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Evaluation of Intra Placental Vascular Pattern in Postdate Placenta, By Casting and Immunohistochemical Evaluation of A-Smooth Muscle Actin

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Abstract.
Placental senescence impairs villous capillaries and intervillous spaces decline, changes in placental vascular structure appear at various stage of pregnancy, many factors act on placental angiogenesis to insure adequate placental vascular function .Study of placental vasculature, and α-smooth muscle actin component of microfilament of contracting cells in villous stemand blood vessels tunics, is of great importance for fetoplacental blood flow assessment, ascontraction and relaxation of myofibroblast regulate inter-villous space volume and control placental hemodynamics.This study aimed to evaluate fetal vascular changes in placental villi, in postdate placenta by casting method and immunohistochemical expression of α-smooth.

Keywords. Postdate, Casting, α-SMA.

1.Introduction

A prolonged pregnancy is defined as a pregnancy extends to or beyond 42 weeks of gestation. While post-maturity, which is a clinical condition thought to be a consequence of a failing in placental functions., that affect both the mother and newborn [1, 2]. Postdate gestation forming 5-10 % of all pregnancies,most cases of postdate pregnancies result from a prolongation of gestation, or resulted from inaccurate dating criteria [3]. Ultrasonographic dating in early pregnancy is more reliable in determining the EDD, reducing the incidence of post-term and minimize unnecessary operative interventions [4]. Post-term pregnancies are at increased risk for numerous adverse outcomes including both maternal and perinatal complications [5], mostly ended by cesarean section (6). Placental dysfunction can occur in aging of the placenta, and fibrin deposition that affect the rate of exchange with the maternal circulation, impairing villous capillaries and intervillous spaces decline (7), use ultrasound to stage placental senescence and noticed regions of raised calcium precipitation within placental compartments, that increases after 40 weeks gestation [8]. Casting technique by injection of a material that's harden into a vascular structure to study these structures, study of casted samples of placenta can give idea on the placental vasculature changesin normal and pathological conditions.(9) Latex material used in casting study of placental samples [10] ,the exchangebetween fetal and maternal circulation occur mainly within cotyledons, therefore investigations are focusing on the peripheral capillaries generations within these cotyledons [11]. The expression of the cytoskeletal proteins α-smooth muscle actin(SMA)was confirmed in placental tissue at different gestational age [12], its detected in perivascular spaces and vascular walls. This specific location could provide a role for α-smooth muscle actin in modulation of the villi , and accordingly the dimensions of intervillous space, ultimately affecting the feto-maternal circulation [13]. Evaluation of theintraplacental (IP) vessels still incomplete in postdate pregnancies. This research attempt to verify the changes in intraplacental vascular pattern and contractility features in postdate placenta by casting technique and α-smooth muscle actinimmunohistochemicalexpression in vascular walls and villous stroma.

