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VEGF Gene Expression and Angiogenesis in the Chorioallantoic Membrane: the Role of Cloprostenol

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BACKGROUND: Cloprostenol, as a common clinically used $PGF_2\alpha$ analogue, is widely employed in veterinary practice to induce parturition. It is main action, as a potent luteolytic, carried by vasoconstriction of the blood vessels that supply the corpus luteum. While vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. **Objective:** This study aimed to investigate the precise effects of $PGF_2\alpha$ analogue (cloprostenol) on the VEGF gene expression. **Methods:** Chorioallantoic membranes (CAM) of local chicken fertilized eggs were used at 6 and 9 days of incubation. The control group, without treatment, and other groups treated with (22.5µg/egg) of cloprostenol sodium (Galapan)® and eggs were re-incubated. CAM blood vessels were observed and documented and samples of them were isolated at 1, 2, 4, 6, 12, and 24 hours after incubation. mRNA was isolated and converted to cDNA, and RT-PCR was done to determine the gene expression of VEGF. **Results:** It shows that the peak of gene expression of VEGF gene was at 24 hours of day 9 of incubation. Furthermore, the best antiangiogenic effects were at the same time of 6 hours of day 6 of incubation. **Conclusion:** It could be concluded that $PGF_2\alpha$ analogue (cloprostenol) has vasoactive properties depend particularly on the VEGF, this action is in a time-dependent manner and may be based on the hypoxia-induced pathways that elicited by vasoconstrictive action of cloprostenol.

Keywords: Angiogenesis, CAM, Cloprostenol, VEGF gene expression.

Introduction

The chorioallantoic membrane (CAM) of a chicken egg is considered a unique experimental model that is applied to trails of blood vessels [1]. Numerous advantages, including simplicity of manipulation, adequacy of naked-eye observations, and inexpensiveness, put this model at the top of experimental approaches were utilized during investigations of blood vessel biology [2]. On the other hand, cloprostenol is a synthetic analogue and structurally related to $PGF_2\alpha$ [3]. It is widely used in veterinary clinics to induce luteolysis (particularly for synchronization of estrus cycle in farms). It is also indicated for induction of labor, for termination of unwanted pregnancies, mummified fetuses, pyometra, or luteal cysts [3].

However, the formation of blood from new blood vessels from pre-existing one (angiogenesis) is a critical physiological process for growth and development, also it›s vital for wound healing and granulation tissue formation [4, 5], but unfortunately, tumors utilize this process, angiogenesis, to «transform» from benign to a malignant state. Thus, angiogenesis inhibitors are widely applied for the conflict of cancers [6]. Though, angiogenesis as a process seems to be under control of a wide variety of biological factors, such as prostaglandins, integrins, and many growth factors, among

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these factors, vascular endothelial growth factor (VEGF) plays a key role in angiogenesis. VEGF also considers signaling protein that is involved in both angiogenesis and vasculogenesis (formation of new blood vessels in the embryo by de novo) [7]. In addition, VEGF is as a soluble glycoprotein exhibit it is effects through binding to its' own receptors, particularly two tyrosine kinase receptors (VEGFR-1 and VEGFR-2), binding of VEGF to these receptors triggers a cascade of signaling events that result in phosphorylation of phospholipase C, leading ultimately to elevated levels of intracellular calcium and inositol 1,4,5 triphosphate (IP3) [8, 9]. So the aim of this study was to investigate the particular properties of $PGF_2\alpha$ analogue (cloprostenol) on the gene expression of VEGF gene.

Material and Methods

CAM manipulation

Local chicken eggs were used in all experiments of this study. Egg weight 50±2 g, and incubated in an ordinary incubator at a temperature of 37 °C and humidity of 46%. Fertilized eggs at age of 6th and 9th day of incubation for groups 2 and 3 respectively, were used. The air space was determined and the hard shell was removed gently leaving a specific window (about 5 cm²) in the shell. A sample without treatment respected control groups was removed and kept in neutral buffer formalin (NBF) at 4 °C for further work.

The treated groups (2 and 3) manipulate by adding (22.5µg/egg) of cloprostenol sodium (Galapan)®, window of the shell was closed with melted parafilm and the melted eggs were re-incubated for 1, 2, 6, 12 and 24 hours for group 2, and 1, 2, 6, and 12 hours for group 3. After that it makes sure that the embryo was alive and immediately the CAM was cut and kept in NBF solution at 4°C for further experiments. In addition, it should be mentioned that in all three groups, and before removing of CAM, photography was done to investigate «gross changes in CAM blood vessels particularly the diameter of intended blood vessels, and remarking the formation of newly blood vessels, angiogenesis.

