

# The Role of Nitric Oxide in the Effects of Ovarian Steroids in the Duodenum <sup>[1]</sup>

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## Summary

The purpose of this study is to determine the distribution and expression of nNOS, eNOS and iNOS and to evaluate the role of ovarian steroids in NOS expressions in the duodenum tissues of rats. Rats in the control group (Ov group) were injected intramuscularly with sesame oil for 10 days, whereas rats in the first, second, and third experimental groups received intramuscular administration of progesterone (P group), 17 $\beta$ -estradiol (E2group) and 17beta-estradiol and progesterone (E2 + P group), respectively. According to the results of an immunohistochemical evaluation, a severe nNOS expression was observed in both the crypts and nerve fibers of the duodenum in the Ov, P and E2 groups but decreased in the E2 + P group. It was determined that iNOS expression increased in the surface epithelial cells and crypts of P group while it only decreased in crypts of E2 group. There was severe eNOS expression in crypts in all treatment groups, while it only increased in the surface epithelial cells in the E2 group. The results of the current experiment suggest that the effect of ovarian steroids on duodenum absorption and microvascular protection can be mediated by crypt and epithelial nNOS, iNOS and eNOS expressions in rats. Besides the estradiol and progesterone administration together inhibit nNOS activity in the duodenum.

**Keywords:** Ovarian steroids, nNOS, iNOS, eNOS, Duodenum, Immunohistochemistry

## Duodenumda Ovaryum Steroidlerinin Etkisinde Nitrik Oksidin Rolü

### Özet

Bu çalışmanın amacı rat duodenum dokularında NOS ekspresyonları üzerinde ovaryum steroidlerinin rolünü değerlendirmek ve nNOS, eNOS ve iNOS ekspresyon ve dağılımlarını belirlemektir. Kontrol grubu ratlara (Ov grup) intramusküler olarak 10 gün susam yağı enjekte edildi. Bunun yanında birinci, ikinci ve üçüncü deney gruplarına sırasıyla progesteron (P grup), 17 $\beta$ -estradiol (E2group) ve 17beta-estradiol ve progesteron (E2 + P group) uygulandı. İmmunohistokimyasal değerlendirme sonuçlarına göre, Ov, P ve E2 gruplarında duodenumun hem kriptlerinde ve hem de sinir fibrillerinde şiddetli nNOS ekspresyonu gözlemlendi, E2 + P grubunda ise reaksiyon şiddeti azaldı. P grubunda kriptlerde ve yüzey epitel hücrelerinde iNOS ekspresyonu artarken, E2 grubunda sadece kriptlerde reaksiyonun azaldığı belirlendi. Tüm gruplarda kriptlerde şiddetli eNOS ekspresyonu bulunurken, E2 grubunda sadece yüzey epitel hücrelerinde reaksiyon şiddetinin arttığı belirlendi. Yapılan çalışmanın sonucu, ovaryum steroidlerinin etkisinde duodenumda absorpsiyon ve mikrovasküler korumanın, sıçanlarda kript ve epitellerde nNOS, iNOS ve eNOS ekspresyonu aracılığıyla olabildiğini, bunun yanında duodenumda östrojen ve progesteronun birlikte uygulanmasının nNOS aktivitesini inhibe ettiğini göstermektedir.

**Anahtar sözcükler:** Ovaryum steroidleri, nNOS, iNOS, eNOS, Duodenum, İmmunohistokimya

## INTRODUCTION

Nitric oxide (NO) is an intercellular and endocellular signal molecule, and has an important role in the physiological

process of the intestines. NO can regulate muscular contraction and blood circulation in the intestine <sup>[1-2]</sup>.



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Endogenous nitric oxide is derived from L-Arginine by the enzyme called nitric oxide synthase (NOS)<sup>[3]</sup>. NOS is widely distributed in the intestine, and has several isoforms, such as constitutive nitric oxide synthase (nNOS and eNOS) and inducible NOS (iNOS)<sup>[4,5]</sup>. iNOS is the NOS isoform most broadly implicated in the processes of inflammation and carcinogenesis in the GI system<sup>[6]</sup>. Neuronal NOS (nNOS) constitutes the predominant source of NO in neurons; once nitric oxide diffuses into smooth muscle cells, it inhibits the contractibility of smooth muscles by activating guanylate cyclase<sup>[7]</sup>. Endothelial NOS (eNOS) is the predominant source in the endothelium<sup>[8,9]</sup>. eNOS expression most prominent in endothelial cells lining vascular channels<sup>[10,11]</sup> throughout the gut, liver, and pancreas<sup>[11]</sup>. eNOS also regulates a number of cellular and physiologic functions within the GI and hepatic systems including vasodilation and protection of the mucosal barrier function.