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2.Materials and Methods

A sample of 20 normal human placentae were collected from Al-Kadhimiya Teaching Hospital/ the obstetric and gynecological department, all were delivered by elective caesarian sections, all mothers agreed to participate with their placentas after delivery with informed written patient consent, the age of the mother ranged from 22 to 35 years old, and gravidity ranged from gravida 1 to 3.The sample is divided into two groups; Term placentae(N: 10) with gestational age from (38 to 41 weeks) , and Postdate placentas (N:10) with gestational age (more than 41 weeks) .Gestational age was confirmed by last menstrual period consistent with a second trimester ultrasound. All mothers were free of underlying medical conditions or placental abnormalities, and all placentae were confirmed to have three vessels cords. Five Placentas from each groups were selected for casting study, where excess blood is squeezed out ; cleaned with tap water ,umbilical cord vessels were canulated (insertion of intravenous canula) then tap water used for irrigation, then the cannula is replaced by pasture pipette which was secured to the vessel wall by surgical threads. Casting material used is (latex) which is a natural rubber polymer from plants origin (Hevea tree) that infiltrate the placenta which then transferred to 10% formaldehyde and kept overnight. They were finely dissected for intra cotyledon vascular pattern that were measured by Hoechst lens (8X) in which (1mm) is subdivided into 10 units each interval equal to (0.1mm). Modified H&E stainfor casted sample donethrough dipping of casted sample into haematoxylin jar for (3 minutes), then under running tap water for (8 minutes), then dipped in water based eosin for (30 seconds), and studied under the dissecting microscope.Samples from the term, and postdate placentae were selected from seven different regions from maternal surface to perform paraffin blocks for histological, and immunohistochemical study for α-SMA. All samples were fixed in 10% neutral buffered formalin overnight, then proceeded to dehydrated in ethanol: 70%,80%, 90%, 100%, cleared in xylene, infiltrated with paraffin and blocking [14]. Sections were placed on glass slides for ordinary H&E, and on positively charged slides for immunohistochemistry for α-SMA monoclonal antibody to alpha smooth muscle actin (orb317295) and detectionkit (biorbyt, orb90443),that provided by biorbyt, negative control was performed on placental tissues in which the primary antibody is replaced by phosphate buffer saline in immunohistochemical staining. Histomorphometric analysis of tunica media thickness in relation to vascular wall thickness,done on placental vasculature by (Image J version 1.52a) where the corrected mean diameter of tunica media in relation to total wall thickness is calculated at three different points (Fig. 1). Aperio scope image analysis software positive pixel count (V12.4), used to counts the number and intensity-sum in each pixel, it has a set of default of parameters that specified for brown color quantification. Positivity is the fraction of positive pixels to total stained pixels is selected for comparison between test groups, markup images allows to confirm that specified measurements in the intensity ranges. α-SMA appeared in blood vessels wall and in myofibroblast that stained positive for α-SMA in stromal core of villi (Figure 2A).

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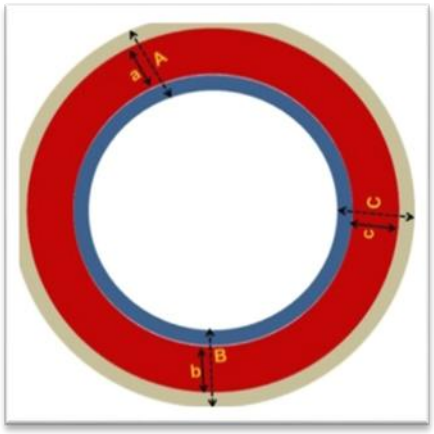

Figure 1. Histomorphometric evaluation of vascular wall tunica media in relation to total wall thickness.

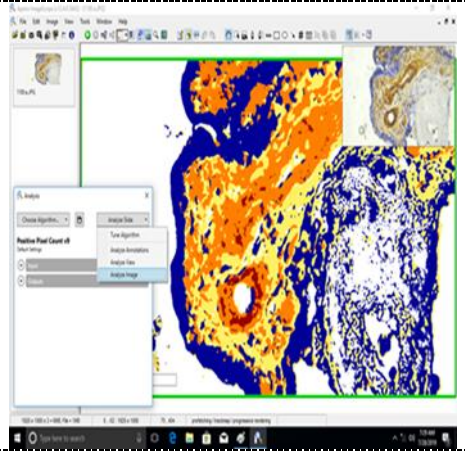

Figure 2. Immunohistochemical evaluation of α-SMA in vascular and stromal core of villi by Aperio scope image analysis software positive pixel count (V12.4).

3. Results

3.1. Casting study

Dissection of casted placentas under the dissecting microscope showed a chorionic vessel within the chorionic plate gives to a number of sub- cotyledon major arteries, this in turns gives rise to another generations of primary arteries , then to a generation of secondary arteries, and final generation of tertiary terminal arteries (Figure 3A&B) and with application of modified H&E stain on casted samples (Figure 4A&B) and the application of Hoechst lens to measure the dimensions of terminal arteries.Vascular generations study in casted samples in postdate placenta showed significant increase in the number of vascular generations that enter the cotyledons (sub-cotyledon major arteries) and significant increase in the terminal (tertiary vessels) length, and significant reduction in their lumen diameter at $p \leq 0.05$ Table (1).

Table 1. Vascular generations and measurements of arteries in terminal villi in casted models of term and postdate placentae.

