Peripheral blood flow in neonatal rats

The d0 rats were intraperitoneally injected with saline, then with U46619 (0.0005, 0.05, or 5.0μg/g) 5min later. Peripheral blood flow in the tail was measured with a laser speckle perfusion imager (MoorFLPI, Moor Instruments, Axminster). During blood flow measurement, each d0 rat was fixed on a hot plate to keep its body temperature.

Microthrombosis in the pulmonary capillary arteries

Lung tissues from PM20 injected with U46619 (0.05μg/g) or arachidonic acid (AA) (Sigma-Aldrich) (100μg/g) were embedded in Paraffin. Paraffin-embedded blocks containing tissues were prepared as previously described[14]. These paraffin-embedded blocks were cut into 5-μm-thick sections and placed on glass slides. Slides were stained with hematoxylin and eosin. We counted the total capillary arteries and the arteries with microthrombosis.

Statistics

Data are presented as mean ± SEM of independent experiments. Statistical analysis was performed among multiple groups by one-way ANOVA followed by Neuman-Keuls multiple comparison test. A *P* value of less than 0.05 was considered significant.

Results

TP stimulation selectively caused vasoconstriction in fetal rat DA

First, we examined the *in vivo* effect of TP stimulation on the fetal rat DA using two types of TP stimulation: U46619, a PGH_2 analogue and I-BOP, a TXA_2 analogue. Both are commonly used as selective TP agonists[7, 15]. Consistent with the previous *in vivo* study by Loftin et al.[11], when U46619 was intraperitoneally injected into the fetal rats at e19 and e21, the DA was significantly constricted in a dose-dependent manner (**Figure 1A-G**). The constriction of the DA by U46619 was greater at e21 than at e19, even though the levels of circulating PGE_2 are supposed to be higher during late gestation (**Figure 1A-G**). Whereas very low-dose of U46619 such as 0.005μg/mg did not showed significant constriction of the DA at e19, effect of U46619 at e21 showed significant constriction even in low-dose. I-BOP also constricted the DA (**Figure 1H**). These results indicated that TP stimulation promoted closure of the DA in the fetal rat.

TP stimulation constricted the DA in two different PDA models

Next, we evaluated the effect of U46619 on two PDA models: premature and hypoxic-induced PDA models. Twenty minutes after delivery, we intraperitoneally injected various doses of U46619 (0.0005, 0.05, and 5.0μg/g) into PM20. The diameter of the DA was measured 10, 20,

and 30min after injection. U46619 at concentrations of 0.05 and 5.0μg/g significantly constricted the premature DA when compared with saline-injection (**Figure 2A**). It should be noted that 75% and 100% of the DA were completely closed 30min after the injection at concentrations of 0.05 and 5.0μg/g, respectively (**Table**).

Regarding hypoxic-induced PDA models, P_{O2} was lower in rats under a hypoxic condition than under a normoxic condition (19.3 ± 1.5 versus 56.2 ± 3.8mmHg, respectively. $p<0.0001$, n=4-7). Under normoxic conditions, within 30min after birth the lumen of the DA shrank by ~91% down from the diameter of the fetal DA on e21. Under hypoxic conditions, on the other hand, DA closure was significantly delayed: the lumen shrank by only ~24% by 30min after birth. Ten minutes after injection (30min after birth) we found that U46619 at a concentration of 5.0μg/g significantly constricted the hypoxic DA when compared with saline injection (**Figure 2B**). In addition, indomethacin at a concentration of 10μg/g constricted the hypoxic DA by ~70% of the DA diameter compared with saline-injection. These results indicated that TP stimulation effectively constricted the DA in two different PDA models.

TP stimulation did not constrict other vessels

We assessed whether U46619 constrict other vessels such as the aorta, the PA, the vertebral

artery, the renal artery, the portal vein, and the marginal artery of the colon (MA) at d0. U46619 at concentrations of up to 0.05μg/g had no significant vasoconstrictive effect on these vessels (**Figure 3A-G**). However, U46619 at concentration of 5.0μg/g significantly constricted the MA (**Supplemental figure 1**), but not other vessels at e21. In addition the aorta and the PA in e19 did not responded to U46619 at concentrations of up to 5.0μg/g (**Figure 3A, B**). These data suggested that the fetal and neonatal DA responded much to U46619 rather than other vessels.