The precise mechanism by which sex steroids modulate changes in gastrointestinal motility is also not known. NO is known to mediate relaxation of gastrointestinal smooth muscle<sup>[12-14]</sup> and to be involved in the pregnancy associated decrease of gastrointestinal motility<sup>[15]</sup>. Besides, there is increased NOS expression in the gastrointestinal tissues during pregnancy<sup>[16]</sup>, and NOS expression also increased by estradiol in various tissues<sup>[16,17]</sup>, including the gastrointestinal tract and neuronal tissues<sup>[16]</sup>.

Therefore, the aim of this study was to determine the effects of ovarian steroids on the distribution of NOS protein expression in rat duodenum.

## MATERIAL and METHODS

### Animals and Experimental Design

Duodenum tissues were obtained from animals used in TÜBİTAK project (Project No. VHAG-2097). In this project, female Sprague-Dawley rats were bought from the Gulhane Military Medical Academy Research Center Department of Laboratory Animals. The experimental protocols were approved by the Animal Care and Use Committee at Afyon Kocatepe University (15.09.2004; B.030.2.AKÜ.0.8Z.00.00/115) and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

The rats in all groups were fed ad libitum with a commercial rat diet. A total of 40 rats were anesthetized by intraperitoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg) and ovariectomized bilaterally. Two weeks after the operation, the rats were randomly assigned to 4 groups of 10 rats each. Rats in Ov group received daily 0.2 ml intramuscular administrations of sesame oil. Rats in P and E2 groups were administered with progesterone (2 mg/rat/day; i.m.) and 17 $\beta$ -estradiol (10 mg/rat/day; i.m.) respectively. Rats in E2 + P group were injected with

both progesterone and 17  $\beta$  -estradiol at the same dosages. The hormone treatments were continued for 10 days. After the treatments, rats were killed by cervical dislocation.

### Determination of Distribution of nNOS, iNOS and eNOS in Duodenal Tissues

The duodenal samples were removed immediately after sacrifice and fixed in 10% buffered formal-saline solution and processed routinely for embedding in paraffin. Immunohistochemistry for nNOS, iNOS and eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using Universal LSAB Kits (Zymed Histostain Plus Broad Spectrum, South San Francisco, CA, US) according to manufacturer's protocol. Briefly, paraffin sections (5 $\mu$ ) were treated with nNOS (1:1000 dilution), iNOS (1:1000 dilution) and eNOS (1: 1000 dilution) primary antibody for 30 min at 37°C. Paraffin sections were washed three times in phosphate buffered saline (PBS), and incubated with the biotinylated secondary antibody for 30 min at room temperature (RT). The samples were then washed three times in PBS and incubated in Streptavidin-HRP for 30 min at RT. Following rinsing in PBS for 3X5 min, the sections were rinsed in distilled water, and incubated in DAB (Zymed, 00-2020) for 5 min to reveal peroxidase. Counterstaining was carried out with haematoxylin. Sections were cleared in xylene and mounted with Entellan. Negative controls were processed using antibody diluent reagent solution instead of the primary antibody. Relative immunoreaction of positive cells was evaluated by two independent observers in a blinded fashion and given a score as followed: 0- no reaction; 1- weak reaction; 2- moderate reaction 3- strong reaction, 4- very strong reaction<sup>[18]</sup>.

### Statistics

Statistical analysis of results was performed by a one-way analysis of variance (ANOVA) followed by Duncan tests. Values are presented as means  $\pm$  SE. Group differences were declared significant at  $P < 0.05$ .

