The effects of *Moringa oleifera* on angiogenic factors in the cervix of pregnant mice

by

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ABSTRACT

One in every nine births in the United States are preterm births (CDC, 2012), and of those, the leading cause is microbial infection. Current pharmacological treatments for these microbial infections have been shown to have serious side effects and low potencies. Alternatively, *Moringa oleifera* (MO) has been shown to exhibit potent anti-inflammatory and -bacterial activities with minimal side effects. Here, we test the effectiveness of MO as an alternative treatment to pharmacological-based inflammation therapies and its potential use in preterm births. Specifically, the effectiveness of (MO) whole leaf extract is tested against inflammation induced by lipopolysaccharide (LPS). Following specific treatments [2 hour pretreatment with MO(4.8 µg/50 µL methanol, *per os*, followed by 2 hour treatment with LPS (100 µg/50 µL 1X PBS, i.p.)] of pregnant mice (day 15), cervical tissues were harvested and mRNA expression of angiogenic factors [vascular endothelial growth factor, VEGF); VEGF receptor 1, Flt-1; and VEGF receptor 2, KDR] were analyzed using real-time PCR (qRT-PCR). The results show that MO whole methanolic extract largely down regulates expression of angiogenic factors VEGF and KDR mRNAs, while it had no significant effect on the expression of Flt-1. Based on this data, we can conclude that MO may be used to prevent inflammation induced preterm labor by decreasing expression of certain angiogenic factors, specifically VEGF and KDR.

KEYWORDS

Moringa oleifera; inflammation; preterm birth; vascular endothelial growth factor (VEGF), Flt-1, KDR.

DEDICATION

To my loving, supportive parents:

I would not be the person I am today without

your guidance and support.

I love you both so much.

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INTRODUCTION

One of the most common causes of perinatal morbidity is premature birth (Read et al., 2007). Along with other adverse events, such as preeclampsia, preterm labor in the USA results in over 400,000 premature births annually and additionally affects 11% of all live births and is the leading cause of neonatal mortality (Boggess, 2005). Of these preterm births, 40%-60% are associated either directly or indirectly with infection (Newton, 2005). Intrauterine infections associated with these pregnancies ascend from the vagina, and may breach the cervical barrier to infect the fetal membranes or the amniotic fluid (Keelan et al., 2016). When this infection occurs, an inflammatory response takes place typically as chorioamniotitis, which is a possible cause of preterm labor and birth (Keelan et al., 2016). In addition to inflammation resulting from bacterial infection, preterm birth can also be caused by environmental factors, genetics, or other unknown causes (Newton, 2005). Of these potential causes, premature cervical ripening precedes the onset of preterm labor in the majority of cases (Mahendroo, 2012). Studies in our lab have also implicated angiogenic factors in the ripening process of the cervix, in particular cytokines, such as vascular endothelial growth factor (VEGF) and its two receptors, KDR and Flt-1, respectively (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2015).

VEGF is an angiogenic factor that induces mitosis and growth of vascular endothelial cells of the arteries, veins and lymphatics (Ferrara, 2001). It is one of the main growth factors involved in the development of new blood vessels from pre-existing ones, a process termed angiogenesis (Mowa et al., 2004). VEGF has two receptors that bind it with high affinity. The first is Flt-1, an *fms*-like tyrosine kinase, and the second is KDR,

which has a kinase domain region (Ferrara, 2001). VEGF and its receptors work together along with other angiogenic factors to induce vascularization and growth in various organs throughout the body (Ferrara, 2001).

Throughout the course of pregnancy the cervix must remain closed and firm in order to prevent the immature passage of the infant through the birth canal resulting in preterm birth (Mahendroo, 2012). As pregnancy approaches full term, the cervix must become flexible and open to allow for delivery. This process is called cervical remodeling (Mahendroo, 2012). Cervical remodeling is one continuous process that can be divided into four different phases, namely softening, ripening, dilation/labor, and post partum repair (Read et al., 2007). The first stage of softening occurs by day 12 in mice and during the first trimester in humans, and is characterized by a slow incremental decrease in tissue compliance that will continue until birth (Timmons et al., 2010). With regards to preterm labor and birth, the most important stage to evaluate is the second stage of ripening. During this stage, there is an observed increase in hyaluronan content, changes in collagen matrix and distribution of inflammatory cells, as well as increased tissue growth and hydration (Read et al., 2007). This ripening process is brief and occurs just before birth, i.e., hours in mice and weeks or days in humans (Timmons et al., 2010). Following ripening, the cervix can now dilate sufficiently to allow passage of the fetus for birth (Timmons et al., 2010). The final phase is postpartum repair, which involves recovery of tissue integrity and competency after birth (Mahendroo, 2012; Stanley et al., 2015).