Low-dose of U46619 did not decrease peripheral blood flow in neonatal rats

Though U46619 did not induce vasoconstriction in large arteries, it should be evaluated whether U46619 constricts a muscular type of arteries or arterioles. Therefore, we measured peripheral blood flow in the tail of d0 rats as an index of microvascular constriction by newly developed methods using a laser speckle measurement technique[16]. Up to a concentration of 0.05μg/g, U46619 did not decrease the peripheral blood flow in the tail (**Figure 4A-G**). However, if the concentration of U46619 was increased to 5.0μg/g, the peripheral blood flow was significantly reduced.

U46619-induced isometric tension of the DA vascular rings was stronger than that of the aorta

To consolidate the *in vivo* data demonstrating that the DA responded to U46619 more than the aorta, we measured the isometric tension induced by U46619 in the DA and aorta vascular rings.

U46619 at concentrations of up to 10^{-7}M developed isometric tension stronger in the DA rings than in the aorta rings from both e19 and e21 (**Figure 5A, B, respectively**). Therefore, it appears that the DA was more sensitive to U46619 than the aorta.

TP stimulation did not induce microthrombosis in the pulmonary capillary arteries

Because one of the most significant adverse effects of TP stimulation is microthrombosis, especially in pulmonary capillary arteries, it is very important to determine whether TP stimulation induces microthrombosis in pulmonary capillary arteries of neonates. U46619 at a concentration of 0.05μg/g apparently did not induce significant microthrombosis in pulmonary capillary arteries at PM20 (**Figure 6B**). On the other hand, consistent with previous studies showing significant microthrombosis in the pulmonary capillary arteries[17], AA induced significant thrombosis in rat lungs (**Figure 6C**), When we counted the ratio of arteries with thrombosis to total capillary arteries, arteries with thrombosis in the lung were 7% in U46619-injected and 4% in saline-injected premature neonates, respectively, whereas those were 42% in AA-injected (**Figure 6D**).

TP inhibition did not dilate the neonatal DA

To clarify the contribution of endogeneous TXA_2 to DA closure, we assessed whether TP inhibition made the closed DA reopened after birth or not. First, to determine the dose of a selective TP

antagonist, SQ29548, we pretreated with SQ29548 (1μg/g) into fetus at e21 10min before injecting U46619. Therefore, SQ29548 at concentration of 1μg/g is sufficient to inhibit the U46619-mediated DA constriction (**Figure 7A**). To observe whether SQ29548 prevents closure of the DA nor not after birth, SQ29548 was injected 40min after birth when the DA is still closing. We found that SQ29548 at concentrations of up to 10μg/g also showed no dilative effect on the DA after birth (**Figure 7B, C**). On the other hand, PGE_2 (15ng/g) significantly dilated the DA until 60min after injection.

Discussion

The present study demonstrated that TP stimulation potently constricted the *in vivo* DA in the following subjects: 1) rat fetuses at e19 and e21; 2) premature rat neonates delivered at e20; 3) mature rat neonates under hypoxic conditions ($5\%O_2$). These results are consistent with the previous study by Loftin et al. using Cox-1/-2 knockout mice with PDA[11]. Previous *ex vivo* studies have identified that TP stimulation produces contraction of ductus smooth muscle through the pathways that control both the concentration of intracellular calcium and the sensitivity of the contractile proteins to changes in intracellular calcium[8]. The former is determined by Ca^{2+} influx through L-type Ca^{2+} channels and the latter is regulated by Rho/Rho-kinase activity[18, 19].

To apply TP stimulation for patients with PDA as a potential alternative pharmacological therapy, it is important to estimate its possible adverse effects, because TXA_2 is endowed with powerful systemic vasoconstrictor, cytotoxic and thrombogenic properties[7]. In this regard, we examined the potential adverse effects of TP stimulation on the rat fetuses and neonates. First, systemic vasoconstriction is an important adverse effect of U46619 to be carefully considered. We found that U46619 even at a concentration of 0.05μg/g, which was sufficient to constrict the DA, did not significantly constrict other vessels including the marginal arteries of the colon and did not decrease

blood flow in the tail. In addition, our *ex vivo* data using the rat DA and aorta vascular ring demonstrated that U46619 produced stronger contraction of the DA than that of the aorta. However, U46619 at a concentration of 5.0μg/g significantly constricted the marginal arteries of the colon and reduced blood flow in the tail (**Supplemental figure 1**). Because continuous U46619 infusion is known to decrease cardiac output[20], the reduction in peripheral circulation may be not only due to vascular constriction but also a decrease in cardiac out by U46619 at a concentration of 5.0μg/g. Further study will be required whether a decrease in cardiac out is responsible for the U46619-mediated reduction in peripheral circulation.