## RESULTS

NOS expressions in duodenal tissue sections are shown in Fig. 1, 2 and 3. nNOS, iNOS and eNOS immunoreactions were identified on the epithelial layers of the villi and crypts. The staining pattern was very similar along the length of the villi. Moreover, nNOS expression was also present in nerve fibers. Because nNOS and eNOS expression were faint in the Brunner glands we did not statistically evaluate this expression in the table. Besides we did not find any iNOS immunoreaction in the Brunner glands.

### Immunohistochemistry Scoring Results (Semiquantitative)

Semiquantitative observations of the nNOS, eNOS and

iNOS expressions in the duodenum are presented in *Table 1, 2 and 3*.

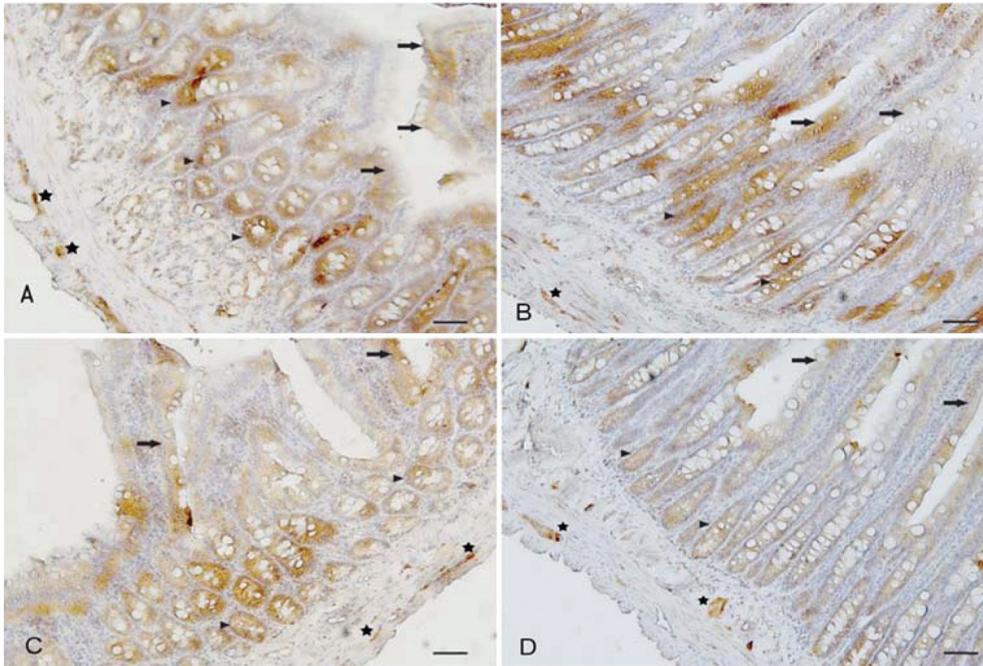
### Neuronal NOS Expression

nNOS expression on the surface epithelium of the villi was moderate in all treatment groups, although the intensity of immunoreaction on crypt epithelium was strong in the Ov, P and E2 groups and moderate in the E2 + P group. The intensity of immunostaining on the surface epithelium of the villi was weaker than that of the crypts (*Fig. 1*). There was no significant difference between nNOS expression in the surface epithelium of the villi among

the groups and in the crypts in the Ov, P and E2 groups, whereas the expression significantly decreased in the E2 + P groups ( $P < 0.001$ ). nNOS expression was strong in nerve fibers among the groups. But the expression ( $P < 0.05$ ) were significantly lower in E2 + P group compared with the Ov, P and E2 groups.

### Endothelial NOS Expression

eNOS expression on the surface epithelium was moderate in the Ov, P and E2 + P groups and strong in the E2 group. The intensity of immunoreaction was strong in the crypt region in all groups. The intensity of immuno-