Based on previous studies from our lab and others, there is a high likelihood that changes occurring in the cervix during remodeling, and ripening in particular, may

involve angiogenesis (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2015, Stanley et al., 2015). During the first trimester, the concentration of VEGF in maternal serum increases (Wheeler, 1999) corresponding to the first stage of cervical remodeling or softening (Timmons et al., 2016). During the second stage of cervical remodeling an inflammation-like process occurs which can in part be attributed to angiogenesis (Mowa et al., 2004). During cervical ripening, the density of immune cells, including neutrophils, eosinophils and macrophages, change (Mahendroo, 2012). Collectively these changes, along with recruitment and mobilization of other leukocytes into the connective tissue of the cervix, are preceded by structural changes to the vasculature (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2015, Stanley et al., 2015). Alterations to the vasculature during this stage resemble VEGF-induced vascular changes, including angiogenesis, vascular leakage and vasodilation (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2015). Subsequently, pathological increase in levels of VEGF and other angiogenic factors in the cervix could lead to premature ripening and preterm birth.

Moringa Oleifera (MO), commonly called the "drumstick" or "horseradish tree," is a perennial angiosperm plant that is distributed throughout the tropics and subtropics around the world (Mbikay, 2012). This member of the monogeneric family is indigenous to India, Pakistan, Bangladesh and Afghanistan (Tan et al., 2015). Historically, MO leaves have been used for traditional medicine, as well as a nutritious food source in Asia and Africa (Waterman et al., 2014). Almost all plant parts of MO contains bioactive compounds, including the leaves, roots, bark, gum, flowers, fruits, seeds and seed oil (Tan et al., 2015). Various bioactive compounds in MO, such as flavonols and phenolic

acids, have been linked to anti-inflammatory, antibacterial and antioxidant properties (Waterman et al., 2014). In addition to these phytochemicals, there are also secondary metabolites, including vitamins, carotenoids, minerals, sterols, amino acids, alkaloids and glycosides, which contribute to a wide range of pharmacological activities from wound healing to protection against oxidative stress in an *in vivo* animal model of Alzheimer's disease (Fard et al., 2015).

In the present study, we evaluate the effects of MO on angiogenesis by examining the mRNA expression of vascular endothelial growth factor (VEGF) and its two receptors in a day 15 pregnant mouse model of preterm labor. We hypothesize that MO will diminish the gene expression of VEGF and its receptors, Flt-1 and KDR, thereby attenuating premature cervical ripening and subsequently preterm birth.

MATERIALS AND METHODS

Animals

Types of animals used and conditions

In the present study, non-pregnant and pregnant ICR [CD-1(R)] were obtained from Harlan and used for experimentation. Pregnant mice used were day 15 of gestation, while non-pregnant were ovariectomized (ovaries were removed) at least seven days prior to the beginning of the study. Housing conditions for animals were maintained at constant room temperature with a 12:12 hour light and dark cycle. The animals were allowed free access to food and water at all times. Treatments were given, as described below, and mice were then killed using a lethal dose of pentobarbital sodium intraperitoneally (IP) (0.2mg/g) (Euthasol®, Virbac Animal Health, Fort Worth, Texas).

This was immediately followed by a trans-cardio perfusion using 0.9% normal saline solution before harvesting of the cervical tissues. After tissue harvest, the cervical tissues were then stored at -80°C until processing. Tissues were processed and analyzed using quantitative real-time polymerase chain reaction (qRT-PCR).