Next, microthrombosis in the pulmonary capillary arteries is expected to be one of the worst adverse effects of U46619. We did not find significant microthrombosis in the pulmonary capillary arteries when U46619 at a concentration of 0.05μg/g was administered into PM20 rats (**Figure 6**). A number of studies have demonstrated that a relatively high dose of U46619 (e.g. 1.0mg/kg, i.v.) causes a shock syndrome resulting in sudden death due to systemic platelet aggregation, pulmonary thrombosis, and coronary spasm in adult animals[21-23]. However, neonatal platelets are known to be less reactive than adult platelets to U46619, thrombin, and ADP/epinephrine[24]. Therefore, thromboembolism may be avoidable when a low dose (up to 0.05μg/g) of U46619 is administered in

newborns.

Furthermore, we need to pay careful attention to administering U46619 into newborns, because neonatal pulmonary hypertension (PPHN) is characterized by pulmonary vasoconstriction, due in part to hypoxia-induced TP hyperresponsiveness[25, 26]. Although PPHN is induced by intravenous infusion of U46619 (~2μg/kg/min)[27, 28], further investigation is required to examine whether a bolus injection of U46619 at a low concentration induces PPHN or not.

Taken together, the present study demonstrated that low-dose TP stimulation induced vasoconstriction of the DA with minimal systemic adverse effects when U46619 is administered at a concentration of up to 0.05μg/g. Although COX inhibitors such as indomethacin and ibuprofen are the current unique pharmacological treatment for PDA[1, 3], the frequent failure rate of COX inhibitors is clinically problematic. COX inhibitors also share the similar adverse effects with U46619. Therefore, we propose that low-dose TP stimulation can be an alternative pharmacological strategy for PDA treatment when COX inhibitors are difficult to be administered.

The mechanism why U46619 constricted the DA more than other vessels is the next important question to be clarified, because a considerable number of *ex vivo* experiments have demonstrated that TP agonists constrict a variety of arteries and veins[7, 29]. We assume that the higher

sensitivity to U46619 in the DA could be due to its artery type (muscular type), because the structure of the DA is considered as a muscular type and most of other arteries that we examined belong to an elastic type. U46619 at a concentration of 0.05μg/g significantly constricted mature fetal DA by ~40% of the control groups, whereas the same dose of U46619 did not reduce the diameter of MA and blood flow of the rat neonatal tail. Because resistant muscular arteries supply the blood flow in the colon and tail, the arterial type may not be the sole reason of the hypersensitivity to U46619 in the DA.

We also examined the abundance of TP expression between the DA and the aorta during development. Although the expression levels of TP mRNA in the DA were higher than those in the aorta in the fetal period, the expression levels of TP protein showed no difference between the DA and the aorta (**Supplemental figure 2**). Therefore, the abundance of TP expression is not the reason of the hypersensitivity to U46619 in the DA. It is then highly possible that TP in the DA has higher binding affinities to TP agonists than that in the other arteries. Several studies have demonstrated that the affinity state of TP is influenced by interaction with $G_{\alpha 13}$ and/or $G_{\alpha q}$[30, 31]. Interestingly, recent studies by Dakshinamurti's group have demonstrated that a change in oxygen tension from normoxia to hypoxia provokes hypersensitivity to TXA_2 in pulmonary arterial myocytes of neonatal piglets[25, 26]. The authors have indicated that hypoxia promotes the membrane localization of TP and increases its ligand

affinity in pulmonary arterial myocytes. The response to oxygen is opposite between pulmonary arteries and other arteries including the DA. The DA is known to be more sensitive than the adjunct arteries to changes in oxygen tension[32]. It should be noted that the response to oxygen is stronger in the mature DA than in the premature DA[33]. The present study also demonstrated that the response to U46619 was stronger in the mature DA than in the premature DA (**Figure 1**). Therefore, this characteristic may be responsible for the DA-specific constriction that results from TP stimulation. Further study is apparently required to understand the mechanism why the DA is hypersensitive to TP stimulation.