Molecular study

a. *mRNA isolation:* mRNA isolated from CAM Samples by using the Hybrid-R™ kit (GeneAll, Korea). Briefly, 100 mg of CAM sample was homogenized in 1ml of RiboEx™ and then incubated at room temp. (about 25 °C) for 5 min., the solution was centrifuged (12000 gx, for 10 min, at 4°C) and the supernatant was get, 0.2 ml of chloroform was added to the 1 ml of supernatant and the procedure was completed according to the guide of manufacturer instruction, in which ultimately the pure mRNA were isolated.

b. *cDNA Synthesis:* After isolation of mRNA, cDNA was made by using WizScript™ cDNA synthesis kit (wizbiosolutions, Korea). Mastermix of synthesis reaction was prepared according to the instruction guide as follows (Table 1), after that reverse transcription reaction was performed (Table 2), and finally cDNA samples get and Kept at -80 °C until executing RT-PCR.

c. *RT-PCR:* Consequently, real-time PCR was carried out by employment of wizPure™ (wizbiosalations, Korea) and according to the manufacturer guide (Table 3), two kinds of primers were utilized, GAPDH primers that consider an internal control (housekeeping gene) and VEGF primers. Ultimately RT-PCR reactions (Tables 4 and 5) were done by employment of applied biosystem™ 7500 fast real-time PCR system.

TABLE 1. Mastermix components used for cDNA synthesis.

Component	Volume (µl)
10X Reaction Buffer	2
20X dNTP mix	1
Random hexamer	2
WizScript™ RTase	1
RNase Inhibitor	0.5
RNase free Water	3.5
RNA Sample	10

TABLE 2. Reverse transcription reaction protocol used for cDNA synthesis.

cDNA Synthesis Reaction Conditions				
	Step 1	**Step 2**	**Step 3**	**Step 4**
Temp (°C)	25	37	85	4
Time	10 min.	120 min.	5 min.	-

TABLE 3. Primer sequences used for VEGF expression.

Primer Name	**Primer Sequence**
VEGF	F-5'AAAGCGAGGAAAGGGGAAGG'3
	R-5'TCTCCTCTCTGAGCAAGGCT'3
GAPDH	F-5'GCAGATGCAGGTGCTGAGTA3'
	R-5'GACACCCATCACAAACATGG3'

TABLE 4. Mastermix components used for Real-Time PCR.

MasterMix component	Volume
2X SYBR-Green MasterMix	10 µl
Forward Primer (10µM)	1 µl
Reverse Primer (10µM)	1 µl
cDNA Template	2 µl
RNase-Free Distilled Water	6 µl
Total	20 µl

TABLE 5. Real-Time PCR Reactions.

PCR Step	**Temp (°C)**	**Time**	**Cycles**
Initial denaturation	95	10 min.	1
Denature	95	15 sec.	40
Anneal	60	60 sec.	
Melting curve	60-95	2-5s/step	1

d. Statistical analysis: The results of RT-PCR were expressed as fold changes (Livak and Schmittgen equation) for determination of gene expression depending on the outcome of RT-PCR (C_t, ΔC_t, and $\Delta\Delta C_t$ values) [10].

Results and Discussion

Figures 1 and 2, and table 5 revealed that the greater gene expression of VEGF gene was in group 2 at the 6 hours of incubation, and this may be due to that this time is adequate for continuous gene activation by cloprostenol, so these effects seem to be as an accumulative response of the gene. As well as, it can be noted that the effect was increased with time (for 1, 2, 6, and 12 hours), these results agree with many authors about the progressive effect of cloprostenol on the VEGF gene expression [11-13].

