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EXOGENOUS L-ARGININE DOES NOT STIMULATE PRODUCTION OF NO OR cGMP WITHIN THE RAT CORPORAL SMOOTH MUSCLE CELLS IN CULTURE

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Abstract

Background and Aim: Nitric oxide (NO) is the intracellular chemical responsible for initiating a penile erection. Despite conflicting clinical data, it continues to be publicized and promoted that orally administered L-arginine, the putative substrate for NO, enhances the erectile response presumably by stimulating NO production by the corporal tissues resulting in an increase in cGMP production. To shed light on this issue, an *in vitro* study was conducted to explore the effect of direct exogenous administration of L-arginine as well as its precursor and metabolite, L-citrulline, on the NO-cGMP pathway within the cavernosal smooth muscle (CSM) cell.

Materials and Methods: CSM cells obtained from 8–10 week old Sprague-Dawley rats were grown in Dulbecco media with 20% fetal calf serum and then incubated with or without L-arginine (L-ARG) or L-citrulline (L-CIT) in a time course and dose-response manner. Sildenafil (0.4 mM), IBMX (1mM), L-NAME (3 μ M), ODQ (5 μ M) and Deta Nonoate (10 μ M) were used as either inhibitors or stimulators of the NO-cGMP pathway. mRNA and protein were extracted and used for the determination of the phosphodiesterase 5 (PDE5). PDE5 activity was determined by luminometry. cGMP content was determined by ELISA. Nitrite formation, an indicator of NO production, was measured in the cell culture media by a colorimetric assay. The cationic (CAT-1) and neutral (SNAT-1) amino acid transporters for L-ARG and L-CIT, respectively, were determined by Western blot.

Results: When compared to untreated CSM cells, incubation with 0.25 to 4.0 mM of L-ARG or 0.3 to 4.8 mM of L-CIT anywhere between 3 and 24 hours did not result in any additional nitrite or cGMP production. The addition of L-NAME, IBMX or ODQ to these L-ARG and L-CIT

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⁶.CONFLICT OF INTEREST

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treated cells did not alter these results. L-CIT but not L-ARG increased PDE5 mRNA and protein content as well as the activity of the PDE5 enzyme. Both CAT-1 and SNAT-1 were expressed in the CSM cells.

Conclusions: This *in vitro* study demonstrates that exogenous administration of L-ARG or L-CIT failed to stimulate production of either NO or cGMP by the corporal CSM cells. A reevaluation of the presumptive role of the exogenous administration of L-ARG in improving the synthesis of NO at least at the level of the CSM cells appears warranted.

Keywords

L-arginine; L-citrulline; cGMP; PDE 5; rat cavernosal smooth muscle cells

1. INTRODUCTION

In 1988, it was discovered that nitric oxide (NO) was the endogenous nitro vasodilator of the vascular smooth muscle cells¹ synthesized from the terminal guanido nitrogen atom of the amino acid, L-arginine.^{2,3} Two years later, it was reported that following stimulation of the cavernosal nerve, the penile tissues formed both NO and cGMP as well as relaxation of the corporal smooth muscle (CSM) cells thereby suggesting for the first time that the NO-cGMP pathway was also the putative biochemical mechanism responsible for the development of an erection.⁴ Although Bult et al were the first to suggest that NO can act like a neurotransmitter,⁵ it was subsequently confirmed that the NO involved in the relaxation of the corporal smooth muscle erectile response was synthesized by the enzyme nitric oxide synthase (NOS) positioned within the axons of the cavernosal nerve but located extracellular to the CSM cells.⁶

Since L-arginine is the nitrogen donor for the production of NO throughout the body and since it has been hypothesized that an increased amount of a substrate, L-arginine, may lead to an increased production of NO, the assumption by many investigators is that exogenously administered L-arginine could act in a pro-erectogenic way. Subsequent human trials, however, have shown conflicting results. In a small non-controlled study conducted in men who ingested large amounts of oral L-arginine for over 2 weeks, about 40% of the participants elicited some improvement in their erectile function.⁷ In another human study, 31% of the men treated for over 6 weeks with oral L-arginine reported an improvement in their erectile response,⁸ but in a third placebo controlled crossover trial no benefit to the oral L-arginine was seen.⁹

Despite this conflicting evidence on the efficacy of oral L-arginine on the human erectile response, there has been a paucity of studies that have addressed the direct effect of the administration of exogenous L-arginine on the ability of the CSM cells themselves to form NO or cGMP. Using an organ bath assay, both Sjostrand et al¹⁰ and Gur et al¹¹ failed to demonstrate the convincing evidence needed to determine unequivocally that exogenously administered L-arginine had any direct stimulatory effect on the NO-cGMP pathway of the CSM cell. Based on the results of a recent *in vitro* study that showed that L-citrulline, the precursor of L-arginine, did not have any effect on either NO or cGMP production in a rat

CSM cell culture,¹² this study was designed to look at the effect of exogenous L-arginine in this same experimental setting while comparing its effect to that of its precursor, L-citrulline.

2. MATERIAL AND METHODS

2.1 Experimental Animals

Eight to ten-week-old male Sprague Dawley rats (B.W 200 ±30 g) from Envigo Laboratories (Livermore, CA) were used for this study, which was approved by the Institutional Animal Care and Use Committee (IACUC) at Charles R. Drew University of Medicine and Science. Rats were housed for one week prior to experimental procedures to allow acclimatization. Animals were sacrificed by CO₂ inhalation. After confirming the death of the animals, they were soaked in alcohol 70% for 5 min. The skin and fascia were first removed, and under aseptic conditions, the penis was then excised. Once the fascia, urethra, and neurovascular bundle of the penis were removed, the remaining corporal tissues were then minced in small pieces and attached to a dry 75-cm² flask.¹³

2.2 Cell Cultures and Incubation with L-arginine and L-citrulline

Primary cavernosal cultures were initiated from small pieces of the corpora cavernosa as described above, by gently adding Dulbecco's modified Eagle's medium (DMEM) with 20% fetal calf serum, without causing the explant to float.¹³ The explants were cultured at 37°C in a 5% CO₂ incubator avoiding handling the plate for a week so as to not dislodge the tissue. After a week, the tissue was removed, and the migrating cells were rinsed with Hanks, and harvested by trypsinization. Cells were transferred to new T75-cm² flasks at approximately 0.5–1.0 × 10⁶ cells per flask. This constituted the first passage. All the experiments were conducted between the second and fifth passages, and cells were never frozen for storage.¹²

2.3 Determination of cGMP

CSM were incubated for 24 hours with either 1) L-ARG at 0.25, 0.50, 1.0, 2.0, and 4.0 mM, 2) L-CIT at 0.3, 0.6, 1.2, 2.4 and 4.8 mM, and 3) vehicle only. A time course (0, 3, 6, 12 and 24 hours) incubation using the highest concentration of L-ARG (4.0 mM) and L-CIT (4.8 mM) was performed. In addition, several inhibitors of the NO-cGMP pathway were employed in these experiments. L-NAME, an inhibitor of NOS, was used at 3 μM; ODQ an inhibitor of the guanylate cyclase was used at 5 μM; sildenafil and IBMX, inhibitors of the PDE enzyme, were used at 0.4 and 1 mM, respectively. DetaNonoate, a NO donor, was used at 10 μM. Incubation was stopped by aspirating the media and by adding 400 μl HCL 0.1 M for 20 minutes, as previously described.¹² Cells were then scraped, homogenized by pipetting, and centrifuged at 1,000g for 10 minutes. The supernatants were used for the determination of cGMP concentration by a colorimetric ELISA (Cayman Chemical Company, Ann Arbor, M), following the manufacturer instructions. Fifty μl of the standard dilutions and samples without acetylation were applied to a plate containing a rabbit antibody specific for cGMP that binds to the wells coated with mouse anti-rabbit IgG. Binding was determined by competition with a cGMP tracer. After overnight incubation, the wells were washed, followed by incubation with Ellman's reagent. The enzymatic reaction

product was determined by spectrophotometry at 405-nm absorbance and expressed as pmol/mg protein.