Ovariectomy (Ovary Removal)

Prior to use in the study, non-pregnant mice, approximately 6 months of age, were ovariectomized. Animals were anesthetized using a mixture of xylazine and ketamine (43–129 mg ketamine and 8.6–26 mg xylazine/g), which were administered intramuscularly (IM). To avoid post-surgical infection, the animals were injected with Baytril® (Bayer, Leverkusen, Germany) (5mg/kg) antibiotic. A seven-day post-surgery waiting period was allowed in order to eliminate possible residual ovarian sex steroid hormones. At the time of tissue harvest, animals with signs of residual sex steroid hormones and large uterine size, were eliminated from the study.

Solvent Optimization Study

Before the use of MO extract in the present study, a series of experiments were conducted to determine the whole leaf solvent extracts with the most potent anti-inflammatory activities. The extracts included 100% ethanol, 80% hydro-ethanolic solution, methanol, butanol, and water. Non-pregnant ovariectomized mice were used in these optimization studies. Using a pre-optimized dosage of 4.8 µg/µL of respective MO extracts, 50 µL (240 mg/mouse) of MO was administered IP (n=4) to non-pregnant mice (*per os* for pregnant mice). The mice were then injected with lipopolysaccharide (LPS) (25 µg/50 µL) IP two hours after treatment with MO extract, and one hour prior to

cervical tissue harvest. The mice were euthanized using a lethal dose of pentobarbital sodium (0.2mg/g) (Euthasol®, Virbac Animal Health, Fort Worth, Texas), followed by trans-cardio perfusion with 0.9% normal saline solution. The tissues were harvested and snap frozen and stored at -80°C until further experimentation and analysis. It was determined that methanol was the optimal solvent, based on the mRNA expression of key pro-inflammatory factors involved in preterm labor, and was, therefore, used in all subsequent studies.

Pregnant Mice Study

At day 15 of gestation, pregnant mice were treated with optimized MO extract (Methanol) prior to LPS administration, overall, as described earlier under non-pregnant. This was done in order to observe the effects of MO on expression of key angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptors one (Flt-1) and two (KDR). The mice were treated as described in the following groups: a) *Negative control:* 1X PBS (50 µL/mouse of vehicle *per os*); b) *Positive control*: LPS (100 µg/50 µL, ip), or c) pretreated with *MO methanolic extract* (240 mg/mouse, *per os*) followed by LPS (100 µg/50 µL, ip) two hours later (n=4). Mice were euthanized one hour after LPS injection using a lethal dose of pentobarbital sodium (Euthasol®, Virbac Animal Health, Fort Worth, Texas) followed by a trans-cardio perfusion with 0.9% normal saline solution before harvesting of the cervical tissues. After cervical tissue harvest, these tissues were stored at -80°C until processing and analysis using quantitative real time polymerase chain reaction (qRT-PCR).

Techniques Used

Gene Expression

Following the animal treatments and tissue harvest, the total RNA from individual mice cervices were isolated using an RNeasy Mini Kit (Oiagen, Valencia, CA). The RNA concentrations were then obtained using a Nanodrop Spectrophotometer (NanoDrop 3000, Thermo Scientific) and recorded. The isolated RNA was then used to analyze changes in the gene expression of angiogenic factors VEGF, KDR, and Flt-1.

Reverse Transcription: Using the previously isolated RNA, complementary DNA was synthesized and amplified through reverse transcription using reagents obtained from Applied Biosystems and an Eppendorf Master Cycler (Hamburg, Germany), as described here: 1 μg of total RNA in 9.5 μL of RNase-free water was incubated for 5 minutes in a water bath set at 65°C and the total RNA was then cooled at RT for 10 minute before adding the following mixture to each sample of total RNA: 9.5 μL of reverse transcription master mix consisting of 2 μL RT buffer, 2 μL $MgCl_2$, 2 μL dNTP, 0.5 μL RNase inhibitor, 2 μL RNase-free water, and 1 μL random hexamers. Finally, 1μL MuLV reverse transcriptase was added to each total RNA sample, excluding a non-template tube, which was used as a control for DNA contamination. The final mixtures were then placed in the Eppendorf thermocycler and allowed to run for 10 min at 25°C, for 2 hours at 42°C, for 5 min at 95°C and stored at 4°C.