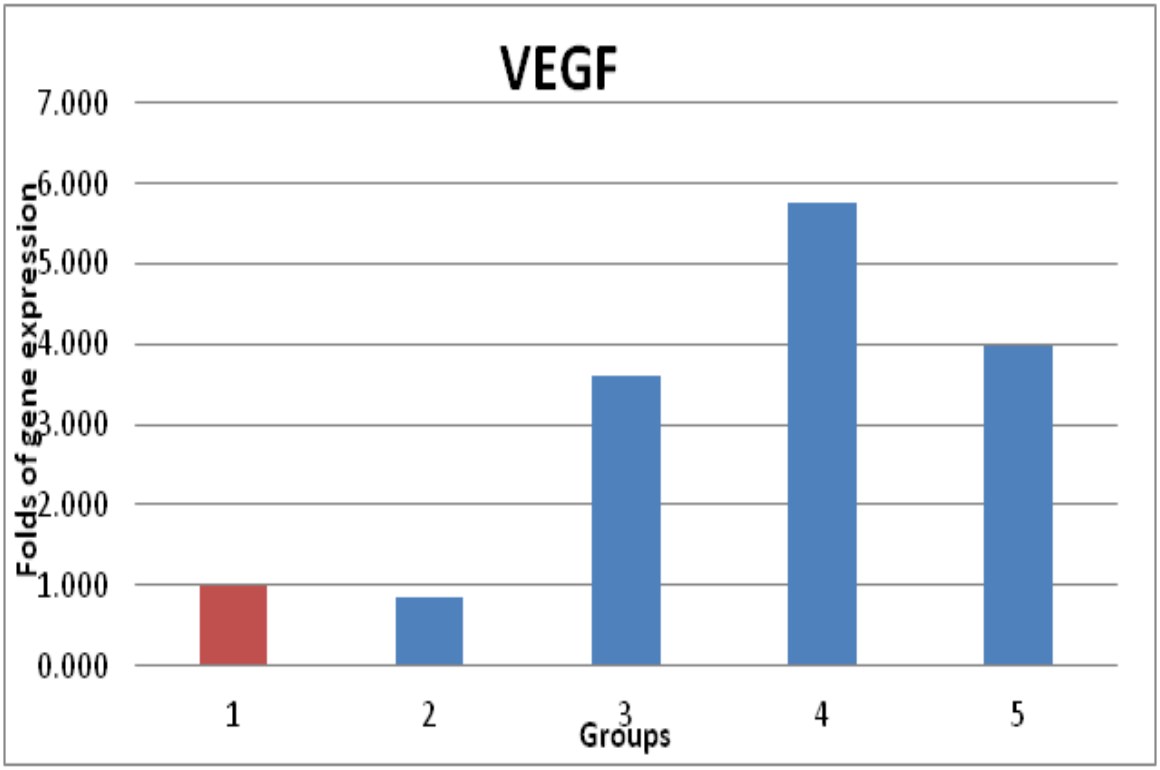


Fig. 1. Gene expression of group 2.