2.4 Real-time quantitative PCR (qPCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) and equal amounts (1 μ g) of RNA were reverse transcribed using High Capacity RNA-to-cDNA PCR kit (Applied Biosystems, Foster City, CA). Rat gene PCR primer sets for phosphodiesterase 5 (PDE5), was obtained from SA Biosciences (Germantown, MD). The Power SYBR green PCR Master Mix (Applied Biosystems) was used with Step-One-Plus real-time PCR System (Applied Biosystems). The protocol included melting for 15 min at 95°C, 40 cycles of three-step PCR including melting for 15 s at 95°C, annealing for 30 s at 58°C, elongation for 30 s at 72°C with an additional detection step of 15 s at 81°C, followed by a melting curve from 55 to 95°C at the rate of 0.5°C per 10 s. The samples of 25 ng cDNA were analyzed in quadruplicate in parallel with RPLP1/3 controls; standard curves (threshold cycle vs log pg cDNA) were generated by log dilutions of standard cDNA (reverse transcribed mRNA from corporal smooth muscle cells in growth media) from 0.1 pg to 100 ng. Experimental mRNA starting quantities were then calculated from the standard curves and averaged using SA Bioscience software as previously described.^{12,14} The ratios of the mRNA for the marker experimental gene for PDE5 to that of RPLP1/3 were computed and normalized with control samples as a ratio of 1.

2.5 Western blotting and densitometry analysis

The cell lysates (80 μ g protein) were subjected to Western blotting analyses by 4–15% Tris–HCl PAGE (Bio-Rad, Hercules, CA) in a running buffer (Tris/glycine/SDS). The proteins were transferred onto Polyvinylidene Fluoride (PVDF) membranes in a transfer buffer (Tris/glycine/methanol) using transblot semi-dry apparatus (Bio-Rad). The nonspecific binding was blocked by immersing the membranes into 5% nonfat dried milk and 0.1% (v/v) Tween 20 in PBS for 3 h at room temperature (RT), as it was previously described.^{12,14} After several washes with the washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 3 h at RT. Primary monoclonal antibodies were used for PDE5 (ABCAM, Cambridge, UK) at 1:1,000, CAT-1 (Proteintech, Rosemont, IL) at 1:250, SNAT-1 (ABCAM) at 1:800 and GAPDH (Millipore, Billerica, MA) at 1:5,000 dilution. After several washes with buffer, the membranes were incubated for 3 h at RT with 1:2,000 dilution of anti-mouse secondary antibody linked with HRP (Cell Signaling Technology, Danvers, MA) and the immunoreactive bands were visualized using the WesternSure PREMIUM chemiluminescent detection system (Li-COR Biotechnology, Lincoln, NE, USA). The scanning of the bands was done with the C-DiGit Blot Scanner (Li-COR Biotechnology) and the images were captured with the Image Studio Software, version 5.2 (Li-COR Biotechnology).¹⁴

2.6 Determination of nitrite formation

After incubation with or without L-ARG and L-CIT for 3, 6, 12 and 24 hours, the cell culture media was collected and frozen at –20°C until the determination of total nitrite concentration by the Griess Reaction (Cayman Chemical Ann Arbor, MI). In brief, nitrite blanks (200 μ l assay buffer), nitrite standards (100 μ l), and each sample (100 μ l) were added

to individual wells on a 96-well plate. Griess reagents R1 (50 μ l) followed by R2 (50 μ l) were added to the standard and sample wells. After 10 minutes incubation at RT, the absorbance was measured with a plate reader at 540 nm. Blank optical density was subtracted from control and samples. Sample Nitrite concentrations (μ M) were based on the optical density of the azo compound and determined as [nitrite (μ M)] = [absorbance at 540 nm – *y*-intercept (standard curve)/slope (standard curve)] * (200 μ l/80 μ l). Samples were run in triplicates of three experiments. Results were expressed in μ M.¹⁵

2.7 Determination of PDE5 activity

The PDE5 activity after incubation with L-ARG and L-CIT was evaluated using the PDE-Glo Phosphodiesterase Assay according to the manufacturer's instruction (Promega Corporation, Madison, WI, USA). Briefly, 5 μ l of 1 \times PDE-Glo reaction buffer containing 10 mU of purified Bovine PDE5 (Sigma-Aldrich, St Louis, MO) was pipetted into 96- white opaque well plate wells (Thermo Scientific, Waltham MA). The tested compounds were dissolved in water, and serial dilutions of L-ARG and L- CIT were created using 1 \times PDE-Glo reaction buffer. Then, 10 μ L of diluted inhibitors and 12.5 μ L of cGMP solution were added to each well. After 90 min of incubation in 25 °C, 12.5 μ L of PDE-Glo™ Termination Solution and 12.5 μ L of PDE-Glo™ Detection solution were added and the plate was incubated for 20 min at RT. Finally, 50 μ L of Kinase-Glo® Reagent was pipetted to each well and after 10 min of incubation, the luminescence was measured using a microplate luminometer (Perkin Elmer, Waltham MA,). All data points were the average of two experiments done by triplicates, and expressed as count per second (CPS).

2.8 Statistical analysis

All data are presented as mean \pm S.E.M., and between-group differences were analyzed using ANOVA. If the overall ANOVA revealed significant differences, then pair-wise comparisons between groups were performed by Tukey's multiple comparison tests. All comparisons were two-tailed, and $p < 0.05$ were considered statistically significant. All *in vitro* experiments were repeated at least thrice, and data from representative experiments are shown.

3. RESULTS.

In order to determine whether the incubation of L- ARG and L-CIT to a pure rat CSM cell culture has any stimulatory effect on cGMP production, a dose response experiment was conducted for both amino acids. Figure 1 shows that in our CSM cell culture, the incubation of either L-ARG or L-CIT for 24 hours fails to elicit any increase in cGMP production. The highest dose of each of the two amino acids used (4.0 mM for L-ARG and 4.8 mM for L-CIT) is comparable to a daily dose in men of about 3000 mg of L-ARG and L-CIT, respectively. The PDE5 inhibitor, sildenafil, was then used as a positive control to compare its result with the highest dose of the two amino acids and, as expected, sildenafil resulted in an increase in the level of cGMP (Figure 2). Similar results were obtained when a 24 hour time course experiment was conducted. No changes in levels of cGMP were observed for either L-ARG or L-CIT at any of the time points (Figure 3).

To determine whether L-ARG or L-CIT was effective in stimulating production of NO by the CSM cells themselves, nitrite levels were measured in the cell culture media after 24 hours of exposure to both amino acids. Similar to what was seen with cGMP expression, no elevation in nitrite levels was observed (Figure 4). The NO donor, detoNonoate, as expected resulted in an increase in nitrite levels. In addition, no changes in the nitrite levels were observed in the cell culture media at any of the time points studied. (Figure 5)

The effect of inhibitors on the NO-cGMP pathway upon incubation of L-ARG or L-CIT was studied. Figure 6A shows the effect of L-NAME on cGMP expression. When compared to the non-treated control, L-NAME showed an inhibitory effect ($p=0.04$) on endogenous cGMP production. However, the co-incubation of L-NAME with L-ARG had no effect on endogenous cGMP production but when co-incubated with L-CIT, there was a further but non-significant ($p=0.07$) inhibition of cGMP production. Figure 6B shows that ODQ by itself also showed a significant ($p=0.0280$) decrease in endogenous cGMP production similar to L-NAME. The addition of ODQ did not modify the cGMP production by either L-ARG or L-CIT. Figure 6C shows that the PDE inhibitor, IBMX, by itself significantly increased cGMP expression ($p<0.001$). However, when either L-ARG or L-CIT were incubated together with IBMX a non-stimulatory effect was observed when compared to control values.