Groups	Generations of vessels enter the cotyledon	Mean length of terminal vessel ±SE	Mean diameter of terminal vessel ±SE
Term	5	5± 0.57	0.1±0.005
Postdate	7	5.5 ±0.4	0.03± 0.008
P-value	0.045	0.005	0.033

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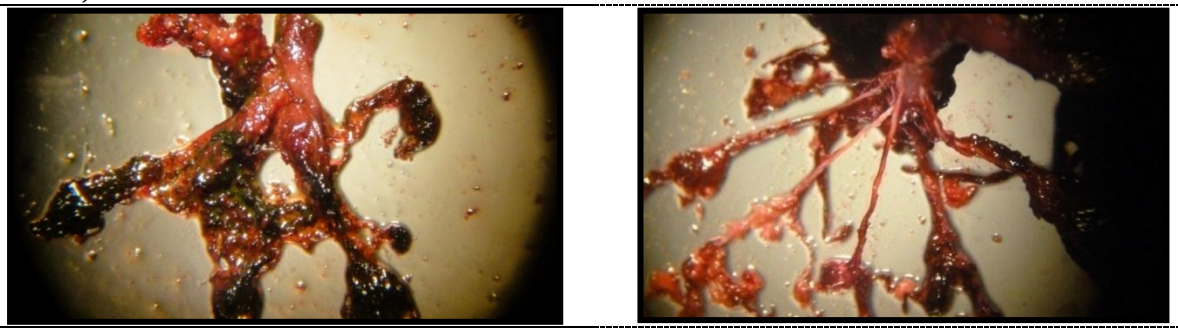
A B

Figure 3. (A and B): Fine dissection of casted placental tissue under dissecting microscope, shows chorionic vessel (black arrow), sub-cotyledon major arteries (red arrows), primary arteries (yellow arrows), secondary arteries(blue arrows), and terminal tertiary arteries (pink arrows).



A **B**

Figure 4.(A&B):Casted placental tissue with modified H&E stain examined under the dissecting microscope, shows sub-cotyledon major arteries (red arrows), primary arteries (yellow arrows), secondary arteries (blue arrows), and terminal tertiary arteries (pink arrows).

3.2. Relative thickness of tunica media

In H&E stained sections the tunica media relative thickness in relation to total vessel wall thickness showed significant increase in subcotyledons major arteries and primary arteries with p values: 0.026, and 0.005 respectively, and non-significant increase in secondary arteries. Terminal arteries are capillaries in type not including tunica media in their wall so not included in this measurements (Figures5A&B, 6A&B, 7) and Table (2).

Table 2. Histomorphometric analysis of tunica media in intracotyledons vascular structures in term and postdate placenta.

Groups	Mean thickness of tunica media in sub-cotyledon major arteries	Mean thickness of tunica media of primary arteries	Mean thickness of tunica media of secondary arteries
Term	0.521± 0.055	0.465±0.043	0.27±0.051
Postdate	0.670±0.043	0.613± 0.230	0.3629± 0.079
P-value	0.026	0.005	0.151

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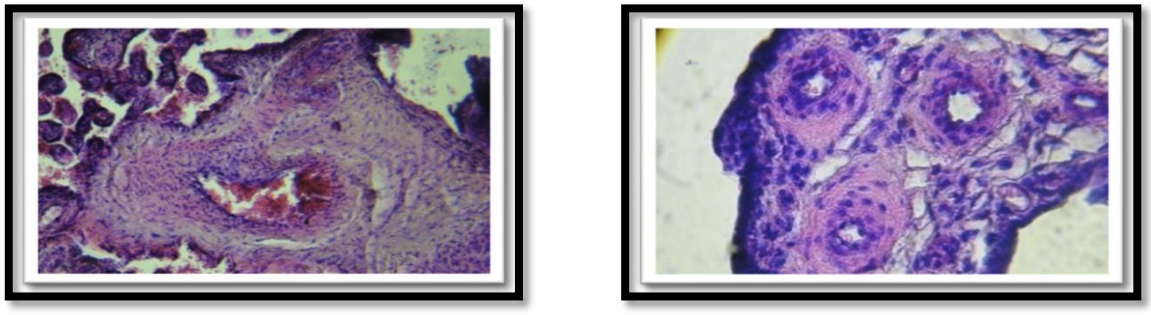
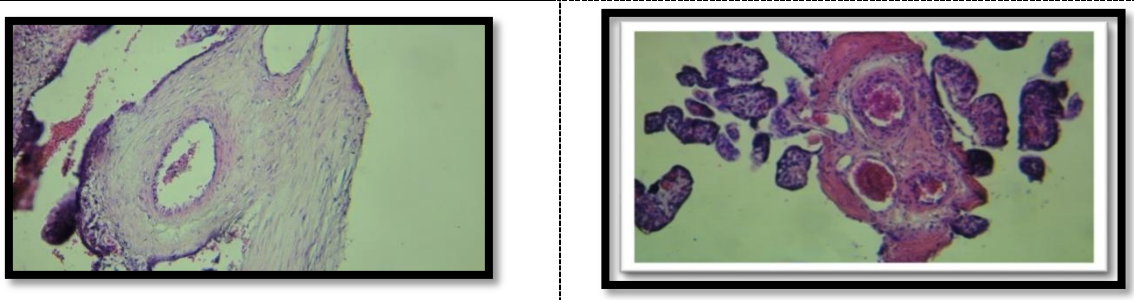


