cell to the maternal deciduas layer to differentiation to trophblast glycogen and that supply a spare weight and volume of placenta maximized function activity and apparent morphometrical enlargement (Figure 1).

Furthermore, these suggestions were encouraged by stereological profile by increased geometrical value of nucleo-cytoplasmic volumes; relative nuclear volume %, relative cytoplasm volume (%), cytoplasm volume (mm^3), nuclear volume (μm^3) and mean cell volume (μm^3)) and decrease nucleus/cytoplasm ratio due to hyper functional activity of storage form and synthesis of cytoplasm.

Finally, increased endocrine function and extended the main location of nutrients and gaseous area through increased labyrinth and junction zones in volume which were play a key role in maternal-fetal transference that underlies fetal growth and weights (Table 5).

Furthermore, surface area and thickness also correlated with capillary length and diameter and volume in the same period 15-21 due to increased capillary length and important factor merged with labyrinth inter-hemal membrane that coincided with the results. The blood volume increased under the facts of increases capillary density according to primary function of L-arginine-nitric oxide system.

The endpoint was improved placental development and reduce shortcomes occur in the cessation of development occur between 15-18 days of pregnancy [60] and undergo the preceded and succeeded by relatively quiescence periods primary that may be caused L-arginine
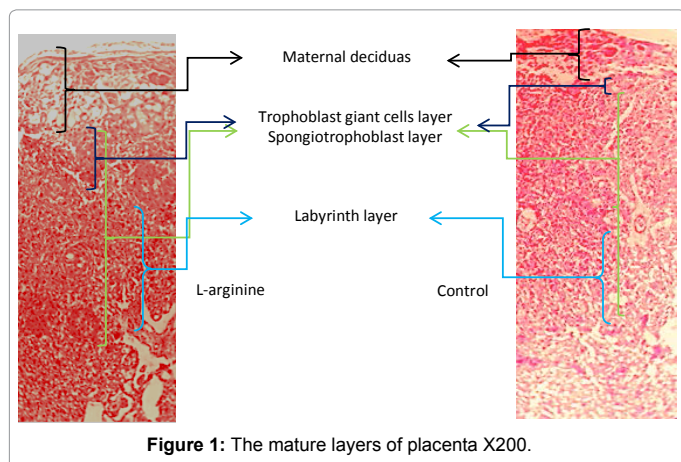

Figure 1: The mature layers of placenta X200.