One of the reasons that cGMP production may be inhibited with L-ARG and L-CIT as was observed in our in vitro assay is the development of tachyphylaxis which would be reflected by an increase in the content of the PDE5 enzyme. Figure 7A shows that there is indeed an increase in the mRNA PDE5 enzyme expression after L-CIT exposure but not after L-ARG. The PDE5 protein content (Figure 7B) was also upregulated by L-CIT, but not by L-ARG, in parallel to what was observed at the mRNA level.

To determine whether the activity of the PDE5 enzyme itself could be impacted by either L-ARG or L-CIT, we compared the results of these two amino acids to sildenafil. Figure 8 shows that LARG had no effect on the activity of the PDE5 enzyme within the CSM cells, while L-CIT demonstrated a slight albeit statistically significant stimulatory effect commensurate with the observed increase in the mRNA and protein expression of the PDE5 enzyme with L-CIT. As expected, sildenafil which acted as our positive control demonstrated a significant inhibitory effect on PDE5 activity.

The ability of L-ARG and L-CIT to enter the CSM cells requires the presence of their individual transporter. Figure 9 shows that both CAT-1 and SNAT-1 are expressed in our CSM cell culture.

4. DISCUSSION

This *in vitro* study clearly demonstrates, at least at the cellular level, that externally administered L-arginine or L-citrulline to rat CSM cells in culture does not result in any increase in the production of cGMP, the putative second messenger in the NO-cGMP pathway that is responsible for the smooth muscle relaxation in both the cavernosal arteries and the corporal tissue. It is this smooth muscle relaxation by cGMP that is an absolute

necessity for both the initiation and the maintenance of an erectile response.¹⁶ Besides the lack of cGMP stimulation in the CSM cells by these two externally administered amino acids, the results of our experiment also fails to provide any evidence that there is an increased production of NO following incubation of the CSM cells with either L-arginine or L-citrulline as suggested by the absence of any increase in the nitrite levels over basal levels. The lack of any increase in NO and cGMP production following incubation with either amino acid cannot be ascribed to an absence of NOS in these CSM cells as there appears to be a measurable basal level of both nitrite and cGMP production by the untreated cells and, as recently reported, the presence of all three isoforms of NOS.¹²

These *in vitro* results demonstrating that the administration L-arginine by itself is incapable of enhancing the production of NO by the CSM cells call into question the reported pro-erectile efficacy of using oral L-arginine⁷⁻⁹ or L-citrulline¹⁷ by themselves in the clinical setting. In men, the oral administration of either L-arginine or L-citrulline both result in an increase in the serum levels of L-arginine although pharmacokinetic studies suggest that for equal amounts of each amino acid ingested orally, the post-ingested L-arginine serum levels seem to be much higher with ingested oral L-citrulline than with L-arginine.¹⁸⁻²⁰ This is believed due to the fact that a large portion of L-arginine that is ingested orally is broken down in the gut thereby minimizing its absorption into the blood stream.²¹ Regardless of whether it was L-arginine or L-citrulline that was utilized in the aforementioned clinical trials, the assumption in the literature has always been that L-arginine itself was capable of enhancing the NO-cGMP pathway and as a result, erectile function. In reviewing these reports, however, it appears as if the proof that is cited for such beneficial claims of orally ingested L-arginine in erectile function, besides the “clinically reported outcome” of such treatment, was based primarily on indirect evidence such as validated questionnaires and never on the levels of nitrite or cGMP formations in the targeted corporal tissue.^{8,9}

The NO that is responsible for the normal erectile response emanates from outside the penis specifically the axons of the cavernosal nerve.⁶ If in the normal setting L-arginine were to drive the NO-cGMP pathway in the penis to enhance erectile function as has been reported,⁷⁻⁹ this increase in NO production would have to be occurring in the cavernosal nerve where the NOS enzyme responsible for normal erectile function is located. If oral L-arginine supplementation was indeed capable of stimulating NO production in the cavernosal nerve, such up-regulation would not be relegated solely to the cavernosal nerve but to all the nerves that specifically have the ability to produce NO. To date, there are no reports of any identifiable clinical effects that suggest such a neuro-stimulatory side effect with either oral L-arginine or for that matter, L-citrulline.

One limitation of this study is that it obviously does not accurately reflect what is operative in the *in vivo* setting. The NO that stimulates the endogenous cGMP pathway within the CSM cells involved in erectile function, as stated previously, emanates from outside of the CSM cells specifically within the terminal axons of the cavernosal nerve.⁶ Despite this important difference between our *in vitro* system and the normal *in vivo* physiology, the CSM cells in our *in vitro* assay are still producing some basal levels of nitrites and cGMP suggesting that there is some ongoing endogenous production of NO within the non-stimulated CSM cells. More than likely these basal levels of NO and cGMP in the CSM

must be due to activity of one or more of the three isoforms of NOS within the CSM cells.¹² Support for this ongoing endogenous NO-cGMP activity within the CSM cells can be inferred from the increase in cGMP levels seen following exposure of the cells to the PDE inhibitors, sildenafil (Figure 2) and IBMX (Figure 6B).

Another potential limitation is the role of arginase in our *in vitro* system. It is known that the expression and activity of arginases, which produce urea and divert arginine from NOS, are positively related to exogenous arginine supplementation^{22,23}. This destruction of the L-ARG by an increase in arginase could in turn lead to a decrease in NO production and this may explain why, as seen in Figure 6B, the control cGMP levels are reduced when L-ARG alone is added. This could also explain why the high levels of cGMP seen with IBMX is reduced by over 60% when L-ARG is added to the IBMX assay. The role of arginase in this *in vitro* system requires further investigation.

The findings of this *in vitro* study certainly require a re-examination of the role of externally administered L-arginine in improving the cellular synthesis of NO within the CSM in the *vivo* setting. Review of the pharmacology literature suggests that the intracellular concentration of L-arginine is usually very high at about 1 mM compared to normal serum levels that is around 80–120 μM .²⁴ The K_m for L-arginine as a substrate for the NOS enzyme which makes NO is in the region of 1–10 μM so there appears to be a vast surplus of L-arginine substrate present in the human body.²⁴ Simply based on what is therefore known about the clinical pharmacology of L-arginine, it is difficult to conclude that patients whose diet includes an adequate intake of protein will become deficient in L-arginine and therefore require supplementation with L-arginine. Despite this, there is indirect evidence in the cardiovascular literature that exogenous L-arginine administration may be beneficial in a variety of conditions e.g. lowering the systolic and diastolic blood pressure in hypertensive individuals, preventing restenosis after balloon angioplasty, and improving endothelium-dependent vasodilatation in humans who have hypercholesterolemia and atherosclerosis.^{25,26} Although these clinical effects have been ascribed at times to the direct role of NO, the evidence to support this conclusion, like that with erectile function, is mostly indirect. Suzuki et al failed to show any increase in plasma nitrite and nitrate levels following oral supplementation with either L-arginine and/or L-citrulline in healthy men.²⁷ Whether the L-arginine acts on asymmetric dimethylarginine, a cellular inhibitor of nitric oxide synthase or some other unknown factor to produce these reported clinical effects, remains unanswered.²⁸

In reality, most of the clinical and laboratory data ascribing the beneficial effects of administered L-arginine seems to have focused on its role in the vascular endothelium where the eNOS enzyme is located.²⁵ The eNOS enzyme is membrane bound as is the active transporter for L-arginine, CAT-1, which allows L-arginine to move from an extracellular to an intracellular location.^{27, 29} Using an endothelial cell line for their assay, Shin et al have demonstrated that nitrite production can be increased intracellularly when the extracellular L-arginine level is increased and this increase in nitrite production can be prevented by inhibiting the CAT-1 transporter.³⁰ It was assumed that the formation of nitrite in this endothelial assay was indicative of NO formation by eNOS but since an inhibitor of NOS was not utilized to block nitrite formation in this study or cGMP levels were not measured, the exact site of action of exogenous L-arginine in the endothelium still remains an open

question. The same can be said about the clinical trials that have utilized oral or infused L-arginine to demonstrate an improvement in whatever metric was being tested, Although it appears as if NO from eNOS could be held responsible for these clinical observations, unequivocal confirmatory testing at the tissue level is still lacking. This is due in part to the fact that in whole corporal tissue, which consists primarily of CSM and endothelial cells, it has been shown that NO activity was distributed entirely to the cytosolic fraction and none to the membrane bound fraction suggesting that at least in the corpora the eNOS membrane bound enzyme, if present, is not active.^{31,32}

In conclusion, oral supplementation of L-arginine by itself to enhance erectile function seems questionable based on the data from this *in vitro* study. To unequivocally determine whether oral L-arginine by itself has a pro-erectogenic effect in men, randomized, placebo controlled studies designed in some way to evaluate the effect of L-arginine at the target tissue will be required.