A B

Figure 5.(A) Relative thickness of tunica media of postdate placenta in sub-cotyledon major artery and (B) in primary arteries in villous cores. H&E, X10.



A **B**

Figure 6. (A) Relative thickness of tunica media of term placenta in sub-cotyledon major artery and (B) in primary arteries in villous cores. H&E, X10.

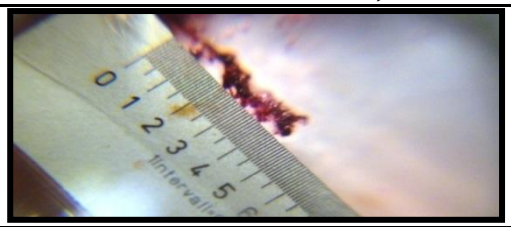


Figure 7. Measurement of the length of terminal artery in terminal villi with Hoechst lens. 8X of the lens.

3.3. Immunohistochemical evaluation of α-SMA

Alpha smooth muscle actin was seen at the smooth muscle layer of tunica media of arteries of placental villi , it showed a significant increase in IHC expression in postdate placenta compared to term in placental villi with p values 0.03 (Figure 8A&B,9A&B). A significant increase in α -SMA stained myofibroblast in postdate placenta that seen in perivascular space with p value 0.022(Figure 10A&B), and Table (3). Negative control performed on placental tissue without the primary antibody for α –SMA Figure (11).

Table 3. The mean positivity of immunohistochemical expression of α-SMA and mean number of positive stained myofibroblast in placental villi in term and postdateplacenta.

Groups	Mean immunohistochemical reactivity	Mean myofibroblast immunohistochemical reactivity
Term	0.631± 0.01	11.21± 6.512
Postdate	0.690± 0.07	19.016±7.343
P-value	0.03	0.022

P-value ≤ 0.05 significant.