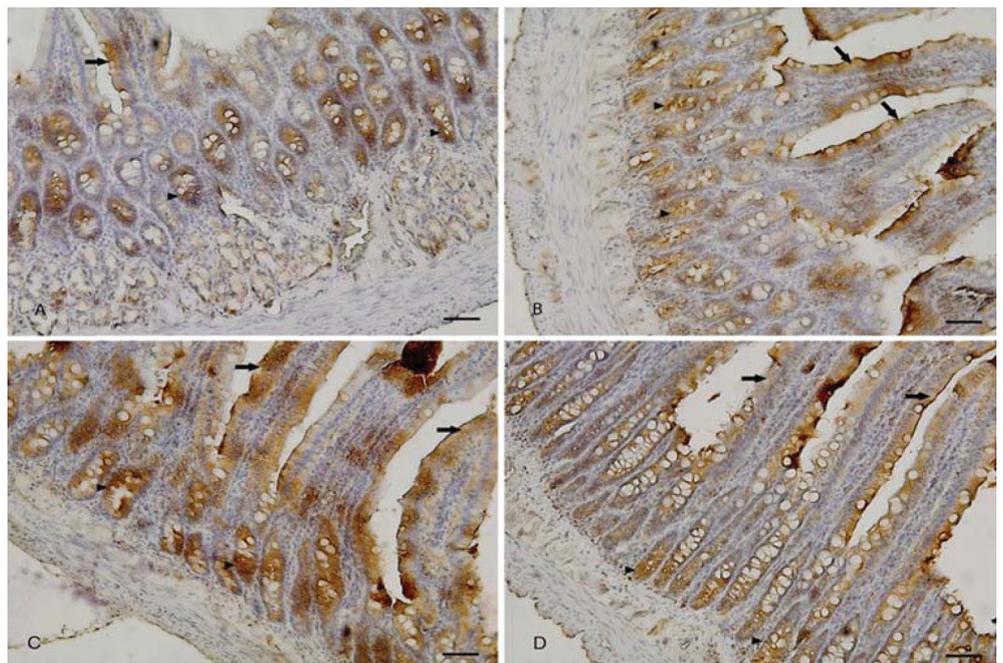


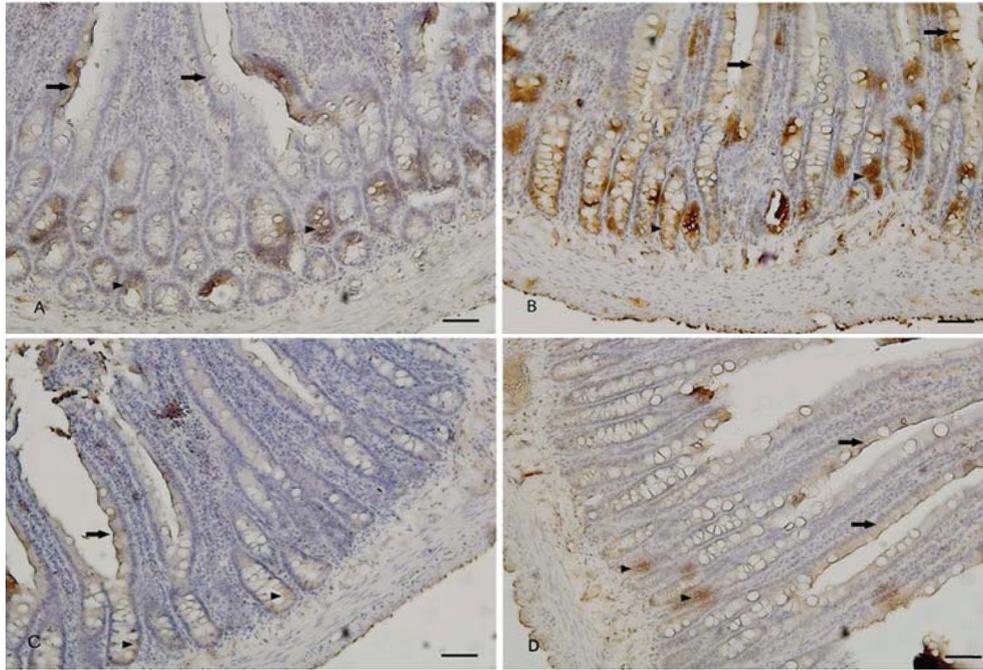
**Fig 1.** nNOS expression in surface epithelium of the villi (*arrows*) and crypts (*arrow head*) and nerve fibers (\*). A- Ov group, B- P group, C- E2 group, D- E2+P group, Bar 50  $\mu$ m

**Şekil 1.** Villus yüzey epiteli (*ok*), kriptler (*ok başı*) ve sinir fibrillerinde (\*) nNOS ekspresyonu. A- Ov grubu, B- P grubu, C- E2 grubu, D- E2+P grubu, Bar 50  $\mu$ m