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Highlights

- In cavernosal smooth muscle cell cultures, incubation with either L-arginine or L-citrulline for up to 24 hours does not enhance the production of nitrite or cGMP.
- Incubation of cavernosal smooth muscle cells with L-citrulline, but not L-arginine, increases phosphodiesterase 5 expression and its activity.
- The use of oral L-arginine or L-citrulline as a sole oral supplement for enhancing erectile function requires re-evaluation.

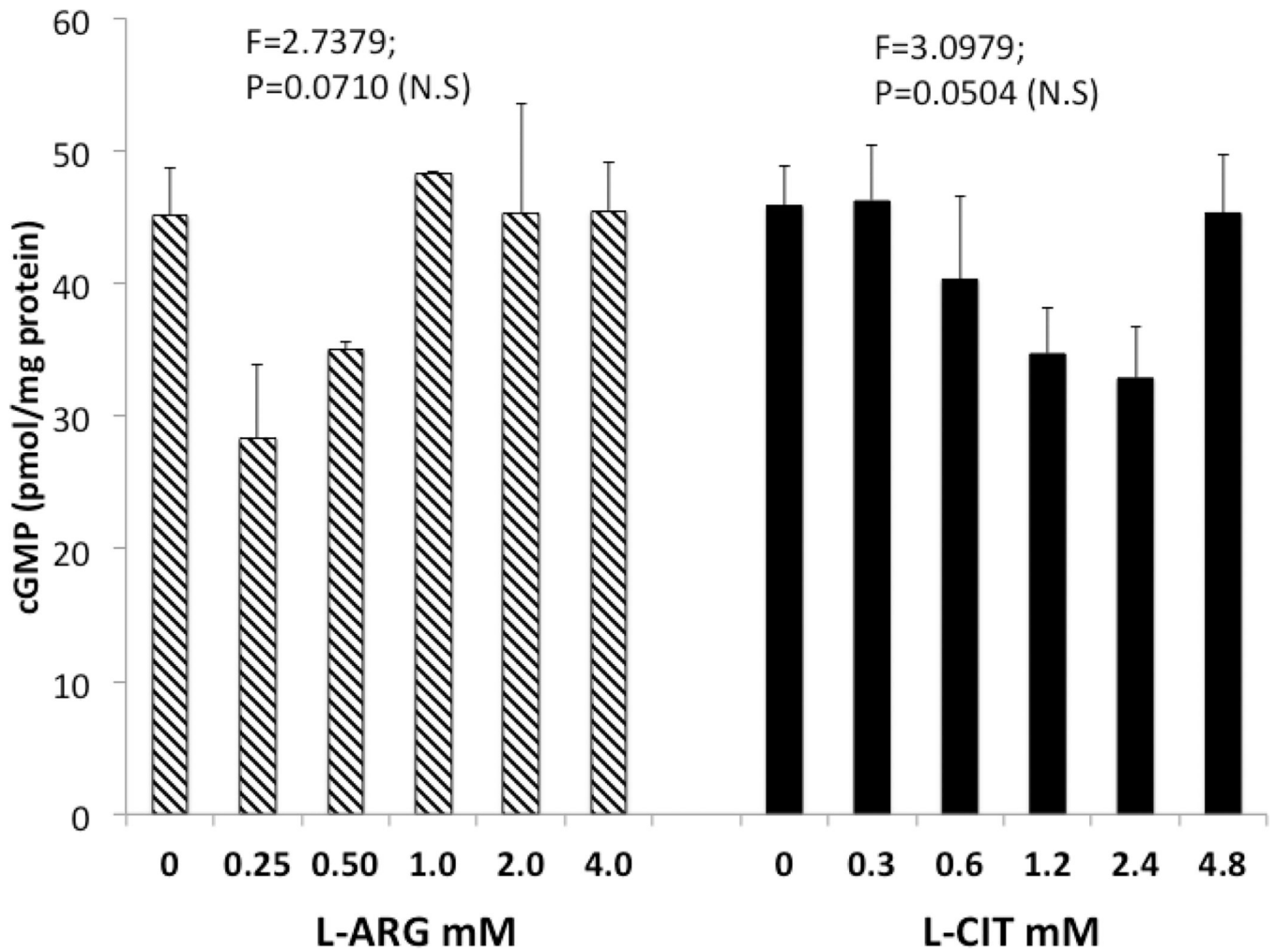


Figure 1: Effect of the incubation of different concentrations of L-ARG and L-CIT on cGMP expression in cavernosal smooth muscle cell culture.

Cavernosal smooth muscle cells (CSM) were incubated for 24 hours with or without different concentrations of L-arginine (L-ARG: 0.25, 0.50, 1.0, 2.0, 4.0 mM), and L-citrulline (L-CIT: 0.3, 0.6, 1.2, 2.4, and 4.8 mM). cGMP expression was determined by ELISA. Results are expressed as pmol/mg protein and represent the mean \pm S.E.M of four experiments done in duplicates. No significant difference was found among the groups.