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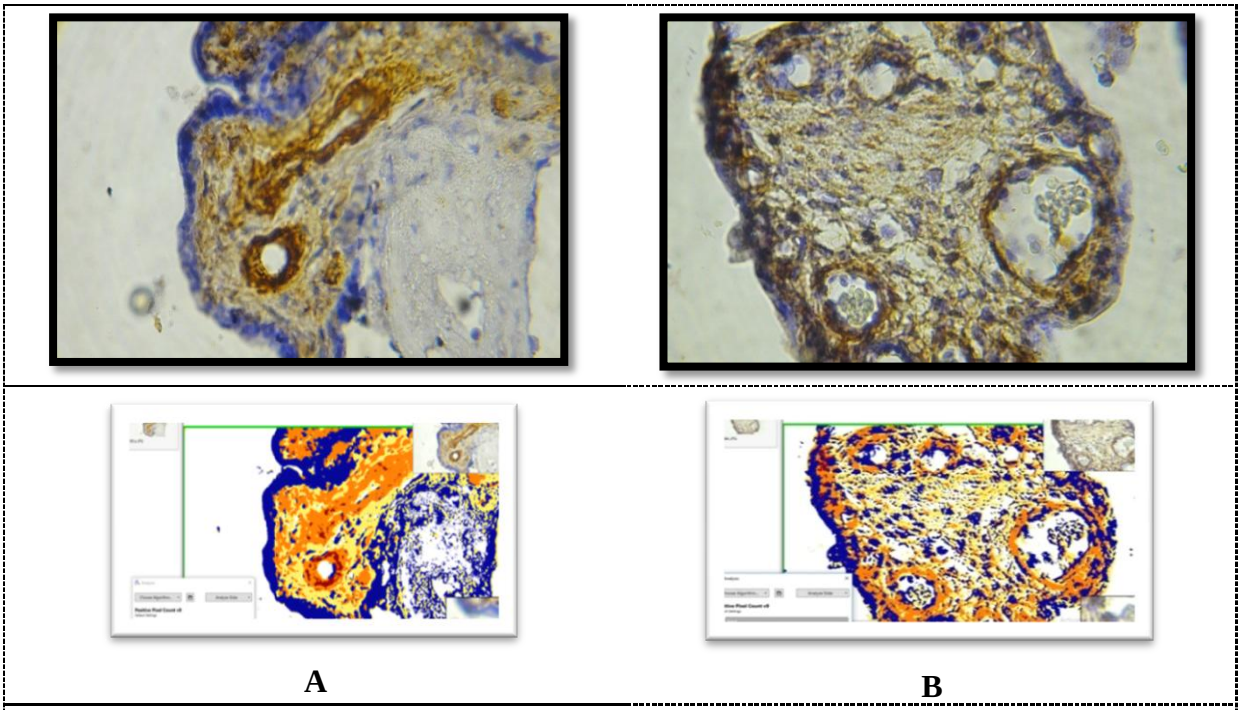

Figure 8. Immunohistochemical expression of α-SMA in primary arteries (A) and secondary arteries in term placenta(B), IHC, assessed by Aperio Scope image X10.