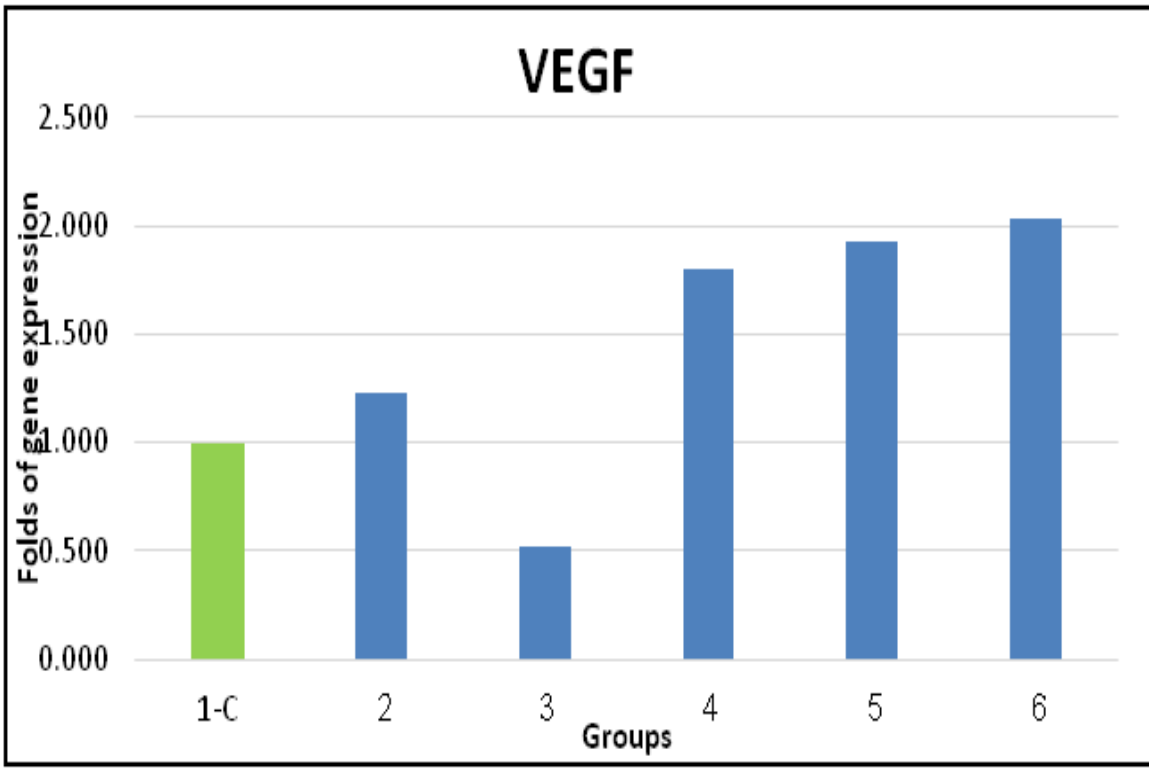


Fig. 2. Gene expression of group 3.

TABLE 6. Gene expression analysis of VEGF gene.

Incubation days		VEGF-Ct	GAPDH-Ct	ΔCT	ΔΔCT	Fold change
6th day	1-Control	29.697	29.024	0.673	0.000	1.000
	2	30.017	29.638	0.378	-0.295	1.227
	3	29.536	27.909	1.627	0.954	0.516
	4	29.919	30.094	-0.175	-0.848	1.800
	5	29.920	30.196	-0.276	-0.949	1.931
	6	29.580	29.929	-0.349	-1.022	2.031
9th day	7-Control	29.884	29.897	-0.013	0.000	1.000
	8	30.761	30.538	0.222	0.235	0.850
	9	29.581	31.441	-1.860	-1.847	3.598
	10	29.305	31.843	-2.538	-2.526	5.758
	11	29.767	31.769	-2.002	-1.989	3.969

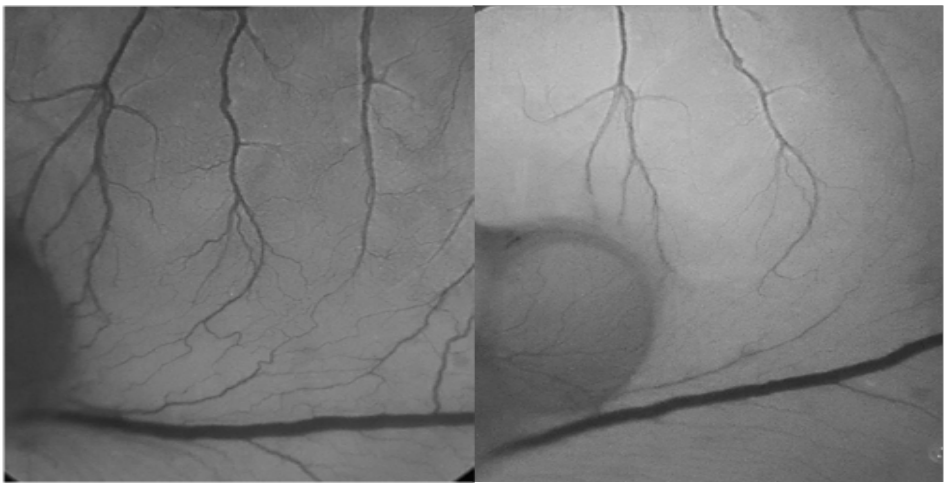
Moreover, the vasoconstrictive action of cloprostenol in CAM blood vessels results in low oxygen supply, thus, hypoxia here consider the leading cause of upregulation of VEGF gene expression, and this effect called hypoxia-induced effect [14]. What is more, the authors demonstrated that the last effect was regulated by a complex interacting factors which called the hypoxia-inducible factors (HIFs) [15]. Indeed, these factors consider a specific sensor that support candidate calls during episodes of hypoxia by various compensatory mechanisms. At the heart of these mechanisms is the angiogenesis, in addition to the metabolic shift, immunosuppression, and extracellular matrix remodeling [16, 17].

HIFs activate more than 200 genes that code for proteins and factors which combat hypoxia [15, 18]. However, HIFs not only promote angiogenesis and cell proliferation, but also enhances the synthesis of glucose transporter 1 (GLUT-1) that increase glucose uptake, thus boosting anaerobic glycolysis [19].. Likewise, HIFs suppress mitochondrial metabolism by downregulation of genes and proteins of oxidative phosphorylation pathway, this will lead to a diminishing in the reactive oxygen species (ROS), so minimizing the cytotoxic oxidative stress [20, 21].