Quantitative RT-PCR: In order to quantify the relative expression of the angiogenic factors VEGF, Flt-1, and KDR qRT-PCR was used. The mRNA levels of these genes in the cervix of pregnant mice at day 15 of pregnancy were analyzed. Using the previously synthesized cDNA, pre-designed TaqMan® Gene Expression Assays

(Applied Biosystems, Foster, CA) were utilized to amplify the genes of interest using an Applied Biosystems 7500 Real-time PCR system (Foster, CA). A qRT-PCR master mix was prepared by carefully mixing the following reactions: 12.5 µL 2X Taqman® Universal PCR Master Mix, 1.25 µL gene-specific TaqMan® Gene Expression Assay, and 6.25 µL qRT-PCR-grade RNase-free water per sample. This mixture was then pipetted into a 96-well plate in a quantity of 20 µL along with 5 µL of cDNA per well. The Real-time PCR system was pre-set to run for 2 min at 50 °C, 10 min at 95°C, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. In order to obtain threshold cycle values, the GeneAmp 7300HT sequence detection system software was utilized. These values were used to calculate the relative expression levels of target angiogenic factors as compared to the mean of the endogenous control gene, Gus b.

Statistical Analysis

Student's *t* test and ANOVA (single factor) were used to analyze the data obtained in these studies. *p*-values equal to or less than 0.05 were considered to be statistically significant.

RESULTS

MO extract down regulates expression of VEGF mRNA in mouse cervix

Following LPS, mRNA expression of VEGF in mouse cervix increased approximately ten fold as compared to the negative control (Figure 1). When pre-treated with MO followed by LPS, there was almost a nine fold decrease in VEGF expression, comparable to levels of the negative control (Figure 1).

MO extract down regulates expression of KDR mRNA in mouse cervix

Treatment of mice with LPS alone increased KDR mRNA expression by approximately 0.75 fold compared to the negative control (Figure 2), based on qRT-PCR analysis. When mice were pre-treated with MO followed by LPS, MO blocked LPS and levels of KDR mRNA were down-regulated to those comparable to the negative control by 0.75 fold (Figure 2).

MO extract has no significant effect on expression of FLT-1 mRNA expression in mouse cervix

Using qRT-PCR data, it was shown that LPS increased Flt-1 mRNA expression approximately three fold when compared to the negative control (Figure 3). Pre-treatment with MO failed to block the effects of the subsequent LPS injection on Flt-1 mRNA expression (Figure 3).

DISCUSSION

Pregnancy is a delicate process and because infection-induced inflammation is one of the leading causes of preterm labor, finding a safe and effective natural-based treatment is essential. The key findings of the present study indicate that methanolic MO whole leaf extract tends to down regulate various angiogenic factors in mice cervices, including VEGF and KDR. While this MO extract down-regulates these angiogenic factors, it has no apparent effect on FLT-1.

In the present study, a decrease in VEGF mRNA was observed when MO was used as a pre-treatment before inducing inflammation with LPS. Our observations are consistent with an earlier study that reported that MO inhibits inflammatory and angiogenic gene expression, including VEGF (Gupta et al., 2013). The aim of the study

by Gupta et al., (2013) was to develop alternative treatments for streptozotocin-induced diabetic rats. They also discovered that MO was able to prevent dilation of retinal vessels in retinae that were treated with the plant extract (Gupta et al., 2013). It is important to note that this study differs from the present study in that the retinae were treated with MO and not the whole animal. Because VEGF is one of the body's most powerful vasodilators, the prevention of vessel dilation following MO treatment was interpreted as an indication of decreased VEGF expression. Equally, during cervical remodeling, our lab has speculated that VEGF could have the same effects, i.e., promoting vasodilation,. (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2015). Such a pattern in VEGF effect is not restricted to the cervix and retina but appears to be more widespread (Mowa et al., 2004). For instance, withdrawal of VEGF has been shown to induce regression of vasculature in a variety of physiological and pathological circumstances (Ferrara, 2001). In contrast, the accompanying changes that occur resemble those in other organs induced by VEGF, including angiogenesis, vascular leakage and vasodilation (Mowa et al., 2004). For now, the underlying mechanism by which MO decreases the mRNA expression of this dynamic molecule (VEGF) is unknown. We speculate that the inhibition of this angiogenic factor, could potentially be utilized to modulate premature cervical remodeling and subsequently preterm birth.