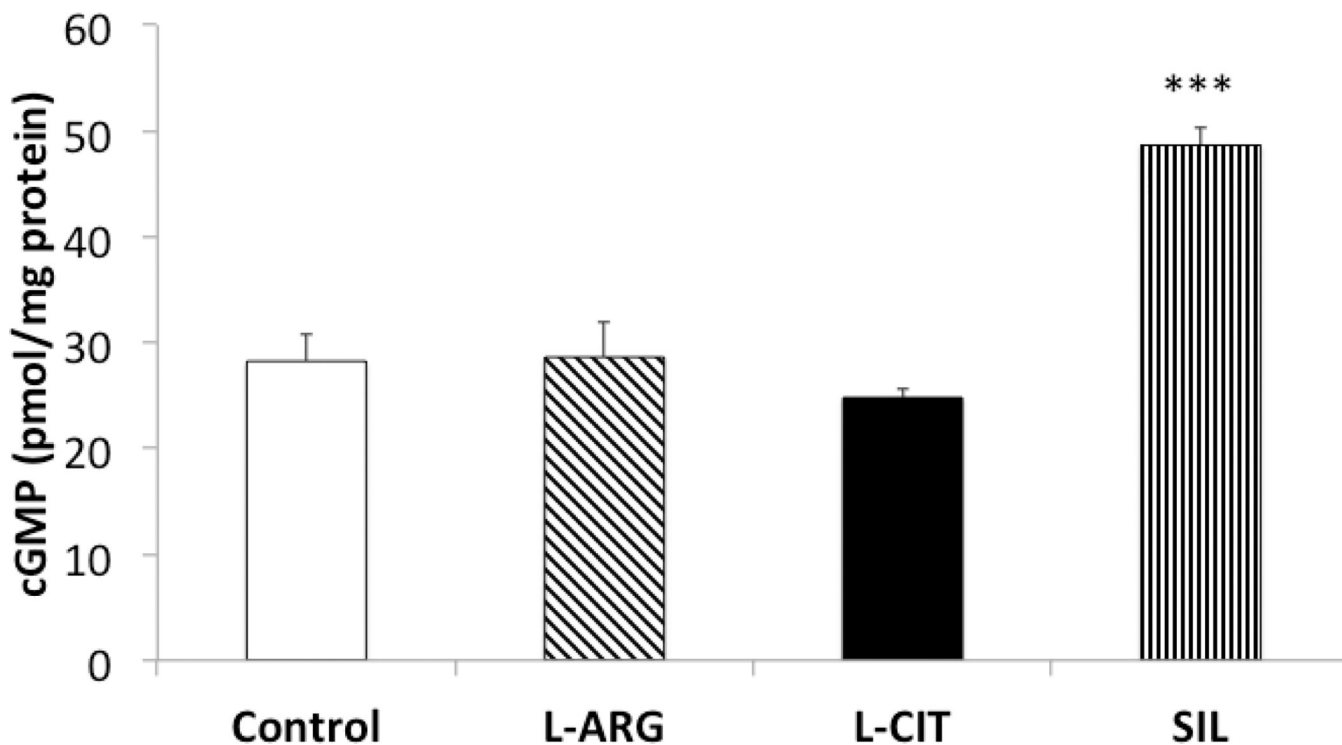


Figure 2: Effect of the incubation of L-ARG and L-CIT in comparison with sildenafil on cGMP expression in cavernosal smooth muscle cell culture.

Cavernosal smooth muscle cells (CSM) were incubated for 24 hours with or without L-arginine (L-ARG: 4.0 mM), L-citrulline (L-CIT: 4.8 mM) and sildenafil (SIL: 0.4 mM). Results are expressed as pmol/mg protein and represent the mean ± S.E.M of four experiments done in duplicate. ***p<0.001 with respect to control.

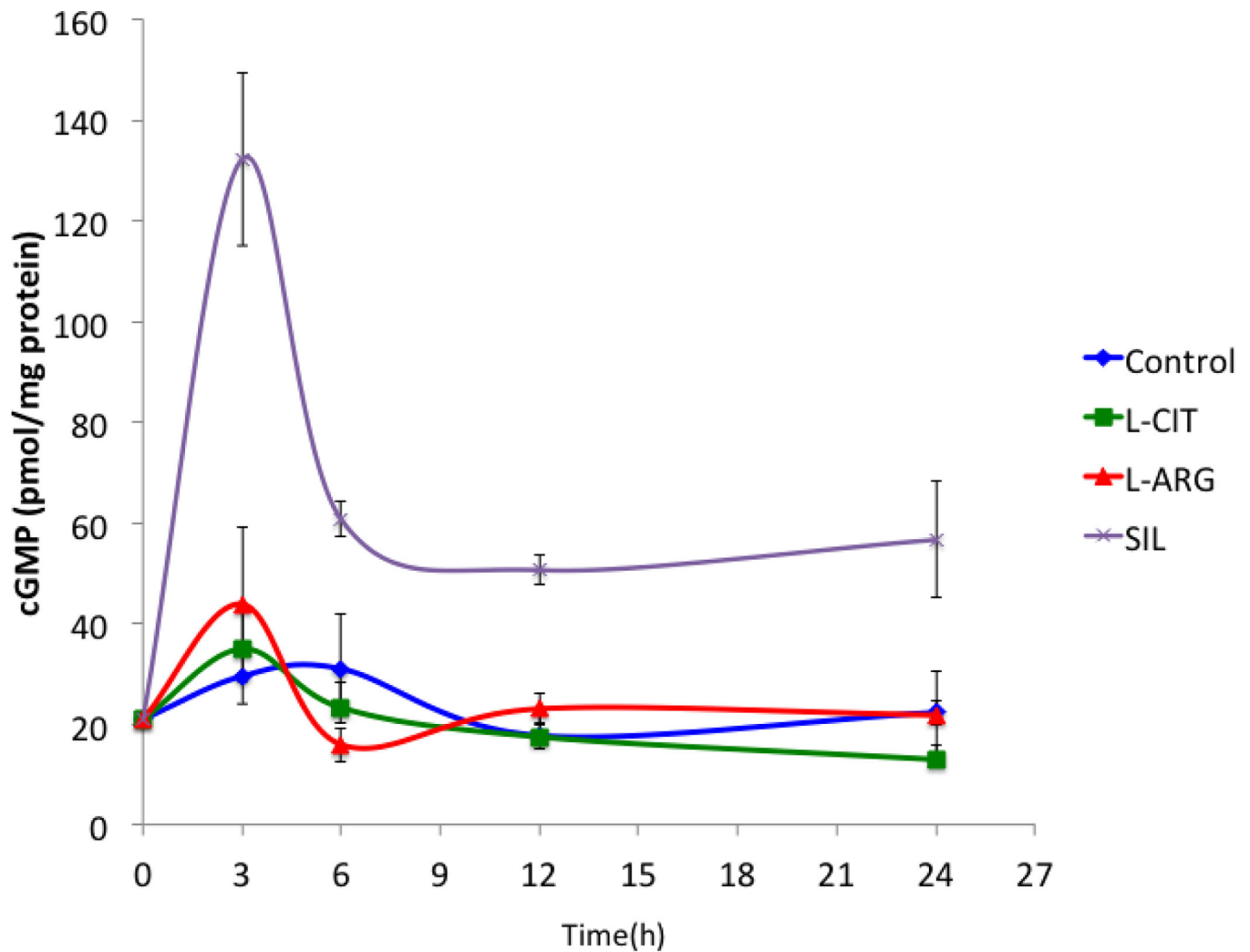


Figure 3: Time course of the expression of cGMP after the incubation with L-ARG and L-CIT in comparison with sildenafil on cGMP expression in cavernosal smooth muscle cell culture. CSM cells were incubated at different time points (from 0 to 24 hours) with or without L-arginine (L-ARG: 4.0 mM), L-citrulline (L-CIT: 4.8 mM) and sildenafil (SIL:0.4 mM). Results are expressed as pmol/mg protein and represent the mean \pm S.E.M of three experiments done in duplicate.