In contrast to exogenous TP stimulation by U46619, our data showed that TP inhibition by the TP antagonist SQ29548 did not have a vasodilatory effect on the neonatal rat DA. Consistent with this observation, a previous study has demonstrated that a native TXA_2 was not synthesized in the DA under physiological conditions[34]. In addition, no PDA phenotype has been identified in TP knockout mice to date. Taken together, the evidence suggests that endogenous TXA_2 and TP are likely to play minor roles in the physiological closure of the DA.

In conclusion, our results demonstrate that TP agonists are a selective and potent vasoconstrictor of the fetal and neonatal rat DA with minimal adverse effects when they were administered at lose dose (up to 0.05μg/g). Although further investigation will be apparently required to

clinically use TP agonists for the patient with PDA, we propose that low-dose TP agonists may serve as a possible pharmacological therapeutic strategy for DA closure.

Acknowledgment

We thank K. Iwai, Q. Jiao, N. Liu (Waseda university), and R. Aoki (Yokohama city university) for technical advice and/or support.

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Figure legends

Figure 1 ***TP stimulation induced vasoconstriction of the fetal rat DA***

(A-F) Effects of U46619 on the fetal rat DA at e19 (A-C) and at e21 (D-F). Each panel showed representative data injected with saline (A, D), U46619 (0.05μg/g: B, E. 5.0μg/g: C, F). Arrows show the constricted DA. Scale bar: 0.2mm. Ao: aorta, LPA: left PA, RPA: right PA.

(G, H) Effects of various doses of U46619 (from 0.0005 to 5.0μg/g) (G) (n=3-12) and I-BOP (0.05 and 5.0μg/g) (H) (n=5-10) on the diameter of the fetal rat DA at e19 and e21. *p* value (vs. saline) $^{\dagger}p<0.05$, $^{**}p<0.01$, $^{*}p<0.001$. (e19 vs. e21) $^{\ddagger}p<0.05$. (n=3-12).

Figure 2 ***TP stimulation caused DA constriction in the premature and hypoxia-induced PDA models***

(A) The ratios of DA constriction in premature rats injected with U46619 (0.0005, 0.05, or 5.0μg/g). The diameter of the DA was measured 10, 20, and, 30min after injection. *p* value (vs. each saline group) $^{\dagger}p<0.05$,$^{*}p<0.001$ (n=4-9).

(B) The ratios of DA constriction in hypoxia-induced PDA model rats injected with U46619 and indomethacin (indo). *p* value (vs. saline on hypoxia) $^{\dagger}p<0.05$, $^{*}p<0.001$, (vs. indomethacin on hypoxia) $^{**}p<0.01$. (n=3-4)

Figure 3 ***TP stimulation caused no vasoconstriction of adjunct arteries and veins***

(A, B) Constrictive effect of U46619 on the aorta (Ao) (A) (n=3-9) and the pulmonary artery (PA) (B) (n=4-10) in e19 and d0.

(C-F) Constrictive effect of U46619 on the vertebral artery (VA) (C) (n=4-5), the renal artery (RA) (D) (n=4-8), the portal vein (PV) (E) (n=4-8), and the marginal artery of the colon (MA) (F) (n=3-6) in d0.

p value (vs. saline) $^{*}p<0.001$

Figure 4 ***U46619 did not decrease peripheral blood flow in neonatal rats***

(A-F) Representative images of blood flow at lower part of the neonates. Left (A, C, E) and right panels (B, D, F) indicate relative blood flow "pre-treated" and "post-treated" U46619 injection, respectively. Upper (B), middle (D), and lower (F) panels indicate U46619-injected group at dose of 0.0005, 0.05, and 5.0μg/g, respectively.

(G) Effect of U46619 on peripheral blood flow in the tails. "pre" indicates "pre-treated" *p* value (vs. pre) $^{\dagger}p<0.05$, (vs. 5.0μg/g) $^{*}p<0.01$ (n=5).

Figure 5 ***U46619-induced isometric tension of the DA and aorta vascular rings***

(A, B) Isometric tension of the DA and aorta rings at e19 (A) or e21 (B), stimulated by various doses of U46619 (10^{-8}, 10^{-7}, and 10^{-6} M). Squares and circles indicate the DA and aorta rings, respectively. *p* value (DA vs. aorta) $^{*}p<0.001$, $^{**}p<0.01$ (n=4-5).