Similarly, MO was also able to down-regulate VEGF's second receptor, KDR. However, it had no apparent effect on the first receptor, Flt-1. Previous studies have shown that the expression of Flt-1 is overall specific to endothelium, while KDR is largely localized in capillaries and endothelial of blood vessels (Waltenberger et al., 1994). Our previous studies and by others, have shown a much broader cell type

distribution beyond the vascular cells, including fibroblasts and epithelial cells (Mowa et al., 2004, 2008, Donnelley et al., 2013, Ohashi et al., 2014, Stanley et al., 2015). With such a broad cell type distribution, both receptors likely play an important role during pregnancy. Indeed, earlier knockout studies have shown that embryonic mice lacking VEGF were unable to develop normal vasculature and resulted in abortions (Wheeler, 1999). Similarly, mice that were deficient in only KDR did not develop differentiated endothelial cells or organized blood vessels. While mice deficient in only Flt-1 were found to possess differentiated endothelial cells, they developed large disorganized vessels (Zachary et al., 2001). Collectively, these earlier findings suggest that both receptors must be present to develop normal vasculature and differentiated endothelial cells. The present findings that MO fails to diminish Flt-1 mRNA expression in mice cervices was unexpected, considering that Flt-1 has a much higher affinity for VEGF than for KDR (Mowa et al., 2004). For now, the underlying reasons for the differential effects of MO on the two VEGF receptors, as well as their implications, are unclear.

As previously stated, in the United States and around the world a significant percentage of live births occur before full term and of those, infection is the leading cause. Unfortunately, there is currently no treatment that is both effective and safe for mother and fetus for these infections. In the present study we have demonstrated that MO can down-regulate both VEGF and its second receptor KDR. Based on other research, the down-regulation of these two molecules should be accompanied by the inhibition of premature cervical remodeling. In our most recent studies, MO was also found to decrease the expression of pro-inflammatory factors, such as TNFa, IL-6, as well as COXII proteins, which have all been linked to preterm birth. Since MO is an edible

vegetable and has been used safely for thousands of years, including in pregnant women, it could potentially become a safe and effective treatment for infection or inflammation-induced preterm birth.

Although the findings of the present study show great promise for MO as a potential treatment option for preterm labor, it had limitations. One limitation is that the effect of MO used as a treatment after inflammation is already present was not analyzed. This data is based on the pretreatment of MO before inducing inflammation with LPS. The mRNA analyzed using qRT-PCR was based on a sample size of only four animals per treatment. While this was sufficient to establish preliminary results, in order to draw any major conclusions, the sample size must be greater, with more animals per treatment group. In addition to a small sample size, the other limitation of the study was that only mRNA expression was analyzed. Future studies should also analyze the expression and activities of proteins using various techniques, such as western blot and confocal microscopy. Lastly, in order to develop an effective treatment, we need to not only know that MO is able to modulate the angiogenic factors, but how it (MO) is able to do this. Also the possible interaction between the angiogenic factors and pro-inflammatory markers, such as Cox-II, TNFa and IL-6 would need to be investigated. Expansion of these studies in a pregnant animals would also be useful to determine the outcome of pregnancies in the treatment groups.

Although there are yet a number of limitations to overcome and improvements to be made going forward before using MO as a preventative of preterm birth, the findings of this study show much promise. This is because VEGF and KDR have been implicated to play a role in cervical remodeling. By modulating these two angiogenic, as well as pro-

inflammatory factors, it is very possible that MO may be used to prevent inflammation induced preterm birth in the future.