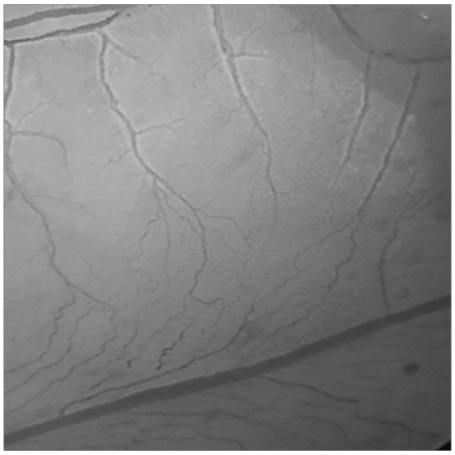
As noted in table 6, and Fig. 3, 4, and 5, the effects of cloprostenol on VEGF gene expression were fluctuating, and this result may be due to that the VEGF receptors play a key role in this response, and it is well known that their receptors have wide variations depending on the age of development (note the difference in response between 6th and 9th days of incubation). The maturity of blood vessels may be contributed to these variations, also the process of angiogenesis requires more quantity and quality of blood vessels in the embryo [22, 23]. Our results reveal that the effects of $PGF_2\alpha$ analogue on the blood vessels may be influenced in a time-dependent manner, so many interacting factors play a key role in VEGF gene expression [24].

In addition, cloprostenol induced vasoconstriction that causes hypoxia, as mentioned above, will induces angiogenesis-promoting cytokines (such as TGF-B and VEGF), and these factors encourage endothelial cells to initiate angiogenesis [25, 26].

Interestingly, during rapid cell proliferation in cancer, the new fastly formed blood vessels seem to be immature and have a poor permeability, thus decreasing anticancer drug targeting [27, 28]. So antiangiogenic drugs (such as anti-VEGF antibodies were applied to reduce the fast