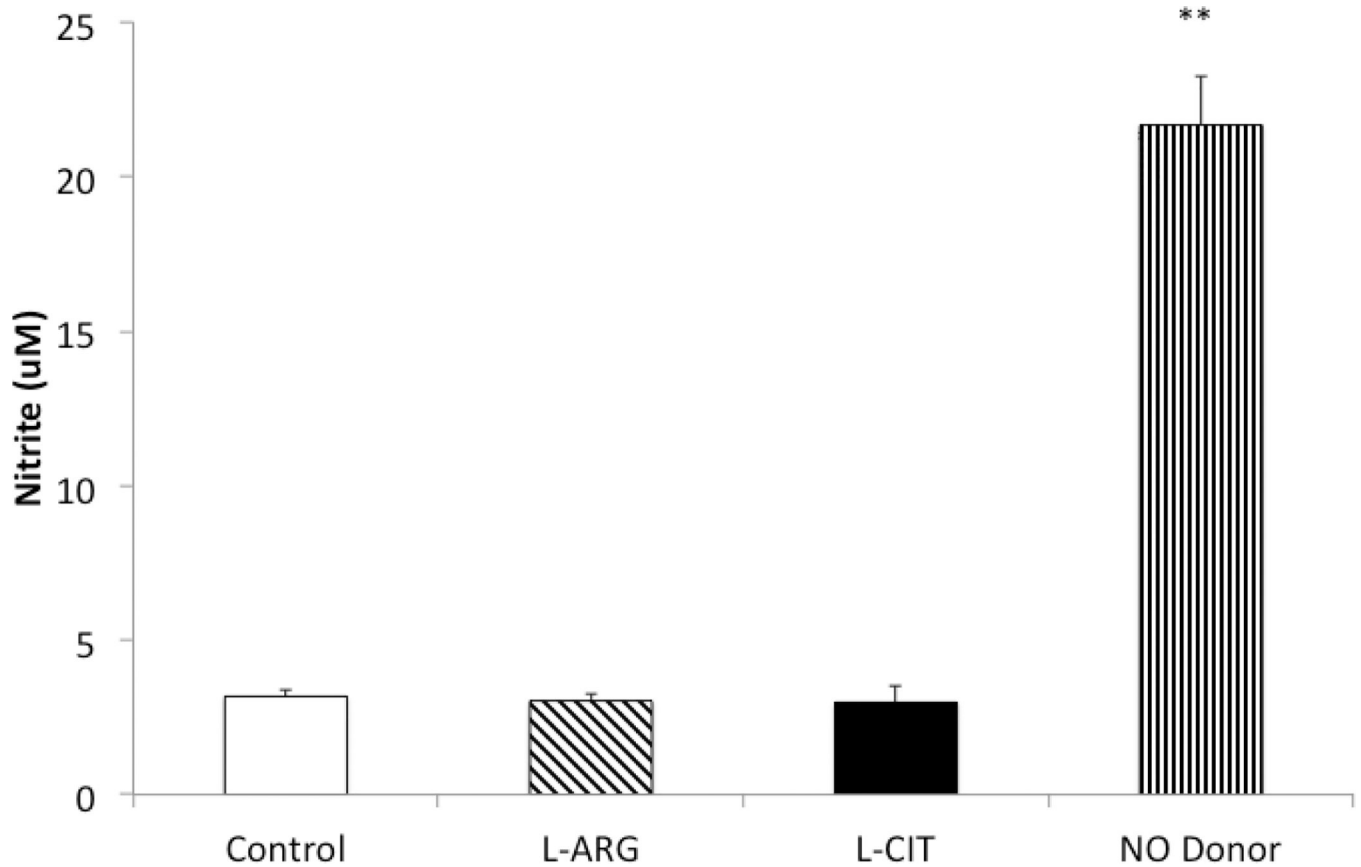


Figure 4: Effect of the incubation of L-ARG and L-CIT on nitrite production in cavernosal smooth muscle cell culture.

CSM cells were incubated for 24 hours with or without L-ARG and L-CIT, at the same concentrations as in Figure 3. The NO donor, DETA-NONOate was used as a positive control in the experiment. The cell culture media from treated and un-treated cells were collected and frozen at -80°C . Nitrite formation was determined by Griess reaction. Results are expressed as μM and represent the mean \pm S.E.M of four experiments done in duplicates. No significant difference was found with respect to control for L-ARG and L-CIT. ** $p < 0.001$ respect to control.

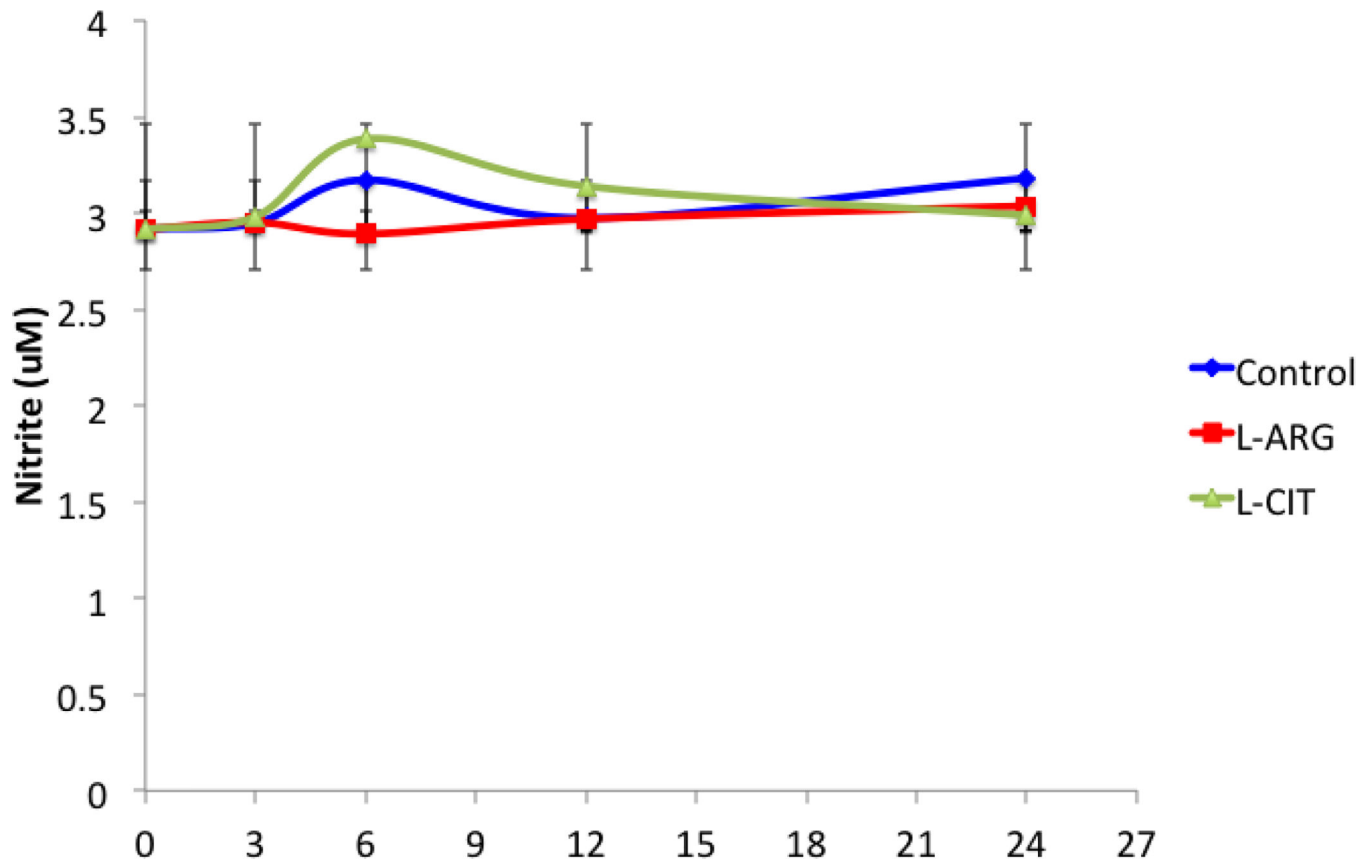


Figure 5: Time course of the nitrite formation after the incubation of the highest concentrations of L-ARG and L-CIT in cavernosal smooth muscle cell culture media.

CSM cells were incubated at different time points (from 0 to 24 hours) with or without L-arginine at the same concentration as figure 3. The cell culture media from treated and control cells were collected and frozen at -80°C . Nitrite formation was determined by Griess reaction. Results are expressed as μM and represent the mean \pm S.E.M of three experiments done in duplicates. No significant differences were found with respect to control at any time point.