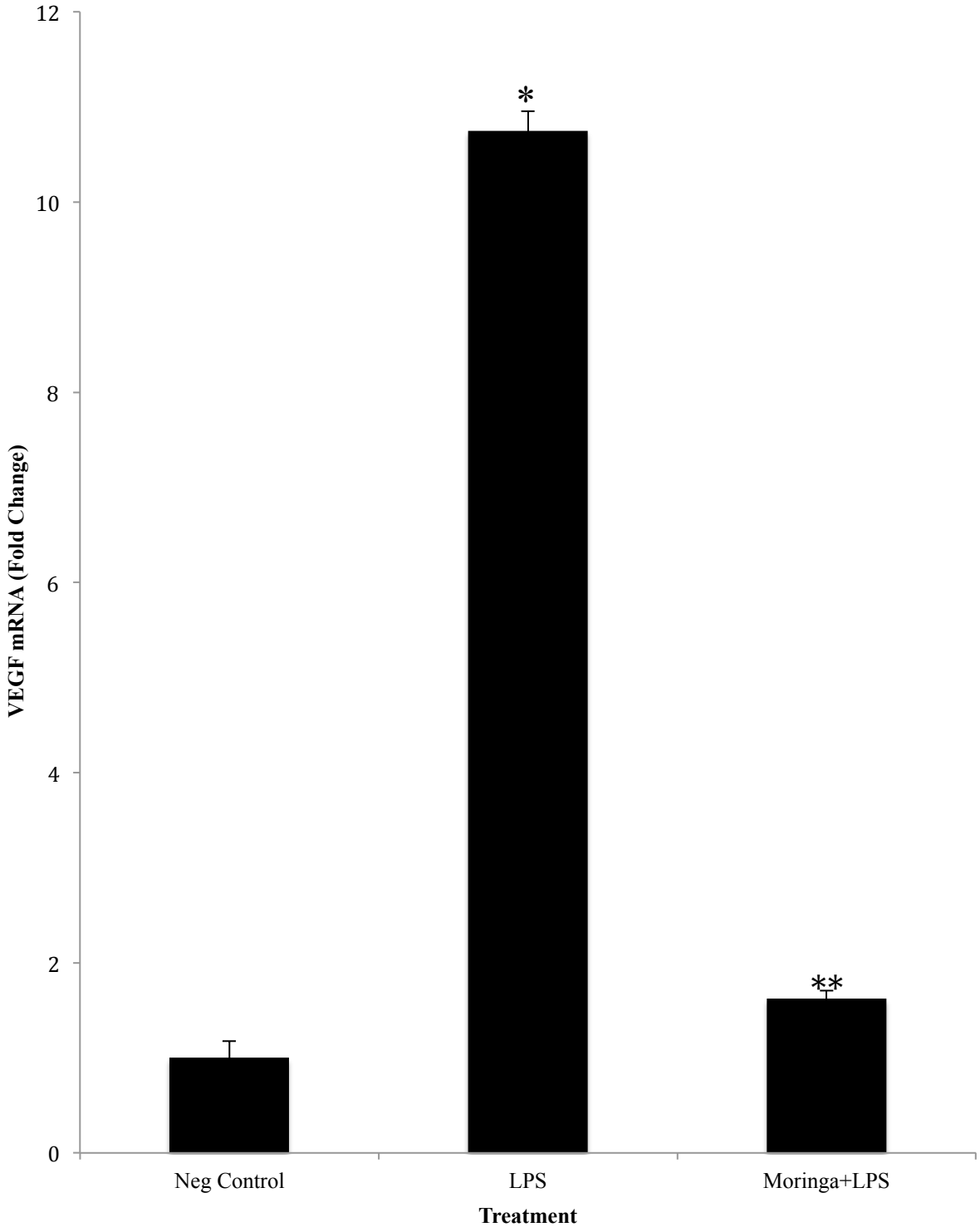

Figure 1. Effects of *Moringa olefeira* (MO) on the mRNA expression of VEGF in day 15 pregnant mouse cervix, as shown by qRT-PCR. LPS up regulates expression of VEGF mRNA in the cervix of pregnant mouse and pre-treatment with MO attenuates the LPS-induced expression of VEGF mRNA. n=4, * $p < 0.05$ LPS vs. Control; ** $p < 0.05$ MO+ LPS vs. LPS.