**Fig 2.** eNOS expression in surface epithelium of the villi (*arrows*) and crypts (*arrow head*). A- Ov group, B- P group, C- E2 group, D- E2+P group, Bar 50  $\mu$ m

**Şekil 2.** Villus yüzey epiteli (*ok*) ve kriptlerde (*ok başı*) eNOS ekspresyonu. A- Ov grubu, B- P grubu, C- E2 grubu, D- E2+P grubu, Bar 50  $\mu$ m





**Fig 3.** iNOS expression in surface epithelium of the villi (arrows) and crypts (arrow head). A- Ov group, B- P group, C- E2 group, D- E2+P group, Bar 50  $\mu$ m

**Şekil 3.** Villus yüzey epiteli (ok) ve kriptlerde (ok başı) iNOS ekspresyonu. A- Ov grubu, B- P grubu, C- E2 grubu, D- E2+P grubu, Bar 50  $\mu$ m

**Table 1.** nNOS expression scores in duodenal surface epithelium and crypts and nerve fibers, in all groups

**Tablo 1.** Gruplarda duodenum yüzey epiteli, kriptler ve sinir fibrillerinde nNOS enzim skorları

Group	Ov	P	E <sub>2</sub>	E <sub>2</sub> +P	P
Surface epithelium	1.30±0.15	1.80±0.13	1.70±0.15	1.60±0.16	N.S
Crypt region	3.20±0.13a	3.30±0.15a	2.80±0.25a	2.10±0.28b	0.000***
Nerve fibers	3.00±0.21ab	3.50±0.17a	3.30±0.15a	2.70±0.15b	0.013*

Letters (a, b, c) in the same line indicate significant differences between different letters, \*  $P < 0.05$ , \*\*\*  $P < 0.001$

**Table 2.** eNOS expression scores in duodenal surface epithelium and crypts in all groups

**Tablo 2.** Gruplarda duodenum yüzey epiteli ve kriptlerde eNOS enzim skorları

Group	Ov	P	E <sub>2</sub>	E <sub>2</sub> +P	P
Surface epithelium	1.60±0.22b	1.70±0.21b	2.70±0.26a	1.70±0.21b	0.004**
Crypt region	3.10±0.10	3.10±0.18	3.10±0.28	2.90±0.10	NS

Letters (a, b, c) in the same line indicate significant differences between different letters, NS: not significant, \*\*  $P < 0.01$

**Table 3.** iNOS expression scores in duodenal surface epithelium and crypts in all groups

**Tablo 3.** Gruplarda duodenum yüzey epiteli ve kriptlerde iNOS enzim skorları

Group	Ov	P	E <sub>2</sub>	E <sub>2</sub> +P	P
Surface epithelium	0.50±0.17bc	1.10±0.10a	0.20±0.13c	0.80±0.13ab	0.000***
Crypt region	1.00±0.00b	3.00±0.21a	0.50±0.17c	0.80±0.13bc	0.000***

Letters (a, b, c) in the same line indicate significant differences between different letters, \*\*\*  $P < 0.001$

staining on the surface epithelium of the villi was weaker than that of the crypts (Fig. 2). The intensity of eNOS expression on the surface epithelium was higher in the E2 group than in the Ov, P and E2 + P groups ( $P < 0.01$ ) and no differences were observed in crypts among groups.

### Inducible NOS Expression

iNOS expression was weak on the surface epithelium in all groups. The intensity of immunoreaction in crypts was weak in the Ov, E2 and E2 + P groups, but strong in the P group (Fig. 3). The intensity of iNOS expression in the

surface epithelium did not change among the Ov and E2 and E2 + P groups. It was greater in the P group compared with the Ov and E2 groups ( $P < 0.001$ ). iNOS expression in the crypt region was significantly increased in the P group compared with the Ov, E2 and E2 + P groups ( $P < 0.001$ ).

## DISCUSSION

Recent studies showed that NO was a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves of the gut<sup>[15,19-21]</sup>. During nerve stimulation, NO generated by nNOS in nerve terminals regulates the release of vasoactive intestinal polypeptide (VIP), which diffuses to muscle cells to participate in muscle relaxation<sup>[22,23]</sup>. Our study showed that nNOS was localized in the nerve fibers, and this expression of nNOS in the nerve fibers correlated with other researches<sup>[10,24-27]</sup>.