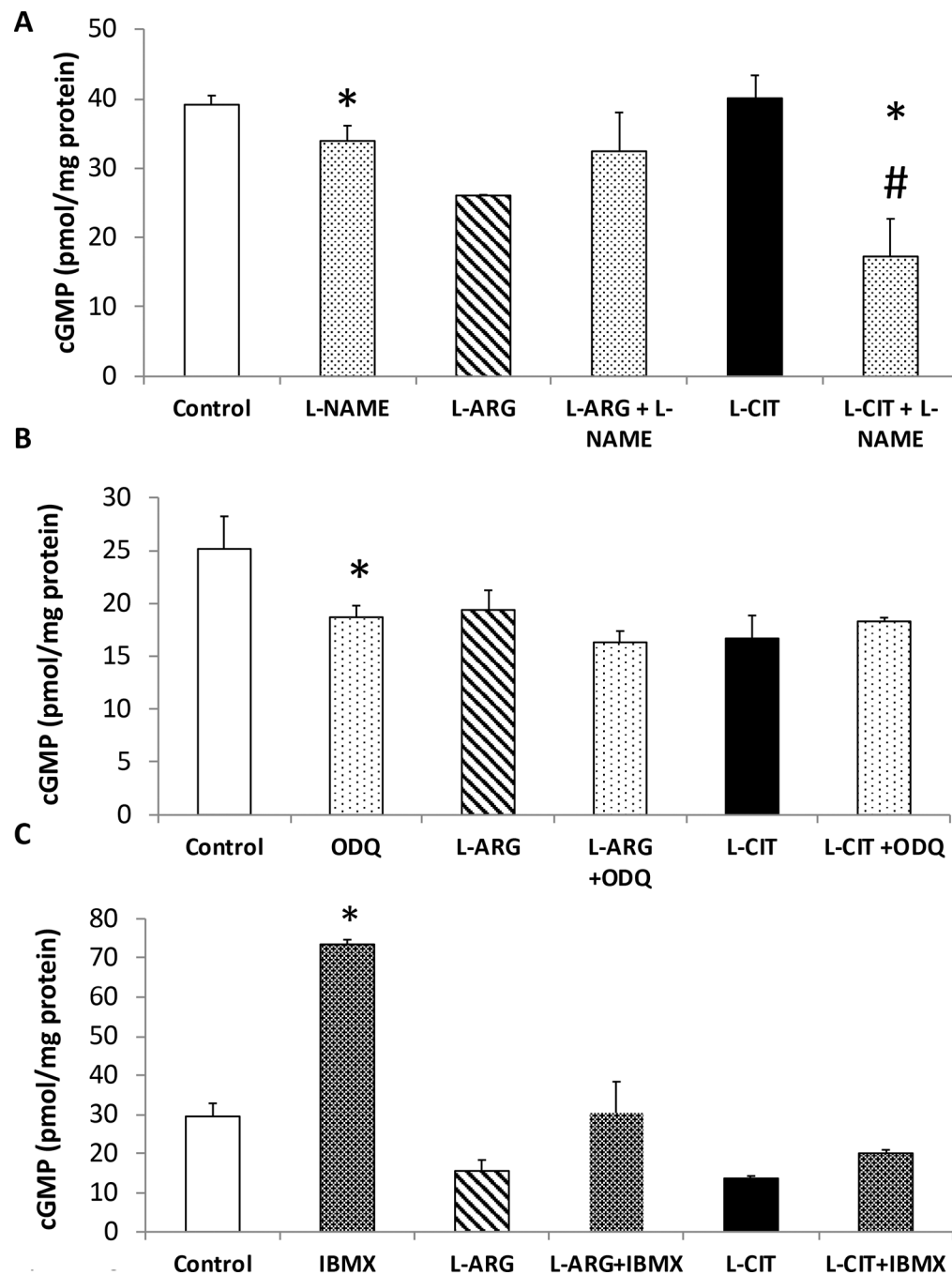


Figure 6: Effects of the inhibition of NOS activity by L-NAME, soluble guanylyl cyclase by ODQ, and phosphodiesterase by IBMX, on the expression of cGMP upon incubation with L-ARG and L-CIT.

CSM cells were incubated with or without L-ARG and L-CIT, at the same concentrations as in figure 3, **Panel A:** with or without L-NAME (3 μ M) for 24 hours. **Panel B:** with or without ODQ (5 μ M) for 24 hours. **Panel C:** with or without IBMX (1mM) for 24 hours. Results are expressed as pmol/mg protein and represent the mean \pm S.E.M of four experiments done in duplicate. * $p < 0.05$ with respect to control. ** $p < 0.001$ with respect to control. # $p < 0.05$ with respect to L-CIT.

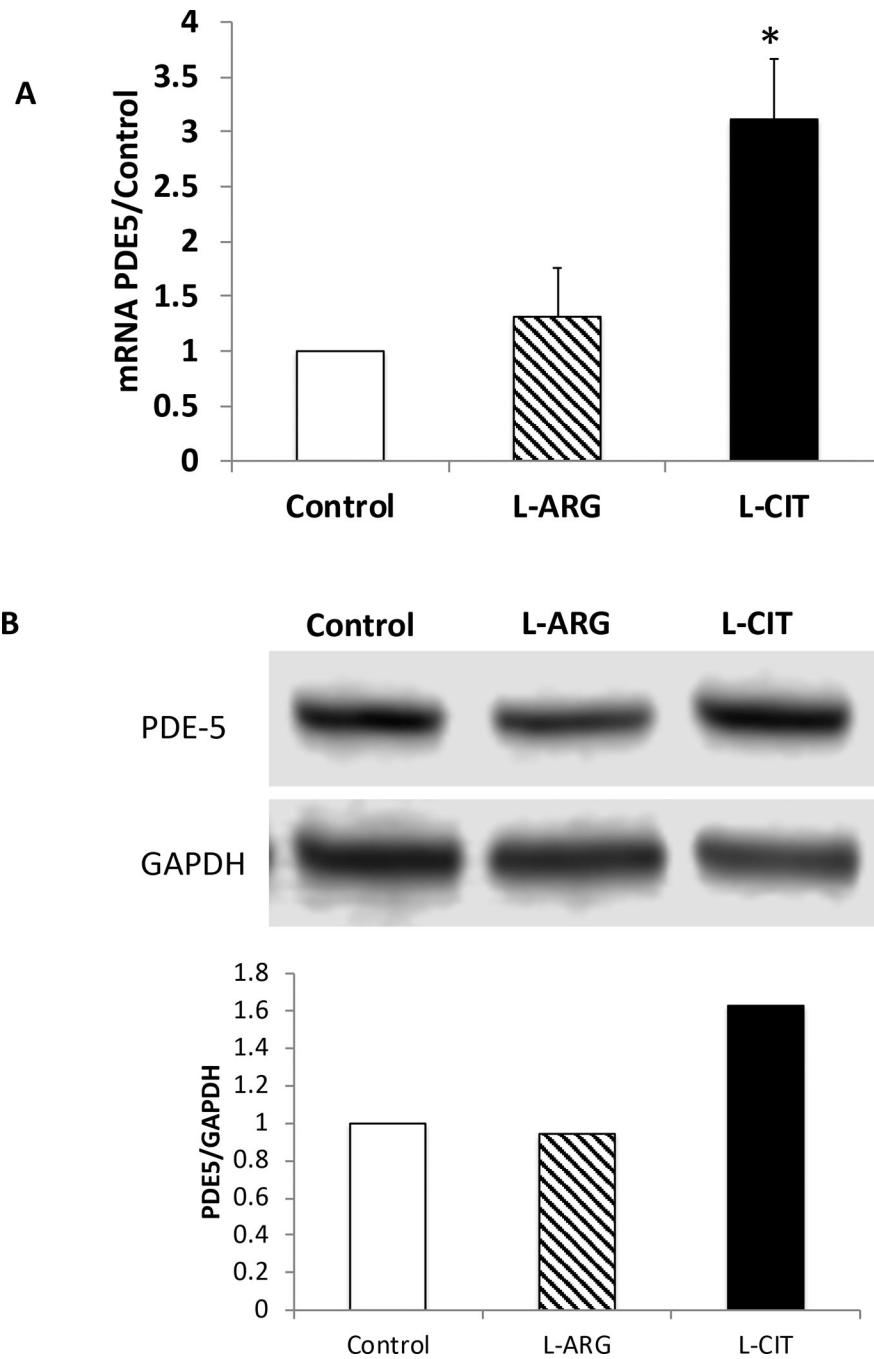


Figure 7: Effect of the incubation of L-arginine and L-citrulline on the phosphodiesterase 5 (PDE5) expression in cavernosal smooth muscle cell culture. CSM cells were incubated for 24 hours with or without L-ARG and L-CIT, at the same concentrations described in Figure 3. **Panel A:** mRNA expression assayed by qPCR. Results are expressed as a ratio of PDE5 mRNA of each group with respect to control. Samples and controls were normalized to the RPLP1/3 housekeeping mRNA. **Panel B:** PDE5 protein expression was determined by Western blot for control, L-ARG, and L-CIT. Results are expressed as a ratio with respect to control, and represent the mean \pm S.E.M of three

experiments. Samples and control were normalized by GAPDH protein expression.
**p<0.01 with respect to the control