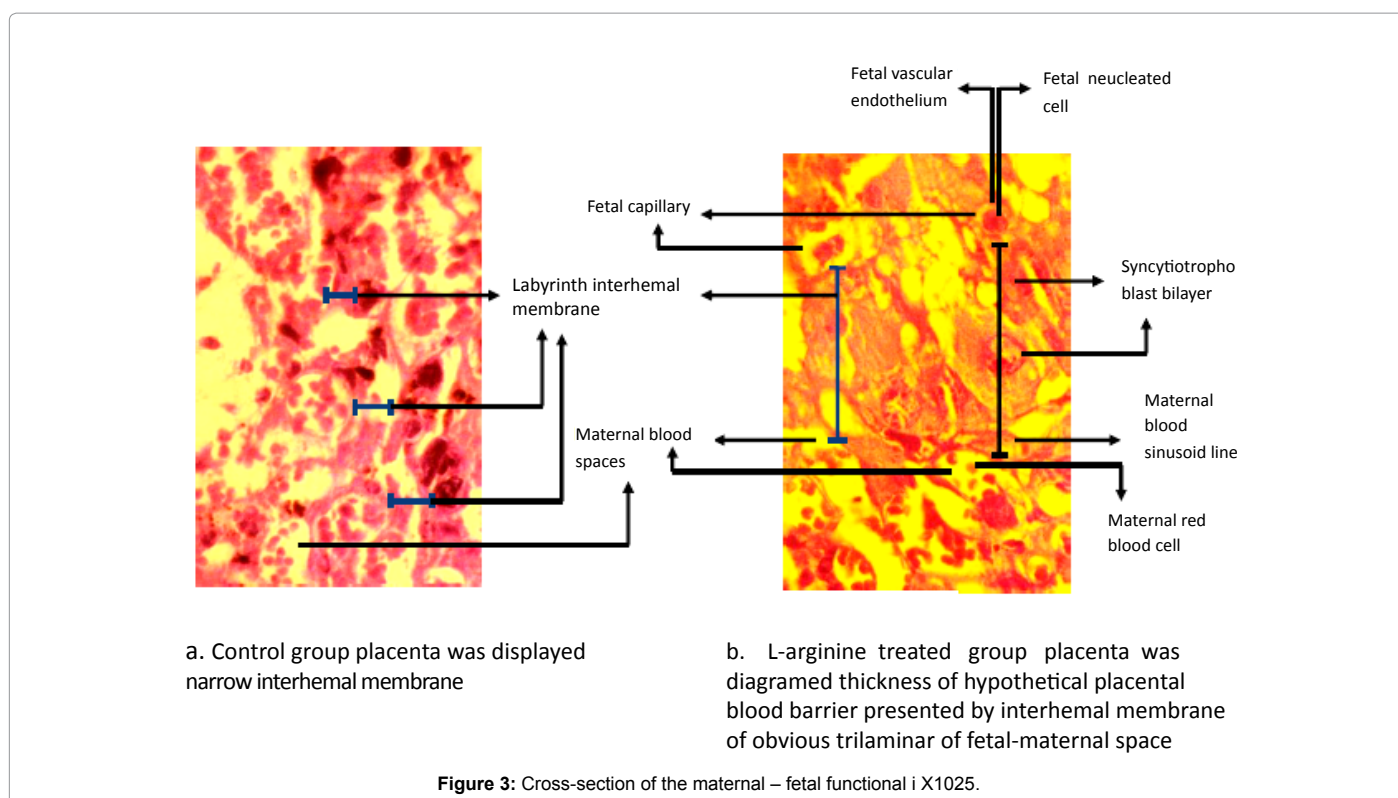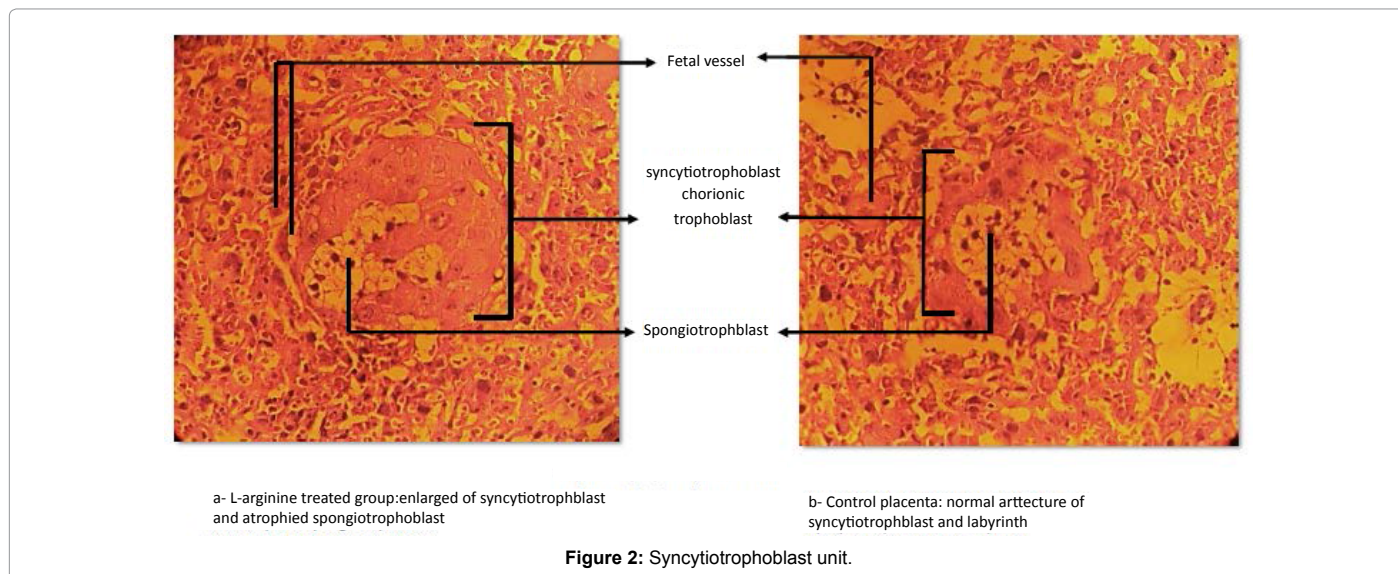| Parameters steriometry	Control groups[2]	L-arginine treated groups
Relative volume of giant cells (%)	1.66 ± 0.21 a	1.85 ± 0.13 b
Absolute volume of giant cells (mm^3)	2.27 ± 0.39 a	3.06 ± 0.21 b
Relative nuclear volume (%)	15.49 ± 1.95 a	16.01±1.04 b
Relative cytoplasm volume (%)	84.51 ± 5.01 a	85.99 ± 3.28 b
Nucleus/cytoplasm ratio	0.1819 ± 0.0096 a	0.1462 ± 0.0065 b
Cytoplasm volume (mm^3)	15037.59 ± 48.91 a	16333.71 ± 33.33 b
Nuclear volume (μm^3)	3512.19 ± 26.43 a	3905.72 ± 14.60 b
Mean cell volume (μm^3)	22306.47 ± 11.92 a	41397.51 ± 12.85 b
Numerical density (n/mm^3)	0.000074± 0.0086 a	0.000044± 0.0055 b
Number of giant cells/placenta	0.000095± 0.0095 a	0.000066± 0.0072 b