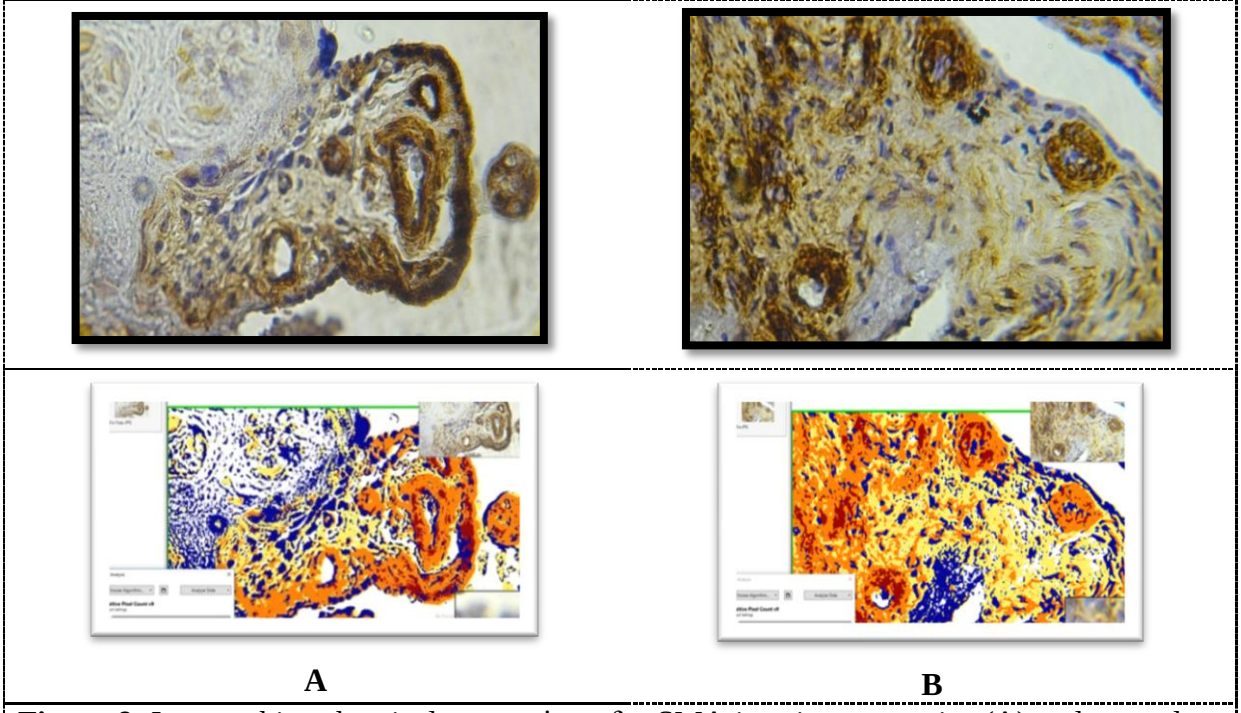

Figure 9. Immunohistochemical expression of α-SMA in primary arteries (A) and secondary arteries in postdate placenta(B), IHC, assessed by Aperio Scope image X10.

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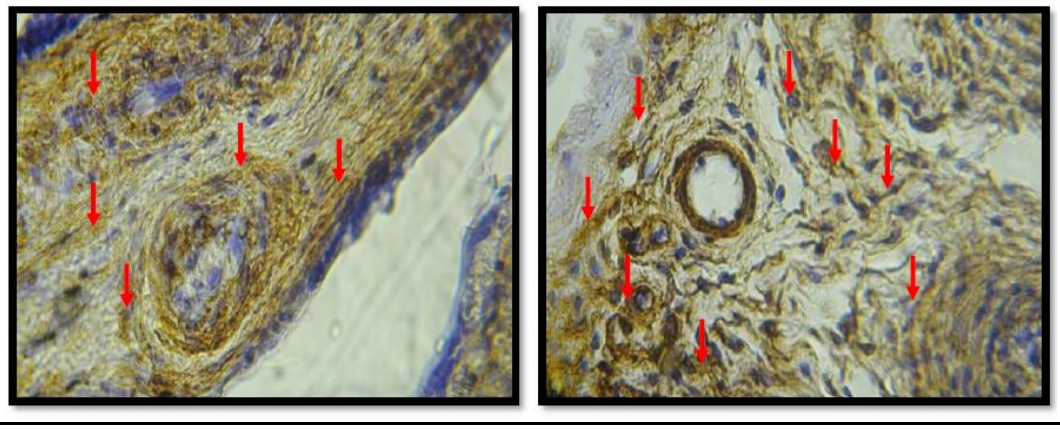

A **B**

Figure 10. Immunohistochemical expression of α-SMA in myofibroblast in term (A) and postdate placenta(B) , IHCfor α-SMA X10**.**

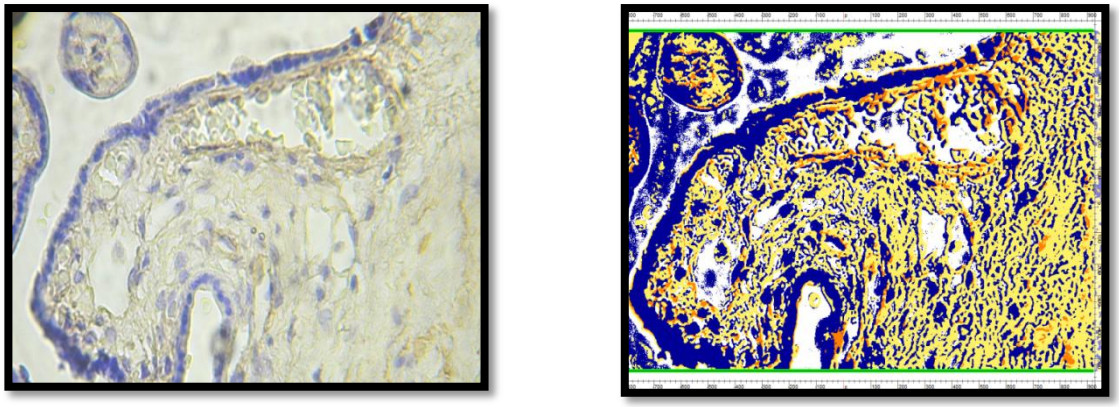

Figure 11. Negative control for α-SMA in postdate placenta,IHCfor α-SMA X10.