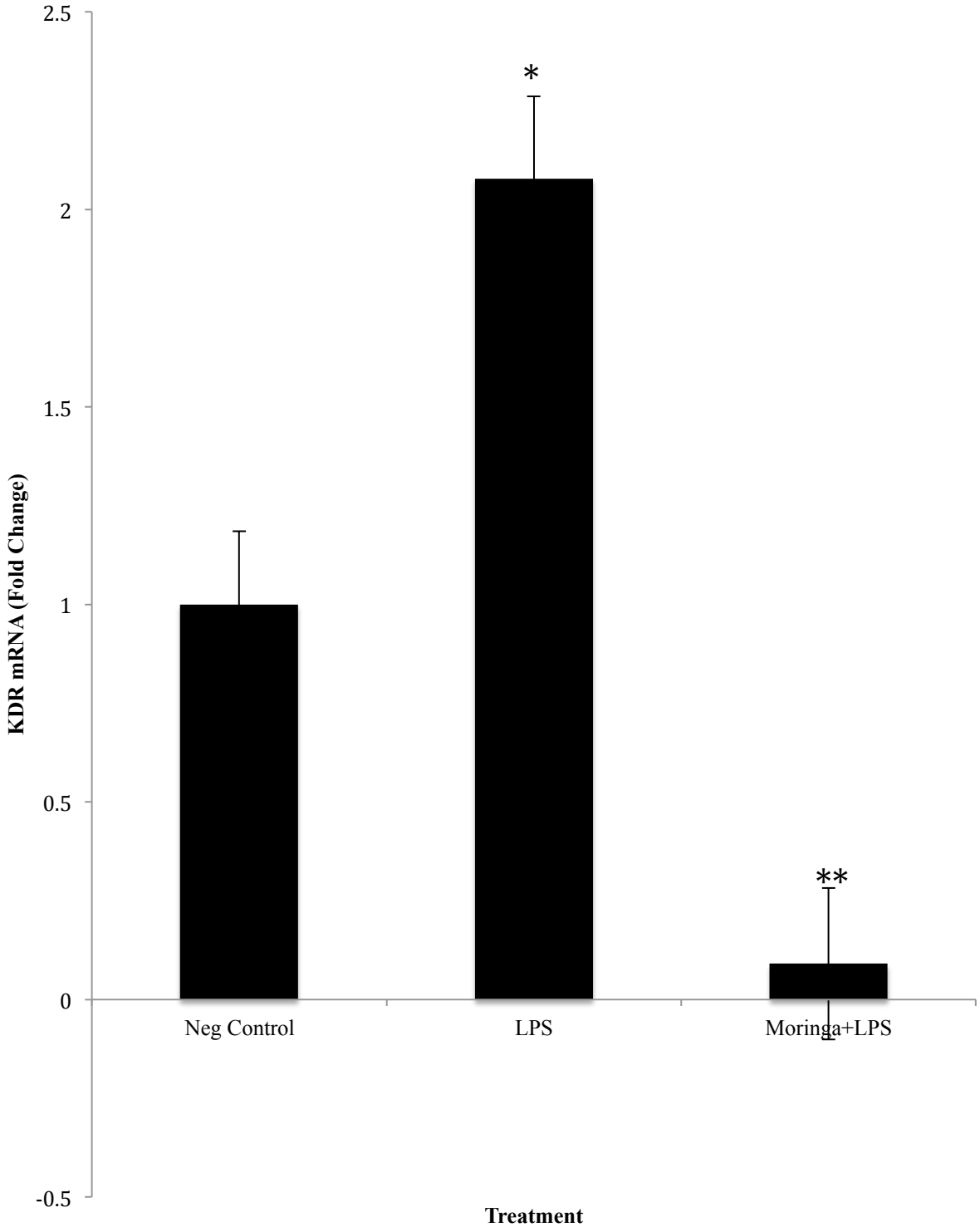

Figure 2. Effects of *Moringa olefeira* (MO) on the mRNA expression of KDR in day 15 pregnant mouse cervix as shown by qRT-PCR. LPS up regulates expression of KDR mRNA in the cervix of pregnant mouse and pre-treatment with MO attenuates the LPS-induced expression of KDR mRNA. n=4, * $p < 0.05$ LPS vs. Control; ** $p < 0.05$ MO+ LPS vs. LPS, MO + LPS vs. Control.