[1]L-arginine 200 mg/Kg BW, daily, orally, 2%; [2]Control normal saline treatment; [3]Gestation periods; Time of loading daily dose of drugs (L-arginine and normal saline); [4]N 12 pregnant mice; Capital letters denoted significant (p<0.05) differences among gestations periods; Small letters denoted significant (p<0.05) differences among L-arginine and control groups

Table 8: Effect of L-arginine[1] loading dose on placental steriometry cm at gestation periods (15-21)3 of pregnant mice[4].

remodeling the discounting changes in maternal blood pressure and hence blood flow. The volume of maternal blood that may be through flow the placenta in the 17 days of pregnancy were is maximized in L-arginine treated group faster than in control group in 15-17 day.

In 17 days of pregnancy this is suggesting that maternal blood space develop more in L-arginine dosed group and sufficient at this stage with satisfactory and necessary maternal blood flow through term. On the other hand, the placental-fetal capillary may be increased in L-arginine dosed group extensively in 18 day of pregnancy than control [48] and extend their function to determine their need as controversy in control suggesting a call for greater fetal blood to exchange with the maternal circulation in order prepare enough nutrient and gaseous agents for fetal growth, that's taking into account the fetal weight [48]. The L-arginine-nitric oxide behaves as an angiogenic factor which acts either elongation and /or branching capillary network. In Figure 2, the control group manifested that thin barrier minimized the diffusion distance and maximized the area of passive exchange L-arginine treated group had a wide barrier proportional with a large surface area in physiologically maximized the diffusion distance and minimized area of passive exchange which play selective barrier not chance passive exchange according the gradient [36,37], in the contrast to compensate reduction of diffusion capacity by increased vasodilation and angiogenic process to overlap the increased, thickness of placenta which is necessary for exponential growth of embryonic tissues which may be harmonic alteration between thickness and vasodilation in L-arginine treated group is a more critical determinant of diffusion capacity of placenta [36,37]. That is interesting finding which might be explained by the difference in growth pattern of the fetus and placenta.