4. Discussion

Examination of casting models of placenta showed sub- cotyledon stem vessels that branched from chorionic blood vessels within the chorionic plate, gives to a subsequent generations of intracotyledon vessels, including primary arteries , secondary arteries found within intermediate mesenchymal villi, which gives to tertiary arteries. Tertiary arteries; considered the functionalcapillary unit that found within terminal villi and projected toward the intervillous space. This study provide evaluation of intracotyledon vascular pattern in postdate gestation, by corporation of vascular casting measurements and immunohistochemical findings of α-SMA.In the present study the cotyledons vessel generations were increased in postdate placentas compared to term, this attributed to the oxidative stress in postdate placenta, as [15] mentioned the importance of oxidative stress in sculpting the villous tree vasculature, maintaining persistent villous growth, and expansion of the villous surface area up to and beyond term. [16]also mentioned the disproportionate growth of the capillaries, and initiating of new terminal villiformation, as overproduction of ROS causes altered placental remodeling and growth ,and the programming of the fetoplacental unit [17,18].The increase in terminal capillary length that demonstrated in postdate placenta compared to term , could be due to increase in demands, as there is deposition of fibrin in some areas of placenta in postdate group, that could render some cotyledons inactive , and a compensatory increase in length of terminal capillaries could overcome these inactive cotyledons. [19] mentioned that the increase in capillary length from the beginning of the second trimester onwards, byincrease in endothelial cells

count towards term. Also [20] mentioned a raise in the count, and dimensions of the capillaries within placental villi with aging,this could explain both the increase in number of vessel generations, and mean length of terminal arteries through nonbranching angiogenesis in postdate group. The mean diameter of terminal arteries are decreased in postdate group, this may be due to oxidative stress effect on endothelial cells, as vascular endothelial cells are affected by oxidative stress, which switch on the apoptotic sequence [15], as placental tissues by themselves contain limited activity and amount of antioxidant enzymes , trophoblastic cells in particular are prone to oxygen-mediated damage [21]. Oxidative damage affect placental growth , maintenance and function.(22)Placental Antioxidant defenses activity of placenta is important for proper function, defect in this defenses activity may lead to intrauterine hypoxia that affect proper fetal growth [23]. In addition the significant increase in the relative thickness of tunica media in sub-cotyledon major arteries and primary arteries could interfere with cotyledon blood supply result inmalperfussionthat could add to the placental oxidative stress,[24] mentioned the increase in contractile proteins in postdate placenta represent an additional change that affects placental perfusion ,as the human placenta contain different isoforms of actin, β actin, α -SMA and γ SMA, α- SMA is localizes in endovascular tissues, and is a biomarker of myofibroblasts.In this study α- SMA expression levels were significantly high in postdate placentas, confirming their role in regulating the permeability and the tone of intracotyledonvascular structures. Myofibroblast showed significant increase in count in postdate placenta , and they stained positive for α -SMA, that could act as local regulator of terminal capillary lumen diameter that lack tunica media, to generate contractile forces through contraction of α- SMA a type of cytoskeletal protein of myofibroblast.Alpha SMA is largely localized in tunica media of intracotyledon arterial vascular branches of villi, and in myofi¬broblastsin villous stroma. Indicating a possible role in modulation of fetal blood circulation, and thus affecting the exchange rate in postdate placenta. This could add to fibrosis of the placenta in postdate period which mentioned by [25], who mentioned in postdate placenta due to oxidative stress actin filament structure may be changed , and the cells that marked by α -SMAantibodies may undergo fibrous tissue formation, leading to dense fibrin deposition , reduced vascular supply for cotyledons rendering some cotyledon out of function of blood exchange between fetal and maternal circulations [26], this Phenotypic switching between fibroblasts and myofibroblast can occur under conditions of oxidative tissue damage [27, 28], and hypoxia [29]. These findings provide a view for the altered vascularization of intracotyledon vascular tree of postdate placenta which contribute tochange in fetal blood supply, and to overcome oxidative stress and hypoxic conditions through changes in their count or local tone by activation of α SMAvascular structure to provide local control of vascular tone within the cotyledon, and affecting feto-maternal perfusion.

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