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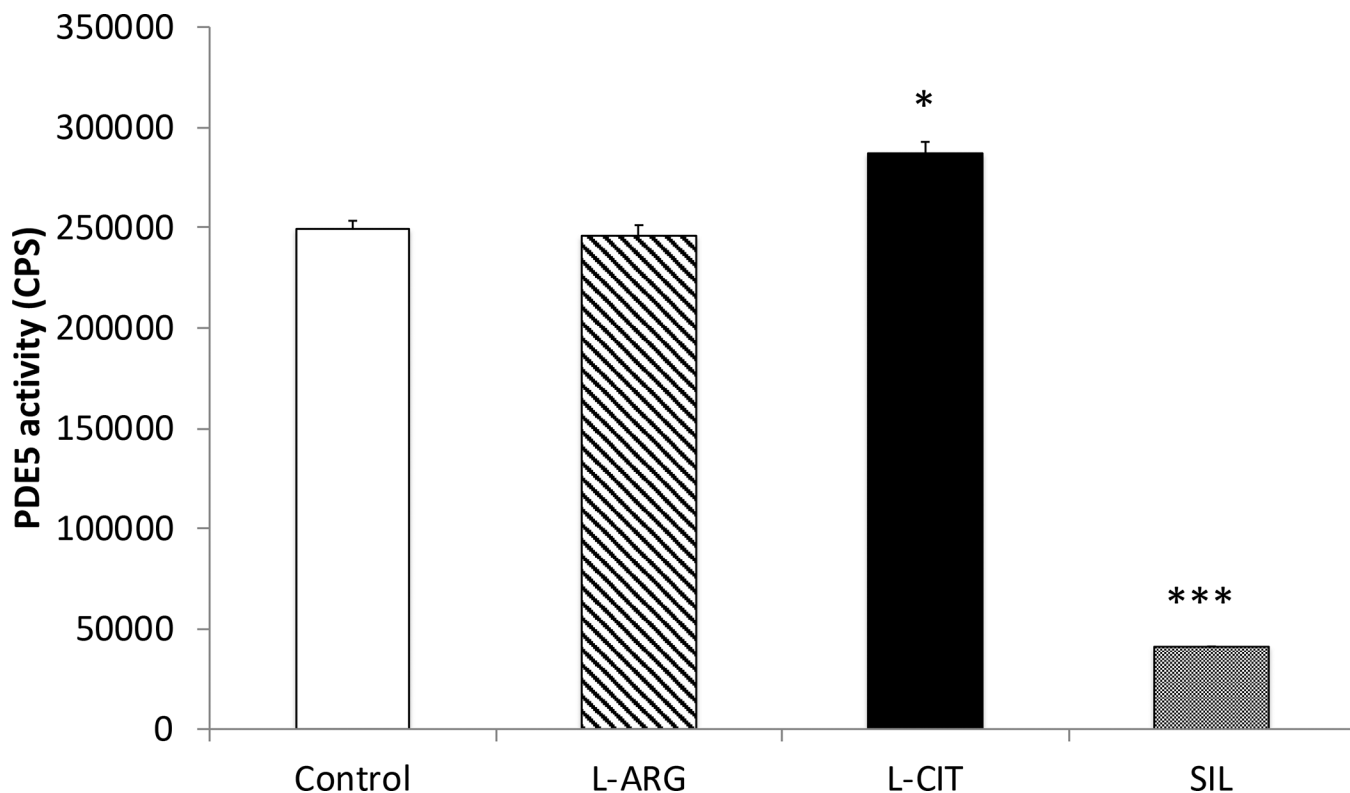


Figure 8: Effect of the incubation of L-arginine and L-citrulline on phosphodiesterase 5 (PDE5) activity in vitro.

The inhibitory activity of L-ARG and L-CIT on PDE5 activity was determined by a luminometry assay. Purified Bovine PDE5 enzyme, cGMP as a substrate, and serial dilutions of L-arginine and L- citrulline were tested. Data represent the activity of PDE5 measured by luminometry at the concentrations used in Figure 3 for L-ARG and L-CIT. Sildenafil (SIL) at a concentration of 0.4 mM was used as a positive control for this assay. Results are expressed as counts per seconds (CPS). * $p < 0.05$; *** $p < 0.001$ with respect to control.

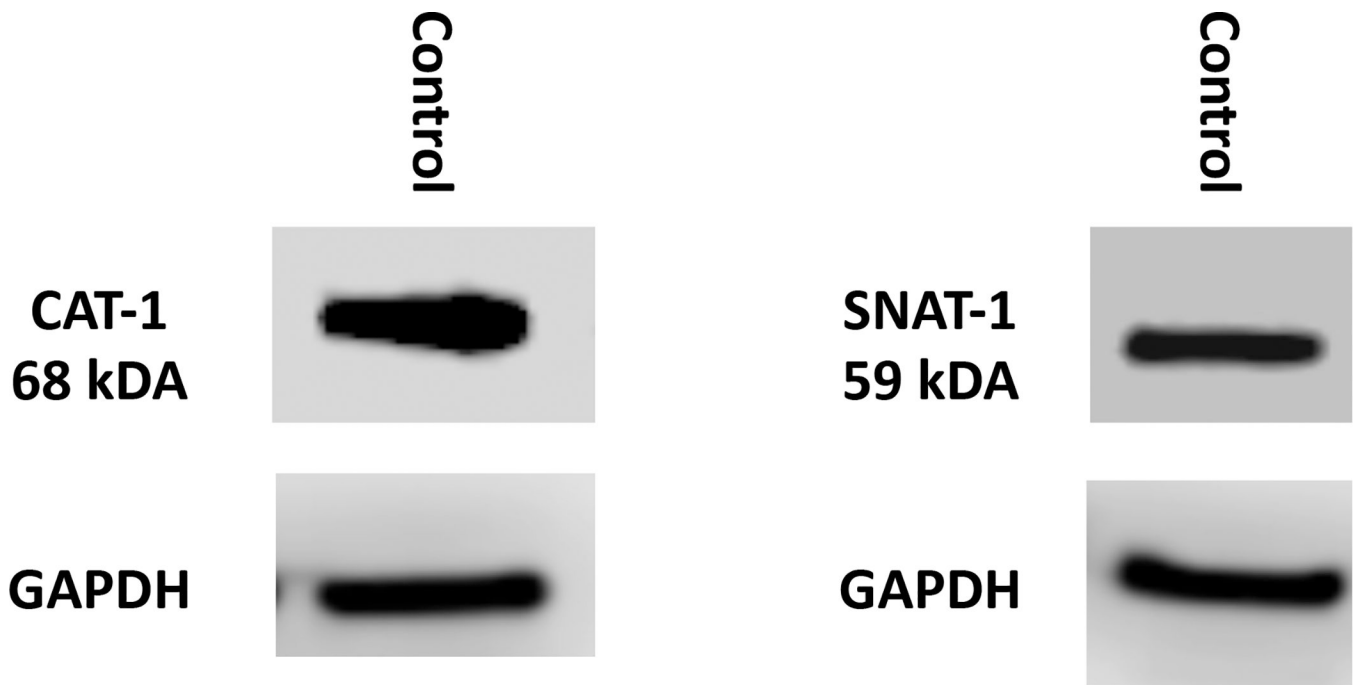


Figure 9: CAT-1 and SNAT-1 expression in cavernosal smooth muscle cells.
 CAT-1 and SNAT-1 expressions were determined by western blot. Samples were normalized by GAPDH protein expression.