Figure 6 ***Thrombosis formation in the microvasculature of the rat lung***

(A-C) Rat lung sections from PM20, injected with saline (A), U46619 (B), and arachidonic acid (AA) (C). Arrows indicate thrombosis formation. Scale bar: 0.1mm.

(D): The ratio of thrombosis formation in all pulmonary capillary arteries. *p* value (vs. saline) $^{*}p<0.001$ (n=4).

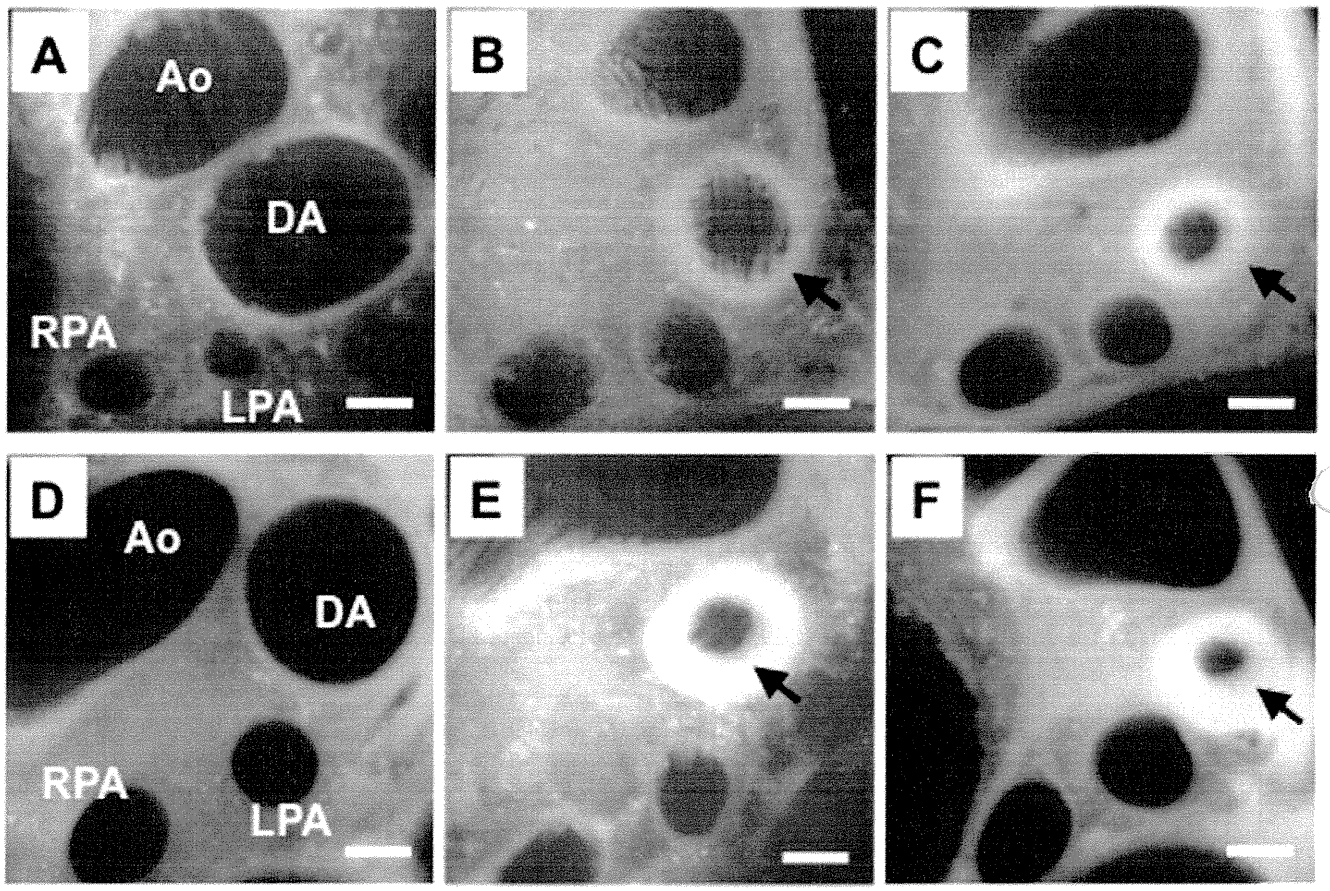
Figure 7 ***The effect of TP inhibition on the neonatal rat DA***