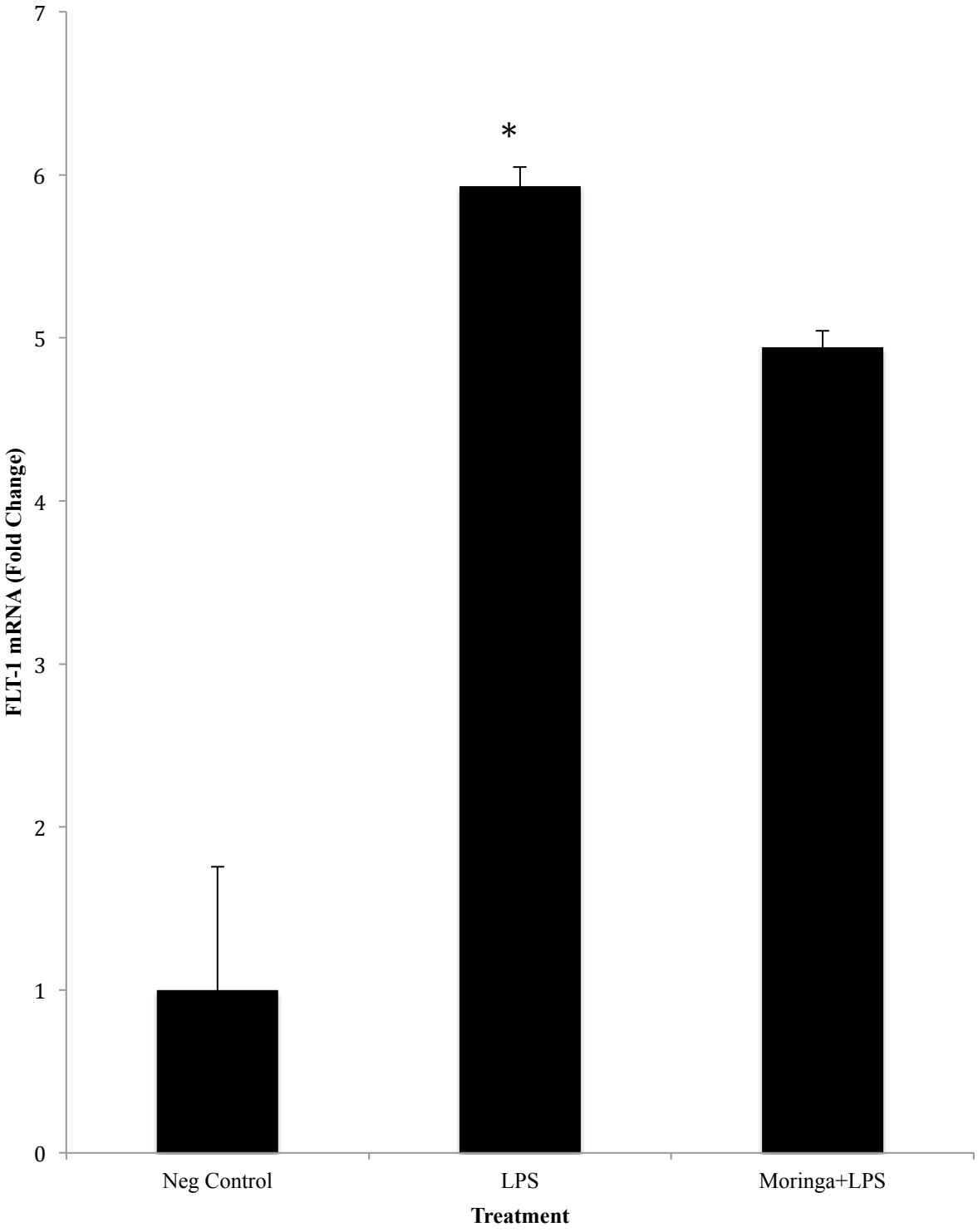

Figure 3. Effects of *Moringa olefeira* (MO) on the mRNA expression on FLT-1 in day 15 pregnant mouse cervix as shown by qRT-PCR. LPS up regulates expression of Flt-1 mRNA in the cervix of pregnant mouse and pre-treatment with MO did not attenuate the LPS-induced expression of Flt-1 mRNA. n=4, * $p < 0.05$ LPS vs. Control.

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