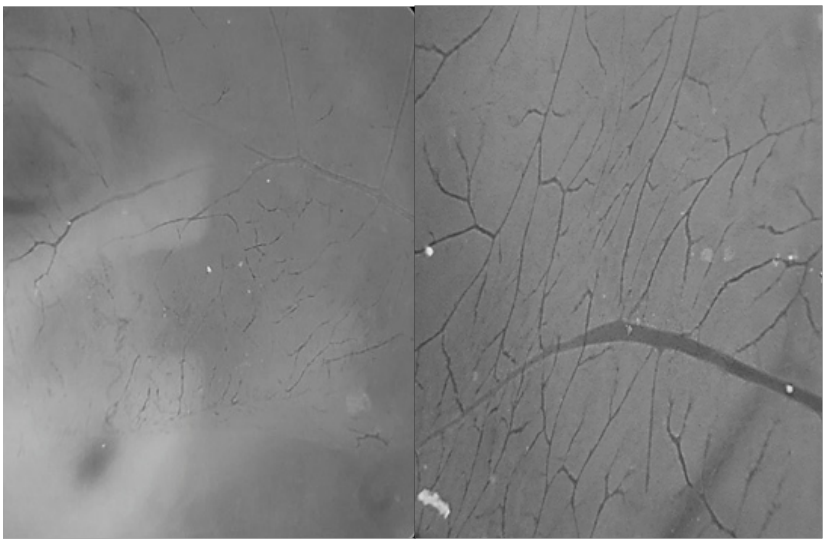


Before **60 minutes**



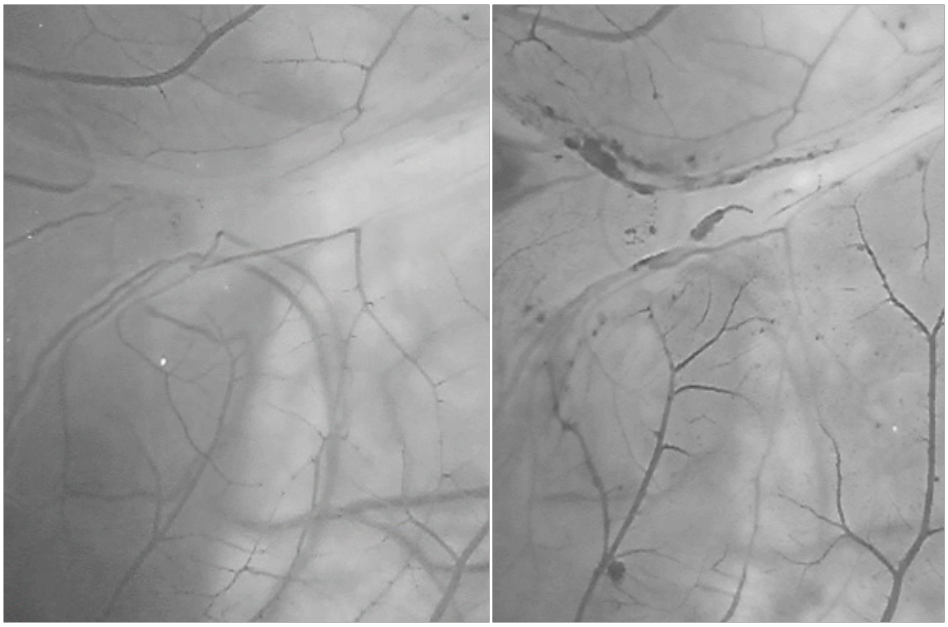
120 minutes

Fig. 3. Group 2. The image showed after 60 minutes the occurrence of antiangiogenesis, while the image after 120 minutes led to the occurrence of angiogenesis, this result was identical with the result of the gene expression.



Before **9 hours**

Fig. 4. Group 3. The image showed a very high angiogenesis occurrence, this result was identical with the result of the gene expression.



Before **60 minutes**

Fig. 5. Group 3: The image showed after 60 minutes the occurrence of angiogenesis, but the effect was less than the image after nine hours, this result was identical with the result of the gene epression.

formation of « immature» blood vessels and then enhance access target anticancer drugs to tumor, therefore antiangiogenic agents widely used (in combination with anticancers) particularly in the treatment of solid tumor (such as breast and ovary cancers) [29-31].

Conclusion

From this work it could be concluded that the cloprostenol as a $PGF_2\alpha$ analogue has had a vasoactive properties and depend particularly on the VEGF, this action seems to be a time-dependent and may be based on the hypoxia-induced pathways that elicited by vasoconstrictive action of cloprostenol.

Conflicts of Interest

The authors declare that they have no competing interests.

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التعبير الجيني لـ VEGF وتكوين الاوعية الدموية في الغشاء المشيمي اللقانقي : دور الكلوبروستينول

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الخلفية العلمية: يعد الكلوبروستينول (وهو مماثل للبروستاكلاندين PGF2α) واسع الاستخدام في الطب البيطري لاحداث الاجهاض. ويكون فعله الرئيسي بتحليل الجسم الاصفر من خلال تضييق الأوعية الدموية المزودة للجسم الأصفر. بينما يلعب عامل النمو البطاني الوعائي VEGF الدور الرئيسي في تكوين الأوعية الدموية. **الهدف:** كان الهدف من الدراسة الحالية هو التعرف على التأثيرات الدقيقة للكلوبوستينول على التعبير الجيني لجين VEGF. **الطريقة:** استخدم الغشاء اللقانقي المشيمي CAM لبيض دجاج محلي مخصب بعمر ٦ و ٩ أيام بعد الحضن، وتركت مجموعة السيطرة بدون معاملة، بينما عوملت باقي المجاميع بجرعة ٢٢,٥ مايكروغرام من الكلوبوستينول صوديوم (كالابان)® واعيد حضن البيض ، وتمت مراقبة الاوعية الدموية لغشاء CAM واخذ عينات منها في الاوقات ١، ٢، ٤، ٦، ١٢، ٢٤ ساعة بعد الحضن، تم عزل mRNA وتحويله الى cDNA ، وأجري تفاعل البلمرة المتسلسل اللحظي RT-PCR لايجاد التعبير الجيني. النتائج: أظهرت النتائج أن أعلى تعبير جيني لجين VEGF كان بعد ٢٤ ساعة من الحضن بعمر ٩ أيام ، بينما كان أفضل تأثير مضاد لتكون الأوعية الدموية بعمر ٦ أيام. **الاستنتاجات:** يمكن الاستنتاج من هذه الدراسة بأن مماثل PGF2α الكلوبروستينول يملك تأثير الفعالية الوعائية ويعتمد بشكل أساسي على عامل النمو الوعائي البطاني VEGF وهذا التأثير يكون بطريقة معتمدة على الوقت ، وربما يحدث ذلك من خلال مسارات استحداث نقص الاوكسجين والتي تحفز عن طريق فعالية الكلوبروستينول المضيقة للاوعية الدموية.

الكلمات المفتاحية: تكون الاوعية الدموية، الغشاء اللقانقي المشيمي، الكلوبروستينول، التعبير الجيني لعامل النمو الوعائي البطاني.