(A) The effect of TP antagonist SQ29548 on the U46619-induced DA constriction. SQ+U indicates the group pretreated with SQ29548 and then injected with U46619. *p* value (vs. saline) $^{**}p<0.05$, (vs. SQ+U) $^{\ddagger}p<0.05$ (n=3-4)

(B) The effect of the TP antagonist SQ29548 on the DA in rat neonates. PGE_2 was injected as a positive control. Circle, square, and triangle indicate group of SQ29548, PGE_2, and saline, respectively. *p* value (vs. saline) $^{*}p<0.001$ (n=3-4).

(C) Effect of a different dosage of SQ29548 (10μg/g) on the DA diameter. *p* value (vs. saline) $^{*}p<0.001$ (n=7-8).

Figure 1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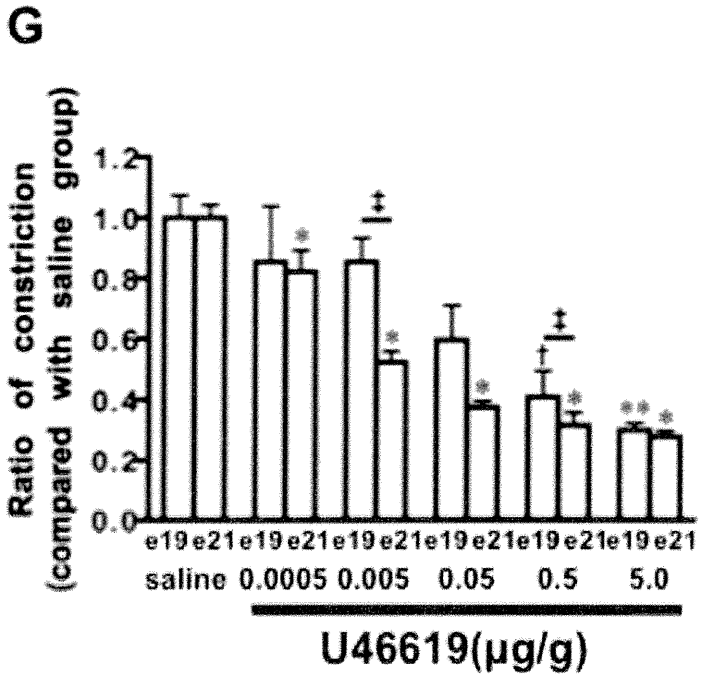
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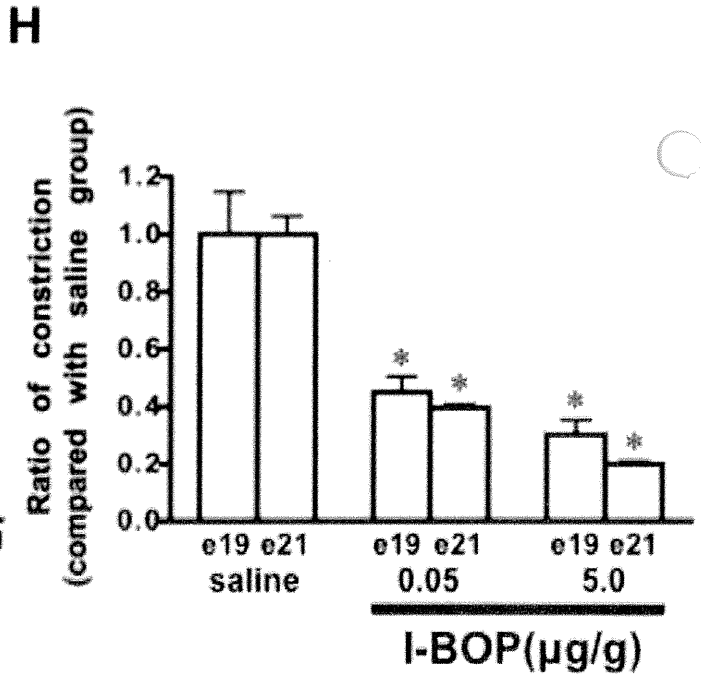
U46619(μg/g)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