Whereas in control group, the decreased in fetal and placental growth in other periods presumably due to the fact that the fetus is small and therefore as a little total volume and reduction capillary with suggesting the inter-hemal membrane and capillary volume is triggered first in this period but not fully working in the control group. In L-arginine treated group, the total capillary network developed showed in well rather than control and inter-hemal membrane remained thick in control group at first due to narrow capillary and volume this would perturb diffusion of substances to the fetus. That's highly density of enlarged fetal capillary in L-arginine treated group enlarged capillary volume for compensation of inter-hemal membrane thickness to accumulate more molecular weight selection agent processes for fetal growth demand which play a supersized roles and expected a

a- L-arginine treated group:enlarged of syncytiotrophblast and atrophied spongiotrophoblast

b- Control placenta: normal arttecture of syncytiotrophblast and labyrinth

Figure 2: Syncytiotrophoblast unit.

a. Control group placenta was displayed narrow interhemal membrane

b. L-arginine treated group placenta was diagramed thickness of hypothetical placental blood barrier presented by interhemal membrane of obvious trilaminar of fetal-maternal space

Figure 3: Cross-section of the maternal – fetal functional i X1025.

greater balance between placental supply and fetal requirements for exaggerated effect needed to fluctuation in local nutrition and reduced placental oxidative stress and prevent hypoxia-reoxygenatal type energy by direct chelating or indirect fating neutralization.

Placental Steriometry parameters: In a histological study of the placenta of mice treated with the L-arginine, cytometry of the giant trophoblastic cells showed that the placentas from the treated group was more positively changed, also in terms of cell volume. Thus, the relative volume, absolute volume, numerical density and total number of giant cells were significantly (P<0.05) superior in the placenta of this group than in control placenta showed in Table 8.

In rodents, giant cells differentiate by endo-reduplication and their functions are endocrine secretion and invasion of the maternal deciduas. These cells are a private source of placental lactogens I and II (PL-I and PL-II) and in the second trimester of pregnancy they likewise synthesize numerous prolactin-like proteins and a different of placental lactogen I (PL-IV) [62]. PL-I, PL-II and PL-IV also served on the fetus. Faria et al. [61] established the cellular derivation of placental lactogen I and the PL-I to PL-II transition at the end of the first half of pregnancy, with these cells starting to express PL-II thereafter.

The changes provoked by the L-arginine are trophoblastic cells and the difference in behavior observed among the cell populations of different placental regions may affect intra-uterine development, probably by efficient production of hormones such as placental lactogen, which acts to motivate a fetal development hormone.

Zybina and Zybina, [63] demonstrated that rat and mouse giant cells have 4c-8c ploidy on the 12th day of pregnancy, whereas on the 13th-14th day ploidy is 8c-16c this increase in ploidy may be important for trophoblast differentiation, allowing invasion of the deciduas. Therefore, L-arginine-NO may play a positive profile to increase ploidy presumably reach to 32-64 ploidy which promote secretory process and increase hormone like protein.

Keighren and West [64,65] did not observe higher order polyploidy in giant cells of the trophoblast of the mouse placenta suggesting that these may be polytene and not polyploidy cells. At a given stage of differentiation, giant cells divide into numerous nuclear fragments forming multinucleated cells that swiftly degenerate into nuclear fragments with 1 to 32c ploidy. Those seen in Figure 3 different multinucleated cells as same area of trophoblastic giant cell that may be L-arginine slowed degeneration of multinucleated cell by promoting anti free radical an ions and engorgement metabolic process to provoked cell long life. So L-arginine could act in promotion of cell cyclic in anaphase sensation telophase.

Furthermore, several areas occupancy were extended and differentiated large zoon of giant cells and given an impression darkely stained and hypertrophy to hyper functional activity for hormonal synthesis.

This result was coincided with hormonal level estimated and displayed higher levels of placental lactogens and alkaline phosphatase activity.

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