Previous studies indicated that the release of NO by NANC nerves was an important factor controlling gut motility *in vivo*<sup>[8]</sup> and *in vitro*<sup>[2,3]</sup>. Some research indicated that the nNOS protein<sup>[19]</sup> and nitrergic activity<sup>[15]</sup> were increased in nerve fibers in the gastric fundus and colon but not in the ileum in late pregnancy. We observed strong nNOS protein expression in nerve fibers but it did not increase with estrogene or progesterone. Conversely, in our study, a combination of estrogene and progesterone decreased the nNOS reaction, this decrease may be associated with longer (10 day) progesterone treatment. We also observed strong nNOS expression in crypts. The expressions decreased in the E2 + P group. Furthermore, nNOS localized weakly in the Brunner's glands in all groups.

In the present study, eNOS localizations were identified on the epithelial layers of the villi and crypts in the duodenum. The crypts showed a stronger immunoreaction than that of the epithelial layer of the villi but there were no differences between groups. eNOS is the key NOS isoform responsible for NO-regulated vasodilation in the GI system and eNOS-derived NO is produced under basal conditions<sup>[28]</sup>. eNOS expression is found throughout the gut<sup>[11]</sup> and lamina propria of villi<sup>[10]</sup>. Previous research has shown that gastrointestinal smooth muscle cells are able to generate NO by a Ca<sup>2+</sup>-dependent constitutive NOS<sup>[29]</sup>. And myogenic NO is responsible for the regulation of smooth muscle contractility<sup>[30]</sup>. Some research claims that NOS isoforms are not expressed by intestinal smooth muscle cells<sup>[31]</sup>. Bani et al.<sup>[27]</sup> showed that eNOS and iNOS immunoreactions occur in the circular and longitudinal smooth muscle layers of the ileum in mice, and also found eNOS protein in intestinal smooth muscle cells<sup>[32]</sup>. In the current study, we did not find any eNOS immunoreaction in duodenum smooth muscle cells. Our results are not consistent with Vannucchi et al.<sup>[32]</sup> because the technique and antibodies they used differ from ours. Research indicated that inhibition of basal NO production enhanced epithelial mucosal permeability, thus implicating

constitutive NO production in the protection of the GI mucosal barrier function<sup>[33]</sup>. In this study there was also a strong expression in the surface epithelium in the E2 group. NO generated by those cells may play an important role in absorption and the protection of microvasculature. Estrogen may have an important role in the duodenal mucosal system and for epithelial function. Furthermore, we observed strong eNOS expression in the crypts and weak immunostaining in the Brunner glands in all groups. It may have a function in stimulating the high rate of macromolecular synthesis in this active secretory tissue.

The intestinal epithelial cells expressed iNOS in a large amount<sup>[34]</sup>. Previous research detected a strong iNOS reactivity in the basal and apical side of enterocytes in villi epithelium, in duodenal crypts, in connective tissue cells and in the endothelial cells of lamina propria vessels in human duodenum<sup>[35]</sup>. iNOS expression in intestinal cells indicates that the isoform of the enzyme is expressed in the small intestine without inflammation. In our study, similarly, an iNOS reaction was found in surface epithelium and duodenal crypts. And these immunoreactions were significantly greater in the surface epithelium and crypts in the P group and it decreased in the crypts in E2 group compared to the Ov group. Crypts represent regions of rapid mitosis and differentiation, providing a continual turnover of cells that differentiate into columnar and goblet cells as they migrate from the crypts towards the luminal end of the villi<sup>[36,37]</sup>. Previous studies also revealed the presence of iNOS immunoreactivity in myenteric neurons, in smooth muscle cells, and in endothelium of the rat duodenum with postembedding immunoelectronmicroscopy<sup>[26]</sup>. These findings are different from our result. This difference may be a function of the techniques used.

In conclusion, ovarian steroids may cause some alterations mediated by nNOS, iNOS and eNOS expression on the duodenal mucosal system, differentiation, mucosal barrier function, and on the absorption and protection of microvasculature.

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